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Human cytomegalovirus genetic variability and its interplay with host innate immunity

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PhD School in Life and Health Sciences

Molecular Medicine

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CONTENTS

PART I

1. Human cytomegalovirus (HCMV)

1.1 General features of herpesviruses

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 (1). Within the *Herpesviridae* family, the viruses are divided into three subfamilies on the basis of their genome sequences: *alpha-, beta- and gammaherpesvirinae*, respectively (Figure 1). 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) (2, 3).

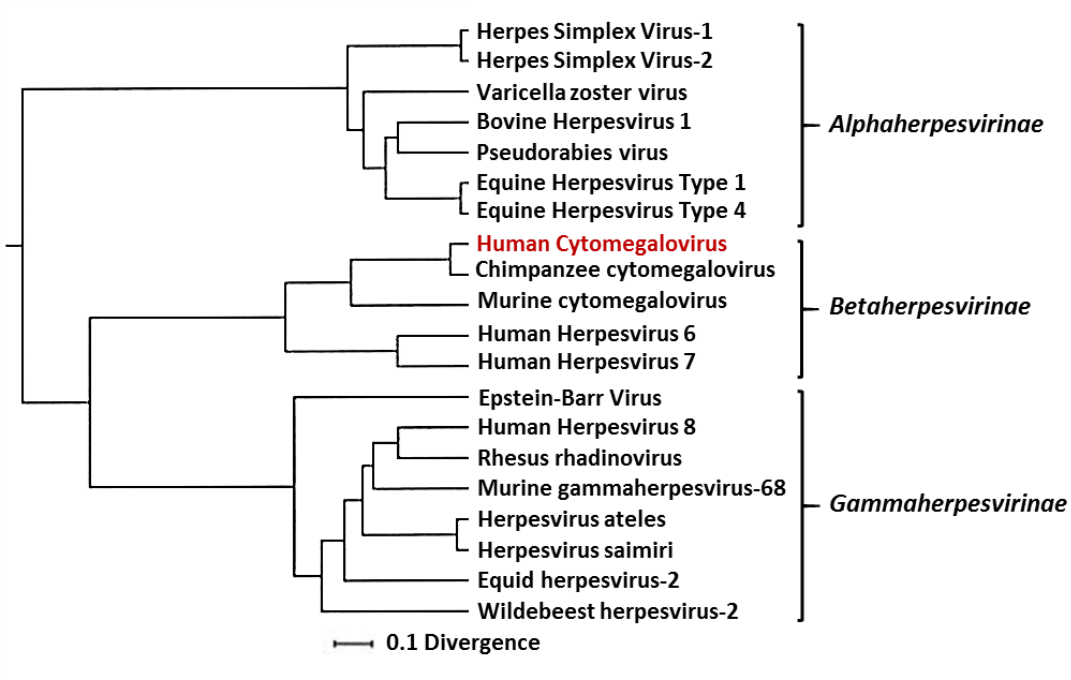


Figure 1. Composite phylogenetic tree for herpesviruses (4). Adapted from McGeoch et al. (4)

Herpesviruses are extremely common within the human population, as about 90% of humans appear seropositive at least for one or even two herpesviruses simultaneously (5). 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 (1, 6), and have been linked to neurodegenerative diseases (7–9) and several malignancies, such as Kaposi's sarcoma and numerous EBV-associated tumors (10–14). Left untreated, severe infections may result in a fatal outcome (15–17). 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 (18–21) 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 (2, 4, 22, 23) and it is highly likely that the process of their evolution is still ongoing (24). 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 (25). 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 co-evolutionary path. Unsurprisingly, herpesviruses possess an arsenal of elaborate strategies to avoid or counteract immune responses of the host (26). Among them human cytomegalovirus, acts as a true master of immune evasion (27, 28).

In this study, we focus on human cytomegalovirus (HCMV), a prototype betaherpesvirus (Table 1), whose virion structure, strict host specificity, genome organization, gene expression, and latency are typical of other herpesviruses. However, HCMV also possesses a range of several distinct intriguing features that differ from other members of the viral family. Firstly, HCMV is the largest human herpesvirus, with a genome size of ~235 kb (29) encoding not only 165 canonical ORFs (30–32) but also engaging multiple alternative transcripts and mechanisms of noncanonical translation (33–36). Thus, the enormous genome reflects the increased coding capacity, which inevitably results in a greater variety of functional proteins and other gene products implicated in viral replication and promotion of viral life cycle. Secondly, despite strict organization of its dsDNA genome, HCMV demonstrates an exceptionally high degree of variability, contradicting

the logical expectation that, being a large double-stranded DNA virus, it should maintain high genome stability (37).

Interestingly, genetic variability has been detected particularly in genes contributing to immune modulation (38). Genetic diversity within a single host may be additionally affected by a high chance of co-infection with multiple strains (mixed infection or superinfection) (39), *de novo* mutations, or reactivation of the latent virus.

1.2 Global burden of HCMV

Human cytomegalovirus is an important clinical pathogen around the globe, with higher seroprevalence in countries with lower socioeconomic status (40, 41). 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 (42–44). Thus, the risk groups for HCMV infection include solid organ or stem cell transplant recipients treated with immunosuppressors (45, 46) and cancer and AIDS patients (44, 47), who commonly display symptoms such as gastrointestinal ulceration, hepatitis, pneumonitis or retinitis, which can lead to blindness (48).

Neonates with immature immune systems are one of the major target groups for HCMV infection, resulting in severe congenital disease (49). HCMV is a leading cause of congenital disease among newborns worldwide due to an infectious agent (50, 51) and the disease prevalence is higher than Down syndrome, spina bifida or fetal alcohol syndrome (40, 52). 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 (53, 54). Furthermore, mounting evidence suggests that HCMV may contribute to immunosenescence in the elderly (55–58) and is linked to a number of autoimmune (59–61), inflammatory and vascular diseases (62–64), as well as some cancers (65–73).

A significant challenge in combating HCMV infection is the absence of a vaccine or antiviral treatment (74–76). Commonly, in addition to immunoglobulin from seropositive individuals (77), ganciclovir and its oral analog valganciclovir are used as antiviral agents that target viral polymerase (pUL54) or viral phosphotransferase (pUL97) (78, 79), despite their significant toxicity, which limits their administration to some categories of patients, such as pregnant women (79, 80). 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 (81, 82). Numerous mutations associated with antiviral resistance were identified through sequencing of both genes, reported by multiple groups upon studying different patient cohorts (83–85). While frequencies of these resistant mutants among transplant recipients vary, they generally range between 5%–10% (86). In our recent study involving a cohort of congenitally and postnatally infected children, the frequency rate of reported mutations potentially associated with drug resistance varied between 5-30% (87).

It is of paramount importance to align and combine the current knowledge on antiviral drug resistance mutations into a publicly available database, which may have value by prognosisticating poor treatment outcome prior to drug administration in clinics and hospitals around the world. If phenotypic and genotypic testing of clinical isolates is available, these data can be used to select drugs based on individual patient isolate susceptibility, optimize the dose within the limits of toxicity, or to select a combination therapy when doing so is considered beneficial. An attempt to generate a public web database has been described (88), in which evaluation of UL97 and UL54 gene sequence of clinical isolates regarding their susceptibility phenotype has been collected.

Finally, the latest proposed antiviral drugs include maribavir and letermovir, however, their use is currently limited and not yet universally available (89).

Although numerous HCMV vaccine candidates have been tested previously, including live attenuated vaccines AD169 and Towne (90), Towne/Toledo chimeric viruses (91), DNA vectors, vaccines based on dense bodies (92, 93) or glycoprotein subunits, eventually they have all failed to provide an efficient antibody response and progress further in clinical trials (Rev in: (94).

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 (95). 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 candidates is complicated by the absence of an HCMV animal model (94).

Nevertheless, the search for an HCMV vaccine currently continues through the engagement of multiple strategies which currently demonstrate some potential (94, 96). For instance, a few AD169 based candidates, such as attenuated vaccine or genetically engineered replication-defective AD169-derivate, contain restored pentameric complexes gH/gL/pUL128-131 and aim at improving potency of neutralizing antibodies (97, 98).

Other experimental vaccine candidates, such as subunit protein (99), DNA vectored (100, 101), and viral vectored vaccines (102–105), 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 (106). More recently, Choi et al. stated that inclusion of the pentameric complex as part of a vaccine design dramatically improves vaccine efficacy in the guinea pig model (107).

Taking into account all the above-fmentioned 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.

1.3 Virion structure

The architecture of the HCMV virion resembles that of other herpesviruses: it contains a linear dsDNA densely packed in an icosahedral capsid (T=16) composed of 12 pentons, 150 hexons, and 320 triplexes (108), surrounded by a tegument matrix of several proteins, and wrapped in a lipid bilayer envelope embedded with numerous glycoproteins (Figure 2). The mature virion particles typically reach 200-230 nm in diameter, similar to other herpesviruses (109). Envelopment of nucleocapsids occurs at the inner nuclear membrane, followed by acquisition of tegument and secondary envelopment in the cytoplasmic viral assembly complex (cVAC), a virally induced perinuclear region in the cytoplasm (110) where the virions bud into the recycling endosomes, and subsequent transportation to the plasma membrane for fusion and virion release.

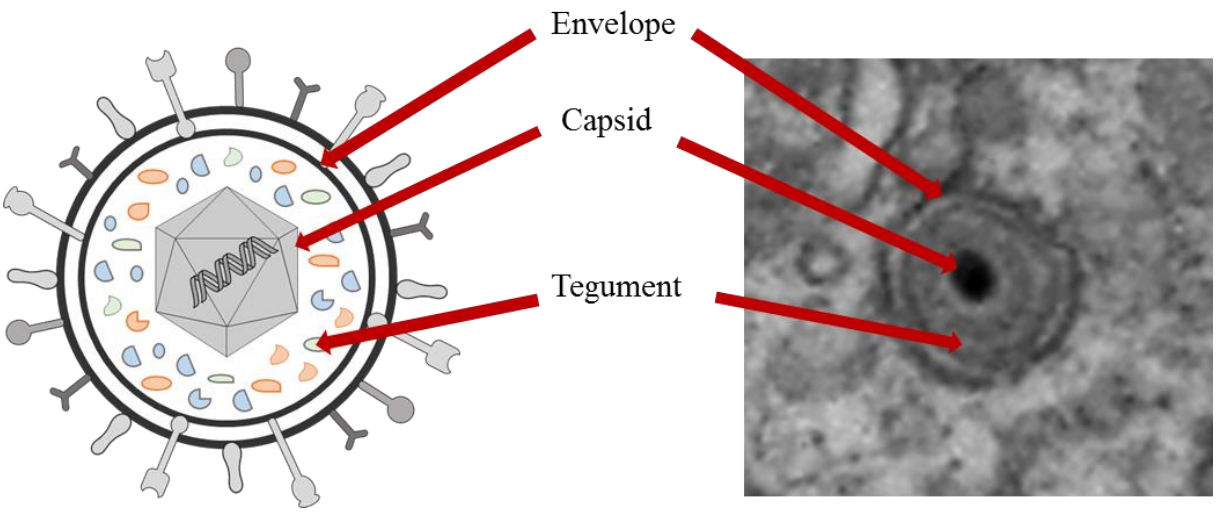


Figure 2. Schematic representation of HCMV virion structure (left panel). Electron microscopy of HCMV mature virion (right panel) (*Kindly provided by Dr. Matteo Biolatti, University of Turin)*.

In addition to mature virions, infected cells release defective viral particles, such as non-infectious enveloped particles (NIEPs) and dense bodies (DB). NIEPs are enveloped genomeless capsids that contain viral assembly protein otherwise not found in mature infectious virions, whereas DB represent enveloped formations lacking both DNA and capsids but containing numerous tegument proteins (e.g. pp65) instead. The structural studies involving purification of all types of viral particles encountered major difficulties in defining the role and mechanisms of such occurrences, although they have pointed out that continuous passaging and virus strain-to-strain variability may also affect protein composition of the viral particles. Interestingly, several groups have shown that DB may serve as favorable low risk vaccine candidates, characterized by complete absence of DNA within particles, albeit an intact glycoprotein composition on their surface and tegument proteins, which may effectively induce both potent neutralizing antibodies and broad cellular immune responses (92, 111–114).

1.4 Genome organization

The dsDNA genome of HCMV is the largest of all known herpesviruses (235 kb) with a high GC content (57.5%) (24). It is comprised of two big unique regions: unique long (UL) and

unique short (US), both flanked by inverted internal/terminal repeats, long and short (terminal/internal repeat long TRL/IRL and internal/terminal repeat short IRS/TRS accordingly), resulting in TRL–UL–IRL–IRS–US–TRS genome organization (1, 32, 115). Interestingly, an inversion between the repetitive regions happens regularly and is mediated by direct terminal repeats (a, b, c) and by inverted repeat elements at the UL-US junction (a', b', c') (Figure 3). The «a» elements contain cis-acting signals, *pac*1 and *pac*2, which are recognized by encapsidation machinery to initiate packaging and direct genome cleavage (116) , that subsequently leads to a change of orientation of each unique region and occurrence of four genomic isomers (117). The organization reflects that of herpesvirus class E genome structure (118), as four generated isomers are packaged with equal efficiency. The exact role of genome isomerization in cytomegalovirus biology remains a mystery.

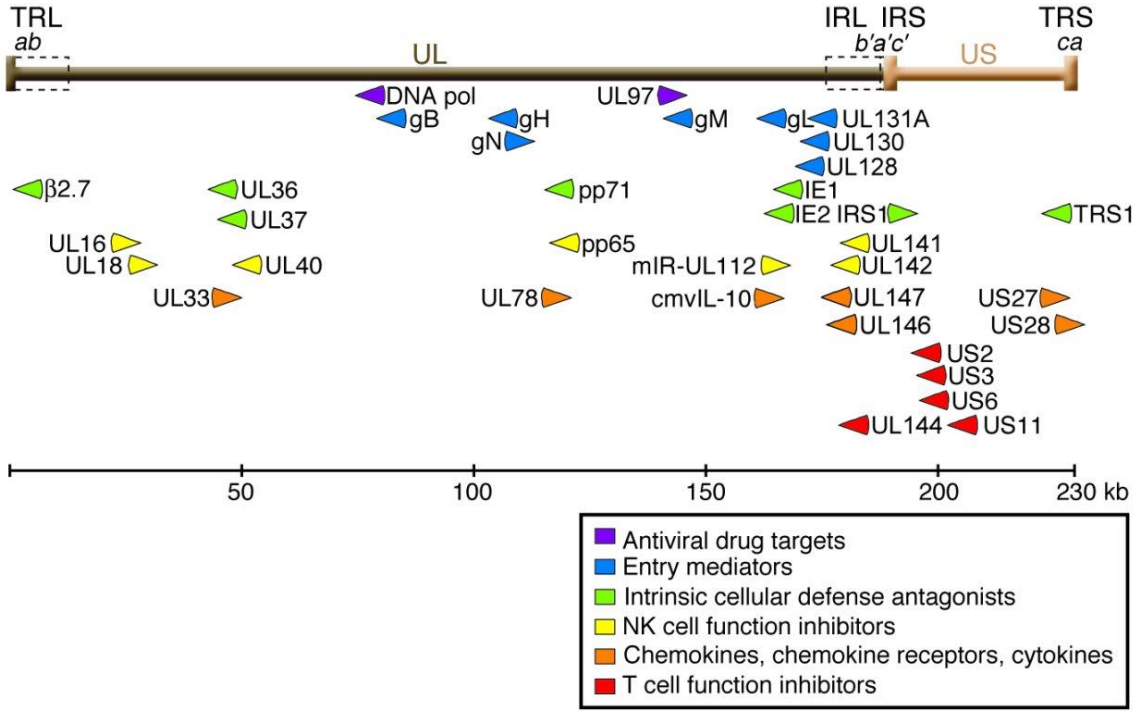


Figure 3. The schematic genome of HCMV (Merlin strain, GenBank accession no. NC_006273). *ab*, *ca* and *b'a'c'* represent inverted repeat sequences.

The genome is packaged in a highly stable icosahedral capsid made up of 162 capsomers surrounded by a thick tegument layer of proteins and enclosed by lipid bilayer, consisting of both

host and viral glycoproteins (115). Although dsDNA appears linear when packed inside the nucleocapsid, upon replication it circularizes via theta-like replication initially and later proceeds to rolling cycle amplification. Upon packaging, the genome is cleaved, linearized and transferred inside the nucleocapsid (115). The genome of wild-type HCMV encodes over 165 ORFs that are orientated sequentially within both unique and repetitive regions, along with extensive alternate mRNA splicing and numerous non-coding RNAs (29).

The first original sequence published and assessed for ORFs was the sequence of laboratory strain AD169. Interestingly, the following sequencing of Toledo and Towne strains, which have been passaged to a more limited extent, revealed the number of additional ORFs absent in the original AD169 sequence. Therefore, it is important to point out that wild-type genome organization differs from that of laboratory strains traditionally passaged *in vitro*, as they undergo profound genetic changes and even the latter differ among each other (119, 120) (Figure 4).

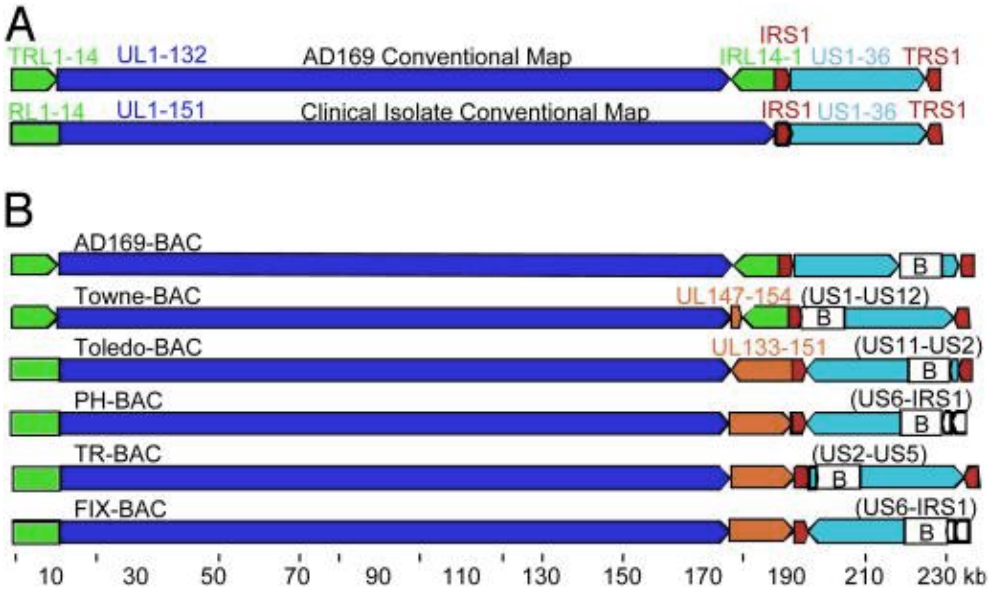


Figure 4. HCMV ORF organization. (A) Conventional ORF maps of the AD169 laboratory strain and clinical isolates. (B) ORF maps of the BAC clones capturing one of the four possible isomers of the viral genome as described in Murphy et al., (119).

The sequencing of HCMV clinical isolates has revealed that the inverted b' has been replaced by an extra UL region ~15 kbp, containing at least 19 additional ORFs that are absent in the AD169 genome (121). This change was attributed to extensive passaging and adaptation of the

laboratory strains to the propagation conditions *in vitro* in contrast to clinical isolates. During passaging in fibroblasts, high-passaged HCMV strains accumulate point mutations and deletions, while retaining a consistent genome size through sequence duplications. As a result, AD169 fails to replicate in several cell types, such as endothelial cells, which are permissive for replication of clinical isolates (122, 123).

Although the HCMV genome encodes over 160 gene products (30, 115, 119, 120), only a quarter of these annotated genes play a role in DNA replication and encapsidation, whereas the majority of genes are responsible for virion maturation, persistence, latency, cellular tropism, and modulation of host immune response (33). HCMV encodes numerous protein homologs of cellular chemokines, chemokine receptors, and cytokines that play a pivotal role in immune evasion (27). Most of the genes located in the UL region are essential for viral replication and are generally conserved among herpesviruses, while repetitive US gene regions are less so. The UL region also contains origins of replication, oriLyt sequence, where DNA replication is initiated (115).

The functional analysis of HCMV genes has generally followed one of these strategies: either direct or random mutagenesis of viral genes or isolation of gene products, which leaves the physiological function of these products in need of further study. Over 200 ORFs have been assigned functions while a number of unknown functional proteins remain (124). It is highly likely that many of the unknown functions are devoted to viral dissemination, tropism and counteracting the host immune system.

1.5 Viral replication and gene regulation

Following entry into permissive cells, the HCMV capsid is deposited into the cytoplasm where cellular microtubules facilitate its translocation into the nucleus where viral DNA is released. Upon productive infection, the HCMV genome is expressed in a temporally regulated manner and the cascade of transcriptional events result in synthesis of several groups of proteins known as immediate-early (IE or α), early (E or β), and late (L or γ). Viral transcription and replication in the nucleus of infected cells are orchestrated by the host RNA polymerase II and related transcription machinery under control of viral transactivators (125) .

Although the replication cycle of HCMV is rather slow, requiring 48 to 72 hours to reach the final stages of maturation and virion release, the expression of IE genes starts within minutes

of infection. Most IE genes are transcribed under the control of the major immediate-early promotor (MIEP) which requires the activity of the tegument proteins pp71 and pp69 brought by the virus (126). The translated IE proteins modulate a number of vital processes, such as intrinsic cell signaling, cell cycle arrest, chromatin remodeling, transport and splicing of RNA, and subsequent activation of early (E/ β) genes (127, 128). Following activation, β-genes are sequentially expressed in two groups, namely β_1 (early-early) and β_2 (early-late) genes (125). The β-gene products include DNA binding proteins and several enzymes that are essential for DNA replication.

Viral DNA synthesis occurs typically after 16 hpi and increases after 24hpi. Replication depends heavily on host cell metabolism in order to ensure continuous supply of dNTPs, as the virus does not encode enzymes for its own dNTP synthesis (e.g. thymidine kinase, thymidylate synthase, dihydrofolate reductase, or an active form of ribonucleotide reductase) (129). As a result, HCMV does not intend to block host synthesis of macromolecules, but rather stimulates cellular production of DNA precursors through multiple strategies via interfering with numerous signaling pathways. The accumulated evidence clearly shows that HCMV infection directly triggers a substantial increase in the expression of enzymes responsible for nucleotide production, including thymidine kinase (130) , ornithine decarboxylase (131) and topoisomerase II (132), dihydrofolate reductase, folylpolyglutamate synthetase, ribonucleotide reductase, thymidylate synthase (133), and deoxycytidylate deaminase (134). Moreover, HCMV blocks cell cycle progression to prevent host DNA replication machinery from competing with the virus for access to DNA precursors (135).

During lytic infection, DNA replication starts from the replication origin (ori_{Lyt}) (136), located between viral ORFs UL57 and UL69. Six highly conserved core proteins form the replisome and are essential for viral DNA synthesis: DNA polymerase UL54 and its processivity factor UL44, single-stranded DNA-binding protein (encoded by UL57), and the helicase-primase complex (encoded by UL70, UL102, and UL105) (1, 115). While the helicase-primase complex unwinds the DNA, pUL57 facilitates strand separation and prevents their reannealing during DNA synthesis (137). Meanwhile, the UL54-UL44 complex synthesizes the leading strand from the primer initiated by the helicase-primase complex at the replication forks.

Moreover, several other proteins additionally contribute to successful DNA replication, including immediate early 2 (IE2) (encoded by UL122), TRS1/IRS1, pUL36-38, pUL84, and four phosphoproteins (pp34, pp43, pp50, and pp84) encoded by UL112-113 (138–141). It is suggested

that initiation of DNA replication from the ori$_{Lyt}$ promoter depends on the complex formed by pUL84 and the key regulator IE2-p86 (142, 143), whereas pUL84 recruits the UL54-UL44 complex through its interaction with the processivity factor (141) (Kim and Ahn, 2010). At last, the four UL112-113 gene products facilitate the assembly of replisome through the association with pre-replicative sites near ND10 (144, 145). A number of additional proteins are also associated with viral replication, such as the ribonucleotide reductase encoded by the UL45 gene, deoxyuridine triphosphatase encoded by the UL72 gene, deoxyribonuclease encoded by the UL98 gene and uracil DNA glycosylase (UNG), an enzyme involved in the base excision repair (BER) and encoded by the UL114 gene (125). The viral UNG is highly conserved among mammalian herpesviruses (146), and presumably evolved to minimize the presence of uracil bases in genomic DNA, therefore preventing damage to the genome (147). The HCMV-encoded UNG, pUL114, associates with viral processivity factor UL44 and is required for efficient viral DNA replication at both early and late stages of infection (147, 148). In contrast to other herpesviruses, the deletion of UL114 ORF results in delayed DNA synthesis, even though the early genes expression is not affected (148). This may suggest that HCMV evolved unique mechanisms to replicate its genome that are independent and distinct of those origin binding proteins of other herpesviruses. Moreover, an interesting hypothesis suggests that the UNG-mediated excision of uracil, which can be incorporated into the viral genome through cytosine deamination early upon HCMV replication, eventually forms convenient substrate sites for initiation of recombination-dependent replication (148). Furthermore, it has been shown that UL114 and its partner UL44 interact with SMARCB1, a factor of the SWI/SNF chromatin remodeling complex, implying their involvement in different DNA transactions (149). However, many of UL114 functions remain to be further investigated.

In addition to viral proteins, some cellular proteins, such as the mitotic checkpoint protein BUB3, polypryrimidine tract protein (PTB)-associated splicing factor, and heterogeneous ribonuclear protein K (hnRNP K), are able to bind to viral oriLyt and thus may contribute to lytic viral replication and/or transactivation. Particularly, hnRNP K has been shown to bind UL84, while UL44 and IE2 enhance this interaction (150). Further investigation of these interactions may be beneficial in expanding our understanding of how the virus utilizes cellular factors to achieve its genome replication.

Finally, the β-gene products activate the expression of γ-genes, transcribed as two groups, namelyγ_1 (leaky late) and γ_2 (true late) genes, respectively (125). The γ-genes encode structural

proteins, necessary for viral assembly and virion maturation (e.g. gB, gD glycoproteins). It is well established that late upon infection γ-genes products are able to regulate the expression of α- and β-genes (125).

Upon late stages of replication, newly synthesized viral genome undergoes inversion and the four genomic isomers are produced in concatemeric units. The synthetized isomers either serve as templates for new cycles of replication or are cleaved during packaging by the terminase complex. The terminase complex consists of UL89, UL56 and UL51 proteins (151, 152) and is responsible for cleavage of concatemeric DNA into monomeric molecules at specific DNA packaging signal sites *pac*1 and *pac*2 in the US region (153). Then the viral DNA becomes packaged into pre-formed capsid and delivered into cytoplasm (154).

Following primary lytic infection, HCMV is able to establish latency in myeloid progenitor CD34+ cells in the bone marrow and their derivative $CD14^+$ monocytes in peripheral blood (155). In these cells, the viral genome is under control of histone repressive markers, which is reflected by a complete absence of viral activators (f.e. pp71) (156), a low level of cellular transactivators and a dominance of cellular transcriptional repressors recruited to the major immediate early promoter (MIEP). As a result, chromatin around viral MIEP becomes repressive and lytic transcription of viral genes is suppressed (157).

Latent state is characterized by a major shutdown of viral gene expression and replication, however some latency-associated transcripts such as US28 (158), LAcmvIL-10 (159), UL138 (160), ORF94 encoding UL126a (161), latency unique natural antigen (LUNA) (162), and UL144 (163) are present. Some roles in promotion of HCMV latency and its maintenance have been ascribed to cellular miRNA and non-coding RNAs (164). Later, HCMV can reactivate upon various stress factors (UV light exposure, fever) or substantial immunosuppression. Along with host-mediated transcriptional repressors, latency may also be affected by differentiation signals, such as cytokines and growth factors (165). Thus, reactivation may occur upon differentiation of progenitor myeloid cells into dendritic cells or macrophages (166). Upon reactivation, the repressed chromatin around the MIEP associates with cellular transcriptional activators, which enables IE gene expression (155). In immunocompromised patients, reactivation leads to virus dissemination to multiple target organs and results in clinical disease.

1.6 Tropism

HCMV can infect an exceptionally broad range of cell types. Epithelial cells, endothelial cells, smooth muscle cells, and fibroblast cells are the cell types most commonly infected for virus replication (167, 168). In this regard, viral glycoproteins dictate the spectrum of susceptible cell types. The virus possesses a remarkable ability to switch its viral ligand on the virion, using the trimer gH/gL/gO for infection of fibroblasts and the pentamer complex (PC) gH/gL/UL128/UL130/UL131 for infection of epithelial, myeloid and endothelial cells, respectively, and leading to membrane fusion (169), also mediated by fusion glycoprotein B (gB). While the trimer gHgLgO complex is known to bind the platelet-derived growth factor receptor α (PDGFRα) expressed on fibroblasts (170, 171), the pentamer complex binds Neuropilin2 (Nrp2) to facilitate entry in epithelial, endothelial and myeloid cells (172).

In detail, gB and a trimer together mediate membrane fusion/macropinocytosis in a pH-independent manner between the virus and the surface of fibroblasts (109, 173). In contrast, entry to the other cells occurs within the endosome and/or by macropinocytosis in a pH-dependent manner mediated by gB, the trimer, and the PC (174). In addition, viral envelope proteins have been shown to interact also with cellular heparin sulfate proteoglycans (HSPGs) (175), integrins (176), epidermal growth factor receptor (EGFR) (177, 178), THY-1 cell surface antigen (CD90) (179), and recently CD147 (180) and OR14I1 (181). It is worth noting that other unreported cellular factors may contribute to entry and cell-to-cell spread, which may also depend on cell type. The identification of these factors is essential to uncover the biology of virus entry and will prove beneficial for vaccine development (182, 183).

Generally, it is assumed that HCMV disseminates primarily via direct cell-to-cell spread, rather than via extracellular cell-free virus release, which would be susceptible to antibody responses. Furthermore, clinical isolates of HCMV spread in a highly cell-associated manner during initial tissue culture passages (122), and the progressive loss of this cell-associated phenotype correlates with disruption of genome regions (184). Thus, several viral genes have been implicated to impact cell-associated versus cell-free spread, such as RL13 and numerous genes affecting gH/gL composition (169). In this regard, RL13 is the most rapidly mutated gene upon viral propagation in vitro, and often acquires nonsense or frameshift mutations after a few passages on fibroblasts,

endothelial cells, or epithelial cells (184, 185). Although it has been shown that ectopically expressed RL13 traffics to the cell surface and binds the FC domain of IgG1 and IgG2 antibodies, followed by internalization (186), potentially indicating its role in immune evasion, the exact functions of RL13 remain elusive. Similarly, the composition of gH/gL envelope complexes is considered to have the largest impact on cell-free versus cell-associated spread. A recent study has demonstrated that the pentamer governs cell-to-cell spread that resists antibody neutralization (187), while it is also well established that restoration of pentamer expression increases cell-associated spread (188). Furthermore, repair of the pentamer in strain AD169 has been shown to promote the formation of syncytia during in vitro cultivation of the virus (174). Contrarily, the trimer is required for cell-free virus spread (189), although the pentamer is nonetheless needed for efficient infection of endothelial and epithelial cells as well as monocytes (169). However, HCMV strain AD169, with a deletion of the essential tegument protein pp28 (UL99) and harboring a frameshift in UL131, which makes it unable to express pentamer, efficiently replicates and spreads in cultured fibroblasts. This fact suggests that the trimer may be sufficient to drive cell-to-cell spread in fibroblasts (190). Moreover, differences in levels of pentameric and trimer complexes displayed by HCMV strains reflect the differences in cell tropism (191, 192).

The gene regions affecting gH/gL composition are to blame. Certain HCMV strains derived from bacterial artificial chromosome (BAC) clones, such as TR (120), TB40/E (168), and VR1814/FIX (193) maintain intact ULb' regions and, at least when reconstituted on fibroblasts, express low levels of pentamer and high levels of trimer. Given their low levels of pentamer expression, it is somewhat expected that these viral strains replicate inefficiently on epithelial cells (174). As TB40/E harbors a mutation in an intron of UL128, it has been shown to dampen pentamer expression, whereas TR and FIX express similar low pentamers level for unknown reasons (194). On the other hand, AD169 strain that has been extensively passaged in vitro carries a ULb' region that has undergone rearrangements and loss of ~14 kbp of coding region (195) is nonetheless able to efficiently replicate on epithelial cells when pentamer expression is restored (174). Interestingly, deletion of UL148 gene within ULb' enhances the ability of TB40/E to replicate in epithelial cells and reduces levels of gH/gL and trimer expression in virions (179, 196).

Recently, the other gene products such as US16 and UL148 have also been shown to modulate the composition of gH/gL complexes (196, 197) in vitro. Additional insights into tropism and its contribution to either cell-associated antibody-resistant or cell-free particles spread (as

more compatible with horizontal transmission via body fluids), may be helpful in shedding light on mechanisms of broad viral dissemination in vivo and applicable in the development of neutralization assays or therapeutics.

1.7 HCMV pathogenesis

Its broad tropism enables the virus to infect multiple organs and contributes to various pathologies associated with the infection (44). HCMV appears tightly restricted to the host, but not to tissues, effectively replicating in epithelial tissues of salivary glands, mucosal tissues, connective tissues in various organs, smooth muscle cells, the gastrointestinal tract, and vascular endothelial cells.

It is estimated that more than half of the human population harbors latent HCMV. Transmission is more frequent in childhood and mostly asymptomatic. Nevertheless, even during asymptomatic infection, the virus may indirectly alter host immune system upon reactivation periods or by expressing several latency products (e.g., LAcmvIl-10). Since a substantial portion of the population is infected with HCMV, this leaves a large pool of people with latent infections. These individuals are potentially susceptible to viral morbidity if they become immunocompromised and HCMV reactivates (6).

The virus spreads through direct contact with infected body fluids, including urine, saliva, tears, breast milk, and genital secretions. Depending on the age and immune status of the host, persistent shedding in saliva and urine may proceed for months to years. In adults, where sporadic shedding of the virus occurs in saliva, cervicovaginal secretions and semen, the sexual transmission mode predominantes (6). Importantly, infection by any route culminates in a leukocyte-associated viremia that deposits virus in sites from which shedding can infect new hosts. When effective cellular immune response comes into play, a latently infected myeloid cell population remains in the bone marrow precursors of monocytes, macrophages and dendritic cells. They serve as a source of latently infected cells that eventually allow viral distribution throughout the body and contribute to the risk of transfer of the virus with organs or tissues during transplantation (1, 6).

In the immunocompromised population, the infection results in active HCMV replication. Depending on the clinical setting, active replication may lead to direct tissue damage, resulting in

an inflammatory response and dysfunction of various organ systems. For instance, HCMV infections following solid organ (SOT) or stem cell transplants (HCT) as well as in AIDS patients are associated with multiple end-organ diseases, such as colitis, renitis, esophagitis, ependymitis, hepatitis, and pneumonitis (198). Furthermore, it has also been shown that graft survival is affected by HCMV infections (199).

Interestingly, there is growing evidence that HCMV may contribute to onset of vascular diseases (64, 200). It is not surprising that the virus may play a role in the development of these diseases, considering its numerous gene products directed at modulation of host immune responses correlate with substantial tissue damage in the setting of profound immune suppression. Besides vascular diseases, HCMV has been associated with a number of other chronic diseases in the general population, including cancer, autoimmune diseases, and immunosenescence. Evidence for a causative role for HCMV based on antibody prevalence or serum level of antibody to HCMV or specific viral proteins is not persuasive and requires further investigation.

1.8 Congenital HCMV

HCMV is a leading cause of congenital infection worldwide due to an infectious agent. It is estimated that the virus causes congenital infection in 0.5–2% of all pregnancies each year in developed countries (201). Virus transmits during primary maternal infection in HCMV-seronegative women in a pattern reminiscent of rubella. However, HCMV causes recurrent maternal infection in HCMV-seropositive women following either reinfection with an additional viral strain or reemergence of persistent/latent infection. Primary infection during pregnancy in HCMV-naïve women is associated with an average 33% risk of transplacental transmission, whereas roughly 1% of recurrent infections result in transmission. The rate of vertical transmission increases with older gestational age at infection, while there is a higher risk of fetal damage when infection occurs in the early stages of pregnancy (202, 203).

Perinatal and postnatal infection of full-term newborns is often acquired from breastfeeding and is of little disease consequence (5). Premature or immunodeficient infants risk acquiring HCMV during delivery, from blood transfusions, and from breast milk and resulting in systemic disease. Once infected, infants and children shed the virus in saliva and urine for months to years and remain an important source of virus, infecting parents as well as other childcare providers (5).

However, the vast majority of infected children (85–90%) present no clinical abnormalities at birth (asymptomatic infection) and therefore, HCMV infection is not diagnosed early in life (50). Up to 15% of those with asymptomatic infection develop long-term sequelae, most frequently sensorineural hearing loss (SNHL) (204). Since sequelae from congenital infection are frequently delayed in onset, eventually a retrospective diagnosis is challenging (50).

The clinical manifestations of HCMV infection vary from moderate to severe symptoms in infected newborns: from mild hepatomegaly, low platelet count or raised levels of alanine aminotransferase to thrombocytopenia, petechiae, severe hepatomegaly, splenomegaly, intrauterine growth restriction, hepatitis (raised transaminases or bilirubin), or central nervous system (CNS) involvement. CNS involvement manifests as microcephaly, radiographic abnormalities consistent with HCMV CNS disease (ventriculomegaly, intracerebral calcifications, periventricular echogenicity, cortical or cerebellar malformations), abnormal cerebrospinal fluid indices, chorioretinitis, and SNHL (205). Currently, it is estimated that almost 25% of hearing loss in children of 4 years of age is attributable to congenital CMV (206). Therefore, it is important that all infants with congenital CMV infection, irrespective of their clinical presentation at birth, receive serial audiological monitoring throughout the first years of life to allow for early detection of possible SNHL (207). Vestibular impairment also is reported frequently, and possibly can show progressive deterioration over time (208). Furthermore, congenital CMV is the leading viral cause of neurodevelopmental delay, with a large proportion of symptomatic children suffering some degree of psychomotor and cognitive disability, and with visual impairment in up to half of symptomatic infants (53, 209, 210). In the worst cases, acute congenital HCMV infection causes severe systemic cytomegalic inclusion disease (CID) that may result in a fatal outcome.

It is worth noting that the diagnostic criteria of symptomatic HCMV infection vary widely in the literature. For instance, some case series consider subjects with abnormalities detected through specific testing, including SNHL, as asymptomatic, while others do not (211, 212). Some studies have categorized newborns with isolated low birth weight as symptomatic, whereas others have not (210, 213). Therefore, these differences may account for some of the differences in the prevalence of symptomatic infection and disease severity across studies.

Testing for primary maternal HCMV infection generally occurs after suspicious ultrasound findings, although it can not serve as a diagnostic method of fetal infection. Such ultrasound find-

ings as echogenic fetal bowel, cerebral ventriculomegaly and calcifications, and fetal growth restriction are the most common indications for ultrasound (214), while hepatic calcifications, microcephaly, and subependymal cysts have also been described (215). Magnetic resonance imaging (MRI) as a noninvasive method is also performed whenever fetal intracranial abnormalities are detected by ultrasound and should be performed during the third trimester (216). MRI was shown to be more sensitive than ultrasound (217), though results might be more difficult to interpret, and specialized neuroradiology consultation is required. It is generally accepted that negative MRI findings concomitant with negative US results reliably exclude severe outcomes for infected fetuses.

Seroconversion is a reliable method for diagnosis of primary infection, but it requires serial serological testing, a strategy unlikely to be feasible for all pregnancies in common practices in many countries. Traditionally, the presence of IgM antibody indicates acute infection, although the high risk of false-positive rate for HCMV-IgM assays indicates that the presence of IgM alone should not be used for diagnosis (218). The IgG avidity assay is a more accurate method that can be used to detect a primary infection than IgM alone, since the antibodies produced upon primary infection have lower antigen avidity than those produced during nonprimary response or later in a primary immune response. Over time, the maturation of the antibody response results in higher antibody avidity. Low to moderate avidity antibodies are encountered for 16-18 weeks following primary infection. Therefore, a low avidity IgG result in combination with a positive IgM antibody is indicative of infection within the preceding 3 months, allowing for a more accurate diagnosis of primary infection during pregnancy (218). Alternate methods of diagnosis are also available and include maternal serum or urine testing for viral load, although this does not correlate well with timing of infection or neonatal outcomes (219). Overall, for women suspected of having primary HCMV infection in pregnancy, the diagnosis by IgG seroconversion or with positive HCMV IgM, positive IgG, and low IgG avidity is highly recommended.

If primary maternal infection is documented, prenatal diagnosis of fetal infection is generally performed by amniocentesis, albeit the severity of HCMV infection cannot be determined. The only other diagnostic option, cordocentesis, provides similar sensitivity and specificity to amniotic fluid HCMV testing, but with a higher complication rate than amniocentesis (220) (Lazzarotto, 2011).

The diagnosis of congenital HCMV infection is currently evolving due to a convergence of recent advances in the field and likely will be changing rapidly over the next few years. Traditionally, virus isolation from urine or saliva in tissue cultures has been the standard method for diagnosis of congenital HCMV. Since this technique is labor- and resource-intensive and requires tissue cultures, it is no longer considered suitable for screening purposes. The optimization of the automatized and low-cost real-time polymerase chain reaction (PCR) has led to important advances in the diagnostic field, and is unlikely to be affected by sample storage and transport conditions (221). At this time and with the available methods, HCMV testing with dried blood spots (DBS) real-time PCR is unsuitable for HCMV screening, and its use is mostly limited to the retrospective diagnosis of congenital CMV infection in children with delayed-onset sequelae. In these cases, a positive result confirms congenital CMV infection but a negative result does not rule out congenital CMV infection. Unlike the DBS specimens, real-time PCR assay on saliva swabs has been proved efficient (221), characterized by high sensitivity and the ease of saliva collection in neonates, which make this specimen advantageous for neonatal CMV screening. The other common specimen widely used for diagnostics of congenital infection is urine, as the virus is constantly excreted in large amounts, but its collection may be complicated in neonates by a number of factors (e.g., inadequate diuresis or sample contamination) (207, 222, 223).

It is important to highlight that many factors contribute to congenital HCMV morbidity and mortality, including the limited awareness of parents and physicians about HCMV infection risk during pregnancy, lack of routine testing of neonates at risk, the absence of effective maternal or neonatal screening programs, the limited efficacy and toxicity of current drugs, and the absence of a licensed vaccine. In part, because of these limitations, congenital HCMV infection and preventive measures for acquiring the virus during pregnancy are not routinely discussed with pregnant women (205). Undoubtedly, preventive measures, early intervention for congenitally infected children, design of effective vaccine candidates for pregnant women and newborns, and safe antivirals remain a priority in research and clinical management (Rawlinson, 2017). The Institute of Medicine has identified the development of an effective CMV vaccine for prevention of congenital HCMV infection as a top priority (224).

1.9 HCMV genetic variability

1.9.1 Genetic phenomenon

The wide spectrum of clinical manifestations of HCMV disease as well as laboratory findings of genetic variability among the HCMV strains sparked an interest in identification of the origin and sources of pathogenicity of HCMV. Additionally, vaccine studies contributed to the hypothesis that distinct HCMV strains have different pathogenic potential. For instance, the highly passaged laboratory strains AD169 and Towne appeared attenuated when administered as vaccine candidates. Meanwhile, the Toledo strain, which had only been passaged several times in culture, caused disease when administered to seropositive individuals. Therefore, the observed differences were hypothesized to be attributed to genetic background of the strains (1) .

The first complete sequence of a HCMV genome was published in 1990, and it was the largest sequence generated at the time (121). The differences between the laboratory-adapted strains, known as AD169 and Towne, and the Toledo strain, which closely resembles the wild-type, were later localized to ORFs located in the UL/b′ region of the genome. These ORFs are thought to play a role in HCMV replication or disease *in vivo* because they were lost on extensive passage *in vitro*. Although most genetic loci within the UL and US regions are highly conserved, proteins produced by genes in the UL/b′ region are among those associated with immune evasion.

Now, clinical HCMV isolates from different cohorts of infected patients have been sequenced as well. The sequencing data revealed that HCMV can be highly polymorphic, among and within hosts (225–229) with a high level of intrahost variability comparable to that of RNA viruses (226). It has been proposed that new mutations occur every time that the virus infects a new host, thereby giving rise to a unique viral strain for each infected individual. Indeed, HCMV infection triggers a selection event where a new genotype becomes dominant due to the selective pressure of the immune response (226). Another possible explanation of this gap comes from the observation that both viral and host factors can contribute to the onset of HCMV genome mutations, thus fostering virus genetic drift during infection (230, 231).

Analysis of the genetic variation, detected particularly in genes contributing to immune evasion (38), requires careful interpretation, as the data from triggered immune responsiveness *in vitro* may appear unreliable. In contrast to wild-type, HCMV laboratory strains lack a large set of gene regions due to the absence of a constant need to retain immunomodulatory functions *in vitro*

(30, 232). In fact, extensive passaging in vitro leads to HCMV mutants lacking genes unnecessary for the replication, which arise within weeks of propagation, and also produces variation over the years between commonly used laboratory strains, evidenced by numerous works (184, 195, 233–235). Thus, to elucidate the relationship between HCMV immunomodulatory mechanisms and genetic variation, the use of clinical isolates instead of immunologically impaired laboratory strains may be necessary to reflect the real clinical picture. Ideally, to define natural populations, sequencing of HCMV genomes should be performed directly from clinical material (236).

Given the fact that HCMV is a large double stranded DNA virus, a high degree of genetic variation contradicts the logical expectation that the virus should maintain high genome stability (37). Initially, intra-host HCMV diversity was mostly attributed to rapid occurrence of *de novo* mutations (226, 227). However, most recent data indicate that a single HCMV strain is no more diverse than that of other DNA viruses, whereas the altered degree of variability is due to mixed infection with genetically distinct strains (39, 236, 237) and extensive recombination (38, 236–238). Moreover, another hypothesis suggests that genetic diversity within a single host can be affected by reactivation of the latent virus. Many of these genetic alterations may ultimately affect cell tropism and evasion of innate and adaptive defenses. Therefore, the variety of ways HCMV is able to modulate innate immune responses, and, as a result, severity, infection mode and diverse clinical outcomes may be ascribed to genetic variation of HCMV. Understanding phenomena of mixed infection (superinfection) and recombination as contributors to viral diversity is critical for distinguishing the role of genetic variation in viral evolution, immune adaptation, and the impact of compartmentalization on infection pathogenesis, especially in congenital or transplant patients, as well as for the development of more effective therapeutics or vaccine design.

It is worth mentioning that HCMV genetic variability poses an emerging issue of drug resistance in clinical practice and represents another major obstacle on the way to predicting clinical outcomes of HCMV congenital infections. Currently, antiviral therapy mostly relies on nucleoside analogs, such as ganciclovir (GCV) and valganciclovir (VAL-GCV) (239). In this regard, evidence from adult transplanted patients has shown that DNA polymerase (UL54) and viral phosphotransferase (UL97), two highly polymorphic HCMV genes, seem to play a role in drug resistance against GCV (240). The frequencies of resistance development have been studied by different groups and show a range of 5%–10% (89).

1.9.2 Targeting genetic variability: the right approach

In the era before sensitive high-throughput sequencing approaches become available, only selected genes of HCMV had been sequenced. Selection of these genes was based on data supporting their potential role in viral pathogenicity and dissemination. Therefore, studies of HCMV genomes in natural infections were mostly limited to Sanger sequencing of polymerase chain reaction (PCR) amplicons, often focusing on a small number of polymorphic (hypervariable) genes (225). The first complete HCMV genome sequence of AD169 strain was derived from a plasmid library, while over a decade later more HCMV genomes were sequenced from bacterial artificial chromosomes (32, 119, 168), virion DNA (30) and overlapping PCR amplicons (184, 241). Despite the fact that the strains derived from different sources, all approaches have initially used Sanger sequencing, but were later followed by many studies increasingly recruiting high-throughput methods (234, 238, 241–244).

In the last few years, the high resolution provided by next-generation sequencing (NGS) has made it possible to study diversity across the entire HCMV genome (30, 243, 245). Several groups have applied NGS approaches to sequencing whole HCMV genomes, albeit mostly using cell-culture passaged isolates or amplicon sequencing (38, 226, 243).

Thanks to the recent introduction of sensitive NGS techniques, the new information on genetic variation among HCMV strains may lead to better categorization of strains. A perspective strategy involves the use of an oligonucleotide bait library representing known HCMV variation to select target sequences from random DNA fragments in clinical samples. This target enrichment technology originated in commercial kits for cellular exome sequencing, and was subsequently applied to various pathogens (246), including HCMV (38, 39, 247).

In comparison to high-throughput sequencing, Sanger sequencing appears as a technically limiting method, whose sensitivity is strongly dependent on the relative frequency of viral variants. In particular, low-abundance viral populations are likely to be missed and the overall viral diversity to be underestimated. Given that Sanger sequencing leaves out most of the HCMV genome, the advantage of use of high-throughput sequencing allows detection and characterization of multiple-strain infections, which eventually may affect the clinical outcomes (236). Extensive high-throughput sequence data are likely to illuminate further the epidemiology, pathogenesis, and evolution of HCMV in clinical and natural settings, thus facilitating the identification of virulence determinants and the development of new interventions.

PART II

1. HCMV and its interplay with innate immunity

1.1 General overview

The innate immune response is a fundamental defense mechanism, shielding the host from constant attacks by invading pathogens of different origin, whether they are bacterial, fungal, transposon or viral (248, 249). Thus, for a virus, successful invasion and efficient subversion of the host immediate immune response are critical steps to achieve productive infection.

In this environment, HCMV succeeds in establishing lifelong persistence in humans by evading immune surveillance, as it has the remarkable ability to manipulate and evade immune detection, literally transforming the host cellular environment into an ideal niche in which to thrive (44). This is achieved through sophisticated manipulation of cellular gene expression or elegant evasion strategies evolved by the virus during its long lasting co-evolution with the host (250, 251). HCMV has an extremely large genome, and its enhanced encoding capacity allows for the generation of multiple viral proteins involved in the modulation and subversion of multiple signaling pathways (33, 252). The exact mechanisms of action and role of this large number of viral proteins has not been completely elucidated, although many of them are likely involved in immune evasion.

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 the earliest reaction measure, innate immunity avails 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 (253, 254). Recently, a prominent role for intrinsic immune mechanisms has been discovered, as a part of the antiviral frontline barrier mediated by a number of cellular proteins, namely restriction factors (RFs). Unlike other innate immunity players, RFs are constitutively expressed and active, including prior to pathogen's entry into a cell. Remarkably, a close interplay occurs between innate and intrinsic immunity, as demonstrated by interferon upregulation of several RFs, increasing their antiviral activity (255, 256).

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 (257, 258). NK cells are widely acknowledged as efficient eliminators of HCMV-infected cells (259, 260). They detect HCMV-infected cells using a plethora of stimulatory and inhibitory receptors on their cell surface that are responsible for NKs activation, proliferation, and their effector functions. Therefore, it is not surprising that HCMV has devised multiple strategies to evade recognition by these cells (259–261).

HCMV components rapidly activate myeloid cells, such as monocytes, macrophages and myeloid dendritic cells (DCs), emphasizing the significance of early virus–host interactions and serving as a trigger in the activation of immediate immune responses (260). Besides, APCs from the myeloid and epithelial compartments, such as monocytes, macrophages and DCs, are well-known targets of HCMV, serving as vehicles upon infection to facilitate viral dissemination (262). 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.

Nevertheless, in spite of multiple evasion strategies of HCMV, the host immune system is still capable of counteracting the infection by building up a robust immune response in wide frontiers, for instance, by involving various immune cells, DNA sensors, and host RFs. This idea is supported by the broad evidence that primary HCMV infection in immunocompetent individuals are generally asymptomatic, while immunocompromised individuals experience the full and severe blast of HCMV disease. Thus, the interplay between HCMV and host innate immune resembles a complex ‘arms race’, reflected in the multiple evasion strategies HCMV has evolved to successfully escape the innate immunity of the host (28). Considering the importance of predicting HCMV infection outcomes, it is key to understand the process and mechanisms of HCMV immunomodulation in order to expand our knowledge of viral pathogenesis, which may eventually contribute to the development of effective HCMV vaccines and/or therapeutic interventions.

1.2. HCMV and the Interferon system

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 (263).

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 (264).

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 (265–268). On the other hand, HCMV has evolved a wide range of proteins with which to manipulate and counteract the host IFN response (26, 264, 269, 270).

In this context, the HCMV tegument protein pp65 - also identified as pUL83 and encoded by UL83 - best exemplifies the multifaceted interplay between viral and host proteins (271). 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 (272).

The recent finding that IκB kinases, the main regulators of NF-κB pathway, exerts antiviral activity (273) adds a level of complexity to this scenario. In this regard, pp65 is able to inhibit NF-κB but not IRF3 nuclear translocation (274). This is in disagreement with findings by Abate et al. (275) 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 (265). 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. 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 (276).

The HCMV tegument protein pp71 (i.e., pUL82, encoded by UL82) also contributes to evasion of the IFN response. According to Fu et al., pp71 interacts with the inactive rhomboid protein 2 (iRhom2) and STING to disrupt STING trafficking (277). Specifically, 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 (278). In this regard, the data suggest that US9 inhibits IRF3 nuclear accumulation by preventing STING dimerization. Moreover, the overexpression of US9 disrupts the mitochondrial membrane integrity and its membrane potential (277).

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 (279). Intriguingly, a recent study by Kim et al. (280) 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 (280).

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. (281) 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.

1.3 HCMV vs. restriction factors

It is well known that susceptibility to viral infection is partly determined by a wide group of RFs that ‘restrict’ viral replication by directly interacting with essential viral and/or cellular genes (249). These intrinsic antiviral factors, which are sometimes regarded as an integral part of

the innate immune response or some other times as an autonomous third branch of the immune system (249). 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 (282, 283).

Similar to what observed for the INF system, during the evolutionary 'arms race' for survival, HCMV has devised clever strategies to sidestep the antiviral activity of RFs, among which IFN-ɣ-inducible protein 16 (IFI16), nuclear domain 10 (ND10) and virus inhibitory protein ER-associated IFN-inducible (viperin) are among the best characterized (284). This list has been recently 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 (284).

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 rather enhances the susceptibility of hematopoietic cells to HCMV infection, thereby favoring viral hematogenous spread (285). 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, HPV-16 and human adenovirus type 5, pointing to an evolutionarily preserved mechanism shared by some DNA viruses to circumvent the antiviral function of IFITMs (286). 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 (287).

1.4 HCMV and NK cells

NK cells play a crucial role in eliminating HCMV-infected cells through cytotoxicity and secretion of several inhibitory cytokines and chemokines (e.g., IFN-γ and TNF-α) or recruitment and/or activation of other immune cells. 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 (254, 288, 289) (Figure 5).

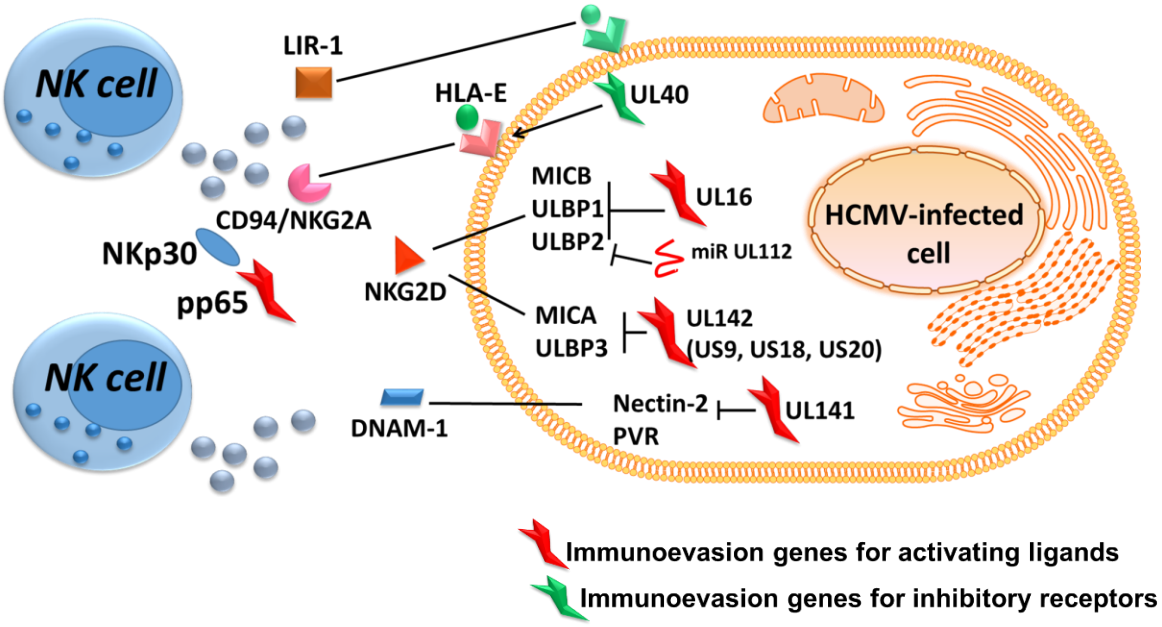


Figure 5. Schematic representation of NK cells interplay with cytomegaloviruses.

The former case is best exemplified by a condition known as human NK cell deficiency (NKD), which inevitably results in high susceptibility to herpesvirus infections (i.e., HCMV, HSV, EBV and varicella-zoster virus (VZV)) (290). In this regard, more than 60% of NKD patients are infected by one of these viruses (291), even in the context of intact cytolytic T lymphocytes (CTL) functions (292). The severity of this condition is demonstrated by the fact that nearly half of patients with NKD tend to die prematurely (291, 293).

The antiviral activity of NK cells against HCMV also appears to be mediated by the NK cell receptor, whose expression is 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 (294–297).

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. 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 (298). However, 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 - (299), 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 (300–304). 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 E (298). 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 (298).

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 (288). Similarly to HCMV, a pathogen-specific recognition mechanism for protection has been described, involving the NK cell-activating Ly49H receptor, which specifically recognizes the MCMV protein m157 (305).

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, γδ and NKT cells) (261, 306). 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 α-domains, 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 upregulated upon different types of stress, including those triggered by viral infection (306, 307). In the absence of a specific viral countermeasure, upregulation of NKG2D ligands (NKG2DLs) would likely result in the killing of infected cells, as has been observed in some experimental conditions (302, 308, 309).

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 were identified only 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 (254, 289). 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, NKG2DL has the highest affinity for its receptor (310), as well as the largest number of variant alleles, with more than 100 identified thus far. 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, there are MICB, a polymorphic gene with more than 40 allelic variants, and 6 ULBP genes boasting a total of 16 allelic variants (311). MICB expression is inhibited by miR-UL112, the only HCMV-encoded miRNA described to date targeting this ligand (33), 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 (312–316). ULBP3 is instead targeted by UL142, also blocking MICA expression (317, 318). 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 (319, 320).

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 (321, 322). Similar to NKG2DLs, DNAM-1 ligands (DNAM-1Ls) are often induced by cellular stresses and can trigger cytotoxicity and cytokine release (321, 323). 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 (304, 324, 325). Of note, UL141 is also able to downregulate the TRAIL receptors R1 and R2, thus preventing TRAIL-dependent NK-cell killing (326, 327). 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 downregulates 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 (328, 329). More recently, CD2 has been shown to play a role in costimulation of adaptive NK cells (314, 330). 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 (331).

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 antiviral NK cell response.

It is also important to point out that development, proliferation and effector functions of NK cells are tightly 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 receptors 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 (302, 308). A plethora of viral molecules evolved by HCMV to escape from NK cell activation, which otherwise would be detrimental for viral fitness.

Furthermore, HCMV can fully accomplish immunoevasion from NK cells thanks to its MHC-I surrogate, called UL18. This protein is markedly similar to cellular MHC-I molecules (332) 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 (312, 324, 333).

To sum up, 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 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.

1.4.1 Impact of HCMV genetic variability on NK cells

Numerous studies have addressed the role of NK cells in controlling HCMV infection, as well as viral immunomodulatory counter-strategies directed against them. However, correlating *in vitro* findings with *in vivo* significance remains tricky, in part due to the interpreting data from experiments using different HCMV strains, many of which do not encode a wild-type complement of viral genes (233, 254). Extensive passaging in vitro leads to HCMV mutants lacking genes unnecessary for the replication, which arise within weeks of propagation, as well as produces over

the years variation between commonly used laboratory strains, evidenced by numerous works (184, 233–235). This is explained by the fact that *in vivo*, HCMV constantly undergoes intense selective pressure from the innate immune system (254, 334), but it is not the case *in vitro*, where such pressure to retain immunomodulatory function is absent. As a result, mutants that are lacking that are not required for replication are rapidly lost during passaging (184, 185). The adaptive mutations often include rather large deletions in gene regions, such as the 13–15 kb U_L/b' region, UL133-UL150, that has been lost from the widely used AD169 and Towne strains (195, 234).

Besides the inconvenience of a loss of numerous genes relevant to the understanding of host-virus interactions, the "same" viruses in different studies may produce different phenotypes because of encoding a different repertoire of genes, such as the multiple genetic variants of AD169 and Towne (234). An example of an incorrect conclusion resulting from the use of a passaged strain is the initial description of HCMV-induced upregulation of CD58 (335) when wild-type viruses impair expression (331). Thus, to elucidate the relation between HCMV immunomodulatory mechanisms and genetic variation, the use of clinical isolates instead of immunologically impaired laboratory strains may be necessary to reflect the real clinical picture.

The aim of our study (Galitska et.al, 2019, unpublished results, manuscript in preparation) described below was to determine whether and to what extent the differences in genetic composition of HCMV clinical isolates affect their ability to modulate NK cell responses. We took advantage of next-generation sequencing (NGS), which enabled us to study HCMV genetic diversity across the entire HCMV genome and assess different aspects of genetic variability. Our work highlights the importance/the need to combine applied wide genetic analyses to immunological assays to shed light on the biological consequences of genetic variations.

For this purpose, we selected five HCMV clinical isolates obtained from pediatric patients with confirmed HCMV congenital infection that we previously characterized as those displaying a high phenotypic heterogeneity (87). In this study, we report that clinical isolates with a different genetic background display a different ability to modulate both NK cell ligands and effector functions. To determine whether the differences in genetic composition and viral fitness (87) influence the ability of HCMV clinical isolates to modulate the immune response, we performed a comparative analysis on the expression of NK cell activating ligands, key molecules in the recognition of infected cells by innate as well as adaptive cytotoxic lymphocytes (261). To this purpose, HFFs infected with selected clinical isolates were cocultured with an excess of HFFs for different time

points post-infection and then subjected to RT-qPCR or FACS analysis to test NK cell ligand mRNA or protein expression, respectively (Figure 6).

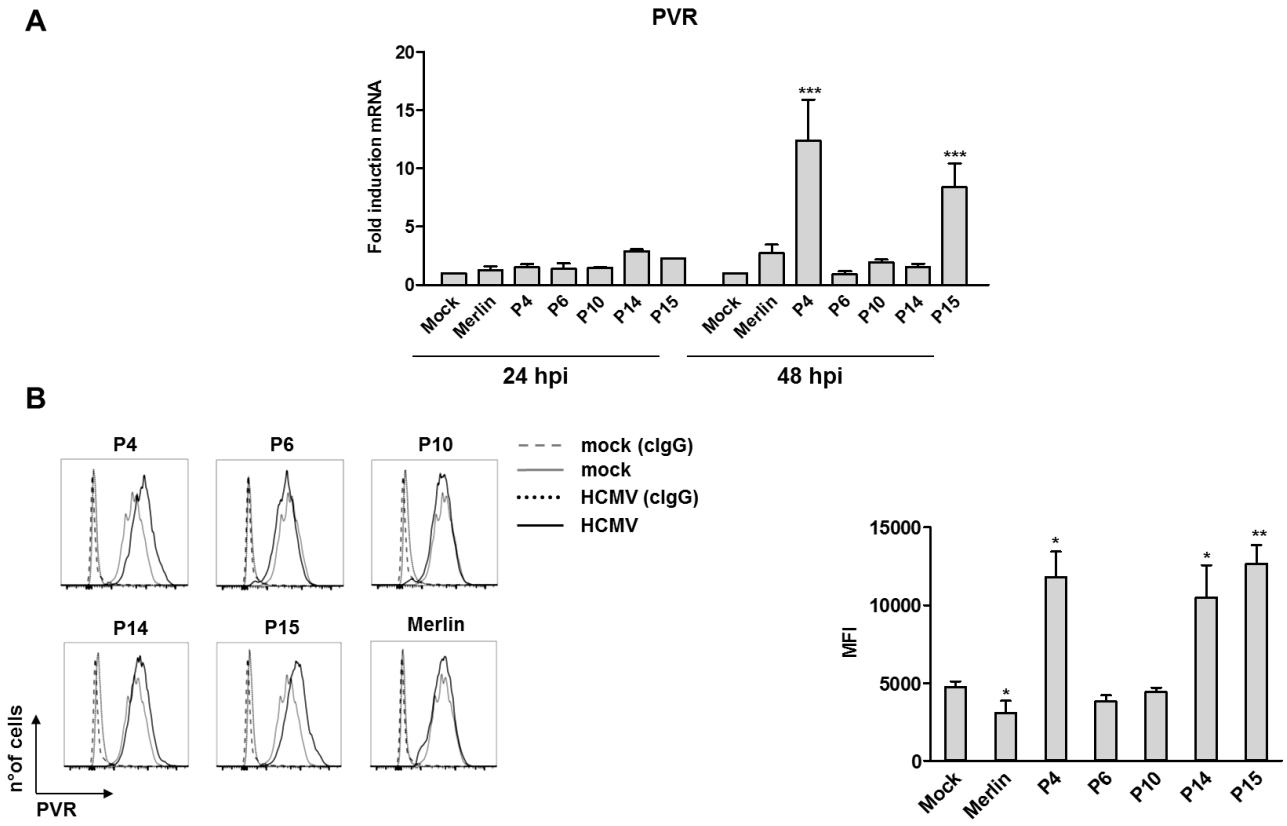
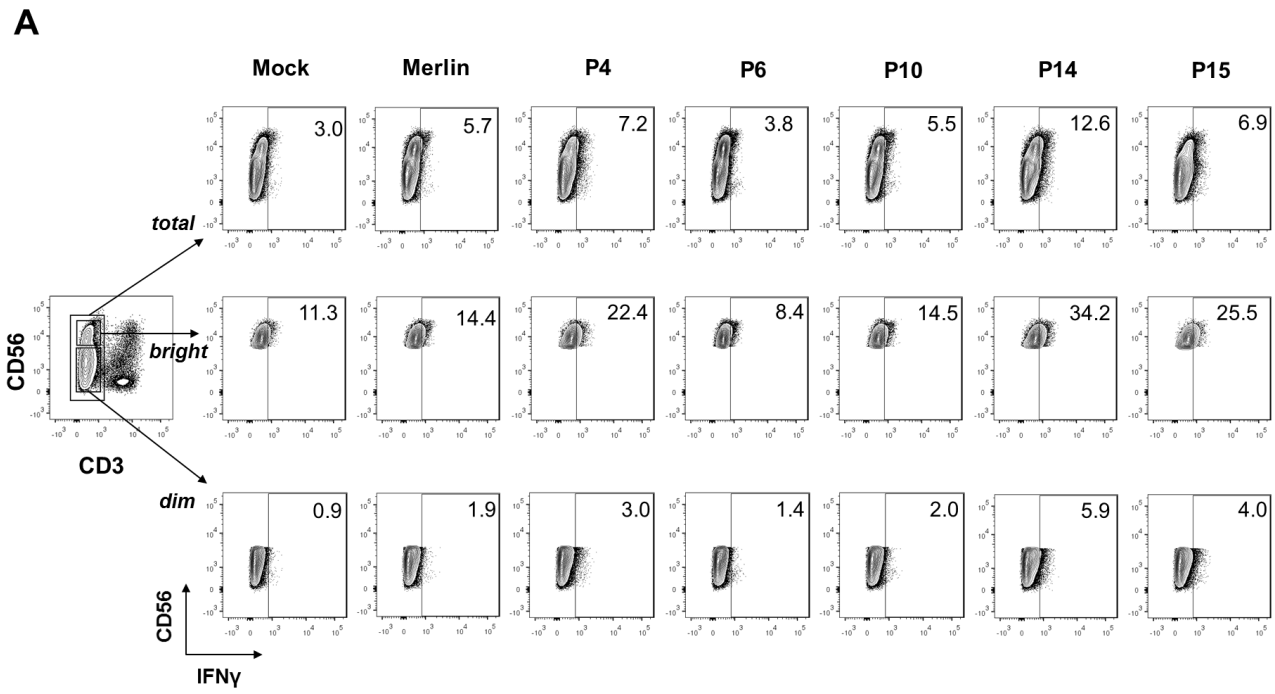


Figure 6. Modulation of the NK cell ligand PVR/CD155 by HCMV clinical isolates. (A) Primary human foreskin fibroblasts (HFFs) infected with the indicated clinical isolates (P), the Merlin strain, or left uninfected (mock) were cocultured with an excess of HFFs and subject to **(A)** RT-qPCR to measure mRNA expression of PVR/CD155. Values were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and plotted as a fold induction relative to mock-infected cells. A representative experiment of three performed at 24 and 48 hours post infection (hpi) is shown. Error bars show standard deviation (SD) (***, P< 0.001; two-way ANOVA followed by Bonferroni's post-tests, for comparison of infected versus mock cells). **(B)** FACS analysis to evaluate PVR/CD155 at 3 days post infection. Left panel: a representative experiment of at least four performed with all HCMV isolates is shown. Dashed and dotted lines indicate isotypic control in mock or HCMV-infected cells, respectively. Right panel: data derived from at least four experiments performed with all isolates. PVR expression levels are presented as mean fluorescence intensity (MFI) ± SE (*P < 0.05; **P < 0.01, paired Student t test for comparison of infected versus mock cells).

Furthermore, to investigate whether the observed modulation of NK cell activating ligands by genetically distinct HCMV clinical strains resulted in differences in NK cell functional activity, and analyzed IFN-ɣ expression by NK cells co-cultured with HFFs infected with different HCMV isolates (Figure 7). The production of IFN-γ by NK cells upon HCMV infection is highly relevant, as IFNs have been known to limit HCMV replication and make uninfected cells resistant to infection, via the so-called "antiviral state".

Compared to uninfected and Merlin-infected cells, a greater percentage of NK cells capable of producing IFN-γ was observed in response to the most aggressive strains P14 and P15, and to a lesser extent with P4 and P10 (Figure 7, panels A-B). By gating on CD3-CD56dim or CD3-CD56bright NK cells, it appeared that the highest percentage of IFN-γ+ NK cells was confined to the CD56bright population.



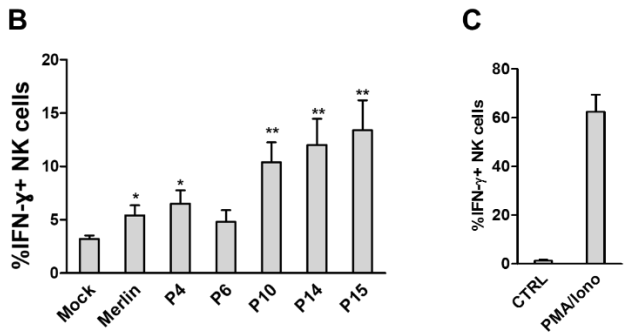


Figure 7. IFN-γ production by NK cells co-cultured with HCMV-infected HFFs. NK cells were plated on HFF mock-infected or cocultured with the indicated HCMV isolates (P), at 2 days post infection. The day after, NK cells were harvested and stained for intracellular IFN-γ. **(A)** A representative experiment of at least four performed with all HCMV isolates is shown. Numbers indicate the percentage of IFN-γ+ cells, in the gate of CD3-CD56+ (total), in CD3-CD56dim (dim), or CD3-CD56bright (bright) NK cells. **(B)** Cells were analyzed as in panel (A), and data are expressed as the mean percentage (%) ± SE of IFN-γ+ cells, in the gate of total CD3-CD56+ NK cells. Data derive from at least four independent experiments. **(C)** Negative (NK ctrl) and positive (NK PMA/iono) controls for IFN-γ production are also shown, and are referred to NK cells cultured alone, or in the presence of PMA plus ionomycin. (*, $P<0.05$; **; $P<0.01$ paired Student t test for comparison of infected versus mock cells).

Altogether, these results demonstrate that genetic variability in HCMV may affect immune responses at different levels and that the most aggressive isolates stand out for several aspects, including cell tropism, replicative capacity (87), and capability to trigger the immune response. We suggest this may be attributed to the *in vivo* pressure exerted by NK cells, leading to the deletion in genes critical for their recognition and activation, or to the presence of superinfection (co-infection) in particular patients (Galitska et.al, 2019 unpublished results, manuscript in preparation). Overall, our results support and expand the hypothesis that the viral genetic background can indeed influence the ability of HCMV to modulate the immune response.

2. HCMV vs. APOBEC

2.1. APOBEC family of proteins: general overview

As discussed above, innate immunity recognizes viral pathogens through the detection of their nucleic acids: packaged viral genome or viral replication intermediates within the infected cell (336). Toll-like receptors are good examples of the former viral sensing mechanisms, while the latter are represented by RIG-I-like or DAI and AIM2 receptors (337, 338). These types of recognition induce the transcription of proinflammatory cytokines and type I interferons (IFNs) that activate the expression of hundreds of IFN-stimulated genes (ISGs) which will engage in counteracting virus replication and spread (339). Among the ISGs, the genes encoding the family of apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) cytidine deaminases have been widely acknowledged as key players in restricting viral infections.

The AID/APOBEC proteins represent a family of zinc-dependent deaminases able to convert cytosine to uracil (C-to-U) in single-stranded DNA (ssDNA) or mRNA substrates. The reaction of C-to-U deamination occurs through a zinc-mediated hydrolytic mechanism, in which a conserved glutamic acid deprotonates water, and the resulting zinc-stabilized hydroxide ion attacks the 4-position of the cytosine nucleobase, with the net replacement of the amine group (NH2) with a carbonyl group (double-bonded oxygen) (340) (Figure 8).

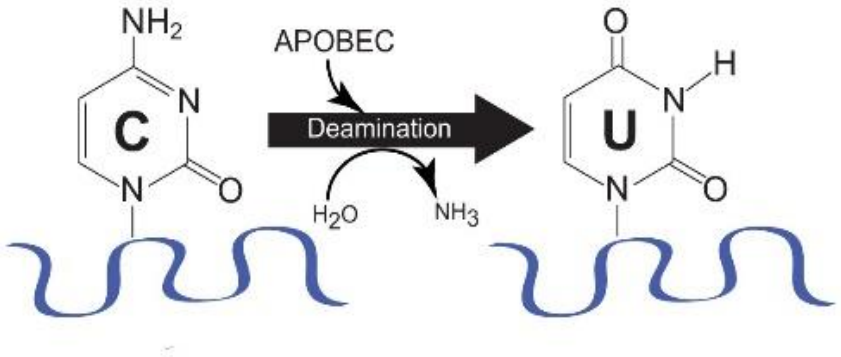


Figure 8. A schematic of the single-stranded DNA cytosine deamination reaction catalyzed by APOBEC family members.

Although AID/APOBEC belongs to a larger superfamily of deaminases, the members are restricted to vertebrates (341), with AID and APOBEC2 being ancestral members of the family

and APOBEC1 and APOBEC3 being more recent, while the origins of APOBEC4 are not clear (342–345). The APOBEC3 enzymes are exclusively found in mammals (345), and their gene copy number is species-specific (i.e., primates have at least seven APOBEC3 genes) (340, 341, 346) (Figure 9).

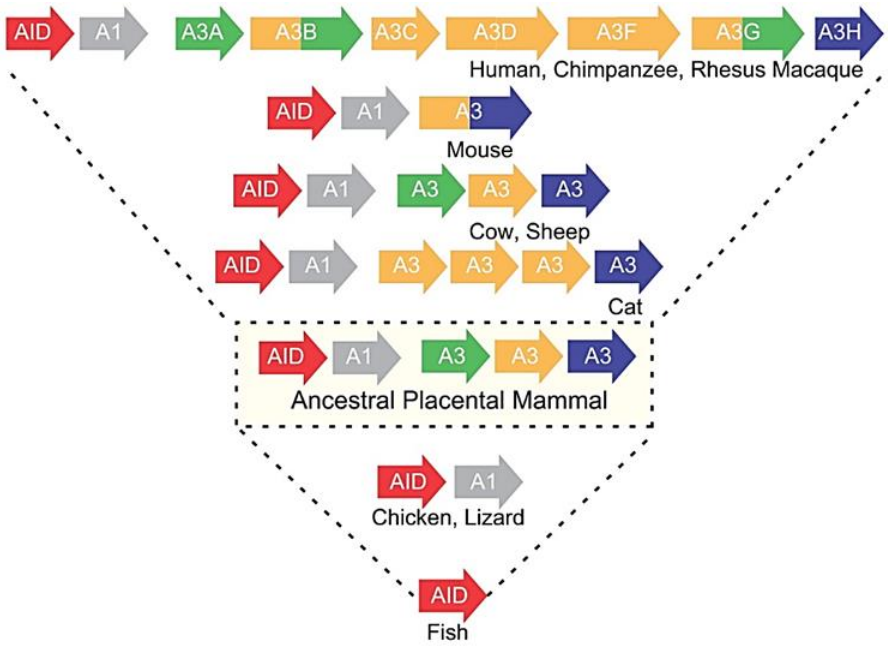


Figure 9. Schematic of the A3 gene composition of several current mammals depicted above the repertoire of a likely common ancestor and current non-mammalian vertebrates. The color scheme distinguishes phylogenetic subfamilies.(340)

In humans, the family comprises eleven members with distinct functions: activation-induced deaminase (AID) and APOBEC1, APOBEC2, seven APOBEC3 genes, and APOBEC4, all located on different chromosomes (except AID and APOBEC1, sharing the chromosome 12).

AID ancestral member, which deaminates ssDNA, is mainly expressed in germinal center B cells (347) and is an essential contributor to the processes of antibody diversification (348, 349) and DNA demethylation (350).

APOBEC1 (A1) member of the family demonstrates its enzymatic activity in both RNA (351) and DNA substrates (352). Mainly, A1 is expressed in the gastrointestinal compartment is involved in posttranscriptional editing of the apolipoprotein B (apoB) mRNA. The synthesized ApoB products then regulate the transport of endogenously produced cholesterol and triglycerides

and the absorption and transport of exogenous dietary lipids in human gut (353). Moreover, A1 has been shown to regulate the stability of specific mRNAs (354).

APOBEC2 and APOBEC4 are expressed in specific tissue compartments and do not possess enzymatic activity (355). APOBEC2 is expressed in the heart and skeletal muscles (356) and most likely contributes to muscle development (357). APOBEC4 is expressed in testicles, and its function remains unknown (358).

The APOBEC3 (A3) is a large group that comprises seven proteins in humans, namely APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3D (A3D), APOBEC3F (A3F), APOBEC3G (A3G), and APOBEC3H (A3H). Human A3 genes are highly polymorphic most likely due to the fact that they have been under strong and continuing selective pressure during primate evolution (341, 359). It is assumed that A3 polymorphisms might influence their specific restriction activity.

Importantly, A3 enzymes are essential players of innate immunity, restricting exogenous viruses and endogenous retroelements (360–362). The restriction may occur both via DNA editing and editing-independent mechanisms (360, 363, 364).

Interestingly, all A3 members are capable of editing single-stranded DNA and recognize specific target sequences (preferred nucleotide contexts, mutational signatures or "hotspots"). For instance, A3G and A3F, edit C's preferentially at CC<u>C</u> and T<u>C</u> dinucleotide contexts (GG<u>G</u> and A<u>G</u> in the complementary DNA strand), respectively (360, 365, 366). Besides the nucleotide preferences, the minus-two and plus-one bases in ssDNA substrates, as well as other factors, such as DNA integrity and secondary structures, may affect the editing process (367–370).

Furthermore, A3 proteins can mutate nuclear and mitochondrial DNA, suggesting roles in DNA catabolism (371). On the other hand, this effect may represent a possible source of mutations driving the onset of cancer progression (372). Indeed, a strong evidence for a specific APOBEC mutational signature has been found in multiple cancers, including bladder cancer, breast cancer, head/neck cancer, lung squamous cell carcinoma and lung adenocarcinoma (373, 374), which suggests that APOBEC family may serve as mutagenic source that fuels cancer heterogeneity and cancer progression (374).

A3 proteins present distinct subcellular localization (375). They localize in the cell cytoplasm and/or nucleus, enabling the protection of both compartments through restriction of nuclear or cytoplasmic replicating elements (375). A3D, A3F, and A3G are known to be cytoplasmic (362,

376), A3B localizes to the nucleus (377), while A3A, A3C, and A3H are found both in the nucleus and in the cytoplasm (362, 378). Noteworthy, different haplotypes of A3H present distinct localizations: the protein encoded by haplotype I is mainly nuclear, while another encoded by haplotype II is predominantly cytoplasmic (378). Regarding A3A, its endogenous version in primary CD14+ monocytes and the monocytic cell line THP-1 has been shown to localize to the cytoplasm, contrasting with its nucleocytoplasmic distribution observed upon A3A transfection, an observation likely explained by artificial overexpression of the enzyme (379).

In addition to distinct subcellular localization, some A3 proteins also appear in distinct forms or specific subcellular structures. APOBECs such as A3C, A3F, and A3H are capable of assembling into HMM complexes (380–382). The form depends on cell types and the switch between the forms can be stimulated by different cytokines (383, 384). The A3G and A3F proteins can accumulate in processing bodies (P-bodies) and stress granules, where they interact with RNAs and several proteins that regulate their metabolism (385, 386). However, the functional consequences of this accumulation remain unclear (387).

The evolutionary history of the APOBEC3 genes involves expansion, divergence, selection and extinction of specific A3 copies (388). It is hypothesized that at least one ancestral APOBEC3 gene was encoded by a mammalian ancestor and that this gene expanded in the different lineages as a response to viral, retroviral, and retrotransposon pressure (345). Interestingly, the rapid expansion of the APOBEC3 locus in primates is correlated with a marked reduction in retrotransposon activity, suggesting an important role in the host genome defense against retroelements (389, 390).

There is strong evidence that A3 proteins can restrict non-LTR and LTR retrotransposons, including both long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) (362, 381, 391–393).

In addition to the restriction of endogenous transposable elements, APOBEC3 enzymes restrict the replication of numerous RNA and DNA exogenous viruses (342).

2.2. APOBEC and virus restriction

2.2.1. Immunity against RNA viruses

The early works regarding APOBEC restriction of viral genomes have demonstrated the potent mutagenic activity of A3G upon HIV infection (340, 352, 360, 394, 395). Particularly, it has been shown that A3G protein becomes incorporated into HIV-1 particles and during reverse transcription of the viral RNA A3G deaminates cytosines in minus-strand DNA to cause G to A mutations, thus creating non-infectious virions (396, 397). In addition, excision of uracil incorporated by APOBEC into viral genomes by the cellular uracil-DNA glycosylase may result in DNA degradation (398). However, the broader studies of cellular UNG proteins and their antiviral activity are required. Interestingly, it has been also demonstrated that HIV encodes the protein virion-infectivity factor (Vif) that abrogates the restriction of HIV by A3G. Particularly, Vif prevents A3G incorporation into the progeny virus and directs its degradation by a proteosome-dependent pathway (398).

A3G-focused studies were then followed by additional studies demonstrating HIV-1 restriction in model cell-based systems using overexpression of A3F and multiple other family members (395, 399, 400). However, conflicting results were reported for all human A3 family members over the next decade, with some studies showing HIV-1 restriction and others not (except A3G) in different models. For instance, A3D/E, A3F, and several A3H haplotypes (II, V, and VII) may also protect against Vif-deficient HIV-1 in tissue culture models (401). Using humanized mouse models it was also shown that several APOBEC3 enzymes (A3G, A3D, A3F) can restrict HIV-1 *in vivo* (357). Several studies have reported that A3A, A3B and A3C are capable of inhibiting HIV infection (395, 402, 403), but their significance is debated (404, 405). In part, this is because some members, including A3B, can inhibit wild-type Vif-proficient HIV, but are not normally expressed in T cells that are the primary targets of HIV infection (403).

Subsequently, HIV-1 restriction was also observed with catalytically defective variants of A3G and A3F, hence a deaminase independent mechanism may also inhibit HIV growth through binding of APOBEC protein to viral RNA and blocking the reverse transcription of the viral genome (340). To sum up, in $CD4^+$ T cells both editing and non-editing mechanisms mostly by A3G, and to a lesser extent by A3F and A3D/E, contribute to the restriction of Vif-defective HIV-1 (406).

Besides HIV, APOBEC proteins, and A3G in particular, have been reported to restrict other retroviruses such as human T-cell leukemia virus type-1 (HTLV-1) (407–411) and human foamy virus (412). Like HIV, these viruses may also express proteins that counteract the A3G activity (408, 413, 414). Of note, the betaretroviruses lack a common mechanism to avoid APOBEC-mediated restriction. For instance, in the animal model, the Mason–Pfizer monkey virus (MPMV) has been reported to be resistant to the expression of rhesus monkey A3G by excluding this enzyme from virions (415).

2.2.2. Immunity against DNA viruses

Although the vast majority of information about APOBEC inhibition of viruses pertains to retroviruses and retroelements, APOBEC has been reported to be a restriction factor for multiple DNA-containing viruses (416). Hepatitis B virus (HBV), a notorious pararetrovirus, is a major cause of liver cirrhosis and cancer (417, 418). Similar to the foamy virus, HBV has a reverse transcriptase that copies packaged pregenomic RNA into DNA within the nascent capsid of the producer cells (419). Unlike retroviruses, the reverse transcriptase is covalently attached to the 5′ end of the minus-strand DNA and does not fully complete plus-strand synthesis within producer cells. The remaining single-stranded DNA region represents a natural target for APOBEC family enzymes (417, 420). Analysis of cell culture models of HBV infection has indicated roles for multiple APOBEC family proteins in virus restriction. AID has been shown to associate with an HBV ribonucleoprotein complex and to deaminate viral RNA in tissue culture experiments (340). Another group reported that both G-to-A and C-to-T substitutions were detected with A3B, A3F, and A3G in a hepatoma cell line (421), suggesting that both strands of HBV DNA may be susceptible to deamination. In another study, cytokine-mediated upregulation of A3A and A3B has led to degradation of HBV covalently closed circular nuclear DNA without apparent damage to the host genomic DNA (422). However, the analysis of patients with chronic HBV infection paints a somewhat different picture of APOBEC restriction, reflecting a significantly lower hypermutation level in HBV than that reported for several retroviruses. Deep sequencing studies by several groups have revealed a small number of G-to-A mutations in minus strands of HBV with a sequence context consistent with A3G activity (421). The other studies revealed sequence contexts more typical of A3G and A3C, rather than AID (417, 422).

Interestingly, transfusion-transmitted virus (TTV), a single-stranded negative-sense DNA virus, extracted from the blood of healthy patients and HBV carriers contains G-to-A hypermutations, indicating that viruses that lack reverse transcriptase can be subjected to APOBEC family restriction (423). Thus, TTV is predicted to be a good target for A3 enzymes (424).

In addition, ssDNA parvoviruses, such as adeno-associated virus (AAV), can be restricted by A3A, but not A3G (425). Curiously, two different parvoviruses are inhibited by A3A, suggesting a conserved mechanism, yet inhibition appears independent of catalytic activity (425).

Recently, APOBEC-mediated restriction has been demonstrated for several double-stranded DNA (dsDNA) viruses, including human papillomavirus (HPV), BK polyomavirus (426) and herpesviruses, such as herpes simplex-1 (HSV-1) and Epstein-Barr Virus (EBV) (375, 427). Several studies reported HPV deamination by different A3 members, including A3A, A3C, A3H, A3F, and A3G deaminate human papillomavirus (HPV) genomes (428–431). Curiously, the recent study provided evidence that PV genomes are significantly depleted in TC dinucleotides, the preferred target sites of several APOBEC3 proteins, which uncovers a viral evasion strategy and acknowledges its driving role in papillomavirus evolution (430).

Genomes herpesviruses such as HSV-1 and EBV are edited by AC3 on both strands. Interestingly, the editing is higher on the minus strand, possibly due to the fact that during discontinued replication the lagging strand exposes more viral ssDNA to nuclear APOBEC3s than the leading strand (375, 427). Edited EBV DNA was also found in infected peripheral blood mononuclear cell lines in association with high levels of A3C expression (427).

Similar to retroviruses, herpesviruses have also evolved counter-mechanisms to evade the antiviral activity of APOBEC. A new example of such counter-restriction is ribonucleotide reductase (RNR)-mediated inhibition of A3B by EBV. Particularly, the γ-and α-herpesvirus subfamilies encode both large and small RNR subunits, which serve the canonical function of synthesizing deoxyribonucleotides by reducing the 2'-hydroxyl from ribonucleotide substrates (432). While the requirement for endogenous viral RNRs differs tremendously across viral families, RNRs are almost ubiquitous among large double-stranded DNA (dsDNA) viruses, such as herpesviruses and poxviruses, presumably due to high dNTP requirements during DNA replication (433). Meanwhile, β-herpesviruses such as HCMV are an exception, however, because they lack a small sub-

unit and the large subunit has a defective catalytic site (434). In addition to ribonucleotide reductase activity, some viral RNRs have been shown to engage in non-catalytic activities that result in proviral phenotypes, such as inhibition of apoptosis and promotion of necroptosis (435–437).

The previous work on mechanisms of APOBEC counteraction (438) focused on the large subunit of the viral RNR, a viral protein known to produce DNA building blocks, namely BORF2, and reported that it causes A3B relocalization from the nucleus to cytoplasmic bodies and thereby protects viral DNA during lytic replication. The most recent work (432) has extended these observations with A3B to include a closely related γ-herpesvirus, KSHV, and to a more distantly related α-herpesvirus, HSV-1. The data demonstrate that different viral ribonucleotide reductases also caused the relocalization of A3A, which is 92% identical to A3B. These studies are important because they suggest a conserved mechanism of APOBEC3 evasion by large double-stranded DNA herpesviruses mediated by the viral RNR large subunit. Strategies to block this host-pathogen interaction may be effective for treating infections caused by these herpesviruses.

2.2.3 APOBEC and HCMV

In light of the last findings regarding the antiviral activity of APOBEC against multiple viral pathogens, the idea of APOBEC possibly counteracting HCMV appeared feasible.

Surprisingly, the role of the APOBEC intrinsic activity has never been before studied in the context of HCMV infection and vertical viral transmission. To fill out this gap, Weisblum et al. (439) have recently reported an important role of APOBEC3A in mediating innate immunity against congenital HCMV infection. In finer detail, it has been shown that A3A is strongly upregulated following *ex vivo* HCMV infection of maternal decidua, and overexpression of A3A in epithelial cells hampers HCMV replication by inserting hypermutations into the viral genome. A3A induction by HCMV has not been observed in HCMV-infected chorionic villi maintained in organ culture, primary human foreskin fibroblasts (HFFs), or epithelial cell cultures, suggesting that HCMV-mediated upregulation of A3A is tissue and cell type-specific. Intriguingly, IFN-β but not IFN-γ induced A3A expression in uninfected decidual tissues, suggesting its potential regulation as an IFN-stimulated gene (ISG) during HCMV infection. Furthermore, the detection of naturally occurring hypermutations in clinical amniotic fluid samples of congenitally infected fetuses further supported the idea of the occurrence of A3 editing of the viral genome in the setting of

congenital HCMV infection. The results revealed a previously unexplored role for A3A as an innate anti-HCMV effector, activated by HCMV infection in the maternal-fetal interface.

However, several issues required further investigation. For instance, the question as to whether HCMV is able to induce other A3 family members besides A3A in different cell types remained open. Resolving of another important issue regarding whether IFN rather than the virus itself mediate A3 induction may provide valuable insight into HCMV induction of IFN production and subsequent expression of IFN-stimulated genes as observed in other viral models.
Moreover, there is a gap in knowledge concerning the possible mechanism of HCMV evasion from A3-induced viral genome mutagenesis.

Toward this goal, our findings (440) reveal that A3G and, to a lesser extent, A3F gene products are induced in HCMV-infected human fibroblasts, and the A3G induction appears to be mediated by IFN-β. Interestingly, the data demonstrate that neither A3G knockout nor its overexpression appears to modulate HCMV replication, indicating that A3G does not act as a restriction factor against HCMV. This may be explained by the fact that throughout evolution, under intense selective pressure, HCMV has shaped its genome nucleotide composition to avoid A3G-mediated restriction. This elaborate escaping strategy has been performed by limiting the A3G target motifs (CCC:GGG), particularly in genes essential for viral replication; whereas, no such pattern has been identified for the other target motifs of A3 family members (440).

Given the fact that not all DNA viruses seem to be susceptible to A3-mediated antiviral activity (i.e. vaccinia virus is not inhibited by APOBEC enzymes, potentially due to the incorporation of its replication complex in cytoplasmic bodies), it may be beneficial to elucidate other potential counteracting strategies employed by the viruses, including HCMV, to limit APOBEC restriction activity (340). Hence, DNA viruses may escape APOBEC3 activity by encoding an undiscovered inhibitor, avoiding induction of A3 proteins, preventing entrapment into virions and/or replicating in privileged subcellular locations or, alternatively, in cells with reduced A3 levels (340). The assumption of whether or not this hypothesis is correct also in the context of HCMV infection remains to be further investigated. Considering the widely differential tissue distribution and biological functions of APOBEC family members, future findings should pave the way to examining the potential impact of these proteins on HCMV pathogenesis.

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PUBLICATIONS

1. Biological relevance of Cytomegalovirus genetic variability in congenitally and postnatally infected children. *Galitska G, Biolatti M, De Andrea M, Leone A, Coscia A, Bertolotti L, Ala U, Bertino E, Dell'Oste V, Landolfo S.*
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3. Catch me if you can: the arms race between human cytomegalovirus and the innate immune system.
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5. Tuning the orchestra: HCMV vs. innate immunity
Valentina Dell'Oste, Matteo Biolatti, Ganna Galitska, Gloria Griffante, Francesca Gugliesi, Selina Pasquero, Cristina Cerboni, and Marco De Andrea
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6. HCMV genetic variability affects NK ligands immunomodulation.
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Biological relevance of Cytomegalovirus genetic variability in congenitally and postnatally infected children

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ABSTRACT

Background: Human cytomegalovirus (HCMV) is the leading cause of congenital infections resulting in severe morbidity and mortality among infected children. Although the virus is highly polymorphic, particularly in genes contributing to immune evasion, the mechanisms underlying its genetic variability and pathogenicity are only partially understood.

Objectives: We aimed to characterize different HCMV clinical strains isolated from 21 congenitally- or postnatally-infected children for *in vitro* growth properties and genetic polymorphisms.

Study design: The growth of various HCMV isolates was analyzed in different cell culture models. Genetic polymorphism was assessed by genetic and phylogenetic analysis of viral genes involved in virulence (UL144, US28, and UL18), latency (UL133-138), or drug resistance (UL54 and UL97).

Results: Here, we report a high degree of genetic and phenotypic diversity in distinct HCMV clinical isolates, as shown by their *in vitro* growth properties. In particular, HCMV isolates displayed the highest degree of genetic variability in the UL144 gene, where we were able to define four distinct genotypes within the cohort based on UL144 heterogeneity. Lastly, among all isolates we were able to identify 36 mutations in UL54 and 2 in UL97.

Conclusions: Our findings indicate that surprisingly high levels of genetic HCMV variability correlate with a high degree of phenotypic polymorphism, which in turn might differentially influence the growth, fitness, and drug susceptibility of HCMV.

1. Background

Human cytomegalovirus (HCMV), a double stranded DNA herpesvirus, is the most frequent cause of congenital malformations worldwide, resulting in neurodevelopmental delay, foetal or neonatal death, and most frequently sensorineural hearing loss [1–3]. It is characterized by a large genome encoding a wide range of gene products, endowed of effective immunomodulatory activity [4–6]. For instance, different viral genes encoding tumor necrosis factor-alpha (TNF-α) receptor (UL144), α-chemokines (UL146-147), β-chemokine receptor (US28) are potential virulence factors associated with severe congenital HCMV infection [7,8].

Mounting evidence suggests that HCMV can be highly polymorphic, among and within hosts [9–13], with a high level of intrahost variability comparable to that of RNA viruses. It has been demonstrated that new mutations occur every time that the virus infects a new host, thereby giving rise to a unique viral strain for each infected individual. HCMV infection triggers indeed a selection event where a new genotype becomes dominant due to the selective pressure of the immune response [10]. Another possible explanation of this gap comes from the observation that both viral and host factors can contribute to the onset of HCMV genome mutations, thus fostering virus genetic drift during infection [14,15].

HCMV genetic variability, an emerging issue in drug resistance, represents another major obstacle on the way to predicting clinical outcomes of HCMV congenital infections. Currently, the only antiviral

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therapy available relies on nucleoside analogs, such as ganciclovir (GCV) and valganciclovir (VAL-GCV) [3,16]. In this regard, evidence from adult transplanted patients has shown that DNA polymerase (UL54) and viral phosphotransferase (UL97), two highly polymorphic HCMV genes, seem to play a role in drug resistance against GCV [17]. However, further research is clearly needed to fill the lack of information on congenitally HCMV infected children.

2. Objectives

Against this background, the aim of our study was to characterize the *in vitro* phenotype and the degree of genetic polymorphism of HCMV virions freshly isolated from congenitally or postnatally infected children, focusing on genes encoding potential virulence factors, such as UL144, US28, UL18, or contributing to viral latency, such as UL133-138. In addition to the aforementioned immunomodulatory genes, we also analyzed UL97 and UL54 to assess the emergence of drug resistant strains within the enrolled group of patients. Finally, we investigated a potential association between genotype and viral fitness.

3. Study design

3.1. Patients and samples

Twenty-one children diagnosed with congenital or postnatal HCMV infection were recruited at the Neonatal Unit of the University of Turin from 2015 to 2017. Infection diagnosis was based on RT-PCR HCMV DNA detection in patients' urine and blood samples. Urine samples were collected during the admission medical examination. The Neonatal Unit created a detailed database on clinical and pathological characteristics of recruited patients (indicated as P), summarized in Table 1. All patients were evaluated for neurobehavioral development, growth parameters, cerebral ultrasound, sight and hearing, antiviral and supportive therapy [18]. In addition, they were subjected to a follow up of one year of clinical and neurobehavioral tests for asymptomatic patients, and two years for symptomatic patients along with 6 years of audiology tests.

3.2. Cells and viruses

Primary human foreskin fibroblasts (HFF, American Type Culture Collection, ATCC SCRC-1041™), human retinal pigment epithelial cells (ARPE-19, ATCC CRL-2302™), and human umbilical vein endothelial cells (HUVEC) were cultured as previously described [19]. For HFF infection with HCMV clinical isolates, urine samples were primarily inoculated in HFF in order to boost the infected cell population. The isolates were then propagated until approximately 60% of cells demonstrated a cytopathic effect. All isolates were used before passage 3 in order to avoid cell culture adaptation [20,21].

3.3. Viral replication analysis

The replication of cell-associated isolates was quantified by focus expansion assay (FEA), as previously described [22]. Plaque area was calculated using ImageJ software. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows.

3.4. Immunofluorescence microscopy

Indirect immunofluorescence analysis was performed at 72 h pi as previously described [23]. The following primary antibodies were used: rabbit polyclonal anti-human von Willebrand factor (vWF) (Sigma-Aldrich), anti-IEA (immediate early antigen; produced in Santo Landolfo's laboratory, University of Turin [24]), mouse monoclonal anti-IEA, UL44 (Virusys Corporation), and pan cytokeratin (Sigma-Aldrich). Signals were detected using goat anti-rabbit or goat anti-mouse conjugated secondary antibodies (Life Technologies). Images were taken with a 40× objective by fluorescence microscope Olympus IX-70, equipped with cellSens Standard - Microscopy Imaging Software (Olympus), and ImageJ software was used for image processing.

3.5. DNA sequencing

Genomic DNA was extracted from infected cells by heating the cell with lysis buffer (1.25 M NaCl, 62 mM Tris-Cl pH 8.0, 9 mM EDTA pH 8.0, 0.5% SDS) for 15 min, followed by ammonium acetate/chloroform treatment, and the target genes were amplified using Q5 High-Fidelity DNA Polymerase (New England BioLabs). Primers designed on human herpesvirus 5 strain Merlin sequence (NC_006273.2) and PCR conditions are listed in Table 2. The amplified products were purified and used for Sanger sequencing (Eurofins Genomics).

3.6. Phylogenetic analysis

Nucleotide sequences were multiple-aligned to match homologue regions along Merlin reference genome (NC_006273) or along the most similar reference genomes. The alignment was performed using Clustal W, included into Geneious software 9.1, and each gene evolutionary model was selected using jModelTest 2.1.7 [25]. Gene sequences were concatenated, and the phylogenetic tree was reconstructed using a Bayesian approach (MrBayes 3.2.5) [26]. The tree was visualized with FigTree 1.4.2 software (Tree Figure Drawing Tool Version 1.4.2 2006–2014, University of Edinburgh). Robustness of the internal nodes was reported as a posterior probability calculated on the consensus of all the equally probable topologies obtained by the heuristic search. Recombination events were evaluated by using DualBrother plugin in Geneious software [27], SpliTree [28] and SimPlot [29], considering both single genes and concatenated alignments. Association between concatenated tree topology and clinical parameters was investigated using BaTS algorithms evaluating Association Index (AI), Parsimony score (PS) and monophyletic clade (MC) size statistics.

3.7. Identification of mutations associated with antiviral drug susceptibility in HCMV clinical strains

To ascertain whether the *in vitro* growth variability of HCMV clinical isolates correlated with different degrees of drug susceptibility, we used the web-based search tool mutation resistance analyzer (MRA) is a platform linking identified HCMV drug resistance mutations to specific phenotypes (http://www.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/hcmv) [30]. Detected mutations are then run through a regularly updated database containing previously published UL97 and UL54 mutations and the corresponding *in vitro* drug susceptibility phenotypes.

4. Results

4.1. Phenotypic characterization of HCMV clinical strains

First, we carried out phenotypic characterization of HCMV isolates from all patients (P), with the exception of P13 and P19, in HFF. Interestingly, we observed a remarkable variation of fibroblast growth properties among the various isolates, with a high value range of infected foci per well (Table 3).

Since cell-free virus transmission is typified by a comet tail phenotype, while cell-associated transmission is characterized by plaques with well-defined edges [22], we sought to determine the transmission pattern of different HCMV clinical isolates by defining plaque morphology. Furthermore, to quantify HCMV replication, we calculated the relative plaque area in HCMV infected HFF. We found that among all isolates, P14 and P15 were those displaying the most aggressive/fast-replicative behavior (Fig. 1A upper panel). These results were also

Table 1
Clinical-pathological characteristics of patients.

MOTHER								FETUS		
Previous history of childbirths		Infection discovery		Type of infection		Trimester of infection		Fetal anomalies	Viral load (amniotic fluid)	
First born	71%	*0 = unsuspected during pregnancy*	50%	*1 = first*	47.6%	*1st*	14.3%	19%	*Neg*	9.5%
		1 = serology	50%	*2 = relapse*	14.3%	*2nd*	33.3%			
Not first born	29%	*2 = ultrasound abnormalities (not IUGR[a])*	0	*3 = acquired after birth*	9.5%	*3rd*	19.1%		*Pos*	0%
		3 = IUGR[a]	0	*N/A[b]*	28.6%	*After birth*	9.5%		*N/A*	90.5%
						N/A	23.8%			

CHILDBIRTH		NEWBORN					
Gestational age (mean week): 37.6	Reanimation at birth: 14.3%	Sex		Birth weight (0 = ≥10°pc; 1 = 3-10 pc; 2 = ≤3°pc)		Head circumference (0 = ≥10°pc/ 1 = < 10°pc)	
Children age at time of sampling (mean month): 2.5		*F*	*M*	*0*	61.9%	*0*	71.5%
		47.6%	52.4%	*1*	9.5%	*1*	19%
				2	19%		
				N/A	9.5%	*N/A*	9.5%

INSTRUMENTAL EXAMS ANOMALIES[c]						LABORATORY ANALYSIS ANOMALIES[f]					
Hearing[c]		Cerebral ultrasound[c]		Cerebral MR[d] or CT[e,c]		Platelets[c]		Neutrophils[c]		Hepatic functionality[c]	
0	76.2%	*0*	52.3%	*0*	38%	*0*	80.9%	*0*	76.2%	*0*	71.4%
1	14.3%	*1*	42.9%	*1*	28.6%	*1*	4.8%	*1*	9.5%	*1*	9.5%
N/A	9.5%	*N/A*	4.8%	*N/A*	33.4%	*N/A*	14.3%	*N/A*	14.3%	*N/A*	19.1%

VIRAL LOAD				ANTIVIRAL THERAPY	
Viral load (urine)		Viral load (blood)		Valganciclovir (os) at time of sampling	Valganciclovir (os) after sampling
Neg	0%	*Neg*	4.8%	4.8%	14.3%
Pos	100%	*Pos*	66.7%		
N/A	0%	*N/A*	28.5%		
Mean (PFU/ml)	2,364,034.9	*Mean (PFU/ml)*	43,318.8		

[a] IUGR: intrauterine growth restriction.
[b] N/A: not available.
[c] 0: normal, 1: pathologic.
[d] MR: magnetic resonance.
[e] CT: computed tomography.
[f] abnormal laboratory indicators: platelet count < 100,000/mm^3, neutrophils count < 1,000/mm^3, ALAT > 80 IU/l, conjugated bilirubin plasma level > 2 mg/dl and > 10% of total bilirubin, per os: oral administration.

Table 2
Primers and reaction conditions for amplifying full length HCMV ORFs.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing T (°C)	Amplicon size (bp)	Nucleotide position
UL144	TCGTATTACAAACCGCGGAGAGGAT	ACTCAGACACGGTTCCGTAA	62	736	182073–182808
UL18	CACACGGCTAAGAGGATACATC	GGTAAAGTAGTGCAGGAACGC	62	1146	23873–25017
US28	ACCGAGGGCAGAACTGGTGC	TACGAAAAGACCGAGGTAGCG	62	1145	225411–226525
UL133-138 A	AGAGTATGTCAGTCAAGGGC	GAGTAGATCGAGCAGAGAAT	52	1390	187371–188760
UL133-138 B	CGACACGGAGTTTGAGATTC	GCCCTTGACT GACATACTCT	58	1070	188741–189810
UL133-138 C	TCGGCAGCCGCTGTAGAGAT	GAATCTCAAA CTCCGTGTCG	62	990	189791–190780
UL54A	ATTCAGATCTCGTGCGTGTGCT	TGTGCCATGATGATGGAAGG	58	1223	79737–80959
UL54B	TGGTGCGCGATCTGTTCAACAC	GCTTCCGAGACCTCGCGATCCT	58	1399	78891–80289
UL97	GGACATGAGCGACGAGAGCT	GTACGCGACACGAGGACATC	58	774	142886–143659

supported by virus plaque morphology analysis showing that P14 displayed larger and comet shaped plaques compared to P9 (Fig. 1A, lower panel). We observed the same infection pattern in HUVEC and ARPE-19 (Fig. 1B and C). Accordingly, in these two cell lines, plaque morphology and area analysis revealed a great heterogeneity (Fig. 1B, C) even though the extent of viral growth in HFF did not exactly mirror that seen in HUVEC and ARPE-19. Indeed, in HUVEC P12 and P18 showed a statistically significant larger plaque area compared to that of P9, albeit to a lower extent with respect to P14 and P15, indicating that HCMV replication not only depends on its genetic background, but also on cell environment.

4.2. Definition of the endothelio- and epithelio-tropic phenotype of different HCMV isolates

Next, we assessed HCMV isolates for viral growth. The FEA in HUVEC and ARPE-19 revealed that all isolates retained their endothelial and epithelial tropism (Fig. 1B, C). To rule out the possibility

Table 3
Growth properties of HCMV clinical isolates.

Patients' code	Mean No. of IEA positive foci/infected cell dilution		
	HFF	HUVEC	ARPE-19
P1	$8*10^2$	$5*10^3$	$4*10^2$
P2	$6*10^2$	$1*10^3$	$5*10^3$
P3	$2*10^2$	$1*10^3$	$7*10^3$
P4	$3*10^2$	$3*10^3$	$2*10^2$
P5	$5*10^2$	$2*10^3$	$3*10^2$
P6	$7*10^2$	$1*10^2$	$7*10^3$
P7	$2*10^2$	$1*10^2$	$3*10^2$
P8	$7*10^2$	$1*10^3$	$1*10^2$
P9	$2*10^2$	$1*10^5$	$8*10^4$
P10	$1*10^3$	$1*10^3$	$6*10^3$
P11	$4*10^3$	$3*10^4$	$3*10^2$
P12	$9*10^3$	$1.3*10^4$	$1*10^1$
P14	$3*10^1$	$1*10^2$	$1*10^2$
P15	$6*10^2$	$2*10^2$	$3*10^2$
P16	$5*10^2$	$1*10^1$	$1*10^2$
P17	$1.9*10^4$	$2.2*10^5$	$4.4*10^5$
P18	$4*10^4$	$2.7*10^5$	$7.9*10^5$
P20	$4*10^3$	$5*10^3$	$7*10^3$
P21	$9*10^2$	$5*10^2$	$1*10^2$

that inoculated infected HFF could overgrow to form infected foci within HUVEC and ARPE-19, we performed an immunofluorescence double staining for von Willebrand factor (vWF) and pan cytokeratin (Fig. 2A and B). Based on HCMV aggressiveness, epithelial cell infection resulted in two distinct morphological phenotypes (Fig. 2). Whereas fast-replicative isolates, such as P14, formed enlarged flower-shaped syncytial foci, slow-replicative isolates, such as P9, were only visible as single mononucleated infected cells (Fig. 2A). These dual phenotypes were not observed in HUVEC, suggesting a distinct replication pattern among different cell lines and viral isolates (Fig. 2B).

4.3. Genetic characterization of HCMV clinical strains

To determine whether phenotypic changes were accompanied by alterations at the DNA level, we performed comparative analysis of a set of genes encoding potential virulence factors (*i.e.* UL144, US28, UL18), or contributing to viral latency (*i.e.* UL133-138), or associated with drug resistance (*i.e.* UL54, UL97) (Table 2). For each region/gene, the sequences were aligned and, based on the best GTR + Γ evolutionary models, the Bayesian trees were drawn (Figs. S1–S6). Given that HCMV often shows recombination events [31], the alignment was used to create a split network (Fig. 3A), and a set of reference sequences was included. Statistically significant evidence of recombination was identified along the concatenated alignment (Φ $p < 0.05$) (Fig. 3A). Moreover, the SimPlot showed a great heterogeneity (Fig. 3B), reaching the highest variability within the UL144 gene (Fig. 3C), found exclusively in clinical HCMV strains.

Remarkably, UL144 amino acid sequence alignment from all HCMV isolates defined four prevalent subgenotypes, namely A, B, C, and A/B (Fig. 4B). The majority of sequences matched with genotype B, whereas those from the isolates P1, P12, and P20, closely related to the Merlin reference strain, were classified under genotype A. Furthermore, sequences derived from the P5, P18 and P21 isolates matched with genotype C, whereas only the P3-derived amino acid sequence was listed under genotype A/B. Interestingly, we noticed that both the P14 and P15 fast-replicative strains belonged to genotype B, indicating that the viral genetic background can indeed determine viral fitness.

Noteworthy, considering both nucleotide (Fig. 3C) and amino acid sequences (Fig. 4A), the most important finding deriving from the SimPlot is that the 5′ region is the key to discriminate the four genotypes. However, despite having a different genotype, we observed that almost all cysteines were conserved along the alignment, suggesting that distinct HCMV isolates may share a similar viral protein folding.

Finally, no statistically significant associations were found between the concatenated tree topology and any clinical parameter reported in Table 1, considering both global (AI and PS) and local (MCs) association parameters.

GenBank accession numbers of all sequences are reported in Table S1.

4.4. Antiviral drug susceptibility of HCMV clinical strains

Complete sequences of UL54 and UL97 from HCMV isolates were uploaded in MRA and compared to the wild-type sequence of the drug-sensitive HCMV strain TB40-BAC4 [32,33]. MRA identified 36 mutations in the UL54 gene associated with genetic polymorphism previously published [34–39], whereas only two mutations were detected

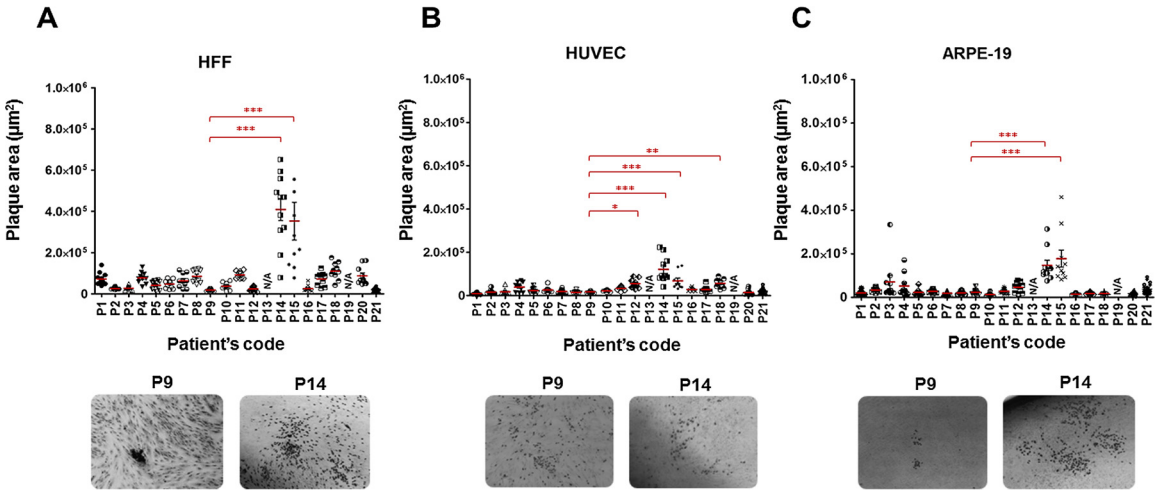

Fig. 1. Replication properties of HCMV clinical isolates. Viral replication was analyzed by focus expansion assay (FEA). Serial dilutions of HFF (A), HUVEC (B), and ARPE-19 (C) infected by clinical isolates were cocultured with an excess of uninfected HFF for 5 days. Monolayers were then fixed, and infected cells were traced by antibodies against HCMV immediate early antigen (IEA), followed by immunoperoxidase assay. Infectious foci were defined as clusters of at least three infected cells, starting from single late-stage infected cells. *Upper panel.* Plaque areas were calculated using ImageJ software. The red bars represent mean values. Differences were considered statistically significant for *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (one-way ANOVA followed by Bonferroni's post-tests, GraphPad Prism version 5.00 for Windows, for comparison of all isolates *versus* P9). *Lower panel.* Representative infectious foci of clinical isolates 9 (P9) and 14 (P14) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

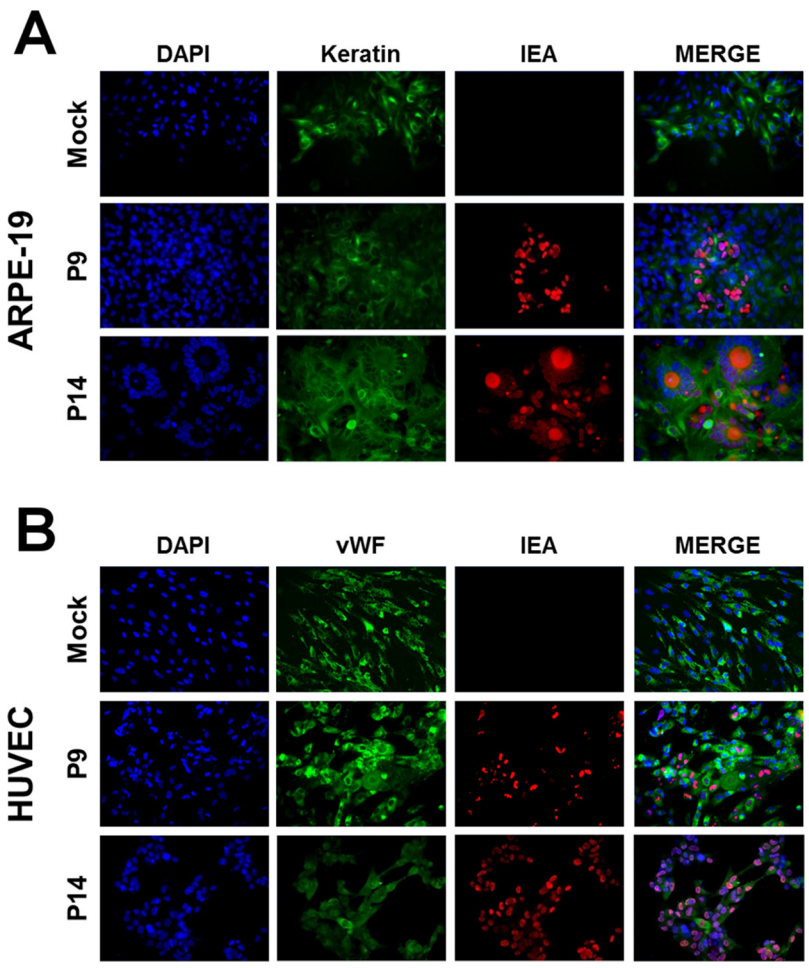

Fig. 2. Definition of the endothelio- and epithelio-tropic phenotype of different HCMV clinical isolates. ARPE-19 (A) and HUVEC (B) were cocultured with an excess of HFF infected with representative HCMV clinical isolates (P9 and P14) or mock infected. Cells were fixed 72 h later for immunofluorescence analysis to detect HCMV immediate early antigen IEA (red) and the inherent cell markers: endothelial vWF (green) or epithelial keratin (green). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Images were taken with a 40× objective by fluorescence microscopy. The most representative infectious foci are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

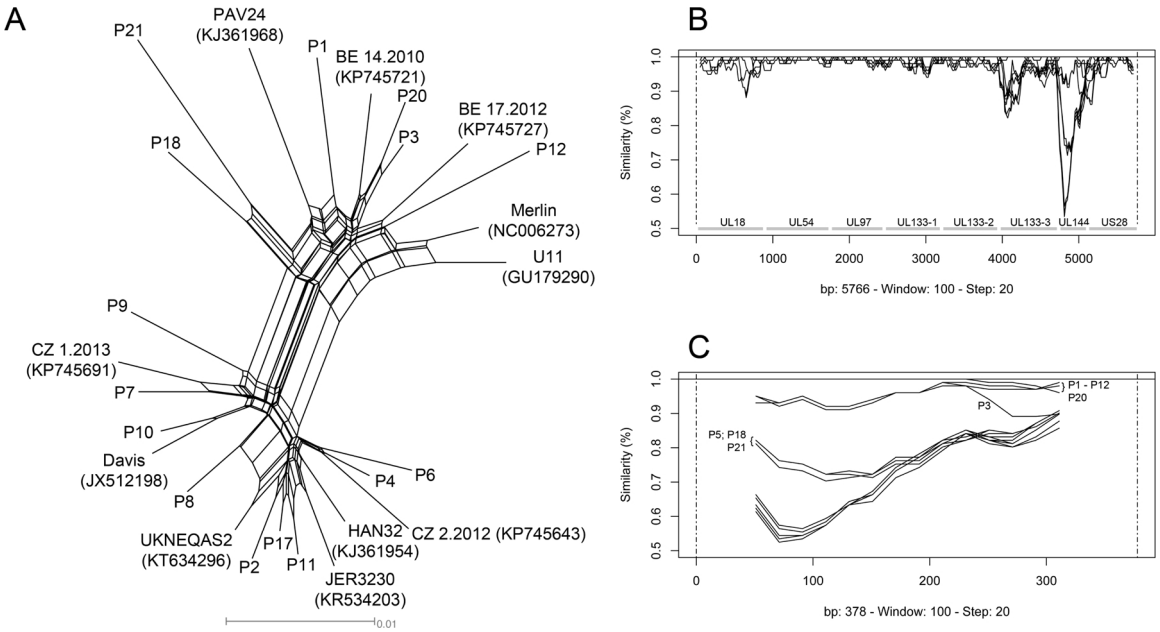

Fig. 3. Genetic analyses on HCMV clinical isolates. (A) Split network; (B) SimPlot of concatenated alignment; (C) SimPlot of UL144 alignment; mean nucleotide diversity among samples = 86.59% (range 73.0%–100.0%). For both the SimPlots, Merlin strain was used as reference sequence.

in UL97, probably due to a major gene stability (Fig. 5A and B) [35,40–43].

Interestingly, drug resistance-associated mutations varied among patients, especially in UL54 and to a lesser extent in UL97 (Fig. 5A and B).

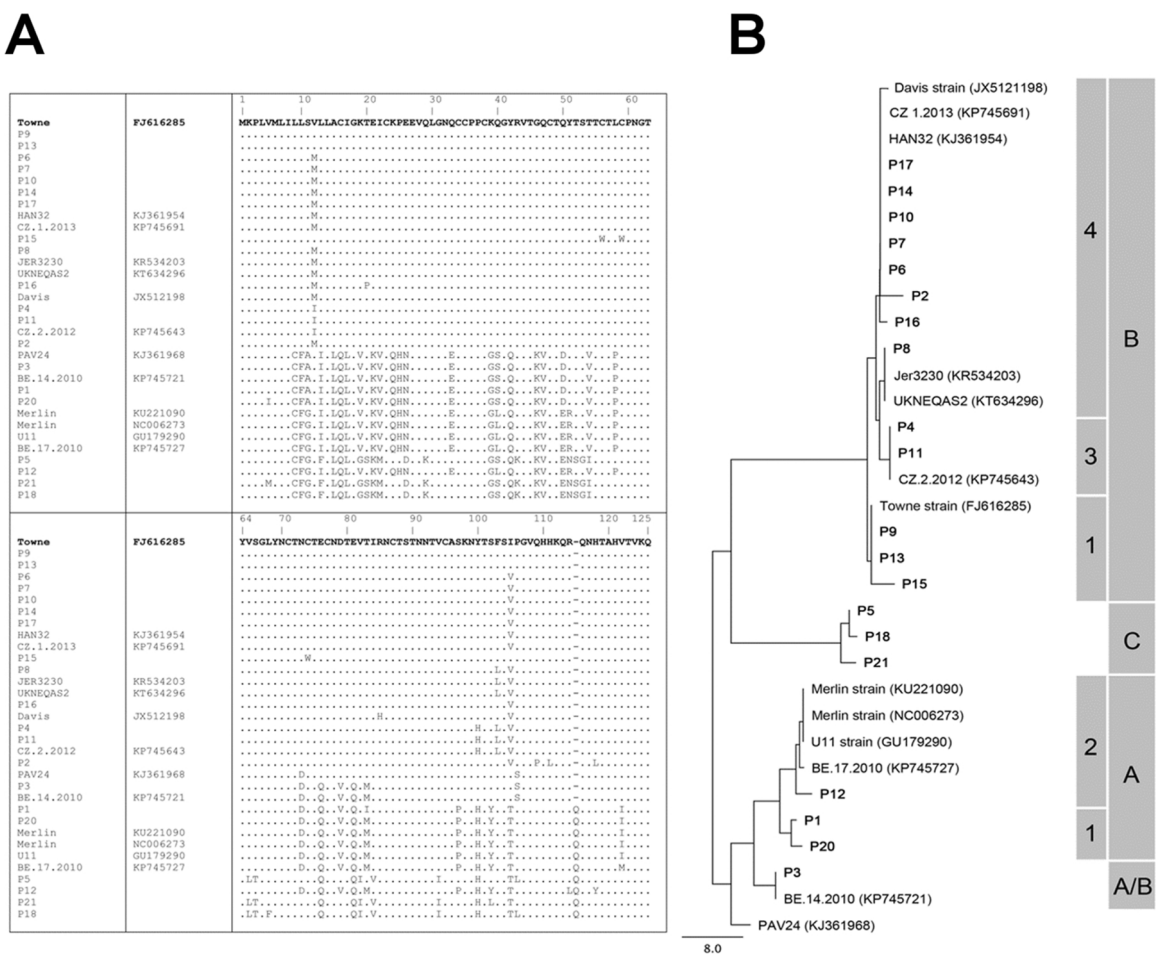

Fig. 4. UL144 gene analysis. Amino acid alignment (A) and neighbor joining tree (B), based on amino acidic sequence alignment, are reported. Dots indicate identical residue. Genotypes are indicated for each tree clade.

5. Discussion

The clinical and biological relevance of HCMV genetic variability in congenitally and postnatally infected children has been the focus of intense research over the past few years. In this study, we sought to determine whether and to what extent the differences in gene composition affected viral fitness. For this purpose, we enrolled 21 pediatric patients with confirmed congenital or postnatal HCMV infection. We evaluated the degree of genetic polymorphism of HCMV clinical strains by genetic and phylogenetic analyses, primarily focusing on viral genes involved in virulence, latency, and drug resistance. In parallel, we ran an extensive *in vitro* analysis of all clinical isolates to characterize viral growth properties and viral tropism in fibroblasts, endothelial and epithelial cells. Our results suggest that HCMV clinical isolates possess phenotypic differences as judged by both viral dissemination rate and replication properties, which define the extent of strain aggressiveness. Particularly, the two strains P14 and P15 were the most aggressive and fast-replicative ones because they could give rise to infectious foci characterized by comet-shaped plaques, typically observed in laboratory strains [22]. Altogether, these results support the hypothesis that HCMV heterogeneity may have an impact on viral fitness, influencing both viral dissemination rate and replication properties.

The reliability of such results could be inferred from the different cell lines employed for virus propagation and their low passage number ($\leq$ 3) to ensure that no cell-culture adaptation had occurred [20,21]. All clinical isolates in the recruited group of patients were able to infect both epithelial and endothelial cells displaying no difference in their cell tropism, while they displayed a unique morphological pattern in cells infected with fast-replicative isolates. Interestingly, the enlarged flower-shaped syncytial foci typical of epithelial, but not endothelial, cells obtained with the most aggressive strains were similar to those observed by Tandon and coworkers in HFF infected with UL96-deleted Towne bacterial artificial chromosome (ΔUL96BAC) [44], which could be partly ascribed to different maturation patterns between isolates.

The analysis of specific HCMV genome regions suggests that genetic variability among HCMV isolates may impact viral fitness. Indeed, here we report enhanced sequence diversity, identified thanks to the 5′ region of UL144 alignment used as a discriminatory criterion. Interestingly, the high degree of nucleotide heterogeneity mirrors in the amino acidic sequence, indicating a considerable difference among UL144 genotypes. This is interesting, as UL144 is a potent NF-κB activator [45] that plays a role in virus-mediated immune evasion [46,47]. This high heterogeneity of UL144 strongly influenced the split tree configuration based on the concatenated alignment, highlighting the role of this gene in the description of genetic relationships between CMV isolates. The same sample clustering has been demonstrated for both UL54 and UL97 genes, known to be involved in antiviral drug resistance. In both cases, the similarity among samples was high even though a number of previously reported mutations were identified in UL54 and to a lower extent in UL97. However, all treated patients so far responded to valganciclovir therapy. Nonetheless, we are not able to fully exclude the possibility that the reported mutations might have an impact on the antiviral therapy over a prolonged time.

It is highly likely that multiple strains of CMV are present in each patient of our group of patients, similar to cases being previously extensively reported in the literature [12]. However, we used an approach

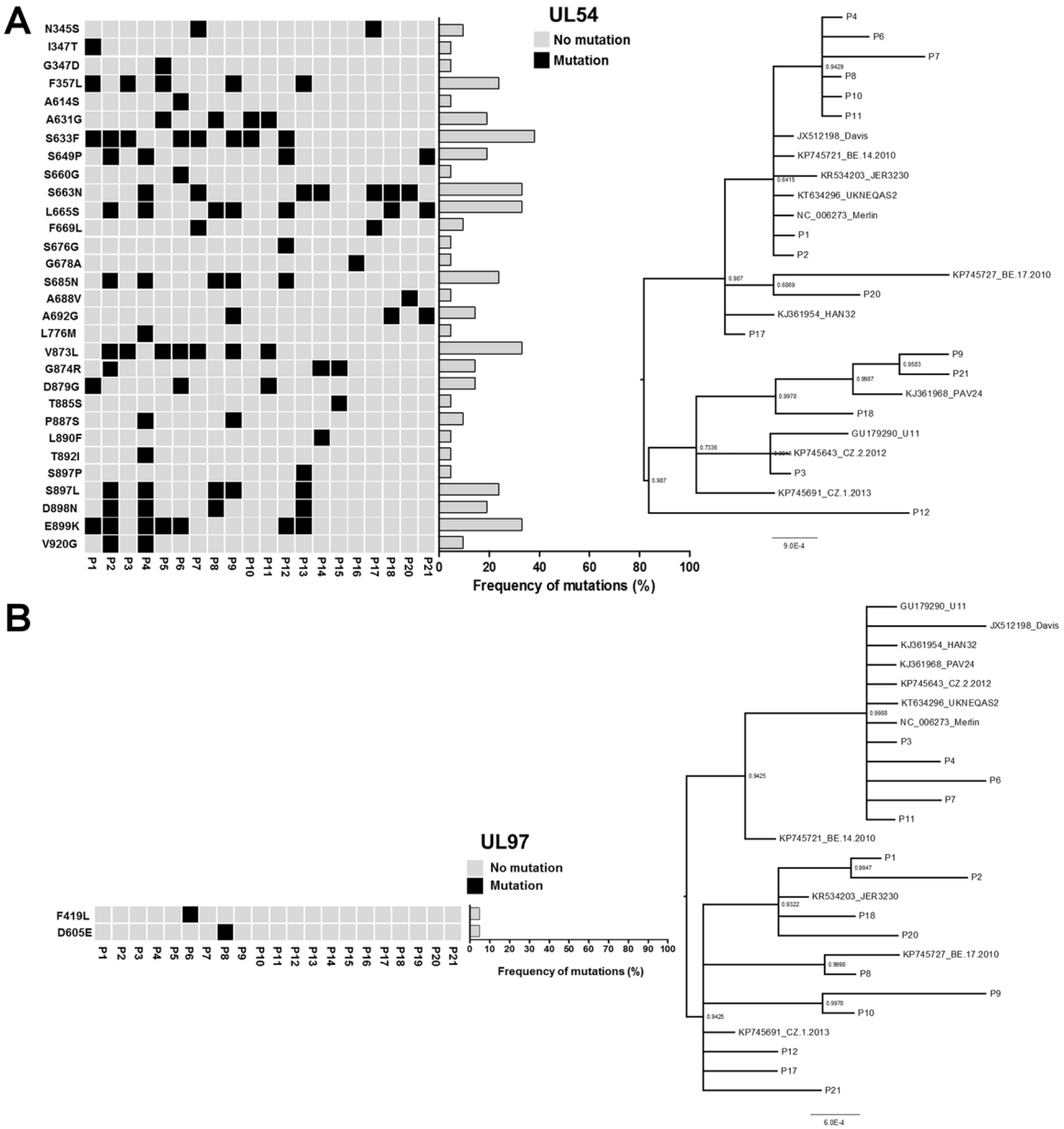

Fig. 5. Antiviral susceptibility testing. The heterogeneity of UL54 (A) and UL97 (B) genes is reported. *Left panel.* The heat maps show the presence and the positions of amino acidic changes, and the histograms show the frequency of each modification. *Right panel.* The Bayesian tree describes the genetic relationship among samples based on nucleotide alignment.

based on the Sanger sequencing of PCR products obtained from cell culture. The sensitivity of this method is strongly dependent on the relative frequency of viral variants. In particular, low-abundance viral populations are likely to be missed and the overall viral diversity to be underestimated. Thus, although sequence electropherograms did not show clear evidence of multiple signals (*i.e.*, double peaks and/or high background signal), we can not exclude the presence of mixed infections. Further investigations can be carried out to evaluate the role of mixed infections in pediatric infected patients, including PCR product cloning strategies or next generation sequencing approaches.

The limits of our study include the small number of the recruited patients, the short collection period (two years) in the limited geographic area. Besides, not all the analysis were available for all the samples, f.i. propagation and isolation of the virus failed for P13 (simultaneous presence of pathogenic bacteria and yeast in the patient's urine, since patient 13 was presented with severe sepsis at admission) and P19 (low viral load in urine sample).

In conclusion, our study may suggest that genotypic variability is associated with *in vitro* phenotypic diversity in HCMV clinical strains isolated from a group of congenitally and postnatally infected patients. In addition, our results indicate that genetic polymorphisms across the UL54 genome might play a role in multidrug resistance HCMV infection, pointing to UL54 as a potential therapeutic target to consider when treating congenital HCMV disease. This is, to our knowledge, the first detailed analysis that tries to associate in the same cohort of patients' genetic polymorphism and viral fitness. Although the results achieved so far do not allow any definitive conclusion, it emerges that a strong genetic HCMV variability is reflected in a remarkable phenotypic polymorphism that could affect virus growth properties and *in vivo* fitness.

Competing interest

None declared.

Authors' contribution

Study design: SL, VDO, MDA; laboratory analyses: GG, VDO, MB; patients' management and clinical data collection: EB, AL, AC; statistical/phylogenetic analyses: LB and UA; manuscript writing: GG, VDO, SL.

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Ethical approval

This study was approved by the Research Ethics Committee of the University Hospital of Turin "A.O.U. Città della Salute e della Scienza di Torino – A.O. Ordine Mauriziano – A.S.L. TO1" (No 007816). Informed consent was obtained from parents of all study participants prior to the collection of demographic and clinical data, along with biological samples. The work was carried out in accordance with the Declaration of Helsinki.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2018.09.019.

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VIRUS-CELL INTERACTIONS

Strategy of Human Cytomegalovirus To Escape Interferon Beta-Induced APOBEC3G Editing Activity

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ABSTRACT The apolipoprotein B editing enzyme catalytic subunit 3 (APOBEC3) is a family of DNA cytosine deaminases that mutate and inactivate viral genomes by single-strand DNA editing, thus providing an innate immune response against a wide range of DNA and RNA viruses. In particular, APOBEC3A (A3A), a member of the APOBEC3 family, is induced by human cytomegalovirus (HCMV) in decidual tissues where it efficiently restricts HCMV replication, thereby acting as an intrinsic innate immune effector at the maternal-fetal interface. However, the widespread incidence of congenital HCMV infection implies that HCMV has evolved to counteract APOBEC3-induced mutagenesis through mechanisms that still remain to be fully established. Here, we have assessed gene expression and deaminase activity of various APOBEC3 gene family members in HCMV-infected primary human foreskin fibroblasts (HFFs). Specifically, we show that APOBEC3G (A3G) gene products and, to a lesser degree, those of A3F but not of A3A, are upregulated in HCMV-infected HFFs. We also show that HCMV-mediated induction of A3G expression is mediated by interferon beta (IFN-β), which is produced early during HCMV infection. However, knockout or overexpression of A3G does not affect HCMV replication, indicating that A3G is not a restriction factor for HCMV. Finally, through a bioinformatics approach, we show that HCMV has evolved mutational robustness against IFN-β by limiting the presence of A3G hot spots in essential open reading frames (ORFs) of its genome. Overall, our findings uncover a novel immune evasion strategy by HCMV with profound implications for HCMV infections.

IMPORTANCE APOBEC3 family of proteins plays a pivotal role in intrinsic immunity defense mechanisms against multiple viral infections, including retroviruses, through the deamination activity. However, the currently available data on APOBEC3 editing mechanisms upon HCMV infection remain unclear. In the present study, we show that particularly the APOBEC3G (A3G) member of the deaminase family is strongly induced upon infection with HCMV in fibroblasts and that its upregulation is mediated by IFN-β. Furthermore, we were able to demonstrate that neither A3G knockout nor A3G overexpression appears to modulate HCMV replication, indicating that A3G does not inhibit HCMV replication. This may be explained by HCMV escape strategy from A3G activity through depletion of the preferred nucleotide motifs (hot spots) from its genome. The results may shed light on antiviral potential of APOBEC3 activity during HCMV infection, as well as the viral counteracting mechanisms under A3G-mediated selective pressure.

KEYWORDS APOBEC3, gene editing, human cytomegalovirus, immune evasion

Human cytomegalovirus (HCMV) is a ubiquitous opportunistic betaherpesvirus, which, despite infecting the vast majority of the world's population, can rarely cause symptomatic diseases in healthy, immunocompetent individuals (1). However,

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reactivation of latent HCMV infection in immunocompromised hosts (e.g., transplant recipients) may result in life-threatening diseases. Likewise, HCMV congenital infection can lead to abortion or dramatic disabilities in the infant, including deafness and mental retardation (2). A hallmark of HCMV pathogenesis is its ability to productively replicate in an exceptionally broad range of target cells such as epithelial, smooth muscle, and endothelial cells as well as fibroblasts (3, 4).

A central component of innate antiviral immunity against HCMV is the rapid activation of multiple interferon (IFN) signaling pathways that upregulate the expression of a rising number of restriction factors committed to counteract virus replication. Such intrinsic immune mechanisms therefore provide a frontline antiviral defense mediated by constitutively expressed proteins, already present and active before the virus enters a cell (5, 6). These intrinsic immune effectors, which were initially discovered as being active against retroviruses, include the apolipoprotein B editing catalytic subunit-like 3 (APOBEC3, or A3) family of cytidine deaminases and tetherin, an IFN-inducible protein whose expression blocks the release of human immunodeficiency virus type 1 (HIV-1) (7). However, it soon became apparent that such effectors were also active against other viruses, such as vesicular stomatitis virus, filoviruses, influenza virus, and hepatitis C virus (8). Moreover, other proteins such as PML, hDaxx, Sp100 (9, 10), viperin, and IFI16 were subsequently identified as restriction factors mediating the intrinsic immune response against HCMV infection (11, 12).

The seven members of the APOBEC3 (A3) family of cytidine deaminases (A, B, C, D, E, F, G, and H) (13–16) catalyze the deamination of cytidine nucleotides to uridine nucleotides in single-strand DNA (ssDNA) substrates. These enzymes are widely acknowledged as fundamental players in the defense against various viral infections (14, 15, 17). Since the identification of APOBEC3G (A3G) as a prototype antiretroviral host restriction factor, A3 subsets have been shown to restrict the replication of retroviruses (18), endogenous retroelements (19), and, more recently, DNA viruses such as hepatitis B virus (HBV) (20, 21) and parvoviruses (22, 23). Moreover, different A3 isoforms deaminate human papillomavirus (HPV) genomes (24) as well as BK polyomavirus (BKPyV) (25). Genomes of some herpesviruses, such as herpes simplex virus 1 (HSV-1) and Epstein-Barr virus (EBV), are edited by APOBEC3 on both strands. Interestingly, the editing is higher on the minus strand, possibly due to the fact that during discontinued replication the lagging strand exposes more viral ssDNA to nuclear APOBEC3s than the leading strand (14–16, 26). Human APOBEC3 proteins are also able to mutate the genome of the murine gammaherpesvirus 68 (MHV68) and, therefore, counteract viral replication. In particular, human A3A, A3B, and A3C proved their ability to restrict MHV68 replication (27).

With regard to HCMV, Weisblum et al. (28) have recently reported an important role of APOBEC3A (A3A) in mediating innate immunity against congenital HCMV infection. In particular, A3A was strongly upregulated following *ex vivo* HCMV infection of maternal decidua, and overexpression of A3A in epithelial cells hampered HCMV replication by inserting hypermutations into the viral genome through cytidine deamination. A3A induction by HCMV was not observed in HCMV-infected chorionic villi maintained in organ culture, primary human foreskin fibroblasts (HFFs), or epithelial cell cultures, suggesting that HCMV-mediated upregulation of A3A is tissue and cell type specific. Intriguingly, IFN-β but not IFN-γ induced A3A expression in uninfected decidual tissues, suggesting its potential regulation as an IFN-stimulated gene (ISG) during HCMV infection.

However, there still remain a number of issues that need further investigation. For example, in contrast to the aforementioned studies, several reports have demonstrated that members of the A3 family are robustly induced in different cell types *in vitro* and in different tissues *in vivo* by either IFNs or viruses (e.g., HIV and HBV). Thus, the question as to whether HCMV is able to induce other A3 family members besides A3A in different cell types remains open. Another important issue stems from the observation that HCMV triggers IFN production during the early steps of infection, but it is still unclear whether A3 induction is mediated by IFN rather than the virus itself. In this

respect, IFN production triggered by HCMV induces expression of IFN-stimulated genes, including the A3 family, which are committed to restrict virus replication as observed in other viral models. Thus, it is conceivable that HCMV has developed strategies to escape from APOBEC3 editing activity. Finally, a major issue concerns APOBEC3 antiviral activity. Although APOBEC3 editing activity has been reported for all the viruses analyzed, it is still a matter of debate whether this is also true for other viruses such as influenza viruses, herpesviruses, papillomaviruses, and polyomaviruses. Thus, there is a gap in knowledge concerning the mechanism of HCMV evasion from A3-induced viral genome mutagenesis.

In the present study, we present evidence of the following: (i) that A3G and, to a lesser extent, A3F gene products are induced in HCMV-infected HFFs; (ii) that the induction of A3G appears to be mediated by IFN-β as it is drastically decreased upon treatment with anti-IFN type 1 receptor antibodies; (iii) that neither A3G knockout nor its overexpression appears to modulate HCMV replication, indicating that A3G does not inhibit HCMV replication; and (iv) that A3G exerted a selective pressure that, during evolution has likely shaped the nucleotide composition of the HCMV genome.

RESULTS

HCMV infection stimulates various APOBEC3 expression patterns in different cell subsets. To assess the role of APOBEC3, we first asked whether HCMV infection could regulate mRNA and protein levels of A3 family members in different cell types. For this purpose, total RNAs from HCMV-infected HFFs, human umbilical vein endothelial cells (HUVECs), macrophage-derived THP-1 cells, or human retinal pigment epithelial (ARPE-19) cells were extracted at 8 and 24 h postinfection (hpi) and subjected to reverse transcription-quantitative PCR (RT-qPCR) analysis. Among all A3 family members analyzed, only A3G and A3F displayed mRNA upregulation in HCMV-infected HFFs compared to levels in mock-infected cells (i.e., ~25 and ~12-fold at 8 hpi; ~10 and ~6 at 24 hpi, respectively) (Fig. 1A). We also observed similar kinetics of mRNA expression for Mx-1, a well-known IFN-inducible gene (Fig. 1A). Human A3F and human A3G share more than 90% promoter sequence similarity and appear to be transcriptionally coregulated (29, 30). In agreement with these findings, we observed a coregulated induction of A3G and A3F expression by HCMV. Notably, A3F and A3G were also induced upon HCMV infection in differentiated THP-1 cells, although several other members of the APOBEC3 family, namely, A3A and A3H, were highly upregulated in this cell line as well (Fig. 1C). In contrast, mRNA expression levels of all A3 family members including A3G and A3F remained unchanged in HCMV-infected HUVEC and ARPE-19 cells, whereas Mx-1 mRNA was potently induced (Fig. 1B and D), suggesting that induction of A3G and A3F is cell type specific.

HCMV infection induces A3G in HFFs. Since A3G was the most potently induced A3 family member by HCMV, we decided to focus our attention on this gene in all further analyses. Consistent with the RT-qPCR results (Fig. 1A), A3G protein expression was significantly upregulated in HCMV-infected HFFs (Fig. 2A). Intriguingly, the kinetics of A3G protein induction, which peaked at 72 hpi, were delayed relative to those of A3G mRNA, which peaked at 8 hpi (Fig. 1A). At the moment, however, the mechanisms responsible for the delay in protein expression have not been explored. To get further insight into HCMV-induced A3G DNA deaminase activity, we used an *in vitro* fluorescence resonance energy transfer (FRET)-based oligonucleotide assay. To this purpose, whole-cell lysates of mock- or HCMV-infected HFFs were incubated with an ssDNA oligonucleotide containing a single CCC trinucleotide, which represents the canonical deamination target of A3G, along with uracil-DNA glycosylase (UDG) and RNase A (31). In the presence of A3G cytosine deaminase activity, the formation of a uracil base results in an abasic site following uracil base excision by UDG. Base hydrolysis of the abasic site then releases a 6-carboxyfluorescein (FAM) signal from the FRET pair. As expected, protein extracted from HCMV-infected cells displayed deaminase activity consistent with the kinetics of A3G protein induction, reaching a peak at 72 hpi, when the deamination activity was ~5-fold higher than that of mock-infected cells (Fig. 2B).

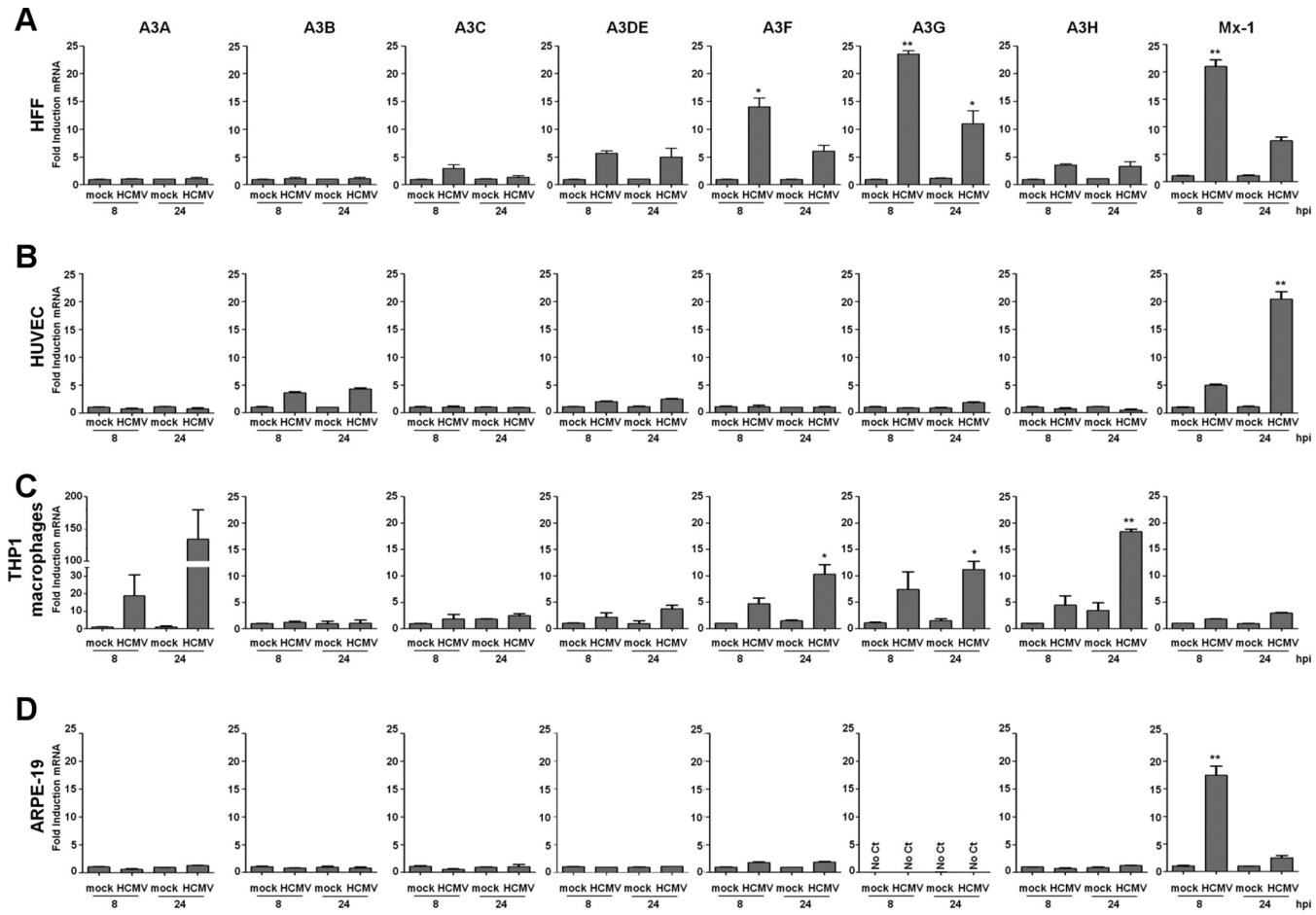

FIG 1 Apolipoprotein B editing enzyme catalytic subunit 3 (APOBEC3) gene expression patterns in human cytomegalovirus (HCMV)-infected cells. Primary human foreskin fibroblasts (HFFs) (A), human umbilical vein endothelial cells (HUVECs) (B), differentiated THP-1 cells (THP-1 macrophages) (C), or human retinal pigment epithelial cells (ARPE-19) (D) were infected with HCMV at an MOI of 1 and subjected to RT-qPCR to measure mRNA expression of various APOBEC3 family members (i.e., A3A, A3B, A3C, A3DE, A3F, A3G, and A3H) and Mx-1. Values were normalized to the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and plotted as fold induction relative to the level in mock-infected cells. Data are presented as mean values of biological triplicates. Error bars show standard deviations, (*, $P < 0.05$; **, $P < 0.01$; one-way ANOVA followed by Bonferroni's posttests, for comparison of infected versus mock-infected cells). Ct, threshold cycle.

Finally, to verify FRET assay specificity, we included an ssDNA oligonucleotide containing the target motif of A3B (TC) (29) as a negative control. As expected, in this case A3G activity was comparable to that in mock-infected cells, confirming that A3G is selectively activated upon HCMV infection (data not shown).

Collectively, these results show that infection of HFFs with HCMV upregulates A3G DNA deaminase activity, which is in good agreement with the increase of A3G mRNA and protein levels.

Although A3G is typically described as a cytosolic protein (32), several groups have shown that A3G is also present in the nucleus of different cell lines (33–35). To determine whether subcellular A3G localization varies during early and late infection with HCMV, we carried out a detailed kinetic analysis using confocal microscopy at time points ranging from 24 to 72 hpi. HFFs were mock infected or infected with HCMV at a multiplicity of infection (MOI) of 1, and intracellular localization of A3G was assessed by confocal microscopy. Consistent with the Western blot results, a substantial accumulation of A3G in the nucleus of HCMV-infected cells was observed at 72 hpi compared to the level in mock-infected cells, where localization of detected A3G seemed evenly distributed among the cytoplasm and nucleus (Fig. 2C). Altogether, these results demonstrate that A3G intranuclear localization is enhanced in HCMV-infected HFFs.

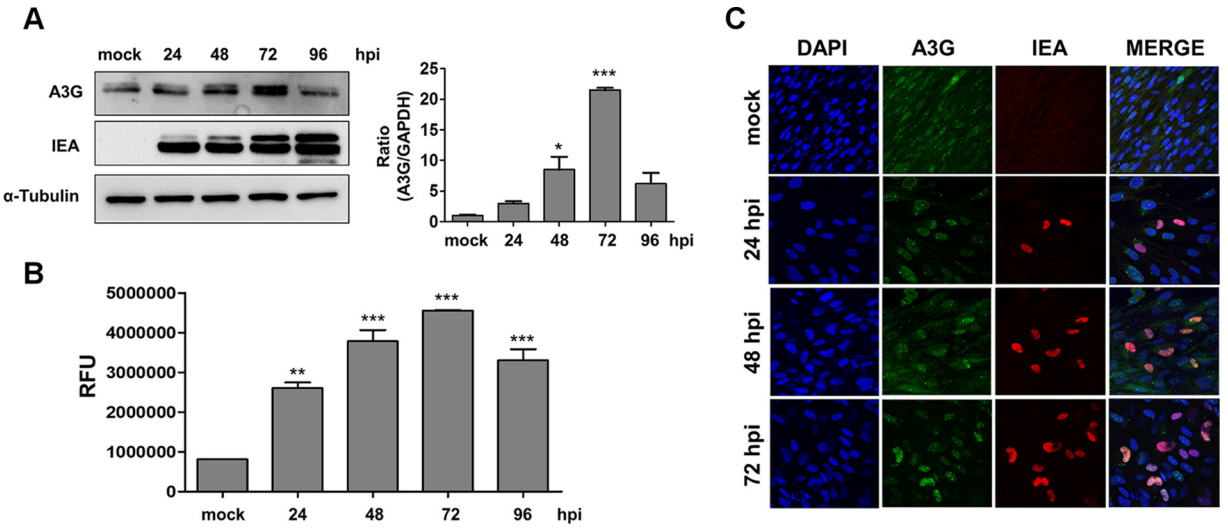

FIG 2 HCMV infection upregulates A3G in HFFs. (A) Lysates were prepared at the indicated time points and subjected to Western blot analysis for A3G, IEA, and α-tubulin (left panel). A3G protein was subjected to densitometry and normalized to α-tubulin (*, $P < 0.05$; ***, $P < 0.001$; one-way ANOVA followed by Bonferroni's posttests, for comparison of infected versus mock-infected cells) (right panel). (B) FRET assay to measure A3G deaminase activity. The average and standard deviation were calculated from three independent experiments (**, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA followed by Bonferroni's posttests, for comparison of infected versus mock-infected cells). RFU, relative fluorescence units. (C) HFFs were infected with HCMV at an MOI of 1 or left uninfected (mock) and subjected to immunofluorescence analysis at the indicated time points. A3G (green)/IEA (red) were visualized using primary antibodies followed by secondary antibody staining in the presence of 10% HCMV-negative human serum. Nuclei were counterstained with DAPI (blue). Images were acquired at ×63 magnification, and representative pictures are shown.

A3G upregulation is IFN-β dependent. The innate immune response against incoming pathogens plays a key role during primary infection, especially in patients with defects in adaptive immunity. Early during infection, HCMV triggers type I IFN production, leading to the induction of a number of IFN-stimulated genes (ISGs), a process that promotes an antiviral state in infected and neighboring cells (36–39). Stimulation of A3 upon IFN production has been observed in different viral models and cell types (40–44). In particular, A3G is strongly induced by IFN-β in response to influenza A virus infection (43). To assess whether HCMV induces A3G expression through IFN-β induction also in our model, HFFs were incubated for 24 h in the presence of serial dilutions of IFN-β (50 to 500 U/ml), and the mRNA levels of A3G were determined by RT-qPCR (Fig. 3A). As shown in Fig. 3A, IFN-β stimulation led to over 30-fold induction of A3G mRNA. Likewise, IFN-β treatment of HFFs led to an increase in A3G protein expression over time, which peaked at the 24-h time point (Fig. 3B).

To definitively prove a causative link between IFN-β production and A3G upregulation, HFFs, pretreated for 18 h with anti-IFNAR antibody (Ab) or an isotype control Ab, were infected with HCMV for 8 h and analyzed by RT-qPCR (Fig. 3C). As expected, suppression of IFN-β production by anti-IFNAR Ab strongly impaired A3G mRNA induction compared to the level with untreated or isotype control Ab-treated HFFs. Altogether, these results indicate that IFN-β released early during HCMV infection triggers A3G expression similarly to what has been reported for other viruses such as orthomyxoviruses and HPV (43, 44).

HCMV replication is not affected by A3G activity. Several reports have shown that A3G is able to counteract the replication of HIV-1 (45–51), human T-cell lymphotropic virus type 1 (HTLV-1) (52–56), and HBV (20, 21, 57, 58). In contrast, A3 deaminases do not appear to affect viral replication or production of infectious viral progeny of two other viruses such as influenza A (43) virus or polyomavirus (59). Thus, we sought to determine whether A3G acted as a restriction factor for HCMV replication. For this purpose, CRISPR/Cas9 systems were used to knock out the A3G gene in HFFs (A3G KO) or a scrambled control (scramble Ctrl). Western blot analysis confirmed that the majority of cells were knocked out for A3G (Fig. 4A). HFFs depleted of A3G were then

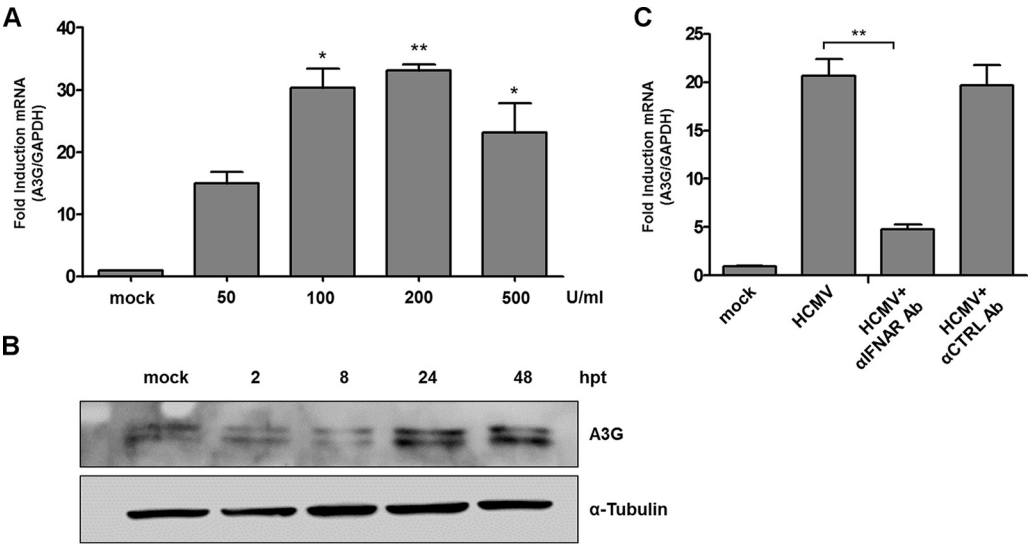

FIG 3 APOBEC3G upregulation is IFN-β dependent. (A) HFFs were stimulated for 24 h with the indicated doses of IFN-β, and the mRNA levels of A3G were determined by means of RT-qPCR. Values were normalized to GAPDH mRNA and plotted as fold induction relative to levels in untreated HFFs. (B) Western blot analysis to assess APOBEC3G protein levels and α-tubulin upon IFN-β treatment (1,000 U/ml) for the indicated time points (hpt, hours posttreatment). One representative experiment of three performed in duplicate is shown. (C) HFFs were mock- and HCMV-infected in the presence of an anti-IFNAR antibody (5 μg/ml) or isotype control. At 8 hpi, cells were processed by RT-qPCR to assess A3G expression. Data presented in panels A and C are mean values of biological triplicates. Error bars show standard deviations (*, $P < 0.05$; **, $P < 0.01$; one-way ANOVA followed by Bonferroni's posttests, for comparison of treated versus untreated cells).

infected with HCMV at an MOI of 0.1 for 24 h, 72 h, and 144 h, and the viral yield was measured by standard plaque assay. As shown in Fig. 4B, the replication of HCMV was not significantly affected following A3G knockout.

To further confirm these findings, we transduced HFFs with an adenovirus-derived vector constitutively expressing A3G protein (AdVA3G) or with a control vector (AdVLacZ) at an MOI of 30. As shown in Fig. 4C, AdVA3G efficiently increased the expression of A3G protein compared to expression with both HCMV and AdVLacZ. After 24 h, cells were infected with HCMV at an MOI of 0.1 for an additional 24 h, 72 h, and 144 h and then analyzed by standard plaque assay. The efficiency of A3G protein overexpression was monitored by Western blotting (Fig. 4C). Consistent with the knockout results, A3G overexpression did not exert any antiviral effects on HCMV replication (Fig. 4D), indicating either that A3G is not a restriction factor for HCMV replication or, alternatively, that HCMV has evolved to escape A3G restriction activity.

A3G-mediated selective pressure shaped the composition of the HCMV genome. Because HCMV infection upregulates A3G expression with no evidence of virus replication restriction, we sought to determine whether, during evolution, A3G-mediated selective pressure might have played a role in shaping the composition of HCMV genomes.

A3G preferentially deaminates the 3′ cytosine within CCC hot spots in single-stranded DNA (60, 61), whereas other members of the A3 family have distinct preferences (TTC for A3F and A3C; TC for A3B and A3H; TCG for A3A) (29, 62–67). We thus assessed the representation of these hot spot motifs in the HCMV genome using the HCMV Towne sequence as a detailed functional map of this strain was constructed by systematic deletion of single open reading frames (ORFs) (68). The representation of CCC-GGG, TTC-GAA, TCG-CGA, and TC-GA motifs was calculated in sliding windows and compared to the expected counts obtained by randomly shuffling the HCMV genome sequence (see Materials and Methods). Results indicated that the CCC-GGG hot spot is strongly underrepresented in several large genomic regions, whereas no such pattern is observed for the other motifs (Fig. 5). In particular, the regions where A3G hot spots are underrepresented broadly correspond to the genomic positions where essential

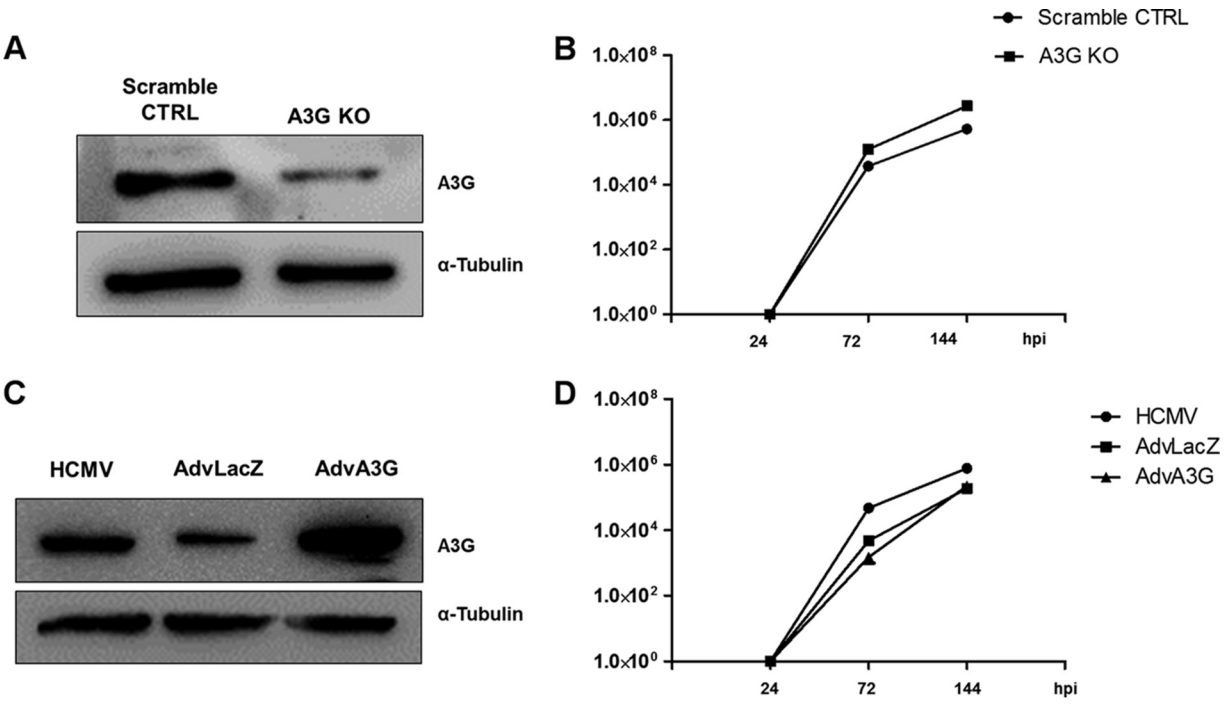

FIG 4 A3G is not a restriction factor for HCMV replication. (A) Knockout gene variants in HFFs for A3G (A3G KO) and the scramble control were generated with CRISPR/Cas9 technology. The efficiency of A3G depletion was measured by Western blotting for A3G and α-tubulin. (B) A3G KO HFFs were infected with HCMV at an MOI of 0.1. The extent of virus replication was measured at the indicated times postinfection by titrating the infectivity of supernatants and cell suspension on HFFs by standard plaque assay. Results are expressed as means $\pm$ SD. (C) HFFs were transduced with AdVA3G or AdVLacZ at an MOI of 30 PFU/cell. The efficiency of A3G overexpression was measured by Western blotting for A3G and α-tubulin. (D) HFFs were transduced with AdV vectors as described in panel C. Subsequently, cells were infected with HCMV at an MOI of 0.1. The extent of virus replication was measured at the indicated times postinfection as described in B. Results are expressed as means $\pm$ SD.

ORFs (i.e., ORFs that impair or strongly reduce HCMV growth *in vitro* when deleted) cluster (68).

To date, only one origin of replication (oriLyt) has been described for HCMV (69). In contrast, the mechanisms of DNA replication remain largely unknown, although a rolling-circle phase is likely to occur (70). When we analyzed the frequency of CCC motifs in the two strands of the viral genomes, we detected no substantial difference (Fig. 6A), suggesting that the A3G hot spot underrepresentation is not mainly determined by preferential deamination of the lagging-strand template (71–74).

Altogether, these observations were consistent with the possibility that HCMV has evolved to limit CCC-GGG motifs in its genome, especially in essential ORFs. To further address this possibility, we used an approach that accounts for the coding capacity of the HCMV genome, as well as for the amino acid composition of single ORFs. In fact, CCC is a codon for proline, and the representation of this hot spot motif in coding genes also depends on the proline content of the encoded proteins. Thus, we counted the frequency of the trinucleotide motifs for A3G, A3A, and A3F/A3C in all HCMV ORFs and obtained expected values by reshuffling codons in each ORF. For each motif in each ORF, we computed a preference index that varies between -1 (underrepresentation) and $+1$ (overrepresentation), with values close to 0 indicating that the representation of motifs is similar to the expected one (see Materials and Methods). Analysis of preference indexes indicated that CCC-GGG motifs are underrepresented in HCMV ORFs and that the median preference index is well below 0. No such pattern was evident for motifs targeted by other APOBEC3 enzymes, which showed preference indexes close to 0 (Fig. 7A). Also, CCT-AGG motifs, which represent the products of A3G deamination without repair, were not overrepresented in HCMV ORFs, and no negative correlation was observed between the preference indexes for CCC-GGG and those for CCT-AGG motifs (Fig. 6B). Thus, the underrepresentation of A3G motifs is not the result of active A3G-mediated deamination and mutation.

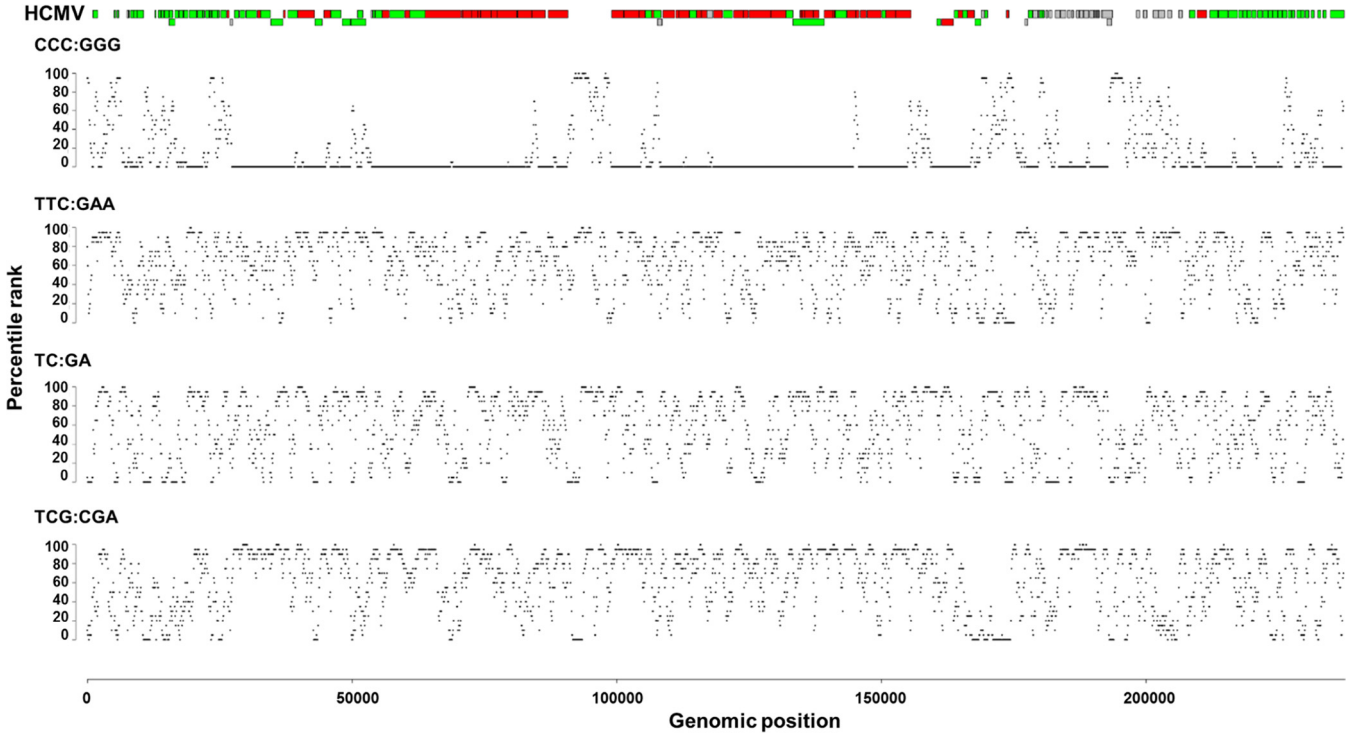

FIG 5 Sliding window analysis of APOBEC3 hot spot motifs along the HCMV genome. The HCMV Towne sequence was used (GenBank accession number GQ121041). Motifs were analyzed in 1,000-bp windows moving with a step of 100 bp. For each window, the percentile rank of the real motif count in the distribution of counts from reshuffled windows is plotted. The lower the percentile rank, the fewer motifs are detected in the window when base composition is accounted for (by reshuffling). A schematic representation of HCMV open reading frames (ORFs) is shown with color codes indicating essential ORFs (red), nonessential ORFs (green), and ORFs with unknown effect when deleted (gray).

We next sought to determine whether essential and nonessential ORFs displayed a different representation of APOBEC3G motifs. ORFs were categorized based on the mutant growth classification proposed by Dunn and coworkers (68), and preference indexes were compared (see Materials and Methods). We found that CCC-GGG motifs are significantly less likely to occur in essential ORFs than in nonessential ones (Wilcoxon rank sum test, $P = 0.014$) (Fig. 7B). As selective pressure is expected to be stronger at essential ORFs, the latter are the most depleted of A3G motifs.

Finally, we verified that the underrepresentation of CCC-GGG motifs is a general feature of HCMV genomes and is not limited to the Towne strain. Thus, the preference index for CCC-GGG motifs was calculated for all ORFs of other HCMV strains (including Merlin) and clinical isolates deriving from different sources. No substantial differences were observed between the Towne sequence and the sequences of any of these strains or isolates (Fig. 7C). Overall, these results suggest that A3G exerted selective pressure on HCMV and that the virus evolved to limit A3G hot spots in its genome.

DISCUSSION

In summary, we report that HCMV infection specifically upregulates A3G and, to a lesser extent, A3F expression in primary human fibroblasts (HFFs) and that the virus has evolved an escape strategy to avoid editing activity. Our findings indicate that human A3G is induced upon viral infection as a part of the antiviral response mediated by IFN-β. In this regard, addition of anti-IFN receptor Abs during HCMV infection ablates A3G gene product induction, demonstrating that A3G induction by HCMV is IFN dependent. Moreover, IFN-β treatment of HFFs can upregulate A3G expression within 24 h in the absence of HCMV infection, confirming that A3G is a bona fide ISG family member. Accordingly, two IFN-sensitive response elements, namely, IFN regulatory factor element (IRF-E)/IFN-stimulated response element (ISRE), located upstream of the first A3G exon have been identified (42). Recently, Weisblum et al. (28) found that A3A

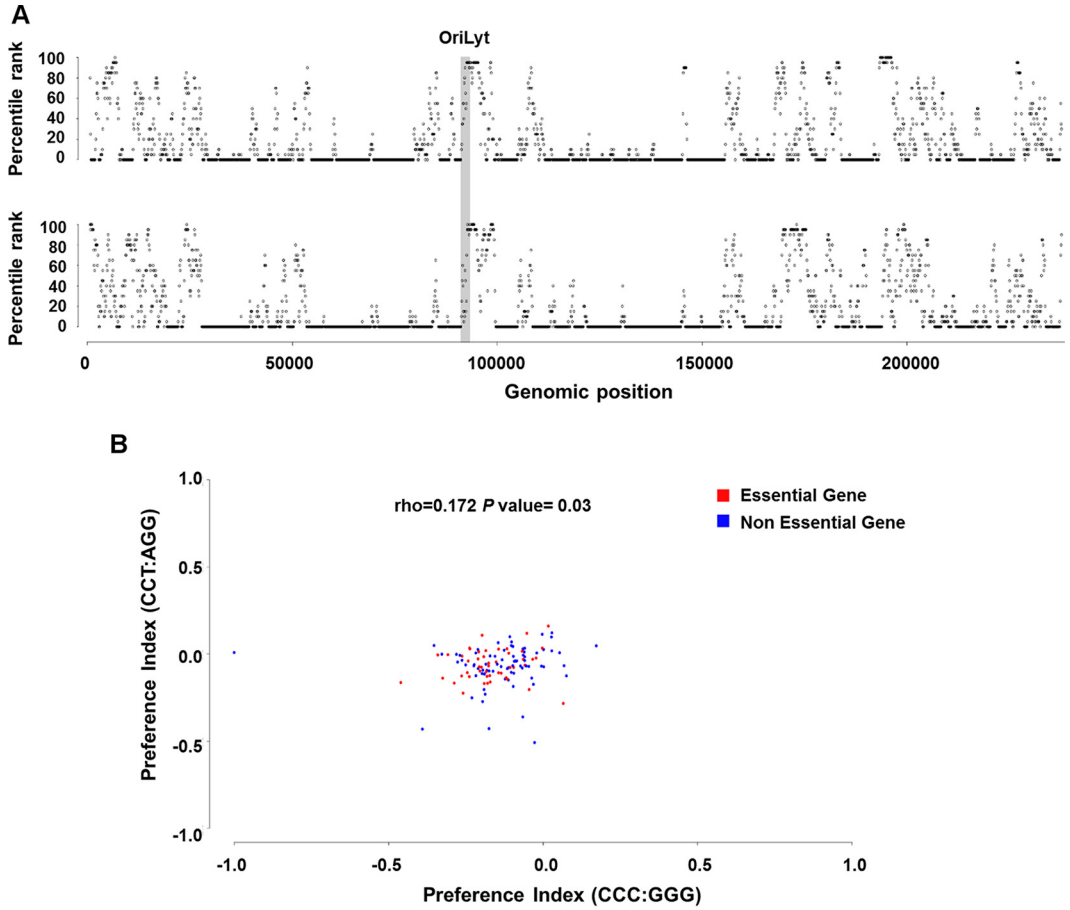

FIG 6 Occurrence of APOBEC3G motif in HCMV. (A) Sliding window analysis of APOBEC3G hot spot motif along the HCMV genome. The APOBEC3G motif (CCC) was analyzed for both strands in 1,000-bp windows moving with a step of 100 bp. For each window, the percentile rank of the real motif count in the distribution of counts from reshuffled windows is plotted. The HCMV Towne sequence was used (GenBank accession number GQ121041). (B) CCC/CCT motif comparison. A preference index calculated for the CCC-GGG motif is plotted against the preference index for the CCT-AGG motif, both calculated for essential (red) and nonessential (blue) Towne ORFs. Spearman's rank correlation coefficient (rho) is also reported, along with the correlation *P* value.

is strongly upregulated following *ex vivo* HCMV infection of human decidual tissues but not upon infection of chorionic villi, primary fibroblasts (MRC-5 and HFFs), and epithelial (ARPE-19) cell cultures. In line with our results, IFN-β significantly induced A3A expression in uninfected decidual tissues, suggesting its potential regulation as an ISG early during HCMV infection. Altogether, these findings demonstrate that A3A and A3G are differentially regulated in HCMV-infected cells.

In the same study, Weisblum et al. (28) demonstrated that overexpression of A3A severely impaired HCMV replication in epithelial cells through cytidine deamination of the viral genome. Moreover, exogenous A3A expression in ARPE-19 cells downregulated the expression of viral genes, such as immediate early (IE1) and delayed early (UL89) genes, and reduced HCMV DNA accumulation, suggesting that in this cellular system A3A does restrict virus replication. In contrast to these observations, here we show that neither knockout nor overexpression of A3G can modulate HCMV gene expression and its replication, indicating that A3G does not behave as an HCMV restriction factor *in vitro*.

Based on this evidence, we hypothesized that during evolution HCMV might have developed strategies to escape A3G editing activity. To test this hypothesis, we assessed whether A3G-mediated selective pressure shaped the composition of HCMV genomes. A3G preferentially deaminates the 3′ cytosine within CCC hot spots in single-stranded DNA, whereas other members of the A3 family have distinct prefer-

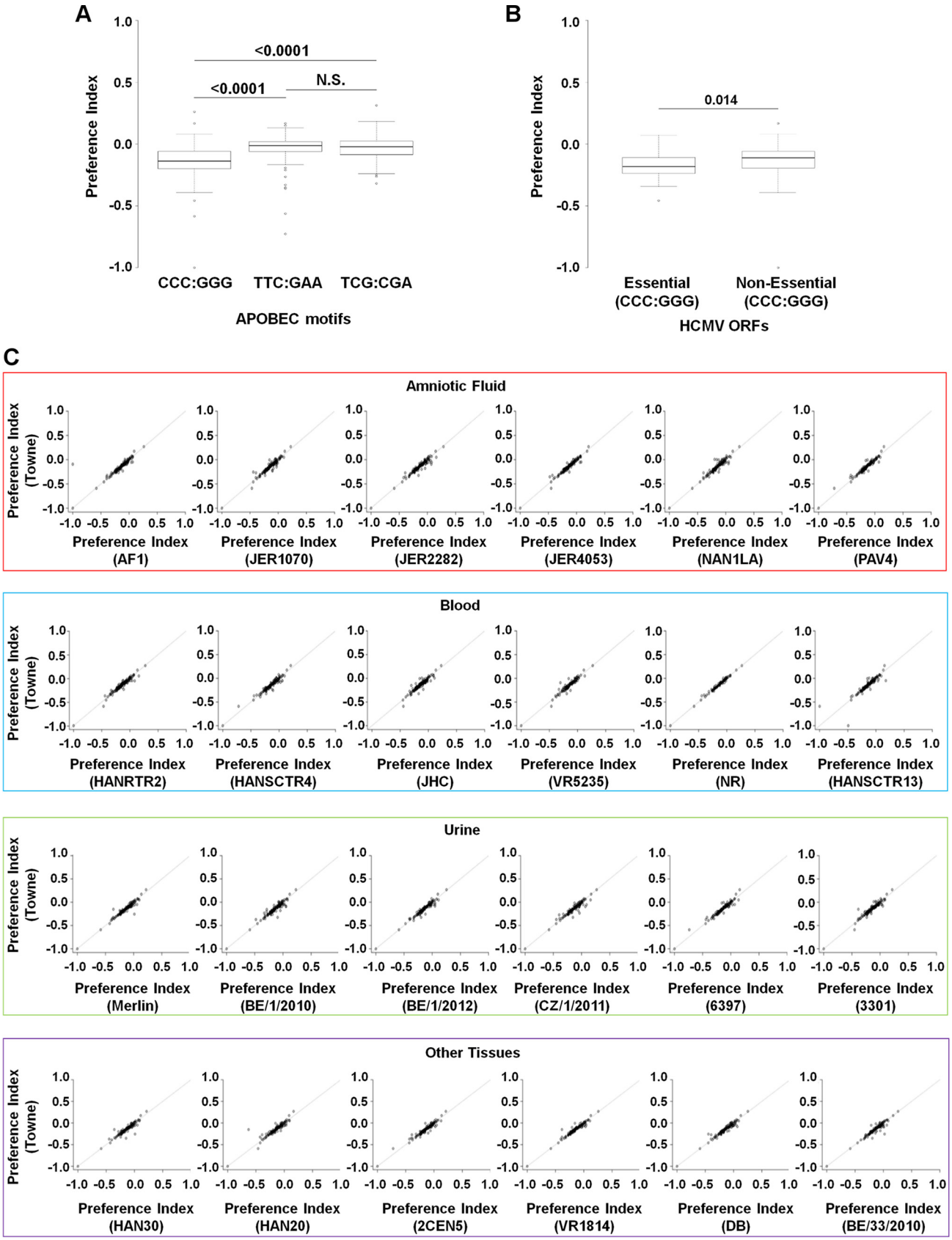

FIG 7 Occurrence of APOBEC3 motifs in HCMV ORFs. (A) The occurrence of hot spot motifs for A3G, A3F/3C, and A3A was analyzed by calculating a preference index. Preference indexes are shown in standard box-and-whisker plot representation (thick line, median; box, quartiles; whiskers, 1.5×

(Continued on next page)

ences. Notably, the CCC-GGG motif, but not other A3 motifs, was found to be significantly underrepresented in several genomic regions where essential ORFs are located. The decrease in CCC-GGG motifs was not paralleled by an increase in their deamination products, and the A3G hot spot motifs were similarly underrepresented in both genome strands. Thus, these observations suggest that A3G no longer affects the HCMV genome composition because the virus has likely evolved to limit the presence of A3G hot spot motifs especially within essential ORFs. In this respect, it is worth mentioning that, albeit underrepresented, some CCC-GGG motifs do occur in HCMV ORFs, including essential ones. Nevertheless, secondary structures and sequence context are also known to modulate A3G preferences (31), suggesting that extant CCC motifs could represent suboptimal targets.

Our findings are in line with previous studies indicating that target motifs for other A3 enzymes are depleted in the genome of alpha papillomaviruses, most likely as the result of viral evolution to avoid restriction (75). Likewise, A3B exerted selective pressure on BKPyV, which shows an underrepresentation of hot spot motifs for this enzyme (59). Nonetheless, the specific knockdown of A3B had little short-term effect on productive BKPyV infection (59).

Recent results have shown that A3A can restrict HCMV replication in human decidual tissues (28). However, we did not find A3A motifs to be underrepresented in HCMV genomes. One possible explanation for this finding is that decidual tissues do not represent the primary target site of HCMV infection, and vertical transmission, despite being clinically relevant, does not contribute significantly to HCMV spread in human populations. Thus, the selective pressure exerted by A3A on HCMV may be limited. In fact, we did not find this enzyme to be upregulated by viral infection in HFFs and other primary HCMV target cell types.

According to these observations, the following scenario could be envisaged. Early during HCMV infection, DNA sensors including cGAS and IFI16 prime IFN-β production, which in turn stimulates expression of ISGs including A3G. To prevent DNA editing by A3G from yielding CCC-GGG hypermutations, the virus has evolved to limit the presence of A3G target motifs in genes essential for its replication.

Various strategies have been adopted by different viruses to prevent the catastrophic consequences of A3-induced hypermutations. While several DNA viruses have evolved to limit the availability of A3 target sites (59, 75), HIV has adopted a completely different evasion strategy based on the ability of its protein Vif to bind A3G and promote its degradation through the proteasome pathway (76–79).

In conclusion, our studies demonstrate for the first time that (i) early during infection, HCMV upregulates A3G in fibroblasts (HFFs) through IFN-β production, (ii) A3G does not restrict HCMV replication, and (iii) HCMV has evolved mutational robustness against IFN-β by limiting the presence of A3G hot spots in essential ORFs of its genome. Our findings reveal a novel immune evasion strategy by HCMV, which further fuels its fame as master in immune evasion.

MATERIALS AND METHODS

Cells and viruses. Primary human foreskin fibroblasts (HFFs; ATCC SCRC-1041), human retinal pigment epithelial cells (ARPE-19, ATCC CRL-2302), and human embryo kidney 293 cells (HEK293; Microbix Biosystems Inc.) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich) as previously described (80). THP-1 cells, cultured as nonadherent monocyte-like cells, were grown in RPMI medium (Sigma-Aldrich), with 10% FCS, 600 μg/ml glutamine, 200 IU/ml of penicillin, and 100 μg/ml streptomycin (Gibco). THP-1 cells were differentiated into macrophage-like cells by addition of 100 nM phorbol myristate acetate (PMA; Sigma-Aldrich). All presented data with THP-1 cells were based on PMA-differentiated cells. Human

FIG 7 Legend (Continued)
interquartile range). The Kruskal-Wallis tests indicated significant differences among motifs ($P < 2.2 \times 10^{-16}$). *P* values from *post hoc* tests (Nemenyi tests) are shown. N.S., not significant. (B) Occurrence of A3G hot spot motifs in HCMV essential and nonessential ORFs. Essential ORFs have significantly fewer CCC-GGG motifs than nonessential ORFs (*P* value from Wilcoxon rank sum test). (C) Occurrence of A3G hot spot motifs in different HCMV strains and isolates. The preference indexes of Towne ORFs are plotted against the corresponding indexes from other HCMV genomes. Isolates derived from different sources or body compartments were analyzed.

umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by chymotrypsin treatment and used for experiments at passage 2 ± 7. HUVECs were cultured in endothelial cell basal medium 2 (EBM-2; Lonza), plus endothelial cell growth medium supplements (EGM-2; Lonza), 2% FCS (Sigma-Aldrich), and 1% penicillin-streptomycin solution (Sigma-Aldrich). HCMV strain Merlin was kindly provided by Gerhard Jahn (University Hospital of Tübingen, Germany), propagated, and titrated on HFFs by standard plaque assay (12, 39).

Recombinant adenoviral vectors. Adenovirus-derived vectors expressing A3G were generated by means of a replacement strategy using recombineering methods (81). Briefly, the A3G gene was amplified using a specific set of primers (forward, 5′-AACCGTCAGATCGCCTGGAGACGCCATCCACGCTGT TTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTGGATCCATGAAGCCTCACTTCAGAAA-3′; reverse, 5′-TATAGAGTATACAATAGTGACGTGGGATCCCTACGTAGAATCAAGACCTAGGAGCGGGTTAGGGATTGGCTTAC CAGCGCTGTTTTCCTGATTCTGGAGA-3′). In order to accomplish homologous recombination, approximately 200 ng of DNA was electroporated into SW102 bacteria harboring pAdZ5-CV5 vector. Cells were then plated on minimal medium agar plates containing 5% sucrose and chloramphenicol and incubated at 32°C for 1 day. The colonies that appeared were inoculated into LB broth containing ampicillin and chloramphenicol and LB broth containing chloramphenicol only. In the colonies grown in chloramphenicol only, the A3G ORF replaced the ampicillin resistance sequence in multiple cloning sites. Colonies were checked by PCR and sequencing. To obtain the recombinant adenovirus, the AdZ vector was transfected into HEK293 packaging cells. Transfected cells were maintained in a 5% CO_2 incubator at 37°C until an extensive cytopathic effect was obtained. Viruses were then purified from infected cultures by freeze-thaw-vortex cycles and assessed for A3G expression by Western blotting. For cell transduction, HFFs were washed once with phosphate-buffered saline (PBS) and incubated with AdVA3G at an MOI of 30. After 2 h at 37°C, the virus was washed off, and fresh medium was applied. For all the experiments, a recombinant adenovirus expressing the *Escherichia coli* β-galactosidase gene (AdVLacZ) was used as a control (12).

RNA isolation and semiquantitative RT-qPCR. Total RNA was extracted using a NucleoSpin RNA kit (Macherey-Nagel), and 1 μg was retrotranscribed using a Revert-Aid H-Minus FirstStrand cDNA synthesis kit (Fermentas), according to the manufacturer's protocol. Comparison of mRNA expression levels between samples (i.e., infected versus untreated) was performed by SYBR green-based RT-qPCR on a Mx3000P apparatus (Stratagene), using the following primers: A3A, GTCTTATGCCTTCCAATGCC (forward [Fw]) and GAGAAGGGACAAGCACATGG (reverse [Rw]); A3B, AATGTGTCTGGATCCATCAGG (Fw) and TGA AGGTCAGCAATTCATGC (Rw); A3C, TCTGCATGACAATGGGTCTC (Fw) and AAACTTGGCTGTGCTTCACC (Rw); A3D, GATCTGGAAGCGCCTGTTAG (Fw) and AGTCGAATCACAGGCAGGAG (Rw); A3F, CCATAGGCTTT GCGTAGGTT (Fw) and AATTATGCATTCCTGCACCG (Rw); A3G, TTCCAAAAGGGAATCACGTC (Fw) and AG GGGCTTTCTATGCAACC (Rw); A3H, AGCTGTGGCCAGAAGCAC (Fw) and CGGAATGTTTCGGCTGTT (Rw); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), AGTGGGTGTCGCTGTTGAAGT (Fw) and AACGTG TCAGTGGTGGACCTG (Rw); Mx1, CCAGCTGCTGCATCCCACCC (Fw) and AGGGGCGCACCTTCTCCTCA (Rw).

Neutralization of type I IFNs. To neutralize the activity of type I IFNs, specific blocking antibodies against interferon receptor (clone MMHAR-2, diluted 1:100; Millipore) were added to culture medium at a concentration of 5 μg/ml for 18 h prior to infection with HCMV Merlin strain at an MOI of 1 and then left in the supernatant until the end of the respective experiment. Mouse IgG2a (clone MOPC-173, diluted 1:100; BD Biosciences Europe) was used as an isotype control. Human recombinant IFN-β was obtained from PBL (catalog number 11415-1).

Transduction of HFFs with lentiviral CRISPR/Cas9. The CRISPR/Cas9 system was employed to generate specific gene knockouts in primary human fibroblasts. Recombinant lentiviruses were packaged in HEK293T cells by cotransfection of APOBEC3G subgenomic RNA (sgRNA) with a CRISPR/Cas9 All-in-One Lentivector set (Human) (Applied Biological Materials Inc.) and 2nd Generation Packaging System Mix (Applied Biological Materials, Inc.) for producing viral particles using Lipofectamine 2000 (Invitrogen). Viral supernatants were harvested after 48 h and used to transduce fibroblasts by infection in the presence of 8 mg/ml Polybrene. Transduced cells were selected with puromycin (1 μg/ml) over the course of 14 days postransduction. After selection, successful knockout was confirmed using immunoblotting. CRISPR negative-control lentiviruses were produced with a scrambled sgRNA CRISPR/Cas9 All-in-One Lentivector (Applied Biological Materials, Inc.) in HEK293T cells as described above.

Western blot analysis. Whole-cell protein extracts were prepared and subjected to Western blot analysis as previously described (82, 83). The following primary mouse monoclonal antibodies were used: anti-A3G (VMA00418, diluted 1:1,000; Bio-Rad), CMV IEA (CH160, diluted 1:1,000; Vyrusis), and α-tubulin (39527, diluted 1:4,000; Active-Motif). Immunocomplexes were detected using sheep anti-mouse antibodies conjugated to horseradish peroxidase (HRP) (GE Healthcare Europe GmbH) and visualized by enhanced chemiluminescence (Super Signal West Pico; Pierce-Thermo Fischer Scientific).

Immunofluorescence microscopy. Indirect immunofluorescence analysis was performed as previously described (82, 84), using the appropriate dilution of primary antibodies for 1 h at room temperature (RT) in the presence of 10% HCMV-negative human serum followed by 1 h of incubation with secondary antibodies in the dark at RT. The following primary antibodies were used: rabbit polyclonal anti-CMV IEA antibody (diluted 1:500) (Santo Landolfo, University of Turin) or mouse monoclonal antibody anti-A3G (VMA00418, diluted 1:200; Bio-Rad). Conjugated secondary antibodies included goat anti-rabbit Alexa Fluor 568 antibody (A-11011, diluted 1:200; Life Technologies) or goat anti-mouse Alexa Fluor 488 antibody (R37120, diluted 1:200; Life Technologies). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, coverslips were mounted with Vectashield mounting medium (Vector). Samples were observed using a confocal microscope (Leica TCS SP2). ImageJ software was used for image processing.

FRET-based *in vitro* A3G deamination assay. A fluorescence resonance energy transfer (FRET)-based assay was used to detect cytosine deaminase activity of A3G (31). Twenty microliters of the cell lysates was used per assay using 96 assay plates. A separate solution of 20 pmol of oligonucleotide, 10 μg of RNase A, and 0.04 U of uracil DNA glycosylase (UDG) were mixed together in 50 mM Tris (pH 7.4)–10 mM EDTA buffer and adjusted to a total volume of 50 μl and then transferred to the assay well. The assay plate was then incubated at 37°C for 5 h. Next, 30 μl of 2 M Tris-acetate, pH 7.9, was added to each well, and the plate was incubated at 95°C for 2 min and on ice for 2.5 min. The fluorescence was then measured at room temperature using a Victor^3 1420 Multilabel Counter (Perkin-Elmer). Experiments were conducted with three independent replicates.

Statistical analysis. Statistical tests were performed using GraphPad Prism, version 5.00, for Windows (GraphPad Software), unless specified differently in the text. The data were presented as means ± standard deviations (SD). Means between two or three groups were compared by using a one-way or two-way analysis of variance (ANOVA) with Bonferroni's posttest. Differences were considered statistically significant at P values of <0.05, <0.01, and <0.001, as indicated in the figure legends.

Analysis of A3 hot spot motif representation. HCMV genome sequences were obtained from the GenBank database. To evaluate the genomic representation of A3 hot spots, we counted the number of each A3 motif in 1,000-bp windows along the HCMV Towne genome, using a sliding window approach with a step of 100 bp, on both genome strands. To assess whether this count is an overrepresentation of A3 motifs, we generated 1,000 shuffled versions of each window and counted the number of each motif within these windows. The number of these occurrences was then used to create distributions of motif counts (in each window), and the percentile rank of the true motif count was calculated. These percentile ranks are plotted in Fig. 5. For instance, a rank of 0 in a window indicates that the real number of motif counts was lower than all those obtained in reshuffled versions of that same window.

To investigate the distribution of A3G motifs in the HCMV genome by also accounting for coding capacity and amino acid composition, we counted the frequency of motifs in each HCMV ORF. We then obtained expected values by reshuffling codons in each ORF; specifically, for each ORF, we generated 1,000 codon-shuffled sequences. We next calculated a preference index for A3 motifs, defined as follows: preference index = (number of motifs observed − number of motifs expected)/(number of motifs observed + number of motifs expected). In practical terms, the preference index varies between −1 and +1, with values equal to 0 indicating that the representation of motifs is equal to the expected; negative and positive values indicate under- and overrepresentation, respectively. ORFs were grouped based on the mutant growth classification proposed by Dunn et al. (68): essential (no growth and severely defective) and nonessential (moderately defective and like wild type).

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Review

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

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

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Background

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

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

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

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

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

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

Innate immunity versus HCMV infection: a brief overview

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

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

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

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

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

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

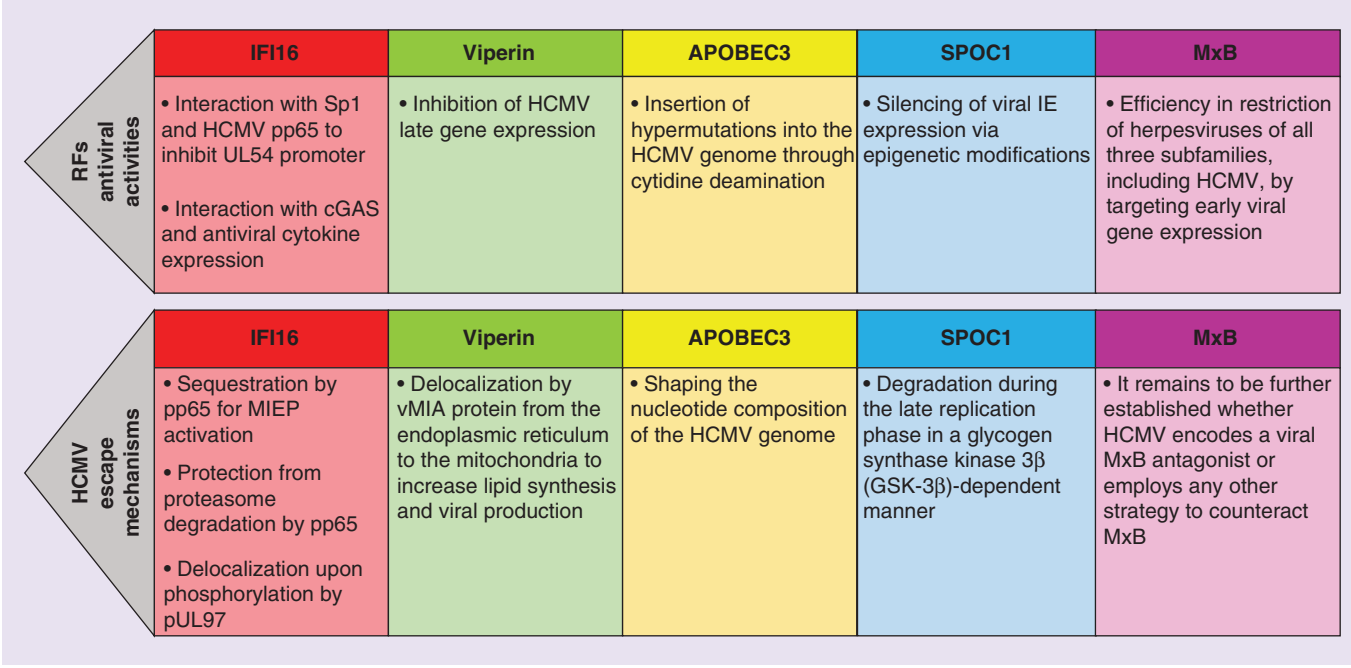

Figure 1. The best-characterized host restriction factors in the defense against human cytomegalovirus and viral countermechanisms.

HCMV restriction factors

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

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

γ-Interferon-inducible protein 16 (IFI16)

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

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

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

Viperin

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

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

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

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

Apolipoprotein B editing catalytic subunit-like 3 (APOBEC3)

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

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

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

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

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

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

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

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

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

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

Myxovirus resistance (Mx)

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

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

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

Cytomegalovirus immune evasion strategies

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

HCMV evasion of the interferon response

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

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

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

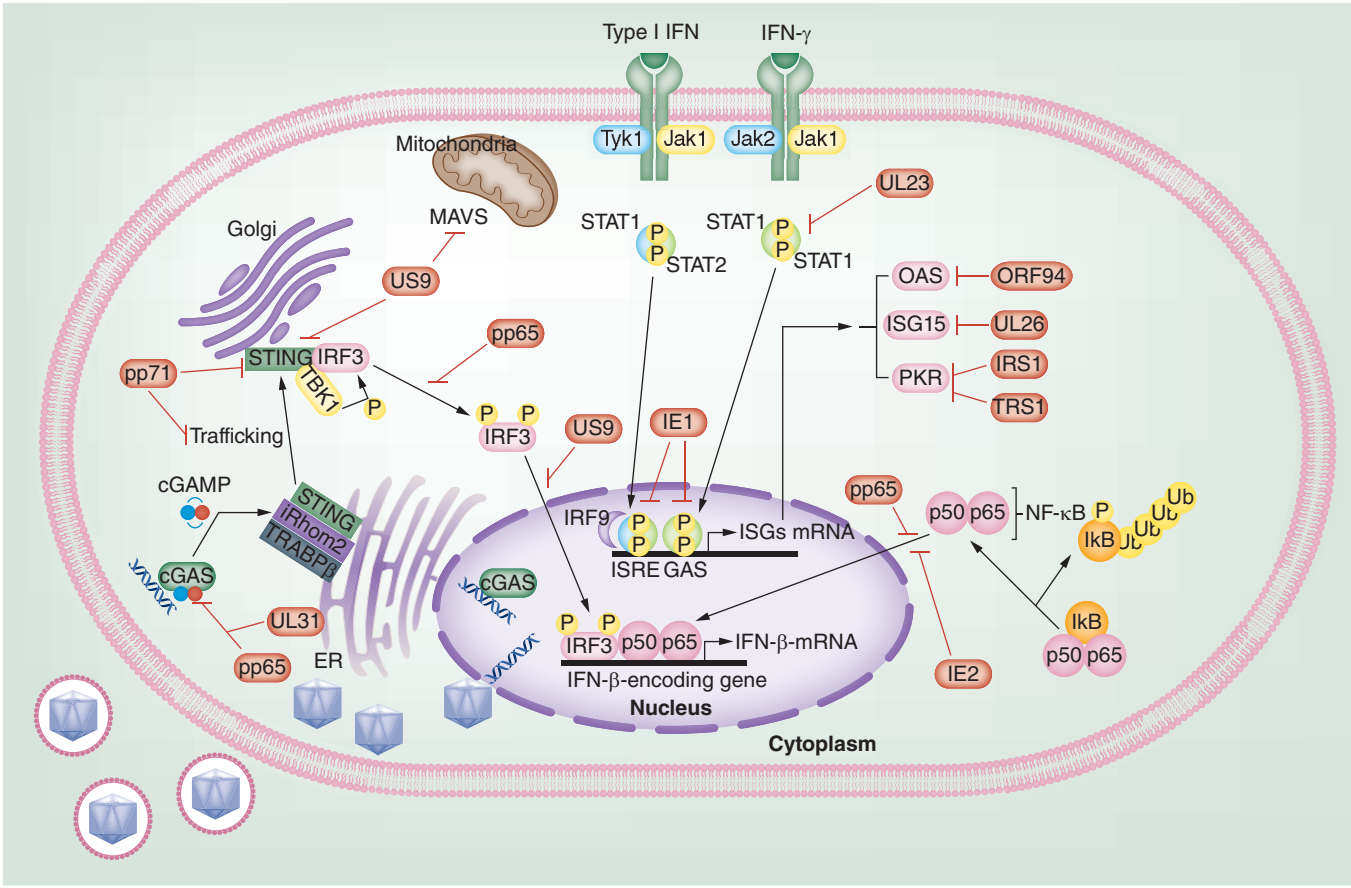

Figure 2. Schematic representation of the human cytomegalovirus evasion strategies from interferon antiviral activity.

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

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

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

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

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

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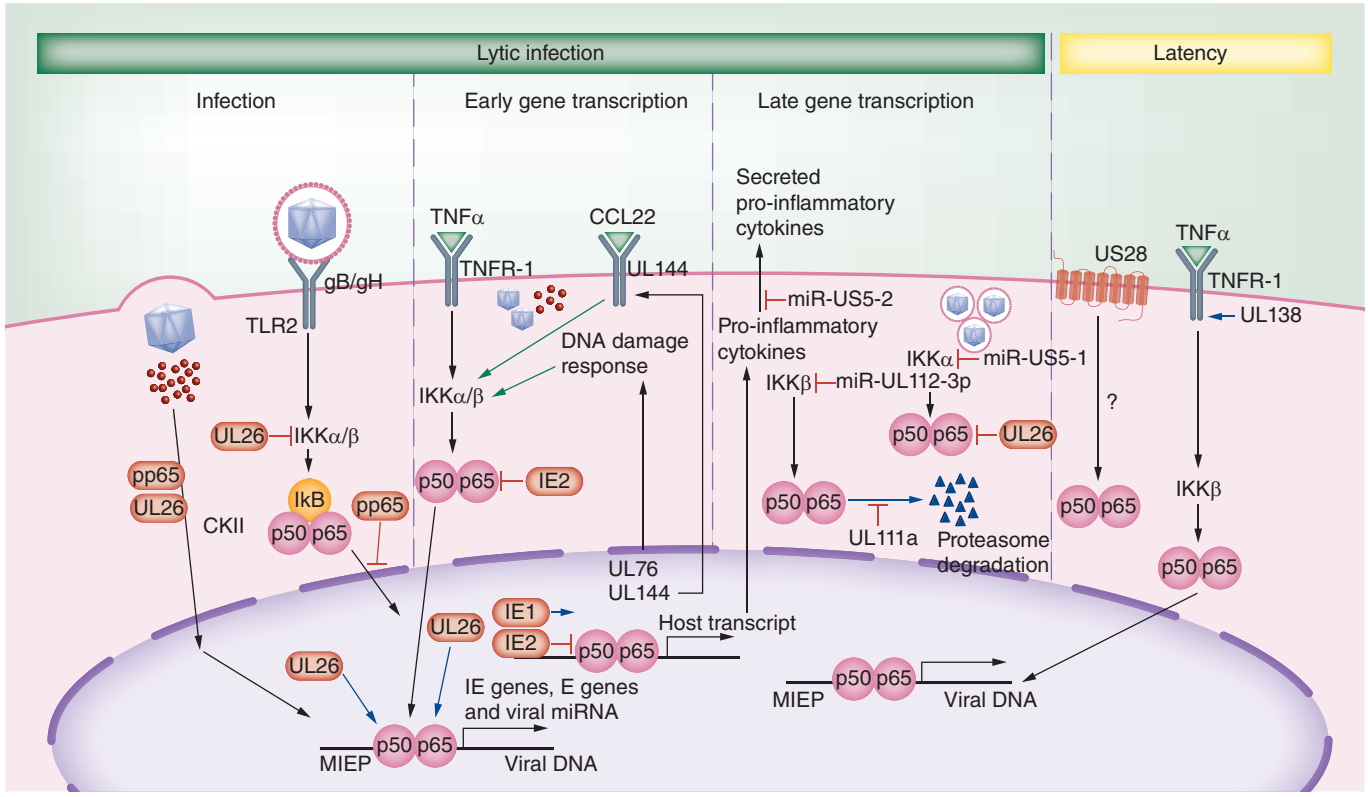

Figure 3. Model depicting the modulation of the NF-κB signaling pathway by human cytomegalovirus.

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

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

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

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

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

HCMV & NF-κB signaling

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

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

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

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

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

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

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

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

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

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

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

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

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

Future perspective

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

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

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

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

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

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

Executive summary

Human cytomegalovirus

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

Immune modulation

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

HCMV restriction factors

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

Evasion from the interferon response

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

Modulation of NF-κB signaling

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

Future perspective

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

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

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A Conserved Mechanism of APOBEC3 Relocalization by Herpesviral Ribonucleotide Reductase Large Subunits

Running Title: APOBEC3 Relocalization by Herpesviral RNRs

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Abstract

An integral part of the antiviral innate immune response is the APOBEC3 family of single-stranded DNA cytosine deaminases, which inhibits virus replication through deamination-dependent and -independent activities. Viruses have evolved mechanisms to counteract these enzymes such as HIV-1 Vif-mediated formation of a ubiquitin ligase to degrade virus-restrictive APOBEC3 enzymes. A new example is Epstein-Barr virus (EBV) ribonucleotide reductase (RNR)-mediated inhibition of cellular APOBEC3B (A3B). The large subunit of the viral RNR, BORF2, causes A3B relocalization from the nucleus to cytoplasmic bodies and thereby protects viral DNA during lytic replication. Here, we use co-immunoprecipitation and immunofluorescent microscopy approaches to ask whether this mechanism is shared with the closely related γ-herpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) and the more distantly related α-herpesvirus, herpes simplex virus-1 (HSV-1). The large RNR subunit of KSHV, ORF61, co-precipitated multiple APOBEC3s including A3B and APOBEC3A (A3A). KSHV ORF61 also caused relocalization of these two enzymes to perinuclear bodies (A3B) and to oblong cytoplasmic structures (A3A). The large RNR subunit of HSV-1, ICP6, also co-precipitated A3B and A3A and was sufficient to promote the relocalization of these enzymes from nuclear to cytoplasmic compartments. HSV-1 infection caused similar relocalization phenotypes that required ICP6. However, unlike the infectivity defects previously reported for BORF2-null EBV, ICP6 mutant HSV-1 showed normal growth rates and plaque phenotypes. These results combine to indicate that both γ- and α-herpesviruses use a conserved RNR-dependent mechanism to relocalize A3B and A3A and, further, suggest that HSV-1 possesses at least one additional mechanism to neutralize these antiviral enzymes.

Importance

The APOBEC3 family of DNA cytosine deaminases constitutes a vital innate immune defense against a range of different viruses. A novel counter-restriction mechanism has recently been uncovered for the γ-herpesvirus EBV, in which a subunit of the viral protein known to produce DNA building blocks (ribonucleotide reductase) causes A3B to relocalize from the nucleus to the cytosol. Here, we extend these observations with A3B to include a closely related γ-herpesvirus, KSHV, and to a more distantly related α-herpesvirus, HSV-1. These different viral ribonucleotide reductases also caused relocalization of A3A, which is 92% identical to A3B. These studies are important because they suggest a conserved mechanism of APOBEC3 evasion by large double-stranded DNA herpesviruses. Strategies to block this host-pathogen interaction may be effective for treating infections caused by these herpesviruses.

64 **Introduction**

65 An important arm of the innate immune response lies in the APOBEC family of single-stranded
66 DNA cytosine deaminases (1-3). Each of the seven human APOBEC3 (A3) enzymes, A3A-D and A3F-H,
67 have been implicated in the restriction and mutation of a variety of different human viruses including
68 retroviruses (HIV-1, HIV-2, HTLV-1) (4-8), endogenous retroviruses (HERV) (9, 10), hepadnaviruses
69 (HBV) (11, 12), small DNA tumor viruses (HPV, JC/BK-PyV) (13-17), and most recently, the γ-
70 herpesvirus Epstein-Barr Virus (EBV) (18, 19). It is difficult, if not impossible, to predict *a priori* which
71 subset of APOBEC3 enzymes has the potential to engage a given virus and, furthermore, how that virus
72 might counteract potentially restrictive A3 enzymes. For instance, the lentiviruses HIV-1 and HIV-2
73 encode an accessory protein called Vif that heterodimerizes with the cellular transcription co-factor CBF-
74 β and recruits a cellular ubiquitin ligase complex to trigger the degradation of restrictive A3 enzymes (20,
75 21).

76 Human herpesviruses can be grouped into three distinct subfamilies (α, β, and γ; phylogeny
77 shown in **Fig 1A**). Pathogenic α- and β-herpesviruses include herpes simplex virus type 1 (HSV-1) and
78 cytomegalovirus (CMV), respectively, and the γ-herpesvirus subfamily includes EBV and Kaposi's
79 sarcoma-associated herpesvirus (KSHV). We recently identified an A3 counteraction mechanism for EBV
80 (18). We demonstrated that the large subunit of the viral ribonucleotide reductase (RNR), BORF2,
81 inhibits APOBEC3B (A3B) by directly binding and relocalizing it from the nucleus to the cytoplasmic
82 compartment. This counteraction mechanism prevents the normally nuclear-localized A3B enzyme from
83 deaminating viral genomic DNA cytosines to uracils during lytic replication. In the absence of BORF2,
84 A3B inflicted C/G-to-T/A mutations in EBV genomes and reduced viral titers and infectivity. We also
85 showed that the homologous protein from KSHV, ORF61, is similarly capable of binding and relocalizing
86 A3B (18).

87 Here, we ask whether the viral RNR-mediated A3B counteraction mechanism is specific to γ-
88 herpesviruses or more general-acting by assessing interactions between γ-herpesvirus BORF2/ORF61 and
89 other human A3 enzymes and by determining whether the more distantly related α-herpesvirus HSV-1
90 has a similar A3 neutralization mechanism (RNR nomenclature in **Fig 1A** and protein domains depicted
91 in **Fig 1B**). We found that, in addition to binding and relocalizing A3B, both BORF2 and ORF61 were
92 also capable of co-immunoprecipitation and relocalization of A3A. Additionally, we found that the HSV-
93 1 RNR large subunit ICP6 similarly binds and relocalizes both A3B and A3A. Overexpression studies
94 showed that ICP6 alone is sufficient for A3B and A3A relocalization. Infection studies with wild-type
95 and mutant viruses demonstrated that ICP6 mediates this relocalization activity in the context of infected
96 cells and that no other viral protein is capable of this relocalization function. However, despite likely

conservation of the A3B/A relocalization mechanism, the infectivity of ICP6 mutant HSV-1 was not affected by A3B or A3A suggesting the existence of a functionally redundant A3 neutralization mechanism.

Results

EBV BORF2 and KSHV ORF61 bind and relocalize both A3B and A3A

Our prior co-immunoprecipitation (co-IP) experiments indicated that EBV BORF2 interacts strongly with A3B and weakly with A3A and A3F [see Fig. 1c in Cheng *et al*. (18)]. EBV BORF2 was both necessary and sufficient to relocalize A3B in a variety of different cell types including endogenous A3B in the AGS gastric carcinoma cell line and the M81 B cell line (18). However, our original studies did not address whether EBV BORF2 could functionally interact with and relocalize any of these related human A3 enzymes. We therefore performed immunofluorescent (IF) microscopy studies of U2OS cells overexpressing A3-mCherry constructs with either empty vector or BORF2-FLAG (**Fig 2A**). As reported, A3B is nuclear, A3A has a cell-wide localization, A3H is cytoplasmic and nucleolar, and the other A3s are cytoplasmic (22-26). Also as expected, BORF2 caused a robust and complete relocalization of nuclear A3B to perinuclear aggregates. Interestingly, BORF2 co-expression with A3A led to the presence of novel linear elongated structures concomitant with normal A3A localization. The localization patterns of the other five A3s were unchanged by BORF2 co-expression. Small BORF2 punctate structures were also noted in all conditions including the mCherry control, which is likely due to transfected BORF2 interacting with endogenous A3B [previously shown to be elevated in U2OS (18)]. Similar A3B and A3A relocalization patterns were evident in Vero cells except that A3A relocalization became whole-cell without elongated structures (**Fig 2B** and data not shown).

Like EBV BORF2, KSHV ORF61 was also shown to co-IP and relocalize A3B (18). However, our original studies did not examine the specificity of this interaction by comparing with related human A3 enzymes. We therefore used co-IP experiments to evaluate KSHV ORF61 interactions with a full panel of human A3 enzymes. ORF61-FLAG was co-expressed with A3-HA family members in 293T cells, subjected to anti-FLAG affinity purification, and analyzed by immunoblotting (**Fig 3A**). The ORF61-FLAG pulldown resulted in A3B recovery as described (18). In addition, the ORF61-FLAG IP also yielded a robust interaction with A3A and weaker interactions with A3D and A3F.

These KSHV ORF61-A3 interactions were then evaluated by IF microscopy experiments to look for changes in A3 localization in U2OS cells (**Fig 3B**). As expected (18), KSHV ORF61 caused A3B to relocalize to perinuclear bodies. Moreover, as above for BORF2 and A3A, ORF61 co-expression caused a portion of the cellular A3A to localize to intense elongated linear structures in the cytosolic compartment (**Fig 3B**). No other A3 proteins showed altered subcellular localization in these experiments. Similar IF

microscopy observations were made using the same constructs in HeLa cells (data not shown). These new results combined to indicate that both A3B and A3A are cellular targets of EBV BORF2 and KSHV ORF61. The potential relevance of these interactions to the pathogenesis of these viruses will be considered in the **Discussion**.

HSV-1 ICP6 binds and relocalizes A3B and A3A

To test whether the RNR-mediated A3B/A relocalization mechanism is more broadly conserved, a series of co-IP experiments was done with the large RNR subunit of the pathogenic α-herpesvirus HSV-1, ICP6. FLAG-ICP6 was co-expressed with each of the seven different HA-tagged human A3s in 293T cells and subjected to anti-FLAG IP as above. The EBV BORF2-A3B interaction was used as a positive control and BORF2-A3G as a negative control to be able to compare the relative strengths of pulldowns between RNRs and A3s. HSV-1 ICP6 showed a strong interaction with A3A and weaker, but detectable, interactions with A3B, A3C, and A3D (**Fig 4A**).

Next, IF microscopy was used to assess functional interactions between HSV-1 ICP6 and each of the human A3 enzymes. Human U2OS osteosarcoma cells were co-transfected with mCherry-tagged A3s and either empty vector or FLAG-tagged HSV-1 ICP6 and analyzed by IF after 48 hours (**Fig 4B**). On its own EBV BORF2 shows a cytoplasmic distribution and, as shown above and previously (18), it was able to completely relocalize A3B from the nucleus to cytoplasm. In comparison, HSV-1 FLAG-ICP6 showed a broadly cytoplasmic localization that did not change significantly with co-expression of any A3. However, co-expression of FLAG-ICP6 and A3B-mCherry or A3A-mCherry led to a near complete relocalization of these DNA deaminases from the nucleus to the cytoplasm. HSV-1 ICP6 did not cause significant relocalization of any of the other A3s. The dramatic relocalization results with A3B and A3A suggested that functionally relevant interactions may be occurring with these enzymes.

HSV-1 infection relocalizes A3B, A3A, and A3C

To address whether HSV-1 infection similarly promotes relocalization of A3B and A3A, U2OS cells were transfected with A3-mCherry constructs 48 hours prior to either mock or HSV-1 infection. We used K26GFP, a HSV-1 strain that has a GFP moiety fused to capsid protein VP26 to allow for identification of infected cells (27). Cells were analyzed by IF microscopy 8 hours post-infection (hpi) (**Fig 5A**). Similar to the ICP6 overexpression experiments described above, HSV-1 infection caused A3A to relocalize to the cytoplasmic compartment and A3B to change from a predominantly nuclear localization to a more cell-wide distribution. A3C also changed from a predominantly cytoplasmic localization to a more diffuse whole cell distribution, whereas A3D, A3F, A3G, and A3H were unchanged by HSV-1 infection. In an independent experiment, quantification was done for HSV-1-

induced relocalization of A3A-mCherry and A3B-mCherry and, as a representative non-altered control, A3G-mCherry (**Fig 5B**). This analysis confirmed that HSV-1 infection leads to significant changes in both A3A and A3B localization, whereas A3G is unaffected. Similar relocalization patterns were found in HeLa cells following HSV-1 K26GFP infection (data not shown). Moreover, time-course experiments showed that relocalization of A3A was detectable as early as 3 hpi, A3B and A3C relocalization became apparent by 6 or 9 hpi, and A3G was not observed to relocalize at any time point (**Fig 6** and data not shown). These kinetic differences may reflect a differential affinity of the viral protein(s) to bind to these cellular A3 enzymes and/or different competitions with cellular interactors.

HSV-1-mediated relocalization of A3B and A3A requires ICP6

To investigate whether the HSV-1 large RNR subunit is required for A3A/B/C relocalization, we next examined A3 localization in cells following infection with an HSV-1 KOS1.1 strain lacking ICP6 due to a deletion of the *UL39* gene (*UL39* encodes ICP6) (28). Vero cells were transfected with A3-mCherry constructs 48 hours prior to mock infection or infection with KOS1.1 or KOSΔICP6. After 8 hours, cells were fixed, permeabilized, and subjected to IF analysis by staining for the HSV-1 immediate early protein ICP27 to mark infected cells, and monitoring A3 localization through mCherry fluorescence. As above, HSV-1 infection caused the relocalization of A3A, A3B, and A3C (**Fig 7A**). However, only the relocalization A3A and A3B was ICP6-dependent, whereas A3C redistributed regardless of the presence of ICP6. Quantification of A3A and A3B relocalization showed that these proteins were not significantly changed upon KOS1.1ΔICP6 infection compared to mock-infected cells (**Fig 5B**). These results provide strong support for mechanistic conservation of the RNR large subunit interaction with A3A and A3B and also indicate that A3C relocalization by HSV-1 is mechanistically distinct.

To further investigate the role of ICP6 in mediating A3A and A3B relocalization, U2OS cells were infected with an HSV-1 KOS mutant with a deletion in the *ICP4* gene (29). ICP4, an immediate early protein, is the major transcriptional activator protein of HSV-1 (29). *ICP4*-null mutants exhibit a strict block to expression of nearly all viral delayed-early and late genes, but are competent to express the viral immediate-early genes (*ICP0*, *ICP22*, *UL54*, and *US12*) as well as the *UL39* gene, a delayed-early gene that is uniquely transactivated by ICP0 (30). In fact, at intermediate and late times post-infection, *ICP4*-null mutants express abnormally high levels of these immediate early proteins as well as ICP6 (29). Similar to what was seen for wild-type HSV-1 infection, infection with the HSV-1 KOSΔICP4 mutant also led to A3A and A3B relocalization, but with noticeably more pronounced phenotypes (**Fig 7B**; also see **Fig 5B** for quantification of data from an independent experiment). For instance, this mutant virus caused A3B-mCherry to form perinuclear aggregates reminiscent of previously observed BORF2-A3B bodies (18) (**Fig 7B**). Interestingly, A3C localization became predominantly nuclear upon HSV-1

KOSΔICP4 infection, suggesting that one of the other four immediate early proteins besides ICP4 induces its relocalization. Taken together, these data show that HSV-1 ICP6 is both necessary and sufficient for the relocalization of A3A and A3B, and that at least one other viral factor is responsible for A3C relocalization. Identification of this factor will be the subject of a future investigation.

Effect of A3B and A3A on HSV-1 replication

We next sought to test the effect of A3 expression on HSV-1 virus replication, with or without ICP6. HFF-1 cells were stably transduced to express HA-tagged A3 constructs and then infected at a low MOI (0.001 PFU/cell) with wild-type HSV-1 KOS1.1 or KOSΔICP6. At 48 hpi, the cultures were harvested, and after freeze-thawing to release infectious progeny, the cell lysates were titered on Vero cells to compare virus production. As previously described, KOSΔICP6 exhibited a 1-2 log defect in virus replication compared to wild-type KOS (28). However, there was no significant difference in either KOS1.1 or KOSΔICP6 virus titers produced from control HFF-1 cells or HFF-1 cells expressing different A3 family members (**Fig 8A**).

To further test whether A3B or A3A can restrict HSV-1 replication, we performed plaque assays on U2OS and Vero cells stably transduced with HA-tagged A3 constructs. Confluent monolayers were incubated with serial dilutions of KOS1.1 or KOSΔICP6 and incubated for 3 days to allow for plaque formation. However, expression of A3A or A3B did not have a discernable effect on the number or size of KOS1.1 or KOSΔICP6 plaques (**Fig 8B**). These data suggest that even without ICP6, HSV-1 is not readily susceptible to restriction by A3A or A3B, possibly because it possesses other defenses against these virus restriction factors.

Discussion

We previously described a novel mechanism for A3B counteraction by the γ-herpesvirus RNR large subunits, EBV BORF2 and KSHV ORF61 (18). These viral proteins interact directly with A3B, inhibit its DNA deaminase activity, and relocalize it from the nuclear to the cytoplasmic compartment. The importance of this A3B counteraction mechanism is evidenced by BORF2-null EBV eliciting lower viral titers, decreased infectivity, and an accumulation of A3B signature C/G-to-T/A mutations. Here, we investigated the question of specificity by comparing interactions with the full repertoire of seven different human A3 enzymes, and we also addressed the potential for broader conservation by asking whether the α-herpesvirus HSV-1 possesses a similar APOBEC3 relocalization mechanism. Although EBV BORF2 and KSHV ORF61 were able to interact with several different A3 proteins in co-IP experiments, these viral RNR large subunits only promoted the relocalization of A3B and A3A. HSV-1 ICP6 showed a similarly broad range of co-IP interactions but also only promoted the relocalization of

A3B and A3A. Wild-type but not ICP6 deletion mutant HSV-1 infections yielded similar A3B and A3A relocalization phenotypes. These studies combine to indicate that human γ- and α-herpesviruses possess a conserved A3B/A relocalization mechanism mediated by the viral RNR large subunit.

The γ- and α-herpesvirus subfamilies encode both large and small RNR subunits (**Fig 1A**). These RNRs are thought to serve the canonical function of synthesizing deoxyribonucleotides by reducing the 2'-hydroxyl from ribonucleotide substrates (31). While RNRs are essential for all cellular life, the requirement for endogenous viral RNRs differs tremendously across viral families. For example, most small dsDNA viruses and single-stranded DNA viruses do not encode RNRs and instead rely on host-encoded RNRs for deoxyribonucleotide production (32, 33). On the other hand, RNRs are almost ubiquitous among large double-stranded DNA (dsDNA) viruses, such as herpesviruses and poxviruses, presumably due to high dNTP requirements during DNA replication (34-36). β-herpesviruses such as CMV are an exception, however, because they lack a small subunit and the large subunit has a defective catalytic site (37). In addition to ribonucleotide reductase activity, some viral RNRs have been shown to engage in non-catalytic activities that result in proviral phenotypes. For instance, the HSV-1 and HSV-2 large ribonucleotide reductase subunits, ICP6 and ICP10, respectively, have unique N-terminal extensions that block caspase-8 activity to inhibit apoptosis and bind RIP3 to promote necroptosis (38-41) (**Fig 1B**). CMV UL45 also has anti-apoptotic and pro-necroptotic functions suggesting this could be its predominant function (41-43).

The question of whether A3B, A3A, or both enzymes is most relevant to γ- and α-herpesvirus pathogenesis is likely to depend, at least in part, on the complex interplay between viral tropism(s) and alternating modes of latent versus lytic replication. For EBV, epithelial cells serve as the source of primary infection which are mandatory for establishing lytic replication cycles for person-to-person spread and enabling secondary infection of B lymphocytes for establishment of long-term latency (44). B cells also support lytic reactivation for reinfection and maintenance of EBV in the blood (45). Here, A3B may be more important than A3A simply because its expression is well-documented in these cell types (46, 47). Likewise, KSHV infects epithelial and B cells, but also engages in infection of clinically relevant endothelial cells which can lead to Kaposi's sarcoma (48). Additionally, because monocytes are likely to be a secondary reservoir for KSHV infection (49-51), it is plausible that this virus requires the capacity to relocalize both A3B and A3A [A3B neutralization for replication in B cells and A3A neutralization for replication in monocytes/macrophages, where A3A is interferon-inducible and capable of being expressed at extremely high levels (46, 52, 53)]. For HSV-1, although neither A3B nor A3A expression has been reported in neural/CNS cells, lytic replication in epithelial cells may require functional neutralization of A3B and/or A3A (54, 55).

The observation that the HSV-1 ΔICP6 mutant replicates at similar levels to wild-type HSV-1 in the presence of A3B or A3A was unexpected, but not entirely surprising. Given the large genomes of herpesviruses, it is possible that other viral proteins may have overlapping redundant functions in A3 counteraction and/or repair mechanisms to overcome A3-mediated hypermutation. One prime candidate is the viral-encoded uracil DNA glycosylase, encoded by the *UL2* gene, which has been shown to associate with the HSV-1 DNA polymerase in the infected cell nucleus (56). Consistent with this idea, we previously found that inhibition the EBV uracil DNA glycosylase (UDG) through expression of a universal UDG inhibitor (Ugi) results in enhanced A3B-mediated hypermutation of EBV genomes (18). It is thus possible that HSV-1 UL2 mediates the repair of uracil lesions generated by A3 enzymes allowing the virus to tolerate moderate levels of mutation in the absence of ICP6. It is also conceivable that HSV-1 encodes an additional, novel A3A/B neutralization or escape mechanism that is able to fully compensate for loss of ICP6 function (at least in the cell types tested here). Alternatively, inherent differences in viral DNA replication between HSV-1 and EBV could account for differences in replication phenotypes. HSV-1 replicates faster than EBV (57), which could result in less accessible single-stranded DNA for A3-mediated deamination. Lastly, the lack of an *in vitro* infectivity phenotype does not preclude *in vivo* disease relevance. Although prior studies have tested the impact of A3A and A3G (and APOBEC1) on wild-type HSV-1 replication in transgenic mice (58, 59), dedicated functional studies with mutants that at least partly cripple each viruses' A3 relocalization mechanism(s) in the most disease relevant *in vivo* systems will be required to fully address the question of whether A3B, A3A, or both enzymes are relevant to the pathogenesis of these herpesviruses.

Materials and Methods

Generation of herpesvirus phylogenetic tree. Amino acid sequences for herpesvirus ribonucleotide reductase large subunits were obtained from NCBI Protein RefSeq with the following GenBank accession numbers: HSV-1 ICP6 YP_009137114.1, HSV-2 ICP10 YP_009137191.1, VZV ORF19 NP_040142.1, EBV BORF2 YP_401655.1, HCMV UL45 YP_081503.1, HHV6A U28 NP_042921.1, HHV6B U28 NP_050209.1, HHV7 U28 YP_073768.1, KSHV ORF61 YP_001129418.1. Alignment was generated using MUSCLE: multiple sequence alignment with high accuracy and high throughput (60) and phylogenetic tree was made using a neighbor-joining tree without distance corrections. Output was made using FigTree using scaled branches (61).

DNA constructs for expression in human cell lines. The full set of pcDNA3.1(+) human APOBEC-HA expression constructs has been described (62) [A3A (GenBank accession NM_145699), A3B (NM_004900), A3C (NM_014508), A3D (NM_152426), A3F (NM_145298), A3G (NM021822), A3H (haplotype II; FJ376615)]. The full set of APOBEC-mCherry expression constructs was PCR amplified

with Phusion High Fidelity DNA Polymerase (NEB M0530) from previously described A3-mCherry constructs (22) and subcloned into pcDNA5/TO (Invitrogen V103320). The forward PCR primers are as follows: A3A (5'-NNN NAA GCT TAC CAC CAT GGA AGC C-3'), A3B and A3C (5'-NNN NNA AGC TTA CCA CCA TGA ATC CA-3'), A3D (5'-NNN NNA AGC TTA CCA CCA TGA ATC CA-3'), A3F (5'-NNN NNA AGC TTA CCA CCA TGA AGC CT-3'), A3G (5'-NNN NAA GCT TAC CAC CAT GAA GCC T-3'), and A3H (5'-NNN NAA GCT TAC CAC CAT GGC TCT G-3'). The reverse PCR primer used was 5'-AGA GTC GCG GCC GCT TAC TTG TAC A-3'. PCR fragments were digested with *Hind*III-HF (NEB R3104) and *Not*I-HF (NEB R3189) and ligated into pcDNA5/TO. The full set of pLenti-iA3i-HA constructs were previously described except the puromycin resistance gene was replaced with a hygromycin resistance gene (63). Briefly, this is a lentiviral construct with an intron spanning the A3 gene with a C-terminal 3xHA tag, arranged in the antisense direction, which is expressed after reverse transcription and integration. This construct bypasses limitation of self-restriction by A3-mediated deamination of its own plasmid.

EBV BORF2 (GenBank accession V01555.2) with a C-terminal 3x-FLAG (DYKDDDDK) tag and EBV BaRF1 (Genbank accession V01555.2) with a C-terminal 3x-HA (YPYDVPDYA) tag was previously described (18). Other viral RNRs were subcloned with Phusion High Fidelity DNA Polymerase from previously described pCMV-3F vectors (18).

KSHV ORF61 (GenBank accession U75698.1) was PCR amplified using primers 5'-NNN NGA ATT CGC CAC CAT GTC TGT CCG GAC ATT TTG T-3' and 5'-NNN NGA ATT CGC CAC CAT GTC TGT CCG GAC ATT TTG T-3', digested with *Eco*RI-HF (NEB R3101S) and *Not*I-HF, and ligated into pcDNA4 with a C-terminal 3x- FLAG. The same construct was PCR amplified using primers 5'-NNN NGC GGC CGC GTC TGT CCG GAC ATT TTG T-3' and 5'-NNN NTC TAG ATT ACT GAC AGA CCA GGC ACT C-3', digested with *Not*I-HF and *Xba*I, and ligated into a similar pcDNA4 vector with N-terminal 3x- FLAG.

HSV-1 UL39 (GenBank accession JN555585.1) was PCR amplified using primers 5'-NNN NGA TAT CCG CCA CCA TGG CCA GCC GCC CAG CC-3' and 5'-NNN NGC GGC CGC CCC AGC GCG CAG CT-3', digested with *Eco*RV-HF (NEB R1395) and *Not*I-HF, and ligated into pcDNA4 (Invitrogen V102020) with a C-terminal 3x-FLAG (20). The same construct was PCR amplified using primers 5'-NNN NGC GGC CGC GGC CAG CCG CCC AGC CGC A-3' and 5'-NNN NTC TAG ATT ACA GCG CGC AGC TCG TGC A-3', digested with *Not*I-HF and *Xba*I (NEB R0145S), and ligated into a similar pcDNA4 vector with N-terminal 3x-FLAG.

Human cell culture. Unless indicated, cell lines were derived from established lab collections. All cell cultures were supplemented with 10% heat-inactivated fetal bovine serum (Gibco 16140-063), 1x Pen-Strep (Thermo Fisher 15140122), and periodically tested for mycoplasma (Lonza MycoAlert PLUS

LT07-710). No cell lines have ever been mycoplasma positive or previously treated. 293T and Vero cells were cultured in high glucose DMEM (Hyclone), U2OS cells were cultured in McCoy's 5A media (Hyclone), and HeLa cells were cultured in RPMI 1640 (Corning).

Co-immunoprecipitation experiments and immunoblots. Semi-confluent 293T cells were grown in 6-well plates and transfected with plasmids and 0.6 µL TransIT-LT1 (Mirus 2304) per 100 ng DNA in 100 µL serum-free Opti-MEM (Thermo Fisher 31985062). A titration series was performed to achieve roughly equivalent protein expression by immunoblot for the A3 panel and RNR homologue co-IP experiments. Growth medium was removed after 48 hrs and whole cells were harvested in 1 mL PBS-EDTA by pipetting. Cells were spun down, PBS-EDTA was removed, and cells were resuspended in 300 µL of ice-cold lysis buffer [150 mM NaCl, 50mM Tris-HCl, 10% glycerol, 1% IGEPAL (Sigma I8896), Roche cOmplete EDTA-free protease inhibitor cocktail tablet (Roche 5056489001), pH 7.4]. Cells were vortexed vigorously and left on ice for 30 minutes, then sonicated for 5 seconds in an ice water bath. 30 µL of whole cell lysate was aliquoted for immunoblot. Lysed cells were spun down at 13,000 rpm for 15 minutes to pellet debris and supernatant was added to clean tube with 25 µL resuspended anti-FLAG M2 Magnetic Beads (Sigma M8823) for overnight incubation at 4 °C with gentle rotation. Beads were then washed three times in 700 µL of ice-cold lysis buffer. Bound protein was eluted in 30 µL of elution butter [0.15 mg/mL 3xFLAG peptide (Sigma F4799) in 150 mM NaCl, 50 mM Tris-HCl, 10% glycerol, 0.05% Tergitol, pH 7.4]. Proteins were analyzed by immunoblot and antibodies used include mouse anti-FLAG 1:5000 (Sigma F1804), mouse anti-tubulin 1:10,000 (Sigma T5168), and rabbit anti-HA 1:3000 (Cell Signaling C29F4).

HSV-1 infections and plaque assays. The HSV-1 strains used were wild-type strain KOS1.1 (64), K26GFP (27), ICP6 deletion mutant ICP6Δ (28), and the ICP4 deletion mutant d120 (29). Titers of viral stocks were determined by plaque assay on either Vero cells (KOS1.1, K26GFP, and ICP6Δ) or ICP4-complemented E5-Vero cells (65). HSV-1 infections were carried out as described (66). For microscopy experiments, cells were infected at a MOI of 5 PFU/cell. To assay HSV-1 replication in A3-transduced U20S cells, cells were infected at a MOI of 0.001 PFU/cell and incubated for 48 h, at which time a volume of sterilized milk equal to the volume of infected cell medium was added to each well, and the cells were frozen at -80˚C. Infectious progeny virus was released by 3 cycles of freeze-thawing and titered on Vero cells. HSV-1 plaque assays were carried out in liquid media supplemented with 1% pooled normal human serum as previously described (66). For the HSV-1 plaque assays, U2OS or Vero cells were stably transduced with A3 constructs prior to carrying out plaque assays.

IF microscopy. For IF imaging of transfected cells, approximately $5x10^4$ Vero, HeLa, or U2OS cells were plated on coverslips and after 24 hrs, transfected with 200 ng pcDNA4-RNR-3xFLAG, 200 ng pcDNA5/TO-A3-mCherry, or both. After 48 hrs, cells were fixed in 4% formaldehyde, permeabilized in

0.2% Triton X-100 in PBS for 10 minutes, washed three times for 5 minutes in PBS, and incubated in blocking buffer (0.0028 M KH_2PO_4, 0.0072 M K_2HPO_4, 5% goat serum (Gibco), 5% glycerol, 1% cold water fish gelatin (Sigma), 0.04% sodium azide, pH 7.2) for 1 hr. Cells were then incubated in blocking buffer with primary mouse anti-Flag 1:1000 overnight at 4 ºC to detect FLAG-tagged RNRs. Cells were washed 3 times for 5 minutes with PBS, then incubated in secondary antibody goat anti-mouse AlexaFluor 488 1:1000 (Invitrogen A11001) diluted in blocking buffer for 2 hrs at room temperature in the dark. Cells were then counterstained with 1 µg/mL Hoechst 33342 for 10 minutes, rinsed twice for 5 minutes in PBS, and once in sterile water. Coverslips were mounted on pre-cleaned slides (Gold Seal Rite-On) using 20-30 µL of mounting media (dissolve 1g n-propyl gallate (Sigma) in 40 mL glycerol overnight, add 0.35 mL 0.1M KH_2PO_4, then pH to 8-8.5 with K_2HPO_4, Q.S. to 50mL with water). Slides were imaged on a Nikon Inverted Ti-E Deconvolution Microscope instrument and analyzed using NiS Elements.

For immunofluorescence imaging of HSV-1-infected cells, approximately $5x10^4$ Vero, HeLa, or U2OS cells were plated on coverslips and after 24 hrs, transfected with 200 ng pcDNA5/TO-A3-mCherry. After 48 hours, cells were infected with HSV-1 K26GFP, HSV-1 KOS1.1, HSV-1 KOS1.1ΔICP6, or HSV-1 KOS1.1ΔICP4 at MOI 5. Cells were fixed in 4% formaldehyde 8 hours post-infection and then IF studies proceeded as above. Time course experiments were fixed at either 3, 6, 9, or 12 hours post-infection. HSV-1 K26GFP experiments did not require primary or secondary antibody staining steps. Cells infected with HSV-1 KOS1.1 and mutants were incubated in primary antibody mouse anti-HSV-1 ICP27 H1113 (Santa Cruz sc69807) 1:1000 overnight at 4 ºC to detected HSV-1-infected cells. Secondary antibody staining, counterstaining with Hoechst, mounting, and imaging proceeded as above.

IF microscopy quantification. For quantification of A3 nuclear to cytoplasmic ratio, IF images were analyzed using Fiji software to obtain mean fluorescence intensities (MFI) of nuclear compartments determined by Hoechst stain outline and cytoplasmic compartments determined by cell outline. MFI values for each compartment were divided and plotted using Prism. Statistical analyses were performed using an unpaired Student's t-test (n.s. = not significant with $p>0.01$).

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591 **Figure Legends**

592

593 **Fig 1. Herpesvirus ribonucleotide reductases conservation.**

594 (**A**) Amino acid sequences from ribonucleotide reductase large subunits were aligned using Multiple
595 Sequence Comparison by Log-Expectation (MUSCLE) and phylogeny was constructed using
596 neighbor-joining tree without distance corrections and scaled for equal branch lengths (scale bar = 1).
597 Shaded boxes indicate herpesvirus subfamilies, which group closely to established phylogenetic trees.
598 Protein names for human herpesvirus ribonucleotide reductase large and small subunits shown on the
599 right.

600 (**B**) Schematic of representative RNR large subunit polypeptides from α-, β-, and γ-herpesviruses with
601 conserved core sequences (colored) and unique N- and C-terminal extensions (gray). Diagram is
602 approximately to scale with a ~190 amino acid portion of HSV-1 ICP6 omitted to fit the figure. Scale bar
603 is 100 amino acids.

604

605 **Fig 2. EBV BORF2 relocalizes A3B and A3A.**

606 (**A**) Representative images of U2OS cells expressing the indicated A3-mCherry construct alone or in
607 combination with a BORF2-FLAG construct. Cells were fixed 48 hours post-transfection, permeabilized,
608 and stained with anti-FLAG antibody and Hoechst.

609 (**B**) Representative images of Vero cells expressing A3A/B-mCherry alone or in combination with
610 BORF2-FLAG. Cells were fixed 48 hours post-transfection, permeabilized, and stained with anti-FLAG
611 antibody and Hoechst.

612

613 **Fig 3. KSHV ORF61 relocalizes A3B and A3A.**

614 (**A**) Co-immunoprecipitation of transfected KSHV ORF61-FLAG with the indicated A3-HA constructs in
615 293T cells. Cells were lysed 48 hours post-transfection for anti-FLAG pulldown and resulting proteins
616 were analyzed by immunoblot. EBV FLAG-BORF2 transfected with A3B and A3G were used as positive
617 and negative co-IP controls, respectively.

618 (**B**) Representative images of U2OS cells transfected with either A3-mCherry or FLAG-RNR constructs.
619 Cells were fixed 48 hours post-transfection, permeabilized, and stained with anti-FLAG antibody and
620 Hoechst. Co-transfection with A3B-mCherry and EBV BORF2-FLAG was used as positive controls for
621 relocalization from nuclear to cytoplasmic aggregates. A3 localization was compared in the presence and
622 absence of KSHV ORF61-FLAG co-transfection.

623

624 **Fig 4. HSV-1 ICP6 binds and relocalizes A3B and A3A.**

(**A**) Co-immunoprecipitation of transfected HSV-1 FLAG-ICP6 with the indicated A3-HA constructs in 293T cells. Cells were lysed 48 hours post-transfection for anti-FLAG pulldown and resulting proteins were analyzed by immunoblot. EBV FLAG-BORF2 transfected with A3B and A3G were used as positive and negative co-IP controls, respectively.

(**B**) Representative images of U2OS cells transfected with either A3-mCherry or FLAG-RNR constructs. Cells were fixed 48 hours post-transfection, permeabilized, and stained with anti-FLAG antibody and Hoechst. Co-transfection with A3B-mCherry and EBV FLAG-BORF2 was used as positive controls for relocalization from nuclear to cytoplasmic aggregates. A3 localization was compared in the presence and absence of HSV-1 FLAG- ICP6 co-transfection.

Fig 5. HSV-1 infection relocalizes A3B and A3A.

(**A**) Representative images of U2OS cells transfected with A3-mCherry constructs, followed by mock or HSV-1 K26GFP infection 48 hours post-transfection. Cells were fixed 8 hpi and stained with Hoechst, then imaged directly. The viral capsid protein VP26 is tagged with GFP which marks infected cells.

(**B**) Quantification of A3 localization patterns in U2OS cells after mock infection or infection with different HSV-1 strains. The mean fluorescence intensity of the nuclear signal was divided by that of the cytoplasmic compartment. Statistical analysis was performed using an unpair Student's t-test between indicated groups ($p>0.01$, n.s. = not significant).

Fig 6. Time course of HSV-1-mediated relocalization of A3B and A3A.

Representative images of U2OS cells transfected with A3-mCherry constructs, followed by mock or HSV-1 KOS1.1 infection 48 hours post-transfection. Cells were fixed at either 3, 6, 9, or 12 hpi and stained with anti-ICP27 antibody to mark infected cells and Hoechst to stain the nuclear compartment.

Fig 7. A3B and A3A relocalization is dependent on HSV-1 ICP6.

(**A**) Representative images of Vero cells transfected with A3-mCherry constructs, followed by mock, wild-type HSV-1 KOS1.1, or HSV-1 KOS1.1ΔICP6 infection 48 hours post-transfection. Cells were fixed 8 hours after HSV-1 infection, permeabilized, and stained with anti-ICP27 antibody to mark infected cells and Hoechst.

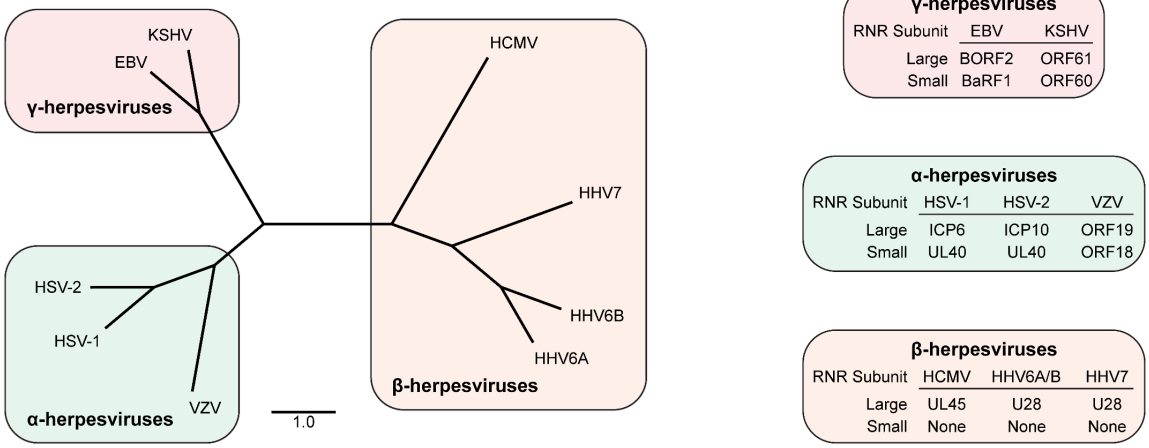
(**B**) Representative images from an experiment similar to that described in panel A, except using U2OS cells and the mutant virus HSV-1 KOS1.1ΔICP4.

Fig 8. A3B and A3A do not impact HSV-1 virus replication or plaque formation.

(**A**) Bar plot of HSV-1 virus titers produced from HFF-1 cells stably transduced with control vector or the indicated HA-tagged A3 constructs. Cells were infected in triplicate at a MOI of 0.002 PFU/cell with either HSV-1 KOS1.1 or KOSΔICP6. The infected cultures were harvested at 48 hpi and titered on Vero cells to determine the level of viral progeny production. Statistical analysis was performed using an unpaired Student's t-test ($p>0.01$, n.s., for all comparisons).

(**B**) Bar plot of KOS1.1 or KOSΔICP6 mutant stock titers determined on U2OS or Vero cells stably transduced with control vector or the indicated HA-tagged A3 constructs. The cells were fixed at 72 hpi and stained with Giemsa for counting.

A



γ-herpesviruses

RNR Subunit	EBV	KSHV
Large	BORF2	ORF61
Small	BaRF1	ORF60

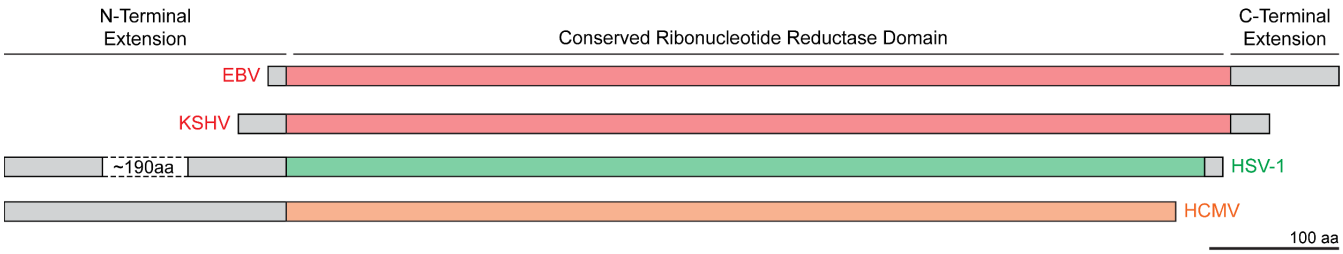
α-herpesviruses

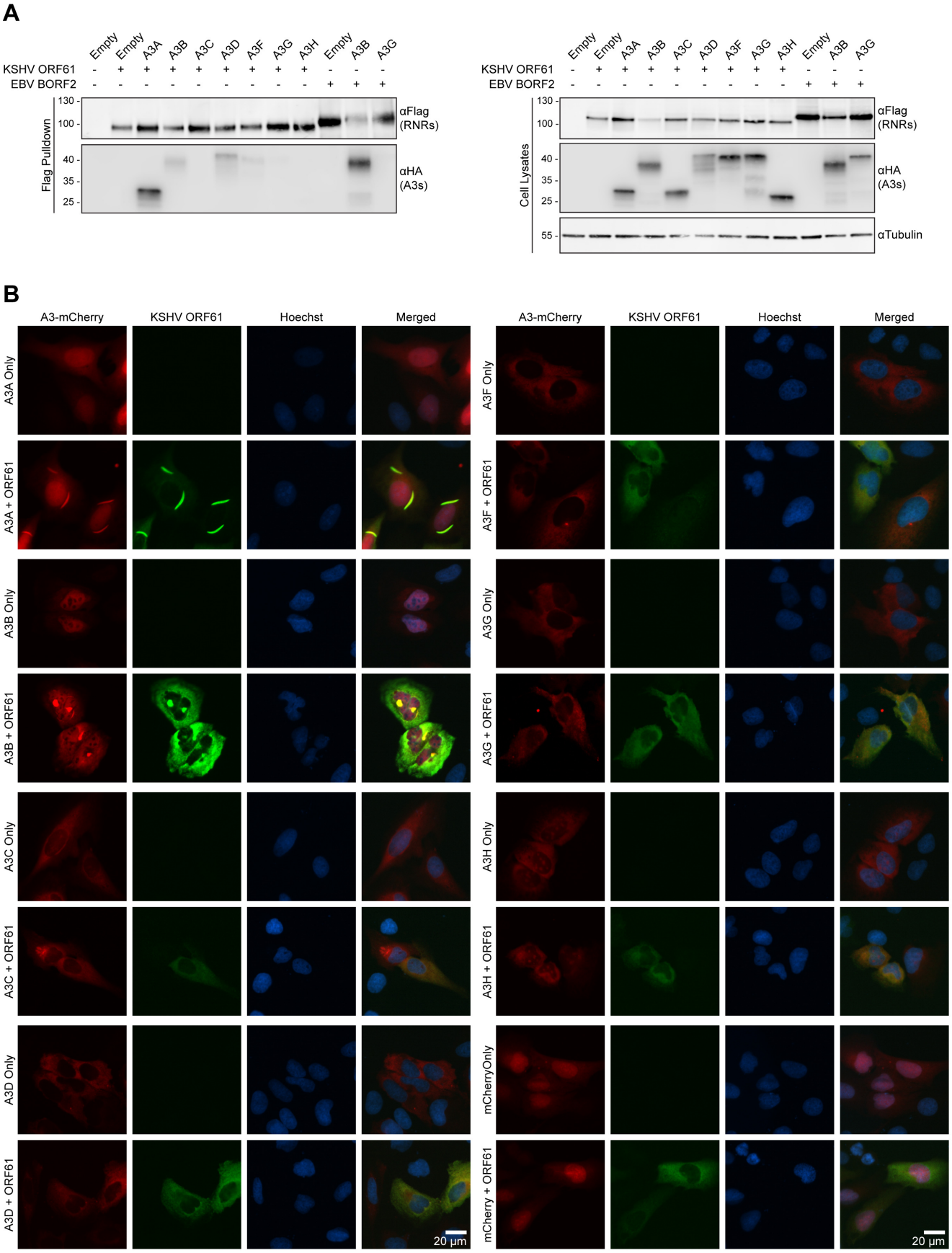
RNR Subunit	HSV-1	HSV-2	VZV
Large	ICP6	ICP10	ORF19
Small	UL40	UL40	ORF18

β-herpesviruses

RNR Subunit	HCMV	HHV6A/B	HHV7
Large	UL45	U28	U28
Small	None	None	None

B





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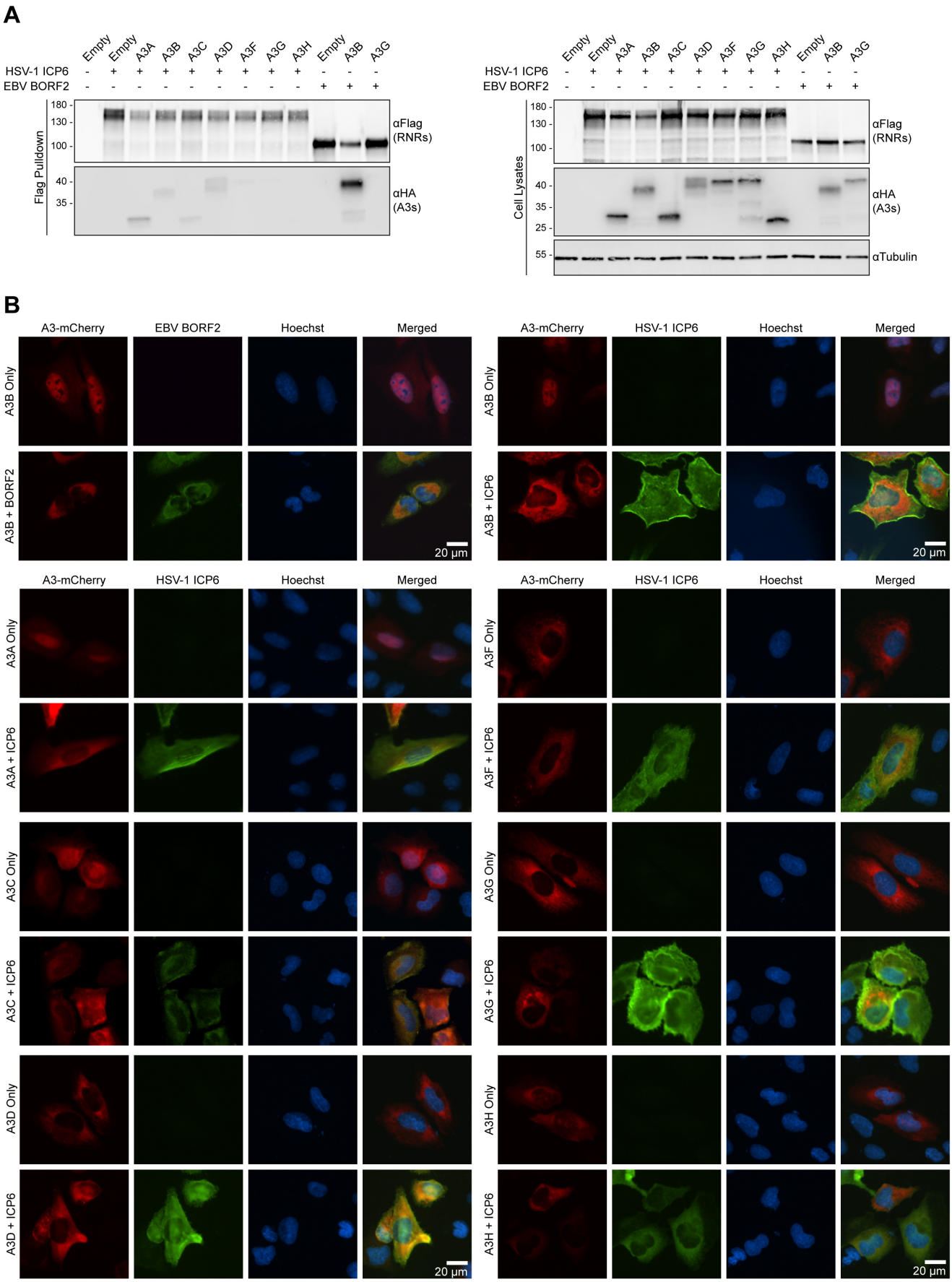

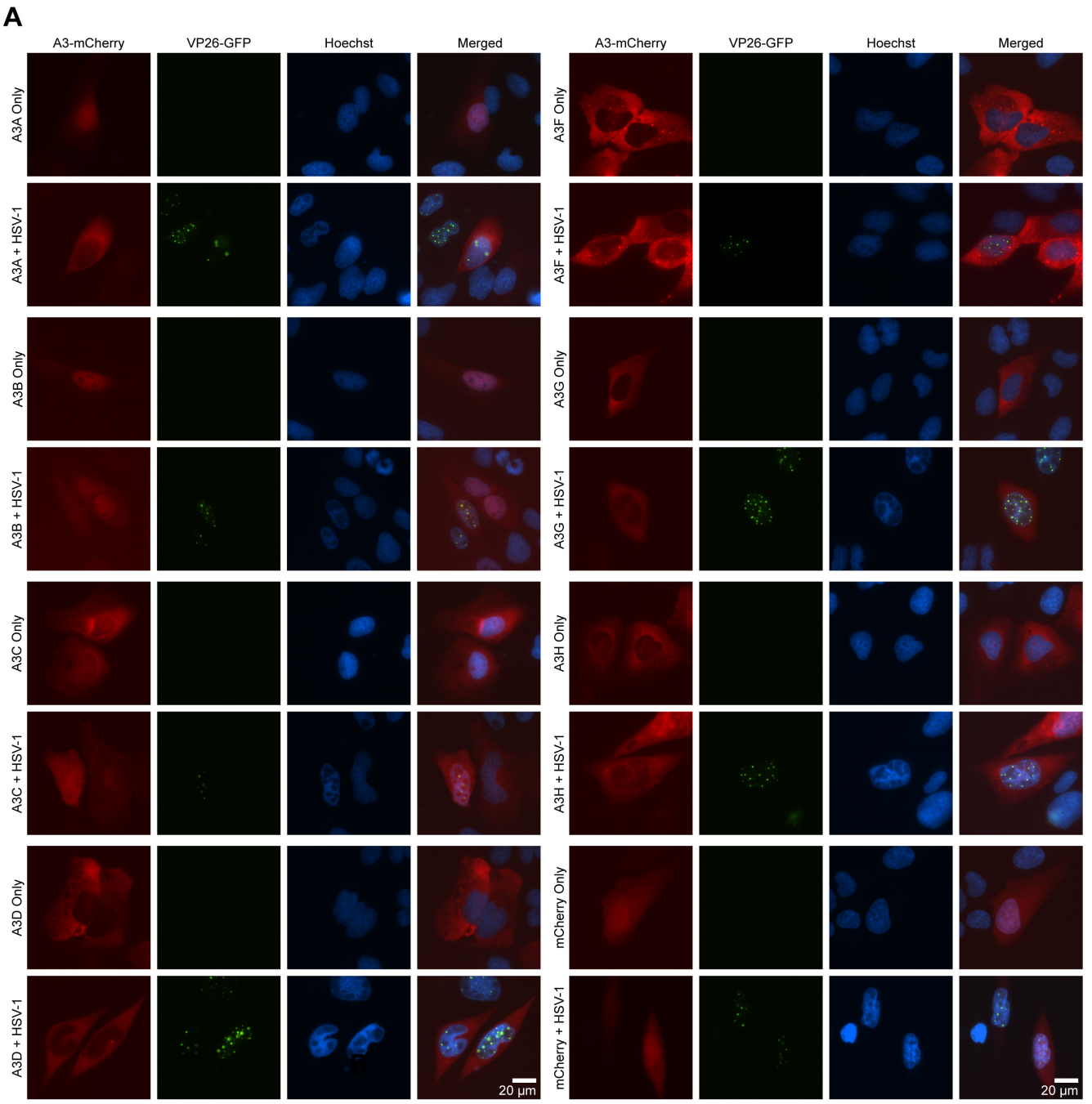

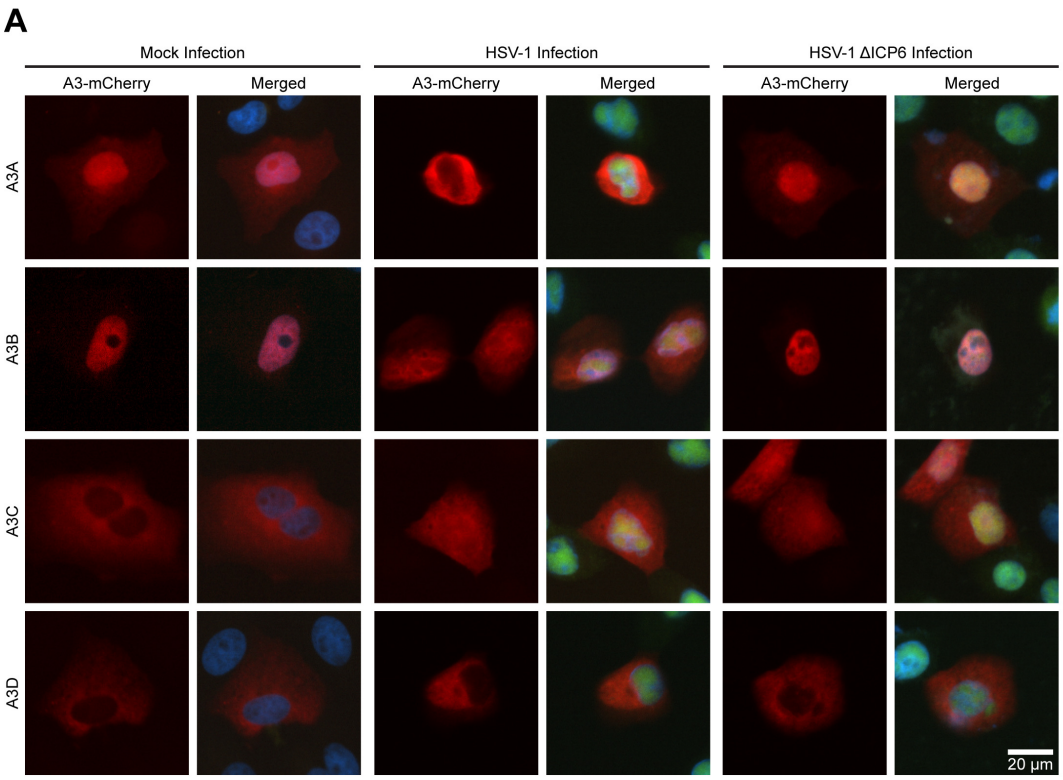

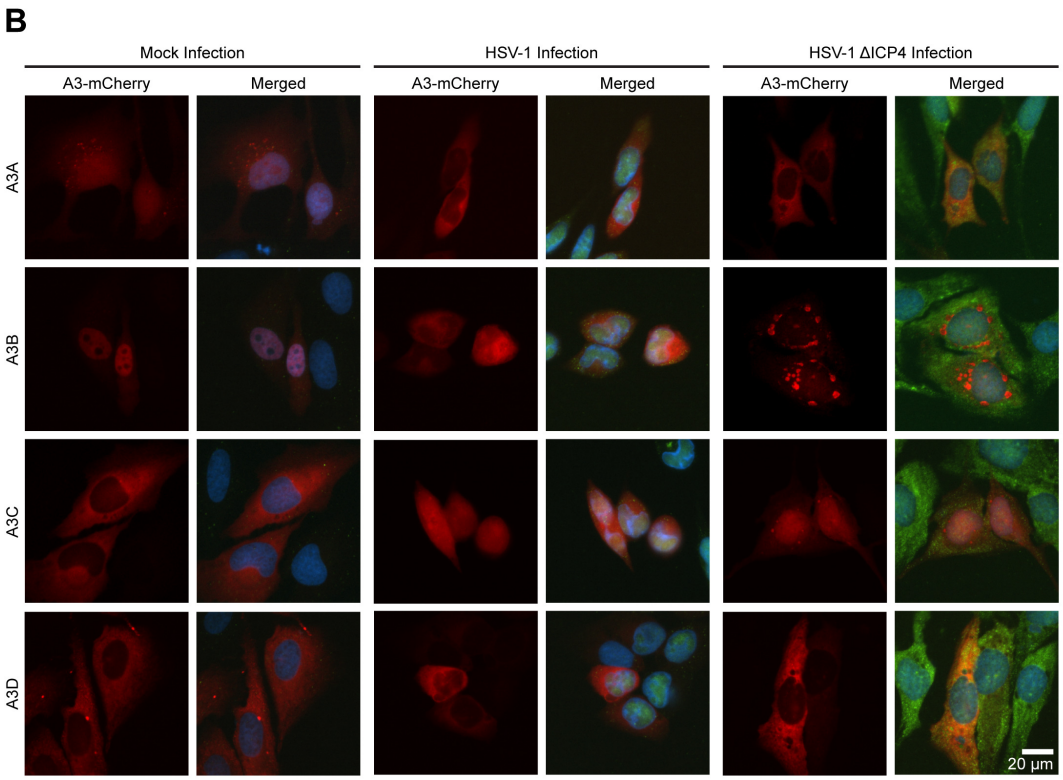

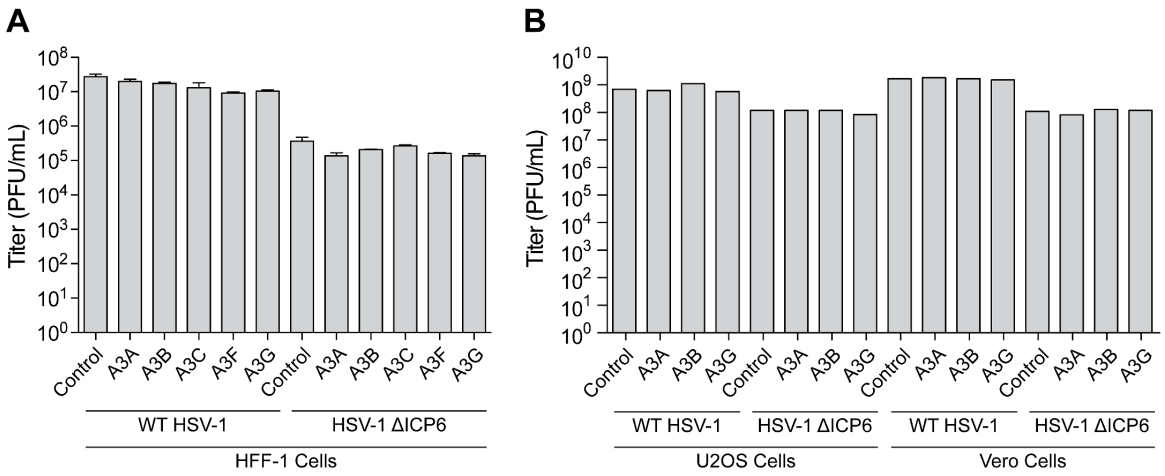

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