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*Review*

# Interplay between autophagy and herpes simplex virus type 1: ICP34.5, one of the main actors

**Inés Ripa [1,2,*], Sabina Andreu [1,2], José Antonio López-Guerrero [1,2] and Raquel Bello-Morales [1,2]**

[1] Departamento de Biología Molecular, Edificio de Biología, Universidad Autónoma de Madrid, Darwin 2, Cantoblanco, 28049 Madrid, Spain; sandreu@cbm.csic.es (S.A.); ja.lopez@uam.es (J.A.L.-G.); raquel.bello-morales@uam.es (R.B.-M.).

[2] Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas (CSIC), Universidad Autónoma de Madrid (UAM), Cantoblanco, 28049 Madrid, Spain

* Correspondence: ines.ripa@cbm.csic.es

**Abstract:** Herpes simplex virus type 1 (HSV-1) is a neurotropic virus that, occasionally, may spread to the central nervous system (CNS), being the most common cause of sporadic encephalitis. One of the main neurovirulence factors of HSV-1 is the protein ICP34.5 that, although initially seemed to be relevant only in neuronal infections, it can also promote viral replication in non-neuronal cells. New ICP34.5 functions have been discovered during the recent years, and some of them have been questioned. This review describes the mechanisms of ICP34.5 to control cellular antiviral responses and debates its most controversial functions. One of the most discussed roles of ICP34.5 is autophagy inhibition. Although autophagy is considered a defense mechanism against viral infections, current evidence suggests that this antiviral function is only one side of the coin. Different types of autophagic pathways interact with HSV-1 impairing or enhancing the infection, and both the virus and the host cell modulate these pathways to tip the scales in its favor. In this review, we will summarize the recent progress on the interplay between autophagy and HSV-1, focusing on the intricate role of ICP34.5 in the modulation of this pathway to gain the battle against cellular defenses.

**Keywords:** ICP34.5; autophagy; herpes simplex virus type 1; neurovirulence.

## 1. Introduction

Autophagy [1] is a highly conserved catabolic process among eukaryotes consisting of the degradation of intracellular components into lysosomes to ensure metabolic homeostasis [2,3]. There are different types of autophagic pathways: macroautophagy, microautophagy, endosomal microautophagy and chaperone-mediated autophagy (CMA) [4]. The most studied pathway is macroautophagy, which involves the *de novo* generation of double-membrane vesicles called autophagosomes, which are subsequently fused with lysosomes for cargo degradation. Autophagosome formation is a highly regulated process carried out by functional units constituted by autophagy-related (ATG) proteins [5]. This process has been extensively reviewed in the recent years [6–10] and consists of four sequential steps, which include initiation, nucleation, elongation and fusion.

First, an isolation membrane, called phagophore, is created at the autophagosome formation site. The primary membrane source of phagophores is the endoplasmic reticulum (ER) [11], but mitochondria [12], cell membrane [13], lipid drops [14], ER-Golgi intermediate compartment (ERGIC) [15] and recycling endosomes [16] have also been raised as possible membrane sources. The first functional unit that takes part in the initiation of the phagophore is the Unc-51-like kinase (ULK) complex, which is composed of four components (ULK1/2, ATG13, FIP200/RB1CC1, and ATG101). ULK complex targets membrane structures to the phagophore formation site and triggers the nucleation of the phagophore by the recruitment of a second autophagy functional unit, the class III phosphati-

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dylinositol 3-kinase complex I (PI3KC3-C1), formed by four elements (VPS34, VPS15, Beclin-1, and ATG14L). The cooperation of these components increases the local concentration of phosphatidylinositol-3-phosphate (PI3P), promoting the recruitment of PIP3-binding autophagy proteins to the phagophore membrane. For initiation and nucleation steps is also essential the role of the multi-spanning membrane protein ATG9A, which is located in vesicles and/or tubules in close proximity to phagophores. Through the transient association of ATG9A with phagophore membranes, the protein supplies key elements to the phagophore [17,18].

The following elongation and maturation of autophagosomal membranes involves two ubiquitin-like conjugations steps: the conjugation of ATG12 to ATG5 (with the subsequent recruitment of ATG16L1 to form a functional complex) and the post-translational modification of the microtubule-associated protein 1 light chain 3 (LC3) with a phosphatidylethanolamine (PE). First, ATG4 cleaves the C-terminal residues of LC3 to expose a glycine residue (LC3-I). Then, LC3-I is activated by the E1-like enzyme ATG7 and transferred to the E2-like enzyme ATG3. The ATG12-5-16L1 complex, which functions as an E3-like enzyme, facilitates the transfer of LC3-I from ATG3 to a PE of the autophagosomal membrane [19]. PE-conjugated LC3B-I, namely LC3B-II, is the most common marker used to monitor autophagy (18). Maturation and enclosure of autophagosomes are followed by the fusion of the outer autophagosomal membrane with a lysosome [20]. Finally, the inner autophagosomal membrane is degraded by lysosomal hydrolases, leading to the formation of a single-membrane, mature autolysosome.

Autophagy can be classified into non-selective or selective depending on the cargo. In non-selective autophagy, bulk portions of the cytoplasm are sequestered by phagophores. This type of autophagy is usually stimulated under starvation conditions, due to its contribution to the maintenance of nutrient levels in cells. On the contrary, selective autophagy is based on the degradation of specific intracellular cargo through the activity of selective autophagy receptors (SARs). SARs interact with ATG8 family proteins on the inner membrane surface of phagophores, mediating the delivery of specific cargo and facilitating the recruitment of autophagic machinery [21,22]. In addition to maintaining the number and integrity of cellular organelles [23], selective autophagy also participates in pathogen clearance. The kind of selective autophagy involved in sequestration and degradation of cytoplasmic viral components and/or entire virions is known as virophagy [24]. This type of autophagy also contributes to the viral antigen processing and subsequent presentation on major histocompatibility complex (MHC) class I and II, which initiates antiviral adaptative immunity [25].

Many viruses can reverse and exploit multiple steps of autophagy, thus evading the immune response and facilitating viral replication [26]. This is the case of herpes simplex virus type 1 (HSV-1), which has acquired the ability to down-regulate the autophagic pathway. HSV-1 is a neurotropic virus that, after a primary infection in epithelial cells, traffics from axon terminals to the trigeminal ganglia and establishes latency [27]. Periodically, the virus can reactivate, being the most common symptoms of HSV-1 infection the painful blisters or open sores in or around the mouth. Occasionally, the virus may spread from the trigeminal ganglia to the brainstem, reaching the central nervous system (CNS) and causing herpes simplex encephalitis (HSE) [28]. This virus has also been proposed as an etiological agent or as risk factor for neurological pathologies, such as Alzheimer's disease [29] and multiple sclerosis [30].

Due to the high adaptability of herpesviruses to their hosts, HSV-1 has developed several mechanisms to evade cellular defenses [31]. In this review, we will focus on the virulence factor ICP34.5, a viral multifunctional protein that blocks many facets of the cellular antiviral responses and plays an essential role in productive HSV-1 infection in several cell types [32], being the inhibition of autophagy one of its most studied and controversial functions.

## 2. The HSV-1 factor of virulence ICP34.5

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*2.1. The HSV-1 γ34.5 gene and the infected cell protein 34.5*

The infected-cell protein 34.5 (ICP34.5) of HSV-1 is encoded by the γ34.5 gene, also known as RL1. This gene is located in the terminal repeat (TR) sequences of the HSV-1 DNA, and therefore is present in two copies per genome [33]. γ34.5 gene is classified as a gamma-1 or leaky late gene. Although maximal levels of ICP34.5 are reaching in the late phase of infection, the expression and function of the protein at early times -3 hours post infection- are essential for the control of host cell environment and for a successful viral replication [34]. γ34.5 gene lacks a canonical TATAA box. Part of its promoter and the transcription initiation site are within the unique repeat junction called the "a" sequence, and its 5′unstranscribed domain (UTR 5′) is GC-rich and has several repeats, lacking the characteristic features of HSV-1 promoters [35].

The structure of ICP34.5 consists of a disordered N-terminal domain, following by a linker region with a variable number of ATP (Ala-Thr-Pro) repeats, and a conserved C-terminal domain (Figure 1). The C-terminal domain of ICP34.5 is highly homologous to the C-terminal of two conserved mammalian proteins implied in cellular stress responses: the growth arrest DNA damage-inducible protein GADD34 [36] and the myeloid differentiation primary response protein MyD116 [37]. These proteins are structurally related, and their corresponding domains are functionally interchangeable with the C-terminal of ICP34.5 in infected cells, being proposed that, in the course of virus evolution, HSV-1 "borrowes" this gene domain to deal with cellular defenses [38].

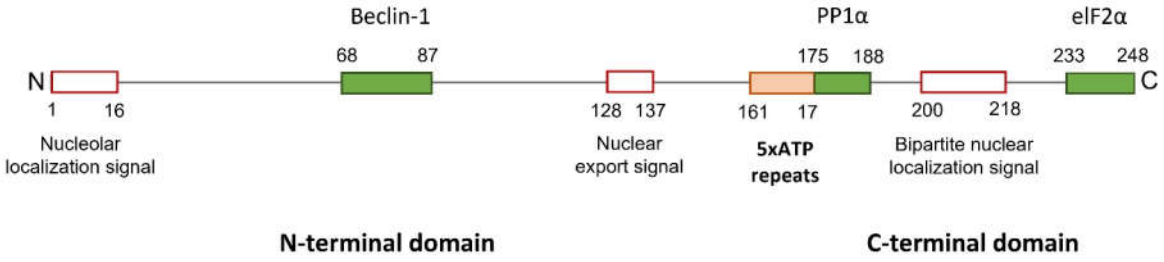


**Figure 1. ICP34.5 of HSV-1 strain 17.** HSV-1 ICP34.5 is a 248 amino acid protein, which is divided into three regions: the amino and the carboxyl terminal domains, and a linked tandem of five ATP repeats (161-175 aa) (orange box). The N-terminal region of ICP34.5 contains a nucleolar localization and nuclear export signals, and the C-terminal domain includes a nuclear localization signal (red boxes). Three binding domains (green boxes) have been characterized: the Beclin-1-binding domain (BBD) in the N-terminus, and the PP1α- and elF2α-binding domains in the C-terminus.

ICP34.5 is a highly dynamic protein that shuttles between the nucleus, the nucleolus and the cytoplasm, being its function dependent on its cellular location [39]. Generally, at early times of HSV-1 infection, the protein is mainly located in the nucleus and, at 8-12 hours post-infection, it is accumulated in the cytoplasm [40]. The N-terminus of ICP34.5 contains an arginine-rich cluster, that primarily targets the protein to the nucleolus, and a leucine-rich cluster, which acts as a nuclear export signal. Besides, the C-terminus contains a bipartite nuclear localization signal, consisting of two clusters of basic amino acids separated by a 9-11 amino acids spacer, which is implied in the import of ICP34.5 into the nucleus [39].

The amino and carboxyl-terminal domains of ICP34.5 are mainly constant, but the number of ATP repeats of the linker region varies between 5 and 10 depending on the HSV-1 strain. Variations in the number of ATP repeats may indirectly affect the cellular location of the protein, likely by masking or unmasking nuclear signals. ICP34.5 with a higher number of ATP repeats tends to be in the cytoplasm [41].Moreover, the number of ATP repeats determines not only the ICP34.5 cellular localization, but also the tissue-behaviour and the virulence of the virus [42].

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### 2.2. HSV-1 ICP34.5: a catch-all

ICP34.5 was primarily described as a neurovirulence factor essential for HSV-1 infection of neurons *in vivo*. HSV-1 lacking the γ34.5 gene was profoundly neuroattenuated in mouse brains [33,43]. However, the role of ICP34.5 in viral pathogenesis is strongly dependent on both cell type and cell stage, and it is not only restricted to neuronal cells. For instance, ICP34.5 is required for HSV-1 infection of stationary state mouse embryo fibroblast 3T6 cells, whereas the replication of ICP34.5-deficient HSV-1 in hamster kidney fibroblasts BHK cells is indistinguishable from wild-type virus [44]. The role of ICP34.5 in HSV-1 infection is extremely complex and the reason of the strong cell type dependence is not fully clarified yet. Even when ICP34.5 is considered an essential virulence factor for HSV-1 CNS infection, ICP34.5-deficient viruses retain the ability to infect and destroy the mouse ependyma (the layer formed by epithelial glial cells that lines the surface of the ventricular system and the central canal of the spinal cord) [45]. In the following sections, we will describe and discuss the multiple functions of ICP34.5 during HSV-1 infection, being these roles classified according to the domain of the protein involved.

#### 2.2.1. Functions of the carboxyl domain of HSV-1 ICP34.5

The first described function of ICP34.5 was associated with its C-terminal domain. Upon viral infection, host cells detect the presence of the virus through pattern recognition receptors (PRRs). PRRs recognize conserved viral structures called pathogen-associated molecular patterns (PAMPs), which include viral proteins, DNA and RNA [46]. PRRs relay signals to TANK-binding kinase 1 (TBK1), which phosphorylates the interferon (IFN) regulatory factor 3/7 (IRF3/7). Phosphorylated IRF3/7 is translocated to the nucleus, where it induces the production of type I IFN and other cytokines. In response to IFN, several molecules are activated through the JAK/STAT pathway, stablishing an antiviral environment in the neighboring cells [47,48]. Among these antiviral molecules is dsRNA-dependent protein kinase (PKR), a cytoplasmic protein capable of detecting viral dsRNA. Its major role is the inhibition of both cellular and viral protein synthesis through the phosphorylation of the translation initiation factor 2 subunit $\alpha$ (elF2$\alpha$) [49]. HSV-1 is able to block this important antiviral mechanism via ICP34.5 [50] (Figure 2). The C-terminal domain of ICP34.5 recruits elF2$\alpha$ and the protein phosphatase 1$\alpha$ (PP1$\alpha$) [51,52]. PP1$\alpha$ dephosphorylates elF2$\alpha$, inhibiting the PKR-pathway and the translational arrest. Alternatively, ICP34.5 induces the translocation of the nucleolar protein NOP53 to the cytoplasm, which facilitates the recruitment of PP1$\alpha$ [53].

#### 2.2.1. Functions of the amino domain of HSV-1 ICP34.5

The functions of ICP34.5 associated to its N-terminal domain (Figure 2) remain obscure. On the one hand, this region is involved in autophagy inhibition by blocking Beclin-1, an essential protein for autophagosome biogenesis. The recruitment of this autophagic protein is carried out by the Beclin-1-binding domain or BBD (68-87 aa) of ICP34.5 [54]. Although it was thought that BBD function was restricted to autophagy inhibition, recently an additional role for this domain was described. BBD not only interacts with Beclin-1, but also with multiple regulators of the antioxidant response, mitochondrial trafficking and programmed cell death. Despite this, BBD does not seem to alter antioxidant or apoptosis genes expression, but it does alter mitochondrial dynamics. In HSV-1 infected primary mouse neurons, the interaction of BBD with the sensor of oxidative stress KEAP1 and the regulator of mitochondrial dynamics PGAM5 causes the formation of mitochondrial clusters near the nucleus, where virions are produced. This mitochondrial relocalization relieves the bioenergetic demands on the cell during infection and facilitates transcription under oxidative stress [55].

On the other hand, the N-terminal domain of ICP34.5 plays an important role in the fight against IFN responses by the dephosphorylation of IRF3. Initially, it was described that TBK1 interacted with ICP34.5 and, thereby, IRF3 phosphorylation was inhibited. ICP34.5 was observed to bind TBK1 in the 76-106 aa region of the N-terminus, called TBK-

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1-binding domain (TBD). HSV-1 lacking the γ34.5 gene replicated efficiently in TBK1(-/-) cells, but not in TBK1(+/+) cells [56,57]. However, later studies demonstrated that TBD binds overexpressed but not endogenous TBK1. Furthermore, TBD shares a region (72-87 aa) with BBD (68-87 aa). The infection with a recombinant virus that lacks amino acids 87 to 106, a portion of the previously described TBD which is outside the region where TBD and BBD overlap, showed no impact on IRF3 phosphorylation or on viral replication and pathogenesis in mice. IRF3 phosphorylation is independent of the TBD, and the ability of ICP34.5 to control IRF3 activation was proposed to be a consequence of the reverse translational shutoff or the expression of other viral IFN inhibitors [58].

Another proposed mechanism of HSV-1 for IRF3 dephosphorylation is the inactivation of the stimulator of interferon genes (STING), a key component in innate immunity. Upon HSV-1 infection, intracellular dsDNA sensors recognize HSV-1 genome and activate STING, which recruits TBK1 to phosphorylate IRF3 and initiate the production of IFN [59]. Alternatively, TBK1 activation by STING can initiate the NF-κB pathway, which promotes antiviral immune responses [60]. The N-terminal region of ICP34.5 can bind to and inactivate STING, triggering an IFN response inhibition and, thus, the blocking of HSV-1 DNA sensing pathway. In addition, N-terminal domain of ICP34.5 disrupts translocation of STING from the ER to Golgi apparatus, a process necessary to promote cell-mediated immunity [61].

The N-terminus of ICP34.5 also mediates the reorganization of nuclear lamins [62]. The nuclear lamina is a meshwork in the inner nuclear membrane composed primarily of type V intermediate filament proteins, lamins A/C and B. This lamina acts as a barrier for the transit of viral nucleocapsids to the cytoplasm. However, herpesviruses can alter this lamina to promote viral nuclear egress [63]. HSV-1 ICP34.5 associates with the protein kinase C (PKC) and the complement component 1 Q subcomponent-binding protein (C1QBP/p32) [64] through its N-terminal domain and redirects these proteins to the nuclear membrane, where UL31/UL34 complex resides. The formation of a multiprotein complex activates PKC, which phosphorylates lamin A/C promoting the disintegration of the nuclear lamina and thus, facilitating nucleocapsid egress [65].

#### 2.2.1. Functions of the amino and carboxyl domains of HSV-1 ICP34.5

Both amino and carboxyl terminal regions are required for certain roles of ICP34.5 (Figure 2). Among these functions is the suppression of dendritic cells (DCs) maturation. Unlike wild-type virus, HSV-1 lacking γ34.5 gene stimulates the expression of MHC-II, CD86 and cytokines in immature DCs [66]. The mechanism by which ICP34.5 disrupts DCs functions is mediated by the dephosphorylation of IκB kinase. ICP34.5 recruits the protein IKKα/β in the N-terminus and PP1α in the C-terminus, forming a complex that dephosphorylates IκB kinase and, therefore, inhibits the activation of NF-κB, a transcription factor that regulates the expression of genes involved in immune response, inflammation and cell survival [67].

In non-dividing cells, the full-length ICP34.5 could also be required to overcome the block of DNA replication. In HSV-1 early phase infection, ICP34.5 is mainly present in the nucleus, where it is able to interact with DNA. This interaction is mediated by the recruitment of HSV-1 DNA replication proteins -such as UL30 polymerase- and by the proliferating cell nuclear antigen (PCNA), an essential protein for the replication and repair of cellular DNA. HSV-1 infection promotes PCNA translocation into the nucleus, where it interacts with ICP34.5 and promotes the initiation of viral replication [40].

It has been recently discovered that, while N-terminus of ICP34.5 blocks HSV-1 DNA-sensing pathway, the full-length protein is implied in RNA-sensing pathway inhibition. The retinoic acid-inducible gene-I (RIG-I) protein is a receptor that recognizes cytoplasmic viral RNA. Upon activation, RIG-I associates with the mitochondrial antiviral signaling protein (MAVS) and activates the IFN response via IRF3 phosphorylation. ICP34.5 precludes the assembly of RIG-I with the cellular chaperone 14-3-3ε. In this way,

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HSV-1 abrogates the RIG-I translocation from cytosol to mitochondria, preventing the interaction of RIG-I with MAVS and, hence, the IFN response initiated through the cellular recognition of viral RNA [68].

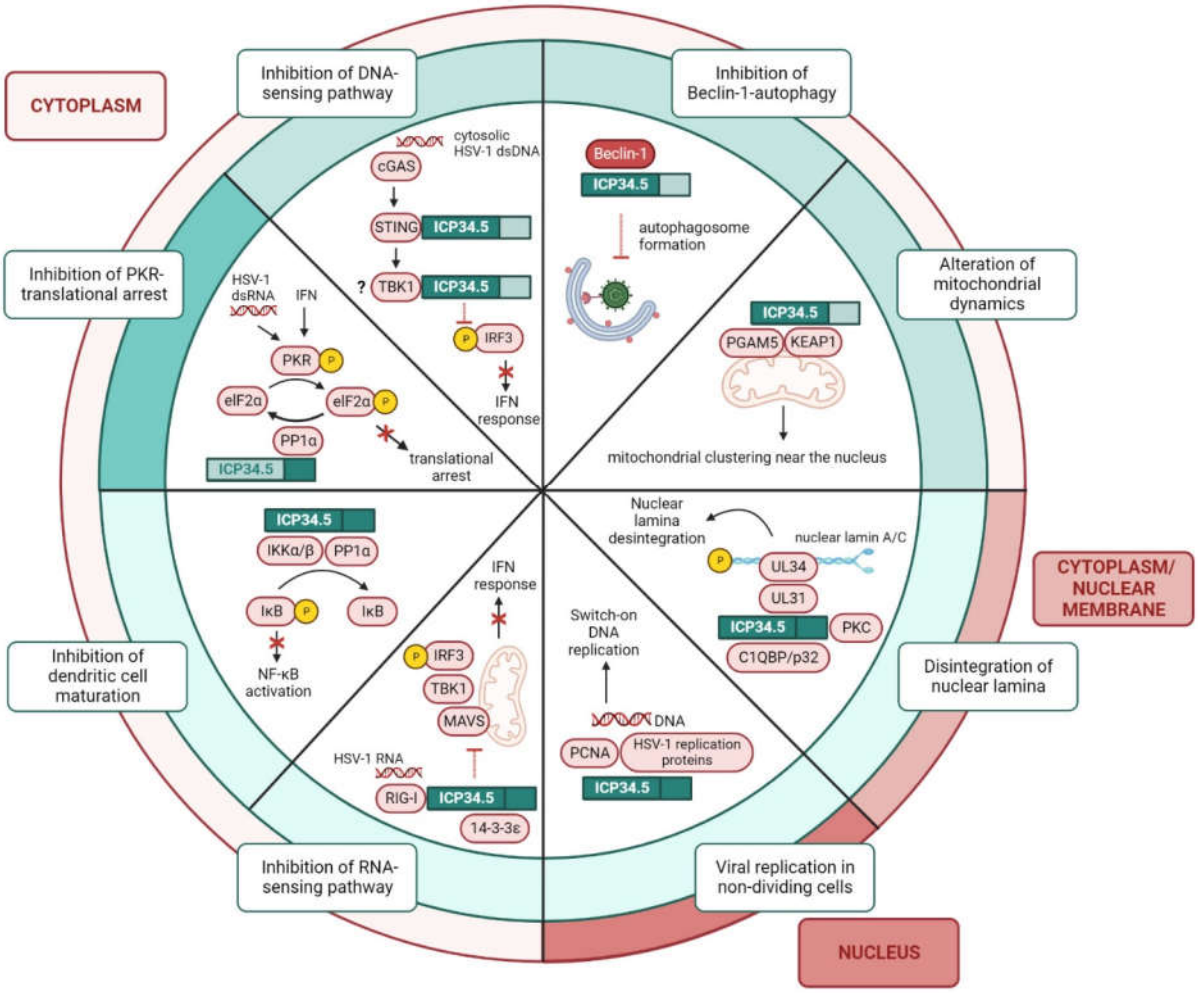


**Figure 2. Functions of ICP34.5 in HSV-1 infected cells.** Functions of ICP34.5 can be classified according to the cellular location of the protein, which shuttles between the cytoplasm and the nucleus. Besides, the amino and the carboxyl domains of ICP34.5 play different roles in infected cells. C-terminus prevents the translational arrest by the binding of PP1$\alpha$ and the subsequent dephosphorylation of elF2$\alpha$. N-terminus is involved in Beclin-1-autophagy suppression, degradation of the nuclear lamina to facilitate nucleocapsid egress from the nucleus and blockade of IFN response by the inhibition of the dsDNA-sensing pathway. The full-length protein plays a role in virus replication in non-dividing cells, prevention of DCs maturation, and suppression of IFN response by blocking the RNA-sensing pathway.

## 3. HSV-1 modulation of autophagy

### *3.1. Autophagy induction in early stages of HSV-1 infection*

Under stress conditions, such as nutrient starvation, oxidative stress, presence of unfolded proteins or pathogen infections, autophagy can be stimulated to promote metabolic adaptation and cellular survival. Because of the role of this pathway in cellular protection

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against pathogens, it is not surprising that in early stages of HSV-1 infection, autophagy flux is usually stimulated (Figure 3). However, the molecular mechanisms involved in autophagy stimulation during HSV-1 infection are still not fully understood. The apparent cell-type dependence of these mechanisms makes their research strongly complicated.

In murine embryonic fibroblasts and sympathetic neurons, the IFN-inducible PKR signaling pathway not only leads to cellular translational arrest, but also to induction of autophagy and HSV-1 degradation into autolysosomes. Stimulation of autophagy by this pathway appears to be dependent on HSV-1 gene expression [69–71]. Viral gene expression is also necessary for the induction of autophagy in monocytic-derived DCs, because infection with UV-inactivated HSV-1 does not lead to the stimulation of this degradative pathway [72]. The relevance of PKR mediating selective autophagy has been demonstrated not only *in vitro*, but also *in vivo*. HSV-1 unable to inhibit autophagy is strongly neuroattenuated in mice brains, but the virulence of the virus is restored in pkr-/- mice [54].

Emerging evidence has shown that toll-like receptors (TLRs) can also mediate the induction of autophagy. TLRs are receptors that recognize PAMPs and reside within the cell surface membrane and in endosomal compartments [73]. HSV-1 may be recognized by the surface TLR2 [74–76]. Upon activation, TLR2 can recruit the myeloid differentiation primary response protein MyD88, causing the dissociation of Beclin-1 from the B cell lymphoma-2 (BCL-2) inhibitory complex, which results in the induction of autophagy [77]. MyD88 protein is necessary to stimulate autophagy in HSV-1 infected monocytic THP-1 cells [78].

The presence of HSV-1 genome in the cytoplasm can also induce autophagy by the DNA-sensing pathway. The DNA sensor cyclic GMP–AMP (cGAMP) synthase cGAS, upon recognition of HSV-1 genome, produces the second messenger cGAMP to initiate the STING pathway, leading to TBK1 activation and IFN production. STING pathway has been proposed to play a role in the promotion of autophagy in HSV-1 infected myeloid cells [79] and fibroblasts [80]. Although STING-autophagy induction is dependent on TBK1, due to the significant impairment of TBK1-deficient fibroblasts in autophagy stimulation, this pathway seems to be IFN-independent. TICAM1/TRIF-null mutant fibroblasts, which are deficient in IFN production, can induce autophagy [81,82].

**4. Selective inhibition of autophagy by HSV-1 ICP34.5**

Upon the initial induction of autophagy, HSV-1 may counteract the host cell by down-modulating the pathway through ICP34.5. The role of ICP34.5 in autophagy inhibition is restricted to a specific domain (amino acids 68 to 87) implicated in the recruitment of Beclin-1 [54], an essential protein for autophagosome formation [83]. This domain is known as “Beclin-1-binding domain” or BBD. The C-terminal region of the protein is dispensable for this autophagy-inhibitory activity, suggesting that the modulation of autophagy is independent on PP1$\alpha$/elF2$\alpha$ binding to ICP34.5 [54]. However, this is not the end of the story…

The effect of HSV-1 ICP34.5 on autophagy is strongly dependent on the cell type. Whereas BBD inhibits the formation of autophagosomes in neurons and fibroblasts [71], in bone-marrow DCs (BM-DCs) it prevents autophagosome maturation, causing accumulation of long-lived proteins and autophagosomes [84]. In some cell types, including corneal epithelial and retinal ganglion cells, the protein has no effect on cellular autophagy [85]. Finally, it is important to make a distinction between inhibition of autophagy –based on the down-regulation of the cellular basal level of autophagy- and the inhibition of its stimulation in response to infection. In monocyte-derived DCs [72] and in BM-DCs, ICP34.5 does not inhibit the induction of autophagy at early times of infection. In BM-DCs, HSV-1 DNA-sensing pathway induces autophagy in a PKR-independent manner that cannot be antagonized by ICP34.5 [79].

Autophagy suppression by the virus not only prevents virion removal into autolysosomes, but also the processing and delivery of intracellular viral antigens to MHC class I

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and II molecules, inhibiting adaptative immunity [86] (Figure 3). When murine DCs are infected with ICP34.5-deficient HSV-1, viral antigen presentation on MHC-I is increased compared to the wild-type virus [87]. In mice, HSV-1 lacking BBD precludes autophagy-mediated MHC-II antigen presentation, decreasing the stimulation of CD4+ T cells [88].

Reduced presentation of viral antigens, a consequence of HSV-1 autophagy arrest, may be compensated by host cells by an alternative Beclin-1-independent autophagic pathway. Recently, the presence of four-layered membrane structures positive for the autophagy marker LC3B was described in HSV-1 infected macrophages. These four-layered structures are formed by rolling up the inner and outer nuclear membrane, and they are accumulated in the cytoplasm about eight hours post-infection. These vacuole structures enhance the presentation of endogenous viral antigens on MHC-I molecules, providing an additional pathway apart from viral antigen degradation by the proteasome. Whereas the formation of canonical autophagosomes can be promoted by pharmacological autophagy inducers, the generation of these four-layered structures is only induced by HSV-1 infection, which means that it is regulated in a different way than macroautophagy [86]. This autophagic pathway was denominated NEDA (Nuclear-Envelope Derived Autophagy) (Figure 3). The first evidence of this process was notified in macrophages, but it has later been observed in many different cell types, indicating that it is a general host mechanism against HSV-1 infection. NEDA is regulated by ICP34.5, but is Beclin-1 and BBD independent. Interestingly, the inhibition of host translation shutoff through the C-terminal domain of ICP34.5 is required for NEDA stimulation [89]. Furthermore, while activation of TBK1 by the DNA-sensing pathway mediates induction of macroautophagy in HSV-1 infected cells, NEDA is a TBK1-independent pathway suggested to be a general cellular response to stress [81]. These results evidence the strong differences in regulation of both processes, and the importance of analyzing them separately. LC3B is a marker of both macroautophagy and NEDA, yet LC3A is only present on NEDA structures, and it can be used to disguise both types of autophagy by confocal microscopy and flow cytometry [89].

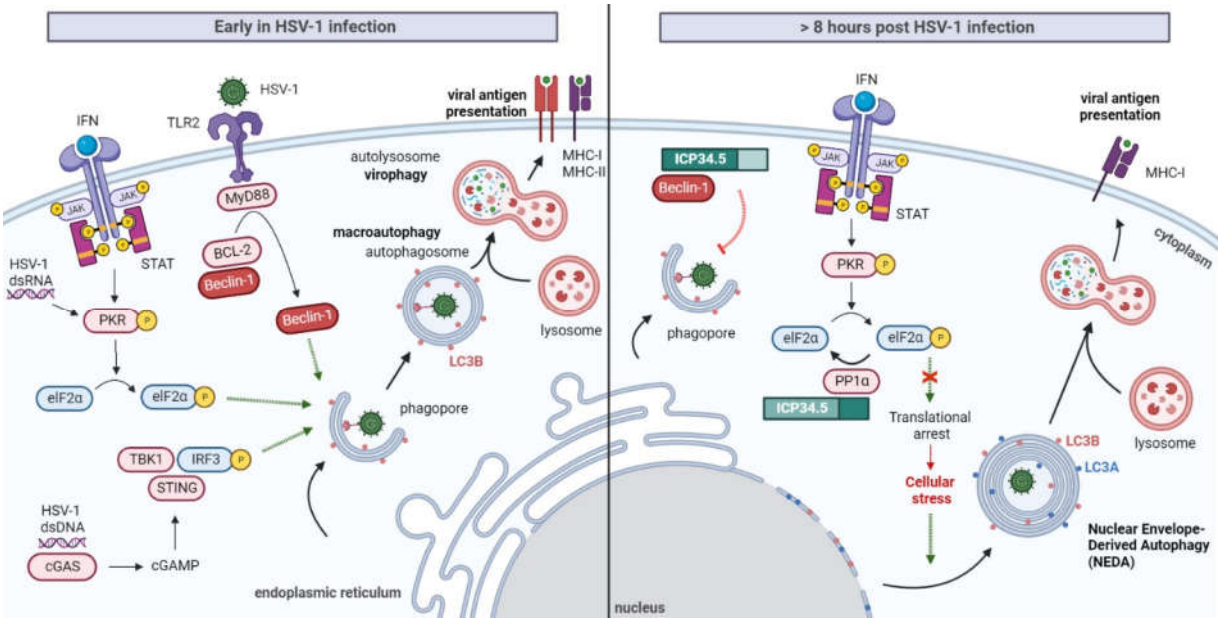


**Figure 3. Autophagy modulation by ICP34.5 in HSV-1 infection.** Early in HSV-1 infection, detection of the virus by host cells promotes the stimulation of macroautophagy. This pathway may act as a cellular defense mechanism involved in virophagy and the processing of viral antigen for MHC presentation. Autophagic flux can be induced in a viral gene expression-dependent manner by the PKR/elF2α pathway. Besides, autophagy may be enhanced through the recognition of PAMPs by

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the surface receptor TLR2. Activated TLR2 recruits the adaptor MyD88, which causes the dissociation of Beclin-1 from BCL-2 inhibitory complex, resulting in the induction of autophagy. Finally, HSV-1 dsDNA may be recognized by the cytosolic dsDNA-sensing cGAS, which produces cGAMP to activate STING and TBK1. Activation of TBK1 has been also related to autophagy stimulation. To fight against autophagy, HSV-1 can suppress this pathway through the Beclin-1-binding domain located in the N-terminus of ICP34.5. Besides, ICP34.5 blocks the PKR-translational arrest by the recruitment of elF2$\alpha$ and PP1$\alpha$ in the C-terminus. In response to this, host cells initiate a general stress response based on an alternative autophagic pathway known as NEDA. This process consists of the Beclin-1-independent formation of four-layered membrane structures by the coiling of the nuclear membrane. NEDA is suggested to have an antiviral role promoting viral antigen presentation, but further research about this type of autophagy is required.

**5. The intricate role of Beclin-1-binding domain of ICP34.5 in HSV-1 virulence**

HSV-1 strains lacking the BBD (68-87 aa) of ICP34.5 exhibit reduced neurovirulence and viral replication in mice compared to wild-type viruses [54,58,88]. However, whereas HSV-1 lacking BBD is strongly neuroattenuated in mice brain, BBD is dispensable for productive viral replication in the neuronal stablished cell line SK-N-SH [54]. Even in primary fibroblast cultures, no difference is noticeable in viral replication between HSV-1 lacking BBD and the wild-type virus [90].

HSV-1 lacking BBD is not so attenuated *in vitro* as data would predict. Recently, it has been proposed that the differences in virulence between *in vivo* and *in vitro* studies could be due to the presence of ferric nitrate in culture media. In the presence of this iron salt, which is involved in redox reactions, the attenuated replication of HSV-1 lacking BBD in primary human fibroblasts is partially restored. The cause of the masked BBD effect could be explained by the fact that this domain not only recruits Beclin-1, but also multiple mitochondrial regulators that play a relevant role in redox reactions and cellular metabolism [55]. Unfortunately, primary neuron cultures do not survive in redox buffer-free medium conditions. Hence, viral replication experiments could not be performed under these conditions, where the effect of BBD is not masked.

Although the role of BBD in virulence may not be restricted to inhibition of Beclin-1-autophagy, it does not mean that suppression of autophagic flux by BBD has no effect on infection. While BBD-deleted HSV-1 is significantly neuroattenuated in mice, the infectivity of this virus is restored in pkr -/- mice, which have an impairment in PKR-autophagy induction. These results demonstrate that the inhibition of PKR-autophagy by BBD is important *in vivo* for neurovirulence [54].

**6. ICP34.5 is not alone in HSV-1 autophagy inhibition**

HSV-1 exploits the factor of virulence ICP34.5 to inhibit Beclin-1-dependent autophagy. However, neither is ICP34.5 the unique HSV-1 protein capable of modulating autophagy, nor is Beclin-1 the only target used by the virus for autophagy suppression (Figure 4).

Us11 is an HSV-1 late protein that binds to dsRNA and physically interacts with PKR [91]. Through the direct association to PKR, Us11 prevents the activation of the PKR/elF2$\alpha$ signalling pathway and, thus, the translational arrest [92,93]. Although Us11 is not able to interact with Beclin-1, its interaction with PKR has a strong anti-autophagic activity on cells. Thus, autophagy inhibition in HSV-1 infected cells could be a result of the activity not only from ICP34.5, but also from Us11 [94]. Us11 also reduces the formation of autophagosomes by the disassembly of the functional complex formed by TRIM23 (tripartite motif protein 23), Hsp90 (heat shock protein 90) and TBK1 [95]. Tripartite motif (TRIM) proteins are essential regulators of both IFN-response and autophagy [96], and current evidence suggest that TBK1 activation through TRIM23 is a key step in selective autophagy in multiple viral infections, including HSV-1 [97]. Nonetheless, the precise mechanism involved in the inhibition of TBK1-selective autophagy by Us11 remains to be fully understood.

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In addition to preventing autophagosome formation, recent research suggests that viruses can evade virophagy by targeting SARs [98]. HSV-1 may down-modulate two important SARs implied in recognition and delivery of specific viral ubiquitinated cargo to the phagophores: the autophagy receptor sequestosome 1 (p62/SQSTM1) and the mitophagy adaptor optineurin (OPTN). Cellular levels of p62/SQSTM1 and OPTN are significantly reduced after 3-6 hours of HSV-1 infection in various cell lines [99]. After activation by TRIM23, TBK1 phosphorylates and activates p62/SQSTM1 and OPTN [100]. However, it has not been established whether there is a relationship between the inhibitory effect of Us11 on TRIM23-TBK1 complex and the early down-modulation of these SARs. In contrast, it has been observed that, upon HSV-1 infection, both SARs are degraded in the proteasome by a mechanism that requires ICP0 expression and calcium mobilization, remaining the role of calcium on ICP0 activity yet unknown [99].

The involvement of ICP34.5 in p62/SQSTM1 and OPTN modulation has been also analysed. In cells infected with ICP34.5-deficient HSV-1, these proteins remain down-regulated, indicating that ICP34.5 is not involved in this process. However, ICP34.5-deficient HSV-1 is not able to inhibit macroautophagy, and SARs could be degraded into autolysosomes [99]. On the other hand, cells infected with HSV-1 lacking the γ34.5 gene show a reduced accumulation of ICP0, so maybe ICP34.5 modulates p62/SQSTM1 and OPTN in an indirect manner [58].

To determine the effect of these SARs on HSV-1 infection, p62/SQSTM1 and OPTN knockdown cells were infected. No viral growth defect was observed, maybe because HSV-1 usually down-modulates both proteins early after infection. However, exogenous expression of p62/SQSTM1 affected negatively viral yields [99]. It remains undetermined if SARs down-regulation by HSV-1 is advantageous for infection because of the role of these receptors in autophagy, or due to other functions they perform in cells. p62/SQSTM1 has also roles in cellular metabolism, cell signaling and apoptosis [101], and OPTN is implied in mitophagy -a type of selective autophagy involved in degradation of damaged mitochondrial components- and modulates multiple cellular processes related to protein trafficking and membrane cargo delivery from the Golgi apparatus to the plasma membrane [102].

It has been observed recently that the HSV-1 protein Ser/Thr kinase Us3 could also suppress autophagy [103]. Us3 may phosphorylate and activate the nutrient-sensing mammalian target of rapamycin kinase complex 1 (mTORC1) [104], which is a negative regulator of ULK complex that keeps autophagy inactive under physiological conditions [105]. Besides, Us3 can modulate autophagy downstream of mTORC1, by the phosphorylation and inactivation of both ULK1 complex and Beclin-1 [103].

HSV-1 has developed multiple mechanisms to prevent autophagy. But, why does the virus require different strategies to inhibit the same pathway? Two options have been considered. On the one hand, HSV-1 could antagonize autophagy in a distinct manner in neuronal and non-neuronal cells. In non-neuronal cells, Us3 seems to have a main role in autophagy inhibition, predominant over the action of ICP34.5. While replication of ICP34.5 null mutant HSV-1 in fibroblasts is not rescued by suppressing autophagy, replication of Us3-deficient and Us3-ICP34.5 doubly deficient HSV-1 are partially restored [103]. On the other hand, HSV-1 may not only inhibit Beclin-1-dependent autophagy, but also Beclin-1-independent or non-canonical autophagic pathways by using other virulence mechanisms [106].

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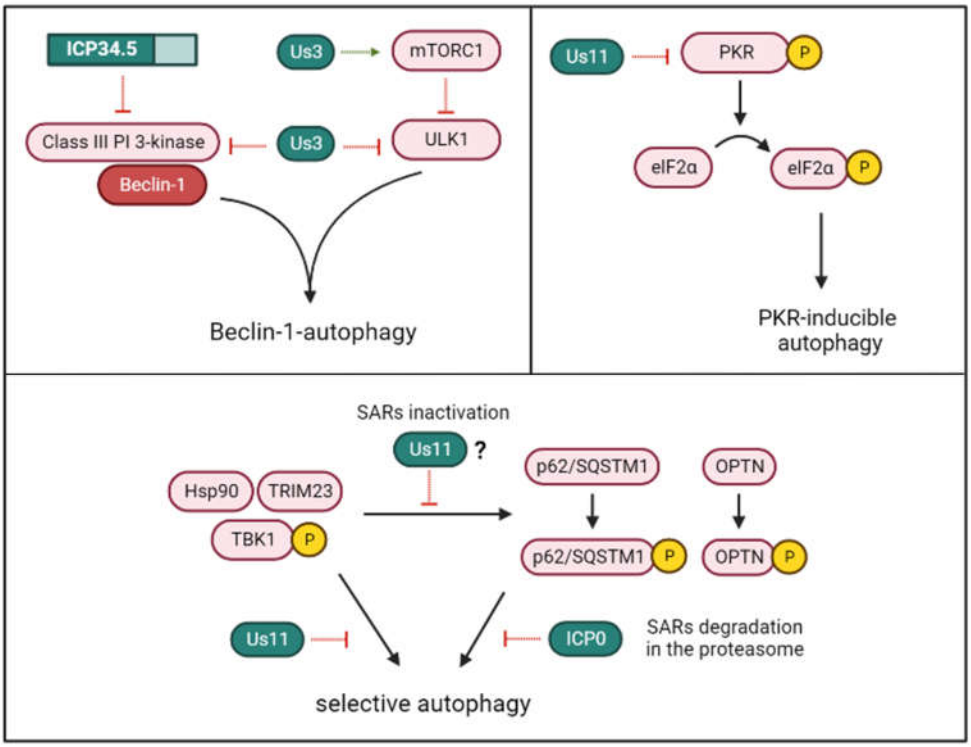


**Figure 4. HSV-1 mechanisms for autophagy inhibition.** HSV-1 proteins Us3 and ICP34.5 can inhibit macroautophagy by binding to Beclin-1, which is required for autophagosome formation. Us3 can also suppress the pathway activating the negative regulator of autophagy mTORC1 or inactivating ULK1 complex, which is involved in phagophore initiation. The HSV-1 protein Us11 can prevent the induction of autophagy mediated by the PKR/eIF2α pathway. Besides, Us11 inhibits virus-induced autophagy by the disassembly of the TRIM23-Hsp90-TBK1 complex. Finally, the HSV-1 protein ICP0 promotes the degradation in the proteasome of the selective autophagy receptors (SARs) p62/SQSTM1 and OPTN. TRIM23-TBK1 complex is involved in phosphorylation and activation of p62/SQSTM1 and OPTN, however, the inhibitory role of Us11 on the complex has not been directly associated with a possible SARs inactivation.

## 7. The two sides of autophagy

Macroautophagy is broadly considered an innate immune response that protect cells from viral infections [107]. Due to the antiviral role of autophagy, pharmacological modulation of this pathway has been proposed as a potential tool to fight HSV-1 infection [108]. Autophagy stimulation by physiological (cellular starvation in a minimal medium) and pharmacological approaches (cellular treatment with autophagy inducers such as MG-132 or trehalose) significantly suppresses HSV-1 infection in neuronal and non-neuronal cell types [99,109]. However, pharmacological autophagy inducers perform multiple cellular functions, which may also influence HSV-1 replication. For instance, MG-132 is a proteasome inhibitor which is not only associated with induction of autophagy, but also with ER stress and apoptosis [110]. Thus, decreased infection could be a consequence not of autophagy stimulation, but of the unspecific effects of the drug. When neuroblastoma cells are transfected with Beclin-1-expressing plasmids to stimulate autophagy, instead of using pharmacological inducers, hyperactivation of the pathway could only slow the rate of HSV-1 replication [111]. These results suggest that the main effect of pharmacological autophagy inducers on HSV-1 infection may not be a consequence of autophagy stimulation. Further research is required about the mechanisms of action of these drugs in the impairment of HSV-1 infection.

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Although the antiviral role of autophagy in neurons has been demonstrated *in vitro* and *in vivo*, in epithelial and other mitotic cells this pathway appears not to be a predominant defense mechanism. Keratinocytes unable to undergo autophagy were generated from Atg5-KO mice, being ATG5 an essential protein for autophagosome formation. These keratinocytes showed no differences in HSV-1 replication compared to those from wild-type mice. The antiviral contribution of autophagy could be masked in wild-type mice by the inhibition of the pathway through ICP34.5. To rule out this possibility, wild-type mice were infected with BBD-deleted HSV-1, but viral replication was indistinguishable from the wild-type virus [112]. Similar results were observed in MEFs of Atg5-KO mice [90]. (113) However, it has been recently proposed that, actually, autophagy does have an important antiviral role in these cells, but the virus suppresses the pathway mainly with Us3, not ICP34.5 [103]. The antiviral potential of autophagy in non-neuronal cells might be underestimated, since previous studies were focus on the role of ICP34.5, and were carried out with HSV-1 expressing Us3, a protein which would inhibit PKR- and Beclin-1-dependent autophagy probably in a stronger manner than ICP34.5.

Autophagy is often considered a double-edged sword, and a possible proviral role of this pathway in HSV-1 infection has also been raised [113]. Suppression of autophagy, both by pharmacological inhibitors and by siRNA knockdown technology, impairs significantly HSV-1 infection in human acute monocytic leukemia (THP-1) cells and in primary human monocytes. These results indicate that the virus may be using the autophagic machinery to its own benefit [78]. One proposed proviral function of autophagy is the degradation of nuclear lamina [72]. The autophagy protein LC3 is not only present in the cytoplasm, but also in the nucleus, where it can interact directly with the lamins. This interaction could lead to the transport of the nuclear lamins from the nucleus to the cytoplasm for lysosomal degradation [114]. Lamina disintegration by nuclear autophagy has been proposed to facilitate the egress of HSV-1 capsids from the nucleus. This kind of autophagy is not down-modulated by ICP34.5, which means that, as NEDA, is Beclin-1-independent [72].

Finally, autophagy may participate in the formation of microvesicles (MVs) during HSV-1 infection [115]. MVs are extracellular vesicles formed by direct outward budding, or pinching, of the plasma membrane. These vesicles could play an important role in viral infections, promoting viral dissemination and evasion of the immune system [116,117]. HSV-1 infected-cells have a significant increased production of MVs compared to mock-infected cells, and non-enveloped HSV-1 virions have been observed inside LC3B-positive MVs. These MVs appear to mediate viral dissemination and cellular tropism, and they contribute to avoid immune surveillance. However, further research would be required to describe the specific role of autophagic membranes in the formation of MVs during HSV-1 infection [118,119].

Most of the research has been focus on the effect of autophagy stimulation or inhibition in HSV-1 infection. However, the knowledge about the influence of cellular basal autophagy on HSV-1 replication is scarce. The level of basal autophagy is strongly dependent on cell type, and it has been proposed as an important host-range determinant. In contrast to immature DCs, mature DCs are non-permissive for HSV-1 infection and it seems to be a consequence of its inefficient autophagic flux. In these cells, nuclear lamina degradation is impaired and fewer viral capsids were released from the nucleus to the cytoplasm [72]. However, very high levels of autophagy also appear to impair HSV-1 infection. Fibroblasts with a higher level of basal autophagy are better protected against HSV-1 infections [120]. Canonical and non-canonical autophagic pathways are highly regulated processes involved in numerous cellular functions, and a fine-tuning of these pathways could be essential for virus replication. In ocular cells, both suppression and induction of autophagy significantly diminish HSV-1 infection. Due to autophagy may function as a dual-edged sword, maintaining an intermediate level of autophagy may be required for a successful HSV-1 infection [121].

## 8. Conclusions

Autophagy is a fine-tune regulated process and, when balance tips to one side, HSV-1 infection is often severely impaired. Because of the strong influence of autophagy in viral infection, modulation of this pathway has been raised as a potential future therapy against HSV-1. However, before getting fully involved in this type of treatments, we should consider that unbalance autophagy could not only negatively affect viral infection, but also cellular homeostasis and survival.

Autophagy is especially important in maintaining the correct functionality of the SNC. Deregulation of this pathway, whether excessive or insufficient, has been related to severe neurodegenerative disorders. Moreover, suppression of autophagy by HSV-1 in the SNC has been proposed as a cause of neurodegeneration. The "autophagic balance" is extremely sensitive, and further research would be required to decode the mechanisms that allow us to modulate autophagy hitting the bull´s-eye.

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