Modulation of the innate immune response by human cytomegalovirus. [*Dell'Oste V., Landolfo S. co-corresponding authors]

Manuscript Details

Manuscript number	MEEGID_2018_11
Title	Modulation of the innate immune response by human cytomegalovirus
Article type	Review Article

Abstract

The interplay between human cytomegalovirus (HCMV) and the innate immune response is a critical process that has attracted the attention of many research groups. The emerging scenario is that the immune response of an HCMV-infected host is mediated by a plethora of viral DNA sensors acting as pattern recognition receptors (PRRs), which are capable of inhibiting indirectly viral infection through the activation of two distinct downstream signaling cascades. The first one triggers the production of cytokines, chemokines and interferons (IFNs), while the second one leads to inflammasome complex formation, which in turn promotes the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β). An additional first line of defense against HCMV is represented by a multiplicity of constitutively expressed restriction factors that inhibit viral replication by directly interfering with the activity of essential viral/cellular genes. Here, we take a closer look at some of the most representative intrinsic restriction factors involved in HCMV infection (e.g. IFI16, ND10 complex, viperin and APOBEC3) and review our current understanding of the mechanisms that HCMV has evolved to counteract both IFN and inflammasome responses.

Keywords	human cytomegalovirus; innate immunity; DNA sensors; restriction factors; interferons; inflammasome.
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HIGHLIGHTS

- Recent advances in the innate immune response against HCMV.
- HCMV DNA sensing mechanisms.
- Impact of interferon and inflammasome pathways on HCMV infection.
- Host restriction factors hijacking HCMV replication and counteracting measures.

Modulation of the innate immune response by human cytomegalovirus

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ABSTRACT

The interplay between human cytomegalovirus (HCMV) and the innate immune response is a critical process that has attracted the attention of many research groups. The emerging scenario is that the immune response of an HCMV-infected host is mediated by a plethora of viral DNA sensors acting as pattern recognition receptors (PRRs), which are capable of inhibiting indirectly viral infection through the activation of two distinct downstream signaling cascades. The first one triggers the production of cytokines, chemokines and interferons (IFNs), while the second one leads to inflammasome complex formation, which in turn promotes the maturation and secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β). An additional first line of defense against HCMV is represented by a multiplicity of constitutively expressed restriction factors that inhibit viral replication by directly interfering with the activity of essential viral/cellular genes. Here, we take a closer look at some of the most representative intrinsic restriction factors involved in HCMV infection (e.g. IFI16, ND10 complex, viperin and APOBEC3) and review our current understanding of the mechanisms that HCMV has evolved to counteract both IFN and inflammasome responses.

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1. Introduction

The human cytomegalovirus (HCMV) is a β-herpesvirus with the largest genome of all known human viruses (~235,000 bp) able to cause lifelong infections in humans. In the developed world, 40-60% of individuals are infected by time they reach adulthood, with seroprevalence approaching 100% in some populations (Cannon et al., 2010; Griffiths et al., 2015).

Although initial HCMV infection is often asymptomatic in healthy individuals, it can cause severe and sometimes fatal disease in immunocompromised individuals and neonates (Britt, 2017). In this regard, HCMV is one of the most common cause of birth defects resulting from an infectious agent, with 20% of congenitally infected infants exhibiting permanent neurological sequelae, including blindness, deafness and/or mental disability (Rawlinson et al., 2017). HCMV can also cause severe diseases in organ transplant recipients and AIDS patients after either primary infection or reactivation of latent infection (Navarro, 2016). To make things worse, immunosuppressed individuals are at potential risk of HCMV primary infection or re-infection and, eventually, reactivation of their endogenous latent virus.

Even though a vaccine is not yet available, HCMV can be treated with several inhibitors of viral replication. Five compounds are currently licensed to treat established HCMV infections: ganciclovir (GCV), its oral prodrug valganciclovir (VGCV), foscarnet (FOS), cidofovir (CDV) and fomivirsen (Ahmed, 2011). However, despite encouraging clinical outcomes, their use has been hampered by major associated adverse effects. One of these is represented by haematopoietic toxicity, which, along with long-term toxicity, low potency and poor bioavailability, limits the therapeutic efficacy of antiviral therapies in neonates and precludes their use in pregnant women (James and Kimberlin, 2016; Rawlinson et al., 2017). Another important issue concerning HCMV-related diseases management is the emergence of antiviral-resistant HCMV strains, especially in severely immunocompromised patients (Komatsu et al., 2014). Moreover, while these drugs are effective against the lytic replication cycle of HCMV, they do not affect the latent virus (Poole and Sinclair, 2015; Wills et al., 2015).

Throughout evolution, HCMV has acquired a number of different strategies to modulate and evade the human immune response, thereby achieving high infection efficiency and widespread dissemination in the host body (Christensen and Paludan,

2017; Noriega et al., 2012). Nevertheless, the human immune system is still capable of building a robust immune response against HCMV infection. This is clearly supported by the observation that all primary infections in immunocompetent hosts are virtually asymptomatic, whereas HCMV disease occurs mostly in individuals with an immature or compromised immune system. (Luecke and Paludan, 2015).

In this review, we will discuss the interplay between HCMV and the innate immune response together with the multiple strategies devised by HCMV to escape from immune surveillance. We will also highlight the different DNA sensing mechanisms and the viral restriction factors (RFs) involved in keeping this virus in check. Finally, we will focus on two of the main players of innate immunity, the interferon (IFN) and inflammasome systems.

2. Sensing HCMV by the host DNA sensors

Infection of host cells by HCMV triggers rapid intracellular innate immune responses largely initiated by pattern recognition receptors (PRRs), germline-encoded molecules able to detect evolutionarily conserved pathogen-associated molecular patterns (PAMPs) (Brubaker et al., 2015). During HCMV infection, viral DNA is detected by a myriad of PRRs that promote the activation of antiviral responses to protect the host cells. Infected cells detect the presence of HCMV very early, and by 4-8 hours post-infection they start producing pro-inflammatory cytokines, such as type I IFN (IFN-I) and activating RFs to antagonize viral replication (Luecke and Paludan, 2015; Orzalli and Knipe, 2014).

PRRs can be divided into two main groups depending on their subcellular localization. The first one consists of PRRs located on the plasma and endosomal membranes able to recognize extracellular PAMPs. These include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (Dambuza and Brown, 2015; Takeuchi and Akira, 2010; West et al., 2012). These membrane-bound PRRs are largely expressed by antigen presenting cells, such as macrophages and dendritic cells. The second group includes intracellular PRRs found in the cytoplasm or nuclei of mammalian cells. These include NOD-like receptors (NLRs) (Kim et al., 2016), retinoic acid-inducible gene-I (RIG-I), I-like receptors (RLRs) (Loo and Gale, 2011), cyclic GMP/AMP synthase (cGAS) (Chen et al., 2016), AIM2-like receptors (ALRs) (Dell'Oste et al., 2015; Huang et al., 2017a), and Z-DNA-binding protein 1 (ZBP1),

also known as DNA-dependent activator of IFN-regulatory factors (DAI) (DeFilippis et al., 2010).

In the following sections, we will review the main PRRs involved in HCMV DNA sensing activity (Figure 1).

2.1. TLR

The first evidence of a role played by TLR signaling during the innate immune response triggered by HCMV was obtained while studying TLR2. In the "classic" TLR2 pathway, PAMP binding to the receptor induces the enrollment of the adaptor protein MyD88 and interleukin (IL)-1 receptor-associated kinases (IRAK-4 and -1) via death domain interactions. The following phosphorylation and ubiquitination cascades switch on the NF-κB and MAP kinase (MAPK) pathways that in turn trigger the transcription of numerous pro-inflammatory cytokines such as IL-6, tumor necrosis factor (TNF)-α and IFN-β (Oliveira-Nascimento et al., 2012). In particular, TLR2 was shown to recognize HCMV gB and gH on the plasma membrane, resulting in the activation of the NF-κB pathway in a MyD88-dependent manner, followed by the production of inflammatory cytokines, such as IL-6, IL-8, IL-12 and IFN-β (Barbalat et al., 2009; Boehme et al., 2006; Compton et al., 2003; Juckem et al., 2008). Consistent with these results, impaired TLR2 function is often correlated with clinical cases of HCMV. Specifically, liver transplant recipients carrying an inactivating point mutation in the Toll-IL-1 receptor (TIR) domain of TLR2 show a higher HCMV load, indicating that TLR2 recognition is critical in controlling HCMV infection (Kijpittayarit et al., 2007). Recently, HCMV miR-UL112-3p (HCMV-encoded miRNA) has been associated with efficient down-regulation of endogenous TLR2 during infection and significant inhibition of its downstream signaling cascade (Landais et al., 2015).

In addition to TLR2, endosomal TLR3 and TLR9 are also involved in HCMV DNA detection. In this regard, a recent study has shown that HCMV infection upregulates TLR2, TLR3 and TLR9 in monocytes in the presence of the human scavenger receptor A type 1 (SR-A1) (Yew et al., 2010). TLR2, Lyn kinase and the p35 subunit of IL-12 were all upregulated within 10 minutes of HCMV infection in THP-1 monocytes. Interestingly, inhibition of Lyn kinase, which is correlated with SR-A1, causes the inhibition of TLR9 signaling and moves the response to both a

primarily TLR3 driven IFN-β response and a non-canonical TLR3 driven NF-κB response. Additionally, CpG-B-mediated stimulation of TLR9 can enhance HCMV infection in fibroblasts through an unknown mechanism, indicating that TLR9 signaling plays an important role during viral replication (Iversen et al., 2009). Finally, a particular polymorphism (T-1237C) altering the TLR9 promoter activity (Novak et al., 2007) has been shown to correlate with symptomatic HCMV infection in stem cell transplants (Carvalho et al., 2009).

Altogether, these results highlight the involvement of multiple TLR-associated pathways in the recognition of and response to HCMV.

2.2. NLRs

The nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs) family was originally reported to induce the NF-κB pathway in response to bacterial pathogens. More recently, induction of alternative signaling reminiscent of antiviral responses, including the IFN pathway and autophagy, has been reported (Kanneganti, 2010). Among NLRs, NLRC5 is involved in IFN-dependent anti-HCMV immune responses. Indeed, infection of human fibroblasts with HCMV, but not heat-inactivated virus, promoted NLRC5 mRNA expression within 24 hours following infection. Consistently, knockdown of NLRC5 altered the up-regulation of IFN-α in response to HCMV (Kuenzel et al., 2010).

Induction of NOD2 and the downstream receptor-interacting serine/threonine-protein kinase 2 (RIPK2) by HCMV, but not human herpesvirus 1 and 2 (HSV-1, HSV-2), is known to up-regulate antiviral responses and suppress virus replication. Upon infection, NOD2 activates downstream NF-κB and IFN pathways, leading to IL-8 and IFN-β production, respectively. Indeed, stable overexpression of NOD2 in human fibroblasts restricts HCMV replication and correlates with increment levels of IFN-β and IL-8. Furthermore, ectopic expression of the NOD2 3020insC mutant, associated with severe Crohn's disease, causes an increased HCMV replication and reduced levels of IFN-β (Kapoor et al., 2014).

Recent findings have also demonstrated a role for NOD1 in HCMV sensing and subsequent inhibition. In contrast to NOD2, which responded efficiently to HCMV infection at a low MOI, activation of the highly expressed NOD1 following HCMV infection was observed over a wider range of MOI (Kapoor et al., 2016). In

agreement with these findings, NOD1 activation by Tri-DAP (NOD1 agonist) suppressed HCMV and induced IFN-β. Signaling through NOD1, resulting in HCMV suppression, was IKKα-dependent and correlated with nuclear translocation and phosphorylation of IRF3. Specific mutations in NOD1 caused differential effects on HCMV replication *in vitro*. In cells overexpressing the E56K mutation, which is involved in caspase activation and recruitment, virus replication was enhanced. By contrast, in cells overexpressing the E266K mutation or the wild-type NOD1, HCMV replication was inhibited. These changes were then shown to be most likely correlated with IFN-β expression (Fan et al., 2016).

2.3. cGAS

cGAS is a DNA sensor directly engaged by HCMV dsDNA genome in the cytosol of infected cells. After DNA binding, it produces the second messenger cyclic GMP/AMP (cGAMP) (Bhat and Fitzgerald, 2014; Gao et al., 2013; Sun et al., 2013). cGAMP, which may also spread via gap junctions to bystander cells, binds to the adaptor protein stimulator of IFN genes (STING) in the endoplasmic reticulum (ER), causing a conformational change in the STING dimer (Zhang et al., 2013). Activation of STING results in its relocalization from the ER to ER-Golgi intermediate compartments (Dobbs et al., 2015), where it associates with the TANK-binding kinase 1 (TBK1). This interaction leads to the subsequent phosphorylation of STING by TBK1, which causes the recruitment of IRF3 followed by its phosphorylation and nuclear translocation (Burdette and Vance, 2013; Liu et al., 2015). The cGAS-STING-TBK1-IRF3 pathway regulates the early IFN response against HCMV. In this regard, CRISPR/Cas9-mediated disruption of STING expression in endothelial cells revealed an essential role of this adaptor protein in eliciting IFN-I responses as well as restricting HCMV replication (Lio et al., 2016; Paijo et al., 2016; Söderberg-Nauclér et al., 2001; Taylor-Wiedeman et al., 1991). Interestingly, although plasmacytoid dendritic cells (pDCs) expressed particularly high levels of cGAS, and the cGAS/STING axis was functional down-stream of STING, pDCs were found to be resistant to HCMV infection in a TLR9 signaling-dependent fashion. Nevertheless, monocyte-derived DCs (mDCs) and macrophages sensed the virus in a cGAS-dependent manner (Paijo et al., 2016).

2.4. IFI16

The DNA sensor IFI16 is an IFN-inducible protein, member of the pyrin and HIN domain (PYHIN) family, with a plethora of different cell functions, including anti-proliferative, pro-inflammatory and pro-apoptotic activities. This family comprises homologous human and mouse proteins that have one or two partially conserved 200-residue C-terminal domains (HIN) and an N-terminal pyrin domain (PYD) (Bawadekar et al., 2015). Crystallographic studies of the HINa and HINb domains of IFI16 have shown that both domains contain two 80 amino acid-long tandem β-barrels, previously characterized as the oligonucleotide/oligosaccharide binding (OB) fold (Albrecht et al., 2005), capable of binding DNA directly (Jin et al., 2012). This is true also for HCMV dsDNA, which is recognized by the IFI16 HIN domain during early stages of infection, leading to STING recruitment and TBK1-IRF3-dependent pathway activation to induce IFN-β (Unterholzner et al., 2010). Interestingly, Diner et al. (2016) demonstrated a multiphasic pattern of IFI16 subcellular localization using live-cell imaging. According to these data, following HSV-1 or HCMV infection, IFI16 first localizes at viral entry sites in the nuclear periphery and then to nucleoplasmic puncta. Furthermore, the IFI16 pyrin domain is required for nuclear periphery localization and oligomerization. During the late stages of infection, IFI16 appears to be mislocalized to the cytoplasmic viral assembly complex (vAC) where it is finally entrapped into mature virions (Dell'Oste et al., 2014). Notably, the HCMV tegument protein pp65 (pUL83) blocks nuclear IFI16-DNA sensing by binding directly the IFI16 pyrin domain, which in turn hinders its DNA-dependent oligomerization, thereby promoting IFI16 nuclear delocalization and inhibition of the immune response (Li et al., 2013).

2.5. ZBPI/DNA

The interferon-inducible protein ZBP1, also known DAI and DLM-1, is a dsDNA sensor that mediates various innate immune responses (Takaoka et al., 2007). After entering into the host cells, HCMV activates ZBP1 by contact with tegument- or nucleocapsid-associated DNA. ZBP1 then promotes TBK1-mediated phosphorylation of both DDX3 and IRF3. Nuclear accumulation and DNA binding of phosphorylated DDX3 and IRF3 proteins lead to the transcription of IFN-β as well as IFN-stimulated genes (ISGs), including ZBP1 itself. Thus, HCMV-mediated activation of ZBP1

causes a positive feedback loop afterward amplified by IRF3 activation and activity (DeFilippis et al., 2010).

3. Manipulation of the interferon and inflammasome systems by HCMV

Upon detection of viral pathogens, intracellular PRRs trigger a series of events leading to the activation of various transcription factors, including MAP kinases (MAPKs), NF-κB, IRF3, and IRF7, which mediates the transcriptional induction of IFNs and the release of pro-inflammatory chemokines to drive immune cells to the site of infection (Hoffmann et al., 2015; Mogensen, 2009). Among pro-inflammatory cytokines, pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) are the main products of PRRs activation. These cytokines need however to be processed into their mature forms by multiprotein complexes known as inflammasomes before being secreted and promote the immune response (Lupfer et al., 2015a). Thus, in recent years the interplay among HCMV, IFNs, and the inflammasome has been addressed in a number of dynamic studies that will be discussed in detail in the following sections.

3.1. Interplay between the IFN system and HCMV

IFNs are grouped in three distinct families, classified as 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) with pleiotropic roles in immunity, autoimmunity and cancer biology (Hoffmann et al., 2015; Pollard et al., 2013). Even though recent evidence has pointed to an inhibitory function of IFN-λ activity against viral infections (Lopušná et al., 2013), IFN-I are generally regarded as the main mediators of the antiviral response.

IFN-I promote cellular resistance against herpesvirus infection of fibroblasts, endothelial or epithelial cells (Trilling et al., 2012) through transcriptional activation of ISGs, which then display a broad antiviral activity (Brinkmann et al., 2015; Schoggins et al., 2014, 2011). These findings are supported by the observation that mice defective in IFN-I signaling are more prone to murine cytomegalovirus (MCMV) infection (Hoebe et al., 2003; Presti et al., 1998). Similarly, administration of IFN-I or inhibition of IFN-I signaling has been shown to alter HCMV replication in human fibroblasts (Paludan, 2016; Rossini et al., 2012). Interestingly, IFN induction

early after HCMV infection does not seem to rely on TLR signaling as levels of secreted IFN-β upon infection are not affected even when TLR2, TLR3, TRL7, TLR8 or TLR9 signaling is inhibited (Marshall and Geballe, 2009a). By contrast, different nuclear or cytoplasmic PRRs are involved in the IFN signaling activation (Unterholzner, 2013). In particular, as aforementioned, the cGAS-STING axis is essential for activating the IFN-I pathway following HCMV infection, (Biolatti et al., 2018; Diner et al., 2016; Jønsson et al., 2017; Paijo et al., 2016).

Another PRR implicated in IFN activation is IFI16 although its exact role in this process has yet to be fully established and still remains controversial. In this regard, recent results have revealed that IFI16 and cGAS cooperate in STING activation upon treatment with exogenous DNA in human keratinocytes (Almine et al., 2017). In addition, IFI16 is essential for early DNA sensing in human macrophages by stimulating cGAMP production (Jønsson et al., 2017). However, the involvement of IFI16 in downstream signaling leading to IFN activation appears to be not as pronounced as that of cGAS, as demonstrated by experiments on IFI16 knock-down fibroblasts where, in contrast to STING- and cGAS-depleted HFFs, a residual IFN-β production could still be detected (Biolatti et al., 2018). Partly consistent with these results, recent findings by Stetson and co-workers (Gray et al., 2016) have shown that IFI16 is not required for the IFN response to HCMV infection.

Different conclusions have been reached in other cellular models. For example, cGAS- or STING-deficient THP-1 monocytes showed a significantly reduced amount of IFN-I production upon HCMV infection (Paijo et al., 2016). In line with these findings, THP-1 monocytes lacking IFI16 were still capable of mounting a robust IFN-I response that was significantly stronger than that of wild-type THP-1 cells.

Taking everything into account, it is likely that all these discrepancies could be ascribed to the different cellular types employed in these studies (e.g. monocytes, keratinocytes, fibroblasts or plasmacytoid dendritic cells), the dissimilar methods used to knock-down IFI16 (e.g. CRISPR/Cas 9, siRNA or shRNA), and finally the different viral strains and synthetic DNA employed to induce the IFN-I response.

3.2 HCMV evasion strategies from IFN antiviral activity

Although HCMV encodes several viral factors able to counteract the IFN pathway, the exact mechanisms that allows HCMV to escape from the host immune surveillance still remain to be fully understood (Marshall and Geballe, 2009) (Figure 2).

Results from different groups (Abate et al., 2004; Biolatti et al., 2018; Browne and Shenk, 2003; Li et al., 2013) have shown that HCMV pp65 is the main inhibitor of IFN-I response. However, it is still a matter of debate at which level pp65 counteracts IFN activation. Abate et al. (2004) have demonstrated that pp65 promotes IRF3 dephosphorylation and its export from the nucleus, affecting the balance of nuclear-cytoplasmic shuttling (Reich, 2002). By contrast, Browne et al. (2003) has shown that pp65 suppresses the induction of some, but not all, IFN-responsive genes by preventing the activation of NF-κB and IRF1. Finally, recent studies by Biolatti et al. (2018) 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.

Another main player of HCMV evasion from the IFN response is the HCMV tegument protein pp71 (pUL82) (Fu et al., 2017), 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.

Recent studies by Choi et al. (2018) have shown that HCMV glycoprotein US9 inhibits the IFN-β response by targeting the mitochondrial antiviral-signaling protein (MAVS) and STING-mediated signaling pathways. In particular, the authors focused on the ability of US9 to disrupt STING oligomerization, STING/TBK1 association through competitive interaction, and to block the IRF3 nuclear translocation and activation.

Finally, the HCMV immediate-early 2 protein (IE86) has also been shown to affect the production of IFN-β by blocking the binding of NF-κB to the IFN-β promoter (DeFilippis et al., 2006; Taylor and Bresnahan, 2006, 2005). In agreement with these findings, reduced protein levels of STING were observed in cells expressing IE86 protein, suggesting that IE86 could also target STING to inhibit IFN-I signaling (Kim et al., 2017).

3.3. HCMV and inflammasome modulation

Multiprotein complex inflammasomes occur in many forms and are mainly activated following recognition of PAMPs (Guo et al., 2015). A number of different inflammasomes have been so far identified; these include NLRP1-, NLRP3-, absent in melanoma 2 (AIM2)- and RIG-I-related inflammasomes (Chen and Ichinohe, 2015). After detecting the presence of specific PAMPs, PRRs act as scaffold proteins for each specific inflammasome complex, leading to the activation of caspases and cytokines. In particular, the inflammasome induces the expression of the inactive pro-forms of pro-inflammatory cytokines IL-1β and IL-18 that trigger the production of adhesion molecules and chemokines involved in immune and inflammatory responses (Chen and Ichinohe, 2015; Garlanda et al., 2013). Maturation of these pro-cytokines requires the proteolytic cleavage of active pro-inflammatory caspases, particularly caspase-1, which is cleaved to give rise to the p10 and p20 subunits, which then form a tetrameric enzyme with a very high affinity for substrates (Martinon et al., 2002; Miller et al., 1993; Srinivasula et al., 2002; Yamin et al., 1996). Interestingly, caspase-8 has recently been identified as an alternative protease that can process IL-1β either in the inflammasome system or independently (Bossaller et al., 2012; Gurung and Kanneganti, 2015; Moriwaki et al., 2015). Indeed, several studies have shown that caspase-8 can directly cleave pro-IL-1β leading to the secretion of the cytokine active form (Bossaller et al., 2012; Gringhuis et al., 2012; Gurung and Kanneganti, 2015).

While the inflammasome involvement in counteracting many microbial infections is widely recognized, more recent evidence has shown that it may also mediate antiviral responses (Lupfer et al., 2015b; Shrivastava et al., 2016). In this regard, studies on MCMV (Rathinam et al., 2010; Shi et al., 2015) have reported inflammasome modulation during MCMV infections, and inflammasome activation has been also observed during monocyte and THP-1 derived macrophages infection with HCMV (Huang et al., 2017a; Yurochko and Huang, 1999). In contrast, Cristea and collaborators (Diner et al., 2016) did not observe any change in caspase-1 cleavage during HCMV infection, which led them to postulate that the canonical inflammasome assembly was not taking place under their experimental conditions. In agreement with their data, our group has recently found that HCMV infection can trigger a non-canonical pathway leading to inflammasome-independent maturation of IL-1β via Fas/caspase-8 activation (Biolatti et al., unpublished results). Also in this case, HCMV pp65 appears to be one of the main players in HCMV immune evasion. This conclusion derives from the observation that, early during HCMV infection, a

mutant virus unable to express UL83-encoded pp65 can upregulate IL-1β transcription in fibroblasts in an NF-κB dependent manner (Biolatti et al., unpublished results).

Previous studies confirmed the impact of pp65 in counteracting impairing inflammasome activation (Huang et al., 2017b). Particularly, the authors focused on the AIM2-inflammasome system, where the N-terminal pyrin (PY) domain of AIM2 binds the adaptor molecule ASC and subsequently recruits pro-caspase-1 via CARD domain for its self-cleavage. Caspase-1 activation then promotes IL-1β maturation followed by its secretion (Huang et al., 2017b; Morrone et al., 2015; Schattgen and Fitzgerald, 2011). In this scenario, pp65 interacts with AIM2 in both HCMV-infected macrophages infected with HCMV and HEK293T cells transfected with pp65 and AIM2 expression vectors. Furthermore, ectopic expression of pp65 in recombinant HEK293T cells stimulated by with poly(dA:dT) resulted in lowered expression and activation of AIM2 inflammasome-associated proteins. However, the impact of pp65 on inflammasome activation is still a matter of debate as other groups have failed to confirm the interaction between pp65 and the AIM2-PY domain, despite being able to detect pp65 binding activity to the pyrin domain of all other nuclear PYHIN proteins (IFI16, IFIX and MNDA) (Li et al., 2013). Notably, a HCMV strain unable to express pp65 did not trigger enhanced inflammasome activity compared to the wild-type HCMV, consistent with the lack of caspase-1 cleavage (Li et al., 2013).

Taken together, these data add further fuel to the controversy surrounding the role of pp65 in the modulation of HCMV evasion mechanisms and clearly highlight the need for further investigations.

4. Host cell restriction factors in HCMV defense

A first line of defense against viruses is represented by RFs, anti-viral proteins produced in the host to counteract or “restrict” viral replication by directly interfering with the activity of essential viral/cellular genes (Bieniasz, 2003; Hotter and Kirchhoff, 2018). Early pioneering studies on retroviruses have allowed us to identify two major RFs: the APOBEC3 class of cytidine deaminases and tetherin (Bieniasz, 2003; Jakobsen et al., 2015; Neil and Bieniasz, 2009; Simon et al., 2015). However, increasing evidence suggests that RFs can also counteract several other viruses, including HCMV (Paludan et al., 2011). So far, different proteins, including IFI16,

nuclear domain 10 (ND10) complex, viperin and APOBEC3 have been identified as RFs of HCMV replication (Figure 3, panel A). At the same time, HCMV has evolved strategies to counteract RF antiviral activities (Figure 3, panel B).

4.1. IFI16

The antiviral activity of IFI16 has been heavily studied for the last decade. The role of IFI16 as a restriction factor has been confirmed for different viruses, including HCMV (Dell'Oste et al., 2015; Landolfo et al., 2016). Our group has demonstrated that the inactivation of IFI16 protein in human embryo lung fibroblasts (HELFs) by transfecting specific siRNAs or lentiviruses expressing dominant negative mutant forms of the protein 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 HCMV DNA polymerase UL54 promoter (Gariano et al., 2012). However, the virus has evolved evasion strategies to counteract IFI16 activity that consists in delocalizing this protein from the nucleus to the cytoplasm during the late stage of infection. The key player mediating HCMV-induced nuclear egression of IFI16 is the viral protein kinase UL97. Upon binding to UL97 phosphoprotein, IFI16 is subject to phosphorylation, which in turn drives its nucleo-cytoplasmic relocalization. The endosomal sorting complex required for transport (ESCRT) regulates thereafter the translocation of IFI16 into the virus assembly complex. Finally, IFI16 becomes incorporated into the newly formed virions (Dell'Oste et al., 2014).

A detrimental partner of UL97 in HCMV escape activity appears to be HCMV pp65 which can interact with IFI16, thus targeting early gene promoters including that of viral DNA polymerase UL54. From the literature, this pp65/IFI16 interaction constitutes undoubtedly a very dynamic and controversial interplay. While in the early phases of HCMV infection pp65 recruits IFI16 to the major immediate-early promoter (MIEP), enhancing viral transcription (Cristea et al., 2010), at later time points, pp65 is able to protect IFI16 from proteasome-mediated degradation, sustaining its inhibitory activity at the level of the UL54 gene promoter (Biolatti et al., 2016). Recent findings have shown that another important interactor of IFI16 is the DNA sensor cGAS, albeit these proteins display different functions. Diner et al. (2016)

445 demonstrated that IFI16 interacts with cGAS through the PY domain, but while IFI16
446 induces antiviral cytokine expression, including IFN-β, only cGAS effectively
447 activates STING/TBK-1/IRF3 and apoptotic responses upon HSV-1 and HCMV
448 infections (Biolatti et al., 2018; Diner et al., 2016).

449 *4.2. ND10*

450 ND10, also known as promyelocytic leukemia nuclear bodies (PML-NBs), is
451 formed by spherical bodies of amassed proteins distributed throughout the
452 nucleoplasm. They regulate diverse cellular key processes, such as oncogenesis, DNA
453 damage repair, apoptosis, senescence and gene expression (Lindsay et al., 2008;
454 Negorev and Maul, 2001). PML, hDaxx and Sp100 are the main constituents of
455 ND10, whose antiviral functions have been demonstrated through gene silencing and
456 overexpression (Adler et al., 2011; Scherer and Stamminger, 2016; Tavalai et al.,
457 2011; Zhang and van Drunen Littel-van den Hurk, 2017). ND10 bodies undergo
458 profound modifications during virus infection of quiescent cells, accumulating viral
459 genome at their periphery or within their central core (Everett, 2006, 2001; Maul et
460 al., 1993; Szekely et al., 1999). Interestingly, HCMV infection of PML-null HFFs
461 induced *de novo* formation of hDaxx and Sp100 (Tavalai et al., 2006), suggesting that
462 HCMV recruitment of ND10 components to the site of viral replication constitutes the
463 first step of the antiviral response (Glass and Everett, 2013; Tavalai et al., 2008;
464 Tavalai and Stamminger, 2008). Interestingly, in cells containing double knock-down
465 combinations of ND10 proteins, HCMV gene expression is enhanced compared to
466 that found in each respective single-knock down (Adler et al., 2011; Cosme et al.,
467 2011; Tavalai et al., 2008). These finding suggest an independent role of these factors
468 in the suppression of HCMV replication.
469 Subsequent studies have shown that the main mechanism involved in the
470 repression of HCMV gene expression by ND10 relies on epigenetic mechanisms.
471 Indeed, chromatin modifying enzymes, such as histone deacetylases (HDACs) and the
472 chromatin remodeling protein alpha thalassemia/mental retardation syndrome X-
473 linked (ATRX), were shown to interact and cooperate with ND10 components,
474 inducing transcriptionally inactive chromatin of the MIEP (Lukashchuk et al., 2008;
475 Preston and Nicholl, 2006; Shin et al., 2012; Woodhall et al., 2006).

While PML, hDaxx and Sp100 act as RFs of HCMV IE gene expression, several controversial findings seem to indicate that these proteins are only marginally involved in the establishment of HCMV latency. On the one hand, hDaxx was shown to act as a restriction factor in cellular settings of latent HCMV infections, such as NT2 and THP-1 cells, primary human CD34+ cells and two myeloblastic cell lines (i.e. KG-1 and Kasumi-3) (Saffert and Kalejta, 2006); on the other hand, knockdown of hDaxx in undifferentiated NT2 cells was not sufficient to trigger IE gene expression (Woodhall et al., 2006), suggesting that hDaxx is not involved in the regulation of the viral MIEP in latently infected cells. Furthermore, Stamminger and co-workers (Wagenknecht et al., 2015) observed that depletion of PML, hDaxx or Sp100 did not affect IE gene expression in non-differentiated THP-1 monocytes, considered a prototype of HCMV latency. In contrast, differentiation of THP-1 cells towards a macrophage-like phenotype, a model of acute infection, in the absence of ND10 proteins significantly increased the number of IE expressing cells (Wagenknecht et al., 2015). Finally, it has recently emerged that the key ND10 components also act as antiviral ISGs during HCMV infection. Specifically, ND10 knockdown cells, but not their normal counterpart, were able to support HCMV plaque formation following IFN-β pre-treatment, indicating that ND10 component upregulation is a crucial mediator of the anti-viral activity of IFN-β in response to HCMV infection (Ashley et al., 2017).

Other strategies adopted by HCMV to counteract the restriction activity of ND10 rely on the viral proteins IE1 and pp71. In particular, IE1 has been recently shown to drive the escape of HCMV from ND10-mediated innate immune response by preventing the *de novo* SUMOylation of ND10 (Lee et al., 2004; Xu et al., 2001), which then leads to the inhibition of ND10 oligomerization, followed by its disruption (Ahn and Hayward, 1997; Korioth et al., 1996; Wilkinson et al., 1998). As for pp71, earlier studies clearly established a functional interaction with hDaxx during recruitment to the ND10 domain (Hofmann et al., 2002). In detail, pp71 binds hDaxx, which then undergoes proteasome degradation, thereby relieving MIEP repression. Moreover, MIEP expression is mediated by the release of the chromatin-remodeling protein ATRX from ND10, which is stimulated by pp71 (Cantrell and Bresnahan, 2005). More recently, two additional tegument proteins, named UL35 and UL35a, have been shown to regulate pp71 activity. UL35 is able to independently remodel ND10 and strongly co-localize with the remodeled ND10 structures (Salsman et al.,

2011, 2008), suggesting that UL35 may facilitate pp71-mediated DAXX-ATRX disruption. Conversely, UL35a is a negatively regulator as it prevents UL35 from remodeling ND10 and directs pp71 to the cytoplasm (Salsman et al., 2011).

4.3. Viperin

Viperin is an IFN-inducible iron-sulfur (Fe-S) cluster-binding protein induced in various cell types by different viruses, including HCMV. It 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 compared with that of control cells (Chin and Cresswell, 2001). Intriguingly, several pieces of evidence seem 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. Firstly, HCMV-encoded viral mitochondrion-localized inhibitor of apoptosis (vMIA) protein 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 (Seo et al., 2011). 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 GLUT4. This leads to 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 (Seo and Cresswell, 2013).

4.4. APOBEC3

The apolipoprotein B editing catalytic subunit-like 3 (APOBEC3) family of cytidine deaminases is formed by seven members (i.e. A, B, C, D, E, F, G and H), catalyzing the deamination of cytidine nucleotides to uridine nucleotides in single-strand DNA substrates (Knisbacher et al., 2016). These enzymes are widely acknowledged as fundamental players in the defense against human immunodeficiency virus type 1 (HIV-1) (Blanco-Melo et al., 2012). However, it soon became apparent that their activities were also directed towards DNA viruses, such as

541 the hepatitis B virus (HBV) (Suspène et al., 2005; Turelli et al., 2004), and the
542 parvoviruses (Harris and Dudley, 2015; Nakaya et al., 2016; Narvaiza et al., 2009;
543 Vieira and Soares, 2013). More recently, Weisblum et al. (2017) reported
544 APOBEC3A (A3A) to be strongly upregulated following *ex vivo* HCMV infection of
545 maternal decidua. Furthermore, overexpression of A3A in epithelial cells hampered
546 HCMV replication by introducing hypermutations into the viral genome through
547 cytidine deamination. In contrast, A3A induction by HCMV was not observed in
548 other HCMV-infected cell models (i.e. chorionic villi maintained in organ culture,
549 foreskin fibroblasts or epithelial cells), suggesting that HCMV-mediated upregulation
550 of A3A is tissue- and cell-type specific. Intriguingly, A3A expression in uninfected
551 decidual tissues is mediated by IFN-β, suggesting that it might function as an IFN-
552 stimulated gene during HCMV infection (Weisblum et al., 2017).

553 Even though these results highlight an important aspect of A3A activity
554 against HCMV, many questions still remain open. For example, it is not known
555 whether HCMV is able to induce other A3 family members besides A3A in different
556 cell types. To start filling this gap, we have recently obtained evidence that A3G is
557 induced in HCMV-infected HFFs, and that this induction appears to be mediated by
558 IFN-β (Pautasso et al., unpublished results). However, in our model, upon gene
559 silencing or overexpression, A3G does not seem to behave as a restriction factor for
560 HCMV replication. Thus, we hypothesize that throughout evolution, HCMV has
561 instead shaped the nucleotide composition of its genome in order to escape from
562 A3G-mediated immune surveillance.

563 **5. Conclusions and future perspectives**

564 In recent years, there has been much progress in our understanding of the
565 immunobiology, diagnosis and treatment of HCMV-related diseases. Nevertheless,
566 HCMV still remains an unmet clinical need for a high proportion of the human
567 population. In this regard, what is most challenging for the scientific community
568 seems to be the development of an anti-HCMV vaccine to fight congenital infections.
569 The reason why an effective cure against HCMV is still in the works is partly due to
570 the lack of mechanistic insights into the interplay among signaling pathways triggered
571 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 others to tackle HCMV replication, focusing particularly on viral DNA sensors, restriction factors and the IFN and inflammasome systems. Furthermore, we have addressed the distinct evasion mechanisms that HCMV has evolved to escape from the host immune surveillance. From this literature, it is clear that forthcoming challenges in HCMV innate immunity rest upon addressing several unresolved issues. For instance, we still do not know how DNA-sensing pathways discriminate between commensal microbiota and invading pathogens. Also, it would be of paramount importance to dissect the real impact of the intricate HCMV restriction and counter-restriction mechanisms on the ultimate outcome of HCMV infection.

Overall, 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.

Acknowledgements

The work related to the topics discussed in the present review is supported by: the Ministry of Education, Universities and Research (PRIN, 2015, to VDO, 2015RMNSTA); Research Funding from the University of Turin, 2017, to SL and VDO.

Conflicts of interest

The authors declare that they have no conflict of interest.

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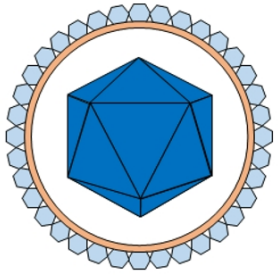
Figure legends

Fig. 1. Proposed HCMV DNA sensors.

Fig. 2. Model depicting the evasion strategy by HCMV against the IFNs signaling.

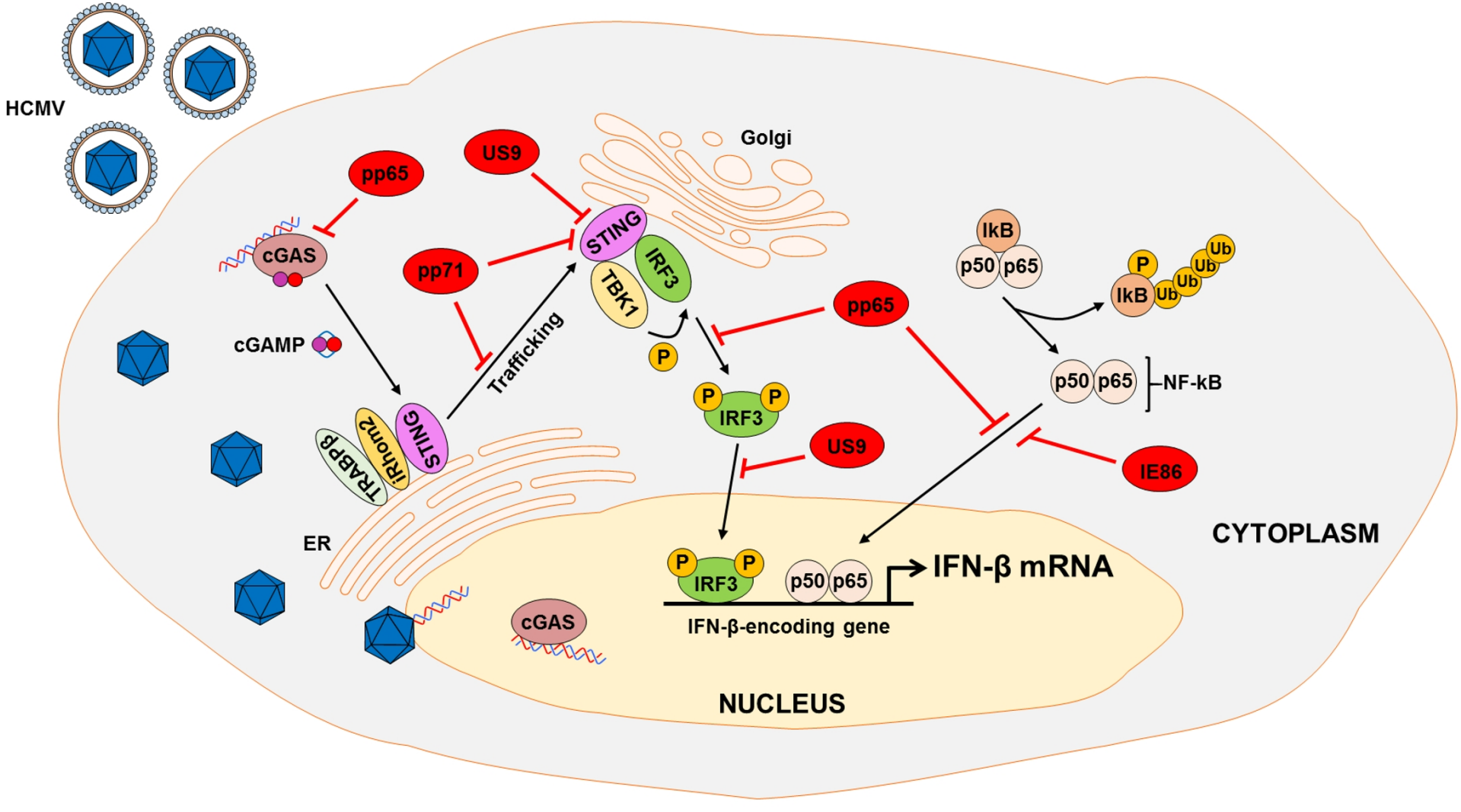
Fig. 3. Schematic representation of the restriction activities played by the major RFs to down-regulate HCMV gene expression (A), and mechanisms exploited by HCMV to overcome RF antiviral activity (B).

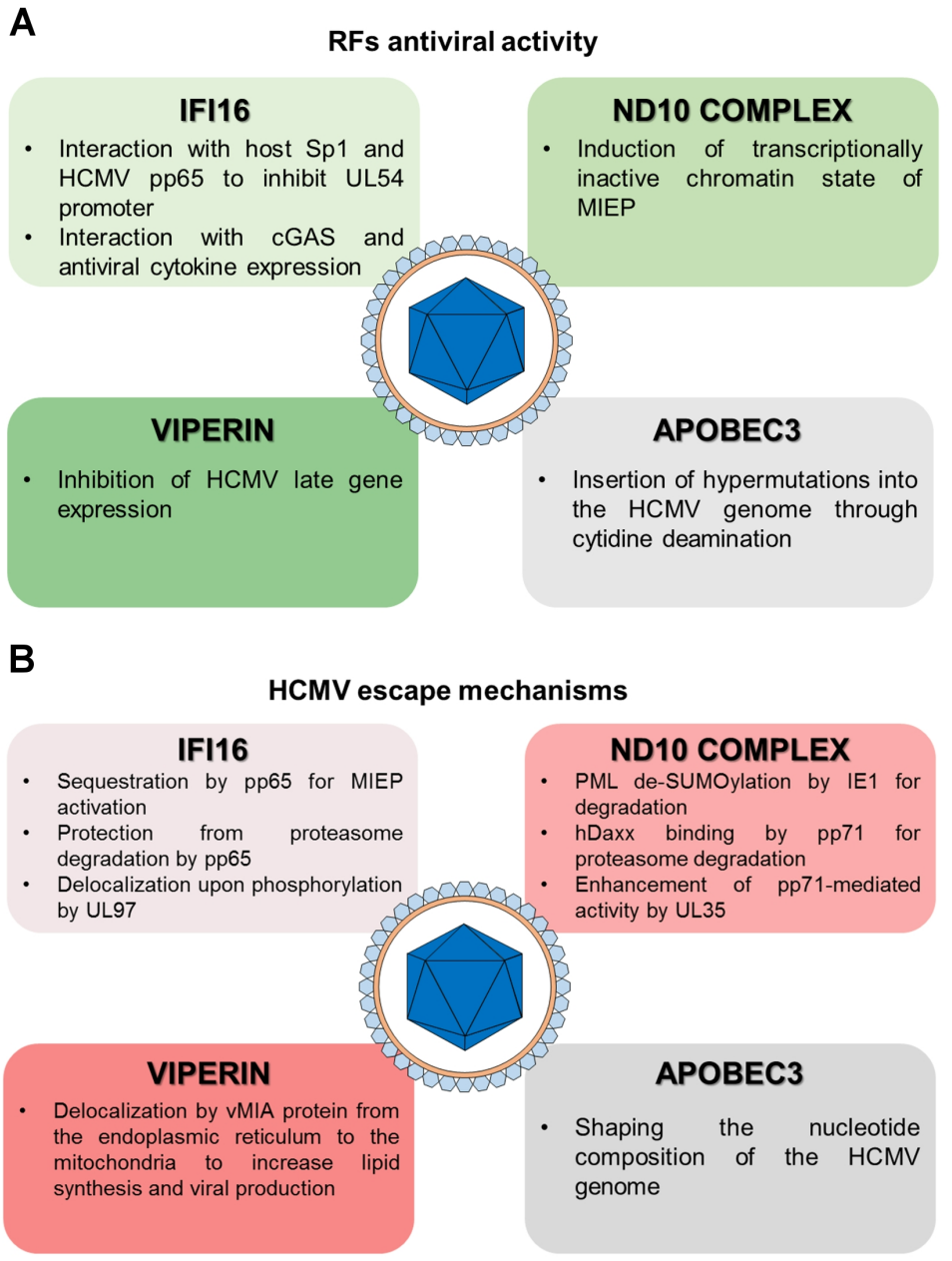
HCMV



DNA sensors

Name	Site of DNA sensing	Immune response pathway
TLR2	Cell surface	IFN-β via MYD88; IL-8, IL-6, IL-12 via NF-κB
TLR3	Endosome	IFN-β via TRIF; IL-8, IL-6, IL-12 via NF-κB
TLR9	Endosome	IFN-β via MYD88; IL-8, IL-6, IL-12 via NF-κB
NLRC5	Cytoplasm	IFN-α
NOD2	Cytoplasm	IFN-β via IRF-3; IL-8 via NF-κB
NOD1	Cytoplasm	IFN-β
cGAS	Nucleus, Cytoplasm	IFN-α, IFN-β via STING
IFI16	Nucleus, Cytoplasm	IFN-β via STING
ZBP1	Cytoplasm	IFN-β via DDX3


HCMV
Golgi
pp65
US9
cGAS
STING
IRF3
TBK1
pp71
cGAMP
Trafficking
P
IkB
p50
p65
Ub
pp65
p50 p65
NF-kB
P
P
IRF3
US9
IE86
STING
IRNOM2
TRABP8
ER
CYTOPLASM
P
P
IRF3
p50 p65
IFN-β mRNA
cGAS
IFN-β-encoding gene
NUCLEUS


A
RFs antiviral activity
IFI16
• Interaction with host Sp1 and HCMV pp65 to inhibit UL54 promoter
• Interaction with cGAS and antiviral cytokine expression
ND10 COMPLEX
• Induction of transcriptionally inactive chromatin state of MIEP
VIPERIN
• Inhibition of HCMV late gene expression
APOBEC3
• Insertion of hypermutations into the HCMV genome through cytidine deamination
B
HCMV escape mechanisms
IFI16
• Sequestration by pp65 for MIEP activation
• Protection from proteasome degradation by pp65
• Delocalization upon phosphorylation by UL97
ND10 COMPLEX
• PML de-SUMOylation by IE1 for degradation
• hDaxx binding by pp71 for proteasome degradation
• Enhancement of pp71-mediated activity by UL35
VIPERIN
• Delocalization by vMIA protein from the endoplasmic reticulum to the mitochondria to increase lipid synthesis and viral production
APOBEC3
• Shaping the nucleotide composition of the HCMV genome