The human cytomegalovirus tegument protein pp65 (pUL83): a key player in innate immune evasion

Review

The human cytomegalovirus tegument protein pp65 (pUL83): a key player in innate immune evasion.

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Running title: pp65 and innate immunity evasion

SUMMARY

The germline encoded proteins serving as "pattern recognition receptors" (PRRs) constitute the earliest step in the innate immune response by recognizing the "pathogen-associated molecular patterns" (PAMPs) that comprise microbe nucleic acids and proteins usually absent from healthy hosts. Upon detection of exogenous nucleic acid two different innate immunity signaling cascades are activated. The first culminates in the production of chemokines, cytokines, and type I interferons (IFN-I), while the second leads to inflammasome complex formation. Human cytomegalovirus (HCMV), a member of the β-herpesvirus subfamily, is a wide spread pathogen that infects a vast majority of the world's population. The virion has an icosahedral capsid that contains a linear dsDNA genome of approximately 240 kb, surrounded by an outer lipid envelope and a proteinaceous tegument containing several viral proteins. Despite the numerous and multifaceted antiviral effects of IFNs and cytokines, HCMV is able to invade, multiply, and establish persistent infection in healthy human hosts. To achieve this goal the virus has developed different strategies to block the IFN-I response and to alter the physiological outcomes of the IFN-inducible genes. This article focuses on HCMV tegument pp65 by reviewing its mechanisms of action in favoring virus evasion from the host innate immune response.

Keywords: human cytomegalovirus (HCMV), pp65, tegument proteins, intrinsic immunity, DNA sensors, restriction factors, antiviral defense, viral escape mechanisms.

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INTRODUCTION

The innate immune response is the first line of defense against human cytomegalovirus (HCMV) and allow the host to rapidly mount antiviral measures (La Rosa and Diamond, 2012; Paludan, 2015). This becomes particularly evident during the perinatal period, because of the immaturity of adaptive immunity (Gibson *et al.,* 2007; La Rosa and Diamond, 2012). The innate immune responses include type I and III interferons, and inflammatory cytokines that immediately set up an antiviral state and initiate an inflammatory response. Innate immunity to virus infection involves recognition of pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs), by germline-encoded molecules termed pattern recognition receptors (PRRs), able to distinguish 'self' from 'non-self'. During virus infection, viral DNA and RNA are detected by a myriad of PRRs whose activation elicits antiviral responses and inflammation (Hoffmann *et al.,* 2015). Generally, these PRRs can be divided into two main categories depending on their subcellular location. PRRs that recognize extracellular PAMPs are essentially found on the plasma or endosomal membranes, and include the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs) (Takeuchi and Akira, 2010; Dambuza and Brown, 2015). These membrane-bound PRRs are largely expressed by antigen presenting cells, such as macrophages and dendritic cells. By contrast, intracellular PRRs are found in the cytoplasm or nucleus of mammalian cells, and include the NOD-like receptors (NLRs) (Kim *et al.,* 2016), RIG-I (retinoic acid-inducible gene I)-like receptors (RLRs) (Loo and Gale, 2011), and a group of intracellular DNA sensors such as cyclic GMP/AMP (cGAMP) synthase (Chen *et al.,* 2016) and interferon-γ-inducible protein 16 (IFI16) (Dell'Oste *et al.,* 2015). Upon the detection of viral pathogens, the PRRs trigger a series of events whose outcome is the activation of various transcriptional factors, including MAP kinases (MAPKs), nuclear factor-κB (NF-κB), interferon regulatory factor 3 (IRF3), and IRF7, required for the transcriptional induction of type I interferon (IFN-I) and the release of pro-inflammatory chemokines to drive immune cells to the site of infection (Mogensen, 2009; Hoffmann *et al.,* 2015). Prointerleukin-1β (pro-IL-1β) and prointerleukin-18 (pro-IL-18) are chief products of PRRs activation, but need to be processed into their functional forms prior to be released from cells through multiprotein complexes known as inflammasomes (Lupfer *et al.,* 2015). Inflammasomes come in many forms and are also mainly activated following recognition of PAMPs (Guo *et al.,* 2015). The common inflammasome platform consists of an inflammasome sensor molecule, an adaptor protein known as ASC (adaptor apoptosis-associated speck-like protein containing a CARD), and a CARD domain containing pro-caspase-1 (Latz *et al.,* 2013). Inflammasome complex assembly results in caspase-1 activation and cleavage of pro-IL-1β and pro-IL-18, leading to their activation and release from cells (Lupfer *et al.,* 2015).

THE HUMAN CYTOMEGALOVIRUS

HCMV, the prototype member of the β-herpesvirus subfamily, is an enveloped virus, with a double-stranded DNA genome, packaged in an icosahedral capsid, consisting of 162 capsomere subunits. The tegument is a protein-rich layer between the capsid and the envelope, containing 50% of the total viral proteins of the virion (*Figure 1*), including a 65-kilodalton (kDa) phosphoprotein (pp) referred to as pp65 (pUL83), one of the dominant targets of the immune response to infection

(Landolfo *et al.,* 2003; Maxwell and Frappier, 2007; Kalejta, 2008; Tandon and Mocarski, 2012; Smith *et al.,* 2014; Griffiths *et al.,* 2015). HCMV slowly grows in cell cultures, displaying a characteristic tissue-specific (blood vessel, lungs, liver, kidney, intestine), strict species-specific tropism, and a broad cell tropism for fibroblasts, epithelial, endothelial, smooth muscle, and myeloid cells (Sinzger *et al.,* 1995; Sinzger *et al.,* 2008; Li and Kamil, 2015). Following primary infection, it establishes a life-long latency in myeloid cells of the bone marrow and then periodically reactivates (Reeves and Sinclair, 2013; Dupont and Reeves, 2016).

HCMV is worldwide distributed, with a seroprevalence ranging from 45 to 100% that is likely to be highest in countries of lower socioeconomic conditions (Cannon, 2009; Cannon *et al.,* 2010). The vast majority of infections are asymptomatic or self-limited in immunocompetent individuals, whereas in immunocompromised hosts and infected fetuses, HCMV produces a high burden of disease (Navarro, 2016). HCMV infection is one of the most common congenital infections worldwide. Its rate in developed countries is estimated to average from 0.6 to 0.7% of all live births, and approximately 60,000 newborns per year are HCMV positive in the United States and the European Union combined (Manicklal *et al.,* 2013; Marsico and Kimberlin, 2017). Symptomatic infections are characterized by mild neurological impairment or deafness, blindness, mental retardation, and premature death (Britt, 2017). Importantly, HCMV is also the most prevalent viral agent in transplant settings with symptomatic infections occur in 20-60% of transplant patients (Brennan, 2001). Patients acquire HCMV from reactivation of latent virus or donor-transmitted virus, leading to impaired graft survival, graft-versus-host disease, and other opportunistic infections such as invasive fungal infections (Kaminski and Fishman, 2016). These patients can also develop life-threatening, multi-organ infectious disease, as the virus disseminates to the lung, liver, pancreas, kidney, stomach, intestine, brain, and parathyroid glands (Potena *et al.,* 2016; Camargo and Komanduri, 2017; López-Botet *et al.,* 2017). HCMV causes severe disease in AIDS patients, including retinitis, gastrointestinal disease, and encephalitis (Shinkai *et al.,* 1997; Sissons and Wills, 2015). Moreover, its association with other syndromes has been reported, such as inflammatory bowel disease (Sager *et al.,* 2015; Siegmund, 2017), Alzheimer's disease (Harris and Harris, 2015), new-onset diabetes, hypertension, immunosenescence, and atherosclerosis (Einollahi *et al.,* 2014; Halenius *et al.,* 2014; Wang *et al.,* 2017). Finally, a link between HCMV and several human cancers, such as glioblastoma and breast cancer has been suggested (Söderberg-Nauclér, 2006; Herbein and Kumar, 2014; Richardson *et al.,* 2015; Quinn *et al.,* 2016; Foster *et al.,* 2017). Nonetheless, it remains elusive whether HCMV is the causative agent or a bystander in these diseases.

The HCMV pp65 (pUL83)

The tegument of HCMV and other herpesvirus virions is a unique proteinaceous layer located between the lipid envelope and the capsid (Kalejta, 2008). In these last few years, the generation of mutant viral strains by BAC technology and the improvement of microscopy techniques offered the chance to better understand the role of tegument proteins in the context of HCMV replication (Smith *et al.,* 2014). For example, the tegument is generally defined as an unstructured and amorphous layer, but recent cryo-electron microscopy studies allowed dissecting

defined structures inside the HCMV nucleocapsid proximal inner, with the binding of tegument proteins to the protein capsid (Chen *et al.,* 1999). Of the 71 viral proteins found within infectious virions, over one-half are tegument components, 10 of which are conserved across herpesviruses (Varnum *et al.,* 2004) (*Figure 1*).

pUL83 (also known as phosphoprotein pp65) is the HCMV most abundant tegument protein and the major constituent of extracellular particles (>2,000 copies per mature virion) (Irmiere and Gibson, 1983; Varnum *et al.,* 2004). Like the other nuclear tegument proteins, pp65 traffics to the nucleus of permissively infected cells after fusion of the viral and cellular membranes immediately after entry, and then relocalizes to the cytoplasm at the later stages of infection (Schmolke *et al.,* 1995; Sanchez *et al.,* 2000). While initial viral growth experiments using pp65-deletion mutants demonstrated pp65 is not essential for viral replication in vitro and for the production of new infectious virus particles (Schmolke *et al.*, 1995), cumulative evidence points that pp65 is important for efficient growth in both monocyte-derived macrophages and fibroblasts (Chevillotte *et al.,* 2009; Becke *et al.,* 2010; Cristea *et al.,* 2010). The primary cause of this growth defect likely involves impaired pp65 functions during the initiation of infection as well as regulation of pp65 localization (Smith, 2014). Accordingly, it has been demonstrated that disruption of pp65 expression can influence the incorporation of viral proteins into the virion tegument, such as pUL25, ppUL97 and ppUL69, by direct or indirect binding, suggesting that pp65 is part of a complex protein interaction network that may be crucial for assembly (Kamil and Coen, 2007; Chevillotte *et al.,* 2009; To *et al.,* 2011). In spite of these modifications in the tegument composition, the virion particles are comparable to the wild type virus, except for the absence of dense bodies. However, it remains to be definitively established if the defect in replication of the pp65 mutant virus in vitro is due to the absence of pp65 per se, or to the lack of important viral proteins such as ppUL97 and ppUL69 during the initial stages of replication (Kamil and Coen, 2007; Chevillotte, 2009; To *et al.,* 2011).

Recently, pp65 protein sequence and its posttranslational modifications (PTMs) have been characterized (Li et al., 2013). The protein displays a bipartite structure, organized in a conserved N-terminal domain (~386 residues), a divergent linker region, and a conserved C-terminus (CTD, ~90 residues) (*Figure 2*). pUL83 is known to be phosphorylated (Roby and Gibson, 1986); however, the phosphorylation sites have been just recently defined, after isolation of the virally or ectopically expressed pUL83 from human fibroblasts followed by mass spectrometry analyses. Eight phosphorylated serine (S) or threonine (T) residues present in both virally and ectopically expressed pUL83, and two sites (T32 and S472) detected only in the latter were identified, suggesting that most phosphorylations are regulated by host kinases. Notably, most sites are within the linker, with S406, T468, and S472 present at casein kinase II-like motifs (S/TXXE/D) (Li *et al.,* 2013). Phosphorylation is directly involved in regulating the localization of pp65 during infection. It has been demonstrated pp65 can shuttle in and out from the nucleus with export in a CRM1 dependent manner (Prichard *et al.,* 2005; Frankenberg *et al.,* 2012), with many tegument proteins such as ppUL69, pUL35, pTRS1 and pUL96 functioning as shuttling protein (Blankenship and Shenk, 2002; Schierling *et al.,* 2005; Toth and Stamminger, 2008; Tandon and Mocarski, 2012). It is possible that phosphorylation of pp65 also plays a role in regulating the localization of this protein during HCMV repliaction. For example, pp65 phosphorylation by ppUL97 regulates pp65

redistribution to the AC at the later stages of infection (Prichard *et al.,* 2005; Becke *et al.,* 2010). These findings are supported by experiments with CDK inhibitors, that affect both the phosphorylation and localization of pp65 (Sanchez *et al.,* 2007). One possible explanation of these data is that phosphorylation of pp65 by ppUL97 blocks recognition of the nuclear localization signals, resulting in a change in distribution to the cytoplasm (Smith *et al.,* 2014). A useful model to understand the impact of pp65 phosphorylation on its localization is the RV-VM1 mutant virus, containing a 30 amino acid insertion within the pp65 open reading frame (amino acid 387, in relatively close proximity to the pp65 nuclear localization signals) (Becke *et al.,* 2010). This virus displays a pp65 protein retained in the nucleus at the late stages of infection, resulting in accumulation of the MCP and large globular structures within the nucleus, enriched of pp65 and ppUL69 proteins, but with a reduced numbers of C-capsids in the cytoplasm, and a lack of dense body formation. Consequently, a different pattern of phosphorylation may result in an active nuclear localization signal that trigger accumulation and aggregation of the protein in the nucleus. Interestingly, two phosphorylation sites were also clustered in the amino terminus in proximity to the self-interaction domain, suggesting that phosphorylation also regulates this behavior of pp65. These data are definitely consistent with the large aggregates of pp65 that occur in the absence of ppUL97 activity (Prichard *et al.,* 2005; Smith *et al.,* 2014).

The most enigmatic characteristic assigned to pp65 is its kinase activity. Early on, it was discovered that virion-associated kinase activity (Mar *et al.,* 1981) is diminished in the UL83-null virus (Michelson *et al.,* 1984; Schmolke *et al.,* 1995), indicating a pp65-associated kinase activity. However, it was not fully understood if pp65 owns an intrinsic kinase activity (Yao *et al.,* 2001) or associates with cellular kinase (Gallina *et al.,* 1999) inside HCMV virions. The putative kinase domain of pp65 shows indeed only poor homology with other kinases (Yao *et al.,* 2001). Therefore, it is possible that the kinase activity observed in immunoprecipitates with pp65 antibodies might not result from an intrinsic kinase activity of pp65 itself but rather from a copurification with other cellular or viral kinases. Consistently, pp65 has been found in association with viral UL97 protein kinase (Kamil and Coen, 2007), and the cellular proteins polo-like kinase 1 (Plk1) and casein kinase II, that have also been found in wild type, but not UL83-null virions (Gallina *et al.,* 1999; Nogalski *et al.,* 2007). Accordingly, recombinant pp65 protein autophosphorylates and phosphorylates casein in vitro on threonine residues only, and a mutation of the predicted catalytic lysine (K436N) abolished this kinase activity (Yao *et al.,* 2001).

pp65 and innate immunity modulation

pp65 is responsible for modulating/evading multiple levels of host cell immune surveillance during HCMV infections in at least three different ways (McCormick and Mocarski, 2007; Kalejta, 2008): i) blocking antigen presentation (Gilbert *et al.,* 1996; Odeberg *et al.,* 2003); ii) modulating the natural killer cells (Arnon *et al.,* 2005); iii) suppressing the induction of antiviral cytokines (Browne and Shenk, 2003; Abate *et al.,* 2004).

First, the capability of pp65 to prevent major histocompatibility complex (MHC) presentation of IE antigens at the start of HCMV infections appears to be dependent on its kinase activity. It was shown that pp65 mediates the phosphorylation of viral IE proteins, which blocks

their presentation to the MHC class I molecules (Gilbert *et al.,* 1996). The kinase activity of pp65 has also been implicated in causing the degradation of the alpha chain in the MHC class II cell surface receptor, HLA-DR, via an accumulation of HLA class II molecules in the lysosome (Gilbert *et al.,* 1996). Furthermore, several studies have presented evidence that pp65 is involved in mediating a decrease in the expression of MHC class II molecules (Odeberg *et al.,* 2003).

Another crucial role of pp65 in immune evasion during HCMV infections is through the inhibition of NK cell cytotoxicity (Arnon *et al.,* 2005; Rölle and Brodin, 2016). In detail, pp65 can act as an antagonistic ligand that can bind to the NKp30 activating receptor to protect the killing of infected cells as well as interfere with the ability of NKp30 to cross-talk between other natural killer cells and dendritic cells (Arnon *et al.,* 2005; Arnon *et al.,* 2006). Moreover, pp65 has been shown to impair host innate immunity by modulating the interferon response and the inflammasome system (*Figure 4*) (Browne and Shenk, 2003; Abate *et al.,* 2004; Li *et al.,* 2013; Huang *et al.,* 2017). This point will be discussed in more details in the following section.

Third, viruses and their host exert a constant evolutionary pressure on each other, leading to mutual adaptation. As an example of pathogen-host co-evolution, AIM2-like receptors (ALR) loci in different mammals are quite distinct and AIM2 and IFI16 appear to have evolved adaptively in primates (Cagliani *et al.,* 2014), suggesting a strong selective pressure (Brunette *et al.,* 2012). Similarly, HCMV has evolved strategies to evade detection by ALRs, mainly by the HCMV tegument protein pp65. It has been demonstrated that early during infection, pp65 suppresses IFI16 mediated detection of HCMV genomic DNA in the nucleus by binding to the IFI16 pyrin (PY) domain through its own pyrin association domain (PAD), thus preventing IFI16 oligomerization (Li *et al.,* 2013). Moreover, previous reports (Cristea *et al.,* 2010; Gariano *et al.,* 2012) have shown that very early during infection, pp65 and IFI16 interact at the viral major immediate-early promoter (MIEP), triggering an increase in IE protein expression, which is accompanied by a concomitant decrease in antiviral cytokine production. MIEP activation could be NF-κB mediated, because four NF-κB responsive elements are present on the MIEP. Moreover, a functional analysis of the ICAM-1 promoter by deletion- or site-specific mutagenesis has indeed demonstrated that NF-kB is the main mediator of IFI16-driven gene induction (Caposio *et al.,* 2007), supporting the hypothesis of a ternary interactome (i.e. pp65/IFI16/NF-kB) at the MIEP promoter (*Figure 3*).

During the late stages of HCMV infection, a different scenario was observed. First of all, consistent with the above reported observations, an increase in IFI16 expression during the early events of HCMV replication has been described also by Biolatti et al. (2016), confirming that HCMV fosters IFI16 expression with the aim of stimulating IE gene expression at the beginning of infection (Biolatti *et al.,* 2016). Of note, the interplay between pp65 and IFI16 was not limited to the very early stage of infection but remained functionally relevant throughout the later stages of viral replication, including the central process of viral DNA synthesis. In this case, the outcome of IFI16/pp65 interaction is downregulation, instead of upregulation, of the UL54 gene promoter, as shown by experiments performed with pp65 mutant viruses (Biolatti *et al.,* 2016). Interestingly, at later time points of HCMV infection, IFI16 is stabilized by pp65, and delocalized from the nucleus to the cytoplasm, to be finally entrapped within newly egressing virions (Biolatti *et al.,* 2016).

Overall, these date confirm and expand the role of IFI16 as a restriction factor for HCMV replication and of pp65 as HCMV evasion mechanism from innate immune response (*Figure 3*).

pp65 and IFN signaling. The IFN system is one of the key component against HCMV infection; conversely, HCMV, like other herpesviruses, encodes viral factors to counteract IFN pathway, even if the exact mechanisms HCMV relies on are only partially defined (Marshall and Geballe, 2009). Results obtained by different groups (Browne and Shenk, 2003; Abate *et al.,* 2004; Li *et al.,* 2013), including those from our lab (unpublished results), and different approaches, such as RT-qPCR, ELISA, and DNA array analysis demonstrated that the HCMV tegument protein pp65 is the main inhibitor of IFN-I expression, even if recently also pp71 has been recognized to neutralize IFN release (Fu *et al.,* 2017). However, it is still matter of debate, at which level pp65 counteracts IFN activation. First of all, one of the main target of pp65 is IRF3, a key component of IFN pathway because it induces IFN-β synthesis at the transcriptional level (Abate *et al.,* 2004). In the milestone paper from Abate et al. (2004), the use of a deletion mutant HCMV strain unable to express pp65 resulted in a greater induction of an IFN-like transcriptional profile after HCMV infection, conforming the inhibitory activity of pp65 (Abate *et al.,* 2004). The hypothesis that IRF3 could be the target of pp65 has been supported by the observation that in the first few hours after infection with the pp65-mutant, but not with wild type virus, IRF3 was converted in the hyperphosphorylated-active form and then translocated to the nucleus of human fibroblasts or PBMC. Moreover, transduction of pp65 alone into human fibroblasts blocked nuclear translocation of IRF3 following an activating stress (Abate *et al.,* 2004) (*Figure 4*). In contrast, the activation of NF-κB, another transcriptional activator of IFN, was pp65-independent, as suggested by the observation that it relocalized to the nucleus of cells by either wild type or pp65-deleted virus. Altogether, these data support the ability of pp65 to block IRF3 activation, by acting in at least two different ways: i) by preventing its cytoplasmic phosphorylation and nuclear accumulation; ii) by promoting its nuclear dephosphorylation and export (Abate *et al.,* 2004; Marshall and Geballe, 2009). The role of pp65 in the counteraction of IFN activation was further supported by the observation that the transcription of IFN-β and several other IFN-stimulated genes (ISGs) was induced to a greater extent by the pp65 deletion mutant virus compared to wild type HCMV (Browne and Shenk, 2003). In addition, the overexpression of pp65 by an adenoviral vector inhibited the induction of MxA by IFN-α (Browne and Shenk, 2003).

The other side of this complex scenario consists of different reports stating that pp65 does not mediate the HCMV evasion from the IFN response. For example, different authors (Browne and Shenk, 2003; DeFilippis *et al.,* 2006; Taylor and Bresnahan, 2006), found that IRF3 relocalized in the same way to the nucleus after infection with wild type or pp65-mutant virus (pp65 deletion or pp65 stop mutants), in contrast to the results of Abate et al. (2004) (Abate *et al.,* 2004). Instead, the absence of pp65 resulted in induction of IRF1 expression and nuclear accumulation of both IRF1 and NF-κB (Browne and Shenk, 2003; Marshall and Geballe, 2009). A very recent work by Fu et al. (2017) partially supports the second idea, because another HCMV tegument protein, pp71, instead of pp65, has been identified as a negative regulator of STING-mediated induction of downstream antiviral genes and innate antiviral response (Fu *et al.,* 2017). This observation could partially

explain the finding that a pp65 nonsense mutant virus, unlike the pp65 deletion virus, retained the capability to block the IFN-β induction (Taylor and Bresnahan, 2006); so it's likely that the impact of the pp65 deletion might be mediated by a reduced expression of pp71, which in turn affects IE86 expression and IFN evasion. The discrepancies among these studies could be explained by several factors. One variable that is seldom considered in experimental work with HCMV is variation among viral stocks. In particular, the ratio of pp65 between virions and dense bodies in viral preparations may vary widely (Jahn *et al.,* 1987). Thus, differences in the dose of pp65 or other virion factors delivered to the cells in different studies might underlie some of the conflicting results (Marshall and Geballe, 2009).

pp65 and inflammasome activation.

The inflammasome is a key component of the innate immune system, and in these last few years, many advances have greatly increased our understanding of its macromolecular activation. The impact of pp65 on inflammasome activation has been evaluated (Huang *et al.*, 2017), focusing on the system driven by the DNA sensor absent in melanoma 2 (AIM2). AIM2 is an interferon inducible protein involved in the foreign double-stranded DNA (dsDNA) recognition by a C-terminal hematopoietic IFN-inducible nuclear (HIN) domain. Then, an N-terminal pyrin domain binds the adaptor molecule ASC and subsequently recruits pro-caspase-1 via CARD domain for its self-cleavage to form a functional inflammasome (Schattgen and Fitzgerald, 2011; Morrone *et al.,* 2015; Huang *et al.,* 2017). Although the impact of AIM2-inflammasome to counteract HCMV infection remains to be fully elucidated, data suggests that the AIM2 HIN domain can recognize HCMV dsDNA through electrostatic interactions, regardless of the DNA sequence and GC content, but in a length-dependent manner (Morrone *et al.,* 2015; Huang *et al.,* 2017). These results are supported by the findings that HCMV infection induces the secretion of the pro-inflammatory cytokines IL-1β and IL-18, the products of inflammasome activation, in both the serum of renal transplant recipients who developed a primary HCMV infection, and in HCMV-infected gingival fibroblasts, respectively (Botero *et al.,* 2008; van de Berg *et al.,* 2010; Huang *et al.,* 2017). In an in vitro recombinant model consisting of HEK293T cells expressing AIM2, ASC, pro-caspase-1, and pro-IL-1β, Huang et al. (2017) proposed that pp65 directly downregulates AIM2 inflammasome (Huang *et al.,* 2017). In particular, pp65 binds to the cytoplasmic AIM2 early during infection, thus partially attenuating the AIM2 inflammasome proteins, followed by a reduction of caspase-1 and IL-1β activation (*Figure 4*). By contrast, Li et al. (2013) showed that pp65 bound the pyrin domain of all nuclear PYHIN proteins (IFI16, IFIX, and MNDA), but not the AIM2-PY domain (Li *et al.,* 2013). Notably, a pp65 deletion mutant HCMV strain is not able to induce a higher inflammasome activity compared to the wild type HCMV, as reflected by caspase-1 cleavage, thus further confirming the inhibitory activity on inflammasome activation (Li *et al.,* 2013). Taken together, this data strength the controversial role of pp65 as a main player in the modulation of HCMV evasion mechanisms (*Figure 4*).

CONCLUSIONS

During millions of years of coevolution with the hosts, HCMV developed distinct strategies to counteract cellular defenses. The main player of HCMV evasion mechanisms against the innate

immune response is the tegument protein pp65, that act at different levels: i) early during infection pp65 interacts with and stabilizes IFI16 at the promoter of immediate-early genes, triggering an increase in IE protein expression, while later pp65/IFI16 interactome inhibits UL54 gene expression blocking HCMV replication; ii) pp65 interferes with the IFN pathway; iii) and with inflammasome activation. Collectively, these data underline the role of tegument protein pp65 as a critical molecular hub to evade the innate immune response induced by HCMV infection.

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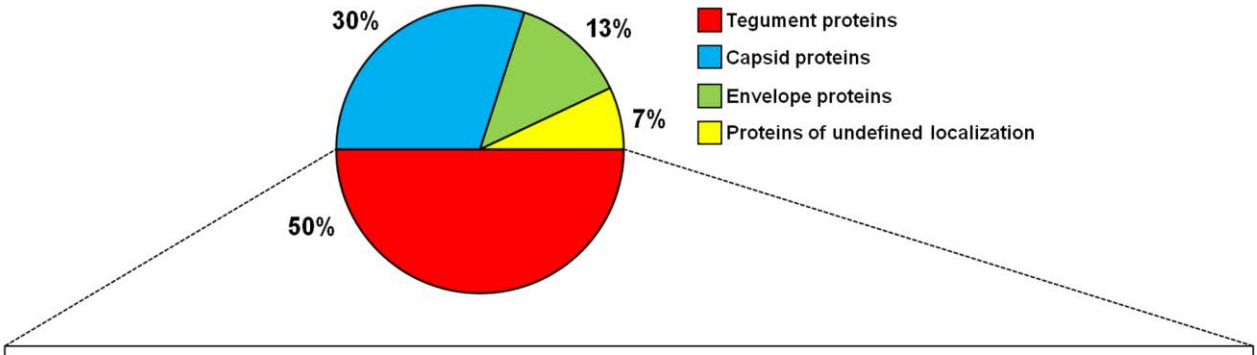
Figure 1 - *Genes that encode representative tegument proteins along with commonly accepted protein names are shown. Data on relative abundance were obtained from Varnum et al.* (Varnum *et al.*, 2004). *Phenotype refers to the ability of virus with a mutation in the indicated gene to replicate in human fibroblasts unless otherwise stated. Phenotypes are listed as augmenting (A), dispensable (D), or essential (E) for virus replication. Functions displays either demonstrated or inferred functions for these proteins.*



Gene	Abundance	Phenotype	Functions
Inner Tegument			
UL32 (pp150)	9.1%	E	Directs capsid to site of final envelopment
UL48	12.6%	E	Deubiquitinating protease/Release of viral DNA from capsid
Nuclear Tegument			
UL26	0.1%	A	Increases stability of virion proteins
UL35	0.5%	A	Activates viral gene expression
UL82 (pp71)	8.9%	A	Degrades Daxx/Facilitates IE gene expression/Degrades Rb Stimulates cell cycle progression/Prevents cell surface expression of MHC
UL83 (pp65)	15.4%	D	Endogenous kinase activity/Associated kinase activity Evasion of adaptive immunity/Evasion of innate immunity
UL94	1.2%	A/E	Putative DNA-binding protein
UL97	0.1%	A	Kinase that phosphorylates ganciclovir/Stimulates DNA replication,assembly, egress Cyclin-dependent kinase-like functions/Disrupts nuclear aggresomes
TRS1	0.6%	A/E	Inhibits PKR antiviral response/Virion assembly
Cytoplasmic Tegument			
UL99 (pp28)	Unknown	E	Directs enclosure of enveloped particles

Figure 2 - *Schematic representation of the viral tegument protein pp65. Sequence revealed a conserved N-terminal Pyrin Association Domain (PAD) (yellow box), a divergent linker region (white box), and a conserved C terminus (CTD, ~90 residues) (brown box), indicating a bipartite structure. The conserved NLS motifs (KRRRHR/KKHR) are also shown. Numbers located above each diagram represent phosphorylation sites.*

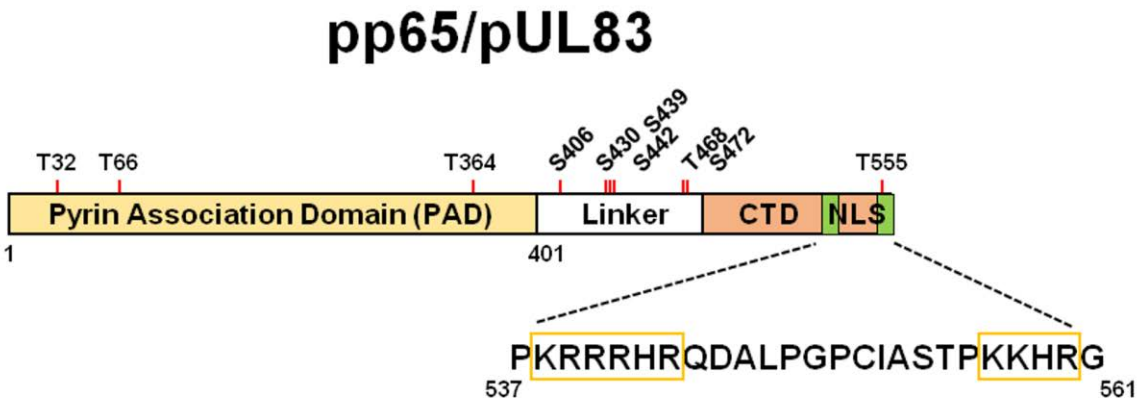


Figure 3 - *Interplay between pp65 and IFI16 during HCMV infection. (A) Wild type infection allows maximal activation of the MIEP through efficient recruitment of IFI16 and probably NF-kB (dashed line) by pp65. (B) In the absence of IFI16, pp65 can still drive expression from MIEP, albeit less efficiently, perhaps through either a weaker interaction with or less efficient recruitment of NF-kB. (C) In the absence of pp65, IFI16 and NF-kB may still be capable of interacting at the MIEP, though MIEP expression is decreased indicating the requirement of pp65 for maximal expression. (D) Wild type infection allows maximal inhibition of the UL54 gene promoter through efficient recruitment of IFI16 by pp65. (B) In the absence of IFI16, pp65 can still drive expression from UL54 gene promoter, albeit less efficiently. (C) In the absence of pp65, and IFI16 may still be capable of interacting at the UL54 gene promoter, though UL54 gene promoter inhibition is absent indicating the requirement of pp65 for maximal inhibition.*

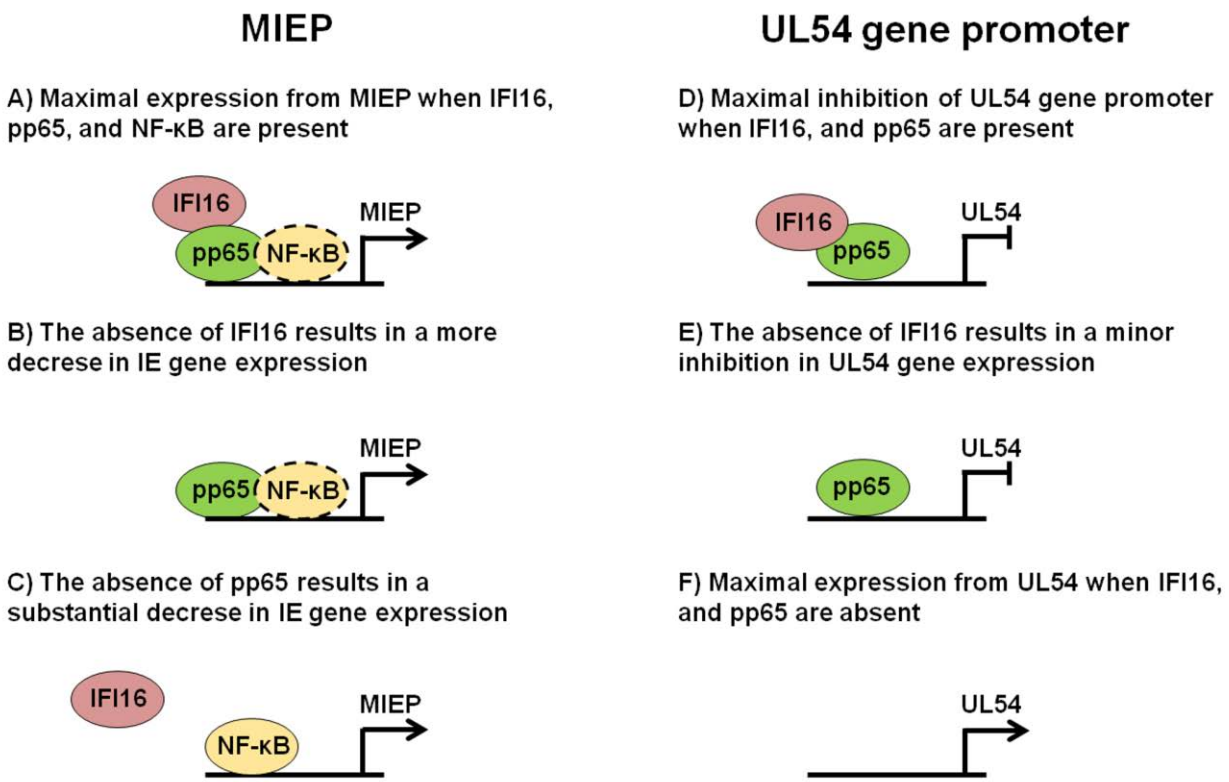


Figure 4 - *Model for the functional role of pp65 in modulating IFNs and inflammasome signaling during HCMV infection.*