Catch me if you can: the arms race between human cytomegalovirus and the innate immune system [Ganna Galitska*, Matteo Biolatti*, equal contribution)

Article title: Catch me if you can: the arms race between HCMV and the Innate Immune System

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Abstract:

Human cytomegalovirus (HCMV) is a common opportunistic pathogen of significant clinical importance, targeting immunocompromised individuals of the human population worldwide. The absence of a licensed vaccine and the low efficacy of currently available drugs remain a barrier on a way of combating the global infection. The HCMV ability to modulate and escape innate immune responses remains a critical step in the ongoing search for potential drug targets. In this review, we discuss the interplay between HCMV and the innate immune system, focusing on different evasion strategies that HCMV has evolved to escape the host immunity. We particularly 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.

Keywords: human cytomegalovirus, antiviral restriction factors, innate immunity, interferon, NF-κB signaling, viral evasion**,** immune modulation

Background

Human cytomegalovirus (HCMV), a prototype member of β-herpesvirus subfamily, is a ubiquitous herpesvirus that contains the largest genome among the all known human viruses, and which is able to successfully establish a lifelong persistence with spontaneous reactivation periods within the infected hosts [1,2]. An important 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 hosts, but it often leads to severe complications and even fatal disease in immunocompromised individuals, such as organ transplant recipients or AIDS patients [3,4]. Neonates with immature immune systems are also at high potential risk of HCMV congenital infection, which often results in severe birth defects and permanent neurological sequelae, including deafness, blindness and long-term mental impairment of an infected newborn [5–8]. Furthermore, HCMV may contribute to immunosenescence in the elderly population [9,10] and is associated with a number of autoimmune diseases [11–14], inflammatory and vascular diseases [15–19], as well as some cancers [20–24].

However, despite its clinical importance, there are currently no vaccines to prevent HCMV infection and only a few antiviral drugs are licensed for treatment, 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-resistant 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 number of elaborate strategies to suppress and evade host immune responses, allowing it to achieve both high infection efficiency and wide dissemination within the host [32]. As a virus with an enormously large genome, encoding over 200 ORF, HCMV potentially employs hundreds of proteins with modulatory functions to facilitate 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 detecting and counteracting the infection by building up a robust immune response in wide frontiers, f.i. by involving various DNA sensing mechanisms and host restriction factors (RFs). This idea is clearly supported by the observation that primary HCMV infections in

immunocompetent hosts are virtually asymptomatic, while severe HCMV disease occurs mostly in immunocompromised individuals.

In this review, we will discuss the complex interplay between HCMV and the host immune system, focusing on different evasion strategies that HCMV has evolved to escape the innate immune response. We will particularly highlight the mechanisms and role of various restriction factors involved in the antiviral response, along with the newest insights into viral modulation of pro-inflammatory nuclear factor-κB (NF-κB) and interferon (IFN) signaling pathway.

Considering the importance of predicting the outcomes of HCMV infection, further elucidating the roles of viral innate immune modulators remains a high priority in expanding of our understanding of viral pathogenesis, which may potentially lead to development of an HCMV vaccine and/or more effective therapeutics.

HCMV restriction factors

It is well known that susceptibility to viral infection and disease is partly determined by antiviral restriction factors (RFs). RFs represent a wide group of host proteins that counteract or "restrict" viral replication by directly interfering with the activity of essential viral/cellular genes and represent a first line of defense against invading viruses. During the evolutionary "arms race" for survival, viral proteins have successfully evolved to modulate or degrade RFs.

Early pioneering studies on retroviruses have identified two major host RFs: the apolipoprotein B editing catalytic subunit-like 3 (APOBEC3) class of cytidine deaminases and tetherin [33–36]. An intense 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 [37]. So far, several host proteins, including IFI16 (γ-interferon-inducible protein 16), nuclear domain 10 (ND10) complex, viperin, APOBEC3, survival time-associated PHD [plant homeodomain] protein in ovarian cancer 1 (SPOC1), and Myxovirus resistance B (MxB), have been identified as RFs of HCMV replication. Interestingly, HCMV, in its turn, has evolved effective countermeasures to resist them (Figure 1). Below, we discuss the abovementioned RFs in detail, leaving out ND10 even though it is a very important RF of HCMV, because this topic has been previously addressed in numerous works [38–45].

1. IFI16.

IFI16 is a widely known key player in the intrinsic resistance against a variety of viruses, acting both as a viral DNA sensor during the early stages of infection and a repressor of viral gene transcription in the later stages. Its antiviral activity has been extensively studied for the last decade. The role of IFI16 as a restriction factor has been reported for several viruses, including HCMV [46–48]. The work of Gariano *et al.* [46] has demonstrated that the silencing of IFI16 expression in human embryo lung fibroblasts (HELFs) by transfecting specific siRNAs or inactivation of IFI16 protein by introduction of lentiviruses expressing a dominant negative mutant form of the protein led to significantly enhanced HCMV replication. Consistent with these results, IFI16 overexpression decreased viral production. The molecular mechanism of IFI16 inhibitory activity relies on its ability to bind and block Sp1-like transcription factors on the viral DNA polymerase UL54 promoter [46]. However, HCMV has evolved evasion strategies to counteract IFI16 activity that result in translocation of this protein from the nucleus to the cytoplasm during the late stages of infection. UL97, a viral-encoded protein kinase, acts as a key mediator of the HCMV-induced nuclear egression of IFI16: upon binding to UL97 phosphoprotein, IFI16 is subject to phosphorylation, which in turn drives its nucleo-cytoplasmic translocation. Later on, the endosomal sorting complex required for transport (ESCRT) machinery facilitates the incorporation of IFI16 into the virus assembly complex. Finally, IFI16 becomes hijacked and trapped in the newly formed virions [49]. Along with UL97, HCMV pp65, another co-partner in crime, has recently been reported to be involved in HCMV escape by interacting with IFI16, thus targeting early gene promoters including that of viral DNA polymerase UL54 [50]. From the literature, this pp65/IFI16 interaction undoubtedly constitutes a very dynamic and controversial interplay, thus remaining a matter of debate.

Previous work by Cristea *et al.* [51] has shown that in the early phases of HCMV infection, pp65 recruits IFI16 to the HCMV the major immediate-early promoter (MIEP), thereby triggering an increase in immediate-early (IE) protein expression, accompanied by a concomitant decrease in antiviral cytokine production, while at later time points, pp65 potentially protects IFI16 from proteasome-mediated degradation, sustaining its inhibitory activity at the level of the UL54 gene promoter [50].

The most recent findings have shown that cellular DNA sensor cyclic GMP-AMP synthase (cGAS) represents another important interactor of IFI16, albeit these proteins display different functions. IFI16 interacts with cGAS through the Pyrin domain [52], but while IFI16 induces antiviral cytokine expression, including IFN-β, only cGAS effectively activates STING/TBK-1/IRF3 and apoptotic responses upon Herpes Simplex Virus type I (HSV-1) and HCMV infections [52,53]. Since pp65 tegument protein interferes with the activities of all the

components of this signaling pathway (i.e., cGAS, STING, and IRF3) to evade the IFN response, this clearly underlines the importance of IFN system in blocking virus replication. Therefore, it may be beneficial to further elucidate the mechanisms through which HCMV inhibits cGAS/STING/IRF3 signaling in order to not only expand the general understanding of this complex pathway but also to potentially facilitate the development of therapeutic interventions aimed at combating multiple diseases in which the pathway is altered.

2. Viperin.

Viperin is an IFN-inducible iron-sulfur (Fe-S) cluster-binding protein, induced in several cell types by a variety of viruses, including HCMV. It appears to have a number of critical functions, from acting as an antiviral protein by modulating signaling events and has recently received increased attention due to its paradoxical role in innate immunity. It has been shown that viperin exploits its antiviral activity in the later phases of HCMV replication, as indicated by the reduced synthesis of early-late (pp65), late (gB) and true late (pp28) genes in stably viperin-expressing fibroblasts [54].

While it has been established that viperin is an HCMV-inducible protein, this brings into question why a virus would directly stimulate the expression of a protein that is known to negatively impact its replication. Intriguingly, evidence seems to indicate that HCMV has evolved additional mechanisms capable of not only subverting the antiviral activity of viperin, but also co-opting the protein to its own advantage by exaggerating its natural function to facilitate the replication. Firstly, HCMV encodes a viral mitochondrion-localized inhibitor of apoptosis (vMIA) protein that binds viperin and translocates it from the endoplasmic reticulum to the mitochondria, where viperin inhibits fatty acid β-oxidation, reduces the generation of ATP, and disrupts the actin cytoskeleton, thereby enhancing viral infectivity [55,56]. This may also potentially reflect a viral sub-strategy to create an inhibitory environment for viruses other than HCMV. Secondly, viperin is also responsible for enhanced lipid synthesis observed in HCMV-infected cells through transcriptional induction of key players of fatty acid metabolism, such as AMP-activated protein kinase (AMPK) and the glucose transporter type 4 (GLUT4). This results in an increase in glucose import along with translocation to the nucleus of the glucose-activated transcription factor ChREBP, followed by enhanced lipid synthesis. The final outcome is the generation of the viral envelope and the optimal production of infectious viruses [57].

Overall, the evidence indicates that viperin is the key regulator of HCMV-induced modulation of lipid metabolism, and it may present a novel target for HCMV therapy.

3. APOBEC3

The APOBEC3 (A3) family of enzymes consists of seven members (A, B, C, D, F, G, and H) that catalyze the deamination of cytidine nucleotides to uridine nucleotides in single-stranded DNA and RNA substrates [58]. These proteins are widely acknowledged as fundamental players in the defense against viruses, particularly against human immunodeficiency virus type 1 (HIV-1) [59] 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, including hepatitis B virus (HBV) [60,61], and parvoviruses [62,63]. Moreover, different A3 isoforms deaminate human papillomavirus (HPV) genomes [64], as well as BK polyomavirus (BKV) [65]. Genomes of some herpesviruses, such as HSV-1 and Epstein-Barr virus (EBV), are edited by A3 on both strands [66]. The identification of new potential A3 targets is currently ongoing.

Recently, Weisblum *et al.* [67] reported the role of APOBEC3A (A3A) editing activity in the context of HCMV infection and vertical viral transmission, and reported that A3A acts as a potent restriction factor 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 A3A induction by HCMV has not been observed in human fibroblasts, epithelial cells or chorionic villi maintained in organ culture, which may suggest that HCMV-mediated upregulation of A3A expression is cell- and tissue-specific. The results of the study greatly contribute to better understanding of the innate mechanisms acting to limit transplacental HCMV transmission. However, even though the results may shed light on important insights regarding A3A activity against HCMV, many questions regarding A3 specificity in different cells and tissues remain unresolved. For instance, it is not known whether HCMV is able to induce other A3 family members besides A3A in other HCMV-susceptible cell types.

To start approaching this issue, we have recently obtained evidence that APOBEC3G (A3G) is strongly induced upon HCMV infection in human foreskin fibroblasts (HFFs) and its induction appears to be mediated by IFN-β. However, upon overexpression or gene knockout A3G did not act as a restriction factor for HCMV replication in HHFs. Thus, we suggest that throughout evolution, under intense selective pressure, HCMV has shaped its genome

nucleotide composition in order to escape A3G-mediated immune surveillance. 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 observed for the other target motifs of A3 members [68]. 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 have been shown to be susceptible to A3-mediated restriction, f.i. vaccinia virus does not appear to be inhibited by APOBEC family members, perhaps due to the sequestration of its replication complex in cytoplasmic bodies [69]. Considering this fact, it is possible that DNA viruses may avoid APOBEC-mediated restriction by encoding an undiscovered inhibitor, preventing incorporation into virions, avoiding induction A3 enzymes, replicating in privileged subcellular locations or, alternatively, in cells with low levels of A3 [70].

4. SPOC1.

The cellular protein SPOC1, also known as PHD finger 13 (PHF13), was initially described 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 [71], cell proliferation [72], and DNA damage response [73,74], acting as a potent regulator of chromatin structure [72,74]. It has been proposed that the interaction of the SPOC1 with chromatin occurs through harboring a C–terminus located PHD, which, in its turn, senses histone marker H3K4me2/3, enabling SPOC1 binding. Upon binding, SPOC1 triggers chromatin compaction by recruiting histone methyltransferases (HMTs), i.e. SETDB1, G9A, or GLP, resulting in an increase of repressive H3K9me3 [74]. 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 centrally located domain, simultaneous with chromatin affiliated polycomb repressive complex 2 (PRC2) and RNA Pol II, thereby acting in a multivalent fashion [75]. This feature of direct binding of DNA, 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 response [74], as it has been recruited to DNA double-strand breaks (DSBs) in an ATM-dependent manner.

In addition to its cellular regulatory functions, SPOC1 also contributes to the intrinsic defense against viral infections [76]. As described by Schreiner et al SPOC1 protein levels 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 ligase complex E1B-55K/E4orf6 [76]. 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 [76].

In a recent study, Reichel and colleagues [77] addressed the role of SPOC1 in HCMV infection. Intriguingly, in contrast to HAdV5 and HIV-1 infection, they observed that SPOC1 protein level is upregulated during the early steps of HCMV infection, whereas in late replication phase it degrades in a glycogen synthase kinase 3β (GSK-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. Interestingly, it has been shown that SPOC1 associates with the HCMV MIEP region, supporting the scenario of SPOC1-induced silencing of viral IE expression via epigenetic modifications [77].

5. Mx

The Mx proteins are interferon-inducible dynamin-like large GTPases that play a significant role in innate immune responses by exhibiting a potent activity against a wide range of RNA and DNA viruses [78,79]. Two human genes, *MX1* and *MX2* encode the MxA and MxB proteins under the strict 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 [78], while the function of MxB has remained unknown until recently when it was identified as a potent inhibitor of HIV-1 [80–83]. In this regard, Mitchell *et al.* [84] analyzed *MX2* evolution in primates, suggesting that MxB most likely extends its antiretroviral activity to a broader antiviral specificity.

Recently, MxB has been described blocking the replication of Murine gammaherpesvirus 68 (MHV68), a member of the gamma herpesvirus family. Schilling *et al.* [85] 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 restriction factor 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 exhibits an antiviral conformation able to recognize and block the herpesviruses, potentially targeting viral capsids accumulating at the nuclear pore and/or affecting components of the nuclear pore complexes (NPC) itself, and, as a result, hindering viral uncoating. This model can be supported by a recent study that showed a defect in the delivery of viral DNA into the nucleus of HSV-1-infected cells, suggesting the role of MxB as a "cytoplasmic gatekeeper" against herpesviruses [86].

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

Undoubtedly, the complex bidirectional relationship between HCMV and host immunity, including virus recognition and the subsequent immune response, is a major determinant of HCMV pathogenesis. The earliest events of the host response typically involve innate immune sensing of infection in non-immune cells, induction of an antiviral alarm state, secretion of antiviral cytokines that both help neighboring cells resist infection as well as recruit and activate defending immune cells. However, many questions currently remain unanswered about the specific mechanisms and biochemical pathways involved in the viral-host interplay, as well as the contexts through which they contribute to viral pathogenesis, replication, persistence, and infection outcome. 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 HCMV evasion strategies that alter interferon (Figure 2) and NF-κB (Figure 3) signaling pathways, focusing on what is currently known about how viral genes modulating them in order to assure successful replication and persistence, as well as the key points that remain unresolved.

1. Evasion of the Interferon Response by HCMV

Upon detection of viral pathogens, intracellular pattern recognition receptors (PRRs) trigger a cascade of events leading to the activation of numerous transcription factors, including mitogen activated protein kinases (MAPKs), NF-κB, IRF3, and IRF7, which mediate the transcriptional induction of IFNs and the release of pro-inflammatory chemokines that drive immune cells to the site of infection [87,88]. IFNs are a subset of cytokine molecules classified

into three distinct groups, namely type I IFN (IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω), type II IFN (IFN-γ) and type III IFN (IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4), that regulate a variety of vital processes, such as cell proliferation, apoptosis, autoimmunity, cancer biology, and defense against viral infections [87,89].

In the context of HCMV infection, the interferon response appears as a complex phenomenon with multiple distinct mechanisms of IFN activation and temporal peaks of IFN activity occurring during the viral life cycle. The initial IFN response to HCMV infection is triggered when the cell detects viral attachment and entry, resulting in early IFN synthesis and secretion. A significant and growing number of newly identified cellular sensors, activated upon HCMV binding and entry, aim to detect the invader. So far, the toll-like receptor2 (TLR2) and CD14 receptors that interact with viral gB and gH, along with intracellular dsDNA sensors Z-DNA binding protein 1 (ZBP1) [90], TLR9 [91] , and cGAS [91] have been shown to be capable of detecting the presence of the viral genomes in the host cell [92]. In conjunction to these cellular defenses, HCMV has evolved to have a line of countermeasures (Figure 1).

Results from several groups [53,93–95] have shown that HCMV pp65 acts as the main inhibitor of the IFN-I response, although it remains unclear at what level pp65 counteracts IFN activation. Browne *et al*. [94] have shown that pp65 suppresses the induction of some, but not all, IFN-responsive genes by preventing the activation of NF-κB and IRF1. In contrast, Abate *et al.* [93] have demonstrated that pp65 promotes IRF3 dephosphorylation and its export from the nucleus, affecting the balance of nuclear-cytoplasmic shuttling [96]. Finally, recent studies by Biolatti *et al.* [53] have shown that pp65 binds cGAS and inhibits the release of a biologically active cGAMP, blocking its interaction with STING, thereby impairing the cGAS/STING signaling pathway. In addition, Huang and colleagues [97] have demonstrated that the human cytomegalovirus protein UL31, similarly to pp65, acts as an inhibitor of cGAS. Specifically, they showed that UL31 interacts directly with cGAS and disassociates DNA from cGAS, thus inhibiting cGAS enzymatic functions and reducing cGAMP production.

Another main player of HCMV evasion from the IFN response is the HCMV tegument protein pp71 (pUL82) [98], which interacts with STING and iRhom2, thereby disrupting the STING-iRhom2-TRAPb complex and blocking STING trafficking. As a consequence, the assembly of the STING/TBK1/IRF3 complex required for the innate antiviral response is severely impaired.

A recent study by Choi *et al.* [99] has shown that HCMV glycoprotein US9 inhibits the IFN-β response by targeting both mitochondrial antiviral-signaling protein (MAVS) and STING–TBK1 signaling pathways during late stages of HCMV infection. In detail, US9

disrupts STING oligomerization and STING/TBK1 association through competitive interaction, thus blocking the IRF3 nuclear translocation and IFN-β production. The study demonstrated that deletion of the C-terminal region of US9 diminishes its ability to dampen the MAVS- and STING-mediated IFN response, suggesting that the C-terminal domain of US9 is critical for protein function of immune evasion [99].

In addition, several studies reported that the HCMV immediate-early 2 protein (IE2) affects the production of IFN-β by blocking the binding of NF-κB to the IFN-β promoter [100–102]. In agreement with these findings, reduced protein levels of STING were observed in cells expressing IE2 protein, suggesting that IE2 could also target STING to inhibit IFN-I signaling [103].

Finally, HCMV tegument proteins have also been shown to impact the modulation of the type II IFN response, which is generally less well-characterized than HCMV-mediated impact on type I IFN signaling. In detail, it is established that upon viral infection, IFN-γ activates Janus kinase/signal transducers and activators of transcription (JAK–STAT) cellular signaling pathway. The transcription of type II IFN-dependent genes is regulated by STAT1, the most important transcription factor that binds and activates transcription at promoters containing gamma-activated sequence (GAS) elements. After binding of IFN-γ to its receptor, JAK1 and JAK2 are activated and regulate the downstream phosphorylation of STAT1 to form STAT1–STAT1 homodimers, which later are transported to the nucleus and bind to GAS elements, thereby inducing transcription of interferon stimulated genes [104]. In this context, human N-myc interactor (Nmi) protein, is an important interactor of STAT1, important for the activation of IFN-γ induced STAT1-dependent transcription. Interestingly, recent work by Feng *et al.* [105] demonstrated that HCMV UL23 protein specifically interacts with Nmi, inhibiting nuclear translocation of Nmi and its associated protein STAT1, leading to a decrease of IFN-γ expression and an increase of viral resistance to IFN-γ to achieve immune evasion from IFN-γ responses. In line with the hypothesis, blocking of UL23 expression led to higher transcription of IFN-γ stimulated genes and significant inhibition of viral growth in infected cells [105].

In parallel, there is significant effort to understand the relationships between individual interferon genes and tegument proteins. For instance, cellular Interferon stimulated gene (ISG15) encodes a ubiquitin (Ub)-like protein that can bind host and viral protein targets in a manner similar to ubiquitin. Several studies reported ISGylation as an anti-viral defense mechanism upon early HCMV infection via cGAS-STING viral DNA sensing, resulting in inhibition of HCMV replication [106,107]. As predicted, HCMV US26 protein has been recently shown to interact with cellular ISG15, as well as with several enzymes which enable

activation and ligation of ISG15 to the target proteins [106]. However, many questions about these interactions and their contribution to the infection outcome remain open.

In addition to proteins involved in cytoplasmic DNA sensing, the host cell also produces proteins that sense cytoplasmic dsRNA and activate similar responses, such as protein kinase R (PKR) signaling [108], that can result in a diverse array of immune responses including stimulation of type I IFN production [109], as well as enhanced NF-κB activity [110]. As per usual, in the ongoing arms race, HCMV employed two HCMV IE gene products shown to specifically target PKR as a means of downregulating the IFN response and maintaining high levels of viral gene transcription during infection: IRS1 and TRS1. A study by Marshall *et al*. [111] demonstrated that deletion mutants of IRS1 and TRS1 both individually and in tandem did not strongly affect viral growth. By contrast, infection with an IRS1/TRS1 double deletion mutant resulted in an extreme reduction in protein synthesis and failure to replicate in HFF [111]. Yet, Ziehr *et al.* [112] reported that infection outcome correlated to levels of PKR activation, and that siRNA silencing of PKR rescued viral growth in the context of simultaneous loss of IRS1 and TRS1. This data suggests that modulation of IFN signaling by IRS1 and TRS1 directly contributes to the infectious outcome.

To sum up, HCMV has developed sophisticated mechanisms to perturb the host IFN response. The recent findings contribute to our understanding of the molecular mechanisms employed by HCMV to evade from the host innate immune system. Knowledge of these mechanisms will greatly assist in future development of therapeutic interventions to treat autoimmune diseases that are characterized by the chronic production of cytokines, including type I IFN.

2. HCMV and NF-κB Signaling

The NF-κB signaling network regulates a variety of pro-inflammatory processes that ultimately shape innate and adaptive immune responses via transcriptional regulation of numerous NF-κB responsive genes. NF-κB signaling can be activated by a myriad of inducers, including paracrine signaling factors, environmental stress, or viral pathogens, 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 the cytoplasm. The viral pp65 protein, as discussed above, likely plays a role in blocking the IFN response during early

infection that is not yet fully understood. Besides the IFN modulatory role, it has been suggested that pp65 may act as a potent regulator of the NF-κB pathway. In support to this hypothesis, Browne and Shenk [94] demonstrated that infection with a pp65-deficient mutant HCMV increases the accumulation of NF-κB target genes and induces the nuclear binding activity of NF-κB transcription factors. However, it remains unknown the exact way pp65 modulates NF-κB or whether its modulation of IFN and NF-κB network may be functionally related.

Another HCMV tegument protein, UL26, has also been shown to impact NF-κB activity. As it has been demonstrated, a UL26 deletion mutant virus is severely attenuated, while UL26 inhibits IKK complex phosphorylation and NF-κB translocation during infection [113,114]. Furthermore, expression of UL26 by itself is sufficient to block TNFα-mediated NF-κB activation [113,114]. Although UL26 is a tegument protein, it seems that it is unable to block activation of NF-κB during early stage of infection, but rather has a strong effect during the late phase of infection, when being detected in cytoplasm, as opposed to the early stage when it localizes in the nucleus [115]. At the same time, it is worth mentioning that the possibility of an interaction between UL26 and NF-κB at the early phase of infection cannot be ruled out, as UL26-deficient virus is more sensitive to challenge with TNFα [113]. Notably, UL26 is capable of inhibiting IκB kinase (IKK) complex activation via diverse upstream stimuli including TNFα signaling, suggesting that it acts at the level of the IKK complex, where these signaling cascades converge [113]. Nonetheless, the exact mechanism of UL26's inhibition of NF-κB signaling remains to be determined.

Interestingly, HCMV tegument proteins are also capable of inducing pro-viral NF-κB signaling. UL76, a viral tegument-associated endonuclease, has been shown to activate the canonical NF-κB pathway through the DNA damage response, inducing IL-8 production which is dependent on the cellular kinases Ataxia-telangiectasia mutated (ATM) and IKKβ [116]. In this regard, induction of IL-8 is particularly important during HCMV infection as neutrophils, primarily attracted by IL-8, play a key role in virus dissemination. Moreover, IL-8 has a positive effect on the replication of HCMV. However, the same study indicates that upon HCMV infection, besides UL76, other genes may be responsible for induction of IL-8 expression, at least in part, through activation of ATM. A UL76 deletion mutant has a significant growth defect [117], but the effect of increased IL-8 production on this attenuation is not fully understood.

It is known that several cellular mRNAs and proteins become incorporated into HCMV virions [118,119]. Potentially, these cellular-derived virion-associated factors could also be modulating NF-κB signaling in addition to viral factors. For instance, the virion packaging of

casein kinase II (CKII) has been found in the virion tegument and has been reported to activate NF-κB signaling through phosphorylation of the IκB repressor, thereby releasing the associated NF-κB subunits to localize to the nucleus and induce NF-κB-dependent transcription [120].

In addition to tegument proteins, the HCMV IE proteins expressed upon MIEP stimulation interact with the NF-κB pathway in diverse ways, f.i. IE1 acts as a potent transactivator of NF-κB constituents and their downstream targets upon infection, and it is involved in the upregulation of p65, IL-6, TNF-α, and IL-8 as well as the induction of NF-κB binding activity in the nucleus [121]. Furthermore, UL144, a TNF-receptor-like transmembrane receptor expressed at immediate-early phase [122], is known to activate expression of the immune cytokine CCL22 by interacting with TNF receptor associated factor 6 (TRAF6) in perinuclear regions of the cell, enabling NF-κB transcription factor translocation and binding [123]). This can be supported by the fact that siRNA targeting UL144, TRAF6, or NF-κB negatively impacted downstream CCL22 expression induced by infection [123]. The CCL22 cytokine is a key chemoattractant, able to recruit Th2 and regulatory T-cells, thereby mediating adaptive immune responses [123]. Moreover, IE2 has been shown to inhibit NF-κB signaling at all phases of HCMV infection either by blocking NF-κB subunit dimer interactions or preventing subunit interactions with specific NF-κB target promoters, e.g. IL-6 [102,124]. Notably, at the same time, the antagonistic effects of IE2 do not prevent UL144 from inducing NF-κB [125].

To summarize, there is strong evidence that at early times of infection the virus seems to act within an optimal pro-inflammatory signaling window with just enough NF-κB transcription factor binding to transactivate the viral MIEP, but staying below a threshold that might trigger a broader antiviral immune response [92].

While NF-κB signaling is activated during the early stages of 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, also known as cmvIL-10 for its functional similarity of about 27% to the human cytokine IL-10, is able to bind as a homodimer to the IL-10 receptor and block NF-κB signaling by preventing IκBα degradation in a manner similar to IL-10 [126–128]. Furthermore, cmvIL-10 also has a significant immunosuppressive effect on interferon signaling [129]. It is currently unknown whether the inhibitory effects of cmvIL-10 on IFN and NF-κB signaling are separable.

The exact mechanisms and signals that lead to the switch of an HCMV infection from the limited lytic phase to the prolonged latency, as well as back to spontaneous reactivations remain only partially understood, although the processes of immunosuppression and

inflammation are considered to play major roles [130]. In line with this hypothesis, several studies indicate that HCMV genes activate the NF-κB network upon reactivation [131] via NF-κB subunit enhancement of MIEP expression [132]. The viral chemokine receptor US28 expressed early during lytic infection, is one of the few viral proteins expressed during latency as demonstrated in latently infected THP-1 monocytes [132]. It has been suggested that US28 is implicated in activating the MIEP via NF-κB signaling: during latent expression, US28 activates the MIEP, thereby assisting reactivation. In greater detail, US28 promotes constitutive NF-κB activation through its interaction with the Gq/11 family of G protein, mediating the release of Gβγ subunits that induce downstream NF-κB activity [133]. Even though in general US28 stimulates NF-κB activity, recent work suggests that US28 attenuates multiple cell signaling pathways including NF-κB, which is required to maintain latency as mutants lacking US28 return to their lytic phase and infected cells are subsequently targeted by T-cells [134]. Intriguingly, this suggests that US28 influences the viral life cycle displaying more than one role that may seem counterintuitive, but likely important in different infectious contexts.

Another HCMV protein, UL138, is expressed during latency and acts by activating and stabilizing the cell surface expression of TNFR1 [135]. The recent study by Lee *et al.* [136] has shed light on UL138 role in maintaining HCMV latency: in addition to UL138 promotion of the sensitivity to TNFα in latently infected cells, reporter assays demonstrated that UL138 strongly represses MIEP transactivation and prevents cellular demethylases from interacting with the MIEP [136].

Along with modulatory proteins, HCMV also expresses miRNAs that interfere with the NF-κB network. HCMV encodes 26 miRNAs that are involved in modulation of a wide range of cellular processes including cytokine secretion, vesicle transport, and immune signaling. Viral miRNAs begin to accumulate during the early stage of infection, reaching the peak of its expression at the later time points [137–139]. HCMV miR-US5-1 and miRUL112-3p have been demonstrated to prevent NF-κB cytokine signaling by specifically downregulating the expression of the key kinases IKKα and IKKβ [121]. In addition, miR-US5-2 has been demonstrated to block secretion of cytokines in infected cells, thus ceasing the positive feedback loop of NF-κB activation and ultimately returning the pathway to its initial inactive state [139]. MiR-UL148D, a miRNA that is highly expressed during latent infection, has been shown to block NF-κB upstream adapters and repress the production of IL-6, thereby permitting the infected cell to escape immune surveillance [140].

In summary, 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 various aspects of NF-κB signaling during different stages of infection. In this way, HCMV gene products, including both proteins and miRNAs, have been shown to inhibit NF-κB signaling and activate constituents of the NF-κB pathway to support lytic replication or induce reactivation from latency. That clearly suggests that NF-κB signaling is capable of multiple transcriptional scenarios depending on specific upstream stimuli depending on viral manipulations. To date, no unifying theory ties all the reported functional aspects together and our understanding of HCMV-mediated modulation of NF-κB is incomplete. Further efforts are needed to better understand the dynamics and mechanisms of such immunomodulation, especially in different biological contexts of HCMV infection, including viral dissemination, persistence, latency, reactivation, and pathogenesis.

Future Perspective

Significant progress has been made in the last few years in our understanding of immunobiology, diagnosis, and treatment of HCMV-related diseases. However, HCMV still remains an unmet need of high clinical importance for a substantial percent of the human population, as the currently available drugs fail to successfully eliminate the infection. Considering the profound effects of HCMV infections on the health and quality of life of immunosuppressed individuals, the elderly, and congenitally infected children, the development of a vaccine against congenital HCMV or therapeutic approaches to limit HCMV disease are considered a high priority. Partly, the reason why an effective cure against HCMV remain a work in progress is potentially due to the lack of insights into the interplay among signaling pathways triggered by HCMV in the modulation of host immune response and evasion.

Here, we have attempted to paint an overall picture of how key players in innate immunity integrate with each other to tackle HCMV replication, focusing particularly on host restriction factors, the IFN and NF-κB signaling. In addition, we have addressed the distinct evasion mechanisms that HCMV has evolved to escape from the host immune surveillance. Over the last years the wide panel of cellular proteins implicated in resisting HCMV has been uncovered and assessed. A number of the new studies, previously reporting cellular factors which are involved in variety of vital cellular processes and now tend to demonstrate an antiviral effect against HCMV, rise yearly. Therefore, one may speculate that numerous works both on identification of the novel restriction factors against HCMV infection, as well as wider insights on the function of the previously reported ones, will take place in the nearest future.

In parallel, we may expect the new reports, shedding light on the remarkable ability of HCMV to evade the intrinsic immunity and pointing out the exact strategies that the virus employed to do so. Given the large numbers of functional HCMV proteins, identification of which viral proteins target a certain cellular restriction factor may represent a challenging, but at the same time, promising task. The new insights into the molecular mechanisms tuning the dynamic balance between RFs and HCMV may provide the rationale for the development of novel therapeutic agents able to target specifically those key players mediating viral immune escape. It is tempting to speculate that agents targeting the early phases of the viral cycle could prevent HCMV from exploiting the host immune system to its own advantage, thereby increasing the immunocompetence of the host.

Finally, the intriguing interplay between HCMV and host immune signaling cascades, represents a wide platform for the future discoveries. The dynamics and tuning of different cascade components by HCMV in variety of ways and in different contexts of infection serve as field of unresolved work. It would be of paramount importance to dissect the real impact of intricate HCMV restriction and counter-restriction mechanisms on the ultimate outcome of HCMV infection, opening new horizons on the way to develop effective therapeutic agents, targeting HCMV both upon lytic and latent phase.

Executive summary:

HCMV

HCMV is a ubiquitous and opportunistic pathogen that causes serious syndrome in newborns and immunocompromised adult patients.

There are currently no vaccines to prevent HCMV infection and only few antiviral drugs are licensed 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 putative immune-modulatory functions and profoundly influences the expression of host immune–related proteins.

- *HCMV restriction factors*

Restriction factors represent a front-line defense against HCMV infections. IFI16, Viperin, APOBEC3, SPOC1 are key 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 major immediate-early proteins IE1 and IE2 counteract antiviral cytokine responses, while HCMV tegument proteins impact the modulation of the type I-II IFN response.

HCMV pp65 acts as the main inhibitor of the IFN-I response, preventing the activation of NF-κB and IRF3 and impairing the cGAS/STING signaling pathway.

- *Modulation of NF-κB signaling*

HCMV encodes both agonists and antagonists of NF-κB signaling in order to aid viral replication and dissemination, establishment of latency and reactivation.

-Antagonists: several HCMV proteins and miRNAs block activation of the IKK complex or downstream binding of the NF-κB transcription factor to its cognate sequences to avoid induction of antiviral and pro-inflammatory genes activated after virus binding and entry.

-Agonists: induction of the NF-κB signaling pathway at early times after infection enhances expression from the major immediate early promoter and thus help initiate 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.

Figure legends

Figure 1. The best-characterized host cell restriction factors in HCMV defense and virus escape mechanisms

Figure 2. Schematic representation of the HCMV evasion strategies from IFN antiviral activity

Figure 3. Model depicting the modulation of the NF-κB signalling pathway by HCMV

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

Figure 1

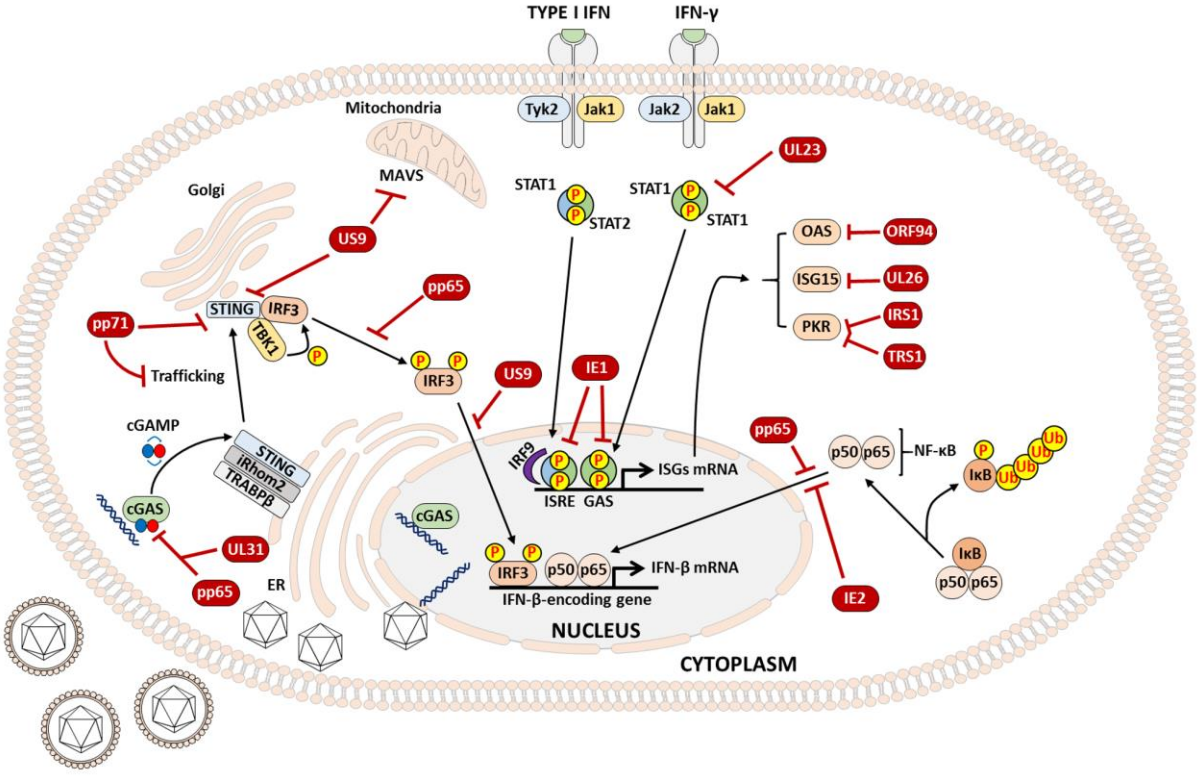

Figure 2

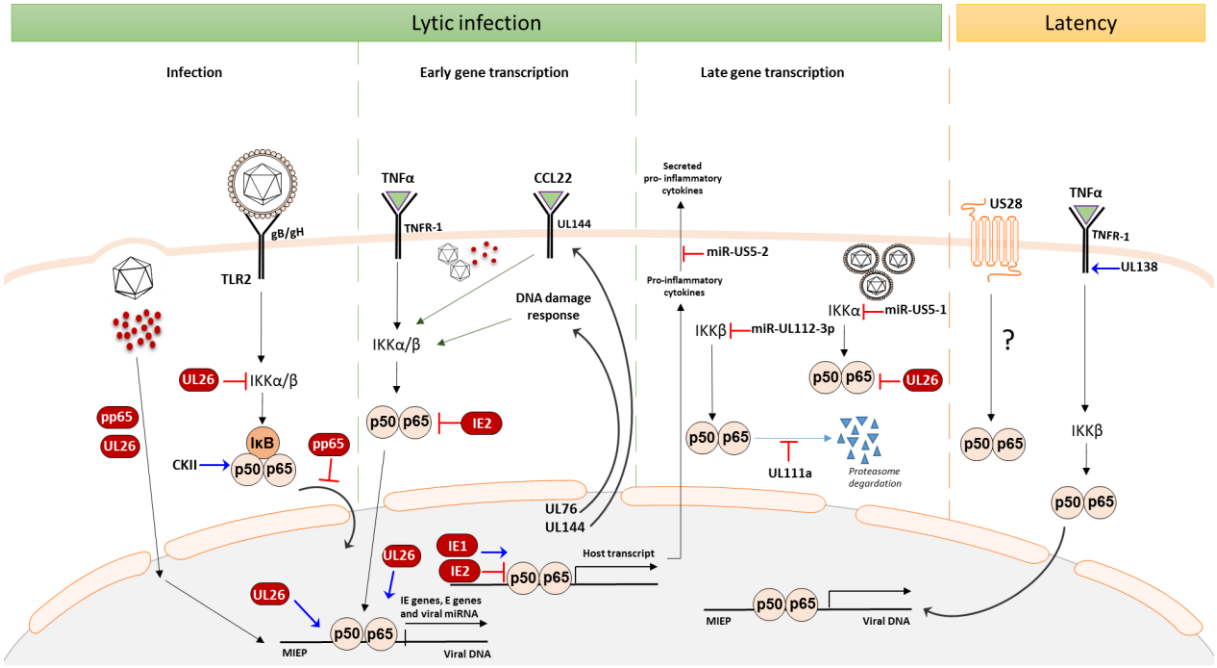
Lytic infection
Latency
Infection
Early gene transcription
Late gene transcription
gB/gH
TLR2
TNFα
TNFR-1
CCL22
UL144
Secreted pro- inflammatory cytokines
miR-US5-2
Pro-inflammatory cytokines
DNA damage response
IKKα/β
IKKα
miR-US5-1
IKKβ
miR-UL112-3p
UL26
IKKα/β
pp65
UL26
IkB
CKII
p50 p65
pp65
IE2
UL111a
Proteasome degardation
US28
TNFα
TNFR-1
UL138
?
IKKβ
UL76
UL144
IE1
IE2
Host transcript
IE genes, E genes and viral miRNA
MIEP
Viral DNA

Figure 3