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THE ROLE OF INTESTINAL IMMUNE SYSTEM IN HERPES SIMPLEX VIRUS TYPE 1–MEDIATED ENTERIC NERVOUS SYSTEM DYSFUNCTIONS

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Definitions

μg	MICROGRAM
Ml	MICROLITRE
μM	MICROMOL
CD	CROHN'S DISEASE
CNS	CENTRAL NERVOUS SYSTEM
CTRL	CONTROL
DD	DIVERTICULAR DISEASE
DMEM	DULBECCO'S MODIFIED EAGLE'S MEDIUM
DNA	DEOXYRIBONUCLEIC ACID
ENS	ENTERIC NERVOUS SYSTEM
FACS	FLUORESCENCE-ACTIVATED CELL SORTING
FBS	FETAL BOVINE SERUM
FITC	FLUORESCEIN ISOTHIOCYANATE
HSV-1	HERPES SIMPLEX VIRUS TYPE 1
HSV-2	HERPES SIMPLEX VIRUS TYPE 2
IBD	IRRITABLE BOWEL DISEASE
ICC	IMMUNOCITOCHEMISTRY
IHC	IMMUNOHISTOCHEMISTRY
IG	INTRAGASTRICAL INOCULUM
IN	INTRANASAL INOCULUM
IFN	INTERFERON
IL	INTERLEUKIN
LMMP	LONGITUDINAL MUSCLE MYENTERIC PLEXUS
mAb	MONOCLONAL ANTIBODY
Mg	MILLIGRAM
mM	MILLIMOLAR
°C	DEGREES CELSIUS
PFPL	PORE FORMING PROTEIN-LIKE
p.f.u.	PLAQUE FORMING UNIT
PDCD1	PROGRAMMED CELL DEATH 1
p.i.	POST INFECTION
PCR	POLYMERASE CHAIN REACTION
PE	PHYCOERYTHRIN
RNA	RIBODNUCLEIC ACID
sec	SECOND
T_A	TEMPERATURE ANNEALING
UC	ULCERATIVE COLITIS
VZV	VARICELLA ZOSTER VIRUS

Abstract

Intestinal neuropathies have been described in a variety of functional and inflammatory gastrointestinal disorders. Although the degenerative alterations and neuronal are often associated to a lymphocytic inflammatory infiltrate, the underlying mechanisms remain obscure. Potential etiologic factors are neurotropic viruses, since they are able to specifically infect neurons and cause cell damage through direct or indirect mechanisms. Among the neurotrophic viruses the HSV-1 shows several interesting features. HSV-1 is most commonly known to latently infect the trigeminal ganglion of cranial nerve innervating the face and oral mucosa but latent HSV-1 has also been found in the nodose ganglia innervating the gastrointestinal tract in humans possibly as a consequence of swallowed viral particles.

The working hypothesis of my PhD work was that swallowed HSV-1 can infect the enteric nervous system and locally trigger an immune mediated response damaging neurons.

To assess the ability of α-herpesvirinae (HSV-1, HSV-2, VZV) to infect human ENS we performed a prospective cohort study using human surgical ileocolonic specimens of patients affected by colon carcinoma (CC), Crohn's disease (CD), ulcerative colitis (UC) and diverticular disease (DD). A total of 121 patients were studies and presence of herpes simplex virus (HSV) types-1 and -2 and varicella-zoster virus (VZV) DNA was examined by RT-PCR in the muscle intestinal layer including the myenteric plexus. The α-herpesviruses DNA was detected in 44,5 % and 52 % of ileum and colon samples, respectively. No significant differences in the viral DNA positivity were observed among the different groups whereas the viral DNA load was significantly higher in samples of patients affected by Crohn's disease. Moreover, HSV DNA was detected more frequently than VZV DNA, 46% vs 3%, respectively.

In order to mimic the human *α-herpesvirinae* infection and spread in ENS, my research group has established an original murine model of persistent ENS infection with HSV-1. Using this model we demonstrated that HSV-1 infection resulted in time-dependent intestinal neuromuscular abnormalities, providing the first *in vivo* evidence for the ability of neurotropic viruses to reach and damage the ENS.

Since significant intestinal motility anomalies and damage of enteric nerves were evident 8 - 10 weeks (W) post intragastric (IG) challenge with HSV-1, I attempted to characterize the adaptive immune response to HSV-1 and whether was causing the neuromuscular abnormalities.

In the longitudinal muscle myenteric plexus (LMMP) I observed an increase in CD3+ lymphocytes starting at 6 W and persisting up to 10 W after viral IG inoculum. At 8 weeks post IG viral inoculum, HSV-1 reactive $CD3^+CD4^+IL4^+$ and $CD3^+CD8^+INF\text{-}\gamma^+$ were detected, whereas at 10 W activated CD8+ T-cells infiltrated the LMMP. By immunohistochemistry CD3+ lymphocytes were demonstrated in the myenteric ganglia of HSV-1 infected mice 6-10 W post IG viral challenge.

To verify the involvement of CD8+ and CD4+ T-cells in HSV-1 induced dysmotility we performed depletion experiments by administration of monoclonal antibody. ENS damage and the neuromuscular anomalies were observed only after depletion of CD8 T cells at 8 and 10 W after viral IG inoculum.

To validate the role of lymphocytes in HSV-1 induced ENS dysfunction, CD3+CD4+ and CD+CD8+ cells were isolated from the LMMP of mice 8-10 W after viral IG inoculum, *in vitro* pulsed with HSV-1, and injected in recipient mice. The effects on isometric muscle tension were determined after one week. Adoptive transfer of $CD8^+$ T cells isolated from LMMP at 8 W post IG HSV-1 inoculum caused significant neuromuscular anomalies (p<0.01 vs control) in recipient mice only if HSV-1 pulsed in vitro. Instead, the adoptive transfer of both CD4+ and CD8+ T cells isolated from 10 W infected mice resulted in dysmotility with or without *in vitro* exposure to viral antigens in recipient mice.

In conclusion, in this study we showed that α-herpesvirinae DNA is present in a large percentage of patients and the presence of HSV-1 in murine myenteric ganglia triggered a strong and specific immune response able to damage the ENS. We speculate that persistent HSV-1 infection in the ENS, in predisposed individuals, may contribute to alter neuronal functional integrity favouring the onset of bowel disorders.

Chapter 1: INTRODUCTION

Section 1. Neuropathies of ENS

The importance of the ENS is highlighted by the wide range of enteric neuropathies that are caused following a failure in one or more of its roles. The set of abnormalities in in the ENS are known as neuropathy. These term describes a heterogeneous group of disorders in which symptoms results from neuromuscular dysfunctions associated with the degeneration or deficiency of enteric neurons. (Knowles et al. 2009). Indeed, ENS neuropathies are described by a damage in motor or sensory (or both) neurons that innervate the gut. Injury in these neuronal areas cause respectively disorders in contractile activity and abnormalities of visceral sensation play a role in symptoms pathogenesis. (Knowles H. et al. 2013).

Considering the wide spectrum of clinical phenotypes that can occur, these disorders are typically difficult to diagnose and to classify: a histological criteria is the current gold standard for diagnosis, seen that unclear evident results only by standard radiologic or endoscopic testing modalities. (Wingate D. 2013). Such, to summarize the complex picture of enteric neuropathies, stand out primary disorders, when the target of the disease is limited on ENS, secondary when neuropathies are associated with other disease states and idiopathic neuropathies whether is known about the etiological factors. Primary neuropathies in turn, may be divided into congenital or those in which prevail acquired disease processes (De Giorgio and Camilleri, 2004, Knowles et al., 2009 and Knowles et al., 2010).

Finally, an additional classification is based on the segments of the gastrointestinal tract that neuropathies can affect, between the oesophagus and rectum (Raj K. et al. 1996). On the basis on these criteria is possible principally identify:

- Achalasia is typical neuropathies recognized in the oesophagus following absence of distal oesophageal peristalsis and abnormal lower oesophageal sphincter relaxation.
- Gastroparesis is defined as a syndrome characterized by abnormal gastric function with delayed gastric emptying in the absence of mechanical obstruction. Most cases of gastroparesis are related to diabetes or have no evident aetiology (idiopathic).

- Pseudo-obstruction describes a condition in which the bowel fails to propel its contents in the presence of an unobstructed lumen. Although probably a diffuse disorder of the gastrointestinal tract, the small bowel is usually worst affected.

However, the commons features that typically characterize abnormalities in the ENS are degeneration or loss of neurons: inflammatory and degenerative cellular mechanisms can contribute to neural changes in primary and secondary neuropathies (Knowles H. 2013).

The most seriously damaged neuronal cells may undergo programmed cell death (e.g. apoptosis) and necrosis and often is associated with increase and accumulation of glial cells. (Nael A. Al-Abdulla and Lee J. Martin 1998). Likewise, alterations in enteric glia, formerly defined as 'supporting' cells within the ENS, are gaining attention with regard to enteric neuropathies .

Growing evidence have indicated that alterations of smooth muscle and interstitial cells of Cajal (ICC) may also contribute to the pathogenesis of gut dysmotility (Garrity MM, et al. 2009). Equally, attention was focused on role of alterations in enteric glia, defined as 'supporting' cells within the ENS, in enteric neuropathies (Bassotti G et al. 2007)

Furthermore, ENS disorders are often associated with an inflammatory immune infiltrate in myenteric plexus (Knowles H. et al. 2013), impaired motor activity and abnormal transit. Experimental data suggest that inflammation, even if mild, could lead to persistent changes in GI nerve and smooth muscle function, resulting in colonic dysmotility, hypersensitivity, and dysfunction even when the preceding infection is restricted to the proximal small intestine (Shaheen E.L. and Kirchgessner A. 2010).

Interesting, evidence show that secondary gastrointestinal motility disorders are common in patients with Parkinson's disease caused by degeneration of the vagal nucleus and myenteric plexus (Wolfgang H. Jost 1997).

Actually, therapeutic opportunities for many of these conditions are inadequate and restricted to palliative interventions aimed to prevent mortality or complications and they are not designed to replace dysfunctional or missing neural components of the ENS (Burns AJ. et al. 2014).

Section 1.2: Enteric Nervous System (ENS)

1.2.1 Structure of enteric nervous system

The enteric nervous system (ENS) is the largest and most complex component of the autonomic nervous system. The enteric nervous system is localized within wall of the gastrointestinal tract and is distributed in the muscles of the esophagus, stomach, and intestine.

The enteric nervous system regulates several bowel functions including intestinal motility, nociception, local blood flow, transport and secretion in the mucosal immune response and endocrine activity (Costa et al. 2000).

The enteric nervous system can function autonomously, but optimal digestive functions require communication between the ENS and the central nervous system. Through these cross connections, the gut can provide sensory information to the CNS, and the CNS can affect gastrointestinal function (Shin F. et al. 2007).

The ENS organized in two concentric circles of ganglia, composed of a complex network of neurons and enteroglial cells, connected by a grid of interglial fibers. These concentric circles establish a complex of ganglia called respectively, myenteric plexus (or plexus of Auerbach) and submucosal plexus (or Meissner's plexus). The first is located between the circular and longitudinal layers of smooth muscle cells and its main function is to regulate the contraction and relaxation of the intestinal wall. The second is located in submucosa layer and has the responsibility to detect signals coming from the lumen, adjust the blood flow and control absorption and secretion of the epithelial cells (Hansen, 2002).

The ENS consists of three classes of neuron: 1) primary afferent neurons, which detect chemical and mechanical stimuli in the environment luminal; 2) interneurons, which send signals ascending and descending; 3) excitatory and inhibitory motor neurons that control the functions of effector cells (Goldstein et al., 2013).

The ENS controls the different cell types present in the gut using a wide variety of chemical messengers, such as neuropeptides, neurotransmitters and gas (NO). The range of neurotransmitters released differs according to the type of neuron and the bowel segment in which it is located. One major excitatory neurotransmitter produced by enteric neurons is acetylcholine that stimulates smooth muscle contraction, increases in intestinal secretions, release of enteric hormones and dilation of blood vessels. In contrast, norepinephrine is used widely for neurotransmission in the gastrointestinal tract, but have an effect always inhibitory and opposite that of acetylcholine.
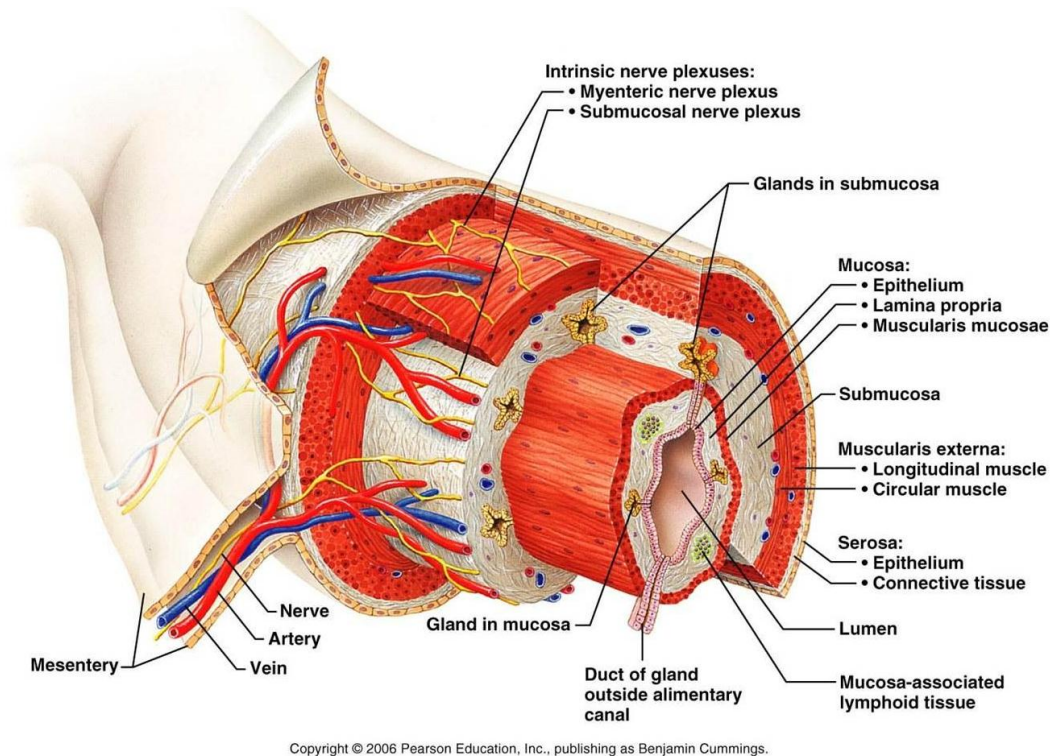
Figure 1. General organization of the gastrointestinal tract

1.2.2 Role of viral infection in enteric nervous system disorders.

The close proximity of immune cells and enteric neurons in the gut wall justify their strong cross-talk and the ability to regulate each other functions. Thus, dysregulated signaling between immune cells and enteric neurons can cause alterations in gut function. Although intestinal inflammation underlies the symptoms associated with IBD, increasing preclinical and clinical evidence indicates that infection and inflammation are also key risk factors for the development of other gastrointestinal disorders.

Several neurotropic viruses have been implicated in the onset esophageal achalasia, gastroparesis, enteric dysmotility, intestinal pseudo-obstruction and chronic constipation because of their ability to disrupt the integrity of the ENS. Among of possible candidate, affecting a significant majority of the world population, herpes simplex virus 1 (HSV-1) represents one of the most studied herpesviruses and that show several interesting features as pathogen involved in ENS neuropathies. Since it is able to establish a persistent neuronal infection, stimulate inflammatory infection and interfere with innate/adaptive immune responses.

Latent HSV-1 has also found in humans in the nodose ganglia innervating the gastrointestinal tract (Gesser 1996). Moreover clinical studies suggests that infection of human gastrointestinal sensory nerves, probably through swallowed viral-loaded oral secretion, may occur and eventually HSV-1 reactivating from theses site may play a role in gastrointestinal disorders (Tracy C. et al. 2009). Other study documented the presence of herpes virus DNA in children with acute appendicitis, suggesting that possible viral infection or reactivation is associated with childhood appendicitis (Katzoli P. et al. 2009). Furthermore, is common in all primary inflammatory neuropathies that infiltrate in the myenteric plexus is predominantly lymphocytic (Gesser R and Koo S. 1997).

In previous studies lymphocytes reactive to HSV-1 antigens have been detected in the esophageal myenteric plexus of patients with primary achalasia (Facco M, Brun P. et al. 2008) whereas altered intestinal contractility and transit has been described in rats infected orally with HSV-1 (Brun et al. 2010). Although it has been shown that HSV-1 elicits the innate immune response with controversial consequences in the central nervous system, there are no data regarding the mechanisms involved in the viral recognition and viral-mediated damage during the ENS infection.

Section 2: The Herpesviridae

2.1 Definition and classification of herpesviruses

The *Herpesviridae* family is constitute of a large double-stranded DNA viruses characterized by present of envelope. The word "herpes," derived from the Greek word *herpein*, means "to creep" and reflects the ability of these viruses to cause lifelong infection in their hosts by entering a latent, quiescent state after primary infection. (Basking et al 2007).

Based on both phylogenetic and biological attributes, *Herpesviridae* are currently divided into three subfamilies of viruses infecting mammalian, reptilian and avian hosts. (Davison et al 2005).

- *Alphaherpesvirinae*: (including herpes simplex virus types 1 and 2, varicella zoster virus and pseudorabies virus). These viruses show a neurotropism and establish latency in neuronal ganglia.

– *Betaherpesvirinae*: (including human cytomegalovirus, guinea pig cytomegalovirus, human herpesviruses -6 and -7 and murine cytomegalovirus). These viruses establish a latent infection in monocytes and spleen cells.

– *Gammaherpesvirinae*: (including Kaposi's sarcoma associated herpesvirus and Epstein Barr virus). Viruses in this subfamily replicate and persist in lymphoid cells but some are capable of establish lytic replication in epithelial or fibroblast cells. The host cell range of these viruses is more limited than either of the other two sub-families (Higgs MR. 2008).

2.2 Features of *Alphaherpervirinae*

Among human alphaherpesviruses, *herpes simplex viruses* (HSV)-1 and HSV-2 cause common, self-resolving diseases of the skin or mucosa, and concurrently establish a persistent latent infection of neuronal nuclei in the sensory ganglia innervating the peripheral site of infection. The third *alphaherpesviruse*s, the *varicella zoster virus* (VZV), following an initial replication phase in respiratory tract reach the skin and establish latency in dorsal root ganglia innervating a dermatome. All three viruses may subsequently reactivate to cause recurrent disease (Kinchington et al.2012).

Herpesviruses have a unique four-layered structure: a core containing the large, double-stranded DNA genome is enclosed by an icosapentahedral capsid originated from both host cellular membranes and viral glycoproteins. A protein coat called tegument encloses the capsid. (Richard J. Whitley, 1996).

A common characteristic shared by human herpesviruses is that after primary infection a persistent lifelong latent phase developed. One reason behind this coexistence between the herpesviruses and their host is a profound ability of these viruses to modulate the host immune response.

The function of the majority of proteins encoded by each of the large herpesvirus genomes is to interfere and interact with different immune effectors, thereby promoting viral persistence in its host (Goodrum et al. 2008; Gianella S. et al. 2012).

Human herpesviruses are ubiquitous agents distributed worldwide. HSV-1, VZV, and EBV are found in 80–99% of the adult population in all countries while the seroprevalence of CMV infection ranges from 50% in Western Europe to 99% in African and Asian countries (Ekstrand MI et al. 2008).

2.3 Life-style Herpes Simplex Virus

HSV-1 and HSV-2 characterized by variety of host range, brief replication cycle, ability to replicate in infected cells causing cell death but also to establish chronic latent infection. Moreover these viruses exhibit exclusive infective properties that influence pathogenesis and subsequent human diseases. Generally, following the initial infection of muco-epithelial cells, HSV invade the sensory nerve fibers innervating the mucosal membranes and reach the neuronal nuclei in sensory ganglia by retrograde axonal transport establishing a latent infection. HSV-1 latency can be defined as a quiescent state in which the viral genome is maintained as episomal DNA in neuronal nuclei for prolonged periods without production of infectious virions (Whitley RJ 1996; Farrell et al. 1991).

However, HSV during its life cycle, can interchange the latent cycle in neuron ganglia with lytic cycle in epithelial cells and fibroblasts (Kinchington et al. 2012).

In the lytic program, the productive replication cycle of HSV takes approximately 24 hours. During replication, the transcription of viral mRNA and DNA synthesis occur in the nucleus of host cells and the HSV genes expression is strictly regulated by events that are triggered in cascade.

Four glycoproteins of envelope are necessary and sufficient to permit virus fusion with the plasma membrane of the host cell: gB, gD, gH, gL. Initial interactions are mediated by heparan sulfate proteoglycans (HSPGs), which interact first with gB and/or gC (O'Donnell et al. 2009). While gC improves HSV binding it is not essential for entry. An additional receptor for gB, are the paired immunoglobulin-like cellular receptors, expressed by monocytes, macrophages, and dendritic cells, that play a role in both binding and fusion of virions (Campadelli-Fiume G. et al. 2007). The next step, the cell entry, requires the specific interaction between gB and gD receptors that include nectin-1 and -2, herpes virus entry mediator (HVEM) and 3-O sulfated heparan sulfate. Most investigators agree that during membrane fusion, gD undergoes a conformational change after receptor binding and then interacts with a heterodimer composed of gH/gL. Fusion domains of the gH/gL complex and gB enable the virus to penetrate into cells. These glycoproteins are also the major targets of humoral and cellular immune responses and viral immune escape mechanisms by binding both complement and anti-HSV IgG molecules (Riley LE. 1998). Upon entry, the viral envelope, capsid and tegument proteins are released into the cytosol. The nucleocapsid travels to the nucleus and release of viral genome. Once inside the cell,

the viral tegument proteins VHS and alpha-TIF start to take over host cell functions. These proteins are important in the initiation of the lytic program. Indeed, viral protein α-TIF also known as VP16, forms a complex which promotes Immediate Early-genes (IE) expression (O'Donnell et al. 2009; Campadelli-Fiume G. et al. 2007).

The IE genes code five α-proteins, designated infected cell polypeptides (ICP) 0, 4, 8, 22, 27, and 47. ICP0 and ICP4 are potent trans-activators of early (E) and late (L) viral proteins, and ICP22 and ICP27 modulate the trans-activating activity of ICP0 and ICP4 (Hagglund R et al. 2004). Moreover, ICP22 and ICP47, have been reported to be involved in evasion of immune recognition by both CD4+ and CD8+ T cells, respectively (Morrison L. et al. 2004). This first phase of gene transcription is followed by the expression of the Early genes, or β-genes, that are either directly or indirectly involved in viral genome replication. Early genes encode polypeptides like thymidine kinase and DNA polymerase, whose expression correlates with viral DNA synthesis. These genes are required for cleavage of viral concatemeric DNA into unit-length genomes and packaging of the virus genomes into preformed capsids (Sheaffer AK.et al. 2001). Finally, upon genome replication, viral structural proteins expressed in high abundance during the late phase. These proteins are involved in assembling the viral capsids: it assembled in the nucleus, and a newly formed nucleocapsid will pass through the nuclear membrane, which contain the viral glycoproteins. Indeed the new virions synthetized fuses with the plasma membrane to release the virus into the extracellular space or to enable cell-to-cell spread. These phases of viral replication are summarized in Figure 2.
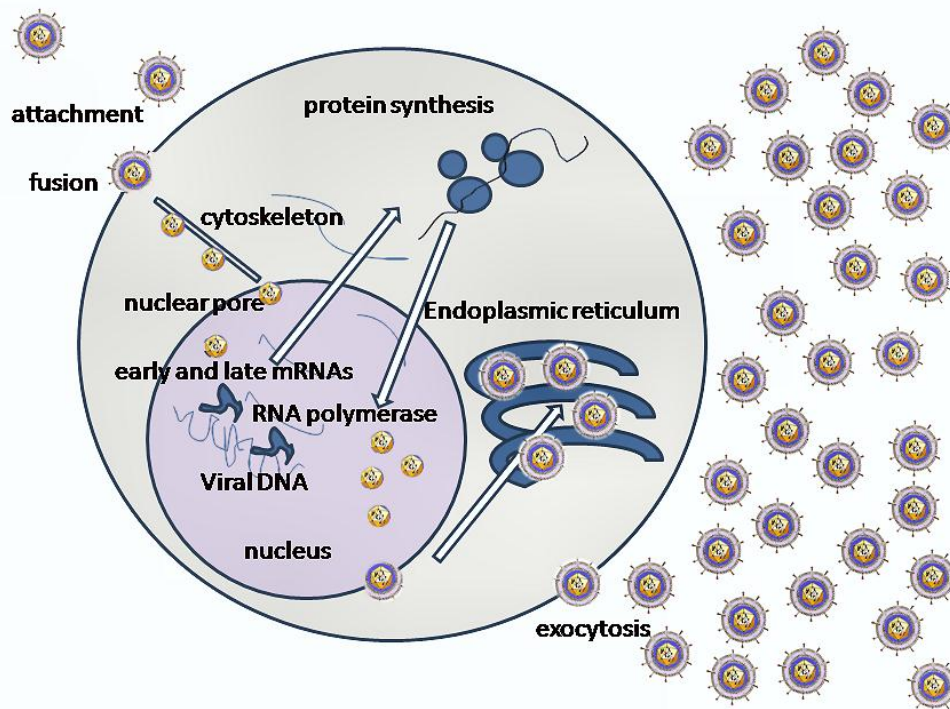
Figure 2. The Herpes simplex virus replication

In contrast to the lytic programme, the only transcripts readily detectable during latency are the latency-associated transcripts (LATs) (Wagner & Bloom, 1996). These have been detected in latently infected neuronal tissues from experimentally infected animals and following natural infection in humans (Stevens et al., 1987).

It is proposed that LAT has an anti-apoptotic activity that could result in a bigger number of neurons surviving infection and therefore favouring establishment of latency. Furthermore, recent data showing that a miRNA encoded by the HSV-1 LAT gene prevents neuronal apoptosis during latency establishment and/or reactivation (Gupta et al., 2006).

Moreover, LAT has been proposed to block IE gene expression (Mador et al., 1998). Studies in the mouse using sensitive RT-PCR, however, demonstrated that transcripts from the ICP4 and thymidine kinase (TK) regions of the genome could be detected in ganglia during latency (Kramer et al., 1998). In particular, evidence led to the hypothesis that the viral TK is required primarily for reactivation from the latent state and not for the establishment or maintenance of latency (Tenser R.B. 1991; Margolis et al., 1992)

In addition, HSV-1 antigen positive neurons were detected and these cells were surrounded by an immune infiltrate. A limited number of neurons seem to support viral gene expression

in the mouse, since IFNγ and CD8+ T cells are present in murine ganglia at latent times to maintain latency (Cantin et al., 1995; Khanna et al., 2003; Chew T. 2009). Therefore, although the majority of latent genomes are maintained in non-transcribed state, the possibility exists that some neurons express HSV-specific proteins and are prevented from producing virus by host's immune responses.

2.4 Herpes simplex and associated infection

Following initial lytic replication at the post of entry, HSV-1 establishes a latent infection in the nuclei of sensory neurons innervating the area of initial exposure, typically the trigeminal ganglion. Following reactivation, HSV-1 reaches the oral mucosa and is transmitted to other individuals through physical contact (Nicoll P. et al. 2012).

The vast majority of HSV-1 infections are oral herpes (infections in or around the mouth, sometimes called orolabial, oral-labial or oral-facial herpes), but a proportion of HSV-1 infections involve central nervous system (Preston CM. 2007). After the neonatal period, most cases of herpes encephalitis are caused by reactivation of HSV-1 (Soares B. and J.M. Provenzale 2015) in immunocompromised host and common symptoms are confusion, fever, and seizures and can be fatal. In addition, HSV-1 can cause both stromal keratitis (HSK) that is a primary cause of corneal blindness and ocular lesions in developed countries. In this case, the infection causes a painful sore, tearing, sensitivity to light and blurred vision. Over time, particularly without treatment, the cornea can become cloudy, causing a significant loss of vision (Herpetic Eye Disease Study Group 1998; Shoji H et al. 2002).

HSV-2 causes primarily anogenital infections. HSV-2 is transmitted to newborns primarily during peripartum period by infected mothers as a result of disrupted membranes, or by direct contact with the mother's vaginal secretions (Karasneh and Shukla 2011). Genital herpes represent a significant problem in immunosuppressive patient causing peri-anal infection (with or without proctitis) in many HIV-infected men that have sex with men. Indeed, Genital herpetic ulcers are known to increase the risk of transmission of infected with HIV.

No current antiviral treatments can eradicate HSV infection, and treatment during primary oral or genital infection not are able to prevent latent infection of nerves. However, during relapses, antiviral drugs, such as acyclovir, valacyclovir, or famciclovir, can relieve distress and placate symptoms.

Section 2.2. Immune response associated HSV-1 infection

2.2.1 Innate immunity response to HSV-1 infection

The pathogenesis of HSV infections strongly influenced by both specific and non-specific host defense mechanisms. However, immunity reflects only one facet of a complex tripartite relationship among immune system, neurons, and virus (Whitley R. 2007)

The initial stages of HSV-1 infection influenced by innate immune mechanisms, such as the activity of type I interferons (IFN), macrophages and natural killer (NK) cells, which serve to limit early virus replication and spread.

The best-known sentinels are Pattern- Recognition Receptors (PRR). To date, three classes of PPRs have been discovered, namely Toll-Like Receptor (TLRs) that are membrane-associated receptors, Retinoic Acid-Inducible Gene (RIG-I), nucleotide- binding oligomerization domain (NOD) Like Receptors (NLRs) that are cytosolic receptors (Kurt-Jones et al. 2004; Trine H. Mogensen 2009).

The effectiveness of these innate immune mechanisms against acute infection could influence the number of neurons harboring virus and the number of viral genome copies per infected neuron: both important factors in determining the probability of reactivation (Divito S et al. 2004; Liu T. 2000). The first line of defense against incoming herpes simplex virions consists of TLR2 and TLR9, located in the plasma membrane and in endosomes, respectively. The virion components that trigger TLR2 are gH/gL and gB whereas TLR9 senses the DNA in endoplasmic compartment (Rasmussen et al. 2007). It is observed that TLR2 and TLR9 induce astrocytes and microglial cells to produce pro-inflammatory cytokines and chemokines and that, the absence of TLR2 expression leads to increased survival in mice model following intracranial infection with HSV-1 in mouse (Wang P.J et al. 2012)

More recently, the important role of the other innate immune effectors, both humoral and cellular, such as macrophages, pDCs, NK cells and γδ T lymphocytes has been re-emphasized, either in direct immune control or via modulation of adaptive immune responses (Kim M. 2012)

NK cells can bridge the innate and adaptive immune responses. DCs and NK cells often colocalize and are able to interact both at sites of inflammation and in lymph nodes (Strowigt T. et al 2008; Moretta A. 2005). In DC-NK cell interaction, cytokines, such as

IL-2, IFN-α, and IL-15 are important, but direct cell-to-cell contact seem to be essential in promoting full NK activation by DCs (Ferlazzo G. 2004). The NK subsets can also act as accessory APCs via upregulated HLA-DR and secretion of IFN-γ, thus enhancing CD4 T lymphocyte responses. Cross talk between DCs and NK cells in viral infection was reported. However, it not been fully explored how the trio of NK cells, DCs and CD4 T lymphocytes interact with each other, especially physically (Andrews DM. et al. 2005; Kim M. et al. 2012).

2.2.2 Adaptive immune response to HSV infection

Although innate immunity thought to play a predominant role in the resistance to HSV, adaptive immune response has a crucial role in maintenance of latency and limiting of viral spread. Clinical observation of immunocompromised individuals and laboratory investigations clearly implicate the host immune system, particularly cell-mediated immunity, in preventing HSV reactivation (Smith et al. 2013)

The concept of a silent HSV-1 latent infection where HSV-1 avoid immune elimination by hiding from the host immune system has been challenged by molecular and immunologic studies suggesting low-level expression of viral transcripts and localization of activated CD8+ T cells to latently infected neurons (Bloom DC. Et al. 2010; Liu T. et al. 2000; Jeon S. 2013).

The adaptive immune system consists of two broad sets of antigen-responsive cells, the B and T lymphocytes. However, experimental evidences suggest that T cells are the major players in maintaining latency. In particular, CD4+ T cells appear to be the principal orchestrators of immunopathology (Kim M. et al. 2012).

In the human herpetic lesions and in murine models was shown that the infiltrate contains mostly CD4+ lymphocytes during the first few days and equal numbers of CD4+ and CD8+ lymphocyte in later lesions. HSV-specific CD4+ lymphocyte proliferative responses and natural killer (NK)-like cytotoxic responses are present at all stages of herpetic lesions (Kim M. et al. 2012). The selective accumulation of HSV-specific CD8+ T cells in the acutely infected TG appears to be due to discriminatory activation of these cells in the draining lymph nodes (Khanna KM. 2003; Leger A. et al. 2011).

IFN-β and β chemokines probably attract monocytes and CD4 and CD8 T lymphocytes into the lesions. IFN-α/β and IL-12 may induce Th1 patterns of cytokine responses and HSV Ag-stimulated CD4 (and CD8) T lymphocytes producing especially IFN-γ (Mikloska ZM. et al. 2000; Krug A. et al.2004).

The antiviral effects of CD8+ T cells been well described, with IFN-γ playing a crucial role in this process (Posavad CM. et al.1997). CD8 cells are generally assumed to kill their target cells by membrane disruption and DNA fragmentation, caused, respectively, by perforin and granzyme B (Gzm B) proteins that are contained within the cytoplasmic granules of cytotoxic lymphocytes (Pereira R. et al. 2000).

Coincident with the initial CD4+ infiltration is the appearance of IFN-γ in infected cells. IFN-γ been shown to increase the susceptibility of infected keratinocytes to CTL-mediated lysis; this effect is maximal at the time when CD8+ cells are infiltrating. Viral clearance from the lesion site is associated with a high level of local HSV-specific CTL activity. The CTL may strike early, before virions produced and before cells disabled by infection. Thus, CTL may be containing the spreading lesion (Klein RS. 2011). In addition, IFN-γ appears to counteract the blocks to antigen presentation mediated by VHS and ICP47, therefore enhancing recognition by CTL (Seliger B. et al. 2005). This may be because IFN-γ upregulates the expressions of class I, TAP, and other components of the antigen presentation pathway (Belicha-Villanueva et al. 2008). The specific signals that regulate the communication between neurons and CD8+ T cells and coordinate an appropriate immune response remain elucidated. Two new exciting areas of research, the neuro-immunologic synapse and neuro peptide influence on the immune system, represent promising avenues of investigation in this field.

2.2.3 Role of Costimulatory Signaling (CS) in the activation of T cells

The antigen-specific response to a viral pathogen is initiated when a T cell recognizes a viral peptide presented in the context of major histocompatibility complex (MHC) on antigen-presenting cells (APC). This primary signal results in the activation of the T cell, the extent of activation being a function of both the affinity and duration of this interaction (Iezzi, G. et al. 1998; Kunding T.M. et al.1996).

Furthermore, several studies show that interactions between CD80 (T-lymphocyte activation antigen CD80) and CD86 (T-lymphocyte activation antigen CD86) expressed on surface of mature antigen-(APCs) presenting cells and CD28 on naive T cells serve as an important costimulatory signal (CS) in the activation of T cells. These molecules are expressed either constitutively or upon activation, since brief primary signals can result in insufficient T-cell activation unless augmented by costimulatory signals (Edelmann K. and Wilson C. 2001). Indeed, engagement of CD28 mediates recruitment of lipids to the

immunological synapse, which lowers the activation threshold of the T cell and modulate the activity of responding CD4+ and CD8+ T-cells (Alegre M. et al. 2001).

However, once T cells are activated, they upregulate expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4), another receptor for CD80/CD86. CTLA-4 negatively regulates T-cell responses by the following mechanisms: sequestering CD80 and CD86 away from CD28 by its high affinity to these molecules (Fuse S. et al. 2006). The CTLA-4 co-inhibitor competes with CD28 for binding to CD80 and CD86. However, CD80 can also bind to PD-L1 (B7-H1) and deliver a co-inhibitory signal (Ford M.L. et al. 2014).

CD28 and CTLA-4 blockade effectively inhibits naïve antigen-specific CD4+ T–cell, but incompletely controls the expansion of antigen-specific CD8+ T cell responses (Figure 3).
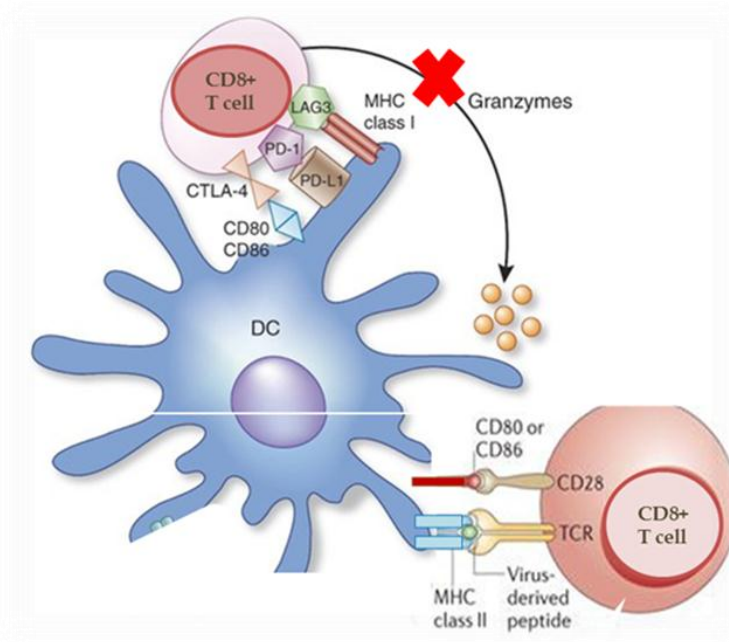
Figure 3. Co-stimulatory and co-inhibitory pathways on T-cells

Abbreviations: CD28, T-cell-specific surface glycoprotein CD28; CD80, T-lymphocyte activation antigen CD80; CD86, T-lymphocyte activation antigen CD86; CTLA-4, cytotoxic T-lymphocyte protein 4; PD-L1, programmed cell death 1 ligand 1.

Antiviral CD4+ and CD8+ T-cell responses are moderately dependent on this CS pathway. As previously demonstrated, CTLA4Ig treatment greatly reduced viral effects during primary acute HSV infection in murine model. This reflected an almost total ablation of the anti-HSV CD4 and CD8 T-cell responses due to anergy and reduced cell numbers, respectively (Edelmann K. and Wilson C. 2001).

Chapter 2: Aim

The pathophysiology of enteric neuropathies remains obscure. Although neurotropic viruses are attractive candidates there is no evidence that enteric neurons are damaged following a viral infection. HSV-1 is an attractive candidate to induced enteric neuropathies since efficiently infects neurons and is able to establish life-long infections, triggering local immune responses. Recently our research group has demonstrated in a rodents model the ability of this virus to reach the ENS and to cause the onset of complex neuromuscular abnormalities characterized by impaired contractility and intestinal transit (Brun et al., 2010).

The purpose of this doctoral thesis were two folds:

I)   To verify whether *α-herpesvirinae* are able to reach human ENS;

II)   To clarify the role of adaptive immune responses in neuromuscular dysfunctions following HSV-1 infection.

Chapter 3. Materials and Methods

3.1. Patients involved in study

A prospective cohort of 121 patients was enrolled to analyze the presence of viral Herpesviridae DNA. Intestinal specimens were obtained from patients with Crohn's disease, ulcerative colitis, diverticular disease or colonic cancer carcinoma undergoing to surgical resection. The study was performed according to the second principles of the Declaration of Helsinki, and all those participating gave informed consent and specific institutional review board approval has been obtained. Characteristics of patients are outlined in Table 1 and 2.

Table 1. Patients involved in study on ileal surgical resections

Disease	Total cases	M	F	Median age (Years)
Crohn's disease	56	39	17	46
Colonrectal carcinoma	24	13	11	63

Table 2. Patients involved in study on colonic surgical resection

Disease	Total cases	M	F	Median age (Years)
Crohn's disease	10	9	6	74,5
Colonrectal carcinoma	11	5	1	33,5
Diverticular disease	12	4	8	60
Ulcerative colitis	8	5	3	65

3.2. Genomic DNA extraction from tissue

Full thickness intestinal specimens were obtained from patients undergoing surgery. The mucosa and the underlying muscle layer were carefully dissected using micro scissors. Then, the mucosal and muscle layers were embedded in Optimal Cutting Temperature (OCT- Kaltek srl) and placed at -80°C. Full thickness ileal and colonic specimens in patients with colorectal carcinoma were obtained at least at 10 cm from the tumor, from a macroscopically normal area. Eight to ten sections (16 µm thick) were cut from each sample using a cryostat. Sections were placed in 300 µl of Lysis Buffer (1,25% p/v Sucrose,0,3% Nonidet P-40, 10 mM NaCl, 3 mM di $MgCl_2$, 20 mM di Tris-HCl pH 7.4). Samples were lysed under denaturing conditions with 62,5 µl Sodium Dodecyl Sulfate (SDS) 10% p/v and 500 µg proteinase K and incubated at 56°C for 30 minutes.

The extraction of DNA was performed by phenol-chloroform method. Chloroform mixed with phenol is more efficient at denaturing proteins than either reagent alone. The phenol-chloroform combination reduces the partitioning of poly(A)+ mRNA into the organic phase and decreases the formation of insoluble RNA/protein complexes at the interphase. (24) In the presence of phenol, the hydrophobic cores of macromolecules interact with phenol causing the precipitation of proteins and polymers (including carbohydrates) that collected at the interface between the two phases (often looking as a white flocculent area) whereas lipids dissolve in the lower organic phase. The pH of phenol determines the partitioning of DNA and RNA between the organic phase and the aqueous phase.

An equal volume of phenol-chloroform (200 µl) was added to the aqueous solution of the homogenized tissues (1:1 v/v), and mixed for 20 minutes at room temperature (RT). Then, the samples were centrifuged at 1000 g for 20 minutes at RT and yielded two phases. The upper aqueous phase containing the DNA was collected and transferred into a sterile microcentrifuge tube (Eppendorf) and NaCl to a final concentration of 200mM was added. Afterward, the products were precipitated by addition of ethanol 96% (v/v) and centrifugation at 13000 rpm for 20 minutes. The precipitated DNA was then suspended in sterile water and the concentration was determine by measuring OD at 260 nm using a NanoDrop 200C spectrophotometer.

3.3. Quantitative Real-time Polymerase Chain Reaction (PCR)

To detect the presence of Herpes virus type I in the specimens we used real time PCR. This technic is made possible by including in the reaction mixture a fluorescent molecule that generates a proportional intensification in the fluorescent signal proportional to the increase of DNA in each cycle. The most commonly used DNA-binding dye for real-time PCR is SYBR Green I, which binds nonspecifically to double-stranded DNA (dsDNA). Therefore, the overall fluorescent signal from a reaction is proportional to the amount of dsDNA present. After completion of the amplification reaction, a melt curve is generated by increasing the temperature in small increments and monitoring the fluorescent signal at each step to verify the specificity of the amplification reaction.

A SYBR Green assay uses a pair of PCR primers that amplifies a specific region within the target sequence of interest and includes SYBR ® Green I dye for detecting the amplified product.

Real-time polymerase chain reaction (PCR) was performed using 50 ng of extracted DNA in 20 µl reaction volume with 10 µl of SYBER Green PCR Master Mix and 20 mM of mix primers in a ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Cycling condition were as follow: stage 1, 95°c for 10 min, and stage 2, that was repeated for 40 cycles, consisted of 2 steps: 95°C for 15 seconds, temperature of annealing (TA) for 1 minutes. The reactions were carried out in triplicates. The number of copies of viral genome in each sample was normalized to the number copies of HUMB human gene. Primers and conditions used are summarized in Table 3. Agarose gel elctrophoresis was performed to visualize RT-PCR products amplified.

Table 3. Human primers used for Real Time PCR

Gene	Sequence	Amplicon size (bp)	T_A
HUMB	F 5'-AGGCAGAGGGAGATTGACTG 3' R 5'-AACCTGTGGCTTCCATTGTC-3'	63	59 °C
HSV family	F 5'-TTCTGCAGCTCGCACCAC-3' R 5'-GGAGCGCATCAAGACCACC-3'	110	60 °C
VZV	F 5'-GAGTACGAGACCCCAAATCG-3' F 5'-TAAAGCTGGGGCCTTTTGTA-3'	100	60 °C

TA= Annealing temperature; F=forward; R=reverse

3.4 Herpes simplex virus 1 preparation

HSV-1 strain 17 was used throughout our studies. Viral stocks were prepared and assayed on Vero cells cultured in modified Dulbecco's medium containing 10% heat inactivated foetal calf serum (FCS) (Altavilla G. et al.; 2002).

To propagate the virus, HSV-1 infections were carried out in RPMI containing 1% heat-inactivated FCS for 2 h at 37 °C in 75 cm^3 flask. After 24 h post infection, Vero cells were collected and lysate by three rounds of freeze-thawing to release viral particle in the supernatant.

Concentration of viral particles was determined by serial dilution technique on Vero cells and reported as plaque-forming units (PFU)/ml. For animal infection, aliquots of viral stocks (10^8 PFU/ml) were stored at -80°C. Male C57/Bl6J mice were infected by intranasal instillation of HSV-1 (10^3 PFU). At least four weeks after the IN inoculum healthy mice received via intragastric gavage of HSV-1 (10^8 PFU).

For stimulation of cultured lymphocytes, viral aliquots (10^8 PFU/ml) were subjected to ultraviolet inactivation (30W UV light; exposure for 5 min). Absence of replication-competent viruses was confirmed in Vero cells. As control, cells were stimulated with equal volumes of conditioned media from mock-infected Vero cells.

3.5. In vivo model of HSV-1 infection of ENS

In this model C57/Bl6J (WT) mice, purchased from Harlan Laboratories S.r.l., were subjected to a double inoculum of the virus. The animals used in the study were housed in the Department of Biomedical Sciences and the Department of Molecular Medicine. The mice were maintained in a controlled environment at room temperature (22 ± 2 °C) and humidity, with a light / dark cycle of 12 hours. The animals had free access to food and water during the study. Male mice 8 –10 weeks old were slightly anesthetized and inoculated with HSV-1 by intranasal (IN) instillation 10^3 plaque-forming units (PFU) contained in 20 µL, by mean of a micropipette. This inoculation route ensures infection of the central nervous system (CNS) through the olfactory nerves and avoids, in the large majority of animals, signs of acute encephalitis. Following the IN inoculum mice were monitored for the appearance of signs of encephalitis in the following 4 weeks. Less than

2% of mice showed signs of disease and were immediately sacrificed. At least four weeks after the IN inoculum healthy mice received via intragastric gavage (using a 24 gauge, 9-cm catheter) 10^8 plaque-forming units of HSV-1 in 100 µl PBS (Figure 4). Animal were monitored for the following 10 weeks for the appearance of signs of systemic infection (i.e. reduced mobility, food avoidance, loss of weight). Animals were sacrificed by cervical dislocation 1 to 10 weeks after the IG viral inoculum. At the same time control animals were injected with equal volume of Vero cells lysate to induce a sham infection and were sacrificed at matching time points.

All experimental protocols were approved by the Animal Care and Use Committees of the Universities of Padua and Bologna, and were in compliance with the national and European guidelines for handling and use of experimental animals.
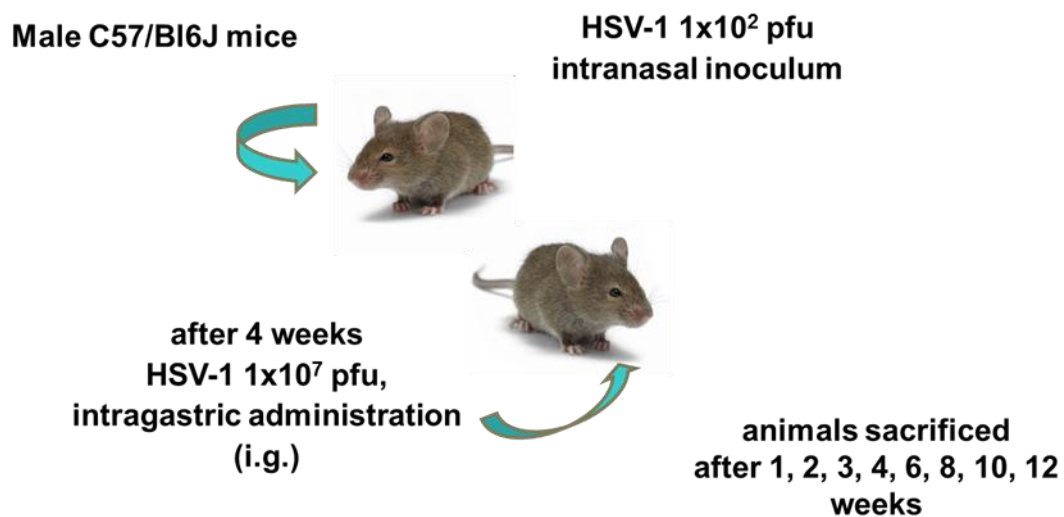
Figure 4. HSV-1 infection in the gut murine model

3.6. Isolation of mononuclear cells from LMMP

Mice were sacrificed at different time post-IG inoculum. The abdomen opened, the whole ileum was removed in sterile condition and placed in sterile ice cold RMPI. Then, the ileum was washed with ice cold sterile RMPI and cut in 6-8-cm pieces. The pieces of ileum were placed on sterile glass rod and the longitudinal muscle layer with the attached myenteric plexus (LMMP) was delicately peeled off using a microdissection scissors and placed in Hank's Balanced Salt Solution (HBSS) on ice. The LMMP was then finely cut with sterile

scissors and then subjected to enzymatic digestion using collagenase type IV (14 mg/ml, Gibco) and DNase (10 mg/ml, Sigma) for 30 min at 37°C. Following enzymatic inactivation, undigested tissue was removed by using a cell strainer and the single cell suspensions were centrifuged (1600 rpm x 8 min) and then resuspended in four ml of sterile RPMI 1640. The cellular suspensions were carefully stratified on the top of 3,5 ml of Ficoll-PaqueTM PLUS (Sigma Aldrich), avoiding to mix the two phases, in 15 ml centrifuge tubes. Tubes were then centrifuged (2000 rpm x30 min without brake, to avoid damage of the cellular layers). The mononuclear cells localize at the interface of RMPI 1640 medium and the Ficoll, whereas dead cells and debris localize to the bottom of the tube. The mononuclear cells forming the visible ring at the interface of medium and Ficoll were carefully collected using a serological pipette, transferred to a fresh tube containing 10-14 ml of sterile RMPI. Cells were then centrifuged (1600 rpm x 8 min) and the resulting pellet resuspended in the appropriate medium.

3.7. Flow Cytometry

Mononuclear cells were isolated from the LMMP of mice 1-10 weeks post IG inoculum, as described in para 4.7. Cells (106 cells/ml) were placed in ice cold in ice cold FACS buffer (PBS+2%FBS) and 0.5 x106 cells were used for each staining condition.

To determine the proportion of T-cells (CD3$^+$) subpopulations, we incubated cells with 1µg/ml of anti-murine CD3 (APC labelled) and anti-murine CD4 (FITC labelled) or anti-murine CD8a (PE labelled). Cells were incubated for 30 minutes at 4°C in the dark. Then the cells were washed 3 times by centrifugation at 1500 rpm for 5 minutes and resuspend in 200µl of ice cold FACS buffer. Flow cytometric analysis was performed by using a FACScalibur based on a CellQuest software (Becton Dickinson) acquiring at least 10^4 events.

3.8. Stimulation in vitro of lymphocytes

To verify the presence of HSV-1 reactive lymphocytes infiltrating the LMMP at different time post IG HSV-1 inoculum, we performed ex vivo challenge studies. Mononuclear cells were isolated from the myenteric plexus as described above (para 4.7) and placed in RPMI 1640 (Invitrogen Life Technologies) containing 10% FBS and 1% penicillin-streptomycin. Cells were cultured for 12 hours at 37°C in presence or absence of UV-inactivated HSV-1 at a final concentration of 10µg/ml. Then, Brefeldin A (GolgiPlug, BD Biosciences), that

inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus preventing formation transport vesicles, was added at 1µg/ml final concentration to each well, for the final 4 hours of stimulation.

Cells were then collected, centrifuged (1600 rpm x8 min) and fixed in 0.01% formaldehyde for 10 minutes. Samples were centrifuged, cells resuspended in FACS buffer and stained with anti-CD4 (FITC labelled) and anti-CD8 (PE labelled) and incubated on ice for 20 minutes. After three washes cells were suspended and incubated in permeabilization buffer (0.1% Triton in PBS) with anti-IFN-γ (FITC labeled) or anti IL-4 (PE labeled). Unbound antibodies were removed by centrifugation (1600 rpm x 8 min) and suspending cells in 100 µl of FACS buffer. Data were acquired on a BD FACSCalibur flow cytometer.

Table 3. Murine antibodies used in flow cytometric analysis

Antigen	Coniugated	Clone	Source	Isotype
IFN-γ	FITC	XMG1.2	eBioscience	Rat IgG1, k
IL-4	PE	11B11	eBioscience	Rat IgG1, k
CD4	FITC	GK1.5	BioLegend	Rat IgG2b, k
CD8a	PE	53-6.7	BioLegend	Rat IgG2a, k
CD3	APC	17A2	eBioscience	Rat IgG2b, k

FITC= Fluorescein isothiocyanate; PE= Phycoerythrin; APC=Allophycocyanin.

3.9. RNA extraction of intestinal tissue

RNA is an unstable molecule due to the ubiquitous presence of RNases which are enzymes present in all tissue and has a very short half-life once extracted from the cell or tissues, therefore special care and precautions are required for RNA isolation. Strong denaturants has always been used in intact RNA isolation to inhibit endogenous RNases.

The extraction of RNA includes several steps which include lysis of plasma membranes by nonionic detergent, a denaturation of protein, removal of protein residues and recovery of cytoplasmic RNA by ethanol precipitation.

Total RNA from LMMP of control mice and mice at different time post-HSV-1 IG infection was extracted using the E.Z.N.A.® Total RNA Kit I (Omega).

The tissues (~30 mg) were placed in 350 µl di TRK Lysis Buffer plus the reducing agent β-mercaptoethanol (20µL/mL of TRK Lysis Buffer) and homogenized for 5 minutes by using metallic beads at 20 Hz (Mixer Mill MM300, Qiagen). The tissue lysate was centrifuged for two minutes at 13000 rpm to remove cellular debris. The clear supernatant was transferred in a clean 1.5 ml microcentrifuge tube and an equal volume of 70% ethanol was added. Samples were then applied to mini-column containing the HiBind® matrix able to binds total RNA. Cellular debris and other contaminants were washed away using several washes with 250 µl of RNA Wash Buffer I. To remove contaminating DNA, the columns were incubated with 75µl di DNasi DNA-free™ Kit (Ambion) at room temperature for 2 minutes. Finally, the The HiBind® RNA Mini Columns were washed with 500µl of RNA Wash Buffer II and total RNA was eluted in DEPC water. Total RNA was stored at -80°C. To determine the concentration of RNA the absorbance at 260 nm was obtained using a spectrophotometer (NanoDrop® ND-1000) whereas RNA purity was assessed by calculating the A260/A280 ratio.

3.10. Retrotranscription (RT) of RNA

Reverse transcription is the process by which a reverse transcriptase enzyme converts RNA into complementary DNA (cDNA). The most commonly used reverse transcriptase is the M-MuLV reverse transcriptase from the Moloney murine leukemia virus. The process is priming dependent and functions at a temperature of 40°C to 50°C, depending on the properties of the reverse transcriptase used. Native reverse transcriptases are multifunctional enzymes. Their DNA polymerase activity allows the transcription of ssRNA and ssDNA. In addition, the endogenous RNase H activity in some reverse transcriptases leads to the degradation of the RNA from an RNA:DNA hybrid. The degradation of the original RNA is a crucial issue for the quality of the cDNA in the subsequent PCR step.

The synthesis of complementary DNA (cDNA) was performed in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 5.5 mM MgCl2, 2 mM dNTPs, 2.5 µM random hexamers, 60 U RNAsi inibitor, 1.25 U MuLV, 200ng of extracted RNA and water MilliQ for a final reaction volume of 20 µl. Samples were placed in a thermocycler to run retrotranscription

(Mastercycler Personal, Eppendorf ®). Cycling condition were as follow: stage I: 25°C for 10 minutes; stage II: 48°C for 60 minutes; stage III: 95°C for 5 minutes.

3.11. Quantification of mRNA levels by Real Time PCR

Levels of mRNA were measured by Real Time RT-PCR. The cDNA template was amplified in the quantitative step, during which the fluorescence emitted by intercalating dyes increases as the DNA amplification process progress.

PCR reaction have been carried in a 20μl of volume with 10 μl SYBER Green PCR Master Mix, 20 mM of mix primers and 3 μl of cDNA template. To study the HSV-1 replication cycle in myenteric ganglia, we used primers (described in Table 4) for immediate early gene (infected cell protein, ICP0 and ICP4), for late gene (VP16 and gC) and latency associated transcripts (LATs). The thymidine kinase (TK) gene was determined on genomic DNA, according to genomic DNA extraction described in the paragraph 3.2., amplified in Real Time PCR and murine β-globin was used for normalization as an internal control (primers used were described in Table 5).

To analyze the expression pattern of activation immunity, specific mRNA transcripts of CD80, MHC-I, MHC-II, CD28, CD69, CTLA4, H2-Q9, IL-10, IFNɣ, Granzyme B (GMZB), PDCD-1, PFPL were quantified using primers and conditions listed in Table 5 in ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Gene specific primers were designed using conventional parameters and specificity of the primers were determined by comparison to the Gene Bank database using the Based Local Alignment Search Tool (BLAST). GAPDH was used for normalization as an internal control. Data are presented as a mean fold change over the control mice.

Table 4. Viral primers used in Real Time PCR

Gene	Sequence	Amplicon size (bp)	T$_A$
ICP0	F 5'-CCCCTTACTCACACGCATCT-3' F 5'-GATCCCGACCCCTCTTCTT-3'	157	62°C
ICP4	F 5'-ATGGGGTGGCTCCAGAAC- 3' R- 5'CTGCCGGTGATGAAGGAG-3'	223	60°C
VP16	F 5'- TACGCGAAGATCCTTGGTTGG-3' R 5'- AGGATGCCGGTTTCGACATTG-3'	128	56°C

LAT	F 5'- GACAGCAAAAATCCCCTGAG-3' R 5'- ACGAGGGAAAACAATAAGGG-3'	175	56°C
gC	F 5'- ATTATCGGCGAGGTGACG-3' R 5'-GTACTCGTGCGGGCTGTC -3'	224	50°C
HSV-TK	F 5'- TAGCCCGGCCGTGTGACA-3' R 5'- CATACCGGAACGCACCACACAA- 3'	215	62 °C

Table 5. Murine primers used in Real Time PCR

Gene	Sequence	Amplicon size (bp)	T_A
GAPDH	F 5'-AGTGCCAGCCTCGTCCCGTA- 3' R 5'-CAGGCGCCCAATACGGCCAA- 3'	71	56°C
CD28	F 5'-ATATCTACTTGGTTTGCTCTTACTTCC- 3' R- 5' AATAAAACAAGGCATGGAGACAGG-3'	103	62°C
CD69	F 5'-GGTGAACTGGAACATTGGATTGGGC-3' R 5'-TCACGGACACGCACCTCCCA-3'	121	66°C
CD80	F 5'-CCCCAGAAGACCCTCCTGATAG-3' R 5'-CCGAAGGTAAGGCTGTTGTTTG-3'	172	62°C
CTLA4	F 5'-ACCTCTGCAAGGTGGAACTCA-3' R 5'-CCATGCCCACAAAGTATGGC-3'	51	60°C
MHCI H2K1	F 5'-GGCGGCGCTGATCACCAAACA-3' R 5'-GTGATGGGTCACATGGGCCTTTGG-3'	163	66°C
MHCII H2Ab1	F 5'-CCAGCCTCCTCCTCTCGGCTG-3' R 5'-TATCGTATGCGCTGCGTCCCG-3'	142	66°C
H2-Q9	F 5'-ATGGCGACCATTGCTGTTGT-3' R 5'-TCCAATGATGGCCACAGCT-3'	51	60°C
PDCD-1	F 5'-TTCAGGTTTACCACAAGCTGG-3' R 5'-TGACAATAGGAAACCGGGAA-3'	52	60°C
IL-10	F 5'-TGGACAACATACTGCTAACC-3' R 5'-GGATCATTTCCGATAAGGCT-3'	81	60°C
GZMB	F 5'-GAAGCCAGGAGATGTGTGCT-3' R 5'-GCACGTTTGGTCTTTGGGTC-3'	183	60°C
IFN-ɣ	F 5'-GCTTTAACAGCAGGCCAGAC-3' R 5'-GCACCAGGTGTCAAGTCTCT-3'	96	60°C
PFPL	F 5'GGGTTTATCAGTTGTGCCGTC-3' R 5'-TAGCAGATGGACAGGGGTGTAG-3'	128	60°C
β-GLOBIN	F 5'- AACTTGGTGTGAGTCCCT- 3' R 5'- GCAAGTGAAGCATGAGGA -3'	232	62°C

TA= Annealing temperature; F=forward; R=reverse

3.12. Immunohistochemistry (IHC)

Immunohistochemical analyses were performed using standard procedures. Four μm thick sections were cut from formalin fixed and paraffin embedded tissue specimens. Sections were deparaffinised and rehydrated (xylene 5 min; ethanol 100%, 95%, 70%, 1 min each), using standard protocols. Samples were then incubated in 10% H_2O_2 to block endogenous peroxidase activity, followed by antigen retrieval by treatment with citrate buffer, and then by incubation with universal blocking solution (Lab Vision Corporation, Fremont,CA). Subsequently, 1 hour incubation at 22°C was performed with either anti-CD3 and CD80 (Table 6). Tissues were extensively washed (3 times x10 minutes each) to remove unbound antibody and then the proper secondary antibody from the Dako Envision+ System-HRP labelled Polymer Detection system (Dako, USA) was incubated for 30 minutes with tissues. Specimens were washed extensively (3 times x10 minutes each) and then incubated with 3-3'diaminobenzidinetetrahydrochloride (DAB) as chromogenic substrate. Finally sections were counterstained with haematoxylin-eosin and mounted. As a negative control, sections were stained with either an isotype-matched antibody of inappropriate specificity or by omitting the primary antibody. TUNEL assay was performed using the TdT FragEL DNA Fragmentation Detection Kit (Merck Millipore) according to the manufacturer's protocol. Tissues were analyzed using an inverted microscope equipped with a high-resolution camera to capture the images

Table 6. Murine antibodies used for immunohistochemistry

Antigen	Dilution	Clone	Source	Isotype
CD3	1:100	Polyclonal	Santa Cruz Bt.	IgG_1
CD80	1:50	Polyclonal	Abcam	IgG_2

3.13. Whole mount staining

Ten cm long segments of the distal ileum were filled with fixative solution (4% PFA, 0.2% saturated picric acid in PBS) for 1 hour at 22°C. Under a dissecting microscope we prepared whole mount specimens containing longitudinal muscle-myenteric plexus (LMMP). For immunohistochemistry LMMP preparations were gently stretched and pinned down on a wax support, washed with PBS and 0.2% Triton X-100 (PBS-T) and incubated with the primary antibodies listed in Table 1 diluted in PBS-T and BSA 2%. Immunocomplexes were visualized by incubating samples with secondary antibody anti-rabbit IgG rhodamine labelled. Nuclei were stained with TOTO-3 iodide (Invitrogen) 1:100 diluted and LMMP preparations were mounted with Prolong Antifade kit and analyzed using a Leica TCSNT/SP2 confocal microscope (Leica Microsystems).

Table 7. Primary antibodies used in whole mountain preparation

Antigen	Dilution	Clone	Source
βIII-Tubulin (rabbit)	1:100	Polyclonal	Couvance
Peripherin (rabbit)	1:150	Polyclonal	Millipore

3.14. Monoclonal antibody purification

Monoclonal antibodies were produced by hybridomas (ATCC) in suspension culture in Iscove's Modified Dulbecco's Medium and fetal bovine to a final concentration of 10%.
The monoclonal anti-CD4 Ab (clone GK1.5 produced by a hybridoma assigned ATCC accession no. TIB-207) and the monoclonal anti-CD8 Ab (clone 2.13 produced by a hybridoma assigned ATCC accession no.TIB-210) were collected and purified using Protein G-Agarose (Thermo Scientific). The sample containing IgG were diluted at least 1:1 with Binding Buffer (phosphate-based contain EDTA, pH 8.0) and applied into the resina in column. Antibodies were eluted with 5ml of Elution Buffer (0.1M glycine, pH 2-3) and immediately eluted fraction were adjusted to physiologic pH by adding 100μL of the Neutralization Buffer (1M phosphate or 1M Tris pH 7.5-9) per each 1 ml of eluate. The

elution were monitored by measuring the absorbance at 280nm and by BCA Protein Assay Kit (Thermo Scientific).

3.15. In vivo depletion of CD4 and CD8 T cells

To better understand the role of different lymphocyte population in HSV-1 induced bowel dysmotility we performed depletion experiments. Mice were injected weekly intraperitoneally with the specific monoclonal antibody starting 4 weeks before the planned sacrifice. Mice received either 200 ng of anti-CD8 mAb and anti-CD4 mAb resuspended in PBS. The last injection of monoclonal antibody was performed six days before sacrifice. To confirm depletion of the desired cell population 100 µl of total blood was collected and subjected to immune staining for CD3+CD8+ or CD3CD4+ cells. Samples were analyzed using a Becton Dickinson (San Jose, CA) FACSCalibur analyzer and CellQuest. This treatment regimen routinely resulted in depletion of 80 to 85% CD8 T and CD4 T cells from the blood.

3.16. Isolation of T-cells subsets using magnetic immuno-sorting

The mononuclear cells isolated using the Ficoll gradient of the single cells suspension of LMMP were selectively selected using a magnetic separator (MACS®). The total cells suspension were incubated with MicroBeads conjugated to monoclonal anti-mouse CD4 antibodies (L3T4; isotype: ratIgG2b; MACS®) or MicroBeads conjugated to monoclonal anti-mouse CD8a antibodies (Ly-2; isotype: ratIgG2a; MACS®), to isolate respectively CD4+ T-cells and CD8+ T-cells infiltrated in LMMP.

The magnetically labelled CD4+ cells and CD8+ cells were retained on the magnetic separator whereas the unlabeled cells run through. After three wash of the column with PBS, the column was removed from the magnetic field and the magnetically retained cells were eluted as the positively selected cell fraction. The selected cell were resuspended in sterile condition in RPMI complete medium.

3.17. Adoptive T cell transfer

To characterize the role of CD3+CD4+ and CD3+CD4+ in the pathogenesis of HSV-1 induces dysmotility, we have performed adoptive cells transfer experiments. In these experiments either LMMP mononuclear cells or CD3+CD4+ or CD3+CD4+ cells were purified from donor mice and transferred to a recipient animal. As donor animals we used mice 8 or 10 weeks post-IG HSV-1 injection. As control we used mice injected IG with Vero cells lysate. Cells were injected in syngeneic C57Bl/6 mice 10 weeks old.

Mononuclear cells from LMMP were obtained by pooling together 2-3 infected mice, and cells were purified as described in paragraph 4.6. Each recipient animal received 5x105 cells by a single intraperitoneal injection.

CD3+CD4+ and CD3+CD8+ cells were immunopurified as described in paragraph above. Each recipient animal received 3-5x105 cells by a single intraperitoneal injection. The recipient mice were sacrificed 1 week post transfer of lymphocytes.

3.18. Western Blot

Whole thickness ileum without mucosa was homogenized in nondenaturing RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 0.25% wt/vol sodium deoxycholate, 0.1% Nonidet P-40, 100 μM NaVO4, 1 mM NaF, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Particulate material was removed by centrifugation. Protein concentration was determined in each sample by the Bradford method with a commercially available kit (Protein Assay Kit; Bio-Rad Laboratories, Hercules, CA). Aliquots of 30 μg-protein were separated through a SDS-polyacrylamide gel electrophoresis. Then transferred onto a nitrocellulose membrane (0.45 μm pore size in roll form, Millipore) and care was taken to remove all air bubbles. The electrophoretic blots were blocked in 5% bovine serum albumin (BSA) in in TBST (120 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.05% Tween 20) for 1 hr at room temperature to saturate additional protein binding sites. Membranes then were incubated overnight a 4°C with monoclonal anti-HSV-1/2gB. After the sheets were washed (about five changes during 30 min, total) and incubated with the proper horseradish peroxidase-conjugated secondary antibody. Bands were visualized using enhanced chemiluminescence (Millipore, Milan, Italy). Images were captured using a Hyper Film MP (GE Healthcare, Milan, Italy). To ensure equal loading and accuracy of changes in protein abundance, protein levels were normalized to β-actin.

Table 8. Primary murine antibody used in WB.

Antigen	Dilution	Clone	Source
gB HSV1/2	1:100	107B	Santa Cruz Bt.
β-actin	1:400	AC-74	Sigma Aldrich

3.19. Isometric Muscle Tension Recording

Isolated tissue bath assays are a classical pharmacological tool for evaluating concentration-dependent responses in a contractile tissue: an isometric twitch contraction is elicited by electric field stimulation and the force response is recorded for analysis.

To evaluate the influence of in vivo lymphocytes depletion on the neuromuscular anomalies induced by HSV-1 infection, and the impact of adoptive transfer of specific lymphocytes subpopulations we measured changes in isometric muscle tension following electric field stimulation.

For these experiments mice were sacrificed 8-10 weeks post IG inoculum or 1 week post IP injection of specific lymphocyte subpopulations. The distal ileum was rapidly removed, placed in oxygenated Kreb's buffer and then segments (~1.5 cm) were mounted vertically by 5-0 silk ligated at each end in organ baths containing 10 mL of Krebs solution, maintained at 37°C and aerated with 95% O_2 and 5% CO_2. For electrical field stimulation (EFS) ileal preparations were positioned between a pair of parallel electrodes connected to a Grass S88 stimulator (Grass Instrument Co.). Frequency-response curves to EFS (2-40 Hz, 1 ms pulse duration, 10 s pulse trains, 40 V) were evaluated in presence or absence of tetrodotoxin (1 µM) or atropine (1 µM). Muscle responsiveness assessed on responses obtained with the cholinergic receptor agonist carbachol (30 µM). The isometric transducers (World Precision Instruments) was connected to a quad bridge amplifier and PowerLab 4/30 data acquisition system using LabChart6 software (ADInstruments)

3.20. Gastrointestinal transit

We administered 70 µl fluorescein-isothiocyanate dextran (70.000 MW, MPBiomedicals LLC) in PBS (6.25 mg/mL) via oral gavage with minimum animal handling. In line with our preliminary experiments, animals were sacrificed 60 minutes after probe administration. Bowel was carefully removed. Stomach, caecum and colon were examined separately. Small intestine was divided into 8 segments. Luminal contents from each segment were collected and clarified by centrifugation (10,000 xg, 15 min, 4°C). Fluorescence analysis was performed at 494/521 nm (Hitachi-F2000). Data were expressed as % of fluorescence per segment. Gastric emptying was determined by percentage of fluorescent probe that emptied the stomach. Gastrointestinal transit was calculated as the geometric center (GC) of distribution of the fluorescent probe

3.21. Beads expulsion time

The animals were slightly anesthetized with halothane. To evaluate colonic motility, a 2-mm glass bead was placed 2 cm proximal to the anus using a plastic Pasteur pipette lightly lubricated with lubricating jelly. The animals were immediately placed into a cage and monitored to observe bead expulsion. Colonic motility was assessed by measuring the amount of time between bead placement and expulsion. Data are reported as the time to expulsion (sec) (Anitha M, et al. 2008).

3.22. Statistical analysis

Data are shown as mean +/- SEM. Non parametric Mann–Whitney's U-test for independent variables, Wilkoxon test for paired matched variables or Kruskall-Wallis ANOVA for multiple variables were used for comparison as appropriate. Data analysis and plots were executed with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, USA).

Chapter 4. Results

4.1. Alpha Herpesviruses are able to reach the enteric nervous system in human gut

To determine the presence of α-herpesviruses sub-family (HSV type 1 and type 2, VZV) in neurons of human intestinal tract we have analysed LMMP from ileocolic surgical resections. We have collected one hundred and twenty surgical resections obtaining 80 ileal and 41 colonic tissues. Furthermore the samples derived from patients affected by Crohn's disease (CD), ulcerative colitis (UC), diverticular disease (DD) and colon carcinoma (CC) in proportion as described in material and methods.

Among the 80 ileal samples, 33 and 2 (37,5% and 3,75 %,) were positive for HSV-1 or VZV DNA, respectively, for a total of 42,5% of ileum virus positive samples (Figure 5.A). Whereas 1 samples was positive for both viruses. Taking into account the 56 ileal samples from Crohn's disease patients, 22 (39%) were positive for HSV-1 DNA and 1 (4%) was positive for VZV DNA, whereas the 24 ileal samples obtained from colon carcinoma resections, 11 (50%) were positive for HSV-1 DNA and 1 (4%). Consequently, we can conclude that incidence of α-herpesviruses DNA in myenteric plexus of ileum samples was detected in 43% of CD patients and in 46% of CC patients (Figure 5. B).

Among the 41 colonic specimens, 18 (43%) and 4 (~ 9%) were positive for HSV-1 or VZV DNA, respectively, for a total of 52% virus positive samples (Figure 5.A.). Whereas none samples were positive for both viruses.

HSV DNA was revealed in 5 (45%) colon specimens from patients with CC, in 3 (30%) specimens from patients with CD, in 6 (58%) specimens from patients with DD and in 4 (50%) specimens from patients with UC. The presence of VZV DNA was demonstrated in 1 (9%) specimen from patients with CC, in 2 (20%) specimens from patients with CD and in 1 (8%) specimen from patient with DD (Figure 5.C).

Overall we can conclude a higher prevalence of HSV (46%) compared to VZV (3%) was found in the samples analysed. Cumulative results of viral detection in surgical specimens summarized in Figure 5.D.

The PCR products amplified by Real Time was loaded in 2% agarose gel electrophoresis to confirm HSV and VZV positivity (Figure 5.E.).

A.

B.

C.
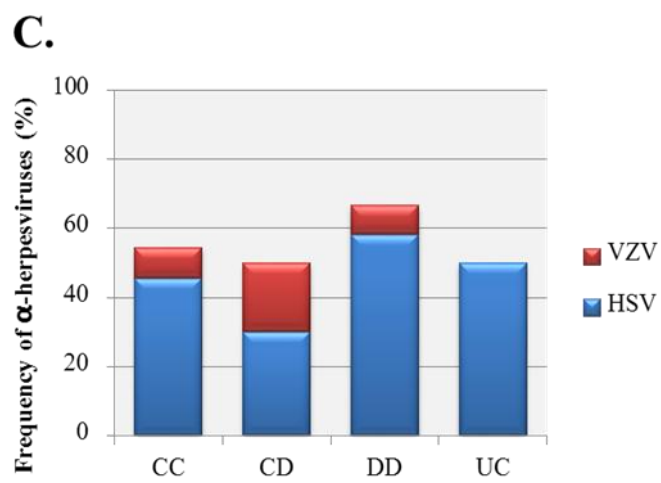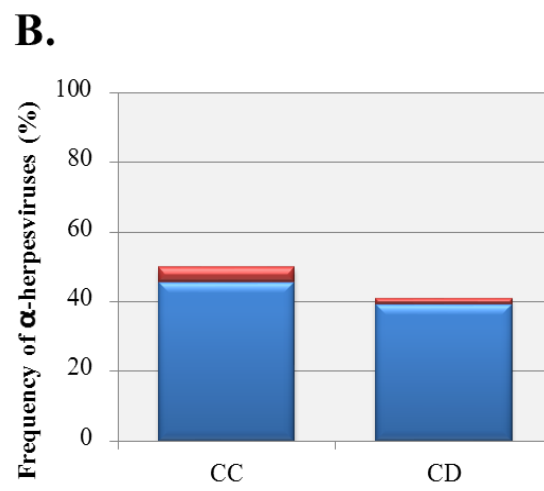
D.		Virus HSV		Virus VZV	
Tissue type	Disease	N_O of samples	Frequency	N_O of samples	Frequency
Ileum	**CD** N=56	Positive 22 Negative 34	39 % 61 %	Positive 1 Negative 55	4 % 96 %
	CC N=24	Positive 11 Negative 13	45 % 55 %	Positive 1 Negative 23	1 % 99 %
Colon	**CD** N=10	Positive 3 Negative 7	30 % 70 %	Positive 2 Negative 8	20 % 80 %
	CC N=11	Positive 5 Negative 6	45 % 55 %	Positive 1 Negative 9	9 % 91 %
	DD N=12	Positive 6 Negative 6	58 % 42 %	Positive 1 Negative 11	8 % 92 %
	UC N= 8	Positive 4 Negative 4	50 % 50 %	Positive 0 Negative 8	0 % 100 %
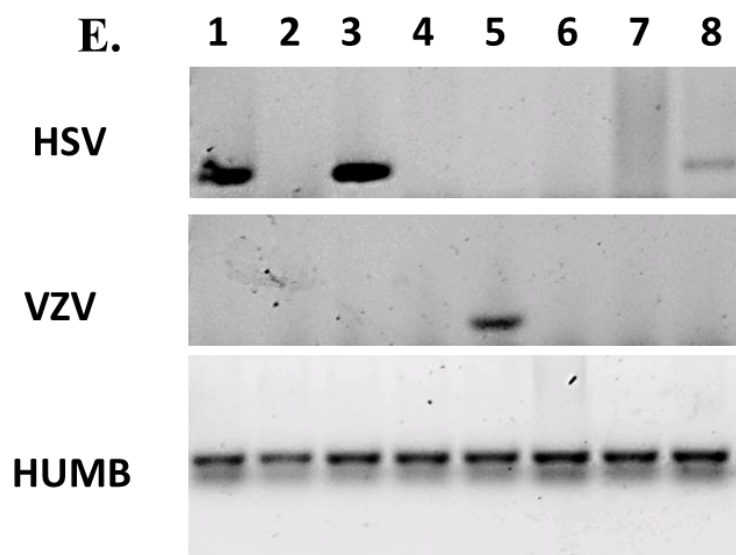
Figure 5. Incidence of α-herpesviranae DNA in human surgical resections.

(A) Cumulative frequency of virus positive samples in ileum and in colon resections. (B) Frequency of α-herpesviranae DNA in ileal specimens: the prevalence of HSV type 1 and 2 DNA (in red) and VZV (in blu) in ileal specimens of patients affected by CC and CD. (C) Frequency of DNA in colon specimens: the prevalence of HSV type 1 and 2 DNA (in red) and VZV (in blu) in colonic specimens of patients affected by CC, CD, UC and DD (D) Cumulative results of viral detection. (E) Agarose gel electrophoresis of PCR products amplified in real time. Results for 8 different samples are shown. N.1 and n.3 samples are positive at HSV, sample n.5 is positive at VZV.

4.2. Quantification of viral genome copies in bowel specimens

As shown previously, α-herpesviruses sub-family DNA was detected in a significant percentage of ileocolic surgical resections. To quantify the relative amount of HSV-1 load, we determined by ratio between amounts of HSV-1 DNA copies detected in positive samples to the copies of the human housekeeping gene (GAPDH).

In the ileum positive specimens the relative viral genome load was significantly higher in Crohn's disease patients compared to ileal tissue obtained from patients with colon carcinoma (Figure 6.A). In contrast in the colon we did not observe any positive correlation between HSV genome level and specific diseases (Figure 6.B). Furthermore, the relative amount of HSV genome in ileum specimens was significantly higher than in colon specimens. (Figure 6.C)

B.

COLON

C.
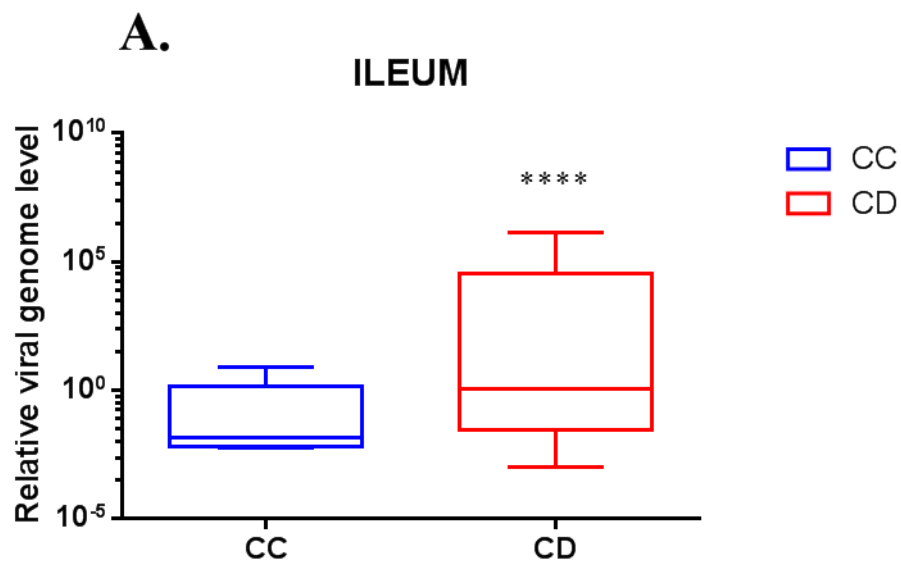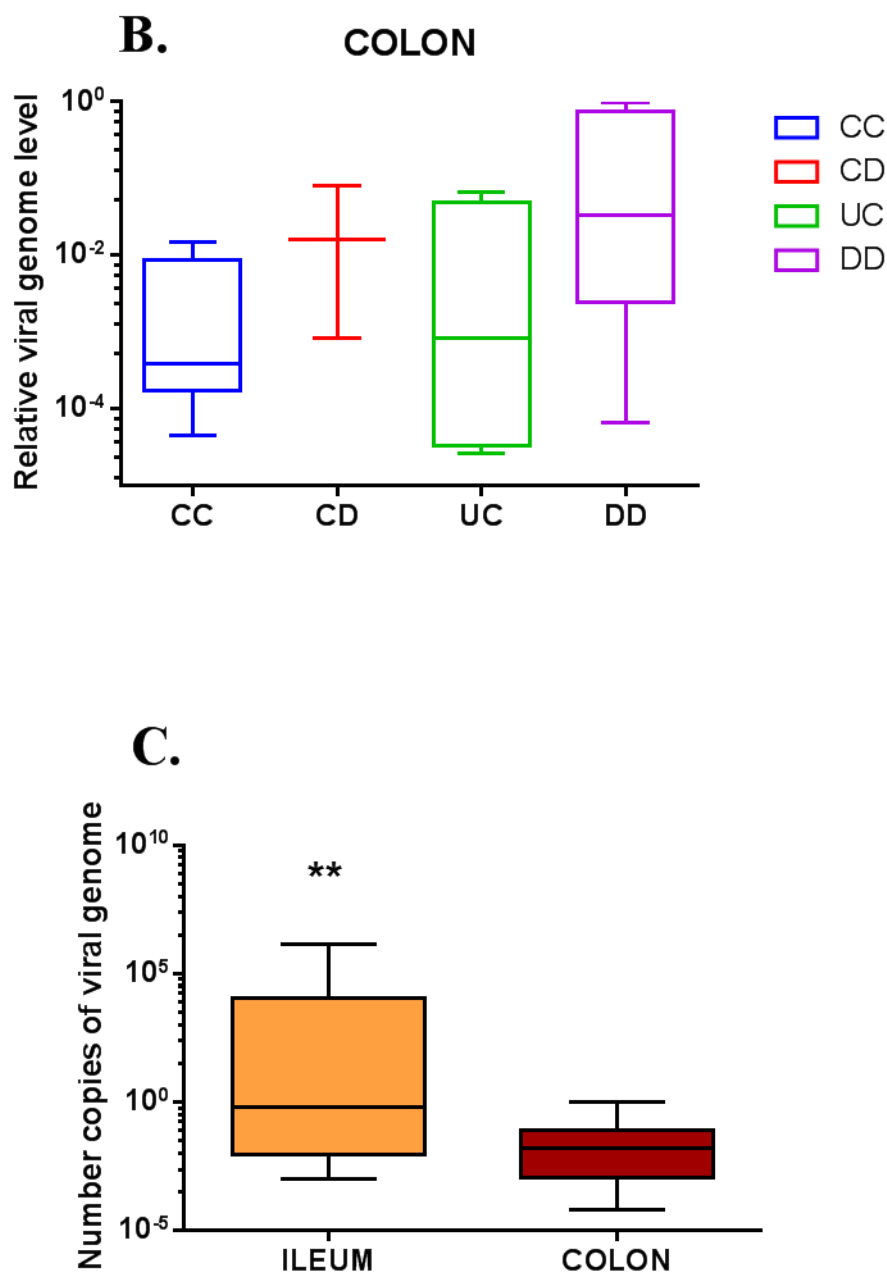
Figure 6. Quantification of HSV genome in human ileocolic specimens

(A) In ileal specimens there was an higher viral genome level in patients affected by CD compared to patients affected by CC (**** P value <0.0001). (B)In colon, specimens not observed any significant difference of relative viral genome level between specific diseases. HSV DNA was detected by real time PCR and was normalized to human housekeeping gene (GAPDH) using the formula: $2^{-(\Delta Ct)}$; $\Delta Ct = (Ct\ HSV-\ Ct\ GAPDH)$. F test to compare variance between CC vs CD; P value <0.05; (C) Number copies of HSV-1 genome in ileum and in colon specimens; Mann Whitney test to compare difference between ileum vs colon; P value <0,005.

4.3. HSV-1 infection causes enteric nervous system dysfunction

Although α-herpesviruses (HSV and VZV) are able to reach the human enteric nervous system it is not known whether contributes to intestinal diseases. To link HSV-1 infection to ENS our research group has recently reported that HSV-1 can infect the ENS of rats following an orogastric inoculum, resulting in time-dependent intestinal neuromuscular abnormalities (Brun et al.; 2010).

To assess the impact of HSV-1 infection on gastrointestinal motility in mice, we used an *in vivo* model. C57/Bl6J mice were inoculated with HSV-1 intranasally and after 4 weeks intragastrically. Then animals were sacrificed at weekly interval up-to 12 weeks post-IG inoculum to evaluate *in vivo* anomalies of gastrointestinal transit, and *ex vivo* alteration of contractility post HSV-1 infection.

We quantified changes in isometric muscle tension following electric field stimulation (EFS) of ileal segments at 20 Hz EFS. A reduced neuromuscular contractility was observed at 2 and 10 weeks post-IG HSV-1 inoculum as compared to sham animals (Figure 7.A).

The gastrointestinal transit was evaluated by administering by gavage a fluorescent non-absorbable probe that reaches the ileocecal valve in 60-90 minutes (Brun et al.; 2010). In mice inoculated IG with HSV-1 we observed a significant reduction of propulsive motor function and a slower intestinal transit at 1, 2, 8 and 10 weeks post-IG inoculum (Figure 7.B).

Whereas, no alterations in electrical- and pharmacological-induced ileal contractility in mice exposed intranasally to HSV-1 were detected (data not shown).

All together these data suggested that it is possible to detect neuromuscular anomalies in early stage of HSV-1 infection, at 1 and 2 weeks post IG infection, and in late stage of infection, at 8 and 10 weeks post IG infection. These evidences provided further support to the role of this neurotrophic virus to induce neuromuscular dysfunction in the gastrointestinal tract and therefore prompted us to explore the underlying mechanisms.

A.

B.
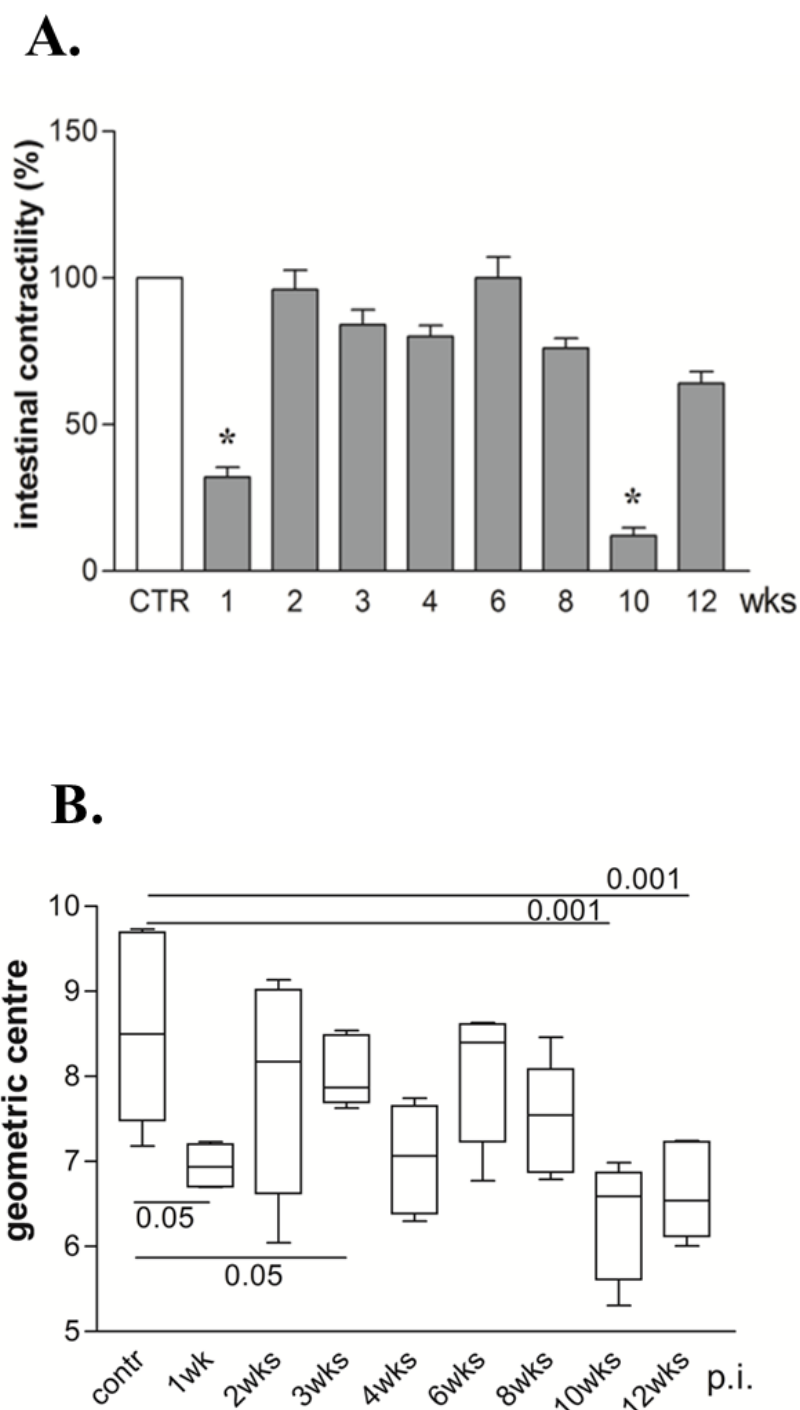
Figure 7. Effects of HSV-1 infection on intestinal contractility and gastrointestinal transit.

(A) EFS-elicited contractions in ileum segments of sham and HSV-1 IG infected mice. (n=6-8 per group). Representative tracings showing EFS evoked responses in ileal segments (20 Hz, 1 ms pulse duration, 10 s pulse trains, 40 V).*P<0.05 compared to sham infected. No alterations in electrical-induced ileal contractility in mice exposed intranasally to HSV-1 were detected (data not shown). (B) Gastrointestinal transit was expressed as percentage of relative distribution of fluorescence probe in the intestinal tract (geometric centre; n=6-8 per group). P<0.01 compared to sham infected.

4.4. HSV-1 damages the enteric neurons within the myenteric plexus

We determined in vivo the effect of HSV-1 infection on ENS integrity evaluating the distribution of peripherin and βIII-tubulin, two proteins that organize in neurofilaments. The analysis was performed on whole mount (WM) preparation from LMMP of control mice and mice infected mice with HSV-1. As shown in Figure 8.A, following HSV-1 exposure peripherin immunoreactivity was significantly reduced 2 weeks post IG HSV-1 exposure, whereas at 6-8 weeks post IG inoculum peripherin network appeared fragmented. Indeed, as regard βIII-tubulin immunoreactivity was reduced at 3-4 weeks post IG HSV-1 inoculum. (Figure 8.B).
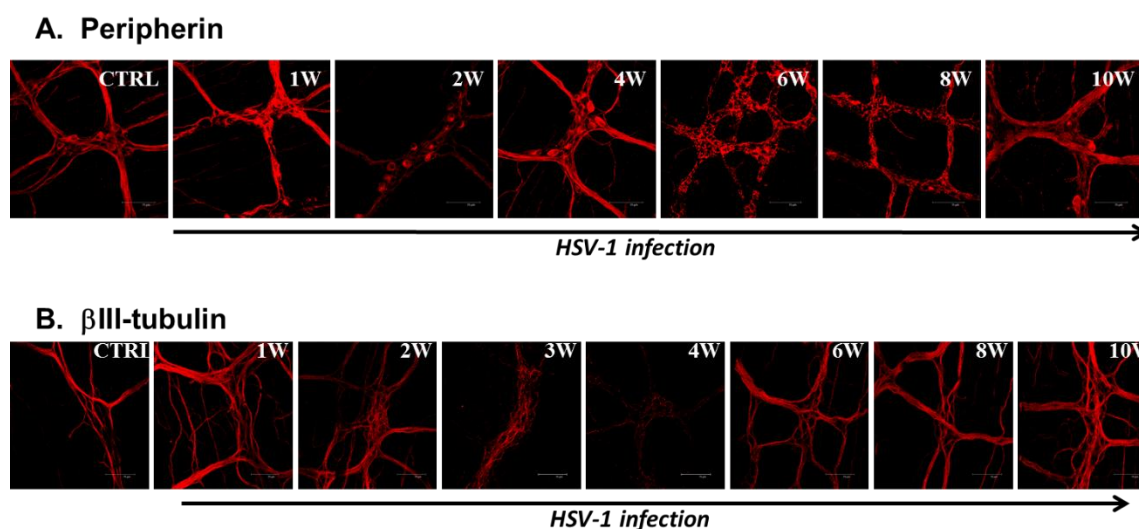
Figure 8. HSV-1 infection causes alterations on expression of ENS neuronal proteins.

Confocal microscopy on WM of murine LMMP stained with anti-peripherin or anti- βIII-tubulin. Control (Ctrl) and HSV-1 infected mice at 1 to 10 W post IG inoculum. The fluorescence in red represents (A) peripherin and (B) βIII-tubulin positive. Scale bar 75 μm

4.5. Markers of viral replication increase at 8 weeks post HSV-1 infection in ENS

To define the nature of HSV-1 infection in the ENS, real time RT-PCR analysis of HSV-1 mRNA transcripts in the myenteric ganglia of sham and infected mice was performed. Presence of viral LATs were detected in isolated myenteric ganglia since the first week of

infection (Figure 9.A) with a peak at 2 weeks after IG inoculation. mRNA transcripts of viral immediate early ICP0 gene (Figure 9.B) were significantly present at 2, 3, 4 weeks whereas ICP4 gene (Figure 9.C) show the highest level of expression at 8 week post-viral inoculum. Furthermore, the mRNA transcripts of viral late gene VP16 were present only at 2, 4, and 8 weeks post HSV-1 infection and gC mRNA transcripts were significantly present at 8 week post HSV-1 infection (Figure 9.D and 9.E). However, gD mRNA were not detected post IG infection. Moreover, an increase of TK-HSV gene was revealed starting from 4 weeks post infection and a peak was observed at 8 weeks post HSV-1 inoculum (Figure 9.F).

A.

B.

C.

D.

E.

F.
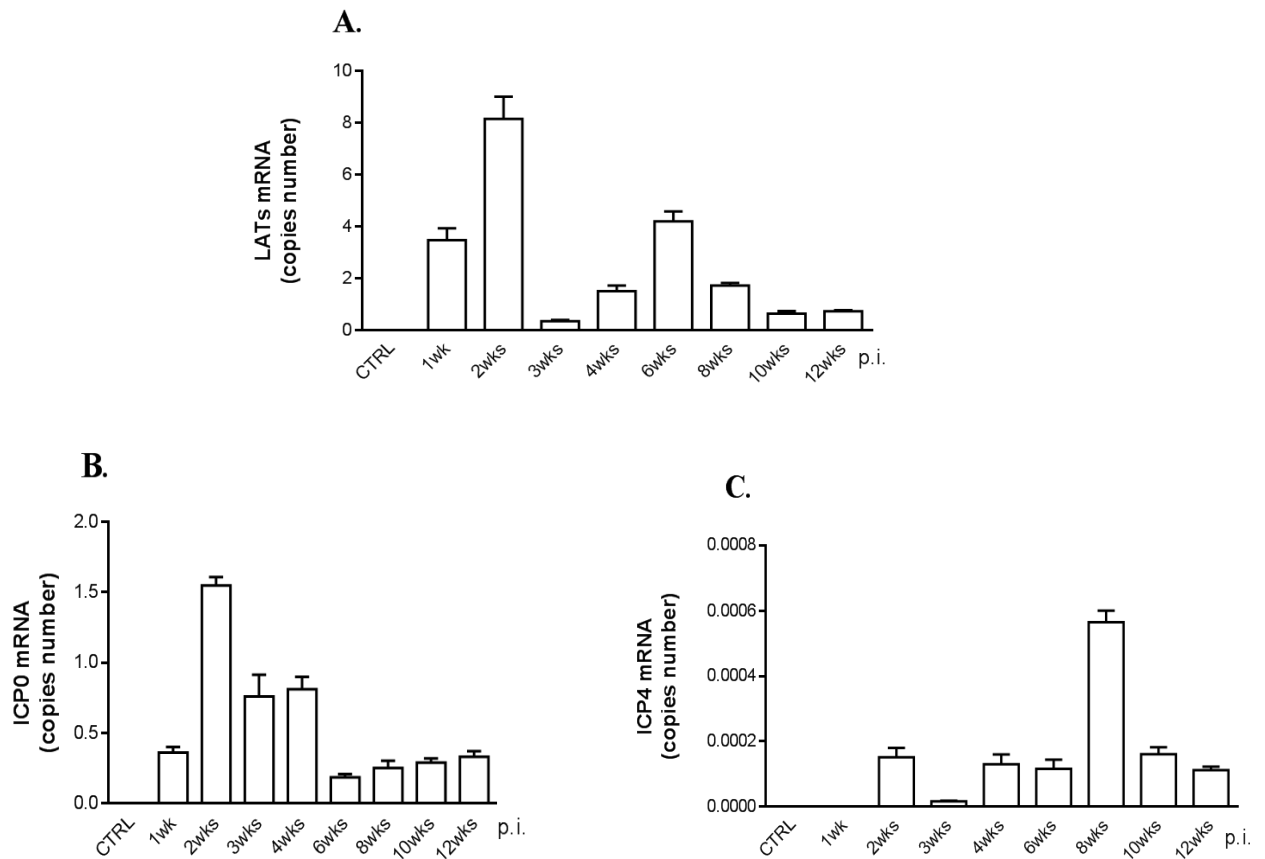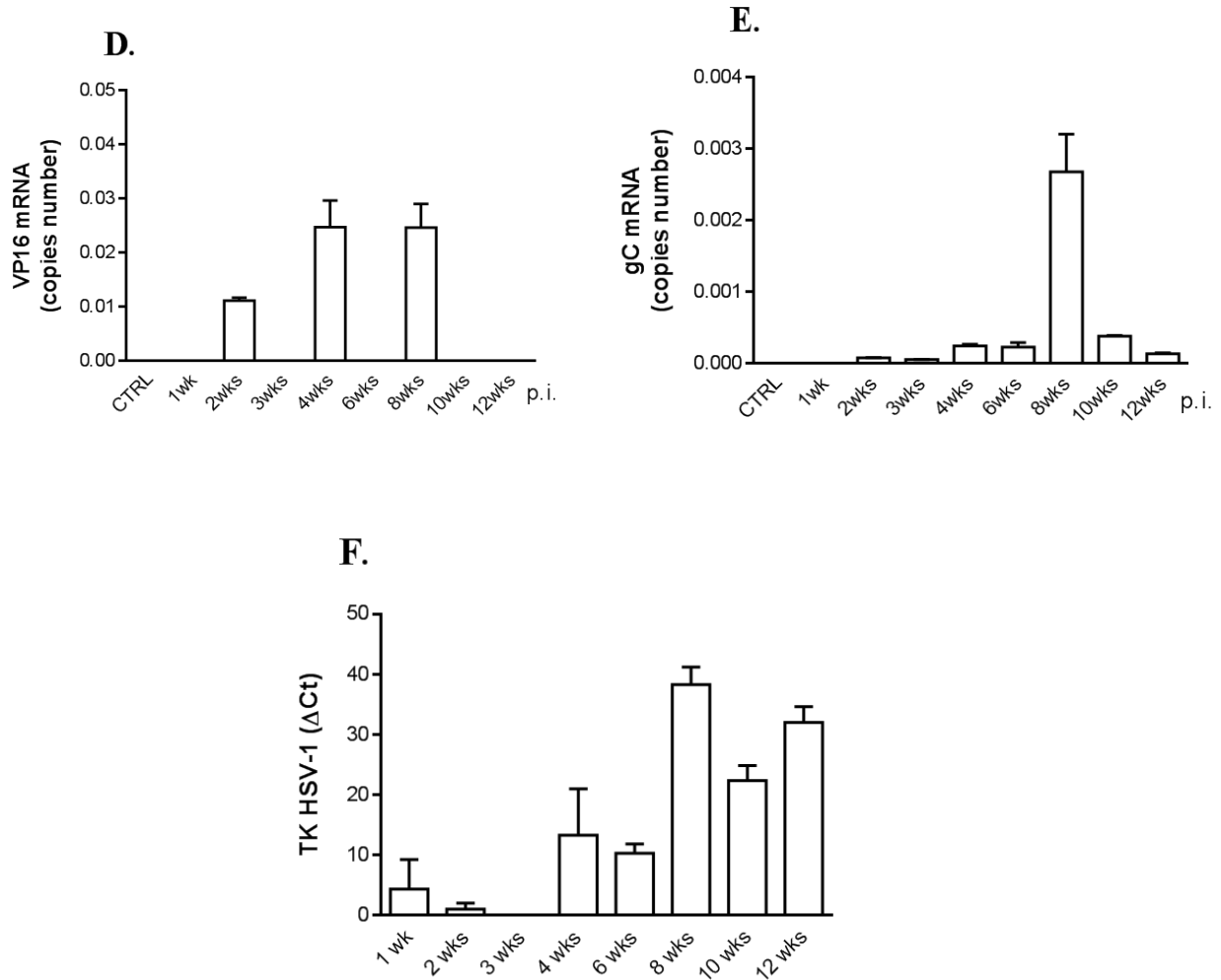
Figure 9. Nature of HSV-1 infection in the ENS.

RNA was purified from myenteric ganglia of sham and HSV-1 infected mice at 1-12 weeks (w) after HSV-1 intragastric administration and the relative expression of HSV-1 LATs (A), very early gene ICP0 and ICP4 (B and C) and late gene VP16 and gC (D and E) was determined by qPCR. (F)TK HSV-1 gene *was* detected viral DNA *and expressed as Δct.* Murine β-globin was used for *as internal control. Data are expressed as means ± SEM (two tissue samples from n=3 animals for each experimental group). *P<0.01 compared to sham mice.*

4.6. CD3+ T cells infiltrating LMMP increase following HSV-1 infection

My group has previously observed that neuronal damage at 1 and 2 weeks post IG infection was associated to an increased level of activated infiltrating macrophages, as compared to control mice. However, activated macrophage were not present at 8-10 weeks post-infection (data not shown).

Therefore, we have focused our attention on the onset of neuronal damage at late stage of HSV-1 IG exposure and the possible correlation between ENS dysfunction and adaptive immunity-mediated damage.

We first quantified the lymphocytes infiltrating the longitudinal muscle myenteric plexus following IG HSV-1 inoculum. The proportion of CD3$^+$ lymphocytes (Figure 10) in infiltrated in the myenteric plexus significantly increased at 6 weeks post-IG inoculum and remained sustained up to 12 weeks HSV-1 post inoculum. The massive presence of CD3+ lymphocytes that match with the neuromuscular anomalies observed 8-10 weeks post-IG exposure suggest that adaptive immune responses may be the main executors of ENS damage.

To determine the distribution pattern of the infiltrating CD3+ cells, ileal sections were probed with an anti-CD3 antibody. Several CD3+ T cells were detected at $6-8-10$ weeks post-IG inoculum within myenteric ganglia whereas CD3+ cells were lacking in control animals or after 1 -3 weeks of IG inoculum (Figure 11).
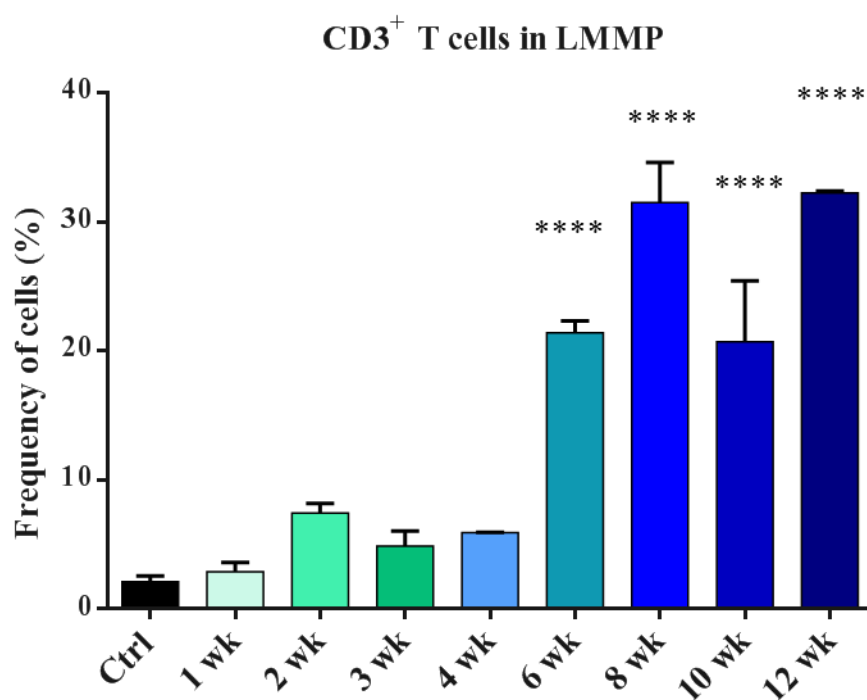
Figure 10. Analysis of CD3$^+$ T-cells infiltrating the LMMP of infected mice

*CD3$^+$ cells infiltrating the LMMP increased significantly (**** P value < 0.0001) at 6 weeks (21%), at 8 weeks (31%), at 10 weeks (20%), 12 weeks (32%) post IG infection compared to uninfected mice (CTRL). The number of CD3$^+$ cells was determined by flow cytometry on the total mononuclear cells isolated from the LMMP. The number of events acquired was 10000 events. Statistical analysis was performed using ANOVA test and Bonferroni's multiple comparisons test.*
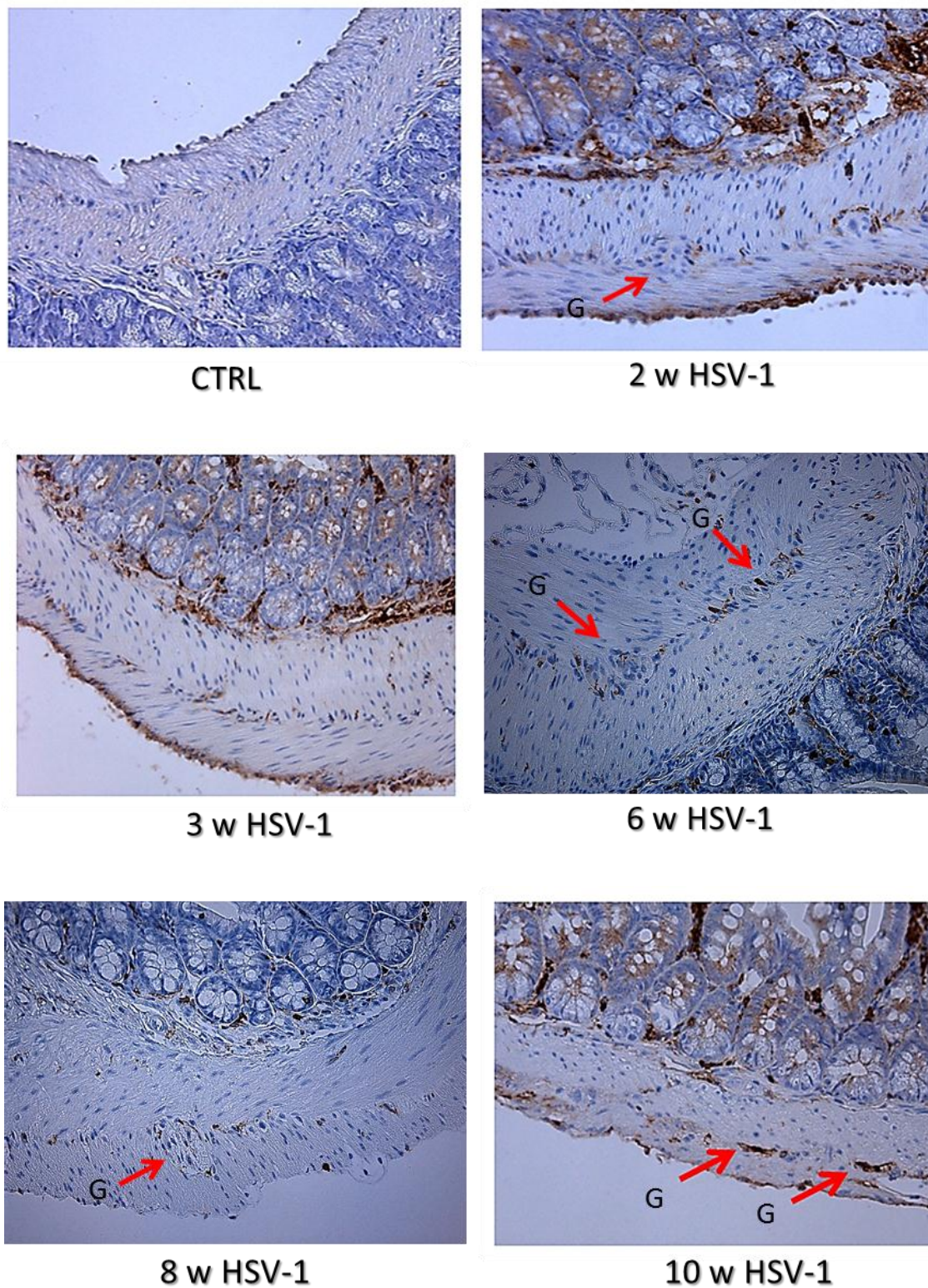
Figure 11. Localization of CD3+ T cells infiltrated in a LMMP in model mouse.

*Representative ileal cross-section in formalin-fixed tissue of the ileum from control mice and infected mice at 2, 3, 6, 8 and 10 weeks following HSV-1 inoculum. Immunohistochemistry with anti-CD3 and counterstained with haematoxylin. Red arrows indicate CD3 staining on the myenteric ganglia (**G**).20x*

4.7. Lymphocytes infiltrating LMMP show time-dependent expression of activation markers following HSV-1 infection

To better understand the immune microenvironment in LMMP post-IG HSV-1 infection we have characterized the expression of lymphocytes' activation markers.

Using real time PCR, we quantified CD69 (pan T cells activation marker), CD28 (T cell receptor for CD80 and CD86, enhancing lymphocytes activation), granzyme B and pore forming protein-like (markers of T-cells citolytic activity), CTLA-4 and PDL-1 (receptors generating an inhibitory signals in activated T-cells) to characterize the lymphocytic infiltrate.

As shown in Figure 12. A and 12.B, CD28 and CD69 mRNA increased after 1 week post-IG HSV-1 infection and persisted augmented up to ten weeks. CD69 levels peaked at 1 week, then slightly reduced to remain stable but at least 100-fold higher than controls. On the contrary CD28 mRNA level showed a time-dependent decrease following HSV-1 exposure. The levels of granzyme B and pore forming protein-like (Pfpl) mRNA peaked at 1-3 weeks post-IG HSV-1 infection and then returned to control values (Figure 12.C and 12.D).

Moreover, CTLA-4 and PDCD-1 mRNA level, two molecules that interact with CD80 generating death signals in lymphocytes, significantly increased in the early phases of HSV-1 infection (1-3 weeks post IG inoculum) but drastically decreased starting at 6 weeks post HSV-1 post infection. Altogether these data indicate the presence of activated lymphocytes in the LMMP with strong cytotoxic potentiality but also the presence of strong inhibitory signals in the first weeks post-infection that completely disappear in the late phases of infection (Figure 12.E and 12.F).

C.

D.

E.

F.
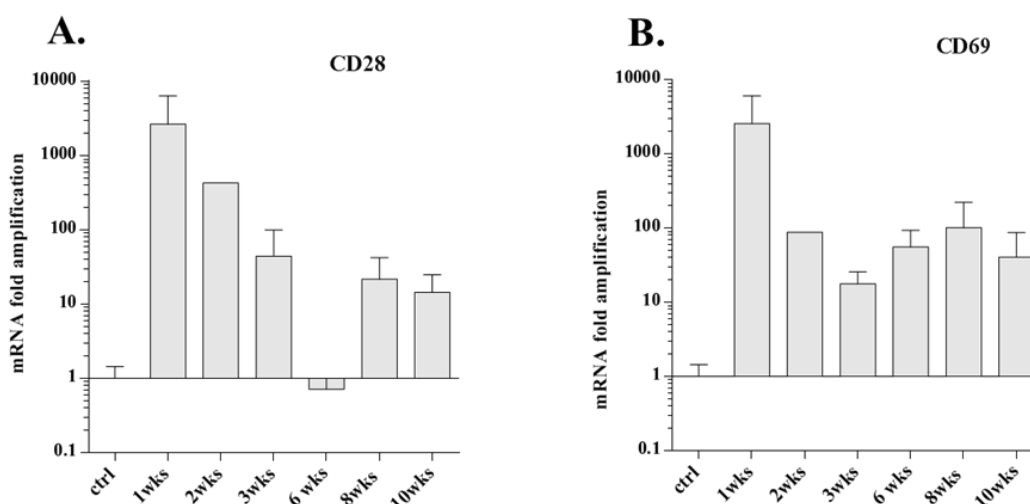
Figure 12. Characterization of expression markers on lymphocytes infiltrated in LMMP. *Total RNA in LMMP was extracted, converted to cDNA, and analysed by real-time RT-PCR for the expression of selected molecule. (A, B) CD28 and CD69 mRNA levels; (C, D) Granzyme B and pore forming protein-like mRNA levels, (E,F) CTLA-4 and PDCD-1 mRNA levels. The data were normalized to GAPDH mRNA levels. The data were expressed as fold increase compared with baseline expression level of uninfected mice and expressed $2^{-\Delta Ct}$. Data represent mean ± SEM (n = 4 and above for each of the analysed time points).*

4.8. Antigen presenting cells show time-dependent expression of co-stimulatory molecules following HSV-1 infection

To further investigate the immune microenvironment in LMMP post-IG HSV-1 infection we characterized the expression of co-stimulatory molecules in antigen presenting cells. We quantified MHCI, MHCII, CD80, H2Q9 mRNA to unveil the expression of activation and inhibitory signals expressed by antigen-presenting cells (APC) in the LMMP.

As regard signals on APC the expression of MHC-I and MHC-II mRNA was up-regulated 1 week post IG HSV-1 inoculum and remained substantially stable up to 10 weeks post-infection (Figure 13.A and 13.B). However, CD80 mRNA levels were up-regulated only at 1-2 and 8-10 weeks post post IG HSV-1 inoculum. Intriguingly, the expression of the inhibitory molecule H2Q9 seemed to follow a similar expression pattern (Figure 13.C and 13.D).

To determine the distribution of CD80 in LMMP, ileal sections were probed with an anti-CD80 antibody. CD80 positive myenteric ganglia were clearly observed at 1 – 8 and 10 weeks post-IG inoculum compared to CD80 expression in control animals (Figure 14).
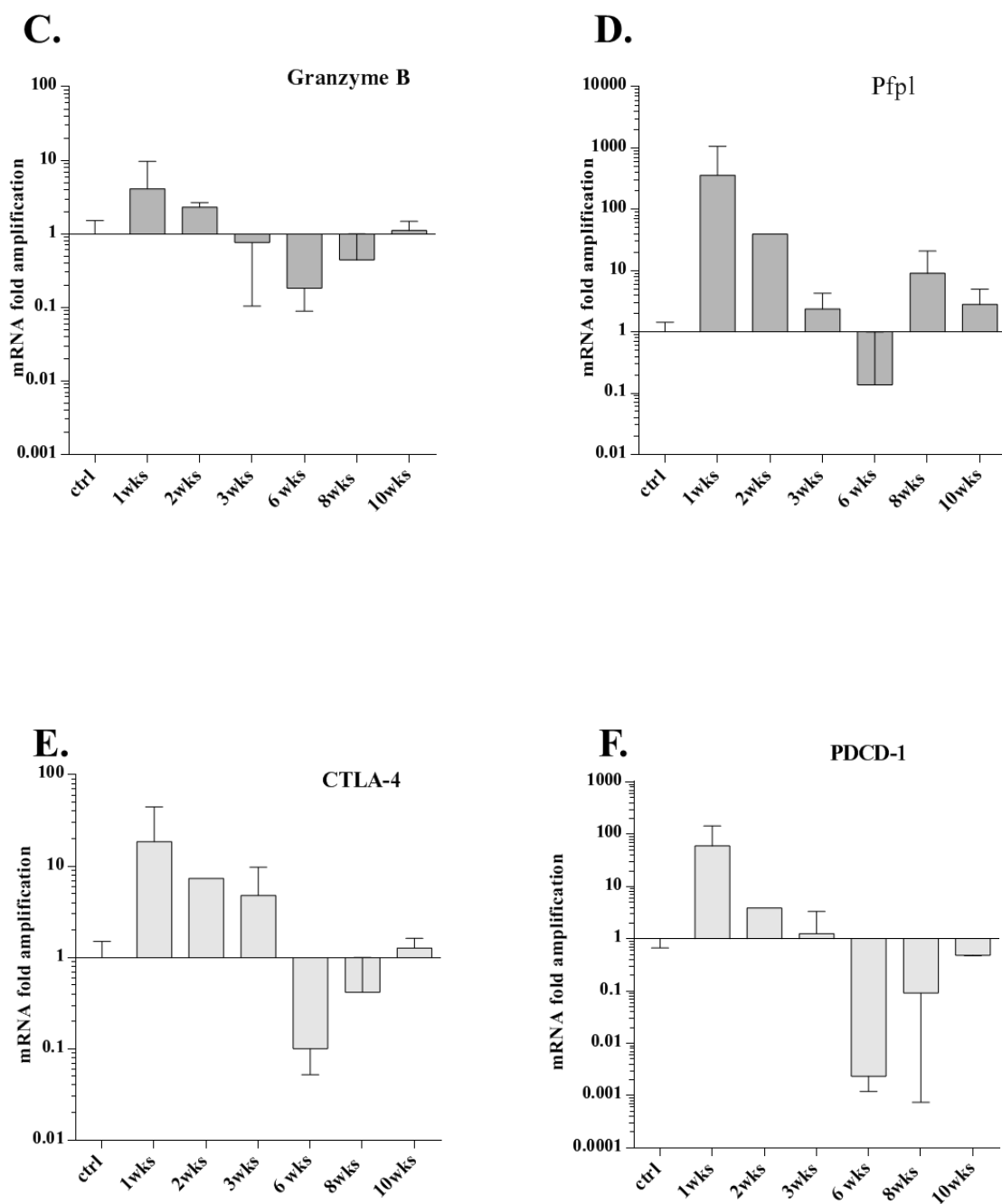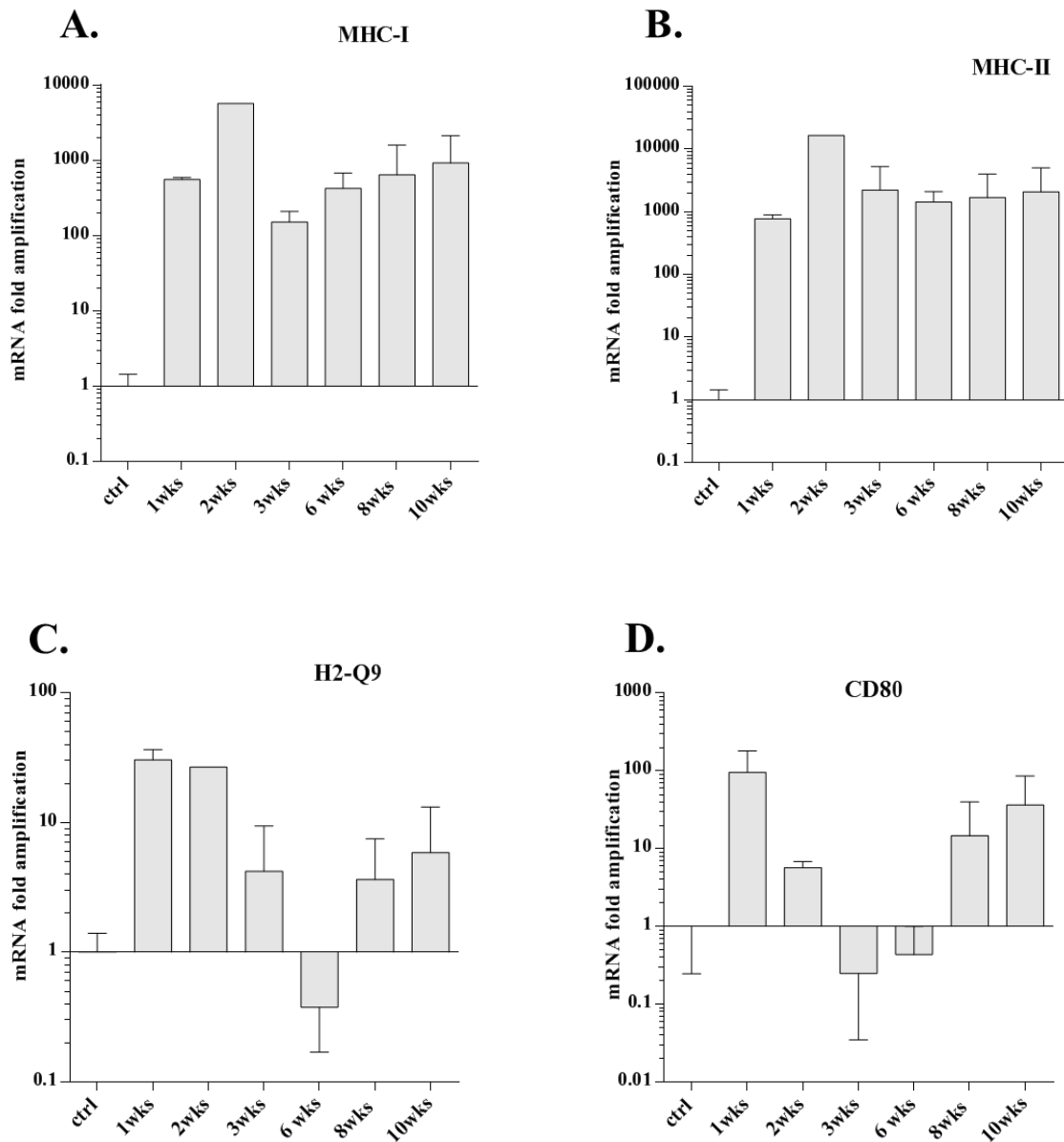
Figure 13. Characterization of expression marker on APC cells infiltrated in LMMP.

*Total RNA in LMMP was extracted, converted to cDNA, and analysed by real-time RT-PCR for the expression of selected molecule. MHC-I, MHC-II mRNA levels (**A, B**) CD80 and H2-Q9 mRNA levels (**C, D**). The data were normalized to GAPDH mRNA levels. The data were expressed as fold increase compared with baseline expression level of uninfected mice and expressed $2^{-\Delta Ct}$. Data represent mean ± SEM (n = 4 and above for each of the analysed time points).*
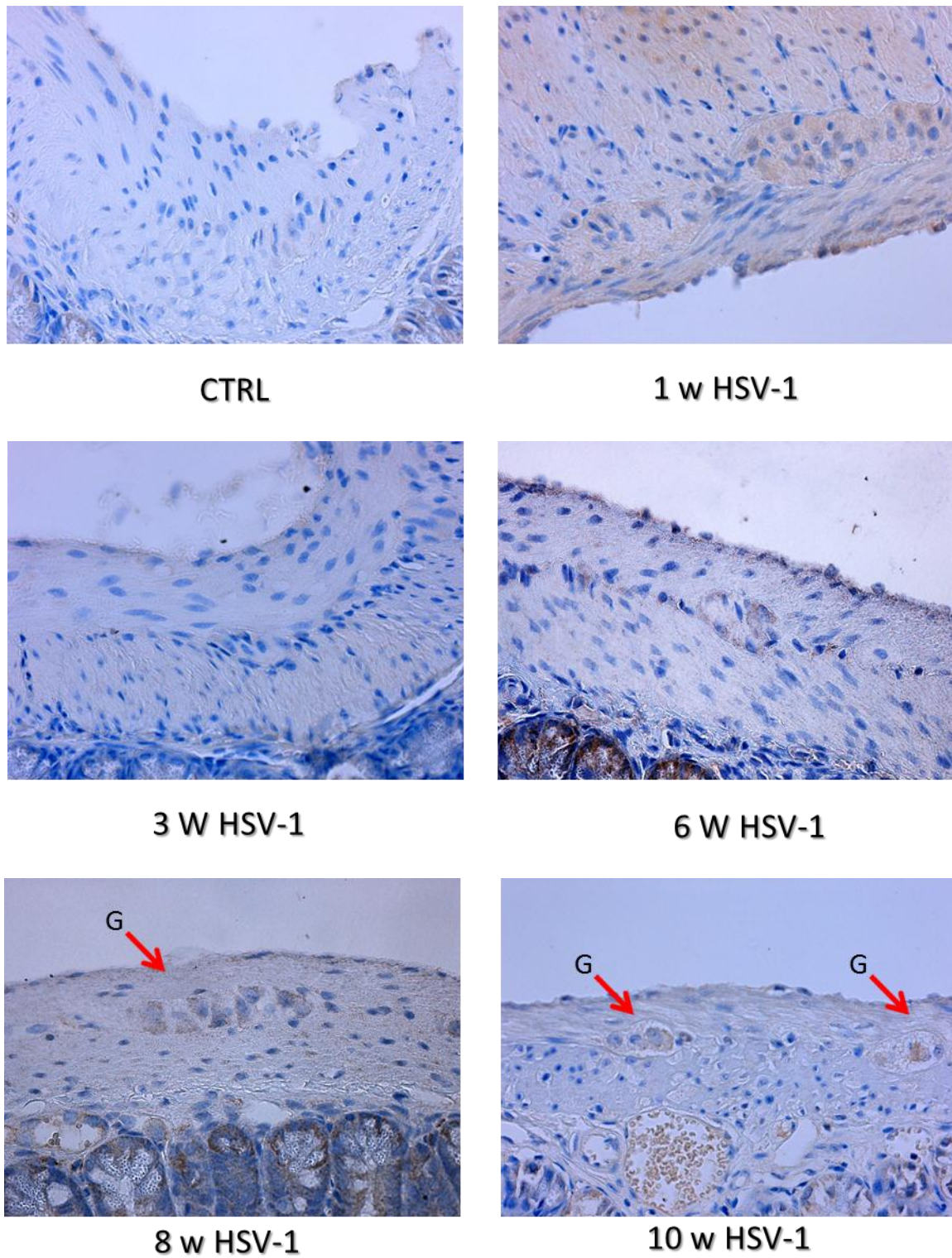
Figure 14. Localization of CD80 expression in LMMP.

Representative ileal cross-section in formalin-fixed tissue of the ileum from control mice and infected mice at 1, 3, 6, 8 and 10 weeks following HSV-1 inoculum. Immunohistochemistry with anti-CD80 and counterstained with haematoxylin. Red arrows indicate CD80 positive myenteric ganglia (G).40x

4.9. HSV-1 reactive T-lymphocytes infiltrate the LMMP

To determine whether the activation status of LMMP T lymphocytes was modulated by local recognition of HSV-1 antigens, we studied the phenotype of LMMP-derived CD3+ T lymphocytes with and without an overnight pulse with HSV-1 antigens.

Activated CD8+ cells, expressing high levels of IFN-γ were detected in the LMMP of mice 6 and 10 weeks post-IG HSV-1 inoculum. Whereas HSV-1 reactive CD8+ T-cells were observed at 8 weeks post-IG HSV-1 inoculum (Figure 15.A).

Activated CD4+ cells, expressing high levels of IL-4 were detected in the LMMP of mice 4 weeks post-IG HSV-1 inoculum. However, HSV-1 reactive CD4+ T-cells were observed at 1, 2, and 8 weeks post-IG HSV-1 inoculum (Figure 15.B).

Summarizing, we found that both CD4+ and CD8+ HSV-1 reactive lymphocytes were present at the 8 week post-IG HSV-1 infection and that activated CD8+ lymphocytes are present at the 10 week post-IG inoculum.
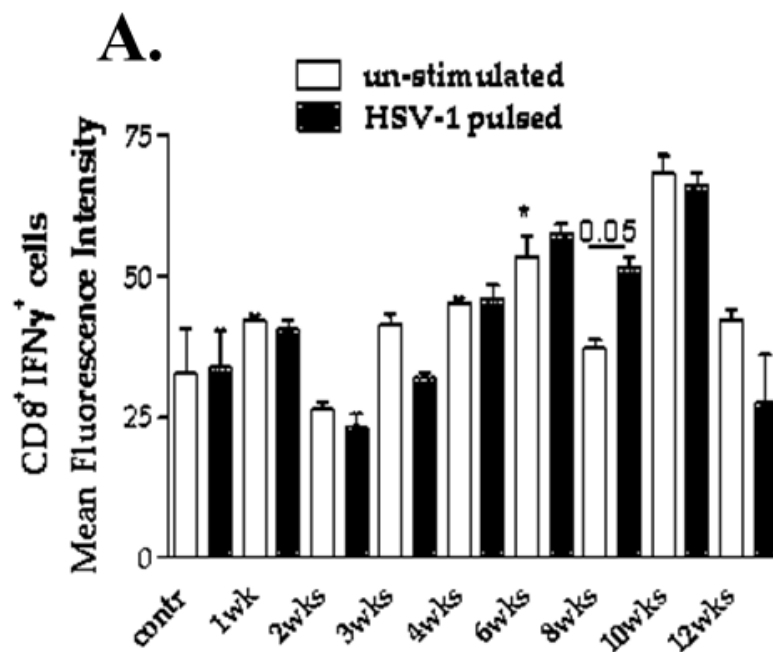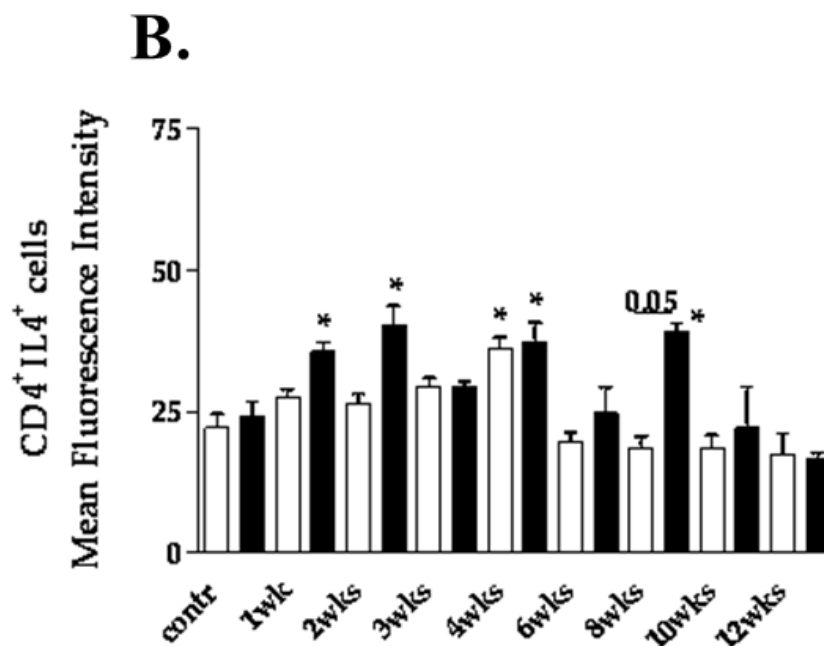
Figure 15. Activation of CD4 and CD8 T-cells isolated from LMMP following challenge with HSV-1 antigens.

Lymphocytes were isolated from LMMP of control and HSV-1 infected mice. Cells were incubated ON with or without UV-inactivated HSV-1 and then IFNγ expression by CD8+ and IL-4 by CD4+ lymphocytes determined by cytofluorimetric analysis. The figure summarizes the data of flow cytometric quantification of CD8+/IFN-ɣ (A) and CD4+/IL-4 (B). The value expressed as Mean Intensity Fluorescence. The data presented as the mean ± S.E.M.; P value< 0.05. n=4 per group

4.10. CD8+ T-cells contribute to ENS damage at 8 weeks post IG HSV-1 infection

To demonstrate the pathophysiologic relevance of CD8+ and CD4+ T cells infiltrating the LMMP in the neuromuscular anomalies observed 8 weeks post IG HSV-1 infection, we performed depletion experiments. Mice were injected weekly intraperitoneally with monoclonal anti-CD8 or anti-CD4 and sacrificed after 4 doses of treatment to determine the consequences on HSV-1 induced neuromuscular dysfunction by measuring the changes in isometric muscle tension following electric field stimulation (Figure 16.A).

As shown in Figure 16.B, although muscle tension remained substantially unvaried at 8 weeks post-IG infection compared to uninfected mice a significant damage in myenteric neurons was revealed by damaged neurofilaments. Depletion of CD8+ T-cells caused the onset of an enhanced neuromuscular contractility following EFS stimulation at 10 Hz. In contrast, CD4+ lymphocytes depletion did not modify HSV1-induced neuromuscular responses (Figure 16.C).
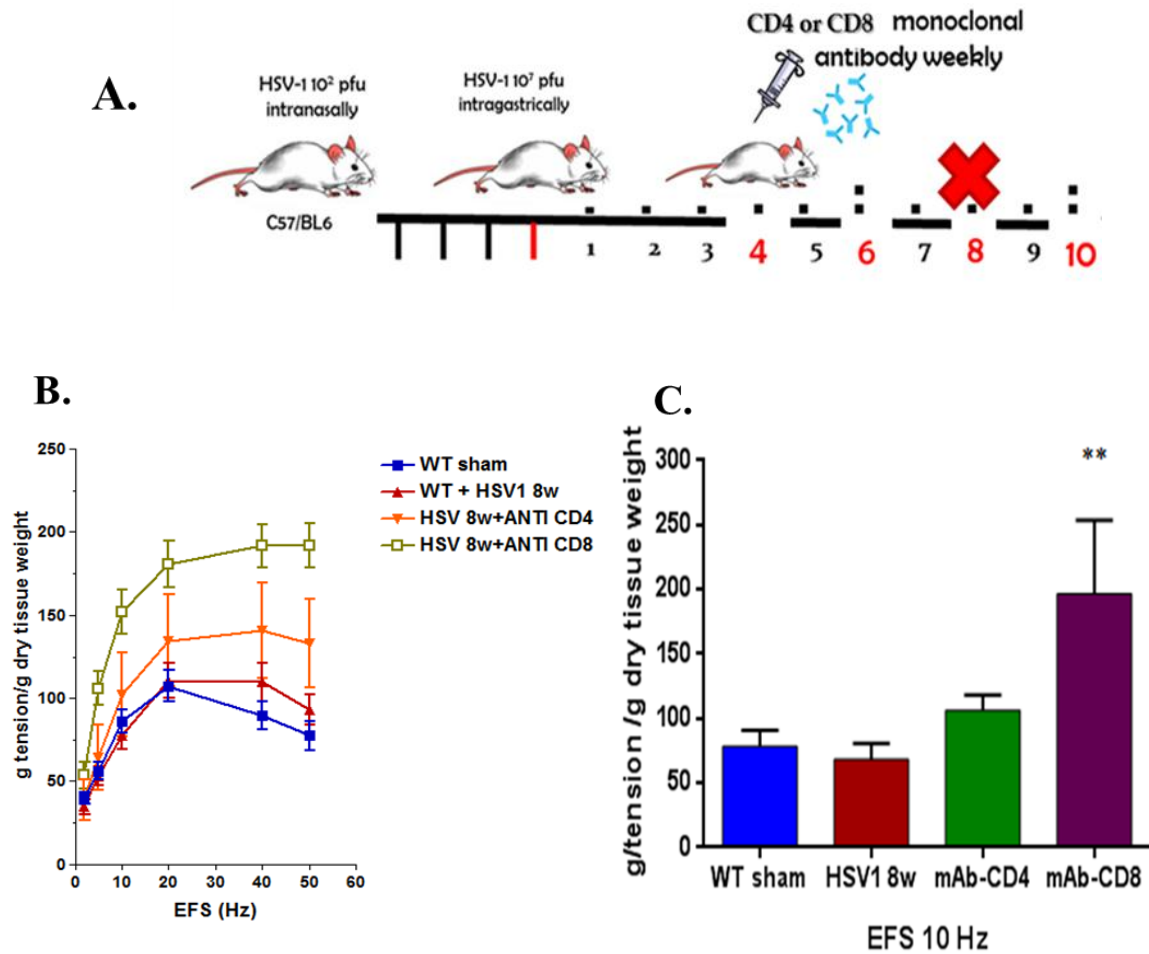
Figure 16. Effect of CD4 and CD8 T-cells depletion at 8 weeks after HSV-1 infection.

(A)Scheme for the experimental depletion in vivo of the CD4 and CD8 lymphocytes model. Mice were injected intraperitoneally with 200 ng of monoclonal anti-CD8 (clone 2.43) and anti-CD4 (clone GK1.5) resuspended in PBS. After 4 weeks the mice were sacrificed. (B) Effect of HSV-1 infection on the ileal longitudinal segment contractions induced by EFS at 0, 10, 20, 30, 40, 50 and 60 Hz after treatment with mAb-CD4 and anti-CD8 inoculation (C) Histogram represents changes of ileal contraction induced by EFS at 10 Hz ; n=at least 3 animals in each group. **P < 0.01 vs sham.

4.11. Lymphocytes from HSV-1 infected mice at 8 week and 10 weeks are negative for viral proteins

To exclude the presence of HSV-1 in lymphocytes extracted by LMMP from infected mice, we performed a Western Blot. Thus, CD3+ T cell infiltrating myenteric plexus at 8 and 10 weeks post infection were lysate and gB HSV1 glycoprotein was detected using specific

antibody. As shown in Figure 17, viral glycoprotein were not present in lymphocytes extracted from LMMP at 8 and 10 weeks post infection, excluding that the virus was inoculated in recipient mice.

Figure 17. Western blot analysis of gB HSV-1 protein on lymphocytes.

The proteins were extracted by lymphocytes infiltrating LMMP of mice (n=3 for each group) infected at 8 and 10 weeks, control mice and compared with positive control, Vero cells infected with HSV-1 in vitro. Membrane was incubated with an anti-β -actin antibody as a control for protein loading.

4.11. Adoptive transfer of CD8+T cells from HSV-1 infected mice at 8 weeks determines neuromuscular damage in naïve C57/B16J mice

To further elucidate whether CD4+ and CD8+ T-cells infiltrating the LMMP at 8 weeks post-IG HSV-1 inoculum are implicated in ENS pathophysiology, we performed adoptive transfer experiments. Thus, CD4+ or CD8+ lymphocytes were purified from LMMP of mice 8 weeks post IG HSV-1 inoculum, incubated overnight with UV inactivated HSV-1 or Vero cell lysate and then transferred in recipient mice (wild-type C57BL/6). After 7 days mice were sacrificed and the isometric muscle tension measured on ileal longitudinal segments (Figure 18.A).

As expected transfer of lymphocytes from control mice to normal recipient mice did not cause any effect on EFS-induced ileal contraction (Figure 18.D). Indeed, CD4+ and CD8+T-cells purified from LMMP of mice 8 weeks post IG HSV-1 inoculum do not influence the neuromuscular contractility following EFS stimulation at 10 Hz.

Alike, CD4+ lymphocytes exposed *in vitro* to inactivated HSV-1, did not cause any effect on EFS-induced ileal contraction when injected in a normal recipient mouse. On the contrary, adoptive transfer of CD8+ lymphocytes, isolated from LMMP of mice 8 weeks post IG HSV-1 inoculum, and exposed *in vitro* to inactivated HSV-1 significantly enhanced neuromuscular contractility (Figure 18.B).

The specificity of effects mediated by LMMP-derived lymphocytes proved by performing adoptive transfer of spleen-derived lymphocytes obtained 8 weeks post IG HSV-1 inoculum. Injection of these lymphocytes did not alter isometric muscle tension (Figure 18.C).
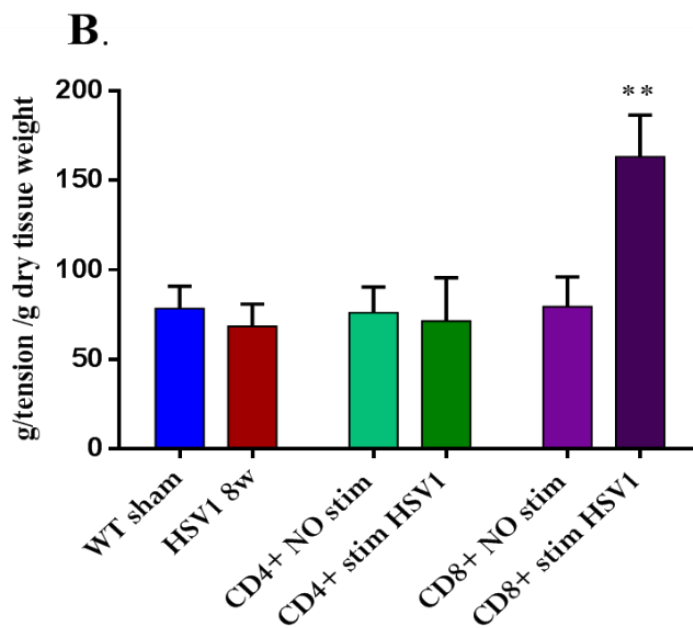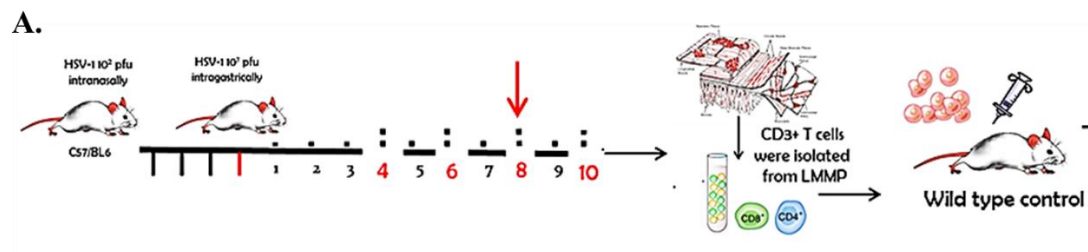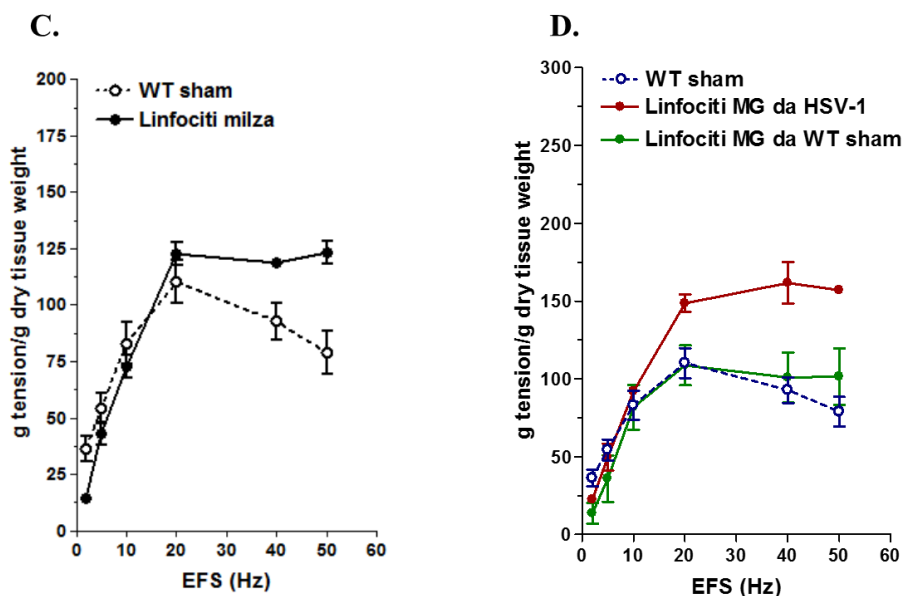
Figure 18. Effect post adoptive transfer of T-cells isolated from LMMP at 8 week on intestinal contractility

(A)Scheme for the experimental adoptive transfer in naïve mice of CD4$^+$ and CD8$^+$ lymphocytes isolated from LMMP at 8 weeks after IG inoculum. After 7 days, mice sacrificed and measured intestinal contractility following EFS (10 Hz). (B) Adoptive transfer of HSV-1 activated CD8+ and CD4+ and inactivated CD8+ and CD4+. Following adoptive transfer of HSV-1 CD8+ exposed we observed alteration of intestinal contraction compared to sham; ** p<0.01 vs WT; n=at least 3 animals in each group. (C) Adoptive transfer of spleen-derived lymphocytes at 8 weeks post infection. (D) Adoptive transfer of lymphocytes from control mice to naïve mice (in green) compared to uninfected mice (in blue).

4.12. Adoptive transfer of CD4$^+$activated T cells at 8 weeks isolated from LMMP reduces colonic motility

To assess the neuromuscular damage mediated by activated T-cells at 8 weeks, we evaluated *in vivo* colonic motility following adoptive transfer by measuring the time taken to expel a glass bead inserted into the distal colon. (Anitha et al., 2008.). The bead expulsion time was recorded at T0, 2 days, 4 days and 6 days.

A significant reduction of expulsion time starting from 4 days measured in mice transplanted with CD4$^+$ lymphocytes HSV-1 compared to the colonic motility of mice transplanted with control lymphocytes (Figure 19).
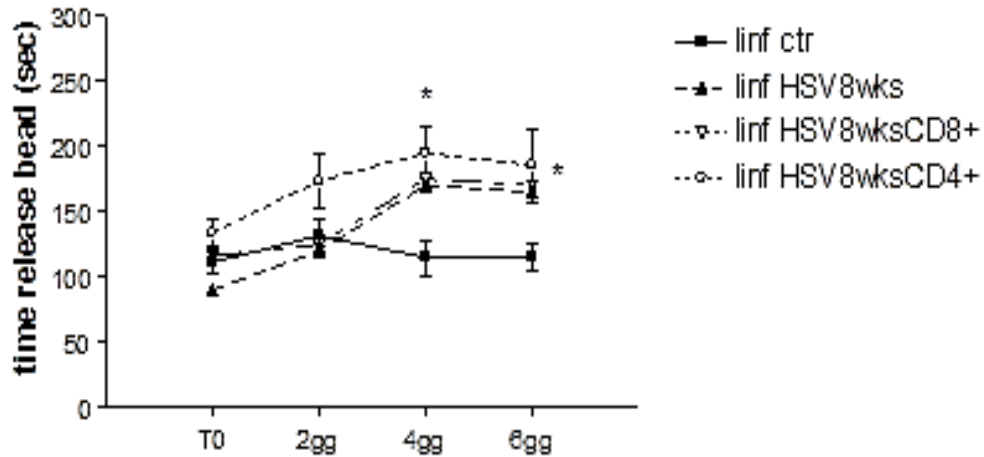
Figure 19. Effect post adoptive transfer of T-cells isolated from LMMP at 8 weeks on colonic motility.

*Colonic motility assessed by measuring the amount of time between bead placement and expulsion of the bead. Measure were recorder at T0, 2, 4, 6 days post transfer. Data are reported as the time to expulsion (sec); n=at least 4 animals in each group; * p<0.05 vs sham.*

4.13. CD8+ T-cells contribute to ENS damage at 10 weeks post IG HSV-1 infection

As previously described, at 10 weeks post IG inoculum we observed CD4$^+$/IL4$^+$ and CD8$^+$/IFN-ɤ$^+$ lymphocytes infiltrating the LMMP (paragraph 4.9). Moreover, at the same time point post viral inoculum an impaired neuromuscular activity was evident (paragraph 4.3). To investigate the pathophysiologic relevance of CD8+ and CD4+ T cells infiltrating the LMMP in the neuromuscular anomalies observed 10 weeks post IG HSV-1 infection, we performed depletion experiments as described above in paragraph 4.10 (model summarized in Figure 20.A).

As shown in Figure 20.B and 20.A, isometric muscle tension following electric field stimulation remained substantially unvaried following CD4+ cells depletion as compared to HSV-1 induced damage at 10 week. In contrast, depletion of CD8+ T-cells caused the onset of an enhanced neuromuscular contractility following EFS stimulation at 10 Hz.
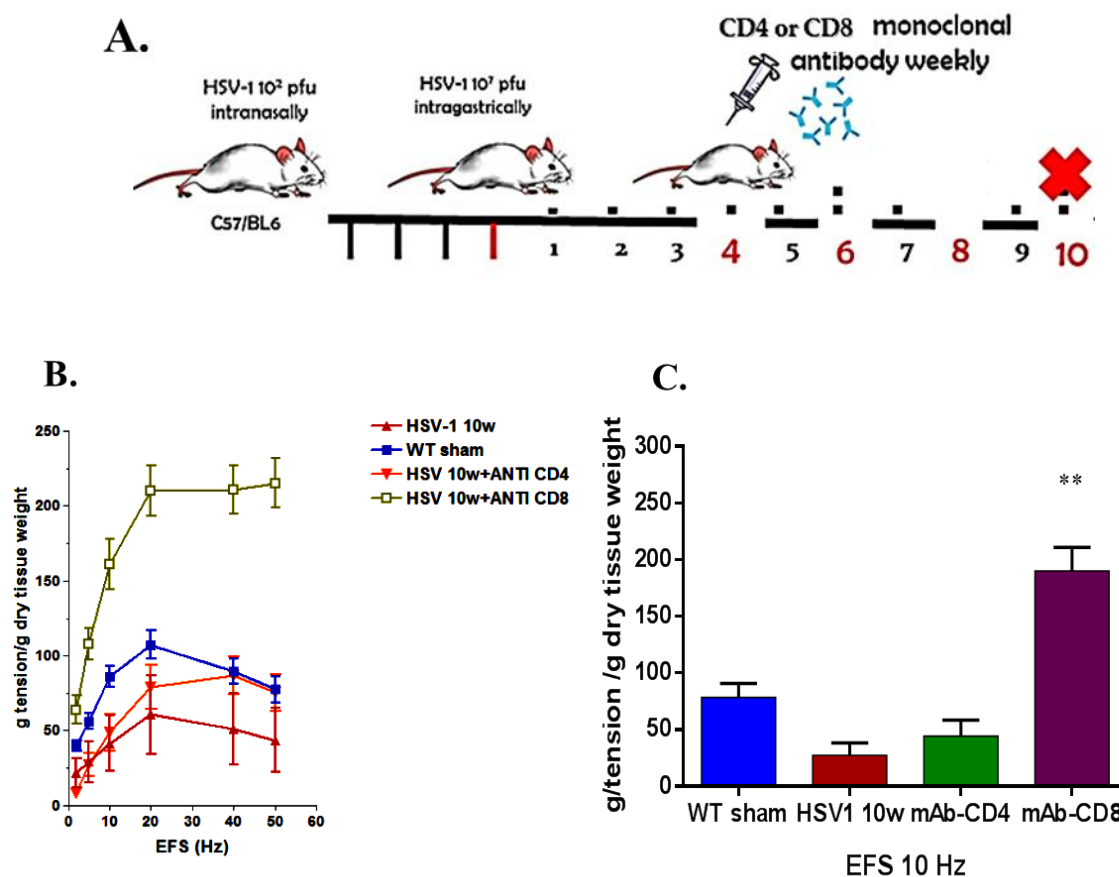
Figure 20. Effect of CD4 and CD8 T-cells depletion at 10 weeks after HSV-1 infection.

(A) Scheme for the experimental depletion in vivo of the CD4 and CD8 T lymphocytes model. Mice were injected intraperitoneally with 200 ng of monoclonal anti-CD4 (clone GK1.5) and anti -CD8 (clone 2.13) suspended in PBS. After 4 weeks the mice were sacrificed. (B) Effect of HSV-1 infection on the ileal longitudinal segment contractions induced by EFS at 0 to 60 Hz after treatment with mAb-CD4 and mAb-CD8 inoculation (C) Histogram represents changes of ileal contraction induced by EFS at 10 Hz,. n=at least 3 animals in each group.*P < 0.05 vs sham.

4.14. Adoptive transfer of CD8+ and CD4+ T cells from HSV-1 infected mice at 10 weeks causes neuromuscular damage in naïve C57/B16J mice

To further elucidate whether CD4+ and CD8+ T-cells infiltrating the LMMP at 10 weeks post IG inoculum are implicated in ENS pathophysiology, we performed adoptive transfer experiments. Thus, CD4+ or CD8+ lymphocytes were purified from LMMP of mice 10 weeks post IG HSV-1 inoculum, incubated overnight with UV inactivated HSV-1 or Vero cell lysate and then transferred in recipient mice (wild-type C57BL/6). After 7 days mice were sacrificed and the isometric muscle tension measured on ileal longitudinal segments (Figure 5.8.A).

Adoptive transfer of CD4+ or CD8+ lymphocytes purified from LMMP of mice 8 weeks post IG HSV-1 inoculum did not influence the neuromuscular contractility following EFS stimulation at 10 Hz. On the contrary, CD4+ lymphocytes exposed *in vitro* to inactivated HSV-1, caused a significant reduction in EFS-induced ileal contraction, comparable to HSV-1 mediated effects *in vivo*. Intriguingly, adoptive transfer of CD8+ lymphocytes, isolated from LMMP of mice 10 weeks post IG HSV-1 inoculum, and exposed *in vitro* to inactivated HSV-1 significantly enhanced neuromuscular contractility.
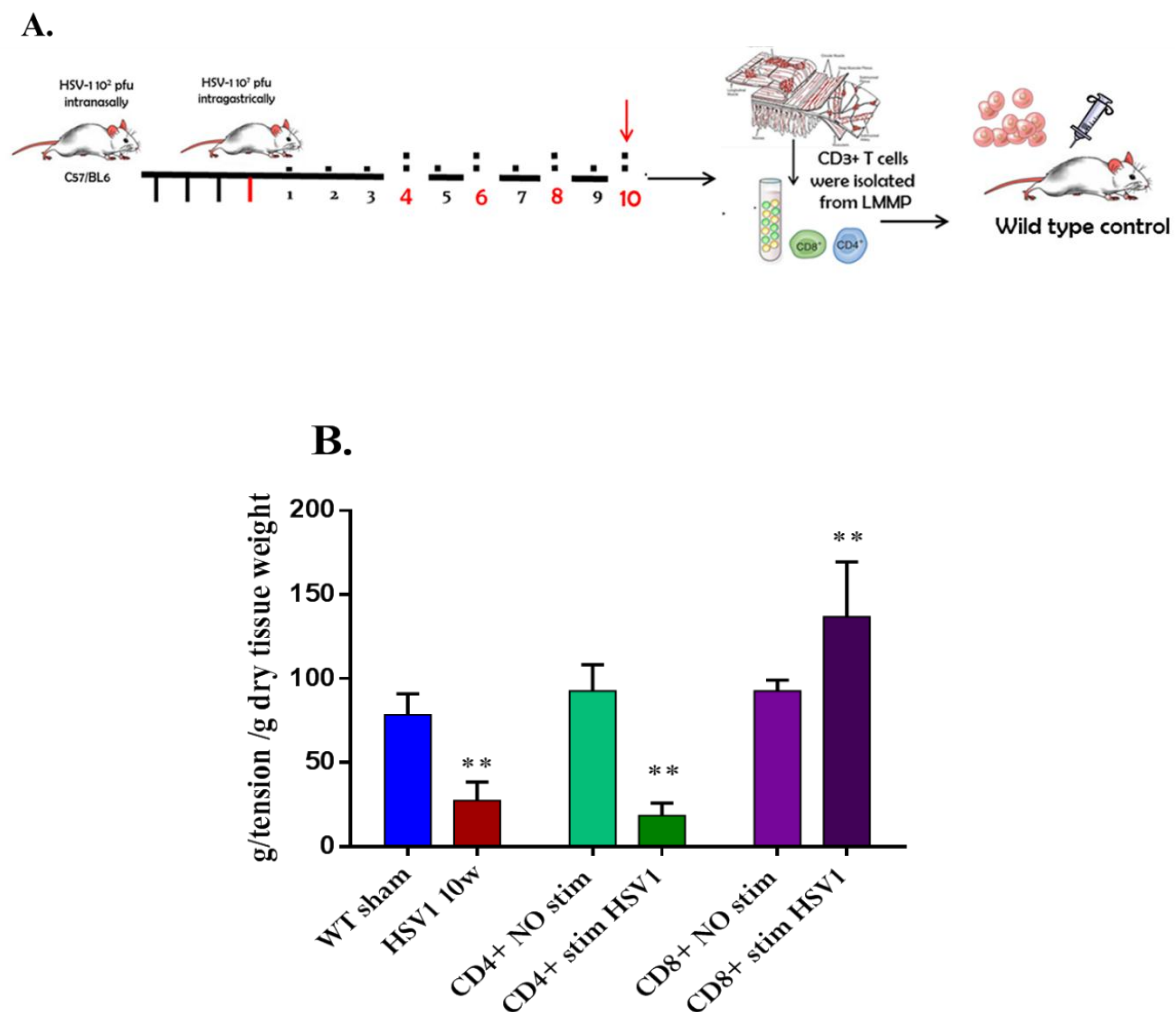
Figure 21. Effect post adoptive transfer of T-cells infiltrated in LMMP at 10 weeks on intestinal contractility.

*(A) Scheme for the experimental adoptive transfer in naïve mice of CD4+ and CD8+ lymphocytes isolated from LMMP at 10 weeks after IG inoculum. After 7 days, mice sacrificed and measured intestinal contractility following EFS (10 Hz). (B) Adoptive transfer of non specific reactive HSV-1 activated CD8+ and CD4+lymphocytes alters intestinal transit compared to sham; ** p<0,01 vs WT; n=at least 3 animals in each group.*

4.15. Adoptive transfer of lymphocytes from LMMP at 10 weeks post HSV-1 infection reduces colonic motility

To assess the neuromuscular damage mediated by activated T-cells at 8 weeks, we evaluated *in vivo* colonic motility following adoptive transfer by measuring the time taken to expel a glass bead inserted into the distal colon. The bead expulsion time was recorded at T0, 2 days, 4 days and 6 days. A significant reduction of expulsion time starting from 2 days measured in mice transplanted with total lymphocytes HSV-1 stimulated, with total lymphocytes not stimulated and with CD8+ no HSV-1 stimulated compared to the colonic motility of mice transplanted with control lymphocytes (Figure 22).
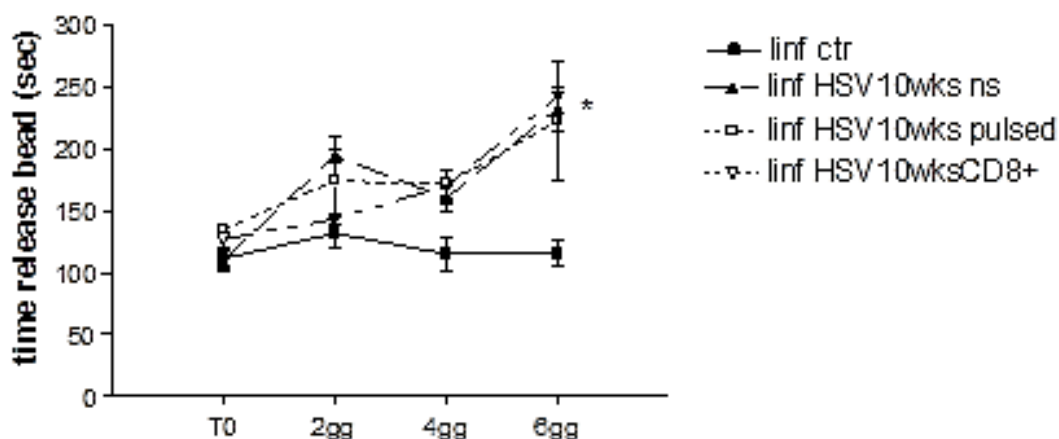

Figure 22. Effect of adoptive transfer of T-cells isolated from LMMP at 10 weeks on colonic motility.

*Colonic motility assessed by measuring the amount of time between bead placement and expulsion of the bead. Measure were recorder at T0, 2, 4, 6 days post transfer. Data are reported as the time to expulsion (sec); n=at least 4 animals in each group; * p<0.05 vs sham.*

Chapter 5: Conclusion

Alterations of ENS play a major role in the onset of gastrointestinal disorders (De Giorgio et al., 2004). This is due to the high complexity of this neuronal network and its ability to control, independently of CNS, all bowel functions (Goyal and Hirano, 1996). Abnormalities of ENS are traditionally linked to motility disorders or aberrant visceral sensitivity, but recent experimental evidence suggest that dysfunctions of ENS are involved also in inflammatory bowel disorders (Giordano et al. 2008; Natale et al. 2008). Although the pathophysiology of enteric neuronal damage is still unclear, the presence of inflammatory cells nearby damaged neurons seem to be a common feature of these

In the first part of my project, I have evaluated the presence of α-herpesviruses, HSV-1/2 and VZVZ, in the ENS in a prospective cohort of patients undergoing bowel surgery. The possibility to collect fresh samples, to avoid tissue damage, the extraction procedure we used and the high sensitivity of the real time PCR employed (Castagliuolo I. et al. 2010; Birgisson S. 1997) allowed to detect the viral genome in 47% of specimens, both in ileal and colonic tract. However, no correlation was found with diseases characterized by enteric neuropathies such IBD, since the incidence of positive specimens in tissues obtained from patients with colon carcinoma and Ulcerative Colitis or Crohn's disease was comparable. These data extend previous reports in experimental animals (Gershon MD. 2005) and in human autoptic specimens indicating the ability of these nerves to reach the enteric nervous system. It is possible that the HSV-1 reaches the ENS diffusing from the gut lumen as a consequence of high viral load swallowed during asymptomatic viral shedding from the oral mucosa, whereas recent studies suggest that VZV may reach the intestine via sensory fibers from the DRG (Zerboni L. et al. 2010). The absence of correlation between the presence of viral genome and enteric neuropathy seem to exclude a direct neuropathic effect. However, these viruses are known to establish persistent latent infections in trigeminal ganglia of animals and humans inducing only a mild infiltrate of immune cells required to prevent reactivation (Theil et al., 2003). We can speculate that in subjects predisposed to inflammatory diseases (i.e. IBD) the persistence of the inflammatory infiltrate might ultimately cause neuronal damage. Indeed, the higher copies number of viral DNA in ileal specimens of CD patients seem to indicate at least a favourable effect of inflammation on viral replication. In addition, we did not evaluated the possibility that the viral infection is segmental, since examining only a small specimen for each patient we

cannot exclude a positivity in different gastrointestinal districts. Overall, our data demonstrate for the first time in a large human cohort that neurotropic viruses are present in enteric nerves, although at the moment we cannot draw any conclusion on the nature of the infection and whether they contribute to the onset of human diseases.

To investigate whether the ENS infection by HSV-1 was able to damage the intestinal neuronal network our research group has developed a new experimental model in mice. In this experimental set-up, following intragastric injection of the virus, a persistent ENS infection is established. Using this model our research demonstrated in rats that HSV-1 infection caused time-dependent neuromuscular abnormalities (Brun et al., 2010) suggesting a complex interplay between the virus and host immune mechanisms. Therefore, in the second part of my project, I validated the model in mice and investigated the involvement of immune cells in ENS damage. Alike in rats, intragastric inoculum of HSV-1 elicited anomalies in the ileal contractility and in slower gastrointestinal transit. These anomalies were compatible with a reduced activity of cholinergic nerves, as previously observed in rats, confirming the existence of a neuronal sufferance (Brun et al. 2010). Intriguingly, the gastrointestinal dysmotility showed a time-dependent course: significant anomalies in ileal contractility and gastrointestinal transit were detected in the early phases of infection (1-2 weeks post IG inoculum) and at late time (6-10 weeks post IG inoculum). Indeed, a morphological analysis of the myenteric plexus confirmed the neuronal sufferance since expression and organization of peripherin and βIII-tubulin neurofilaments were altered in at the same time points of gastrointestinal dysmotility. Peripherin is a type III intermediate filament protein expressed in neurons of the enteric nervous system and peripheral, which seem to be involved in the growth and shape maintenance of neuronal extensions. During the development of the nervous system in combination with the formation and growth of axon, significantly increases the expression of peripherin indicating that this neuronal protein is involved in regenerative processes and remodelling of the cytoskeleton (Helfand et al., 2003). In fact, an abnormal accumulation of peripherin appears to be a common feature of neurodegenerative diseases although the neuropathological role of these aggregates is still unknown (Lariviere and Julien, 2004; Liem and Messing, 2009). The βIII-tubulin is a protein of microtubules that mediate transport of various cellular components within cells and often is considered the main neuronal marker associated with the cytoskeleton during development. The expression of βIII-tubulin is greater during the period of growth and neuronal maturation; levels decrease

in the adult central nervous system, and remain high in the peripheral nervous system (Jiang and Oblinger, 1992).

The time-dependent gastrointestinal dysfunctions and neuronal damage strongly suggested the existence of different underlying mechanisms. Since viral-mediated tissue damage is dependent on the replication cycle (Rautemaa R. et al. 2002) we evaluated the nature of HSV-1 infection in the ENS. By real-time PCR we detected both LATs transcripts and several viral mRNA coding for very early and early viral proteins. LATs are the main factors involved in latency and interfere with apoptotic processes inhibiting the activation of caspases 8 and 9 to promote the survival of infected cells (Henderson G. 2002). Moreover, LATs are also the precursor of four different miRNAs that inhibit the expression of two key proteins in the viral replication and thereby stabilize the state of latency (Perng et al., 2000; Knipe and Cliffe, 2008). However we detected also mRNA transcripts of viral immediate early gene ICP0 and ICP4, essential for the activation of viral genes (Perng and Jones, 2010) as well as viral late genes such VIP16 and gC although gD was never detected. Overall, these data suggest that HSV-1 infection in the murine ENS is in a dynamic condition. Neurons with a latent infections coexist with neurons in which the virus is attempting to replicate, although it is unable to complete the replication cycle. These data strongly resemble the picture reported in the human trigeminal ganglia where the presence of lymphocytes reactive to specific HSV-1 proteins suggest the ability of the virus to express a few proteins even if "in latency" (Zoppellaro C. et al. 2013). Furthermore, these data seem to support the view that HSV-1 latency is not completely silent in matter of expression of viral proteins, but rather it is a dynamic condition in which the virus expresses few proteins probably stimulating immune cells.

The presence of HSV-1 attempting to replicate in neurons of the myenteric plexus might trigger an adaptive immune response. While this response is necessary to suppress virus replication, it may eventually damage infected neurons causing cell death and foci of tissue pathology like previously shown in the central nervous system (Conrady C.D. et al. 2009). We detected a CD3+ infiltrate in the LMMP with a similar time-course of late intestinal dysmotility post-IG viral inoculum. Thus, starting at 6 weeks post-intragastric viral inoculum we observed an increase in activated lymphocytes in LMMP combined to CD3+ lymphocytes directly infiltrating the myenteric ganglia. Interestingly, CD3+ infiltrate within myenteric ganglia have been described in several gastrointestinal neuropathies (Facco et al. 2008; Lakhan S.E. et al. 2010; De Giorgio 2004), often expressing activation markers (Clark SB. et al.; 2000; Cerf-Bensussan N. 1985). The presence of activated CD3+

has been widely described during HSV-1 acute infections (Smith PM. et al. 1994) but also in latently infected trigeminal ganglia (Theil D. et al. 2003) and their presence has been linked to the control of viral infection. The presence of CD4+ and CD8+ HSV-1 reactive lymphocytes in the LMMP of infected mice further supports the view that a continued and/or repeated exposure to HSV-1 antigens activates locally an immune response eventually producing the neuronal damage and/or dysfunction associated to gastrointestinal dysmotility.

To clarify the pathophysiologic relevance of CD8+ and CD4+ T cells infiltrating the LMMP in the onset of the neuromuscular anomalies observed at 8 and 10 weeks post IG HSV-1 infection we performed immune-depletion and immuno-transfer experiments. We observed that CD8+ T lymphocytes depletion was associated to hypercontractile responses, whereas CD4+ depletion did not modify the neuromuscular contractility. Moreover, CD8+ lymphocytes transferred to recipient naïve mice caused intestinal dysmotility only if challenged in vitro with HSV-1 antigens. Overall these data suggest that CD8+ cells are the main players in controlling HSV-1 infection in the ENS but also mediating the neuronal damage. These data are in agreement with previous studies reporting the dominant role of this T-lymphocytes subset in mediating neuronal damage during HSV-1 infections (Melzer N. et al. 2009; Zhu Y. et al.2006).

It is well known the ability of HSV-1 to modulate host innate and adaptive immune responses (Chew T. et al. 2009), however both in this experimental model as well as in other models of murine infection (Theil D. et al., 2003) it is not completely clear how activation of lymphocytes is manipulated locally. In vivo, T cells are activated after receiving two signals from an antigen-presenting cell (APC), either professional or not professional. The first signal originates from their T cell receptor upon ligation with MHC class I or II molecules containing TCR-specific peptide, and the second signal derives from a costimulatory molecule, such as CD28 (Smith-Garvin J. et al. 2009; Medina M.A. et al. 2012).

To better understand immunologic status of CD3+ infiltrating LMMP to 1-10 post-IG HSV-1 inoculum, we investigate the expression of co-stimulatory molecules involved in the immune response regulation. Whereas MHC-I and MHC-II mRNA levels was up-regulated starting from 1 week post IG HSV-1 inoculum, the key co-stimulatory molecule CD80 was up-regulated only transiently at 1-2 and 8-10 weeks post IG HSV-1. Moreover, at 8-10 weeks post IG HSV-1 inoculum myenteric neurons seem to express this potent co-stimulatory molecule possibly contributing to activate CD3+ lymphocytes. In the CNS up-

regulated expression of CD80 during inflammatory processes has been described in microglia (Bechmann et al., 2001), neurons (Issazadeh et al., 1998) and neural stem cells (Imitola et al., 2004).

Moreover, CD69 and CD28 mRNA were increased in the LMMP following HSV-1 exposure. The receptor CD69, has been described as the earliest activation antigen of lymphocytes, inducing TGF-β expression and suppressing the production of pro-inflammatory cytokines IL-17 and IFN-γ (Sancho D. 2003; Radulovic K. 2013) while membrane receptor CD28 play a role to enhance TCR-induced proliferation and differentiation of naive T cells. Activated T cells produce toxic molecules, including Granzyme B and pore forming protein-like (Pfpl) that are able to induce intracellular cascade of caspases killing the target cells. In addition, CTLA-4 and PDCD-1 mRNA level, that negatively regulate T-cell activation following interaction with CD80, significantly increased in the early phases of HSV-1 infection but drastically decreased starting at 6 weeks post HSV-1 post infection. Altogether these data indicate the presence of activated lymphocytes in the LMMP with cytotoxic potentiality but also the presence of strong inhibitory signals in the first weeks post-infection that completely disappear in the late phases of infection. The down regulation of CTLA-4 in the late phase of infection, in light of the strong clinical impact of its blockade, further support a strong activation of CD4+ and CD8+ effector cells (Wolchok JDand Sanger Y., 2008) in agreement with its modulatory effect on anti-viral responses (Fuse S. et al. 2006).

In this scenario, we would expect a strong immune response, able to eradicate the viral infection eventually causing massive neuronal damage. However, we never detected a massive tissue damage, but rather several data indicated that the immune responses is at least partially blunted by the virus. The up-regulation of the inhibitory molecule H2Q9 and the reduced levels of lymphocyte-effector molecules Granzyme B and Pfpl, strongly suggest that the immune response in the myenteric ganglia is depotentiated. CD8+ T cells exhaustion phenotype was associated during the chronic infection as reported in experimental models and human infection including HIV (Fuller MJ et al. 2004) and HCV (Gruener NH. Et al. 2001; Shin H. et al. 2007;)

In summary, in this study we demonstrated for the first time the presence of neurotropic virus in the enteric nerves of about 50% of the population. Although this presence does not directly correlate to gastrointestinal disorders, using a mouse model we have demonstrated that HSV-1 infects the enteric nerves establishing a latent infection. However, several viral genes and proteins are expressed in enteric neurons, triggering a local adaptive immune

response characterized by recruitment of virus specific CD4+ and CD8+ T cells. Virus reactive lymphocytes are responsible of neuromuscolar dysfunctions mediating gastrointestinal dysmotility. Within the infected myenteric ganglia the neurons express both co-stimulatory molecules and molecules to inhibit T lymphocytes activity, modulating the amplitude of the immune response able to control viral replication with a limited neuronal damage. Indeed, virus-activated lymphocytes are known to drive microtubule axonal destabilization in independent manner of neuronal death (Miller, 2013), according to inadequate levels of granzyme and perforin produced in our study. In conclusion, α-herpesviruses can easily reach the ENS in humans and, according to the data in the mouse model, a complex interplay with the immune cells takes place, eventually leading to neuronal damage or dysfuntion. How and whether HSV-1 induced neuronal harm is the triggering event of clininical pictures in humans needs to be further investigated.

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