
**PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE**
**Facultad de Ciencias Biológicas**
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**Mención Genética Molecular y Microbiología**

# EVALUATING THE IMPACT OF ASYMPTOMATIC HERPES SIMPLEX VIRUS TYPE 1 INFECTION ON MULTIPLE SCLEROSIS DISEASE IN A MOUSE MODEL

**Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias con mención en Genética Molecular y Microbiología**

**Por**

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## ABSTRACT

Multiple sclerosis is a demyelinating autoimmune disease of the central nervous system (CNS) that severely impairs the individual's motor and sensory functions. At present, its cause or causes are unknown, and the available treatments only decrease the frequency of inflammatory relapses but do not prevent chronic damage and neurologic decline. There is evidence that suggests that viruses may play roles in multiple sclerosis onset and pathogenesis by acting as environmental triggers. Importantly, viruses belonging to the *Herpesviridae* family, which are acquired at early stages of life, and cause lifelong infections have been defined as major candidates for triggering or exacerbating this disease. Currently, only a few studies have assessed a potential role for herpes simplex viruses in multiple sclerosis. Noteworthy, herpes simplex virus type 1 (HSV-1) DNA has been found in cerebrospinal fluid and peripheral blood of patients with multiple sclerosis relapses, as well as more frequently in post-mortem brain samples of individuals with multiple sclerosis than healthy controls. Notably, HSV-1 infections are mainly asymptomatic, and this virus may reach the brain throughout life without evident clinical symptoms. Moreover, accumulating data suggests that persistent HSV-1 infection in the brain could produce prolonged neuroinflammation due to continuous subclinical reactivations leading to neurodegenerative disorders in susceptible individuals. The goal of this thesis was to determine whether asymptomatic HSV-1 infection favors the onset of multiple sclerosis and its severity. We studied this question by using animals that recapitulate several aspects related to multiple sclerosis disease and HSV-1 infection in humans. First, we infected mice with a sub-lethal dose of HSV-1, waited for their recovery from acute infection at least 30 days, and then we induced experimental autoimmune encephalomyelitis (EAE), the main animal model used

for studying multiple sclerosis disease. The onset and severity of multiple sclerosis symptoms in the EAE mouse model was compared with non-infected animals. We determined the populations of immune cells infiltrating the CNS of mice after HSV-1 infection and EAE induction, as well as cytokines produced in this tissue once autoimmunity was initiated. We also assessed the permeability of the blood-brain barrier (BBB) 30 days post-HSV-1 infection. Our results show that a previous infection with HSV-1 accelerates the onset of EAE and enhances disease severity. Moreover, the animals previously infected with HSV-1, and induced to develop EAE undergo increased CNS inflammation as compared to uninfected animals, which was characterized by prolongated microglia cell activation, an elevated infiltration of $CD4^+$ T cells in the brain and increased infiltration of neutrophils in the spinal cord, as well as significant levels of IL-6 and IL-1β mRNA expression in these tissues. Notably, we also found that after asymptomatic HSV-1 infection, the BBB remains disrupted for up to 30 days when virions are not detectable. We expect that this study will help to better define the possible contribution of HSV-1 infection in multiple sclerosis disease and warrant future studies and trials with antiviral interventions as a potential treatment for this disease to slow its progression.

# RESUMEN

La esclerosis múltiple es una enfermedad autoinmune desmielinizante del sistema nervioso central (SNC) que perjudica severamente las funciones sensoriales y motoras del individuo. Hoy en día, la causa o causas de esta enfermedad son desconocidas y el tratamiento disponible solo disminuye la frecuencia de las recaídas inflamatorias, pero no previene del daño crónico y el declive neurológico. Existe evidencia que sugiere que los virus pueden tener un papel importante en el inicio y la patogénesis de la esclerosis múltiple por actuar como gatillantes ambientales. Notablemente, virus que pertenecen a la familia *Herpesviridae,* los cuales son adquiridos en etapas tempranas de la vida y causan infecciones latentes de por vida, han sido definidos como principales candidatos para iniciar o exacerbar esta enfermedad. Actualmente, pocos estudios han evaluado el potencial papel de los virus del herpes simple en esclerosis múltiple. Cabe resaltar, que el virus del herpes simple tipo 1 (VHS-1) se ha detectado en líquido cefalorraquídeo y en sangre periférica de pacientes con esclerosis múltiple durante recaídas inflamatorias, así como también en mayor frecuencia en muestras de cerebro post muerte de individuos con esclerosis múltiple que en individuos sanos. Además, las infecciones producidas por VHS-1 son principalmente asintomáticas y este virus podría alcanzar el cerebro a lo largo de la vida sin síntomas clínicos evidentes. Además, datos acumulados sugieren que la infección persistente con VHS-1 en el cerebro produce prolongada neuroinflamación debido a continuas reactivaciones subclínicas que conduce a desordenes neurodegenerativos en personas susceptibles. El objetivo de esta tesis fue determinar si la infección asintomática con VHS-1 favorece el inicio de la esclerosis múltiple y su severidad. Nosotros abordamos esta pregunta usando animales que recapitulan varios aspectos relacionados con la enfermedad de la esclerosis

múltiple y la infección con VHS-1 en humanos. Primero, infectamos ratones con una dosis no letal de VHS-1, esperamos a la recuperación de la infección aguda, al menos 30 días, y luego inducimos la enfermedad de encefalomielitis autoinmune experimental (EAE), la cual es el principal modelo animal usado para estudiar la enfermedad de esclerosis múltiple. El inicio y severidad de síntomas de esclerosis múltiple en el modelo murino de EAE fue comparado con animales no infectados. Determinamos las poblaciones de células inmunes infiltrando SNC de ratones después de la infección con VHS-1 e inducción de EAE, así como también las citoquinas producidas en este tejido luego del inicio de la autoinmunidad. También evaluamos la permeabilidad de la barrera hemato-encefálica 30 días post infección con VHS-1. Nuestros resultados muestran que una infección previa con VHS-1 acelera el inicio de EAE y aumenta la severidad de la enfermedad en el modelo murino. Además, animales previamente infectados con VHS-1 e inducidos a desarrollar EAE padecen una mayor inflamación de SNC que los animales no infectados, lo cual se caracterizó por prolongada activación de microglía, una elevada infiltración de células T $CD4^+$ en el cerebro y neutrófilos en la médula espinal, y niveles de expresión significativos de mRNA de las citoquinas IL-6 e IL-1β en estos tejidos. Notablemente, también encontramos que después de la infección asintomática con VHS-1, la barrera hemato-encefálica permanece alterada hasta por 30 días cuando no son detectados viriones. Esperamos que este estudio ayude a definir mejor la posible contribución de la infección por VHS-1 en la enfermedad de la esclerosis múltiple y a garantizar futuros estudios y ensayos con intervenciones antivirales como un potencial tratamiento de esta enfermedad para retardar su progresión.

# 1. THEORETICAL BACKGROUND

## 1.1 Epidemiology and life cycle of Herpes simplex virus type-1 (HSV-1)

HSV-1 is an enveloped double-stranded DNA virus belonging to the *Herpesviridae* family, that has a genome of approximately 152 Kbp with more than 80 different open reading frames (ORFs) (Boehmer and Nimonkar, 2003). Importantly, all herpesviruses cause lifelong latent infections in their hosts with sporadic reactivations (Perng and Jones, 2010). HSV-1 is a neurotropic pathogen with a wide spectrum of clinical symptoms ranging from harmless manifestations, such as oral and facial lesions to severe infection of the eyes and the central nervous system (CNS) (Suazo et al., 2015). This virus is the most common cause of sporadic encephalitis in adults, as well as infectious blindness due to herpetic keratitis (Lairson et al., 2003; Whitley, 2015). HSV-1 is usually acquired during childhood, and worldwide approximately 65% of people have antibodies against this virus. However, only 20–40% of infected individuals develop symptoms (Dobson et al., 2003), but they are reservoirs that contribute to viral transmission towards new hosts through asymptomatic shedding (Miller and Danaher, 2008; Ramchandani et al., 2016).

HSV-1 can alternate between a lytic infection phase that produces virions, or a latent state characterized by transcriptional repression of all viral lytic genes (Whitley and Roizman, 2001). HSV-1 enters epithelial cells at the initial site of infection by fusing its envelope with the cell membrane, through a process that is mediated and assisted by several viral glycoproteins. The fusion of membranes leads to the release of the viral capsid surrounded by tegument proteins into the cell cytoplasm, then travels associated to microtubules, to the cell nucleus. The viral DNA is delivered into the nucleus and transcribed in a cascade-dependent manner, with

three major waves of transcription: first, the expression of immediate early genes (IE or alpha genes), followed by the expression of early genes (E or beta genes) and lastly, late genes (L or gamma genes). Furthermore, the latter are sometimes sub-divided into late-early and late genes (or gamma-1 and gamma-2 genes, respectively) (Honess and Roizman, 1974; Ibáñez et al., 2018) (Figure 1). For IE mRNAs, a viral transactivator called VP16 plays an important role in promoting their transcription by binding to cellular factors namely the octamer-binding protein 1 (Oct1) and the host cell factor-1 (HCF-1) (Herrera and Triezenberg, 2004). Some IE viral genes play key roles in the evasion of the host cellular antiviral response. As IE proteins are expressed, some of them will act as transcription factors for E viral genes, and then is promoted the synthesis of E proteins that play roles in viral processes, such as DNA replication (Suazo et al., 2015). Finally, late gene expression occurs thanks to the transactivation properties of viral IE genes (Honess and Roizman, 1975). These later genes encode, among others, for structural components of the virion, such as capsid, tegument, and viral surface proteins (Herrera and Triezenberg, 2004). Once viral DNA is replicated, it is packaged into new capsids that are released into the cytoplasm, where they are complemented with viral tegument and glycoproteins. Finally, new infectious viral particles are released to the extracellular and the virus gains access to the termini of sensory neurons that innervate the skin reaching the cell body of these cells by retrograde transport through neuronal axons (Antinone and Smith, 2010). Here, the virus can spread through a lytic cycle or enter latency (Figure 1). During facial infections that affect the mouth, face or eyes, viral progeny from HSV-1 replication in the epithelium will reach the cell bodies of sensory and autonomic nerve terminals of neurons in the trigeminal ganglia (TG). Virus within neurons can enter in a latency phase in which viral DNA remains as an episome in the nucleus with reduced-to-none virus protein expression

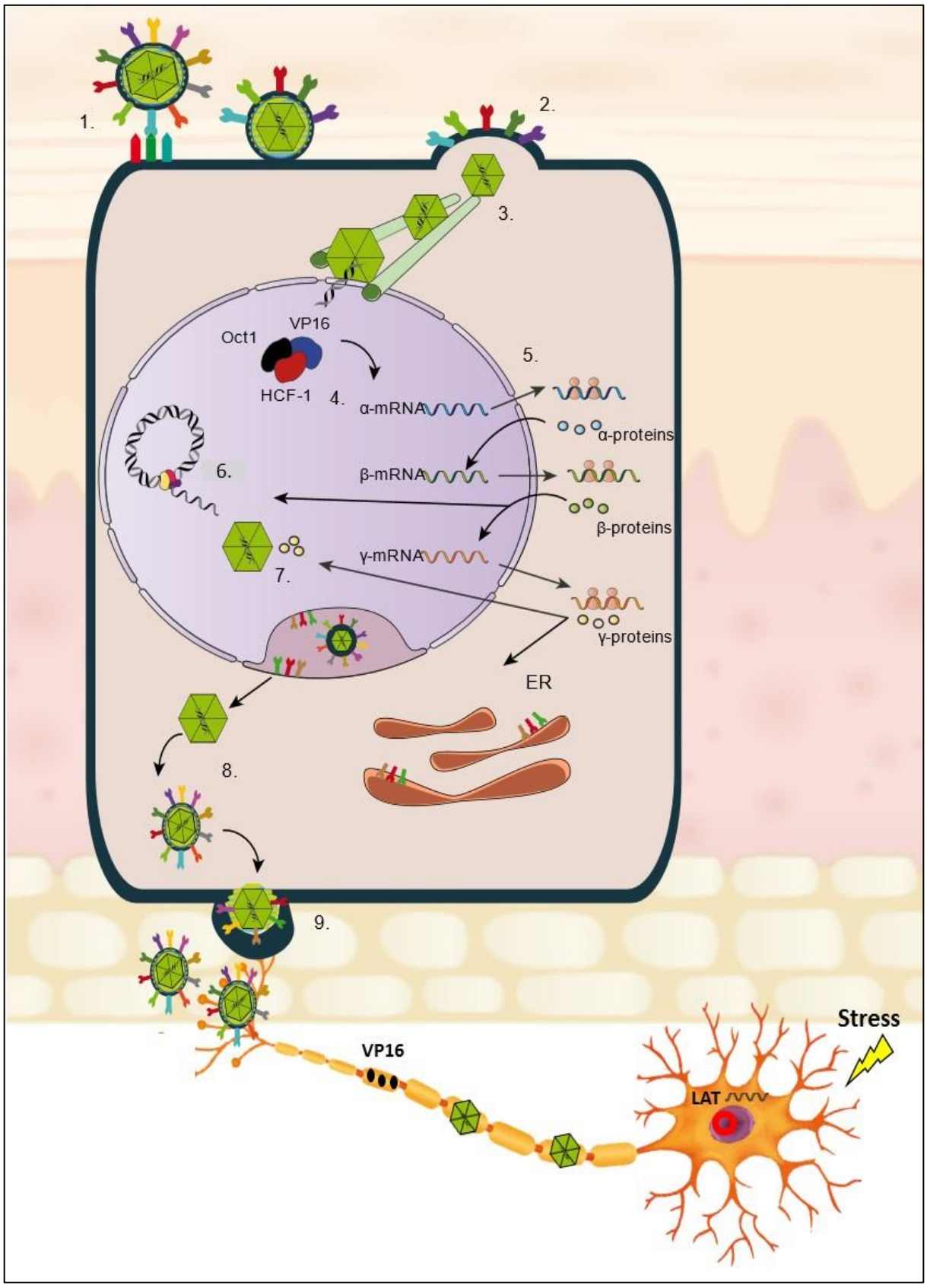
1.
2.
3.
VP16
Oct1
HCF-1
4.
5.
α-mRNA
α-proteins
6.
β-mRNA
β-proteins
7.
γ-mRNA
γ-proteins
ER
8.
9.
VP16
LAT
Stress

**Figure 1. Life cycle of HSV-1:** 1) Binding of viral glycoproteins to receptors on the cell surface. 2) Virus entry through the fusion between the cell membrane and viral envelope. 3) Capsid transport to the nucleus through microtubules. 4) Interaction of VP16 with host cell factors HCF-1 and Oct-1 to start viral gene transcription in a cascade manner: alpha genes, beta genes and then gamma genes. 5) Translation of viral proteins: alpha proteins, beta proteins and gamma proteins. 6) Genome replication. 7) Capsid assembly and exit to the cytoplasm. 8) Envelopment of capsids with viral tegument and glycoproteins, which have been glycosylated in the Golgi apparatus. 9) Viral particle release. The resulting virus can reach nerve termini of sensory neurons innervating the site of primary infection and travel by retrograde axonal transport to the cell body. After DNA is injected into neuron nuclei it can enter into a latency state and remain as an episome until stress or other conditions reactivate it. VP16 (viral protein 16), HCF-1 (host cell factor-1), Oct1 (octamer-binding protein 1), ER (endoplasmic reticulum). LAT (latency associated transcript). Modified from *Ibañez et al, 2018.*

(Nicoll et al., 2012). It has been hypothesized that VP16 may be lost during axonal transport and latency state is favored due to the lack of a viral transactivator (Kim et al., 2012). Remarkably, latency is mainly characterized by the transcription of only one viral transcript from the viral genome, which is non-coding and is termed the latency-associated transcript (LAT) (Nicoll et al., 2016). Importantly, in latently-infected cells LAT is processed into miRNAs that silence the expression of viral genes that are required for productive virus replication (Umbach et al., 2008). In addition, LAT promoter in neurons has been associated with epigenetic markers of active transcription during the latent state (i.e. particular acetylation patterns at histone H3) (Kubat et al., 2004). In contrast, the promoters of lytic viral genes were found to display methylations associated to heterochromatin (Cliffe et al., 2009; Cliffe and Wilson, 2017). Nevertheless, sporadic expression of lytic viral genes in neurons during latency in the form of mRNA has been reported by several groups (Feldman et al., 2002; Margolis et al., 2007; Ma et al., 2014), which was followed in some cases by protein synthesis without production of new viral particles suggesting that HSV-1 persistence is a dynamic process that includes not only a latent state with sporadic productive reactivations, but also spontaneous molecular reactivations without productive progeny production (Du et al., 2011; Kim et al., 2012; Martin et al., 2014a). Ultimately, under stress conditions HSV-1 can reactivate from neurons releasing new viral particles that can cause recurrent lesions close to the initial site of infection, spread asymptomatically to new hosts, or enter into the CNS by anterograde transport (Halford et al., 1996).

### 1.2 HSV-1 at the central nervous system

HSV-1 can invade the brain and replicate in neuronal cells causing herpes simplex encephalitis (HSE) (Gnann and Whitley, 2017) or creating a reservoir for virus production with asymptomatic reactivations. About 30% of HSE cases are related to primary infection (more commonly in children and adolescents), while 70% of cases are attributed to viral reactivation from previous infection (mainly adults). Figure 2 shows the different strategies used by HSV-1 to infect the brain. One of them is associated with a primary infection via olfactory tracts (Burgos et al., 2006; Jennische et al., 2015). In fact, studies using animal models have shown the spread of HSV-1 from the nasal cavity to the CNS after infection of the olfactory epithelium, which is connected with the olfactory bulb and consequently the limbic system, resulting in focal encephalitis in the brain (Figure 2A) (Twomey et al., 1979; Dinn, 1980). Regarding neonatal HSV-1 infections, the olfactory route is frequently considered responsible and widely described as the result of close contact between the newborn olfactory tissue and HSV-1 virions present in the birth canal of the mother at the time of birth. However, a study in mice suggests that vertical transmission is predominantly hematogenous (Burgos et al., 2006). This study showed that placenta had high number of viral genomes, indicating that HSV-1 could reach the brain of fetuses by this route through the maternal bloodstream (Burgos et al., 2006). Another route by which HSV-1 may gain access to the brain, is peripheral viral reactivation with subsequent anterograde axonal transport, associated with latent virus in TG acquired in a previous orolabial or eye infection (Figure 2B) (Whitley et al., 1982). Finally, latent HSV-1 in the brain may be a source of productive reactivations that seed infection to other sites within this tissue, or cause HSE in some susceptible individuals (Figure 2C). In the past, sensory ganglia was understood to be the only place of HSV-1 latency, but autopsy studies have

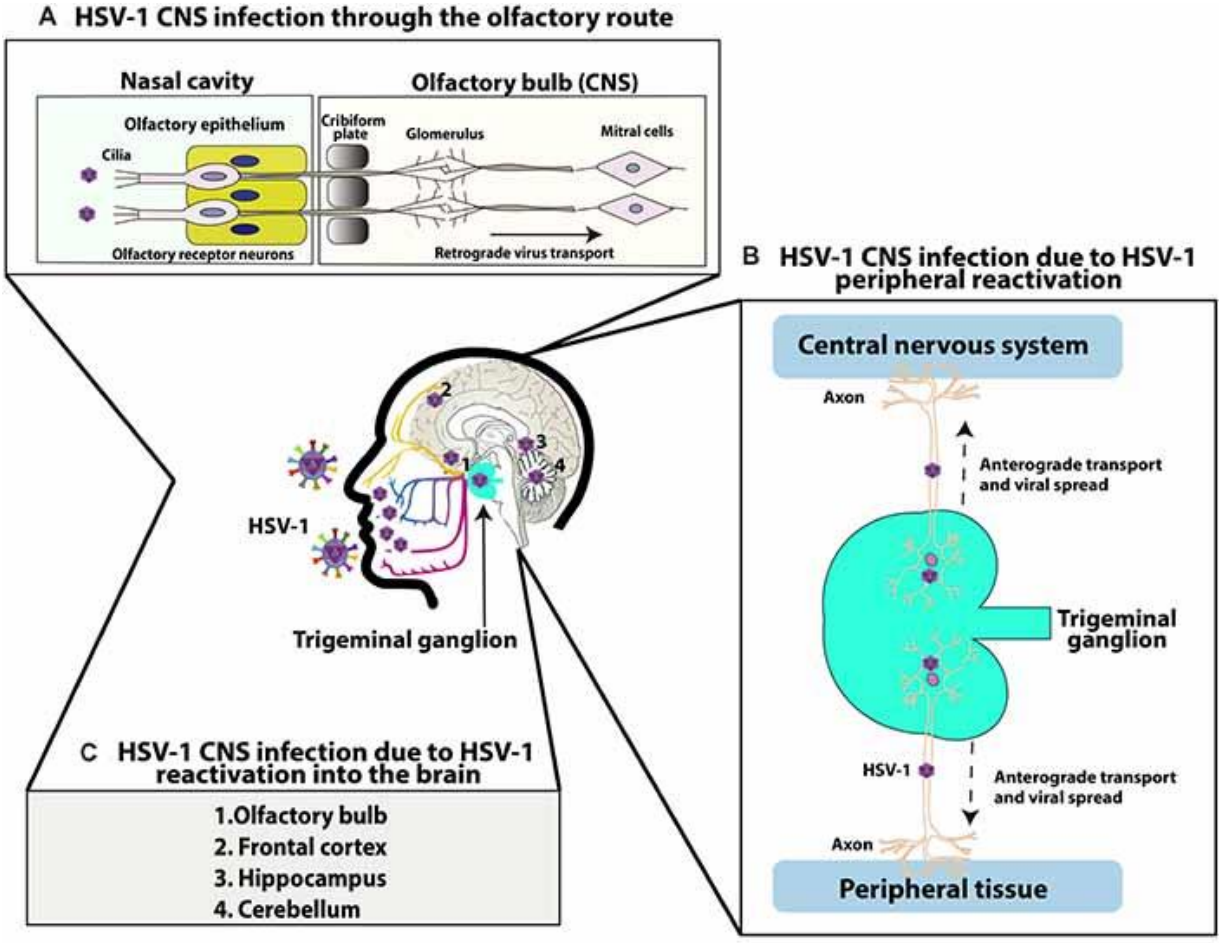


**Figure 2. Central nervous system infection with HSV-1**. A) HSV-1 CNS infection through the olfactory route: HSV-1 can infect the termini of olfactory neurons enervating the nasal epithelium and access the CNS by retrograde axonal transport through neurons until reaching the olfactory bulb in the brain. B) HSV-1 can also infect the CNS because of HSV-1 peripheral reactivation. HSV-1 can reactivate from neurons in the trigeminal ganglia (TG) and reach either the skin or CNS through anterograde axonal transport. C) HSV-1 can also reach different regions of the CNS because of HSV-1 reactivation within the brain. Reactivation of latent virus within the CNS has been reported to reach the cerebellum, olfactory bulb, frontal cortex, or hippocampus. Modified from *Duarte et al, 2019.*

demonstrated the presence of HSV-1 DNA in brain tissue in individuals with no known neurologic disease, suggesting the possibility that HSV-1 could establish latency in the CNS (Baringer and Pisani, 1994). Moreover, some studies have reported viral reactivation in *ex vivo* brainstem tissue explants following latent infection with HSV-1 in mice (Chen et al., 2006). Also, infectious virus was recovered in the brainstem of latently infected mice, which were induced to viral reactivation by hyperthermia and latent viral genomes were detected in the cerebellum, olfactory bulbs, frontal cortex, and hippocampus (Yao et al., 2014). That study indicates that this virus can reach the brain and remain there in a latent state, from where it can reactivate after stress conditions leading to a symptomatic or an asymptomatic spread.

### 1.3 HSV-1 and neurodegeneration

There is accumulating evidence suggesting that HSV-1 infection of the brain both, in symptomatic and asymptomatic individuals could lead to neuronal damage and eventually, to neurodegenerative disorders, such as multiple sclerosis or Alzheimer´s disease (extensively reviewed in Duarte et al.,2019). Indeed, neurological sequelae, such as epilepsy, amnesia or cognitive and behavioral alterations are common after HSE despite treatment with antivirals that limit virus replication (Misra et al., 2008; Riancho et al., 2013). Noteworthy, immune-related mechanisms have been defined as main players that induce chronic neurologic damage (Marques et al., 2008). In addition, subclinical reactivations from brain neurons may eventually occur and produce local and regional effects in this tissue which may ultimately lead to neurodegenerative manifestations (Perng and Jones, 2010; Martin et al., 2014b).

Importantly, studies using mouse models support the above-mentioned notions and have allowed to deepen our knowledge on the chronic alterations elicited by HSV-1 infection over the CNS both, in mice that are more susceptible of undergoing severe viral encephalitis (Marques et al., 2008; Martin et al., 2014a), as well as in C57BL/6 mice that are resistant to HSV-1 encephalitis under certain experimental conditions, given by their rapid and effective innate alpha/beta interferon (IFN-α/β) response that reduces viral pathogenesis and increases their survival, leading to asymptomatic brain infection (Halford et al., 2004; Kastrukoff et al., 2012; Zimmermann et al., 2017).

A study using BALB/c mice showed that early during HSE, the immune response in the brain is dominated by the influx of macrophages and neutrophils, which play a critical role in viral clearance (Figure 3A) (Marques et al., 2008; Terry et al., 2012). Moreover, macrophages secrete TNF-α and microglial cells express high levels of IL-1β, which affect the blood-brain barrier (BBB) by upregulating endothelial cell adhesion molecules (Fields et al., 2006). Non-productive HSV-1 infection of microglia can also lead to the expression of others cytokines and pro-inflammatory molecules, such as TNF-α, IL-6, IL-8, CCL5 and chemokine CXCL10 (Lokensgard et al., 2001). After 14 days post infection T lymphocytes begin to be a predominant leucocyte cell type infiltrating the brain, which is composed mainly by $CD8^+$ T cells that persist in this tissue up to 30 days post-infection without detectable viral replication (Figure 3B) (Marques et al., 2008; Terry et al., 2012). Importantly, infiltrating $CD8^+$ T cells express IFN-γ which is known to synergize with TNF-α to increase NO-induced neurodegeneration and demyelination in the brains of mice (Blais and Rivest, 2004). Moreover, prolonged microglial activation has also been reported in the brains of mice latently-infected with HSV, as indicated by high MHC class-II expression levels up to 30 days post-infection

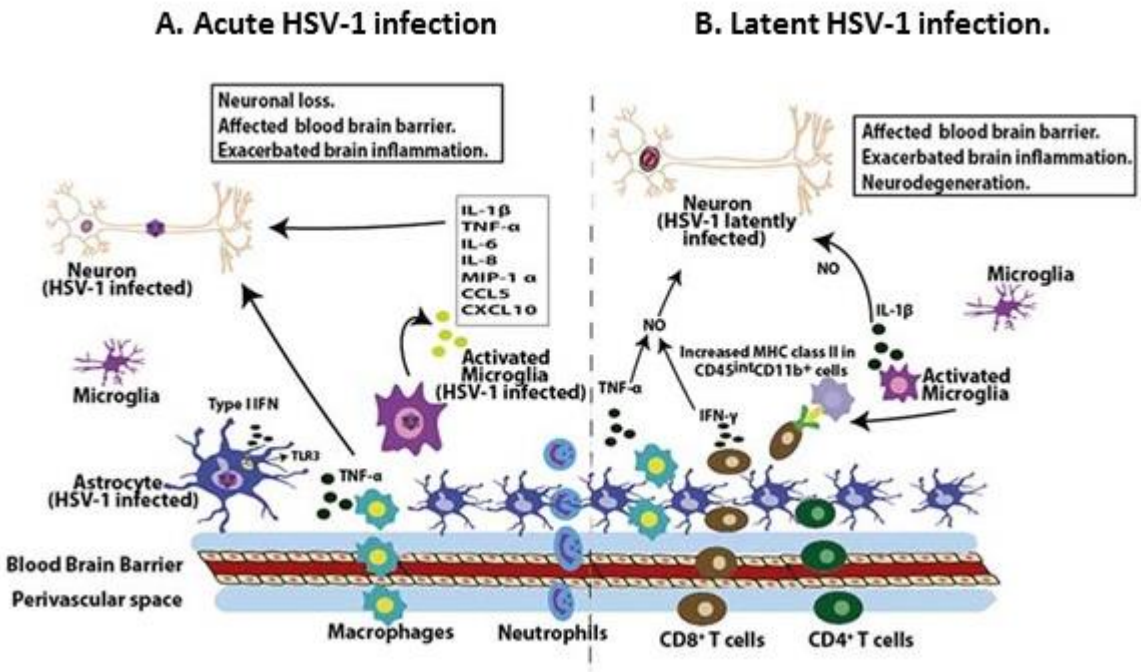


**Figure 3. Acute and chronic neuroinflammation by HSV-1 brain infection:** A) During acute infection of the brain, HSV-1 leads to the infiltration of macrophages and neutrophils and the expression of pro-inflammatory molecules by microglia. Astrocytes in turn produce type-I interferon (IFN) mediated by TLR3 engagement in response to HSV-1. These soluble molecules will affect the permeability properties of BBB) and potentially exacerbate brain inflammation, potentially leading to neuron insult. B) HSV-1 latent CNS infection is characterized by the infiltration of $CD8^+$ and $CD4^+$ T cells. Importantly, these T cells are localized near latently infected neurons and are detected in a 3:1 ratio ($CD8^+$ to $CD4^+$ T cells). Moreover, $CD8^+$ T cells can secrete IFN-γ. Prolonged microglial activation in the brain by HSV-1 infection produces increased MHC-II expression in $CD45intCD11b^+$. As a consequence of immune cell infiltration into the brain during both, acute and persistent HSV-1 infection of the brain, cytokines such as TNF-α and IL-1β can affect the BBB, which can exacerbate brain inflammation. Importantly, synergistic effects between TNF-α and IFN-γ can lead to increased nitric oxide-induced neurodegeneration and demyelination in the brain of susceptible mice. IL-1β: interleukin-1β, TNF-α: tumor necrosis factor-α, MIP-1α: macrophage inflammatory protein 1-α, CCL5: chemokine (C-C motif) ligand 5, CXCL10: chemokine (CXC motif) ligand 10. Modified from *Duarte et al, 2019.*

(Figure 3B) (Marques et al., 2008) . In addition, asymptomatic reactivation in BALB/c mice was demonstrated by the detection of viral ICP4 protein in the TG and cerebral cortex of mice 60 days post-infection, and was accompanied by the up-regulation of markers of neuroinflammation, such as toll-like receptor (TLR) 4, interferon $\alpha$/β, and phosphorylated interferon regulatory factor 3 (p-IRF3) (Martin et al., 2014a).

On the other hand, another study using C57BL/6 mice that survived an acute phase of ocular infection accompanied with virus dissemination to the CNS, showed that LAT was mainly concentrated within the lateral ventricles and the hippocampus (ependymal zone), as well as the brainstem 30- and 60-days post-infection (Menendez et al., 2016). Surprisingly, the ependymal region in the brain evidenced HSV-1 lytic gene transcripts being expressed at these time-points post-infection, in contrast to the brainstem and TG, in which the expression of lytic genes was decreased (Menendez et al., 2016). Interestingly, this study proposes the hypothesis that a specific tropism of HSV-1 to the ependymal zone may be linked to chronic inflammatory responses in the brain and that this zone may have particular conditions that provide an environment that enhances viral persistence, potentially leading to neurodegeneration (Webb et al., 1989; Conrady et al., 2013). A more recent study showed that the ependymal zone harbors neural progenitor cells that are vulnerable to acute HSV-1 infection and viral lytic-associated proteins were detected in these cells during latency (Chucair-Elliot et al., 2014). Importantly, viral persistence in the ependymal zone of the brain was related to T cells expressing exhaustion markers (LAG-3, TIM-3, PD1, CD160 and KLRG-1), which were unable to control HSV-1 infection *ex vivo* and secreted less IFN-γ and granzymes in comparison to T cells isolated from TG (Wherry and Kurachi, 2015; Menendez et al., 2016).

At the molecular level, the matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) have been shown to be elevated in the brains in both, acute and latent HSV-1 infections. These MMPs could degrade the extracellular matrix and cell surface proteins of the BBB and modulate its permeability, which could lead to persistent cell infiltration increasing neuroinflammation (Martínez-Torres et al., 2004; Weiser et al., 2007).

Finally, it has been reported that HSV-1 negatively modulates apoptosis-related pathways in neurons favoring its persistence in the brain (Du et al., 2012; Carpenter et al., 2015), and can disrupt autophagy-related processes leading to protein accumulation and cellular toxicity in this tissue (Lussignol et al., 2013; O'Connell and Liang, 2016). Moreover, HSV-1 infection can produce mitochondrial dysfunction, which increases the production of reactive oxygen species (Wnek et al., 2016). Therefore, HSV-1 could significantly contribute to the pathogenesis of neurons, by interfering with these processes in the brain (Lussignol et al., 2013; Carpenter et al., 2015; Wnek et al., 2016). On the other hand, because the immune system of an individual tends to decay upon aging, opportunities arise for HSV-1 to reactivate in the organism and spread to tissues such as the brain contributing to neurodegenerative disorders in humans (Dobson et al., 2003; Otth et al., 2009; Martin et al., 2011; Buscarinu et al., 2017).

### 1.4 ICP34.5 is a neurovirulent factor of HSV-1

To productively replicate in the host nervous system, HSV-1 encodes several viral proteins that counteract the host antiviral response (Suazo et al., 2015). The gamma-34.5 gene encodes a neurovirulence factor named infected cell protein 34.5 (ICP34.5 or gamma-34.5), which is present in two copies in the viral genome and is located in the inverted repeats of the

regions flanking the unique long ($U_L$) sequence (Wilcox and Longnecker, 2016). This viral protein has several binding-domains that target specific host proteins that are involved in several effector pathways, such as type-I interferon (IFN-I) induction, host shutoff of protein synthesis, and the inhibition of autophagy (Figure 4) (Wilcox and Longnecker, 2016).

Host cells respond to HSV-1 infection through the recognition of pathogen-associated molecular patterns (PAMPs) that trigger IFN-I production, which in turn induces the expression of an array of antiviral genes (Rasmussen et al., 2009). Recognition of PAMPs by host sensors, such as toll-like receptors (TLRs), retinoid acid-inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5) or DNA-dependent activator of IFN-regulatory factor (DAI), leads to downstream signaling events that ultimately activate TANK-binding kinase 1 (TBK1), which is responsible of phosphorylating and activating IRF3, the primary transcription factor regulating the induction of type-I IFNs (Fitzgerald et al., 2003). Importantly, ICP34.5 abolishes the induction of IFN-I production through direct binding to TBK1 through its amino terminus (Ma et al., 2012). This hijacking of TBK1 prevents IRF3 phosphorylation and its consequently nuclear translocation for the transcriptional activation of IFN-I genes (Figure 4A) (Verpooten et al., 2009). Nevertheless, if type-I IFNs are produced, they are detected by the IFN-I receptor (IFNAR), which activates the JAK-STAT signaling pathway and initiates the transcription of interferon stimulated genes (ISGs), which enhance their antiviral state (Ivashkiv and Donlin, 2014). One of such ISGs is the double-stranded RNA–dependent protein kinase R (PKR), which inhibits protein synthesis by phosphorylating the translation initiation factor eIF2a (Mohr, 2004). Importantly, the carboxyl terminus of ICP34.5 binds to the host phosphatase PP1α, which in turn binds to eIF2α and leads to eIF2α dephosphorylation and the

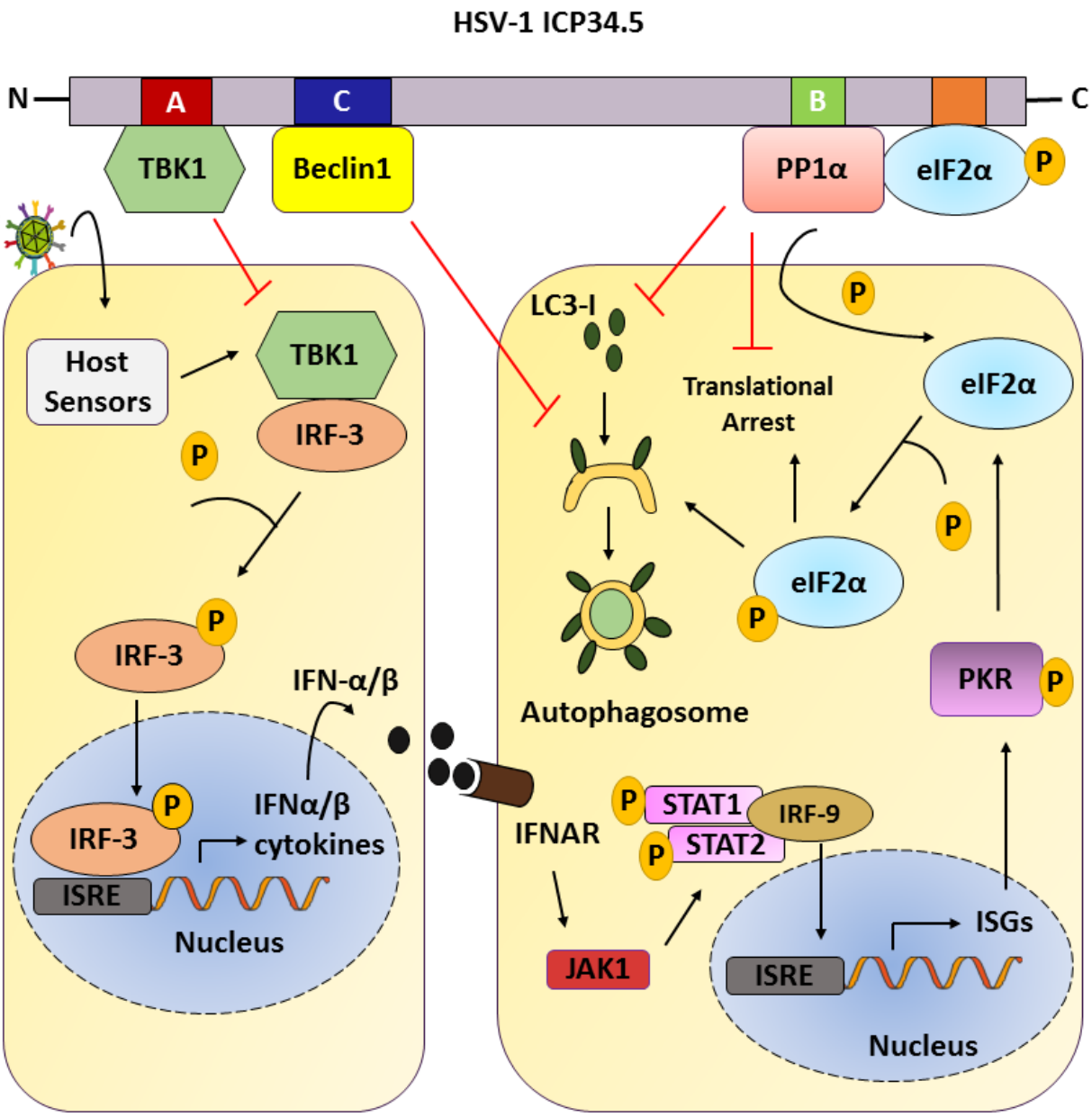


**Figure 4. Functions of the neurovirulence factor ICP34.5**. ICP34.5 has several domains that play key roles for HSV-1 evasion of the innate immune response. (A) This protein inhibits the induction of type-I IFNs (IFNα/β) through its TBK1 binding domain in its amino terminus, (B) it also inhibits the host shut-off of protein synthesis and autophagy through the PP1α and eIF2α binding domains in its carboxyl terminus, (C) and it also inhibit autophagy through a beclin-1-binding domain.

reversing of protein synthesis shutoff in the cell (Figure 4B) (Wilcox et al., 2015a). In addition, eIF2α phosphorylation promotes the induction of autophagy (Acevo-Rodríguez et al., 2020).

Autophagy acts as a defense mechanism against different infectious agents, promoting lysosomal degradation of microorganisms, as well as playing key roles in immune signaling. It also plays roles in antigen processing for pathogen-derived peptide presentation in MHC molecules and for the delivery of viral nucleic acids to endosomal TLRs (Lussignol and Esclatine, 2017). Importantly, autophagy has been reported to be key for controlling HSV-1 infection in neurons (O'Connell and Liang, 2016). This finding is in sharp contrast with epithelial cells, where an IFN-I response is sufficient alone to control HSV-1 infection and in which case autophagy is not required (Yordy et al., 2012). However, although autophagy protects the adult brain from viral encephalitis, contrasting results have been reported in newborn mice, where autophagy seems to be detrimental for the host and was described to promote neuronal apoptosis. Interestingly, these findings suggest an age-dependent role for autophagy during HSV-1 brain infection (Wilcox et al., 2015b). Notably, ICP34.5 inhibits autophagy indirectly through eIF2α dephosphorylation by PP1α, as well as directly through its interaction with the autophagy-inducing protein beclin-1 and interfering with the formation of autophagosomes and antigen presentation in dendritic cells (DCs) (Figure 4C) (Orvedahl et al., 2007; Gobeil and Leib, 2012; Wilcox et al., 2015a).

Because of the aforementioned functions of this protein, previous investigations have studied HSV-1 mutant viruses that have the ICP34.5 gene deleted. Interestingly, these viruses can replicate at the mucosae and epithelial tissues, although yielding lower titers and lasting for fewer days as compared to the wild type virus (Whitley et al., 1993). These results indicate ICP34.5 positively modulates the replication ability of HSV-1 early during infection when the

virus challenges the innate immune response. Moreover, these mutants did not cause lethal encephalitis due to its impaired ability to evade the antiviral response, reporting a reduced ability to replicate in the nervous system, and also to establish latency and reactivate as determined *ex vivo* (Orvedahl et al., 2007).

Nevertheless, some studies have shown that despite the apparent attenuated phenotype of ICP34.5-deleted viruses, these mutants can cause the destruction of ependymal cells, as well as neurons that are exposed to high amounts of the virus, which lead to inflammation in the brain (Kesari et al., 1998; Markovitz and Roizman, 2000). A study evaluating the effect of the Δ34.5 mutant HSV-1 in the brain of different strains of rats and mice reported robust immune responses consisting of macrophages and T cells in the brain in all the animal strains tested, yet significant weight loss was only seen in some animals, which was accompanied by signs of clinical disease (McMenamin et al., 1998). These results suggest that the dose of the virus used, as well as the host immune system can impact the overcoming of the infection and limit or not the severity of the infection and related disease. This is an important observation, as these mutant viruses have been exploited for the delivery of disease-limiting cytokines in cancer and tumor therapies, yet the immune responses elicited against these HSV-1 vectors have not been fully elucidated (Broberg and Hukkanen, 2005). More recently, another study evaluated the replication efficiency of numerous Δ34.5 HSV-1 mutants in nervous system tissues after intranasal, corneal or intralabial infection routes, as well as the effects of the viruses over the immune response after intranasal infection (Broberg et al., 2004). Importantly, this study reported that the intranasal inoculation of HSV-1 mutants is an effective route for viral spread into the CNS, with poor replication of the virus in this tissue, but viral DNA persistence even 21-days post-infection. Regarding the immune response, the infection with HSV-1 mutants

alone, or encoding IL-4 or IL-10 transgenes induced Th2-type cytokine responses (Broberg et al., 2004). However, viruses encoding the IL-10 transgene or without any transgenes produced Th1-type cytokines, namely IFN-γ and IL-23 in the brain. Additionally, the transgene-free mutant virus elicited a higher number of lymphoid T cells and $CD11c^+$ antigen presenting cells in the spleen as compared to WT HSV-1 (Broberg et al., 2004). Taken together, these results suggest an additional immunomodulatory role for ICP34.5 and calls for further studies of the immune responses produced by these mutants viruses that are being used as vectors in gene therapy (Broberg and Hukkanen, 2005; Hukkanen and Nygårdas, 2013). It is important to guarantee desired immune responses that aid as therapies, while avoiding possible adverse effects.

### 1.5 Multiple sclerosis disease

Multiple Sclerosis (MS) is a neurodegenerative disorder affecting the CNS, where the protective myelin sheath that covers the nerve cells in the brain, spinal cord and optic nerves are damaged, inflamed and hardened by attacking of myelin-specific autoreactive T cells or B cells, and myeloid cells that infiltrate the CNS mediating an inflammatory response that results in demyelination and axon degradation, that disrupts the ability of neurons to transmit the nerve impulse, resulting in a widespread of signs and symptoms depending of the site of lesion, including physical, sensorial, cognitive and sometimes psychiatric problems (Compston and Coles, 2008; Thomas, 2012; Dobson and Giovannoni, 2019). MS is the most common cause of non-traumatic neurological dysfunction affecting principally young adults between the age of 20 and 50 with an average age of onset of 29, which generate a great socio-economic burden because the disease may hinder ability to maintain studies and work (Msif, 2013). It is estimated

that approximately 2.3 million people suffer from this disease worldwide, with highest prevalence in countries in North America and Europe (140 and 108 cases per 100,000 individuals, respectively) and lowest in African and Asian countries (2.1 and 2.2 cases per 100.000 individuals, respectively) (Msif, 2013). Chile is considered a low to medium risk country for MS, because in the Magallanes region there is a prevalence of 13 to 14 cases per 100,000 individuals (Melcon et al., 2013), with all geographical regions in Chile showing a cumulative prevalence rate of 5.69 per 100,000 individuals and an annual incidence rate of 0.90 (Díaz et al., 2012).

MS exhibits a heterogeneous progression and symptomatology. The first evident sign of its appearance is called clinically isolated syndrome (CIS), an event with observed demyelination involving the optic nerve, brain or spinal cord (Miller et al., 2005; Filippi et al., 2018). 85% of newly diagnosed patients present a relapsing-remitting form (RRMS) of MS, which is display a worsening of neurological function called relapse or exacerbation. Disease is followed by periods of remission in which the neurological functionality is partially recovered within weeks to months. It has been estimated that up to 80% of these individuals will develop secondary progressive MS (SPMS), one to two decades post-diagnosis. In SPMS, the inflammation of CNS is reduced, however progressive neurological decline and CNS atrophy are observed. Finally, approximately 10% of patients with MS are diagnosed with primary progressive disease (PPMS), which shows a progressive decline from the onset and an absence of relapses (Dendrou et al., 2005; Filippi et al., 2018; Dobson and Giovannoni, 2019).

The pathology of the disease is characterized by focal demyelinated plaques caused by activated self-reactive cells that recognize myelin antigens and migrate to the CNS after disruption of the BBB. These infiltrating cells may also lead to reactive gliosis, loss of

oligodendrocytes, and axonal damage (Dendrou et al., 2005; Haider et al., 2016). The mechanisms underlying the BBB breakdown are not entirely determined but seem to be mediated by the direct effects of proinflammatory cytokines, such as IL-1β and IL-6, or chemokines released by resident CNS cells (microglia, astrocytes and endothelial cells) or lymphoid and myeloid infiltrating cells (Argaw et al., 2006; Aubé et al., 2014; Wang et al., 2014).

There is no cure for this disease because its cause is unknown. Currently, two models have been proposed to explain the development of MS. Whereas in the first model, autoreactive T cells are activated by a peripheral stimulus and then migrate to the CNS by crossing the BBB, in the second model the demyelination is caused by an inflammatory response mounted against an infection in the CNS, and the activation and infiltration of self-reactive T-cell occur as a secondary phenomenon (Dendrou et al., 2005). However, what triggers the loss of peripheral immunologic tolerance leading to the activation of these autoreactive immune cells in an individual and what determines their infiltration into the CNS remains at present somewhat unknown. It is thought that MS develops as an interplay between multiple factors, such as genetic predisposition, the host immune system and environmental factors (Beecham et al., 2013). Importantly, viral infections have been defined as environmental triggers that could play an important role in disease development and progress.

### 1.6 Animal models to study the relationship between virus and multiple sclerosis disease.

Animal models of demyelinating diseases have allowed advances in the understanding mechanisms involving virus in autoimmunity. As support to the intrinsic theory, some viral

infection in the CNS can produce demyelinating disease by epitope spreading or bystander activation. As an example, Theilers´s murine encephalomyelitis virus (TMEV) causes a persistent infection in CNS without complete viral clearance and reactivity to myelin antigens emerges after the onset of viral-induced clinical symptoms, which is due to epitope spreading after initial virus-specific Th1-mediated demyelination (Karpus et al., 1995; Miller et al., 1997). In contrast, during CNS infection by neurotropic mouse hepatitis virus (MHV), infectious virus is not detected in the brain tissue, and MHV persistence is characterized by presence of viral RNA and proteins, which have been associated with T cell retention. Likely, chronic inflammation releases myelin antigen leading to the bystander activation of myelin-specific T cells (Bergmann et al., 2006).

Because of the difficulty in identifying direct causal effectors over MS initiation in humans, animal models that mimic MS or share disease traits with this disease are highly valuable for this purpose. Experimental autoimmune encephalomyelitis (EAE) is a disease in animals that shares numerous molecular and cellular signatures with MS and can be actively induced using different CNS antigens and peptides, as well as through passive adoptive transfer of activated $CD4^+$ T cells that recognize such self-antigens (Baxter, 2007). One such model is based on peripheral immunization of mice with oligodendrocyte glycoprotein-derived peptide ($MOG_{35-55}$) and the disruption of the BBB with pertussis toxin (Kastrukoff et al., 1987). Approximately 12 days after treatment, mice develop ascending paralysis due to spinal cord inflammation, which leads to demyelination, neuron dysfunction and death in its severe form when using high doses of MOG peptide and pertussis toxin (Constantinescu et al., 2011; Robinson et al., 2014). Immune cell infiltrations in the brain are atypical in this mouse model of MS and if present, are restricted to the meninges. Infiltrating $CD4^+$ T cells are re-activated in

the CNS by antigen-presenting cells (APCs), with the resulting inflammatory response leading to monocyte recruitment into the CNS. Currently, Th1 and Th17 are considered the main $CD4^+$ T cell sub-sets implicated in this disease (Figure 5) (Constantinescu et al., 2011).

Interestingly, some herpesviruses such as Epstein-Barr virus (EBV) and human herpesvirus 6 (HHV-6) have received particular attention for their ability to remain latent in lymphoid cells and potentially to modulate the onset and relapse of MS in humans. Animals models have contributed to study the molecular and cellular events that could interfere with the disease course (Casiraghi et al., 2012; Reynaud and Horvat, 2013; Casiraghi et al., 2015; Leibovitch et al., 2018). In fact, a study investigated the role of the murine gamma-herpesvirus γHV-68 (a homologue of EBV in humans), on the pathogenesis of relapsing-remitting EAE in SJL mice. Importantly, this study found that infection with live γHV-68, but not UV-inactivated virus exacerbated EAE disease (Peacock et al., 2003). Additionally, a follow-up study found that latent-infection with γHV-68 virus, prior to EAE induction was capable of increasing the pathogenesis of active EAE, which was associated with increased $CD4^+$ and $CD8^+$ T cell responses in the brain and spinal cord, yet was independent of viral reactivation (Casiraghi et al., 2012). On the other hand, human herpesvirus-6 (HHV-6) has also been investigated as an environmental trigger of EAE. As rodents are not susceptible to HHV-6 infection, a recent study used non-human primates to examine the impact of HHV-6 infection on EAE disease. Although the viral infections were asymptomatic, MS-like disease in these animals was significantly accelerated in all virally-inoculated animals with detection of viral antigens in the brain, which showed a marked colocalization with $CD3^+$ cells, suggesting that this virus may participate in MS in humans (Leibovitch et al., 2018). However, the mechanism underlying this potential relation and its impact in MS patients requires further studies.

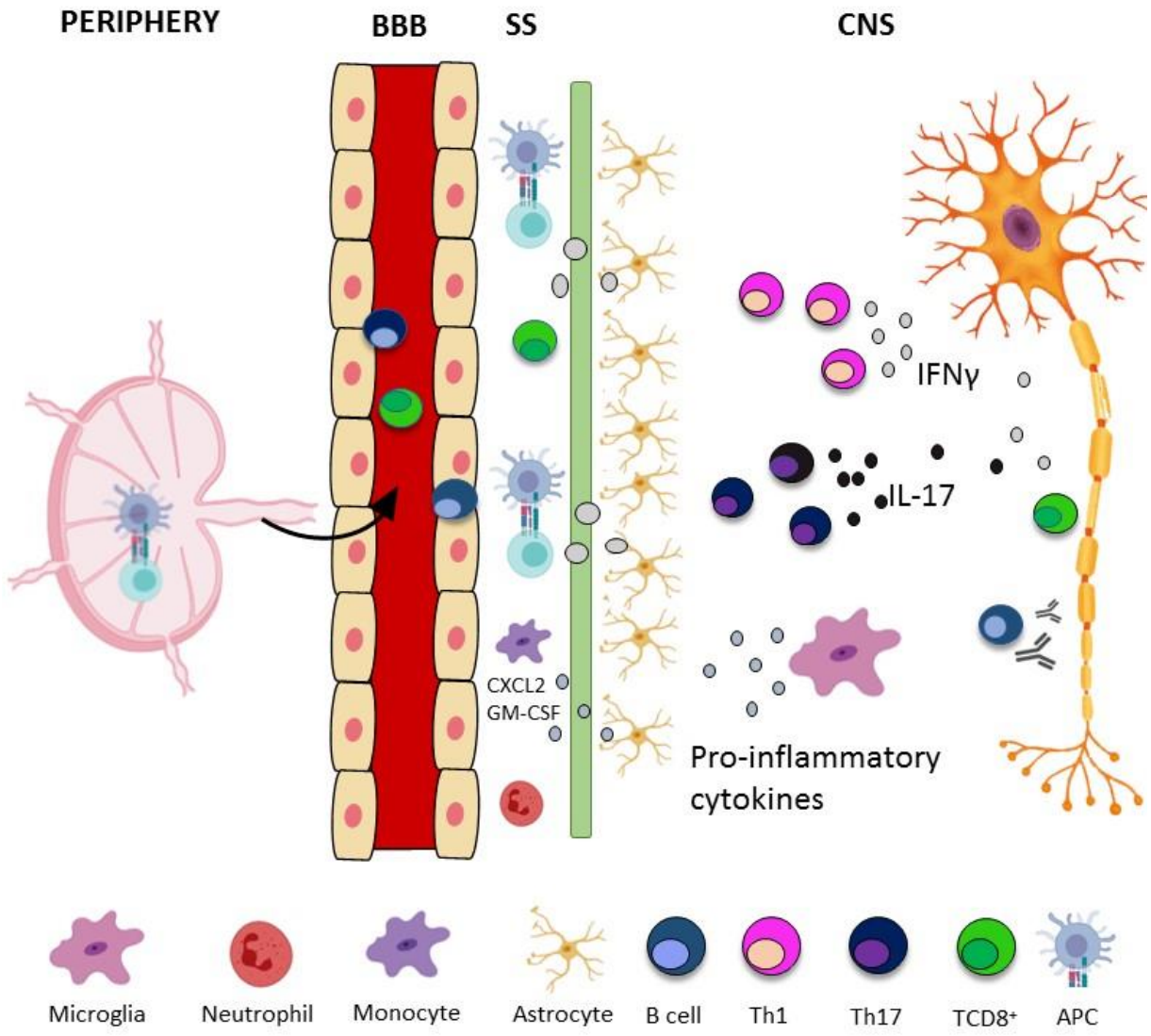


**Figure 5. Inflammatory process after EAE induction**. MOG-peptide is presented by antigen-presenting cells (APCs) to self-reactive cells in the peripheral lymphoid node. Self-reactive cells become activated and migrate into CNS through of BBB, where they are reactivated by CNS-resident APC in the subarachnoid space. At the beginning, the main infiltrating cells are T CD4+ cells, which acquire a Th17 or Th1 phenotype releasing soluble mediators that produce demyelination. Then, other resident cells, such as astrocytes and microglia are activated leading to increased BBB disruption and migration of myeloid cells, B cells and CD8+ T cells that contribute with CNS inflammation and myelin damage. BBB: Blood-brain barrier, SS: subarachnoid space, CNS: central nervous system.

### 1.7 HSV-1 and multiple sclerosis disease

At present, an association between HSV-1 and MS disease may be considered based on the finding of virus genetic material in tissue samples or in body fluids of patients with MS. In 1964, HSV-1 was isolated for the first time in the brain of a postmortem patient with MS (Gudnadottir et al., 1964). Then, HSV-1 was isolated from the cerebrospinal fluid in alive patient during the first episode of MS (Bergstrom et al., 1989). More recently, a case-control study evaluated the prevalence of HSV-1 in peripheral blood mononuclear cells (PBMCs) of patients with RRMS, and HSV-DNA was founded in 45.1% of patients with MS, in comparison with 3.4% of healthy subjects (Najafi et al., 2016). Another study also detected DNA and mRNA of HSV-1 in the peripheral blood of patients with MS during clinical acute attack, and it probably play a role in the triggering of MS relapses (Ferrante et al., 2000). Finally, HSV-DNA has been reported more frequently in postmortem MS brain tissues than control subjects, and HSV-DNA was found more in active plaques than inactive plaques in these tissues (Sanders et al., 1996).

On the other hand, HSV-1 seropositivity has been associated with increased risk of MS in those individuals that do not have the DRB1*15 allele, or decreased risk in those that have it (Waubant, 2011). Importantly, these observations somewhat support the idea that this virus may play a role in MS in individuals with particular genotypes (Kastrukoff et al., 2012). Moreover, another study showed that depletion of macrophages causes CNS demyelination in mice ocularly infected with HSV-1 (Mott et al., 2011; Zandian et al., 2011). Likewise, a recombinant HSV-1 expressing IL-2 produced autoreactive T cells and CNS demyelination, supporting the hypothesis that within an environment that promotes T cell activation, HSV-1 may be enough for initiating processes that end with the destruction of the myelin in the CNS (Osorio et al.,

2005; Mott et al., 2013). A subsequent study determined that the mechanism that led to CNS demyelination in these HSV-1-infected mice was the suppression of IL-12p70 formation by IL-2 or after macrophage depletion (Lee et al., 2017). Moreover, a recent study showed that the HSV-1 host-pathogen interactome is highly concentrated in susceptibility genes associated with neurological disorders, such as MS with enrichment values at 4-fold (Carter, 2017). Additionally, microorganisms may also contribute to the pathogenesis of MS by inducing the activation and clonal expansion of self-reactive lymphocytes by mimicry molecular (Wucherpfennig and Strominger, 1995). For instance, the Hy.1B11 T cell receptor (TCR) originated from a patient with MS showed to be cross-reactive with a peptide derived from HSV-1 (UL15154-166) (Sethi et al., 2013).

Taken together, although some studies support a role for HSV-1 infection in MS (Ferrante et al., 2000; Najafi et al., 2016), this has been poorly studied in animal models which could help define whether HSV-1 infection plays a direct role in MS. In 1977, a study performed in rats showed that repeated inoculations of HSV-1 elicit clinical and histological evidence of recently exacerbated EAE. However, the authors did not determine the mechanism behind this observation (Hochberg et al., 1977). Moreover, the approach available in that time of EAE disease in rats was characterized by inflammation and edema leading to paralysis without demyelination, which differs from what happens in MS (Robinson et al. 2014). In contrast, MOG-induced EAE is characterized by CNS demyelination and can follow a relapsing–remitting or chronic disease course as MS, depending on the induction conditions (Berard et al., 2010). Importantly, this model has been widely used to develop and evaluate therapies to treat MS (Robinson et al., 2014). For this reason, for this thesis we proposed to assess the impact of asymptomatic HSV-1 infection over MOG-induced EAE in C57BL/6 mice to determine the

possible roles of HSV-1 infection on multiple sclerosis disease. First, we infected mice with a neurovirulent strain of HSV-1 that reaches the brain after intranasal inoculation. Notably, C57BL/6 mice can be resistant to acute encephalitis after CNS infection by HSV-1, which we consider can recapitulate several aspects of asymptomatic HSV-1 infection in humans, which undergo infection without clinical manifestations, despite having this virus in the brain (Kastrukoff et al., 2012). Moreover, we also evaluated the effects of an attenuated viral strain of HSV-1, which does not cause encephalitis and has an impaired ability to establish latency and reactivate from the nervous system. This study could help better understand the relationship between HSV-1 infection and multiple sclerosis disease, as well as help identify new factors contributing to the progression of this disease.

# 2. HYPOTHESIS AND AIMS

According to the previous evidence described above it is possible that HSV-1 may modulate the severity and susceptibility to MS because:

1. HSV-1 infects an important percentage of the population.
2. HSV-1 is acquired early in life and causes lifelong persistent infection.
3. HSV-1 infects neurons and can remain in a latent state from which it may reactivate periodically causing symptomatic or asymptomatic shedding.
4. HSV-1 can reach the brain throughout life without inducing clinical symptoms.
5. Recurrent subclinical reactivations during a persistent brain infection may produce neuroinflammation and chronic neuron damage.
6. Acute and latent brain infection elevates the MMP-2 and MMP-9 expression, which could affect the BBB integrity.

To assess a possible relationship between HSV-1 and MS, we proposed to evaluate the following hypothesis and aims:

**Hypothesis:**

*"Asymptomatic HSV-1 infection enhances MOG-induced EAE disease severity in the mouse model by increasing the permeability of the blood-brain barrier".*

**Main goal:**

To assess the impact of asymptomatic HSV-1 infection on the onset and severity of multiple sclerosis in a mouse model.

**Specific Aims:**

1. To evaluate the clinical and histopathologic score after EAE induction in HSV-1-infected and non-infected animals.
2. To determine the immune cells infiltrating the CNS after EAE induction in HSV-1-infected and non-infected animals.
3. To determine the cytokine environment in the CNS after EAE induction in HSV-1-infected and non-infected animals**.**
4. To quantify MOG or HSV-1 specific antibodies levels in the sera of HSV-1-infected and non-infected animals after EAE induction.
5. To investigate whether asymptomatic HSV-1 infection increases BBB permeability.

# 3. ASYMTOMATIC HERPES SIMPLEX VIRUS TYPE 1 INFECTION CAUSES AN EARLIER ONSET AND MORE SEVERE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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**Keywords: HSV-1, viral infection, multiple sclerosis, experimental autoimmune encephalomyelitis**

### 3.1 Abstract

Herpes simplex virus type 1 (HSV-1) infection is highly prevalent in the human population, yet its presence is generally unnoticed as the virus can establish asymptomatic infection and remains latent in the host with periodic reactivations. Importantly, the virus may undergo subclinical reactivations and shed onto other tissues or individuals. Noteworthy, HSV-1 infects neurons and may eventually reach and expand within the central nervous system (CNS) with no apparent disease. Multiple sclerosis (MS) is an increasingly prevalent progressive autoimmune and debilitating chronic disease that involves the recognition of CNS antigens by the immune system. Although significant progress has been made in the last decades on the biology of MS and the identification of novel therapies to treat its symptoms, the triggers of this disease remain unknown. However, recent studies have suggested that viral latent infections may contribute to disease onset. Interestingly, a potential association between HSV-1 infection and MS have been reported, yet a direct relationship between both has not been conclusively demonstrated. Experimental autoimmune encephalomyelitis (EAE) recapitulates several aspects of MS in humans and is widely used to study this disease. Here, we evaluated the effect of asymptomatic brain infection by HSV-1 on the onset and severity of EAE in C57BL/6 mice, as well as by an HSV-1-mutant that is attenuated in neurovirulence and does not cause encephalitis. Importantly, we observed a more severe EAE in mice previously infected with either, with the wild-type (WT) or the mutant HSV-1, as compared to uninfected control mice. These findings support the notion that a previous exposure to HSV-1 can accelerate and enhance EAE, which suggests a potential contribution of HSV-1 to the onset and severity of MS.

### 3.2 Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory disorder of the central nervous system (CNS) that affects both, the brain and spinal cord in which multifocal autoreactive lymphocytic infiltrations lead to damage of the myelin and the axons of neurons (Karandikar et al., 2004; Dendrou et al., 2005). Defining what triggers the loss of immunologic tolerance to CNS antigens and the onset of autoreactivity with infiltration into the associated tissues remains elusive (Compston and Coles, 2008; Steelman, 2015). Likely, MS develops as an interplay between genetic predisposition, the immune system and environmental factors, such as viral infections (Beecham et al., 2013).

Herpes simplex virus type 1 (HSV-1) infection is highly prevalent in the human population with nearly two thirds of the world population infected with this virus (Suazo et al., 2015). HSV-1 is neurotropic and causes a wide spectrum of clinical manifestations, ranging from mild symptoms such as oral and facial lesions (e.g. *herpes labialis,* herpetic gingivostomatitis), to more severe more diseases affecting the eyes and CNS (e.g. herpetic keratitis, retinitis, encephalitis and meningitis) (Arduino and Porter, 2008; Rechenchoski et al., 2017). Importantly, HSV-1 can access the CNS with no apparent pathology (asymptomatic) establishing a persistent latent infection (Looker et al., 2015). Accumulating evidence indicates that healthy individuals frequently have HSV-1 in the brain, which could eventually favor the development, or enhance the severity of neurodegenerative disorders by altering normal neuronal cell function (Duarte et al., 2019). Subclinical HSV-1 reactivations within CNS neurons may also contribute to local and regional dissemination of the virus, as well as long-term detrimental effects in this tissue(Marques et al., 2008; Duarte et al., 2019). Importantly, HSV-1 infection of the CNS is characterized by persistent lymphocytic cell infiltrations and

elevated levels of cytokine transcripts (e.g. IFN-γ, TNF-α), as well as increased amounts of chemokine mRNAs (e.g. CXCL10, CCL5), suggesting that latent HSV-1 infection can be accompanied by a chronic inflammatory process in this tissue (Theil et al., 2003). Moreover, increased levels of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) have been detected in HSV-1 latently-infected CNS, which could contribute to the degradation of the surrounding extracellular matrix and cell surface proteins leading to a partial breakdown of the blood-brain barrier (BBB), which plays an important role in MS (Martínez-Torres et al., 2004; Weiser et al., 2007). This inflammatory response could be in response to low-level expression of viral genes during HSV-1 latency of the CNS (Feldman et al., 2002), which could facilitate an inflammatory environment that modulates the onset and severity of neurological disorders (Steiner and Benninger, 2013).

Importantly, viruses belonging to the *Herpesviridae* family have been suggested as potential triggers and positive modulators of MS (Wuest et al., 2014). For instance, human herpesvirus 6 (HHV-6) was recently shown to increase the severity of MS-like symptoms in non-human primates treated to undergo experimental autoimmune encephalomyelitis (EAE) (Leibovitch et al., 2018). In another study, latent-infection with the homologous of Epstein-Barr virus in mice (γHV-68 virus), prior to EAE induction was shown to enhance the pathogenesis of active EAE, which was associated with increased $CD4^+$ and $CD8^+$ T cell responses in the brain and spinal cord, yet was independent of viral reactivation (Casiraghi et al., 2012, 2015). On the other hand, a study performed in rats showed that repeated inoculations of HSV-1 elicited clinical and histological evidence of exacerbated EAE, but the possible mechanisms behind this observation were not determined (Hochberg et al., 1977). Additionally, HSV-1 genetic material has been found more frequently in the cerebrospinal fluid and blood of MS patients than control

subjects, suggesting an association between this virus and MS (Sanders et al., 1996; Ferrante et al., 2000; Najafi et al., 2016). However, a direct relationship between both, as well as the mechanisms underlaying a role of HSV-1 over MS, or vice versa has not been elucidated. Here, we assessed whether a sub-lethal infection of the CNS with HSV-1 that produces an asymptomatic infection in the mouse, modulates the severity of MS-like symptoms upon the induction of EAE, which is widely used as a surrogate model for multiple sclerosis. Importantly, we used C57BL/6 mice, which are resistant to HSV-1 acute brain infection and to HSV-1-induced demyelinating lesions throughout the brain (Kastrukoff et al., 2012), to facilitate the assessment of asymptomatic brain infection by HSV-1 over EAE disease. We also performed experiments with an HSV-1 mutant virus that has the gamma-34.5 gene (*ICP34.5*) deleted. This mutant has been reported to replicate in peripheral tissues, but is attenuated in neurons and does not cause encephalitis (Whitley et al., 1993).

Noteworthy, we found that HSV-1 infection with the wild-type (WT) virus accelerated the onset of EAE. Furthermore, previous infection with both, the WT and the attenuated mutant virus elicited a more severe EAE disease in mice, which was accompanied by increased CNS inflammation, as well as histological alterations in these tissues. Additionally, infected animals induced to undergo EAE showed an increase in activated microglia in the brain and spinal cord, more infiltrating $CD4^{+}$T cells in the brain and higher amounts of neutrophils in the spinal cord. We also found significantly higher levels of IL-6 and IL-1β mRNA in these tissues. Interestingly, we found that infection with either viruses elicited prolonged alterations to the BBB, which may account for some of the effects described above. Taken together, our results suggest a direct relationship between asymptomatic HSV-1 infection after intranasal viral

inoculation and an increased susceptibility to undergo a more severe form of EAE. The implications of these findings are discussed.

## 3.3 Material and methods

### *3.3.1 Mice and Viruses*

Five-week-old C57BL/6 female mice were obtained from The Jackson Laboratories (Bar Harbor) and maintained with environment enrichment, sterile food and water *ad libitum* at the central animal facility at the Pontificia Universidad Católica de Chile. Virus stocks were prepared and titters were determined in Vero cells (ATCC® CCL-81) and kept at -80°C until use. WT 17syn+ HSV-1 and the R3616 HSV-1 mutant used in this study were kindly provided by Dr. Carola Otth (Universidad Austral de Chile, Chile). R3616 lacks the gamma-34.5 gene (Δ*ICP34.5*) and was generated and generously donated by Dr. Bernard Roizman (University of Chicago, USA) (Chou et al., 1990). All procedures in this study were approved by the Scientific Ethical Committee for Animal and Environmental Care of the Pontificia Universidad Católica de Chile and the Biosafety Committee of the same institution (Protocol #170705018) and were performed according to the National Institutes of Health Guide for Care and Use of Animals (National Research Council (US), 2011).

### *3.3.2 Infections and EAE Induction*

Five-week-old C57BL/6 female mice were infected intranasally with a sub-lethal dose of $10^6$ plaque forming units (PFU) of 17 syn+ or Δ34.5 HSV-1, as previously described (Broberg et al., 2004; Zimmermann et al., 2017). Mock (vehicle)-inoculated mice were used as controls. During the first two weeks post-infection, mice were clinically scored daily based on

physiological parameters, appearance, posture, and neurological signs of herpes simplex encephalitis (i.e. seizures, paralysis). EAE was induced 30-35 days post-infection after asymptomatic HSV-1 infection. Briefly, mice were anesthetized with a mixture of ketamine and xylazine, and injected subcutaneously with 50 μg of myelin oligodendrocyte glycoprotein-(MOG)-derived peptide ($MOG_{35-55}$, sequence MEVGWYRSPFSRVVHLYRNGK; Pan Web, Stanford University) emulsified in complete Freund's adjuvant (Thermo Scientific) supplemented with heat-inactivated *Mycobacterium tuberculosis* H37 RA (DIFCO). Mice also received two intraperitoneal injections of 350 ng of pertussis toxin (List biological laboratories, Inc) at the time of induction and 48 hours later. Mice were scored daily based on an EAE scale as follows: 0, no changes in motor function; 0.5, tip of tail is limp; 1, limp tail; 2, limp tail and weakness of hind legs; 2.5, limp tail, and one hind limb paralyzed; 3, limp tail, and complete paralysis of hind limbs; 3.5, hind limbs and one fore limb paralyzed; 4, hind limbs and forelimbs completely paralyzed; 5, moribund.

### *3.3.3 Blood-brain barrier integrity assay*

The integrity of blood-brain barrier (BBB) of HSV-1-infected mice was evaluated using an Evans blue (EB, Sigma-Aldrich) dye exclusion test, as previously reported (del Valle et al., 2008). 30 days post-infection, mice were transcardially perfused with 50 mL of phosphate-buffered saline (PBS, pH 7.4), followed by 50 ml of the EB 2% in PBS under lethal ketamine/xylazine dose. Brains and spinal cords were dissected, fixed in 4% of p-formaldehyde (PFA) and cryopreserved in PBS with 30% sucrose for 24 h. Later, organs were embedded in cryostat-embedding compound (OCT, Sakura), cut into 20 μm thick sections on a cryostat at −22°C and mounted on Superfrost slides (Thomas Scientific). Slides were examined under a

confocal laser microscope (Leica TCS LSI), and EB extravasation was visualized as red fluorescence using a 543-nm laser. Additionally, the amount of EB entering the CNS was quantified by spectrophotometry at 620 nm after tissue homogenization in 50% of trichloroacetic acid in PBS and normalized according to the weight of the tissue (EB ng/mg tissue) (Wang and Lai, 2014).

### *3.3.4 Histological analysis and immunohistochemistry*

Mice infected with HSV-1 and induced to develop EAE were transcardially perfused with 50 mL of PBS to remove intravascular leukocytes. Lumbar regions in the spinal cords and corpus callosum in the brain were dissected and carefully processed for histological analyses. Briefly, tissues were fixed for 24 h in 4% PFA, dehydrated with ethanol and embedded in paraffin. 6-µm thick sections were obtained using a microtome, and slices were stained with Luxol Fast Blue solution (LFB) (0.1%, 2 h at 60 °C) and counterstained with Cresyl violet (0.1%, 6 min) to evaluate demyelination and cell infiltrates, respectively. Four to five sections per mice were analyzed using an Axio Vert.A1 microscope (Zeiss) with a 10X and a 20X objective, and histopathologic score was determined as follows: 0, no detected inflammation or demyelination; 1, one inflammation focus with slight demyelination; 2, two inflammation foci with moderate demyelination; 3, three or more inflammation foci with severe or complete demyelination, as previously described (Paintlia et al., 2009). Additionally, immunohistochemistry against the myelin basic protein (MBP) was carried out using the Mouse-on-Mouse HRP-Polymer Bundle kit (Biocare Medical). The procedure was carried out following the manufacturer's instructions. Briefly, sections were deparaffinized with xylene and rehydrated with decreasing concentrations of alcohol. Endogenous peroxidase was quenched

with 3% $H_2O_2$ in PBS for 20 min, followed by several washes in PBS. Antigen retrieval was performed using the reagent Rodent Decloaker 1X (Biocare medical) at 95°C for 40 min in a steamer. Then, slides were incubated for 30 min at room temperature (RT) in Rodent Block M for 30 min (Biocare medical), followed by 60 min of incubation at 37°C with a dilution 1:1000 of primary anti-MBP antibody (SMI-99P, Biolegend) in 1% bovine serum albumin (BSA, Winkler) in PBS and 0.1% Triton X-100. After washes with PBS pH 7.4, Mouse-on-Mouse HRP-Polymer was added for 30 min. Finally, immunostaining was performed using 0.05% diaminobenzidine and 0.015% $H_2O_2$, and counterstained with hematoxylin for 5 min. Slides without primary antibody were used as controls.

### *3.3.5 Western blot analysis*

Western blot analyses were performed to evaluate the expression of MBP in lumbar regions in the spinal cord and corpus callosum in the brain of mice infected with HSV-1 and induced to develop EAE. Samples were homogenized, placed in lysis buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, 1 mM phenylmethanesulfonyl fluoride, 0.5% NP40, 0.5% Sodium Deoxicholate, and 0.1% SDS), and total protein was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) following the manufacturer's instructions. Proteins were resolved using 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). After blocking with 5% BSA, membranes were incubated overnight at 4°C with a 1:300 dilution of mouse anti-MBP (SMI-99P, Biolegend) or a 1:1000 dilution of anti-β-actin (2F1-1, Biolegend) for 2 h at RT. A horseradish peroxidase (HRP)-conjugated anti-mouse antibody was used as secondary antibody (GenScript), and proteins were visualized by chemiluminescence using a ChemiDoc®MP

Imaging System (Bio-Rad). Band intensity was calculated using ImageJ (U.S. National Institutes of Health).

### *3.3.6 Mononuclear cell isolation, staining and flow cytometry*

Single cells suspensions were generated from the spinal cord and brain of HSV-1-infected EAE-induced mice perfused with PBS, as previously reported (Manglani et al., 2018). Infected and uninfected mice without EAE were used as controls. Tissues were incubated with 1 mg/ml collagenase IV (Thermo Scientific) and 50 µg/ml DNAse I (Roche) in RPMI (Thermo Scientific) at 37°C for 30 min. Mononuclear cells (MNCs) were isolated using 30/70% Percoll gradients (GE healthcare). For staining, MNCs were treated with CD16/32 Fc-block (BD Biosciences) to inhibit nonspecific antibody binding and incubated with anti-mouse immune cell surface markers for 45 min at 4°C. The following antibodies were used: anti-CD3 (Clone 17A2), anti-CD4 (clone 6K1.5), anti-CD8 (clone 53-6.7), anti-CD19 (clone 1D3), anti-CD45 (clone 30-F11), anti-CD11b (clone M1/70), anti-Ly6C (clone HK 1.4), and anti-Ly6G (clone RB6-8C5) and anti-MHC-II (clone AF6-120.1) (BioLegend). Dead cells were detected using the fixable Zombie Violet kit (BioLegend) and excluded from the analyses. Cells were enumerated by adding CountBright™ absolute counting beads (Thermo Scientific) to each sample before acquisition using a FACSCanto II flow cytometer (BD Biosciences) and data was analyzed using FlowJo software (Tree Star, Inc). This work was supported by the Cytometry Core UC (FCC UC).

### *3.3.7 Quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR)*

Total DNA from brain and trigeminal ganglia tissues was isolated using phenol-chloroform (Winkler) for quantifying the number of viral genomes. 200 ng of DNA was used for qPCR analyses with the following primers and probe for the viral polymerase *UL30* gene: Fwd-GGCCAGGCGCTTGTTGGTGTA, Rev-ATCACCGACCCGGAGAGGGA and Probe-CCGCCGAACTGAGCAGACACCCGC (Integrated DNA Technologies) and an Applied Biosystems StepOnePlus thermocycler, as previously described (Retamal-Díaz et al., 2017). Total RNA was isolated from tissues for cytokine expression analysis using TRIzol reagent (Thermo Scientific) according to the manufacturer's instructions. cDNA synthesis from total RNAs was performed using SuperScript™ II Reverse Transcriptase (Thermo Scientific) and random primers. RT-qPCR reactions were carried out using PowerUp™ SYBR™ Green Master Mix (Thermo Scientific) and primers for the detection of IL-1β, IFN-γ, TNF-α, IL-10, IL-17 and IL-6 (Zaheer et al., 2007) using a Mx3000P™ QPCR System (Stratagene) with the following cycling conditions: one cycle of 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 57° for 15 s and 72°C for 1 min. The abundance of each target mRNA was determined by relative expression to the β-actin housekeeping gene and the 2^-delta delta cycle threshold ($2^{-\Delta\Delta CT}$) method (Rao et al., 2013).

### *3.3.8 ELISAs Assays*

Antibodies against HSV-1 were detected by ELISA using sera obtained before and after EAE induction. MaxiSorp ELISA plates (Nunc/Thermo Scientific) were coated with 20 µg/mL of protein extracts from uninfected-Vero cells or 10 µg/mL of protein extracts from infected-

Vero cells and incubated at 4°C overnight in a humidity chamber. Plates were blocked with PBS-BSA 1% and then incubated with serial dilutions of the sera. To reduce non-specific antibody binding to the infected protein extracts, the sera were pre-adsorbed over plates with uninfected-Vero protein extracts for 2 h at RT and then transferred to plates with infected-Vero protein extracts and incubated at 4°C overnight in a humidity chamber. After three washes with PBS-Tween 20 0.05%, the wells were incubated with an HRP-conjugated anti-mouse-IgG antibody diluted 1:2000 (Thermo Scientific) for 1 h at RT, washed 3 times with PBS-Tween 20 0.05%, developed with 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Scientific) for 10 minutes, and read on a Multiskan ELISA plate reader at 450 nm after adding $H_2SO_4$ 2N to stop the enzymatic reaction. Anti-MOG antibodies were also detected in the sera from uninfected-EAE and infected-EAE mice carrying out the steps mentioned above and using 10 µg/mL of MOG peptide to coat the ELISA plates.

### *3.3.9 Statistical Analyses*

Statistical significance between experimental groups was assessed by one-way analysis of variance (ANOVA) with Dunn's multiple comparisons post-test for parametric data, Kruskal-Wallis with Dunn's multiple comparisons post-test for non-parametric data (three or more groups) or two-way ANOVA with Tukey's multiple comparison post-test (two independent variables) using GraphPad Prism software (GraphPad Software, La Jolla California USA).

## 3.4 Results

### *3.4.1 Asymptomatic HSV-1 infection alters the permeability of the blood-brain barrier*

To assess a potential effect of asymptomatic HSV-1 infection of the CNS over the onset and severity of experimental autoimmune encephalomyelitis (EAE) in the mouse model, we performed experiments with C57BL/6 mice. These mice have been reported to be resistant to acute HSV-1 encephalitis and hence could better reflect circumstances related to asymptomatic CNS infections reported in humans that do not display clinical manifestations despite having the virus in the brain (Baringer and Pisani, 1994; Wozniak et al., 2005). Thus, C57BL/6 mice were infected intranasally with a sub-lethal dose of HSV-1 and followed for 30 days. As expected, the weight of animals did not vary significantly after HSV-1 infection and overall paralleled that of mock-infected animals (Supplementary Figure 1A). Latent brain infection by the WT virus was corroborated using a virus plaque assay and by qPCR 30 days post-infection. As expected, no viral PFUs were recovered from brain tissue homogenates overlaid onto Vero cells, while the qPCR evidenced the presence of viral genome copies both, in the trigeminal ganglion and brain of inoculated mice (Supplementary Figure 1B). Additional to the use of WT HSV-1 virus, we also included in the following experiments an HSV-1 mutant that has the gene encoding the virulence factor gamma-34.5 deleted (*ICP34.5* gene, Δ34.5 mutant virus). This mutant virus does not cause encephalitis and has been reported to be hampered at replicating in neurons, although it can elicit an inflammatory response in the brain of mice, which may somewhat homologate the case of humans undergoing asymptomatic HSV-1 infection of this tissue (McMenamin et al., 1998; Broberg et al., 2004).

Because previous reports indicate that acute HSV-1 infection of the brain alters the BBB, we sought to assess whether this was also the case in asymptomatic animals infected with HSV-1 30 days post-infection. For this, we used Evans blue (EB), a dye that when is administered systematically cannot access the CNS in normal conditions unless the BBB is altered (del Valle et al., 2008). Hence, extravasation of this dye into the CNS is indicative of increased BBB permeability. As shown in Figure 6, mice infected with WT virus presented increased EB diffusion into the brain and spinal cord at 30 day post-infection, as compared to mock-inoculated animals, suggesting that the BBB is altered in these mice long after infection and in the absence of detectable infectious virus. Notably, mice infected with the mutant HSV-1 virus also showed significantly increased EB diffusion into the brain as compared to uninfected animals, evidencing BBB disruption independent of viral replication in neurons in the brain. Future studies should help determine how long the BBB is disrupted after HSV-1 infection.

### *3.4.2 Asymptomatic HSV-1 infection accelerates the onset and increases the severity of EAE*

To determine if HSV-1 infection impacts the onset and severity of CNS autoimmunity, we carried out an EAE induction protocol in mice that had been previously infected with HSV-1 (Figure 7A). As a control, EAE was also induced in mock-infected animals. As shown in Figure 7B, previous infection with WT HSV-1 accelerated the onset of EAE in 2 days approximately, while infection with the Δ34.5 mutant displayed a similar disease onset as the mock-infected animals (Table 1). Importantly, mice infected with WT HSV-1 displayed a higher

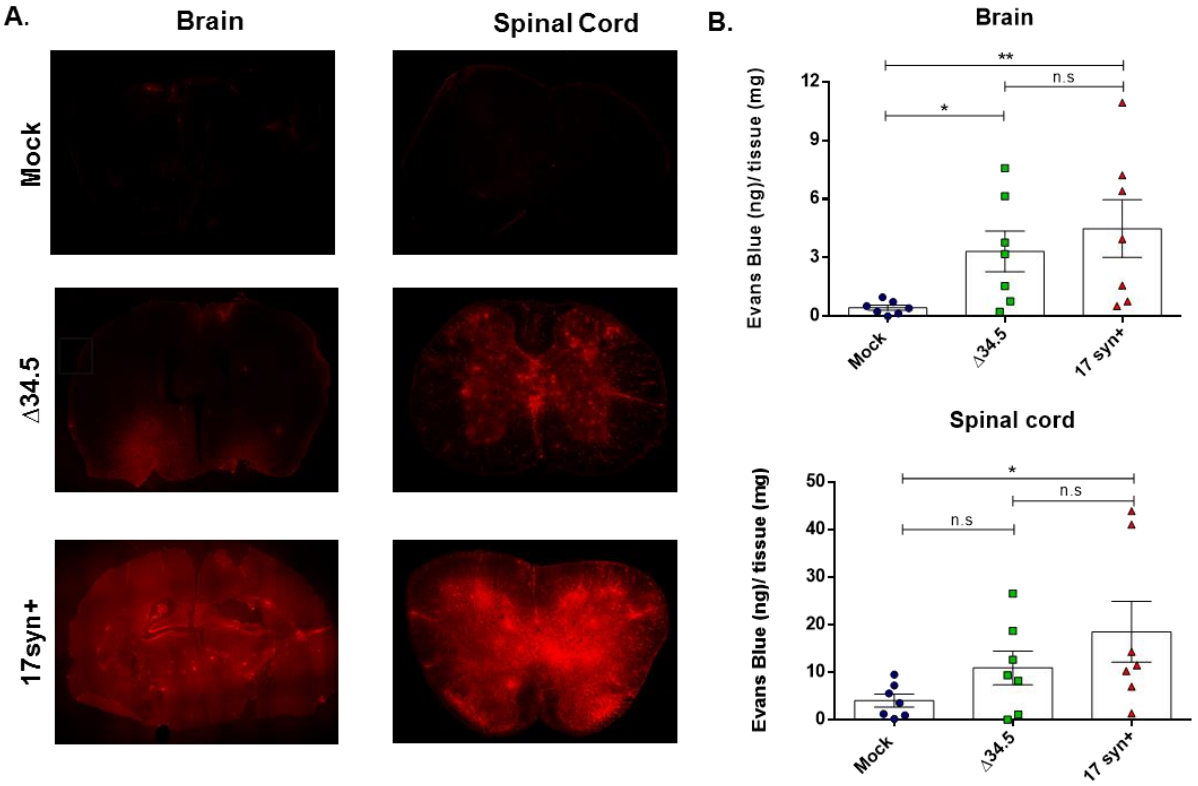



**Figure 6. Asymptomatic HSV-1 infection increases BBB permeability in vivo.** 30 days post-infection mice were transcardially perfused with Evans Blue dye (2% w/v). **(A)** Evans blue visualization by confocal microscopy in brain (left panels) and spinal cord sections (right panels) in uninfected mice or animals inoculated with Δ34.5 HSV-1, or 17syn+ HSV-1. Representative images of two independent experiments are shown. The original magnification of the photomicrographs is 10x. The brain image is a composite of 10 serial images. **(B)** Quantification of Evans blue incorporated into the brain (upper panel) and spinal cord (lower panel) by spectrophotometry at 620 nm. Values represent means ±SEM of two independent experiments (n=7/group). Data were analyzed using Kruskal-Wallis and Dunn's multiple comparisons post-test; **p<0.01; *p<0.05.

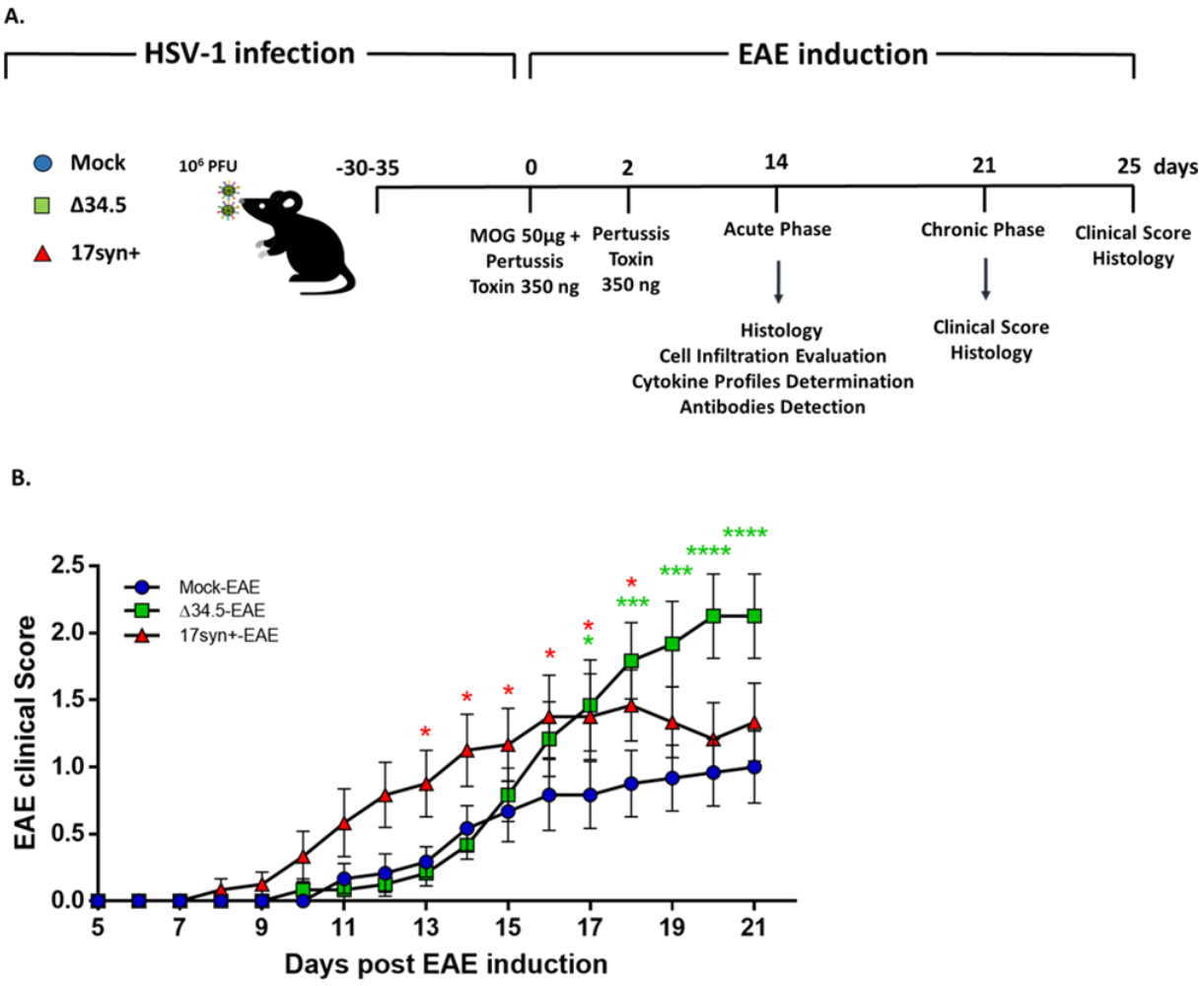



**Figure 7. Asymptomatic HSV-1 infection accelerates the onset and increases the severity of EAE. (A)** Schematic representation of the experimental design carried out in this study. **(B)** EAE was scored for each mouse after EAE induction, which was carried out 30-35 days post-HSV-1 infection. Mice were followed until day 21 post-EAE induction. The graph shows the means of disease scores ± SEM for mice mock-treated (blue circles), infected with Δ34.5 HSV-1 (green squares), or infected with 17syn+ HSV-1 (red triangles) in three independent experiments (n=12/group). Data were analyzed using two-way ANOVA followed by Turkey's post-test; **** $p<0.0001$, *** $p<0.001$, * $p<0.05$.

**Table 1. Summary of EAE disease parameters after HSV infection and EAE induction**

| *Group* | *Incidence of EAE symptoms* | *Mean day of disease onset* | *Maximum clinical score of EAE reached* | *Mean clinical score at day 14 (disease peak)* | *Mean clinical score at day 21 (remission stage)* |
|---|---|---|---|---|---|
| ***Mock-EAE*** | 66.7% (8/12) | 13.6 | 2.5 (2/12) | 0.5 | 1 |
| ***17syn+-EAE*** | 91.7% (11/12) | 11.9 | 3 (2/12) | 1.1 | 1.3 |
| **Δ*34-5-EAE*** | 100% (12/12) | 14.1 | 3.5 (2/12) | 0.4 | 2.1 |

incidence and scores of EAE symptoms than non-infected animals with EAE (Table 1, and Figure 7B). On the other hand, mice infected with the Δ34.5 mutant virus had a higher incidence and increased EAE clinical scores than WT HSV-1-inoculated animals (Table 1, and Figure 7B). In addition, a subset of animals was monitored for an extended period of time (25 days post-EAE induction) to evaluate the remission stage. Unlike the mock-EAE treated animals, which showed mild EAE symptoms, the animals infected either, with the WT or mutant HSV-1 showed a chronic progressive course of EAE symptoms up to permanent paralysis, which would normally be observed in C57BL/6 mice after severe $MOG_{35-55}$-induced EAE (Supplementary Figure 2) (Berard et al., 2010).

To characterize the impact of asymptomatic HSV-1 infection on the integrity of CNS tissues after EAE induction, we performed histological and molecular analyses of brain and spinal cord samples. Histological analyses with Luxol Fast Blue (LFB), which stains the myelin was contrasted with Cresyl violet to evidence cellular infiltration. Additionally, we performed myelin basic protein (MBP) expression analysis by immunohistochemistry and western blot for this protein. As shown in Figures 8A-C, histology analysis of spinal cord tissues revealed morphological alterations after staining with LFB and performing MBP immunohistochemistry, that were more evident for the experimental group infected with the Δ34.5 mutant virus induced to undergo EAE. In these animals, this tissue displayed significant cellular infiltration and loss of myelin, consistent with more severe EAE than the other groups at day 21 post-EAE induction (Figure 8D). Importantly, histological samples of mice infected with WT HSV-1 and treated to undergo EAE did not display significant differences respect to mock-infected group, possibly because these animals experienced fewer maximum disease score than the Δ34.5-inoculated group. Surprisingly, the expression of the MBP protein in western blot assays was lower in mice

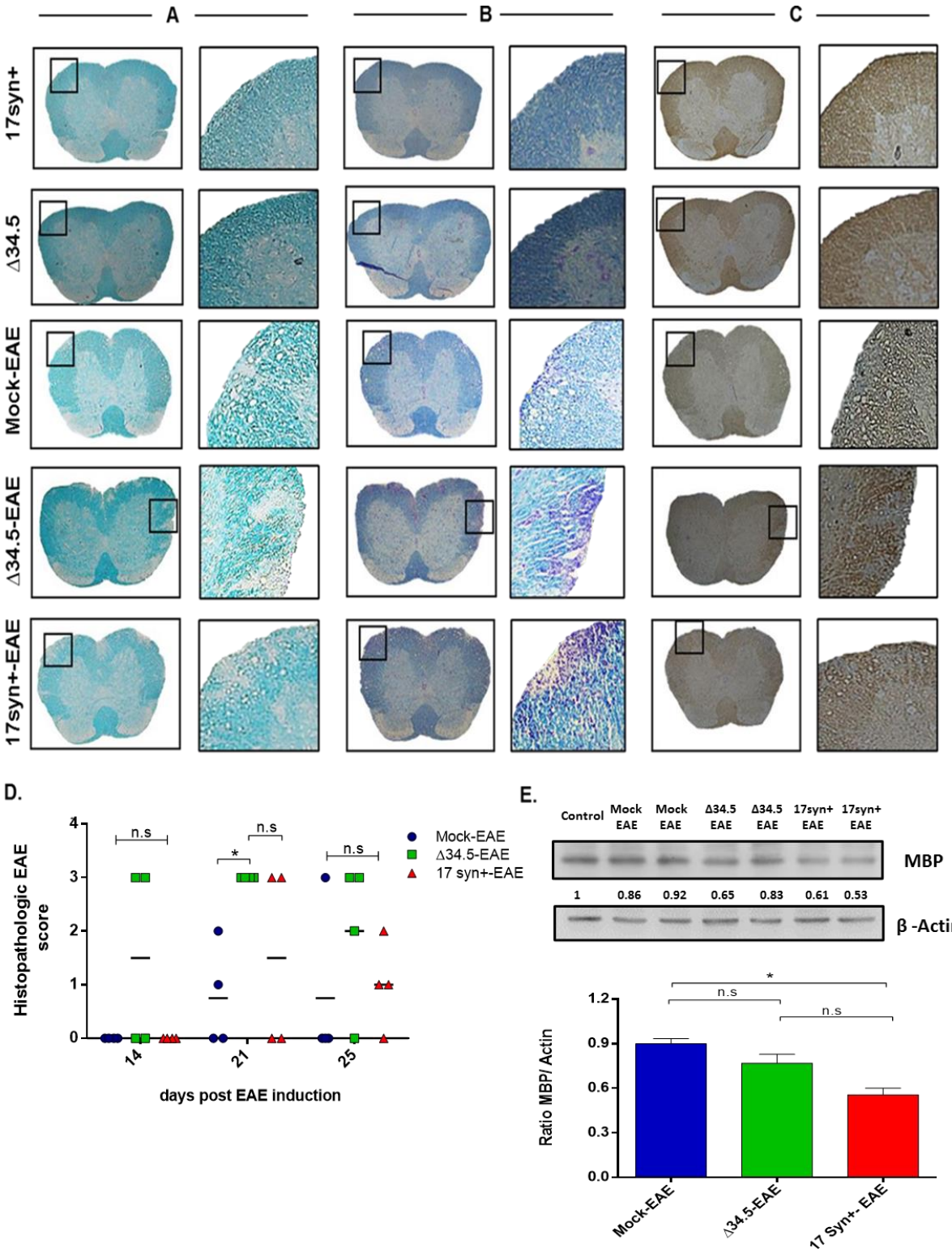

A
B
C
17syn+
Δ34.5
Mock-EAE
Δ34.5-EAE
17syn+-EAE
D.
Histopathologic EAE score
n.s
*
n.s
n.s
Mock-EAE
Δ34.5-EAE
17 syn+-EAE
14
21
25
days post EAE induction
E.
Control
Mock EAE
Mock EAE
Δ34.5 EAE
Δ34.5 EAE
17syn+ EAE
17syn+ EAE
MBP
1
0.86
0.92
0.65
0.83
0.61
0.53
β -Actin
Ratio MBP/ Actin
*
n.s
n.s
Mock-EAE
Δ34.5-EAE
17 Syn+- EAE

**Figure 8. Asymptomatic HSV-1 infection increases spinal cord demyelination after EAE induction. (A)** Representative images of lumbar sections of spinal cords stained with Luxol Fast Blue showing tissue demyelination. **(B)** Representative images of Luxol Fast Blue staining contrasted with Cresyl violet showing cellular infiltration. Myelin staining is observed in blue in the white matter and cell nuclei are colored purple. **(C)** Representative images of immunohistochemistry performed against the MBP protein. Representative images of three independent experiments are shown. Image magnifications are 10x (left) and 20x(right) and correspond to day 21 post-EAE induction. **(D)** Quantitative histopathological analyses of spinal cord lumbar sections. Values represent the mean ± SEM of three independent experiments. Data were analyzed with two-way ANOVA followed by Turkey's post-test; *$p<0.05$ (n=12, 4/group per day evaluated). **(E)** Representative western blot images for MBP (upper panel) and actin (lower panel) in the spinal cord at day 14 post-EAE induction. The graph shows densitometric analyses for MBP bands that were normalized to actin. Data represent the mean ± SEM. Comparisons between ratios were performed using one-way ANOVA with Dunnett's multiple comparison post-test; *$p<0.05$.

previously infected with the WT virus, as compared to the Δ34.5 mutant virus-infected group, which is somewhat unexpected, as the latter displayed increased histological pathology as compared to the animals infected with the WT virus (Figure 8E). These differences may be due to more regional damage in this tissue in the Δ34.5-EAE group, as compared to the WT-EAE group.

On the other hand, as shown in the Supplementary Figure 3, brain tissues showed some regions of evident demyelination only in HSV-1-infected animals induced to develop EAE. This was not the case for HSV-1-infected mice without EAE induction which were used as controls. Similarly, mock-inoculated animals treated to undergo EAE did not show significant histological alteration, which was expected as the protocol used for inducing EAE in our experimental setting was mild, consistent with mild disease score values and no significant demyelination in the brain in the absence of previous viral infection (Supplementary Figure 3). Regarding the western blot assays in the brain, animals infected with HSV-1 either, with the WT or mutant virus and treated to develop EAE, showed a decrease in the expression of MBP.

Taken together, these results indicate that asymptomatic infection with HSV-1 either, with a WT virus or mutant virus that cannot replicate in neurons significantly affects the outcome of EAE, suggesting a direct relationship between both, the virus and this autoimmune disease.

### *3.4.3 Asymptomatic HSV-1 infection increases EAE-associated inflammation*

To determine if previous asymptomatic infection with HSV-1 favors the infiltration of immune cells into the CNS after EAE is induced, we performed flow cytometry analysis of the brain and spinal cord at day 14 post-EAE induction and assessed the presence of $CD4^+$ T cells

($CD3^+/CD4^+$ cells), $CD8^+$ T cells ($CD3^+/CD8^+$ cells), or B cells ($CD19^+$ cells) (Supplementary Figure 4), as well as myeloid cells, namely monocytes ($CD45hi^+CD11b^+Ly6C^+$ cells), neutrophils ($CD45hi^+CD11b^+Ly6G^+$ cells), and activated microglia ($CD45lo^+CD11b^+MHC\text{-}II^+$) (Supplementary Figure 5). As shown in Figure 9A, the brains of mice infected with WT HSV-1 and induced to undergo EAE displayed significantly more cellular infiltration of lymphoid cells than other groups. In contrast, those previously infected with the Δ34.5 mutant virus had more infiltration of myeloid cells in this tissue, although the differences were not statistically significant. Because HSV-1 latent brain infection has been reported to be accompanied by persistent T cell infiltration (Marques et al., 2008), we sought to determine if this would be the case in our HSV-EAE model. As a control, mice infected with WT or the mutant virus alone, without EAE induction were evaluated at equivalent time-points as mice infected and then treated to undergo EAE (6 weeks post-infection). As shown in Supplementary Figure 6A and 6B, animals infected with HSV-1 alone did not display increased amounts of T cells in the brain or spinal cord as compared to healthy mice. Surprisingly, mice infected with WT HSV-1 and treated to undergo EAE displayed a significantly higher number of $CD4^+$ T cells in the brain as compared to the mock-EAE group (Figures 9B, and 9D). Regarding the myeloid cells analyzed in the brain, significant differences were observed for activated microglia expressing the MHC-II surface marker, which was higher in the WT HSV-1-EAE group than in the other groups (Figure 9C). On the other hand, no significant differences were observed between the different groups in terms of the number of infiltrating lymphoid cells in the spinal cord (Figures 10A and 10B). However, HSV-1-infected mice induced to experience EAE had a greater number of infiltrating myeloid cells than mock-EAE group (Figure 10A), which were mainly neutrophils as shown in Figures 10C and 10D. Moreover, the amount of activated microglia in the spinal

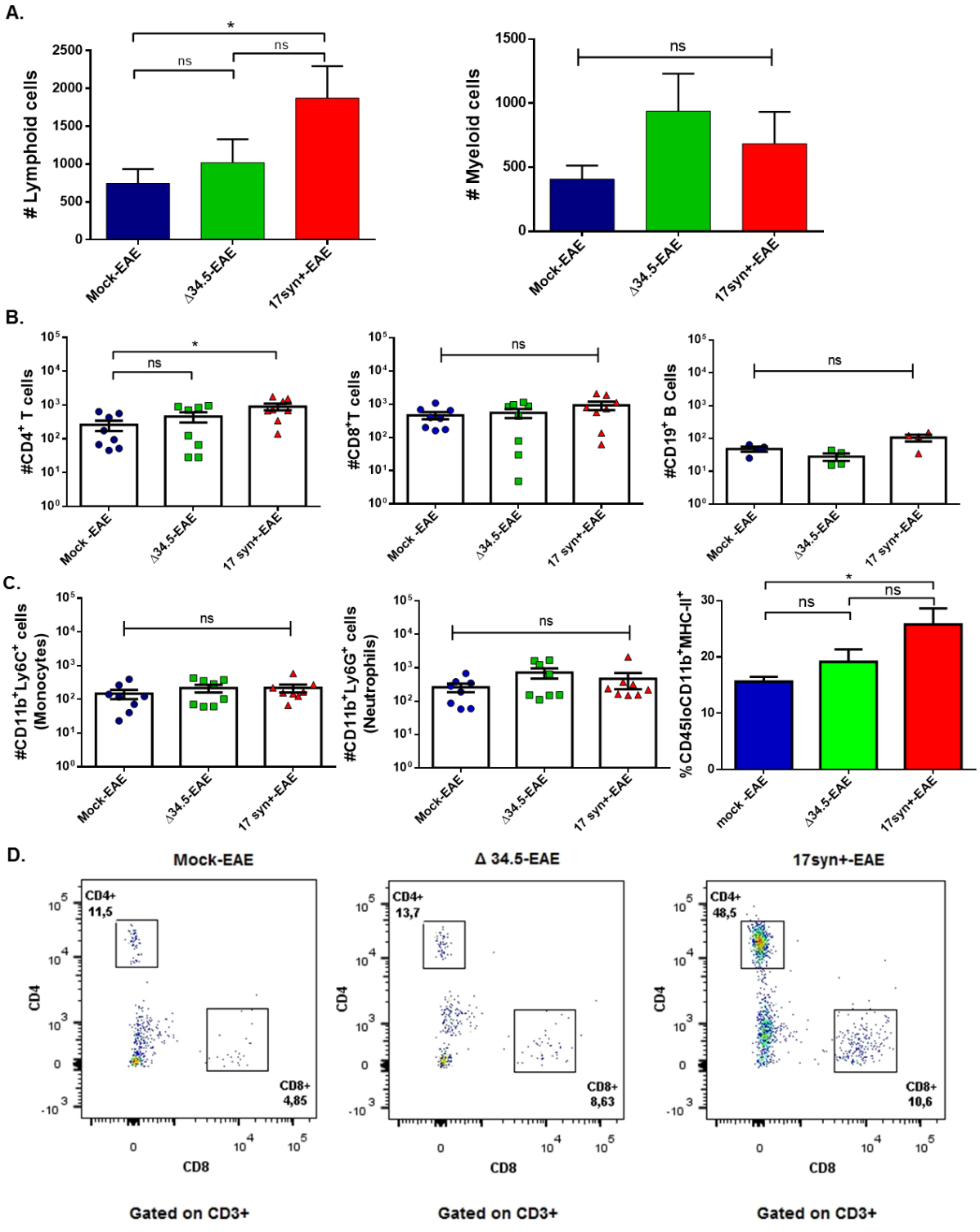

A.
# Lymphoid cells
# Myeloid cells
Mock-EAE
Δ34.5-EAE
17syn+-EAE
ns
*
B.
#CD4+ T cells
#CD8+T cells
#CD19+ B Cells
Mock -EAE
17 syn+-EAE
C.
#CD11b+Ly6C+ cells (Monocytes)
#CD11b+Ly6G+ cells (Neutrophils)
%CD45loCD11b+MHC-II+
mock -EAE
D.
Mock-EAE
Δ 34.5-EAE
17syn+-EAE
CD4+ 11,5
CD8+ 4,85
CD4+ 13,7
CD8+ 8,63
CD4+ 48,5
CD8+ 10,6
CD4
CD8
Gated on CD3+

**Figure 9. Animals infected with WT HSV-1 and treated to undergo EAE show increased number of CD4+ T cell infiltration in the brain**. Mice were mock-treated, infected with HSV-1 Δ34.5, or infected with HSV-1 17syn+. EAE was induced four weeks post-HSV-1 infection. At day 14 post-EAE induction, mice were perfused and the brain was harvested and processed to isolate immune infiltrating cells. **(A)** Total lymphoid cells (left) and myeloid cells (right) infiltrating the brains of mice induced to develop EAE. Values represent the mean ± SEM of two independent experiments (n=8/group). Data were analyzed using Kruskal-Wallis and Dunn's multiple comparisons post-test *$p<0.05$. **(B)** Infiltrating T cells, $CD4^+$ (left), $CD8^+$ (middle), or B cells $CD19^+$ (right) plotted individually. **(C)** Infiltrating myeloid cells $Ly6C^+$ (left) and $Ly6G^+$ (middle) plotted individually, data are means ± SEM of two independent experiments n=8/group. For the percentage of activated microglia $CD45loCD11b^+MHC\text{-}II^+$ (right), the data are means ± SEM of n=4/group. Data were analyzed using Kruskal-Wallis and Dunn's multiple comparisons post-test; *$p<0.05$. **(D)** Representative FACS plots showing the frequencies of lymphoid T cells in the brain. Live single cells were pre-gated on $CD3^+$ and $CD19^+$. $CD3^+$ T cells were subdivided into $CD4^+$ and $CD8^+$.

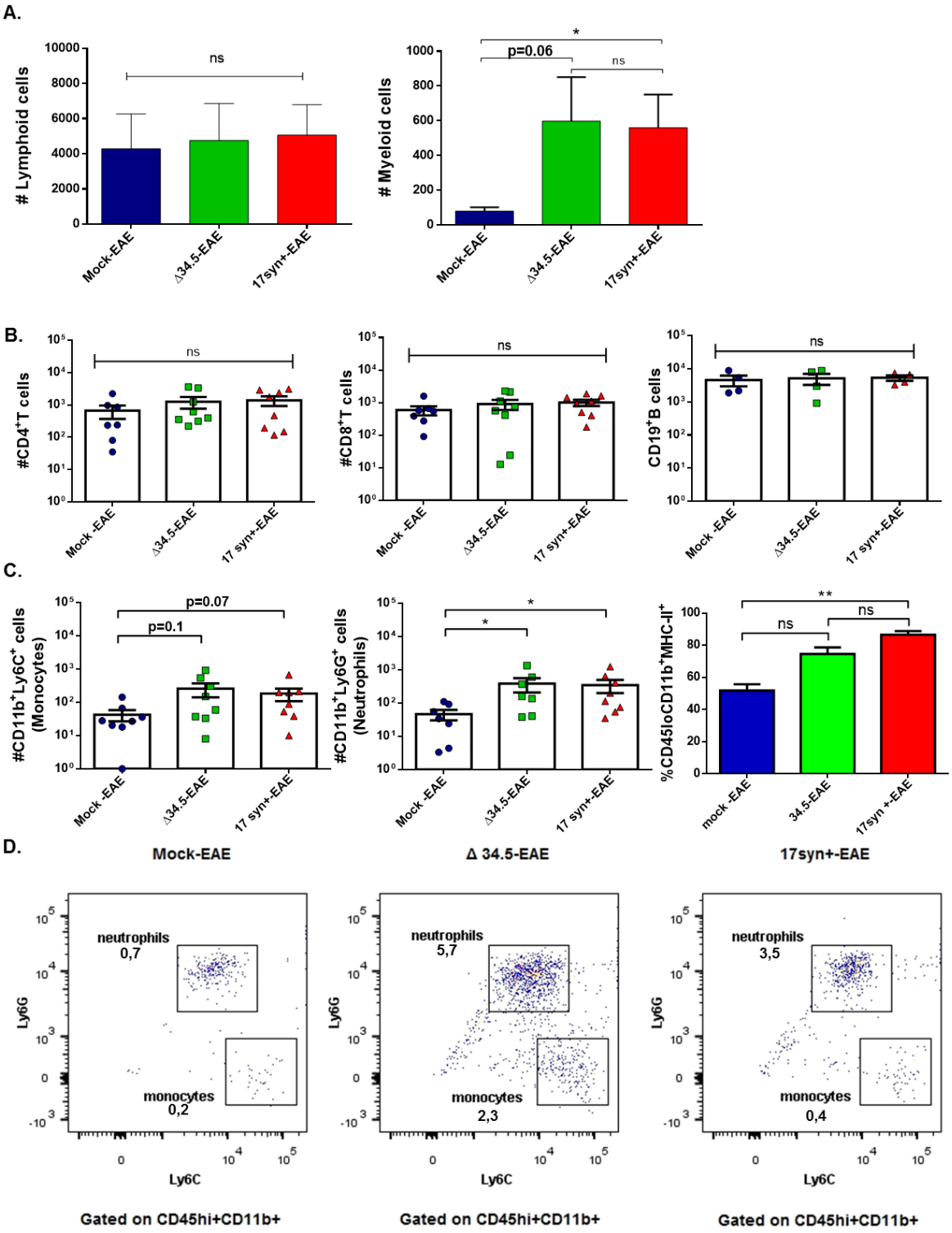

A.
# Lymphoid cells
ns
Mock-EAE
Δ34.5-EAE
17syn+-EAE
# Myeloid cells
p=0.06
ns
*
B.
#CD4+T cells
#CD8+T cells
CD19+B cells
Mock -EAE
17 syn+-EAE
C.
#CD11b+Ly6C+ cells (Monocytes)
p=0.07
p=0.1
#CD11b+Ly6G+ cells (Neutrophils)
%CD45loCD11b+MHC-II+
mock -EAE
34.5-EAE
17syn +-EAE
D.
Mock-EAE
Δ 34.5-EAE
17syn+-EAE
neutrophils 0,7
monocytes 0,2
neutrophils 5,7
monocytes 2,3
neutrophils 3,5
monocytes 0,4
Ly6G
Ly6C
Gated on CD45hi+CD11b+

**Figure 10. Animals infected with HSV-1 and treated to undergo EAE display increased number of neutrophils infiltrating the spinal cord.** Mice were mock-treated, infected with HSV-1 Δ34.5, or infected with HSV-1 17syn+. EAE was induced four weeks post-infection. At day 14 post-EAE induction, mice were perfused and the spinal cords were harvested and processed to isolate immune cells infiltrating this tissue. **(A)** Total lymphoid cells (left) and myeloid cells (right) infiltrating the spinal cords of mice induced to develop EAE. Values represent the mean ± SEM of two independent experiments (n=8/group). Data were analyzed using Kruskal-Wallis and Dunn's multiple comparisons post-test *$p<0.05$. **(B)** Infiltrating T cells, $CD4^+$ (left), $CD8^+$ (middle), or B cells $CD19^+$ (right) plotted individually. **(C)** Infiltrating myeloid cells $Ly6C^+$ (left) and $Ly6G^+$ (middle) plotted individually. Data are means ± SEM of two independent experiments n=8/group. For the percentage of activated microglia $CD45loCD11b^+MHC\text{-}II^+$ (right), the data are means ± SEM of n=4/group. Data were analyzed using Kruskal-Wallis and Dunn's multiple comparisons post-test *$p<0.05$. **(D)** Representative FACS plots showing the frequencies of infiltrating myeloid cells in the spinal cords. Live single cells were pre-gated on $CD45^+$ and $CD11b^+$. $CD45hi^+/CD11b^+$ infiltrating myeloid cells were subdivided into neutrophils ($Ly6G^+$) and monocytes ($Ly6C^+$).

cord of WT-infected mice was significantly higher than in uninfected mice in this tissue (Fig 10C).

Next, to evaluate whether asymptomatic infection with HSV-1 modulates the cytokine environment in the CNS upon EAE induction, we performed RT-qPCR for a set of ytokines that either, promote an inflammatory state in this tissue (i.e. IL-1β, IL-6, IL-17, TNF-α and IFN-γ) or an anti-inflammatory environment (i.e. IL-10). As shown in Figure 11A, the brain of mice infected with WT HSV-1 or the Δ34.5 mutant virus and treated to undergo EAE showed increased expression of all the cytokines evaluated, as compared to mock-infected animals. Notably, more IL-1β mRNA was expressed in the brain of infected animals with EAE than equivalent tissue obtained from mice induced to develop EAE without a previous HSV-1 infection (Figures 11A). Moreover, IL-6 mRNA levels were also significatively increased in the brain of mice infected with Δ34.5 mutant virus (Figure 11A). Cytokines mRNAs in the spinal cord displaying important variations, as compared to mock-infected animals were IL-6 and IL-10 in the WT HSV-1-EAE group, as shown in Figure 11B. IL-17 and IFN-γ also showed some differences among the evaluated groups, and although these changes were not-significant these cytokines also showed a tendency to be increased in the brain and spinal cord of mice infected with either virus and treated to undergo EAE (Figures 11A and 11B).

#### *3.4.4 Asymptomatic mice infected with WT HSV-1 display increased amounts of anti-HSV-1 antibodies after EAE induction*

Given the results obtained above, it is possible that asymptomatic infection with HSV-1 predisposes the animals to undergo increased EAE severity, but it is also possible that the

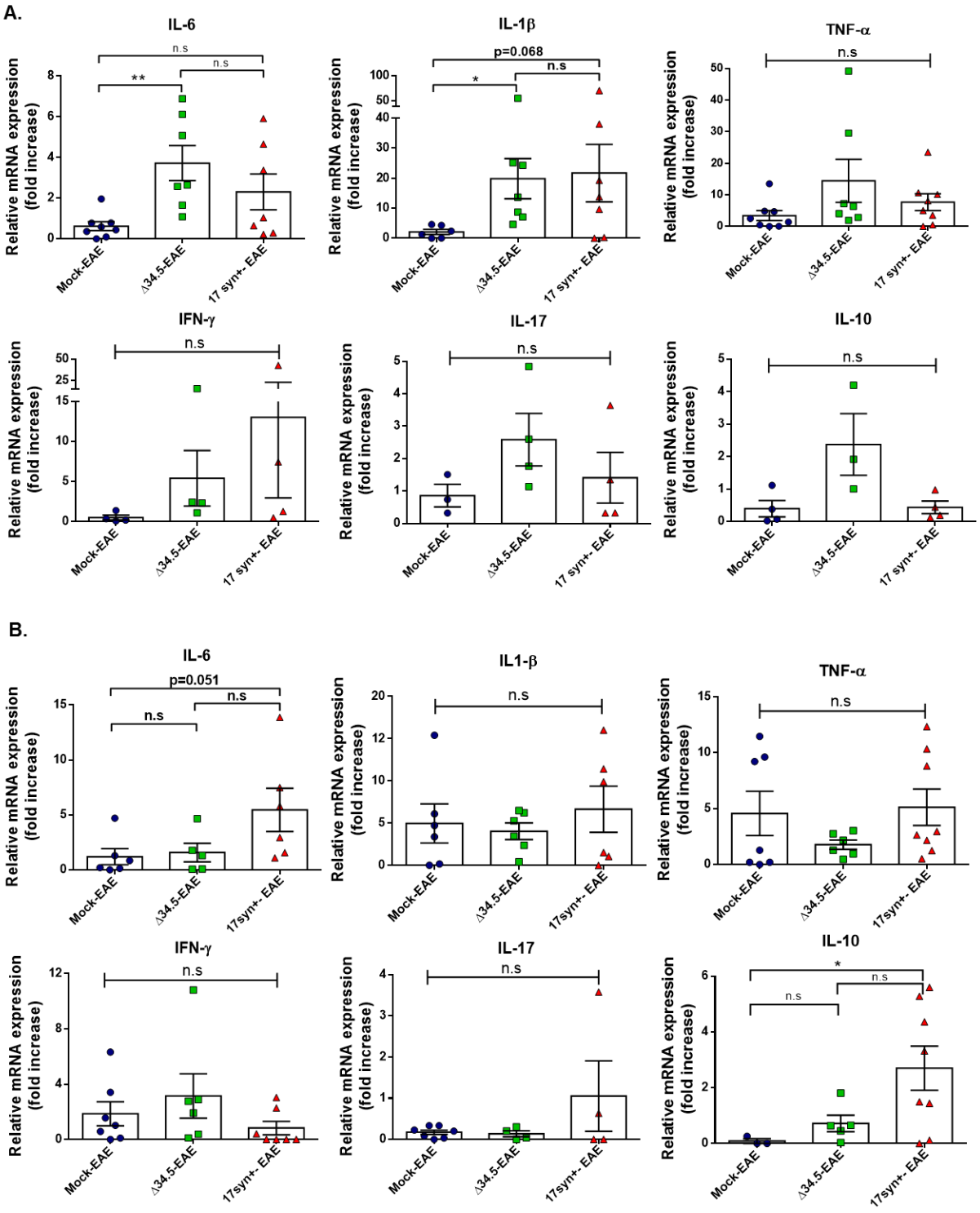

A.
IL-6
n.s
n.s
**
Relative mRNA expression (fold increase)
Mock-EAE
Δ34.5-EAE
17 syn+- EAE
IL-1β
p=0.068
n.s
*
TNF-α
n.s
IFN-γ
n.s
IL-17
n.s
IL-10
n.s
B.
IL-6
p=0.051
n.s
n.s
17syn+- EAE
IL1-β
n.s
TNF-α
n.s
IFN-γ
n.s
IL-17
n.s
IL-10
*
n.s
n.s

**Figure 11. Asymptomatic HSV-1 infection increases the expression of pro-inflammatory cytokines in the CNS of mice with EAE.** Mice were mock-treated, infected with HSV-1 Δ34.5, or infected with HSV-1 17syn+. Four weeks post-infection EAE was induced. 14 days post-EAE induction, tissue homogenates were evaluated by RT-qPCR to assess cytokine expression at the mRNA level using the $2^{-\Delta\Delta CT}$ method with actin as a reference gene. **(A)** Relative expression levels of proinflammatory cytokines (IL-6, IL-1β, TNF-α, IFN-γ and IL-17), and the anti-inflammatory cytokine IL-10 in the brain of HSV-1 17syn+-infected mice (red triangles), HSV-1 Δ34.5-infected mice (green squares), and mock-treated mice (blue circles) plotted individually. **B)** Relative expression levels of cytokines in the spinal cord of HSV-1 17syn+-infected mice (red triangles), HSV-1 Δ34.5-infected mice (green squares), and mock-treated mice (blue circles) plotted individually. Values represent means ± SEM of two independent experiments (n=8/group). Data were analyzed using Kruskal-Wallis and Dunn's multiple comparisons post-test; $**p<0.01$, $*p<0.05$.

induction of EAE in previously-infected animals may promote virus reactivation in the CNS or periphery and facilitate enhanced neurodegenerative disease. To preliminarily assess this latter scenario, we assessed the concentrations of circulating antibodies against HSV-1 in the serum of infected animals before- and 14 days after EAE induction. Interestingly, we found that those animals that were previously infected with WT HSV-1 and then treated to undergo EAE displayed a modest, yet significantly increase in the quantity of anti-HSV-1 antibodies in the serum (Figure 12A). Although these differences are not substantial, this result suggests possible viral reactivation, either productive (new infectious particles) or at the molecular level (expression of HSV-1 antigens without the release of new infectious particles), which requires further attention. However, because infections with the Δ34.5 mutant virus previous to EAE induction did not increase the quantity of HSV-1-specific antibodies after EAE induction, suggest the increased amount of anti-HSV-1 antibodies in the WT HSV-1-EAE group may be due to viral reactivation (Figure 12A).

Additionally, we assessed the quantity of MOG-specific antibodies in the sera of animals infected or not with HSV-1 and then treated to undergo EAE. As shown in Figure 11B, although mice infected with WT HSV-1 displayed significantly higher amounts of anti-MOG antibodies after EAE induction as compared to control healthy mice, no significant differences were observed between the animals of the WT HSV-1 EAE, Δ34.5 HSV-1-EAE mice or mock-EAE group (Figure 12B).

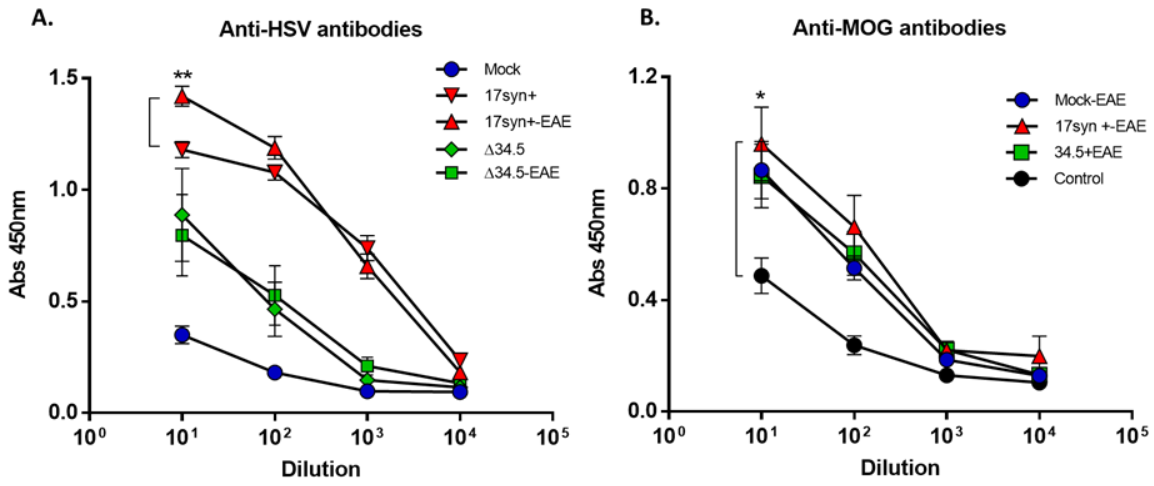



**Figure 12. Animals infected with WT HSV-1 and then treated to undergo EAE display increased anti-HSV antibodies after EAE induction.** Mice were mock-treated (blue), infected with HSV-1 Δ34.5 (green), or infected with HSV-1 17syn+ (red). EAE was induced in the indicated groups (EAE) four weeks post-infection. At day 30 post-HSV infection and 14 post-EAE induction, sera were harvested and levels of **(A)** anti-HSV-1 IgG antibodies (n=8/group) were quantified by ELISA. **(B)** anti-MOG IgG antibodies (n=10/group) were quantified in sera harvested at day 14 post-EAE induction by using ELISA. Data are means ± SEM of two independent experiments. Data were analyzed using two-way ANOVA followed by Turkey's post-test; **p<0.01, *p<0.05.

### 3.5 Discussion

Infections with human herpesviruses has been suggested as potential triggers or enhancers of MS in recent reports (Casiraghi et al., 2015; Leibovitch et al., 2018), yet studies that assess or support a role for HSV-1 infection are relatively scarce and a direct relationship between this virus and this disease has not been reported before (Ferrante et al., 2000; Ferrò et al., 2012; Rizzo et al., 2016; Buscarinu et al., 2017). Although the fact that HSV-1 infects the CNS makes this virus a suspect candidate in MS, the fact that HSV-1 infection is highly prevalent in the human population, unlike MS somewhat argues against this idea. However, asymptomatic HSV-1 infection in the CNS may be insufficient for developing MS *per se* and the initiation of the disease likely requires other contributing elements, such as genetic and environmental factors (Briggs et al., 2010; Kakalacheva et al., 2011; Waubant, 2011). However, the prevalence of CNS infection with HSV-1 in otherwise healthy individuals is somewhat unknown, as this is not a routine analysis to be performed after death. Despite the fact that CNS infection with HSV-1 in healthy individuals is undetermined, it is possible to foresee that the chances of having HSV-1 infection of the CNS will likely increase with aging, as progressive senescence of the immune system may allow HSV-1 to reactivate from peripheral tissues, such as the trigeminal ganglia and spread within the brain (Jamieson et al., 1991; Wozniak et al., 2005; Itzhaki and Lathe, 2018). Furthermore, repeated HSV-1 reactivations throughout the life of an individual may provide opportunities for increased number of neurons to be infected with this virus as a person gets older. Additionally, neuronal senescence may also facilitate neurodegenerative disorders by HSV-1 and eventually facilitate MS initiation and progression (Menendez et al., 2016; Duarte et al., 2019).

Here, we observed that a previous infection with HSV-1 after intranasal virus inoculation can predispose the host to an earlier onset and more severe EAE disease. Our results showed a significant increased demyelination of spinal cords in animals previously infected with HSV-1, which was more evident for those infected with Δ34.5 mutant virus. Surprisingly, these results suggest that viral replication in the brain may not be necessary for experiencing increased EAE severity after infection with HSV-1.

Although we did not observe significant histological alterations in the brain tissues obtained from mice that displayed an earlier onset in EAE, or increased EAE severity after a previous infection with HSV-1, several molecular markers associated with inflammation and cellular infiltration in the CNS of these animals could be detected by other means. As reported above, we found that IL-6 mRNA was elevated in both, in the brain and spinal cord of infected animals. Importantly, this cytokine has been reported to be a key player in the development of autoimmune diseases by differentiating autoreactive proinflammatory $CD4^+$ T cell responses towards a Th-17 phenotype, as well as by inhibiting the induction of regulatory T cells (Tregs) (Maimone et al., 1997; Kimura and Kishimoto, 2010). Studies performed in humans with RRMS show that IL-6 supports T cell effector function resistance to regulation by Tregs, which may contribute to disease severity (Schneider et al., 2013). This could explain why although there were increased levels of IL-10 mRNA in the spinal cord of the WT HSV-1 group, these animals suffered a more severe disease than the mock-EAE group. It is possible that the anti-inflammatory effect of IL-10 may be disrupted by the high levels of IL-6 in this tissue, thus favoring a Th-17 phenotype. However, this remains to be evaluated. Moreover, the elevated levels of IL-1β mRNA observed in the brain may also promote BBB permeability, possibly through previously reported mechanisms over astrocytes (Wang et al., 2014; Lin and Edelson,

2017). An interesting finding was the fact that the BBB of asymptomatic HSV-1-infected mice remained permeable to the Evans blue dye 30 days after infection. Although alterations in the BBB during HSV-1 infection had been reported before, this phenomenon was only observed in *in vitro* BBB models, or during acute CNS infection with this virus (HSV-1 encephalitis) (Liu et al., 2019b, 2019a; He et al., 2020). Our results show that the disruption of the BBB occurs independent of encephalitis and persists in the absence of infectious virus in the CNS. Moreover, these results suggest that intranasal virus inoculation is enough to disrupt the BBB for a long period. However, it remains to be determined how long these alterations last and whether they are key for the observations reported herein.

On the other hand, while $CD4^+$ T cells have been shown to play a key role over EAE onset and severity (Constantinescu et al., 2011), and that we observed that these cells were increased in the brain of WT-HSV-1-EAE mice, relevant roles for other immune cells, such as neutrophils are emerging as a relevant immune component contributing to CNS damage (Aubé et al., 2014; Rumble et al., 2015; Woodberry et al., 2018). Importantly, we found that these cells were increased in the CNS of the experimental groups infected with HSV-1, as compared to mock-infected mice. It would be important to characterize the phenotype of these cells to determine if they are contributing to the enhanced disease severity observed, which would support the notion of a detrimental role for neutrophils in EAE, and eventually MS pathogenesis. Additionally, it will be interesting to assess the contribution and role of virus-specific $CD4^+$ and $CD8^+$ T cells in these experiments, as these cells may be contributing to CNS inflammation by promoting immune cell access to the CNS, cytokine secretion in these tissues or direct neuron damage (Steinbach et al., 2019). Previous reports suggest that viral infections can increase the susceptibility to autoimmune diseases by eliciting bystander inflammation and the activation of

autoreactive cells, which can lower the threshold for disease development (Miller et al., 1997; Daniel R. Getts et al., 2013).

Although our findings suggest a role for asymptomatic brain infection by WT HSV-1 on the onset and severity of MS, it remains unknown whether EAE induction in these animals reactivates HSV-1, leading to active viral replication and potentially HSV-1 replication-related disease in the CNS, which could account *per se* for some of the observed symptoms or directly contribute to the severity of the EAE induced. The fact that animals infected with WT HSV-1 and then induced to undergo EAE displayed increased amounts of anti-HSV antibodies, although modest suggests that HSV-1 reactivation may be occurring in these mice, although this remains to be further assessed. As discussed above, because increased amounts of anti-HSV-1 antibodies were only observed in the WT HSV-1-EAE group and not with the mutant virus (Δ34.5-EAE group), such potential reactivation may be related to the generation of infectious particles, although a molecular activation of HSV-1 may also be the case (Feldman et al., 2002; Martin et al., 2014a). Because the mutant virus elicited enhanced EAE symptoms, even more than the WT virus for some of the analyzed parameters, it is also possible that the main mechanism behind enhanced EAE by HSV-1 infection may be a consequence of a long-lasting signal of the virus over infected cells early after virus inoculation, or even adjacent cells, which could trigger an inflammatory response that increases the host susceptibility to undergo this autoimmune disease with increased severity (Steinbach et al., 2019).

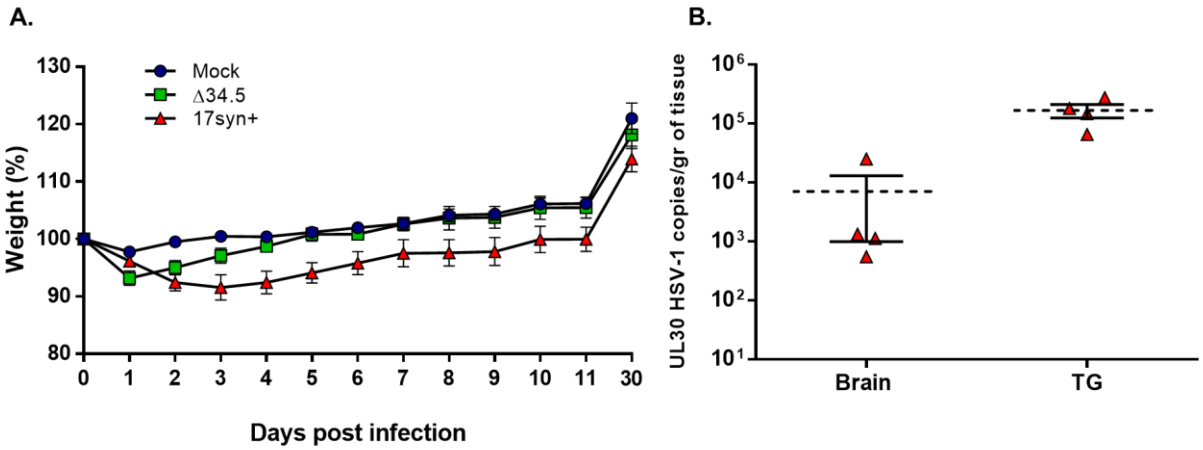
Given the existence of antivirals specific for herpesviruses, such as acyclovir, it is tempting to speculate that such compounds may delay the onset of EAE in animals previously-infected with HSV-1, or reduce the severity of the disease in these mice once initiated. However, because the Δ34-5 mutant virus is attenuated in neurons and that the animals inoculated with

this virus displayed more severe EAE, the use of such drugs may not necessarily have therapeutic effects. Nevertheless, it will be interesting to perform the experiments carried out in this study in the presence of drugs such as acyclovir after virus infection to determine the contribution of HSV-1 replication in the different stages of EAE.

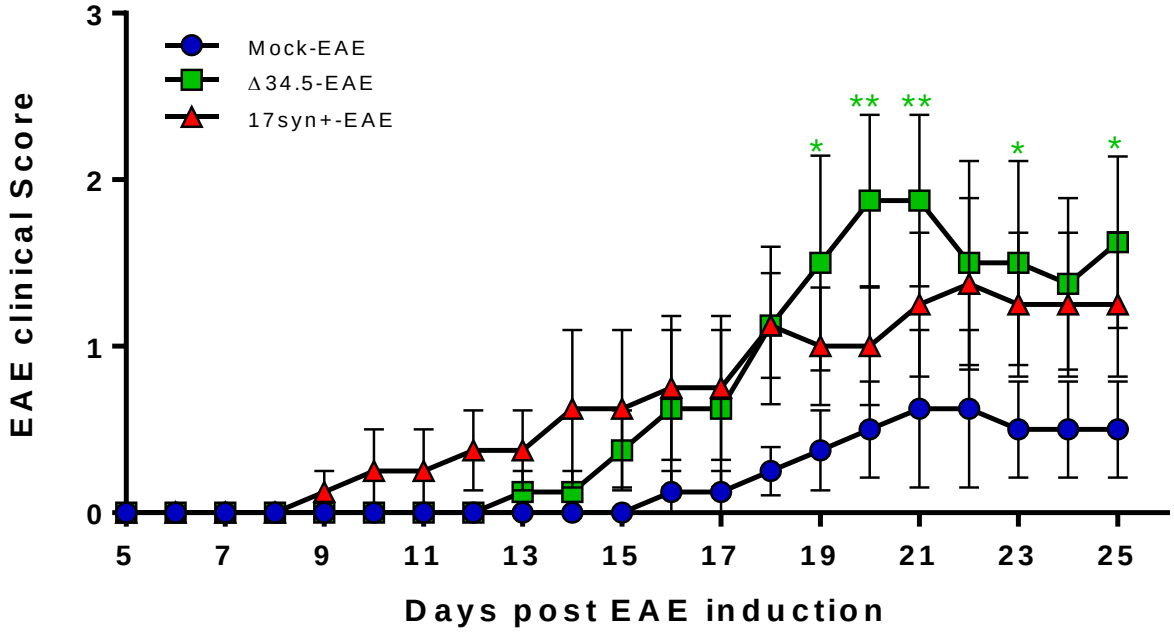
### 3.6 Acknowledgements

We are grateful to Dr. Luis Larrondo for sharing equipment for visualizing western blots. This work was supported by the Millennium Institute on Immunology and Immunotherapy (P09/016-F) from the Millennium Science Initiative of the Agencia Nacional de Investigación y Desarrollo (ANID, Chile); and FONDECYT (ANID, Chile) grant #1190864.

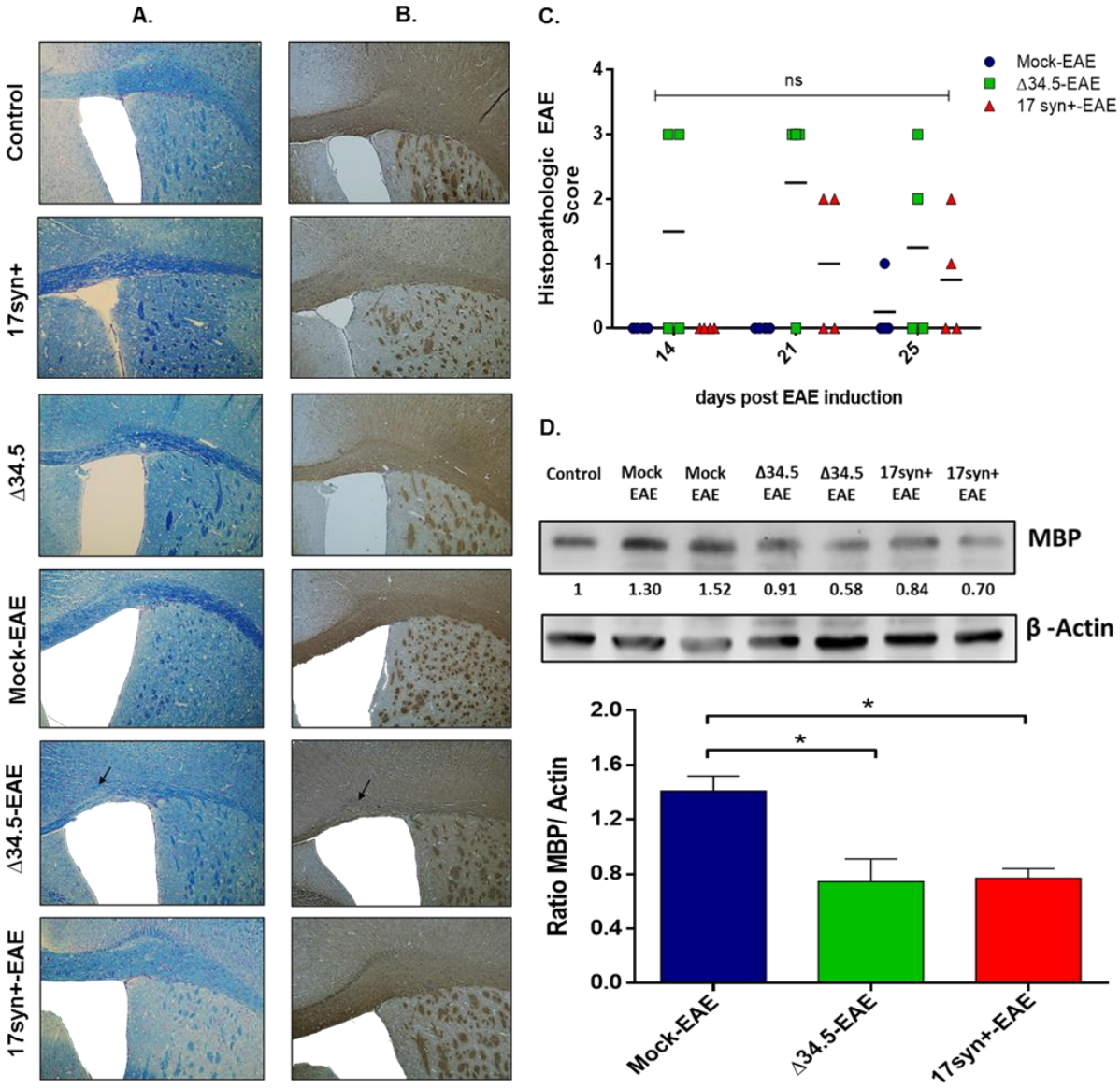
### 3.7 Supplementary figures



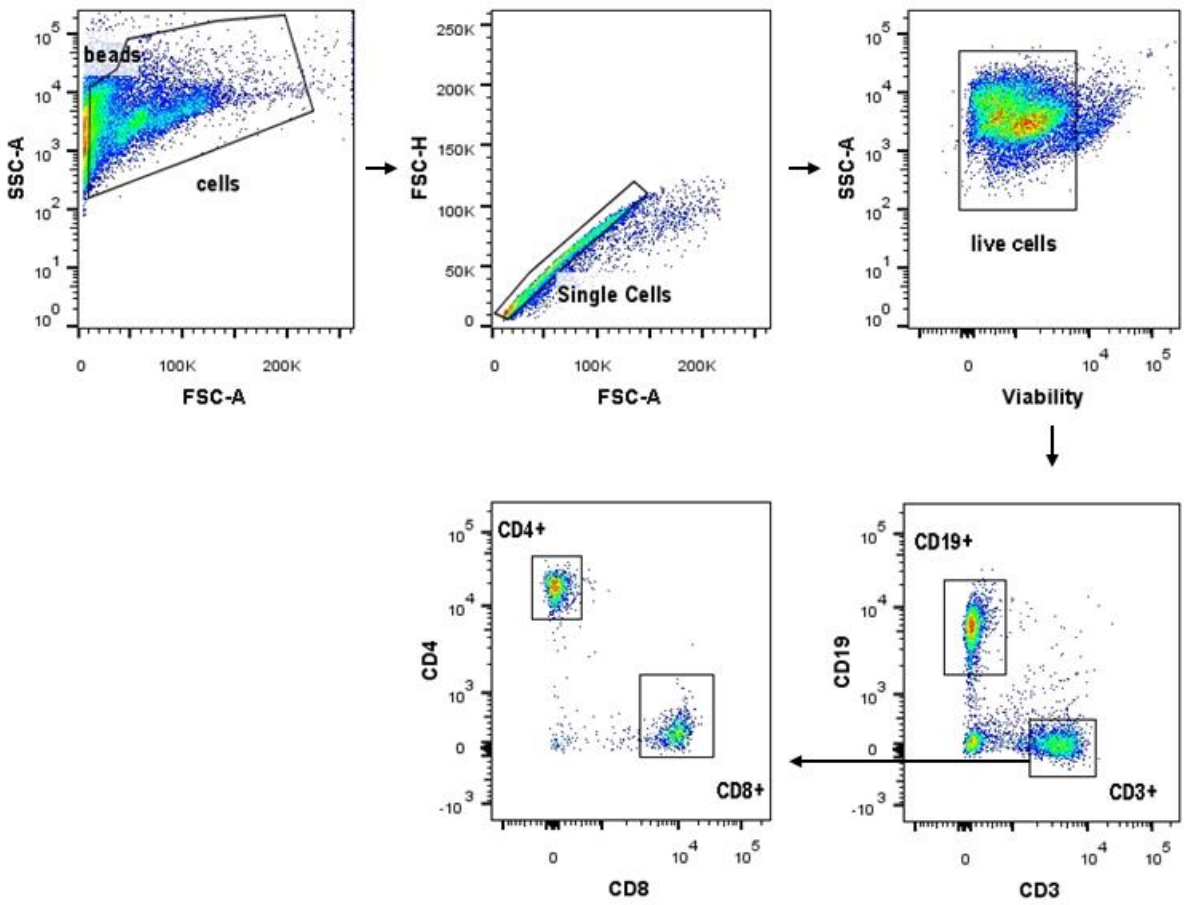

**Supplementary Figure 1. Asymptomatic brain infection with WT HSV-1 after intranasal virus inoculation.** C57BL/6 mice were intranasally mock-inoculated or infected with HSV-1 17syn+ or HSV-1 Δ34.5 and weighted daily until day 30. **(A)** Weight curves of infected and non-infected mice. Values represent means ±SEM from three independent experiments (n=12/group). **(B)** HSV-1 UL30 gene copies per gram of brain or trigeminal ganglia from a subset of WT infected-mice obtained at 30 days post-infection and normalized with values from uninfected mice. Values represent means ±SEM of four animals per group.



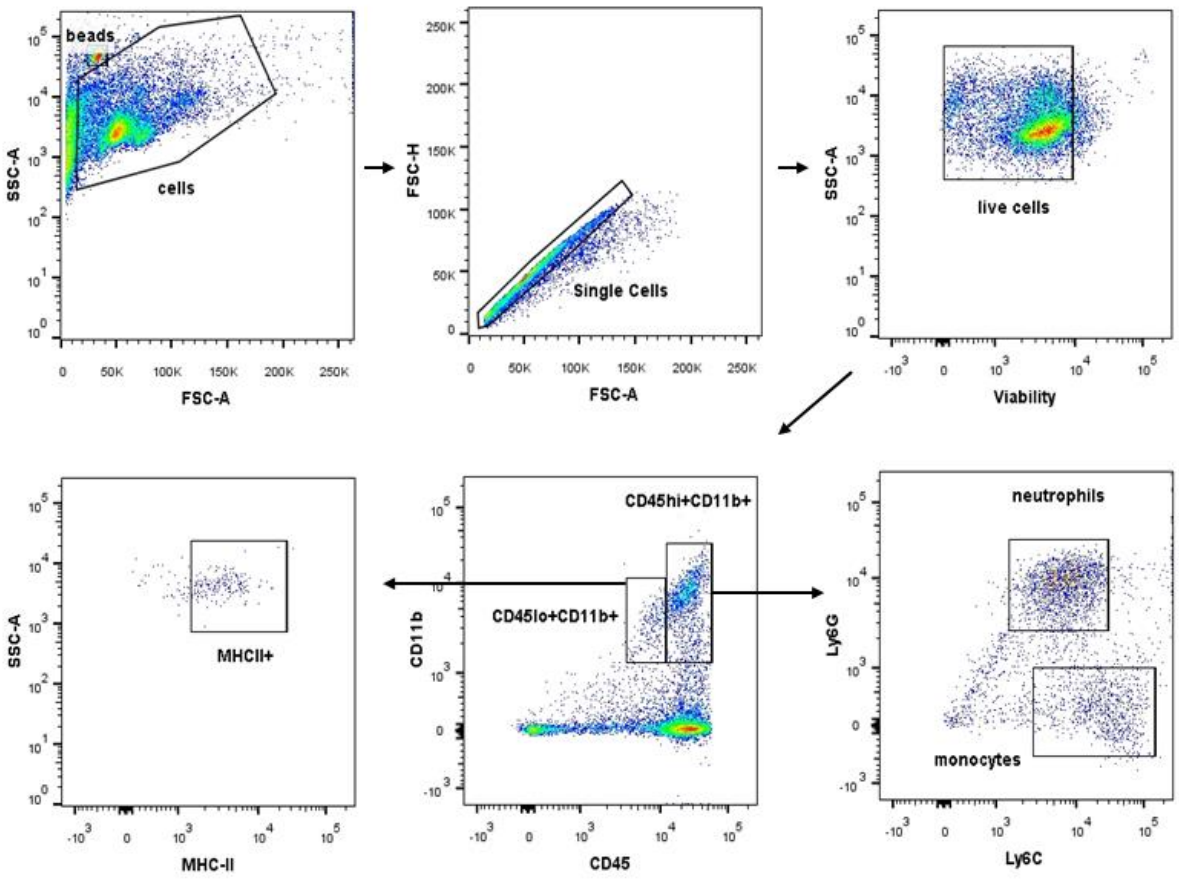

**Supplementary Figure 2. Asymptomatic HSV-1-infected mice show a chronic course of EAE disease.** EAE disease was scored for each mouse after EAE induction, which occurred 30 days post-HSV-1 infection. Mice were followed until day 25 post-EAE induction. The graph shows the mean ± SEM of EAE disease scores for mice mock-treated (blue circles), infected with HSV-1 Δ34.5 (green squares), or HSV-1 17syn+ (red triangles) (n=4/group). Data were analyzed using two-way ANOVA followed by Turkey's post-Test; ** $p<0.01$, * $p<0.05$.


A.
B.
C.
Control
17syn+
Δ34.5
Mock-EAE
Δ34.5-EAE
17syn+-EAE
Mock-EAE
Δ34.5-EAE
17 syn+-EAE
ns
Histopathologic EAE Score
days post EAE induction
D.
Control
Mock EAE
Mock EAE
Δ34.5 EAE
Δ34.5 EAE
17syn+ EAE
17syn+ EAE
MBP
1
1.30
1.52
0.91
0.58
0.84
0.70
β -Actin
Ratio MBP/ Actin
Mock-EAE
Δ34.5-EAE
17syn+-EAE

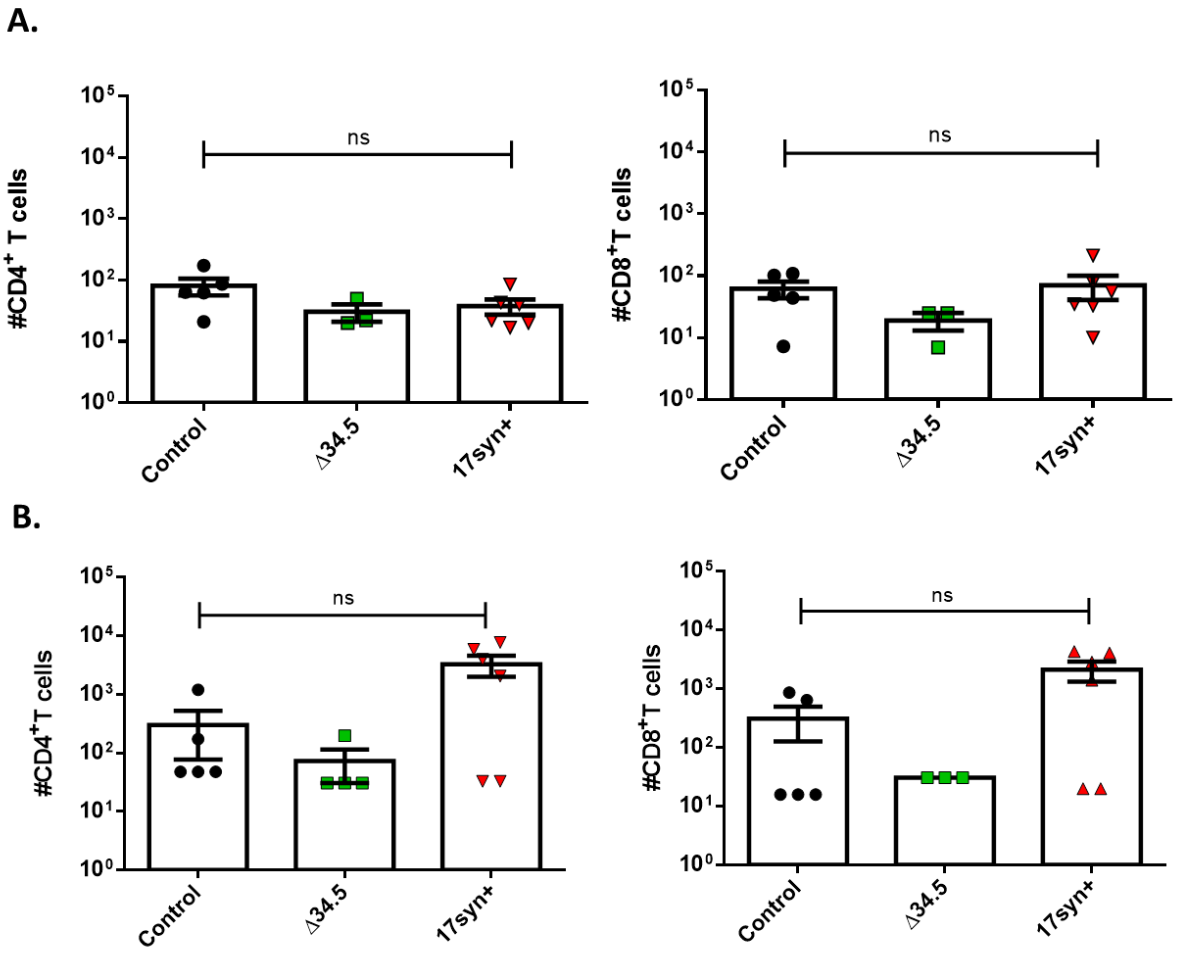
**Supplementary Figure 3. Asymptomatic HSV-1 infection contributes to brain demyelination after EAE induction. (A)** Representative images of brain sections stained with Luxol Fast Blue showing corpus callosum demyelination. **(B)** Representative images of immunohistochemistry against the MBP protein in brain samples. Images are representative of three independent experiments. Image magnification is 10x and correspond at day 14 post-EAE induction. Arrows show demyelination sectors with reduced myelin in the corpus callosum. **(C)** Quantitative histopathological analyses of brain tissue samples. Values represent means ± SEM of three independent experiments. Data were analyzed using two-way ANOVA followed by Turkey's post-test; (n=12, 4/group per day evaluated). **(D)** Representative western blot images for MBP (upper panel) and actin (lower panel) in brain tissue at day 14 post-EAE induction. The graph shows densitometric analyses for MBP bands that were normalized to actin. Data represent the mean ± SEM. Comparisons between ratios were performed using one-way ANOVA with Dunnett's multiple comparison post-test; *$p<0.05$.




**Supplementary Figure 4. Flow cytometry gating strategy to phenotype lymphoid cells isolated from CNS tissues.** Infiltrating cells were selected on the forward versus side scatter (FSC vs SSC) gating. Then, exclusion of doublets was performed by plotting the height against the area for forward scatter, and the live single cells were pre-gated on $CD3^+$ and $CD19^+$. Finally, $CD3^+$ T cells were subdivided into $CD4^+$ and $CD8^+$.




**Supplementary Figure 5. Flow cytometry gating strategy to phenotype myeloid cells isolated from CNS tissues.** Infiltrating cells were selected on the forward versus side scatter (FSC vs SSC) gating. Then, exclusion of doublets was performed by plotting the height against the area for forward scatter, and the live single were pre-gated on $CD45^{+}$ and $CD11b^{+}$. $CD45hi^{+}/CD11b^{+}$ infiltrating myeloid cells were subdivided into neutrophils ($Ly6G^{+}$) and monocytes ($Ly6C^{+}$). On the other hand, $CD45lo^{+}/CD11b^{+}$ (microglia) was evaluated for the activation marker MHC-II.




**Supplementary Figure 6. Asymptomatic HSV-1 infection per se does not increase T cell infiltration in the CNS of C57BL/6 mice.** Mice were mock-treated (black circles), infected with HSV-1 Δ34.5 (green squares), or infected with HSV-1 17syn+ (red triangles). Six weeks post-infection, mice were perfused, and tissues were harvested and processed to isolate immune infiltrating cells in the tissue. **(A)** Infiltrating T cells, $CD4^+$ (left) and $CD8^+$ (right) in the brain. **(B)** Infiltrating T cells, $CD4^+$ (left) and $CD8^+$ (right) in the spinal cord. Data are means ± SEM of two independent experiments (n=3-6/group). Data were analyzed using Kruskal-Wallis and Dunn's multiple comparisons post-test. No significant differences were observed between the analyzed groups.

# 4. DISCUSSION

Despite significant advances in the identification of immune system components that participate in MS disease, there is still a poor understanding on the initial events that lead to the onset and progression of this disease. Autoreactive T cells that have escaped negative selection in the thymus are frequently detected in the blood of healthy individuals but only rarely induce autoimmune disease, because they are controlled by different regulatory mechanisms and usually do not have access to the CNS (Raddassi et al., 2012; Cao et al., 2015). Therefore, it is thought that environmental factors in genetically susceptible individuals could play important roles in MS development (Beecham et al., 2013). Noteworthy, viral infections have been identified as potential environmental triggers that could lead to disease onset and/or exacerbation (Kakalacheva et al., 2011; Steelman, 2015).Thus, studying their effects in MS may help identifying determinants that contribute to the onset and progression of the disease, as well as help in the development novel strategies to prevent or treat MS.

Our current results show for the first time that previous infection with HSV-1 alters the BBB increasing its permeability to small compounds, such as the dye Evans blue, for at least 30 days post infection in the absence of infectious virus, and independent of viral encephalitis. This finding is highly relevant in the context of MS, as for developing this disease autoreactive cells need to enter the CNS. In the animal model, pertussis toxin is used to permeabilize the BBB, followed by MOG peptide immunization and interference with the BBB is key for initiating an autoimmune response to MOG. The severity of EAE is also somewhat proportional to the amounts of pertussis toxin used (Iruretagoyena, 2004; Berard et al., 2010; Albornoz et al., 2013). Importantly, EAE induced in our experiments represent mild- to moderate- scenarios of disease

as compared to other models, specifically with the aim of assessing in a more physiological context the potential relationship between HSV-1 infection and EAE. Moreover, the dose of HSV-1 used herein was sub-lethal and the mouse strain we used is considered resistant to HSV-1-induced encephalitis under the experimental conditions applied, which was evidenced by the fact that the virus-inoculated mice rapidly recovered from infection and did not succumb to death (Kastrukoff et al., 2012; Martin et al., 2014a). Furthermore, the amount of MOG peptide (50 µg) and pertussis toxin (350 ng) used in our study for inducing EAE are comparatively low side by side to other studies, in such a way to induce a mild form of EAE, which is evidenced by the fact that not all animals manifest disease (i.e. 67% in the mock-EAE group) and the maximum scores (mean of maximum score 2) are overall below those generally reported for severe EAE, where total paralysis is observed with clinical scores of 4 or death (Iruretagoyena, 2004). Several studies have reported that the breakdown of the BBB is an early event in EAE development, which causes cell infiltration into the CNS with subsequent myelin damage (Bennett et al., 2010). Therefore, the induction of EAE in infected animals would occur in the context of an previously altered BBB enabling facilitated and faster migration of immune cells into the CNS, which could shorten the inductive phase of EAE and explain why WT HSV-1-infected animals present symptoms before the uninfected animals, as well as higher scores. Importantly, our findings suggest that because of HSV-1 interrupts the permeability of the BBB for long periods, EAE may be induced in mice previously infected with HSV-1 simply by immunizing with the MOG peptide. However, not all animals showed BBB alterations after HSV-1 infection and it is unknown what is the impact of the timing between HSV-1 infection and the induction of EAE disease over the latter. HSV-1 modulation of the BBB could be temporal, and once enough time has passed since infection, the BBB may recover and require

its disruption again with pertussis toxin to induce EAE, but further studies are needed to evaluate that. Moreover, although at a lower extent than the WT HSV-1, the Δ34.5 mutant virus also showed significant BBB permeability in the brain. The attenuated phenotype of the Δ34.5 mutant virus could explain its decreased ability to affect the BBB. As reported by several studies, the gamma-34.5 protein inhibits IFN-I responses, autophagy, and host-mediated shut-off of protein synthesis in order to evade the host immune response, and the targeting of these host pathways and processes by HSV-1 is also required for its dissemination and disease, and contributes to HSV-1-related pathogenesis in the brain (Orvedahl et al., 2007; Wilcox and Longnecker, 2016). Given that Δ34.5-infected mice displayed a significant increase of demyelination in the spinal cord without BBB alterations in this tissue, particularly at 30 days post-infection, the hypothesis of this thesis on the role of the BBB in the increased onset or severity of EAE can only be partially validated, as infection with the mutant HSV-1 disrupted the BBB in the brain and not the spinal cord. Given this result, it is possible that disruption of the BBB at the brain is sufficient for EAE-related damage in the spinal cord. Alternatively, other additional mechanisms, different from increased BBB permeability may also play a role in the EAE exacerbation by HSV-1 infection.

EAE is associated with increased immune cell infiltration into the CNS, likely due to the recruitment of CNS antigen-specific T cells that recognize autoantigens and secrete soluble factors that recruit more immune cells into this tissue (Zamvil, 1990). Although the type and nature of immune cells infiltrating the CNS during EAE have been well documented, it is unknown whether the same amounts and type of immune cells are recruited to these tissues when previous infection with HSV-1 exists. In this study, we determined the amounts and types of immune cells infiltrating the CNS of animals infected with HSV-1 and in which EAE has

been induced and compared them with animals in which EAE has been induced without previous HSV-1 infection. Notably, we found some differences that could partially explain the increased severity of the disease. On the one hand, inflammation accompanied by T cell infiltrations were observed in the brain, which is rarely found in mild EAE, where ascending paralysis is mainly due to spinal cord inflammation and demyelination. $CD4^+$ T cells were the predominant T cell type invading the brain of WT HSV-1 infected mice. However, the specific antigenicity and phenotype of these cells remain to be identified. It could be possible that activated $CD4^+$ T cells that are not specific for CNS epitopes (i.e. HSV-specific T cells) are also able to enter the brain parenchyma and participate in sustaining a pro-inflammatory environment that recruits additional immune cells. On the other hand, myeloid cells seem play a key role in spinal cord demyelinating during EAE after HSV-1 infection. Importantly, some studies have confirmed the pathogenic role of neutrophils in MS in humans and animal models, which is related with BBB breakdown and augmented Th17 immune responses (Aubé et al., 2014). Indeed, neutrophils have been found in the cerebrospinal fluid in MS patients during relapse both in adults and children (Chabas et al., 2010; Kostic et al., 2014). Moreover, post-mortem CNS tissues revealed neutrophil infiltration associated with regions of BBB leakage in a MS patient (Aubé et al., 2014), and the neutrophil-to-lymphocyte ratio in peripheral blood has been proposed to be a marker of MS disease activity (Bisgaard et al., 2017). Notably, in some cases neutrophils can have an immune suppressive functions depending on the inflammatory environment (Ioannou et al., 2012; Ma and Xia, 2018), for which in our case it would be important to characterize the phenotype of these cells to determine their contribution during disease development and progression, including the production of key mediators of effector functions, such as ROS, neutrophil elastase, myeloperoxidase, peptidylarginine deiminase 4

(PAD4), neutrophil extracellular traps (NETs), and the anti-inflammatory cytokine IL10, arginase-1 (Arg-1) and inducible nitric oxide synthase (iNOS) as suppressors factors. Studies in Alzheimer's disease discuss the possibility that BBB breakdown is mediated through NETs (Zenaro et al., 2015). Similarly, we could increase our panel of soluble mediators including the quantification of the levels of important chemokines involved in neutrophil recruitment to the CNS, such as CXCL2 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Notably, we observed that HSV-1-infected animals tend to have increased levels of IL-17 mRNA, which is also a cytokine that favors neutrophil migration (Simmons et al., 2014; McGinley et al., 2020).

Moreover, the obtained profile of cytokines in our study provides an overall picture of what inflammatory events are occurring in the CNS of the infected animals. We found significatively higher levels of IL-6 and IL-1β mRNA in the brain and spinal cord of previously infected mice as compared to uninfected animals induced to undergo EAE disease. However, because several regulatory processes occur after mRNA expression, such as post-transcriptional modifications, translational regulation, and protein degradation control, the results obtained in our experiments should be corroborated at the protein level. Regarding the role of IL-6 in MS, this cytokine has been reported exacerbates clinical manifestations and spinal cord pathology in EAE, mainly by promoting the differentiation of $CD4^+$ T cells toward a Th17 phenotype, which initiate and perpetuate neuroinflammation and demyelination in this model (Samoilova et al., 1998). Importantly, IL-6 can be produced by several cells in the CNS and it could be important to know which cells would be producing this cytokine in high amounts in the context of HSV-1 infection, as well as after EAE induction in that infected-animals. A study in mice with IL-6 deficiency in astrocytes (Ast-IL-6 KO) induced to develop EAE showed that lack of astrocytic IL-6 produces

a delay in the onset of clinical symptoms with fewer inflammatory infiltrates and decreased demyelination (Erta et al., 2016). These attenuated symptoms of EAE are likely observed in our mock-infected mice and suggest that IL-6 could be released by chronically activated astrocytes and elicit EAE enhancement in previously infected animals. However, further studies are needed to evaluate this hypothesis. On the other hand, IL-1β is also found augmented in the blood and cerebrospinal fluid of MS patients, and post-mortem CNS tissues from ill people with MS (Hauser et al., 1990; Dujmovic et al., 2009). In addition, clinical EAE is significantly attenuated in IL-1 receptor-deficient and IL-1β-deficient mice (Schiffenbauer et al., 2000; Li et al., 2011). Moreover, similar to IL-6 several immune cell types serve as critical producers of IL-1β during EAE, with this cytokine inducing responses in hematopoietic and CNS resident cells (Di Paolo and Shayakhmetov, 2016). A recent study using an IL-1β reporter mouse identified neutrophils and monocyte-derived macrophages as the main cells subsets expressing IL-1β in the spinal cord after EAE (Lévesque et al., 2016). Furthermore, some studies have shown that Th17 cells polarized *in vitro* express higher levels of the IL-1β receptor than Th1 or Th2 cells, and that IL1β enhances GM-CSF production by Th17 cells, which as mentioned above is important for neutrophil recruitment and the pathogenicity of EAE (Chung et al., 2009; Guo et al., 2009). Regarding CNS resident cells, there is evidence supporting BBB breakdown in EAE by IL-1β over astrocytes or directly over endothelial cells. This cytokine can lead to the production in astrocytes of hypoxia inducible factor-1 (HIF-1) and vascular endothelial growth factor-A (VEGF-A), which are potent inducers of BBB permeability and angiogenesis (Argaw et al., 2006). More specific activities of IL-1β over astrocytes were also reported by others and include the stimulation of chemokine production (CCL2, CCL20, CXCL2), which might recruit and activate leukocytes (Wang et al., 2014; Rothhammer and Quintana, 2015). Although microglia

have been defined as a key producer of IL-1β in CNS, a recent study showed that during acute EAE infiltrating macrophages are activated and are the main producers of this cytokine, whereas microglia remained suppressed (Vainchtein et al., 2014). In contrast, we found that microglia displayed a significant increase of the activation marker MHC-II in mice infected previously with WT HSV-1. Therefore, microglia could be playing an important role enhancing the BBB breakdown, which was more pronounced in these animals than those infected with the mutant virus that did not show a significant increase of activated microglia. Our results open the possibility for assessing, later on, the contribution of particular cell types over the release of specific cytokines and modulate the observed phenotypes by cell depletion or cytokine neutralization with antibodies, or alternatively using knock-out mice.

To further evaluate the dependence of viral replication in the CNS, or HSV-1 reactivation over EAE initiation and severity, we tested a mutant HSV-1 virus that lacks a gene associated to neurovirulence (ICP34.5 gene), which is attenuated for replication in neurons and does not cause acute encephalitis, yet elicits an inflammatory response in the brain (Broberg et al., 2004). Moreover, this virus has also shown be attenuated in the establishment of latency, as well as in its capacity to reactivate (Whitley et al., 1993). Importantly, we observed that Δ34.5-infected animals showed a worse EAE score than non-infected, and WT HSV-1-infected animals, which could be relevant for figuring out mechanisms behind the modulation of MS disease by HSV-1 infection. Although some studies in the past have characterized the replication, establishment of latency and reactivation of HSV-1 Δ34.5 mutants in some mice models, differences have been found between the reported results and data regarding the course of infection of C57BL/6 mice with this virus are lacking (Whitley et al., 1993; Broberg et al., 2004). Some time ago, *Whitley et al.* reported that the Δ34.5-mutant virus assessed herein had lost the capacity to spread from

the nasal mucosae to the CNS and replicate in this latter tissue, as well as displayed a reduced ability to establish latency and reactivate *ex vivo*. Indeed, after intranasal infection of Swiss Webster and BALB/c mice with $10^5$ to $10^6$ PFU of the mutant virus, no infectious viral particles were detected in the brain or TG at any of the evaluated time-points (1, 3,5 and 7 d.p.i.) and latent viral genome was only detected in the TG of a single animal out of five at 28 d.p.i; also the amount of virus detected in this tissue was much lower than that recovered with the WT virus (Whitley et al., 1993). Later, another study using BALB/c mice reported that intranasal infection was an effective way to spread the Δ34.5-mutant virus in the CNS. While the virus did not grow in cultures derived from brain samples, the viral DNA was detected in brain preparations up to 21 d.p.i. Viral reactivation from the trigeminal ganglia in the explant cultures was not detected (Broberg et al., 2004). The differences observed between the different studies evaluating viral spread in the nervous system seem to be associated with the amount of virus used during infection, as the last study compared intranasal infections with either, $10^6$ or $10^7$ PFUs of the mutant virus and only found a significant increase in viral spread to both, the trigeminal ganglia and brain when using $10^7$ PFU (Broberg et al., 2004). Given that our experiments were performed using $10^6$ PFUs of the Δ34.5 mutant virus, it is possible that viral spread to the nervous system was limited and that the virus was not able to establish a latent infection. This notion is further supported by the fact that a previous study reported that HSV-1 enters, replicates, spreads and establishes latent infections similarly in C57BL/6 and BALB/c mice (Halford et al., 2004), and that the resistance to HSV-1 encephalitis in the C57BL/6 mouse strain is conditional and depends on the amount of the inoculum, the viral strain used, and viral resistance to the host IFN response (Lopez, 1975; Zawatzky et al., 1981; Halford et al., 2004). Because the mutant virus lacks ICP34.5, which is important for inhibiting the IFN-I pathway,

the infection by this mutant may be rapidly contained by the a strong innate IFN α/β response elicited in C57BL/6 mice that impairs viral progression into the nervous system (Zawatzky et al., 1982; Halford et al., 2004).

Although it is currently unknown to us if the mutant virus was completely cleared in the infected mice, or if the virus reached the nervous system establishing a latent infection, based on previous studies discussed above we suggest that the enhanced EAE severity observed in our experiments after asymptomatic HSV-1 infection could be due to an inflammatory signature imprinted in infected tissues early after infection, rather than an effect of latent virus in the nervous system or viral reactivation from this tissue. Interestingly, our results may reinforce data reported in previous studies, in which mice showed increased susceptibility to severe EAE after a cleared viral infection (Chen et al., 2017; Steinbach et al., 2019). A study reported that a transient brain viral infection induces the formation of tissue-resident memory T cells ($T_{RM}$) clusters with a persisting chemotactic signal with CCL5, which increased autoimmune lesions in the brain after EAE induction by a virus-independent mechanism (Steinbach et al., 2019). Importantly, similar $T_{RM}$ have been reported in various mucosal and epithelial tissues after peripheral infections, which could also predispose the host to a long-term permissive inflammatory environment that may modulate autoimmune diseases (Steinbach et al., 2018). Despite the well-characterized protective function of $T_{RM}$ acting as sentinels to trigger an antigen-specific response against reinfections (Gebhardt et al., 2009; Iijima and Iwasaki, 2014; Mueller and Mackay, 2016), evidence of a possible harmful role of these cells in autoimmune diseases is emerging. Recently, it has been proposed that these cells could contribute to the recruitment and reactivation of self-reactive cells through bystander mechanisms (Park and Kupper, 2015; Steinbach et al., 2018, 2019). In addition, a long-term exacerbation of EAE in

mice was observed after a resolved influenza infection. In this study, the researchers attributed the increase in EAE severity to an inflammatory environment in the lung and mediastinal lymph nodes 50 days post-influenza virus inoculation, which likely modulated the course of EAE leading to a higher amount of Th1 T cells infiltrating the CNS in the animals (Chen et al., 2017).

However, on the other hand our results differ from those reported with another herpesvirus, in which case the modulation of the course of EAE disease was suggested to depend on the latent virus in B cells (Casiraghi et al., 2012, 2015). Mice latently infected with the herpesvirus γHV-68, a murine homolog of EBV, showed an earlier onset, and a worse clinical EAE outcome that was accompanied by enhanced T cell infiltrations inside the CNS with a potent Th1 response (Casiraghi et al., 2012). Here, EAE was induced during the acute phase of infection with the WT virus or in animals infected with mutant γHV-68 virus that is deficient in latency in order to evaluate the role of latency in the observed overcome (Casiraghi et al., 2015). This study showed a delay in the onset of EAE when the disease was induced during acute infection, and that the disease scores were similar to those reported in the uninfected mice. In line with this observation, mice infected with the virus deficient in latency also displayed a less severe disease course and lower amounts of T cells infiltrating the CNS. No viral DNA was detected in the splenocytes of mice infected with this virus, indicating that the virus was cleared before latency was established (Casiraghi et al., 2015). Interestingly, enhanced EAE disease was associated with STAT-1 and CD40 upregulation in uninfected dendritic cells, which was abolished in mice infected with the virus deficient in latency.

Noteworthy, we cannot rule out that asymptomatic brain infection with WT HSV-1 could be modulating the outcome of EAE disease by other virus-dependent mechanisms. In this regard, because the induction of EAE, and EAE *per se* is associated with CNS inflammation

and alterations of the BBB which allows the infiltration of immune cells into this tissue that secrete pro-inflammatory cytokines (i.e. MOG-specific T cells) (Bennett et al., 2010), it is possible that latent WT HSV-1 in the CNS may be reactivated during EAE. Furthermore, increased BBB permeability during EAE likely favors the infiltration of bystander T cells into the CNS (Liu et al., 2019b), which could favor the infiltration of T cells into this tissue that recognize HSV-1 antigens and hence further increase CNS inflammation. Moreover, although our findings indicate that asymptomatic CNS infection with WT HSV-1 before EAE induction increases the onset and severity of EAE, it remains unknown to us whether the observed effects over EAE in WT HSV-1-infected animals may also be due to replicating virus, after viral reactivation, or molecular viral reactivation with the expression of some viral proteins (Feldman et al., 2002; Nicoll et al., 2012).

Importantly, it is unknown whether HSV-1 infection in humans could either, initiate or aggravate the progression of MS or be a consequence of MS disease. Numerous studies have reported reduced percentages of $CD8^+$ T cells in peripheral blood of MS patients, which could be associated with impaired responses against viral infections in these persons (Thompson et al., 1986; Pender et al., 2012). Additionally, a recent study showed that EBV-specific $CD8^+$ T cells in individuals suffering MS displayed limited cytokine production, evidencing an exhaustion-like phenotype (Pender et al., 2017). Others have found that $CD8^+$ $CD57^+$ T cells have increased expression of the inhibiting surface molecule programmed death-1 (PD-1) in patients with MS, as compared to healthy individuals, and was associated with a negative regulation of cytotoxic responses against EBV (Cencioni et al., 2017). Thus, it is possible that a defective control of HSV-1 infection by T cells in MS or the EAE model, together with T cell exhaustion may lead to HSV-1 reactivation. Further studies should be performed regarding the

specific immune cells infiltrating the CNS under the conditions described in our study to draw a more comprehensive picture of the events occurring after EAE induction in mice previously infected with HSV-1 and elucidate possible interrelationships between EAE and HSV-1 latent infection.

Taken together, we report that a previous asymptomatic HSV-1 infection enhances EAE disease, even in the absence of latent or reactivated virus, and that the mechanism seems be mediated by an inflammatory environment permissive for autoimmunity, which remains to be further investigated in future studies. Although similar inflammatory environments could be generated by other stimuli, our study could help to elucidate the participation of HSV-1 over MS, revealing some of the pathways involved in this interrelationship, which could aid find new pharmacological targets to treat or prevent the progression of this disease.

# 5. CONCLUDING REMARKS

Based on the results obtained during the development of this thesis, we conclude the following:

- Asymptomatic infection with WT HSV-1 accelerates the clinical symptoms of EAE and enhances EAE severity. Infection with the Δ34.5 mutant virus increases the clinical course of EAE.
- Asymptomatic infection with WT HSV-1 produces an increased infiltration of T $CD4^+$ cells into the brain after EAE induction. Moreover, a previous infection with either, WT HSV-1 or the Δ34.5 mutant virus, lead to a higher infiltration of neutrophils into the spinal cord after EAE induction.
- A previous infection with HSV-1 either, WT or the Δ34.5 mutant virus elicits a higher expression of pro-inflammatory cytokines after EAE induction both, in the brain and spinal cord.
- Asymptomatic infection with WT HSV-1, after intranasal inoculation elicits prolonged BBB disruption in both, the brain and spinal cord at least up to 30 days post-infection. BBB disruption also occurs after infection with the Δ34.5 mutant virus, but to a lesser extent and only in the brain.

These results indicate that under certain conditions that predispose the development of EAE, which is a murine model for multiple sclerosis disease in humans, HSV-1 infection could play a role on the onset and severity of the disease. Finally, although the breakdown of the BBB may explain the increased number of T $CD4^+$ cells infiltrating the brain of the WT HSV-1-infected

animals together with a faster onset of EAE symptoms, as well as exacerbated demyelination in this tissue in animals infected with both viruses (WT and mutant), the alteration of the BBB does not seem to be a determining factor regarding disease severity at spinal cord level, as a worst course of EAE was observed in the animals infected with the mutant virus. This latter virus did not significantly increase the permeability of the BBB in the spinal cord, as compared to the uninfected group.

Overall, given the findings described above we partially validate the hypothesis *"Asymptomatic HSV-1 infection enhances MOG-induced EAE disease severity in the mouse model by increasing the permeability of the blood-brain barrier"*, because other mechanisms besides BBB disruption could be responsible for the increased disease observed in HSV-1-infected animals. Furthermore, since the mutant virus used herein is defective in the establishment of latency and reactivation from the nervous system, we suggest that HSV-1 enhances the severity of EAE by an indirect immune-mediated mechanism, likely mediated by an inflammatory signature in the infected tissues that is imprinted early after HSV-1 infection of the host.

# 6. APPENDIX

## 6.1 Contribution in scientific publications during this thesis and PhD training.

Castillo, E.*, **Duarte, L. F.***, Arriagada, J., Corrales, N., Álvarez, D. M., Farías, M. A., et al. (2020). Anti-herpetic activity of *Macrocystis pyrifera* and *Durvillaea Antarctica* algae extracts against HSV-1 and HSV-2. *Frontiers in Microbiology*. ***Equal contribution. *Accepted.***

Alvarez, D. M., **Duarte, L. F.,** Corrales, N., Smith, P. C., & González, P. A. (2020). Cetylpyridinium chloride blocks herpes simplex virus replication in gingival fibroblasts. *Antiviral research*, 179, 104818.

Álvarez, D. M., Castillo, E., **Duarte, L. F**., Arriagada, J., Corrales, N., Farías, M. A., et al. (2020). Current Antivirals and Novel Botanical Molecules Interfering With Herpes Simplex Virus Infection. *Front. Microbiol.* 11, 1–19.

**Duarte, L. F.,** Farías, M. A., Álvarez, D. M., Bueno, S. M., Riedel, C. A., & González, P. A. (2019). Herpes Simplex Virus Type 1 Infection of the Central Nervous System: Insights Into Proposed Interrelationships With Neurodegenerative Disorders. Frontiers in cellular neuroscience, 13, 46.

Ibáñez, F. J., Farías, M. A., Gonzalez-Troncoso, M. P., Corrales, N., **Duarte, L. F.,** Retamal-Díaz, A., et al. (2018). Experimental dissection of the lytic replication cycles of herpes simplex viruses in vitro. Front. Microbiol. 9, 2406.

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Research paper

# Cetylpyridinium chloride blocks herpes simplex virus replication in gingival fibroblasts

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ABSTRACT

Infections with herpes simplex viruses are lifelong and highly prevalent worldwide. Individuals with clinical symptoms elicited by HSVs may suffer from occasional or recurrent herpetic lesions in the orofacial and genital areas. Despite the existence of nucleoside analogues that interfere with HSV replication, such as acyclovir, these drugs are somewhat ineffective in treating skin lesions as topical formulations only reduce in one or few days the duration of the herpetic ulcers. Cetylpyridinium chloride (CPC) is a quaternary ammonium compound present in numerous hygiene products, such as mouthwashes, deodorants, aphtae-treating formulations and oral tablets as an anti-septic to limit bacterial growth. Some reports indicate that CPC can also modulate host signaling pathways, namely NF-κB signaling. Because HSV infection is modulated by NF-κB, we sought to assess whether CPC has antiviral effects against HSVs. Using wild-type HSV-1 and HSV-2, as well as viruses that are acyclovir-resistant or encode GFP reporter genes, we assessed the antiviral capacity of CPC in epithelial cells and human gingival fibroblasts expanded from the oral cavity and its mechanism of action. We found that a short, 10-min exposure to CPC added after HSV entry into the cells, significantly limited viral replication in both cell types by impairing viral gene expression. Interestingly, our results suggest that CPC blocks HSV replication by interfering with the translocation of NF-κB into the nucleus of HSV-infected cells. Taken together, these findings suggest that formulations containing CPC may help limit HSV replication in infected tissues and consequently reduce viral shedding.

## 1. Introduction

Herpes simplex viruses (HSVs) type 1 (HSV-1) and type 2 (HSV-2) are highly prevalent worldwide. While HSV-1 infects nearly 70% of the world population (Looker et al., 2015), HSV-2 is present in more than 10% of humans (Looker et al., 2008). Both viruses produce diverse clinical manifestations, from mild to severe and infections are lifelong (Suazo et al., 2015a). Common clinical manifestations produced by HSV consist of orofacial and genital skin lesions, as well as herpetic gingivostomatitis (Arduino and Porter, 2007; Ballyram et al., 2016; Bernstein et al., 2013; Suazo et al., 2015b). Herpetic skin lesions are usually treated with topical creams containing acyclic nucleoside analogues, such as acyclovir which selectively inhibits the viral DNA polymerase (Skoreński and Sieńczyk, 2014; Kukhanova et al., 2014; De Clercq, 2013). However, acyclovir formulated as a cream only reduce in 1–2 days the duration of skin lesions (Arduino and Porter, 2007; Moomaw et al., 2003; Sauerbrei, 2016).

Cetylpyridinium chloride (CPC) is a cationic ammonium compound with surfactant properties that is safe to be used in humans. It is often found in products at concentrations ranging 0.05–0.1% (0.5–1 mg/ml), such as mouthwashes, oral tablets, deodorants and aphtae-treating products, and its use is indicated as an anti-bacterial agent (Gerba, 2015; Teng et al., 2016; García et al., 2011; Jenkins et al., 1994; Altenburg et al., 2014; Garcia-Godoy et al., 2014). However, CPC has also been reported to modulate signaling events associated with the cellular transcription factor nuclear factor-kappa B (NF-κB) (Kim et al., 2005; Zheng et al., 2013).

Previous reports suggest that NF-κB may play important roles in the replication cycle of HSVs (Goodkin et al., 2003; Patel et al., 1998; Zhang et al., 2013a). Importantly, this host factor has been shown to be translocated to the nucleus during HSV infection and reportedly, bind to the promoter of the HSV immediate early viral gene *ICP0* to promote

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# Current Antivirals and Novel Botanical Molecules Interfering With Herpes Simplex Virus Infection

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Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) are highly prevalent within the human population and are characterized by lifelong infections and sporadic recurrences due to latent neuron infection. Upon reactivations, HSVs may manifest either, symptomatically or asymptomatically and be shed onto others through mucosae body fluids. Although, HSVs can produce severe disease in humans, such as life-threatening encephalitis and blindness, the most common symptoms are skin and mucosal lesions in the oro-facial and the genital areas. Nucleoside analogs with antiviral activity can prevent severe HSV infection, yet they are not very effective for treating skin manifestations produced by these viruses, as they only reduce in a few days at most the duration of lesions. Additionally, HSV variants that are resistant to these antivirals may arise, especially in immunosuppressed individuals. Thus, new antivirals that can reduce the severity and duration of these cutaneous manifestations would certainly be welcome. Here, we review currently available anti-herpetic therapies, novel molecules being assessed in clinical trials and new botanical compounds reported in the last 20 years with antiviral activities against HSVs that might represent future treatments against these viruses.

**Keywords: HSV-1, HSV-2, natural antiviral compounds, antiviral extracts, phytopharmaceuticals, therapy**

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## INTRODUCTION

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 virus (HSV-2) are viruses belonging to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily and *Simplexvirus* genus. HSV-1 and HSV-2 belong to the same family and subfamily than varicella zoster virus (VZV), yet VZV belongs to the *Varicellovirus* genus (McGeoch, 2009; Kinchington et al., 2012; Ibáñez et al., 2018). HSV-1 and HSV-2 are highly prevalent in humans, with global infections ranging 70 and 10% of the world population, respectively (Smith and Robinson, 2002; Schillinger et al., 2004; Looker et al., 2008; Chayavichitsilp et al., 2009; Doi et al., 2009) (World Health Organization, Regional Estimates[1].

[1]http://www.who.int/mediacentre/news/releases/2015/herpes/en/

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# Herpes Simplex Virus Type 1 Infection of the Central Nervous System: Insights Into Proposed Interrelationships With Neurodegenerative Disorders

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Herpes simplex virus type 1 (HSV-1) is highly prevalent in humans and can reach the brain without evident clinical symptoms. Once in the central nervous system (CNS), the virus can either reside in a quiescent latent state in this tissue, or eventually actively lead to severe acute necrotizing encephalitis, which is characterized by exacerbated neuroinflammation and prolonged neuroimmune activation producing a life-threatening disease. Although HSV-1 encephalitis can be treated with antivirals that limit virus replication, neurological sequelae are common and the virus will nevertheless remain for life in the neural tissue. Importantly, there is accumulating evidence that suggests that HSV-1 infection of the brain both, in symptomatic and asymptomatic individuals could lead to neuronal damage and eventually, neurodegenerative disorders. Here, we review and discuss acute and chronic infection of particular brain regions by HSV-1 and how this may affect neuron and cognitive functions in the host. We review potential cellular and molecular mechanisms leading to neurodegeneration, such as protein aggregation, dysregulation of autophagy, oxidative cell damage and apoptosis, among others. Furthermore, we discuss the impact of HSV-1 infection on brain inflammation and its potential relationship with neurodegenerative diseases.

**Keywords: herpes simplex virus, neurodegeneration, neurological disease, apoptosis, autophagy, mitochondrial damage, oxidative stress, neuroinflammation**

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## INTRODUCTION

Herpes simplex virus type-1 (HSV-1) is an enveloped double-stranded DNA virus belonging to the *Herpesviridae* family, that has a genome of approximately 152 kbp encoding more than 80 different open reading frames (ORFs; Boehmer and Nimonkar, 2003). Importantly, HSV-1 is a neurotropic pathogen with a wide spectrum of clinical disorders ranging from harmless skin manifestations, such as oral and facial lesions to severe infection of the central nervous system (CNS). HSV-1 is the most common cause of sporadic encephalitis in adults, as well as the leading cause of infectious blindness in developed countries due to herpetic keratitis

frontiers in Microbiology

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# Experimental Dissection of the Lytic Replication Cycles of Herpes Simplex Viruses *in vitro*

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Herpes simplex viruses type 1 and type 2 (HSV-1 and HSV-2) produce lifelong infections and are highly prevalent in the human population. Both viruses elicit numerous clinical manifestations and produce mild-to-severe diseases that affect the skin, eyes, and brain, among others. Despite the existence of numerous antivirals against HSV, such as acyclovir and acyclovir-related analogs, virus variants that are resistant to these compounds can be isolated from immunosuppressed individuals. For such isolates, second-line drugs can be used, yet they frequently produce adverse side effects. Furthermore, topical antivirals for treating cutaneous HSV infections usually display poor to moderate efficacy. Hence, better or novel anti-HSV antivirals are needed and details on their mechanisms of action would be insightful for improving their efficacy and identifying specific molecular targets. Here, we review and dissect the lytic replication cycles of herpes simplex viruses, discussing key steps involved in cell infection and the processes that yield new virions. Additionally, we review and discuss rapid, easy-to-perform and simple experimental approaches for studying key steps involved in HSV replication to facilitate the identification of the mechanisms of action of anti-HSV compounds.

**Keywords: life cycle herpes simplex viruses, replication cycle herpes simplex virus, herpes simplex virus infection steps, antivirals, acyclovir, antiviral drugs**

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## INTRODUCTION

Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) are two *Alphaherpesvirinae* viruses that are highly prevalent in the human population and are known to produce numerous clinical manifestations after the infection of different tissues within the host. While the world prevalence for HSV-1 nears 67%, estimates for HSV-2 fluctuate between 11 and 20% (http://www.who.int) (Looker et al., 2015). Infections with HSVs mainly occur after these viruses have gained contact with the mucosae or micro-lesions in skin epithelia; dissemination in turn ensues from oral and genital secretions (Kaufman et al., 2005). Similar to other herpesviruses, HSV infections are lifelong and generally asymptomatic, yet the viruses can be shed from infected individuals independent of the occurrence of clinical manifestations (Wald et al., 2000). Additionally, HSVs can infect neuronal prolongations enervating peripheral tissues and establish latency in these cells, namely in the trigeminal ganglia and dorsal root ganglia of the sacral area from where they can sporadically reactivate (Gillgrass et al., 2005; Margolis et al., 2007; Huang et al., 2011).

## 6.2 Scientific meetings attended during this thesis and awards.

- Awarded with the ***"2nd place of three-minute thesis 3MT® competition 2019 Award"*** from Pontificia Universidad Católica de Chile.
- SOMICH Congress 2019 and XLI SOMICH anual metting. 5th to 8th of November 2019. Puerto Varas, Chile (Poster presentation). Awarded with the *"Metting Attendance Award 2019"* from the Sociedad de Microbiología de Chile.
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- 4th Innovative Approaches for Identification of Antiviral Agents Summer School. Cagliari, Italy. 24th to 28th of September 2018 (Oral presentation). Awarded with the ***"Best oral communication award"*** from the European Society for Virology.
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