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The Effects of HSV-1 Infection of the ENS on Beta Amyloid Deposition during Gut Dysbiosis

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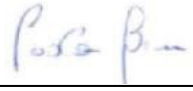
I certify that I have read this thesis and that in my opinion, it is fully adequate in scope and quality as a thesis for the degree of Masters of Pharmaceutical Biotechnologies.



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This Thesis project is the result of the independent work of Ahmed Hablass. All other work reported in the text has been attributed to the original authors and is fully cited in the text and listed in the bibliography section.

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(Applicant)

Abstract

There is a growing interest in understanding and mapping the effects of potential neurotropic viruses such as HSV-1, infecting the enteric nervous system (ENS). Tremendous efforts and studies are committed to linking HSV-1 to neurodegenerative diseases such as Alzheimer's disease as well as the role of enteric glial cells (EGCs) in the ENS that contribute via the microbiota-gut-brain-axis (MGBA) cross talk to neurodegeneration. In the present study, C57BL/6J mice are employed as the animal model of infection, in which HSV-1 is found to infect and persist in the enteric neurons. The innate immune response is stimulated upon HSV-1 infection and through CCL2/CCR2 pathway, it results in the recruitment of inflammatory macrophages and release of reactive oxygen species that cause GI dysmotility with an apparent upregulation in inflammatory mediators including IL2, TNFα, and IFNγ in the longitudinal muscle myenteric plexus (LMMP). The adaptive immune response is stimulated but considered as a secondary factor to dysmotility while the innate response is considered primary. The EGCs markers S100β and GFAP upregulation indicated the presence of the switched-on also known as an activated phenotype that leads to the cascade of neurodegeneration with an expected detection of β-amyloid in the ENS backed by Alzheimer's animal model simulations. The gut dysbiosis simulations show an expected decrease in the anti-inflammatory and gastroprotective effect of gut microbiota that is consistent with the changes exhibited in the ENS as well as explaining the etiology behind gastric dysmotility as the gut microbiome emerges as a key factor in maintaining gut barrier integrity.

Acknowledgements

In the name of God, the beneficent the merciful

to whom all praises due

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Chapter 1

Introduction

1.1 Herpes simplex virus 1

Herpes simplex virus 1 (abbreviated HSV-1) is one of eight *homo sapiens* herpes viruses (HHV-1), in which the family includes HSV-2 also labelled HHV-2, varicella zoster virus (VZV) which is HHV-3, Epstein Barr virus HHV-4, cytomegalovirus HHV-5, beta human herpes virus HHV-6, 7 and Kaposi's sarcoma associated herpes virus HHV-8. Herpes infections had been recognized ever since the ancient time of Greece, the term herpes translates into crawling or creeping and is employed as a reference to herpetic skin lesions (Bradshaw and Venkatesan, 2016). Out of all the herpesviruses the most closely related and similar are HSV-1 and 2, they have almost 70% homology and are regularly benign however they can also be severe in which each case is presented with an initial lesion that appears to be the same moreover, the most common mild clinical manifestations of HSV-1 and 2 are mucocutaneous infections which include:

Oral herpes in the form of cold sores, it is caused by both HSV-1 and 2, a clear vesicle that contains the infectious virus having a red base referred to as erythematous lesion in the vesicles base, it is characteristic as it looks like a dewdrop on a rose petal, in which the pustular (pus containing) encrusted ulcers and lesions develop (Hunt, 2016).

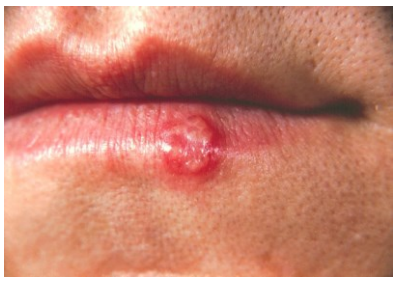


Figure (1) HSV-1 oral cold sore lesion of the lower lip

Gingivostomatitis which is usually caused by HSV-1, it happens most frequently in children less than the age of 5, it is characterized by fever, erythema, sore throat, and pharyngeal edema, which is followed by the development vesicular or ulcerative lesions on the pharyngeal and oral mucosa, the recurrent HSV-1 infections of the oropharynx could be manifested as cold sores (herpes simplex labialis) and mostly appear on the vermillion border of the lip, intraoral

lesions are quite uncommon in normal hosts as a manifestation of recurrent HSV infection on the other hand it occurs frequently in immunocompromised individuals (Whitley, 1996; Hunt, 2016).

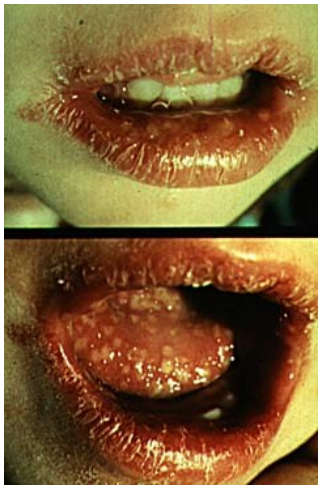


Figure (2) HSV-1 gingivostomatitis appears different than that of the cold sore in which it occurs once and is mostly very mild to go unnoticed

Genital herpes is mostly caused by HSV-2 with around 10% of the cases contributed for HSV-1, its primary infection is often asymptomatic however many painful lesions may develop on the glans or shafts of the penis in men while on the vulva, cervix, vagina and perianal region in women, the urethra may be involved in both sexes, in women the infection is associated with vaginal discharge, HSV transient viremia can be accompanied by several symptoms including myalgia, fever and glandular inflammation of the groin area, in the case of recurrence the infection is less severe with a shorter period of time, patients may first experience a burning sensation in the area about to erupt (Hunt, 2016).

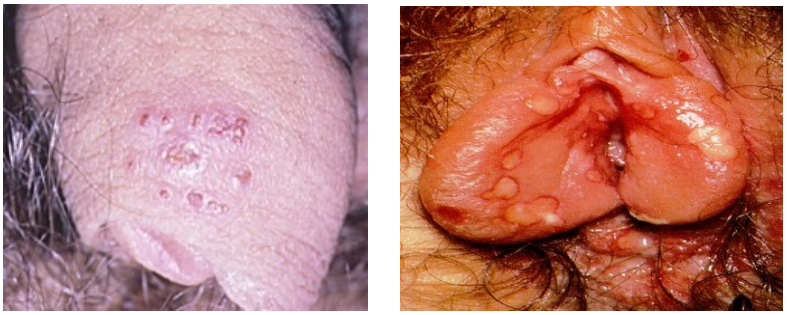


Figure (3) Genital herpes on the penis and vulva respectively

There are other HSV-1 infections including:

Herpes keratitis is an infection that is primarily caused by HSV-1, it can be recurrent, it is also accompanied by conjunctivitis in many cases; it may cause blindness in which it is the leading cause of blindness in the United States. The characteristic HSV keratoconjunctivitis lesions are dendritic ulcers which are best detected by fluorescein staining, there has been reports of deep stromal involvement and it may result in visual impairment (Whitley, 1996; Hunt, 2016).

Herpes whitlow known as the disease that occurs in individuals who come into contact with herpes infected bodily secretions which is caused by both HSV-1 and 2, in which it enters the body via small wounds on wrists or hands, it may also be caused by transfer from genitals to hands (Hunt, 2016).

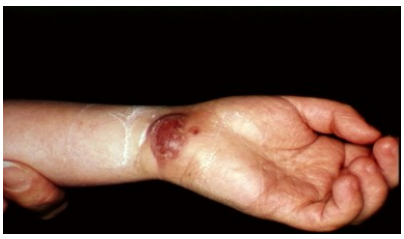


Figure (4) Herpetic whitlow on the wrist

Herpes gladiatorum is mostly contracted wrestlers, apparently it spreads through direct contact of the skin lesions from one wrestler to another (opponent), it usually appears in the head and neck regions as in the frequent sites of contact in wrestling, the lesions are more frequently on the body's right side which could be due to the fact that most wrestlers are right handed, other contact sports such as rugby the infections are also reported (Hunt, 2016).

Eczema herpeticum is discovered in children with active eczema, where they have a preexisting atopic dermatitis in which the virus can spread over to the site of eczema lesions, it also may spread to other organs such as the liver and adrenals (Hunt, 2016).

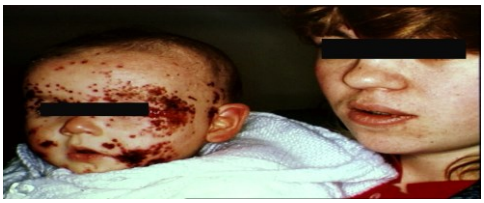


Figure (5) Eczema herpeticum in a baby

HSV proctitis characterized by inflammation of the anus and rectum (Hunt, 2016).

Moreover, there are severe HSV-1 infections including:

Neonatal HSV infections

This category of infections is usually caused by contact of the fetus with infected maternal genital secretions at the time of delivery, the manifestations of neonatal herpes include skin, mouth and eye infections, encephalitis and disseminated infection, as a result of an underdeveloped immune system in neonates the virus can spread rapidly to many peripheral organs and induces infection in the central nervous system manifested as neonatal encephalitis (Whitley, 1996; Hunt, 2016).

HSV-1 induced herpes simplex encephalitis (HSE)

In 1920s it has been demonstrated that the introduction of material from the skin lesions into scarred skin or cornea of rabbits induced encephalitis suggesting that HSV induces encephalitis in *homo sapiens* (Bradshaw and Venkatesan, 2016). HSV-1 being an endemic worldwide, it is capable of causing a severe life threatening condition that is known as herpes simplex encephalitis (HSE) in which more than 50% of HSE survivors develop life-altering neurological deficits in spite of available anti-viral therapies (Menendez, Jinkins and Carr, 2016).

HSV-1 induced HSE is characterized by hemorrhagic necrosis in the inferomedial part of the temporal lobe, the pathological condition initiates unilaterally and consecutively it spreads contralaterally to the temporal lobe, it is identified as the most common cause of sporadic and focal encephalitis in the United States, HSV-1 being the causative agent in most cases, the clinical manifestations include fever, headache, altered consciousness and abnormalities of behavior and speech, focal seizures may happen as well, the mortality and morbidity remain high regardless of antiviral therapy (Whitley, 1996).

HSV-1 infections in immunocompromised patients

HSV-1 in immunocompromised patients is clinically severe, progressive and demands more time in the healing process, some of the manifestations associated with HSV-1 infection in immunocompromised patients include esophagitis, pneumonitis, hepatitis, colitis and disseminated cutaneous disease, individuals suffering from HIV (human immunodeficiency

virus) are at high risk of exhibiting extensive orofacial or perineal ulcerations moreover, HSV-1 possesses higher severity upon burned patients (Whitley, 1996).

Overview

HSV-1 is identified as the leading cause of viral corneal blindness and encephalitis in developed countries. Anti-viral medications and supportive therapeutic approaches combat the extremely severe manifestations of the pathological condition. Instant identification and treatment could be lifesaving in the case of patients suffering from herpes simplex 1 encephalitis as it is the most common recognized cause of sporadic encephalitis worldwide. Clinicians must have the ability to identify the clinical signs and symptoms of the infection and be familiar with the diagnostic and therapeutic approaches as early identification and treatment are vital for an improved prognosis and overall outcome (Bradshaw and Venkatesan, 2016). The clinical manifestations are variable, they are dependent upon the nature of the disease as in primary or secondary; they are also affected by the route of contraction as in portal of entry, as well as the degree of the hosts immune competence. The HSV infection ranges from asymptomatic to mucocutaneous conditions including orolabial, ocular, genital herpes, eczema herpeticum, herpetic whitlow, herpes gladiatorum, as well as central nervous system complications as discussed earlier the most dangerous are encephalitis and neonatal encephalitis, fatal dissemination which is a threat specifically for immunosuppressed patients (Fatahzadeh and Schwartz, 2007).

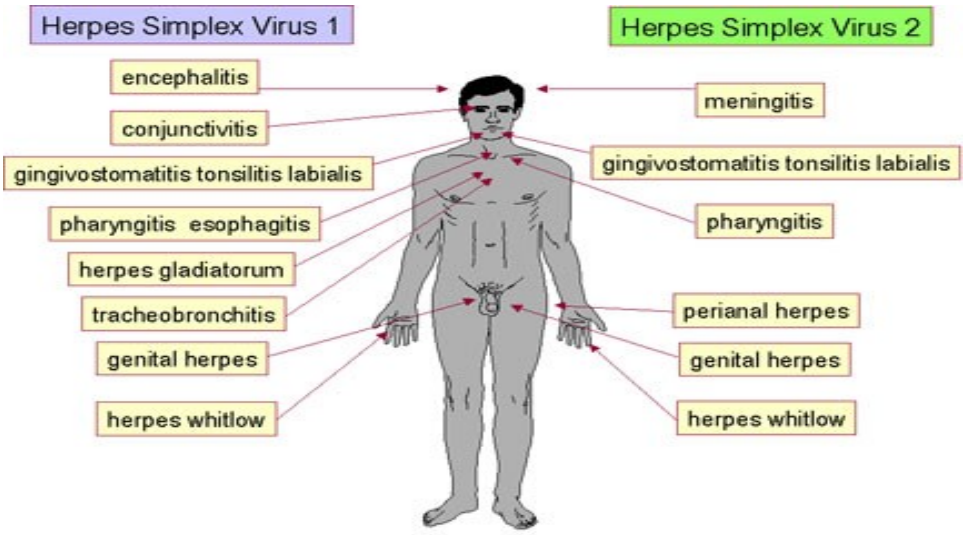


Figure (6) Disease induction chart of HSV-1 and 2

1.1.1 Structure and replication of HSV-1

HSV-1 has a four layered structure which includes a large core that contains a double stranded DNA genome (152kb) that is enclosed by an icosapentahedral capsid that is composed of capsomers. The capsid is surrounded by an amorphous protein coat referred to as tegument; it is enveloped in a glycoprotein bearing lipid bilayer derived from the membrane of host cells (Whitley, 1996; Sabeti, 2009). HSV-1 structure in detail contains the following:

Envelope in which HSV-1 is enveloped in, it buds from the inner membrane which is modified through the insertion of herpes glycoproteins. In the mature virus, these glycoproteins are the determinants of which cell is going to be infected as a result of the availability of the appropriate receptors, HSV-1 viral membrane is quite fragile and in the case of a damaged envelope the virus is non-infectious, a non-enveloped HSV-1 is readily falling apart and thus, can only be obtained through direct contact with an infected individual mucosal surfaces or secretions moreover, it cannot be obtained from toilet seats, HSV-1 envelope is sensitive to acids, organic solvents and detergents as well, as it is expected of its lipid bilayer based envelope (Hunt, 2016).

Tegument known as the space between the capsid and the envelope, it consists of the enzymes and encoded viral proteins that are essential for the initiation of replication, the various tegument proteins include virion host shut-off protein “VHS”, alpha- transducing factor “α-TIF” (VP16). The tegument’s bulk is not ordered in an icosahedral fashion but, a small part appears to be filamentous structures that surround the pentons, it interacts with the capsid extensively, the tegument’s interactions and locations with cellular transport proteins propose several functions of the tegument in guiding DNA transport into the nucleus (Jacobs et al., 1999; Hunt, 2016).

Capsid in which herpes viruses possess a doughnut shaped capsomere that ranges from 100 to 200 nanometer in diameter with an icosahedral nucleocapsid of 162 capsomeres, 150 hexons and 12 pentons that is encased by the tight adhering tegument (Jacobs et al., 1999; Hunt, 2016).

Genome that is composed of a large double stranded DNA moreover, the HSV-1 genome is composed of two segments one that is unique long U_L and another unique short U_S segments, they are both flanked by inverted repeats R, the L segment repeats are designated as ab and b' a', while that of the S segment are a' c' and ca, the genomic double stranded DNA is large, located centrally and linear with 152kb, it encodes 80 viral genes, with an approximate

percentage of 50% of those genes are essential for viral replication in cell cultures, while the other 50% encode for accessory functions that includes contribution to the viral life cycle in certain tissues or cell types The HSV-1 genes are classified into 3 categories depending on the transcription kinetics that involve a cascade of 3 temporal phases immediate early “IE or α”, early “E or β” and late “L or γ”, IE genes encode for infected cell proteins ICP0, ICP27, ICP4, ICP22 and ICP47, they mostly possess regulatory functions and they initiate early viral gene expression, E genes are essential for viral DNA synthesis and most E gene products are enzymes essential for DNA metabolism and serve as signals for the onset of DNA replication, L genes encode for structural components of the virus, 11 identified viral proteins “VPs”, they are located on the surface of the virion labelled as glycoprotein spikes, all the other VPs are located in the tegument as in the space between the capsid and envelope such as VP16 (Jacobs et al., 1999).

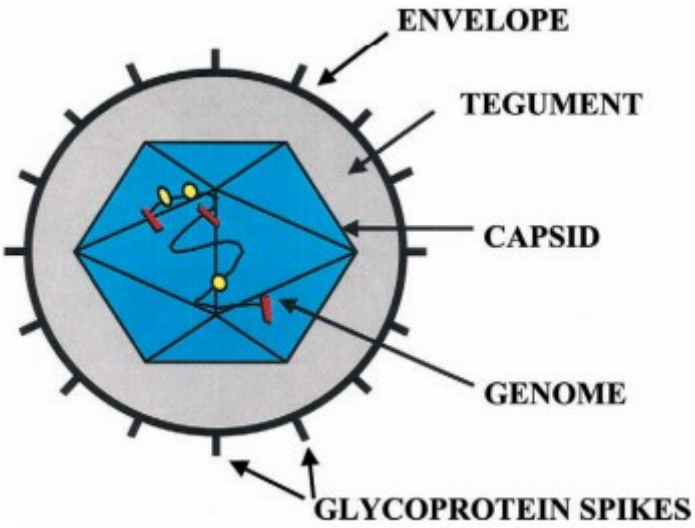


Figure (7) HSV-1 virus structure

The HSV-1 virion structure diameter ranges from ~120 to 300 nanometers, while the capsid is around ~100 nanometers, its DNA genome is organized regularly spaced ~26 Å in concentric layers within the capsid, as observed in figure 7 there are glycoprotein spikes on the envelope’s surface that vary in their amounts and numbers, in addition to multiple non glycosylated viral proteins, polyamines and lipids (Jacobs et al., 1999). These glycoprotein spikes are particularly essential for their interactions with the surface proteins of host cells mediating HSV-1 entry into the cells, the glycoproteins identified alphabetically start from gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN making a total of 12 identified VPs, several glycoproteins exist in

heterodimers such as gE-gI and gH-gL, they may associate with each other and they also possess the ability to function as oligomeric complexes (Karasneh and Shukla, 2011).

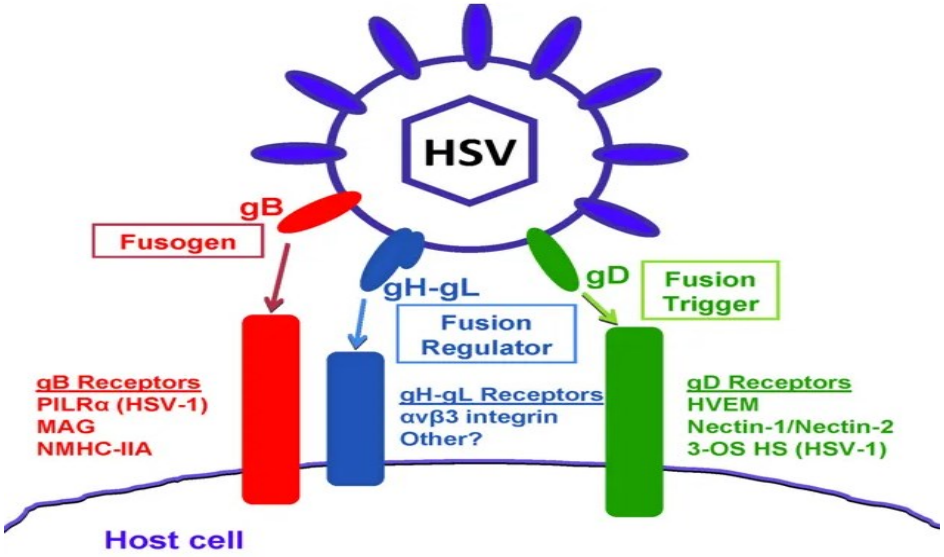


Figure (8) HSV-1 identified receptors

Out of all the glycoprotein spikes (VPs) only four glycoproteins namely gB, gD, gH and gL are essential and enough to permit viral recognition, interaction, fusion and integration into the plasma membrane of host cells (Karasneh and Shukla, 2011).

Virulence

The HSV-1 virus is well adapted to *homo sapiens* as it results in a lifelong infection that rarely causes mortality and it is readily transmitted amongst individuals (Derfuss and Held, 2011). HSV-1 undergoes productive infection in nonneuronal and neuronal cells in a way where the viral genes are transcribed in an ordered cascade, HSV may also establish quiescent or latent infection in peripheral neurons (Harkness, Kader and Deluca, 2014).

Replication cycle (lytic and latent)

The HSV-1 infection in *homo sapiens* initiates in epithelial cells in which the virus replicates within and it undergoes the typical lytic life cycle with the production of viral genes, this is all followed by the generation of infectious virions and lysis of cells (Held and Derfuss, 2011).

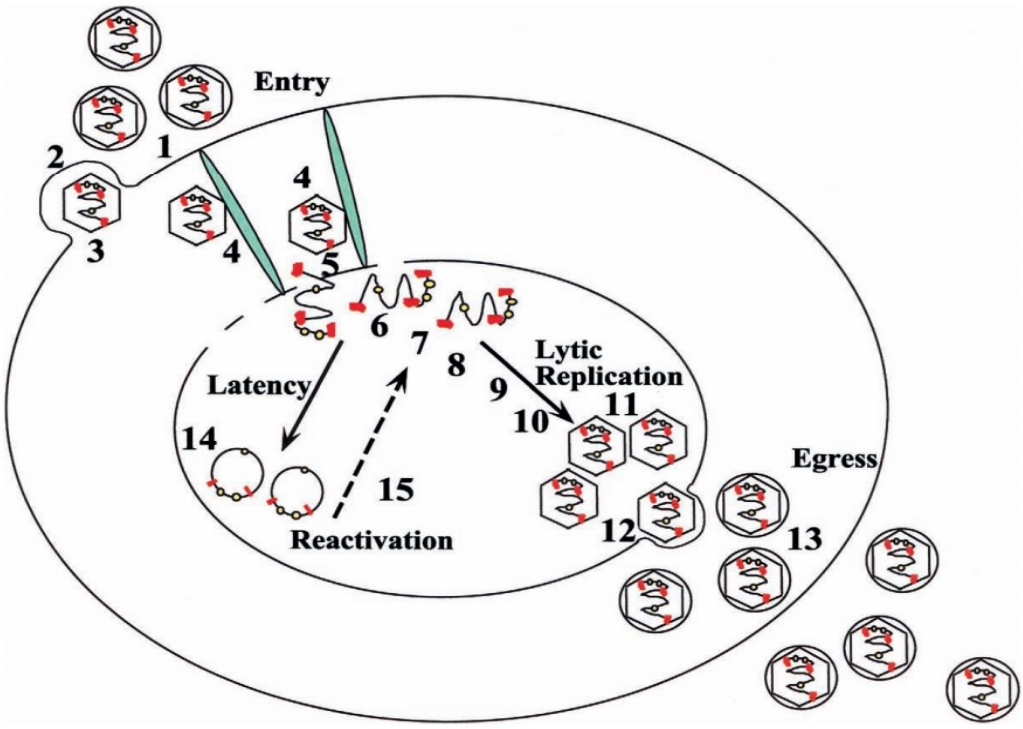


Figure (9) HSV-1 replication cycle (lytic and latent)

The lytic cycle of HSV-1 takes ~18 hours and it includes the following 15 steps:

1st step is the attachment of HSV-1 through its envelope's glycoprotein spikes (VPs) specifically gB to heparan sulfate proteoglycans HSPG found on the host cell's surface, this serves as a step to concentrate HSV-1 on the surface of host cells by tethering it (Jacobs et al., 1999; Karasneh and Shukla, 2011).

2nd step involves an entry specific fusion based interaction of HSV-1 gD with the gD receptor, the interaction permits tight anchoring of HSV-1 to the plasma membrane of host cells by bringing them into close juxtaposition, it is reported that the gD receptor undergoes conformational changes and transmits a signal to gB and gH-gL receptors which leads to membrane fusion hence, fusion requires the formation of a fusogenic complex a multiprotein complex (Jacobs et al., 1999; Karasneh and Shukla, 2011).

3rd step is post-fusion in which HSV-1's capsid is released into the cytoplasm of host cells (Jacobs et al., 1999).

4th step is the active transport of the capsid along with the host cell's microtubular cytoskeleton to nuclear pores (Jacobs et al., 1999).

5th step involves the release of HSV-1's DNA into the nucleus which is facilitated by tegument proteins. In the nucleus HSV-1's genome is circularized and transcribed in the cascade of 3 temporal phases IE, E and L (Jacobs et al., 1999).

6th step is the induction of transcription of IE genes via α-TIF (VP16) which in the presence of cellular transcription factors binds indirectly to viral DNA at an identified consensus sequence and induces transcription of IE genes via host RNA polymerase (Jacobs et al., 1999).

7th step in which transcription of E genes occurs, HSV-1 IE has 5 gene products four of them encoding for ICP0, 27, 4 and 22 which regulate the productive cycle by initiating transcription of E genes while the fifth product ICP47 blocks the presentation of antigenic peptides on the surface of infected cells (Jacobs et al., 1999).

8th step involves DNA synthesis, in which E gene products ICP6 (viral ribonucleotide reductase RR), ICP8 (DNA binding protein), ICP36 (thymidine kinase TK) and DNA polymerase signal the onset of viral DNA synthesis, where the chromatin gets degraded and DNA synthesis starts.

9th step is the transcription of L genes, a 3rd round of transcription yields L gene products which are essential for viral DNA encapsulation and envelopment (Jacobs et al., 1999).

10th step involves capsid assembly, **11th step** involves packaging of DNA into preformed capsids, **12th step** involves capsulation and envelopment, finally the **13th step** is the egression of HSV-1 out of host cells (Jacobs et al., 1999).

14th step is identified as the latency phase where the HSV-1 genome as a circular or concatemeric molecule is bound by nucleosomes and it does not express viral genes however, it is involved in generating latency associate transcripts, while the **15th step** involves the reactivation of HSV-1 from latency through a variety of factors including viral, host cell and external factors (Jacobs et al., 1999).

HSV-1 latency mechanisms and signals

HSV-1 causes a lifelong chronic infection of the TG (trigeminal ganglion) neurons which is referred to as neuronal HSV-1 latency (Velzen et al., 2013). Around 52 to 84 percent of *homo sapiens* population is latently infected by HSV-1, persistence and latency have been demonstrated in TG mainly, vestibular ganglia, facial ganglia, geniculate ganglia and the brain (Held and Derfuss, 2011). It's latency gene expression in the neurons is restricted to one part

of the double stranded DNA in which transcription from this region results in a number of viral RNAs referred to as latency associated transcripts (LATs), the sensory fields across the entire GI tract surface are represented by the neurons of the nodose ganglia, as the ganglia innervates into the gastrointestinal tract, there is reported HSV-1 latency in the nodose ganglia detected via in situ hybridization of LATs, it is sensible to conclude that a number of recurrent inflammatory and functional GI disorders may indirectly or directly be attributed to latent viral reactivation in the nodose ganglia which results in enteric HSV-1 infection (Gesser and Koo, 1997). A study confirms that HSV-1 infection results in the accumulation of immediate early IE transcripts with greater abundance in neuronal cells compared to non-neuronal cells, indicating the presence of a greater transcription across the genome in neuronal cells, specifically LATs (Harkness, Kader and Deluca, 2014).

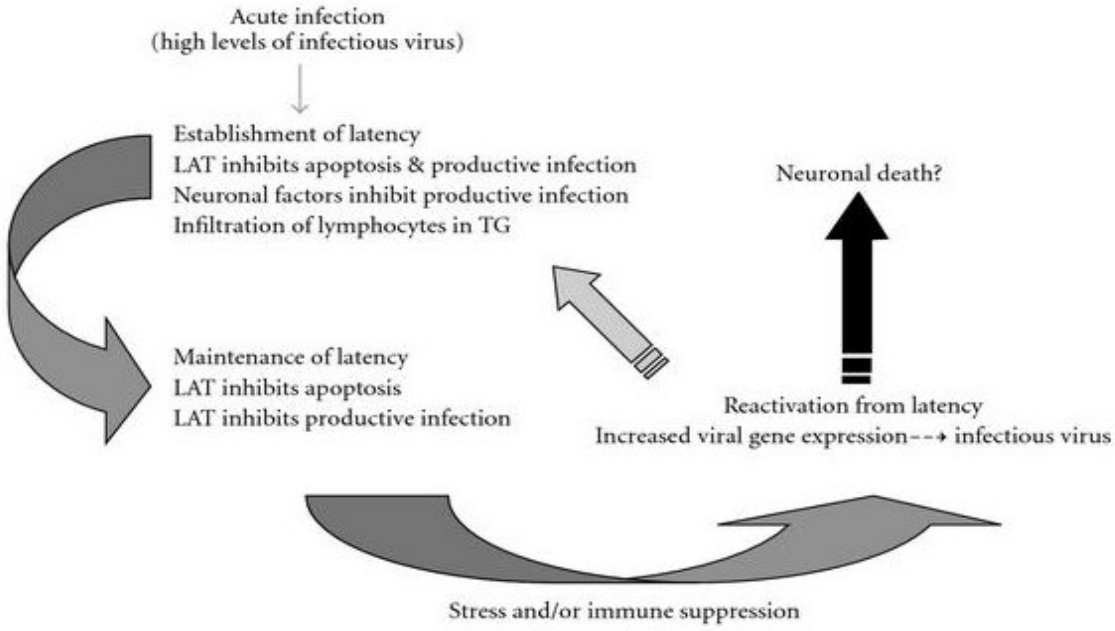


Figure (10) HSV-1 latency-reactivation cycle

HSV-1 latency-reactivation cycle is classified into three main steps including establishment, maintenance and reactivation, in which the establishment step involves the entry of HSV-1 viral genomes into sensory neurons while in the maintenance step is when the HSV-1 cannot be detected and LATs are abundantly expressed and finally the reactivation step which involves an external stimuli such as stress or immunosuppression which stimulates HSV-1 expression in which there is abundant HSV-1 gene expression, it can be detected and isolated in the trigeminal ganglia, eye swabs or nasal swabs (Perng and Jones, 2010).

Reportedly the most common trigger for latent reactivation of HSV-1 is stress, it has been shown that in mice through decreasing the number and functionality of $CD8^+$ T-cells, stress induced immunosuppression has been shown in *homo sapiens* that led to increased activation of latent infection, Glucocorticoids decrease the amount of T-cells in the TG of mice latently infected with HSV-1, it also regulates gene expression and induces modification of chromatin status therefore, may possibly activate viral gene expression, it also has been shown that neuronal excitation affects the efficiency of HSV-1 replication whereby, the increased neuronal excitability inhibited replication while the decreased neuronal activity enhanced replication (Held and Derfuss, 2011).

There are studies that indicate different triggers to latency and reactivation of HSV-1, in which one study reports that the blockade of HVEM-LIGHT-BTLA-CD160 contributes to reduced latency and reactivation therefore, suggesting that the complex network of interactions of HSV-1 and its ligands gD, HVEM, LIGHT, BTLA, CD160 and LATs regulate its latency and reactivation (Wang et al., 2018). Another study reports that glucocorticoid drugs such as dexamethasone induced HSV-1 reactivation in a dose dependent manner (Halford, Gebhardt and Carr, 1996).

The potential of HSV-1 to effectively infect a wide variety of hosts and cell types implies its evolution to utilize multiple receptors, pathways and strategies that facilitates its entry into multiple cell types (Karasneh and Shukla, 2011). HSV-1 possesses the ability of reactivation post latency due to LATs in the TG signaling for survival as in inhibition of apoptosis and as a result of multiple pathways, external stimuli, drugs, immunosuppression or stresses it is capable of reactivating and causing different complications (Perng and Jones, 2010). Addedly its capability to spread further which is confirmed by the high prevalence of HSV-1 induced complications of blindness, encephalitis, and inflammation (Karasneh and Shukla, 2011).

1.1.2 Epidemiology

HSV-1 is a common infection that has seropositivity that is among older adults that is estimated to be between 60% to 90% worldwide, there is a survey that dates from 2005 to 2010 which includes Americans from the ages of 14 to 49 that had an estimation of HSV-1 seropositivity at ~54% while HSV-2 at ~16% in the U.S, although HSV-2 is able to cause encephalitis especially in immunocompromised patients HSV-1 is the major cause of encephalitis accounting to ~90% of herpes simplex virus encephalitis in adults and children, in spite of rarely being manifested as encephalitis in infected individuals it still is the most consistently common cause of sporadic encephalitis worldwide. The incidence of herpes simplex encephalitis (HSE) is estimated to be between 2 and 4 cases for every million worldwide with similar figures in USA, the HSV has a bimodal distribution with a peak incidence in juveniles up to 3 years old in the very young on the other hand in over 50 years old adults however, the majority of cases happen to be in those over 50 with no distinction amongst different sexes the HSV infections occur equally (Bradshaw and Venkatesan, 2016).

In 1990 to 2004 HSV-1 seroprevalence in the U.S was 57.7% whilst that of HSV-2 was 17%; in a study regarding U.S college students of the percentage of genital herpes samples, it was recorded that HSV-1 increased from a percentage of 31% in the year 1993 to 78% in 2001, in a more recent study it had been shown that HSV-1 was responsible for nearly 60% of genital herpes. In a recent U.S study that estimates the seroprevalence of HSV-1 and 2 employing data from the national health and nutrition examination survey (NHANES) it was found that there are large declines in HSV-1 seroprevalence among the ages of 14 to 19 year olds, within this age group HSV-1 seroprevalence declined by nearly 23% from a percentage of 39% to 30.1%, from the years 1999-2004 to 2005-2010 respectively and there was a decline by more than 34% ever since peaking in 1988-1994, ages among 14 to 49 year old HSV-1 seroprevalence declined by nearly 7% from a percentage of 57.9% to 53.9% dating from 1999-2004 to 2005-2010 respectively, and nearly 13% decline ever since 1988-1994. The seroprevalence of HSV-2 was 15.7% in the years 2005-2010 that had no significant difference from the estimates recorded for the years 1999-2004. Ever since the years 1976 to 1980 the seroprevalence of HSV-2 has increased significantly, that large increase is primarily recorded from the years 1976-1980 to 1988-1994. There is a statistically essential decline in HSV-2 seroprevalence which was observed from the years 1988-1994 to 1999-2004 however, since that time the findings indicate that the seroprevalence has plateaued (Bradley et al., 2014).

1.1.3 Inflammatory response against HSV-1

Studies conducted in HSV-1 models are suggestive of a central role of TG infiltrating viral specific CD8 T-cells that control reactivation, There is a selective retention of HSV-1 reactive T-cells in TG of *homo sapiens*, which is suggestive of their role in modulating reactivation by identifying HSV-1 locally expressed proteins. There is a reported characterization of HSV-1 proteins identified by viral specific CD4 and CD8 T-cells that were recovered from *homo sapiens* TG, the TG sample source was human cadavers in which the sample was obtained within hours after death in order to characterize the local HSV-1 T-cell specific response in HSV-1 latently infected *homo sapiens* TG, juxtaposition of CD4 and CD8 to TG neurons was detected, clusters of T-cells that consist of CD4 and CD8 surrounded HSV-1 infected neurons that were identified via in-situ recognition and anti-viral function tests (Velzen et al., 2013).

An immunohistochemical characterization of *homo sapiens* TG infiltrates during HSV-1 latent infection consisted primarily of $CD3^+$ T-cells that belong to the subset of $CD8^+$ T-cells also CD68 macrophages, $CD4^+$ T-cells were mostly found spread amongst the axons, the majority of infiltrating T-cells have the effector memory phenotype as they express CCR5 and CXCR3 also their corresponding ligands CCL5 and CXCL10, these ligands and their receptors are essential for T-cells migration into the CNS and they are identified to be expressed on memory effector $CD8^+$ T-cells (Held and Derfuss, 2011).

HSV-1 onset of latency triggers immune cell infiltration of the ganglia, a persistent CD8 population remains and is maintained for the life of the host, with current evidence the $CD8^+$ T-cells can affect reactivation and lytic/latent decisions, the ganglionic $CD8^+$ T-cells surveys the ganglia for reactivation events, the study concludes that the latent ganglionic HSV-1 viral antigen expression kinetics plays a role in shaping $CD8^+$ T-cells responses, as latency progresses the sporadic HSV-1 viral gene expression becomes an important risk factor (Treat et al., 2019).

A study that links the HSV-1 antigens in the myenteric plexus of achalasia patients reports the presence of lymphocytic infiltrates suggesting an immune mediated mechanism. Achalasia is characterized by loss of myenteric neurons in the lower esophageal sphincter (LES) thus, it is known as an esophageal motor disorder; through flow cytometry and spectratyping analysis, they were able to characterize a significantly high lymphocytic infiltrate of $CD3^+$ and $CD8^+$ T-cells in comparison to controls. The data they accumulated suggest that the oligoclonal lymphocytic infiltrates observed in achalasia patients represent an immune inflammatory

reaction that is triggered via HSV-1 antigens. The cytokines released may contribute for the neuronal damage that accounts for the clinical manifestations exhibited in achalasia patients, the lymphocyte derived cytokines alter the local generation of neurotropic factors that are detrimental for regulating neuro-mediators and supporting neuronal survival, as a consequence, specific neuro-mediators may be lost in the early stages of inflammation due to the direct effects of $CD3^+$ and $CD8^+$ T-cells infiltration whereas, in the late stages of the disease the neurons die as a result of persistent exposure to inflammatory cytokines and lack of neurotrophic factors, they reported that the lymphocyte infiltration in achalasia patients had 3 peculiar characteristics including increased $CD3^+$ and $CD8^+$ T-cells lymphocytic infiltration in the LES, skewed TCR repertoire and strong reactivity towards HSV-1 antigens. T lymphocytes located in the LES represent the outcome of an immune reaction that is initiated via HSV-1 antigens (Facco et al., 2008).

1.2 Inflammatory bowel disease

Inflammatory bowel disease abbreviated IBD is an idiopathic chronic inflammatory condition of the alimentary tract which includes two mainly closely associated yet heterogeneously different pathological entities, ulcerative colitis and Crohn's disease. They are affecting genetically predisposed individuals, IBD is characterized by relapsing or chronic inflammation and immune activation in individuals who have genetic predisposition to trigger factors that interact with the flora of the gut inducing primary alterations of the digestive tract. Dr. Burrill Crohn, Dr. Gordon Oppenheimer and Dr. Leon Ginzburg are the first to describe Crohn's disease in 1932 as a terminal or regional inflammation of the ileum ileitis, Dr. Samuel Wilks recognized ulcerative colitis in 1859 as a discrete entity however, it was Sir Arthur Hurst who described the endoscopic pattern and distinguished the disease from other common pathological conditions such as bacillary dysentery, in terms of pathology Crohn's disease basically consists of transmural inflammation of all the layers from the mucosa to serosa and it may discontinuously include other areas such as any portion of the alimentary tract from the anus up to the mouth, whereby ulcerative colitis is more characterized to be submucosal inflammation that is limited to the colon, in approximation about 1.5 million Americans and 2.5 million Europeans have IBD taking into consideration the conditions are half represented in each of the two subtypes of IBD, the pathogenesis of IBD is attributed to an uncontrollable inflammatory response that is mainly immune mediated, as a result of environmental triggers in which it interacts with the intestinal microbiota, there are a variety of determinants of IBD including gut microbiota, environmental triggers, immune mechanisms of the intestine, hereditary factors, diet, nicotine, mesenteric fat, infectious agents, medications, hygiene, immunization breastfeeding, lifestyle, pregnancy and stress (Arora and Malik, 2016).

IBD, ulcerative colitis and Crohn's disease are recognized as essential etiological factors of gastrointestinal disease in children and adults, they occur worldwide, they have different predominance across some regions where they are more common such as in the UK, U.S and Scandinavia, they are mostly diagnosed between the third and fourth decades of one's life, 20% of all patients with IBD approximately develop the symptoms during the years of childhood, about 5% before the age of 10 and 25% have been shown that they have positive family history of IBD but, there are no differences whatsoever noted amongst children with versus without IBD in all regards. Ulcerative colitis (UC) can be defined as a condition in which the inflammatory response and morphological alterations remain within the colon, in 95% of the patients the rectum is involved, the proximal extension has varying degrees, the inflammation

is primarily limited to the mucosa. It contains a continual involvement of varying severity accompanied by edema, ulceration and hemorrhage along the length of the colon, the histology reports findings which are characteristic of acute and chronic inflammation of the mucosa via mononuclear cells and polymorphonuclear leukocytes, distorted mucosal glands, crypt abscess and goblet cell depletion. Crohn's disease (CD) can be defined as a condition of inflammation of any part of the GI tract from the anal area up to the oropharynx contrastingly with ulcerative colitis which is limited to the colon. In Crohn's the diseased segments are segregated by an intervention of the normal bowel thus, the commonly used term skipped areas, the inflammation can be transmural frequently with extension through to the serosa which results in fistula and sinus tracts formation, the histological findings report small superficial ulcerations over which is a Peyer's patch referred to as an aphthoid ulcer and also chronic focal inflammatory extension into the submucosa, which is sometimes coexistent with noncaseating formation of granuloma, that may be followed by the terminal part of the ileum alone, either diffused in the small bowel or isolated disease of the colon in an order of frequency that is decreasing (Hendrickson, Gokhale and Cho, 2002).

1.2.1 Epidemiology

The now worldwide recognized IBD was thought to be a condition that affected Caucasians across Europe. North American and Australia therefore, most of the available epidemiological studies conducted and data gathered is within these geographical regions, the incidence and prevalence of Crohn's disease and ulcerative colitis have stabilized in the previously mentioned regions but, it still persists higher than the other regions of the world, the incidence and prevalence of IBD and in which case there is predominance of CD have increased in the developing regions of the world as in Asia pacific regions and the Middle East. South American and Africa have the incidence and prevalence are significantly low although some reports hint to an increase of incidence, even in the west IBD has become an increasingly recognized disorder among the minority of populations, the highest significant increase has happened to immigrants of the second generation coming from areas of low risk to areas of high risk, this could potentially support the concept of a higher contribution of environmental alterations when comparing it with genetic predisposition, it has been noted that there is an increasingly higher incidence of IBD among immigrants and their families, when comparing with the minorities of the west the recent immigrants tend to have a milder disease course, the incidence and prevalence data may vary across the globe according to the geographic regions and the environments, ethnicity, immigration trends even within the same geographic region, it has been attributed to regional differences that are related to sunlight and vitamin D exposure in which high levels of exposure are inversely correlated with IBD risks. The yearly incidence rates of Crohn's disease are comparable across the developed regions where it is estimated to be 20.2 per every 100 thousand person-years, 12.7, 29.3, and 16.5 in North American, Europe, Australia and New Zealand respectively, contrastingly with Asia where there is a low incidence rate of approximately 0.54, closely the incidence rates of ulcerative colitis in North American, Europe and Asia range from 7.6 to 19.5 per every 100 thousand person-years, 1.7 to 13.6, 0.3 to 5.8 respectively. Ulcerative colitis was thought to be slightly more prevalent in the past but, as of the past few decades the increased incidence of Crohn's disease had resulted in the reversal of this trend, most recently the prevalence estimates of Crohn's disease in North American are 25 to 300 per 100 thousand person-years, while that of ulcerative colitis are 170 to 250 per 100 thousand person-years and 43 to 294 in North America and Europe respectively, the incidence and prevalence of UC and CD seem to be increasing as a function of time, this is attributable to the considerable load of factors that include diet, medications, sanitation and IBD awareness, even-though IBD could occur at any age, the peak age for the onset of UC and

CD is generally between the years of age 30 to 40 and 20 to 30 respectively, some European cohorts suggested the second peak to be between 60 to 70 years of age especially for ulcerative colitis, it is probably due to the increased checkup and health care access available to older patients, in a study the majority of the population in North America show a median and mean age of diagnosis of Crohn's and ulcerative colitis ranging between 30 to 45 years and 40 to 45 years of age respectively, the studies especially conducted in adults have suggested that there is a predominance of Crohn's in females and a predominance of ulcerative colitis in males, this gender based disparity is attributable to life style and hormonal factors yet, the variability is not consistent, especially in regions of low IBD incidence where Crohn's appear to be more prevalent in Men, men tend to be diagnosed with IBD particularly ulcerative colitis at a later age in comparison to females. On the other hand, the population of pediatrics this trend is reversed with gender distribution showing Crohn's in boys more than girls (Arora and Malik, 2016).

A recent study reported the incidence and prevalence of IBD across different areas of the globe, Europe had the highest reported prevalence values of ulcerative colitis 505 per 100 thousand in Norway and Crohn's in Germany accounting for 322 per 100 thousand, in North America specifically USA had 286 per 100 thousand ulcerative colitis and in Canada Crohn's represented 319 per 100 thousand, the prevalence of IBD had exceeded a value of 0.3% in Oceania, North America and many countries in Europe, 72.7% of Crohn's disease studies and 83.3% of ulcerative colitis studies had reported a stable or rather decreasing incidence of IBD in Europe and North America, since the 1990s the incidence of IBD has been rising in newly industrialized countries in Asia, South America and Africa, in Brazil CD and UC had increasing values of 11.1% and 14.9% respectively while in Taiwan 4% and 4.8% respectively (Ng et al., 2017).

1.2.2 HSV-1 mechanisms of infection and pathology in inducing IBD

Herpes simplex viruses are increasingly linked with inflammatory bowel disorders and there is a study by Wakefield and colleagues reporting the detection of herpesviruses DNA in the intestine of patients suffering from ulcerative colitis and Crohn's disease employing nested polymerase chain reaction (PCR), DNA was extracted in the form of resection biopsy samples from the intestine of patients suffering from Crohn's, ulcerative colitis and non-inflammatory bowel disease as control, the nested PCR was employed to detect the viral DNA using primers specific for multiple subtypes of HSV, herpesviruses do have the ability of infecting the endothelium in vitro hence, inducing multiple functional alterations that are consistent with the pathological abnormalities observed in ulcerative colitis. HSVs have shown to induce the expression of cytokines and promote leukocyte adherence in cultured vascular epithelium, these processes are both active in the case of ulcerative colitis, the detection of herpesvirus DNA in the tissues and peripheral blood mononuclear cells is a first step towards clarifying the role of HSV in the chronic inflammatory disease cascade, the following figure represents the detection of HSV DNA PCR products (Wakefield et al., 1992):

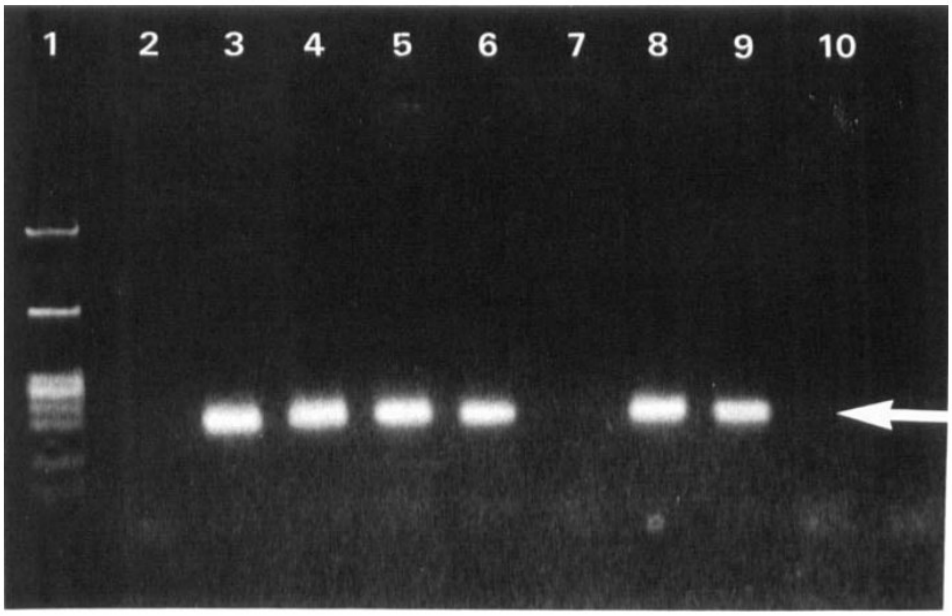


Figure (11) HSV PCR products

In the previous figure lane 1 is the reference ladder, lane 2 is empty while from 3 to 6 are the DNA samples obtained from patients with inflammatory bowel disease, 7 is DNA from a patient with Crohn's disease, 8 and 9 are positive controls while 10 is negative control, the high prevalence of DNA from multiple herpesviruses suggests a possible synergistic role of these

viruses in the pathogenesis in ulcerative colitis and inflammatory bowel diseases (Wakefield et al., 1992).

A study by Schunter and colleagues which links herpes simplex virus with ulcerative colitis involves a case report of a 35 year old female with ulcerative colitis and frequent flare up of 15 to 20 bowel movements per day, the histo-pathological analysis of the colonic resection confirmed the presence of severe ulcerative colitis in addition to providing a histological evidence of viral superinfection accompanied by ballooning and opaque infectious antibodies, following an immuno-histochemical analysis employing anti-HSV-2 antibody, they were able to detect herpes simplex virus 2 within the submucosa of the colon as represented in the following figure (Schunter et al., 2007):

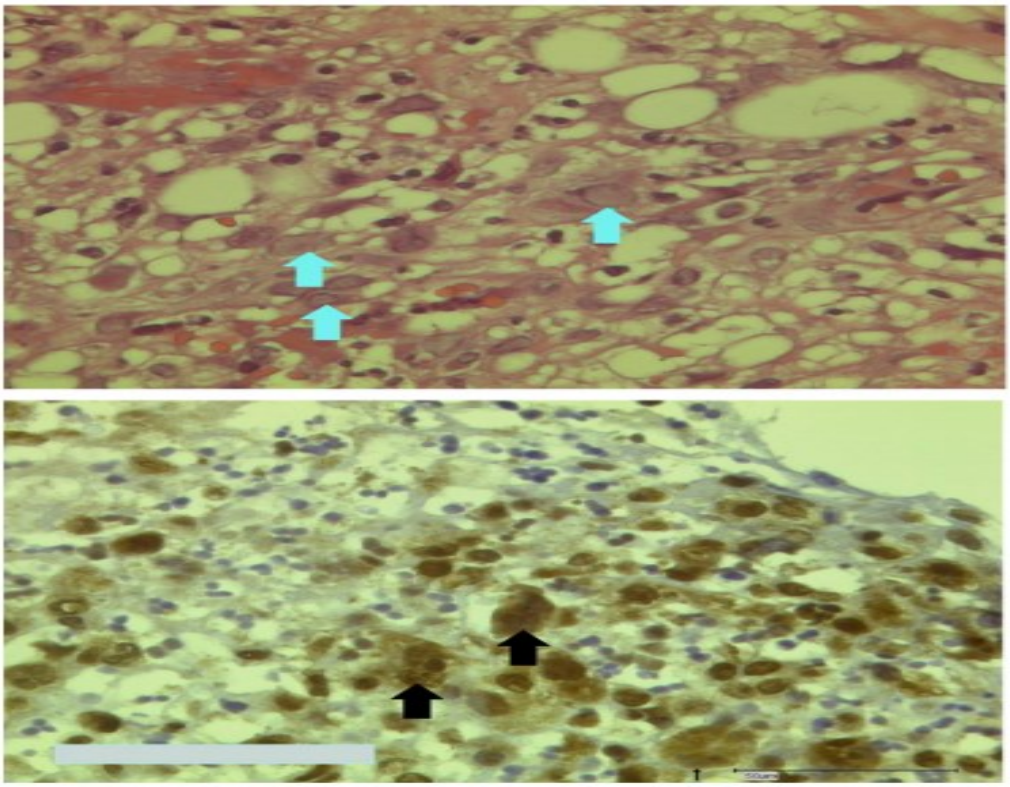


Figure (12) Histologic samples of colon

In the previous figure the anti-HSV-2 revealed positive staining and the blue arrows indicate lymphatic cells infiltration, the polymerase chain reaction was evidently positive for HSV DNA in the mucosa of the colon. In the serum Schunter and colleagues were able to detect HSV IgM and IgG immunoglobulins which verified a recent acquired HSV infection, their remarks indicate that HSV infection should be considered in patients with inflammatory bowel disease and a PCR of the rectosigmoidal biopsy and immunohistochemical analysis of the colonic

tissue is recommended as it is the most sensible method for the detection of HSV infections (Schunter et al., 2007).

Moreover, there is a study by Phadke and colleagues regarding herpes simplex colitis and hepatitis in a case of ulcerative colitis, the patient was a 43 year old male who developed symptoms of a colitis flare up that was non responsive to corticosteroids, he later developed orolabial ulcers and progressive hepatic dysfunction, the pathology obtained from the colon and liver was consistent with herpes simplex viral infection, a viral culture of the orolabial lesions in addition to a serum PCR analysis confirmed the identification of herpes simplex, colon specimens revealed HSV deep ulceration with hematoxylin and eosin while liver biopsy with the same stain revealed HSV hepatocytes patches of necrosis, HSV infections are common in patients with inflammatory bowel disease therefore, HSV should be considered in the differential diagnosis. The following on the left is the immuno-histochemical analysis of colon specimens while on the right immunohistochemical specimens from hepatocytes (Phadke et al., 2016):

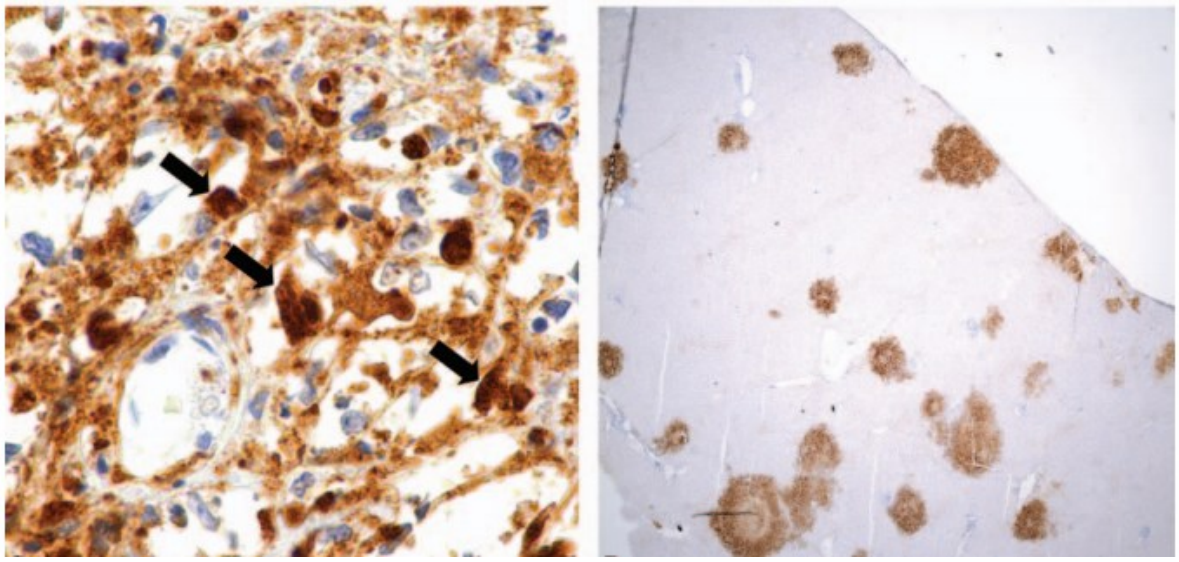


Figure (13) Colon and hepatocytes immunohistochemical assay respectively

Herpes simplex virus 1 is linked with evoking bowel neuromuscular abnormalities, it is proposed to cause disruptions of the enteric neuromuscular system, a study by Brun and colleagues establishes a link between HSV-1 enteric neuronal infection with dysmotility of the gut, it reports that after 1-10 weeks of intragastric administration, they were able to detect HSV-1 latency associated mRNA transcripts in the brain and ileal neurons with no signs of disease or histological anomalies, they were able to detect HSV-1 in the myenteric ganglia, $CD3^+$ and significantly increased levels of interleukin 2 and IFNγ 1 to 6 weeks post administration, smooth muscles response indicated a significantly increased gastric residence time (↑transit), the detection of significantly reduced release of acetylcholine 1 to 6 weeks post administration furthermore confirms the abnormalities exhibited, the following figure confirms the detection of HSV-1 alongside $CD3^+$ and T cells in the mice ileum (Brun et al., 2010):

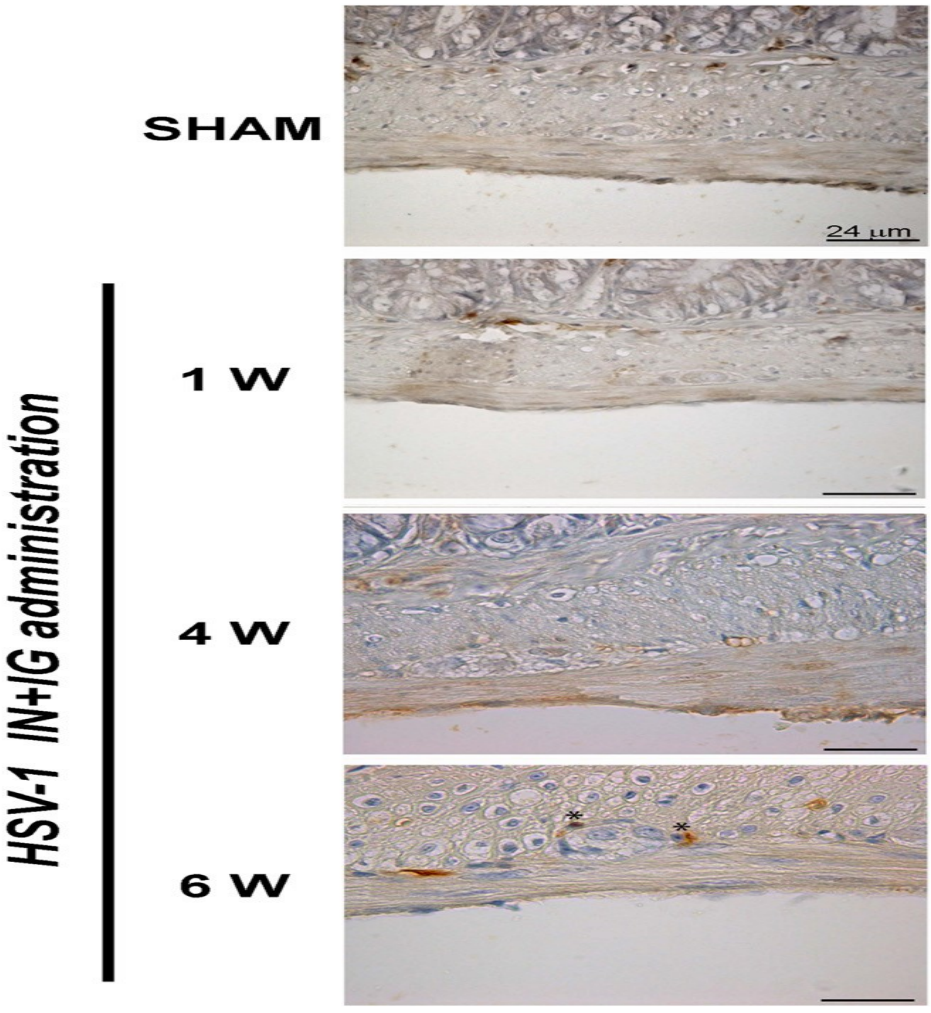


Figure (14) HSV-1 in mice ileum

The study concludes a novel model of HSV-1 infection mechanism that shows abnormal intestinal contractility and gastrointestinal transit. HSV-1 is confirmed to establish a latent infection in rat's myenteric ganglia that progressively leads to gut dysmotility (Brun et al., 2010).

Adenosine has an essential role in the regulation of intestinal motility and inflammatory processes, a study by Zoppellaro and colleagues reports that adenosine mediated neuromuscular functions are affected during HSV-1 infection of the ENS, HSV-1 orally inoculated rodents exhibit ENS infection that affects gut motor function without signs of systemic infection, in this study through isolated organ bath assays they were able to discover impairment of adenosine receptors A_1 or A_{2A} responsible for contraction mediation at 1 and 6 weeks post viral administration, furthermore it revealed the redistribution of adenosine receptors A_1 and A_{2A}, they were confined to muscle layers while A_{2A} or A_3 receptors were mainly expressed in the myenteric plexus, HSV-1 induced ENS neurodysfunction which affected adenosine metabolism via increasing adenosine deaminase (ADA) and CD73 levels in the longitudinal myenteric plexus without a sign of inflammation, the following figure represents the distribution of enzymes in sham versus wild type 1 and 6 weeks post HSV-1 infection (Zoppellaro et al., 2013).

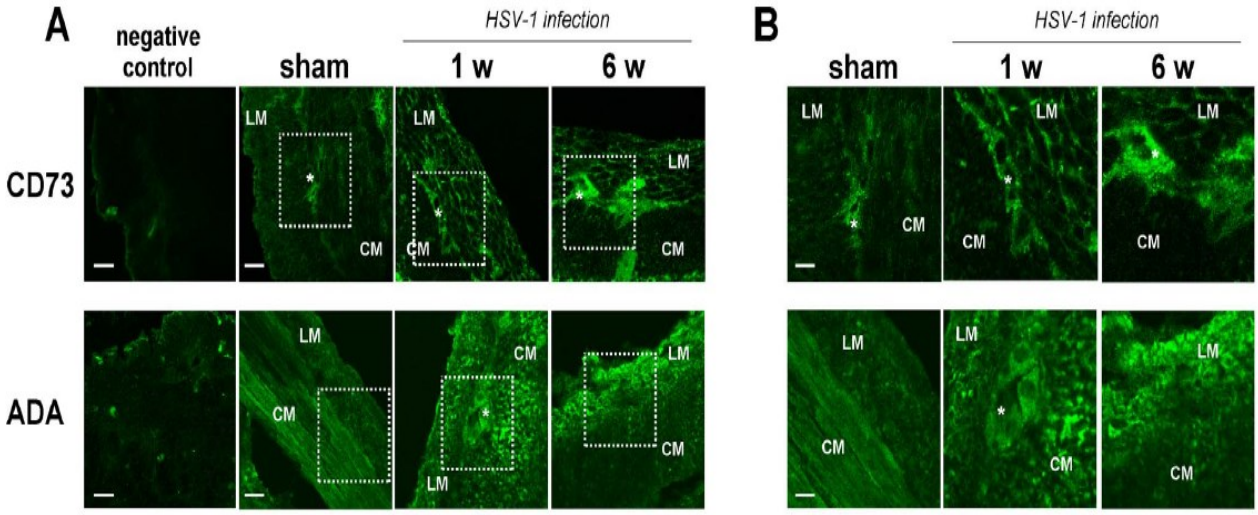


Figure (15) Distribution of CD73 and ADA during HSV-1 infection of rat ileum

The previous figure A demonstrates increased level of CD73 in the longitudinal smooth muscle myenteric plexus and myenteric ganglia 6 weeks post HSV-1 infection moreover, B also shows ADA increased in the myenteric ganglia at 1 week post infection followed by the longitudinal smooth muscle myenteric plexus 6 weeks post HSV-1 infection (Zoppellaro et al., 2013).

HSV-1 is reported to infect the enteric neurons and plays an important role in triggering gut dysfunction, Brun and colleagues study reports following intragastrical (IG) administration of HSV-1, leads to infection of the neurons within the myenteric plexus which results in structural and functional alterations of the enteric nervous system, through using mice and infecting them with a replication defective strain of HSV-1. The infection had revealed gastrointestinal neuromuscular abnormalities that were independent of HSV-1 replication, the enteric neurons that were exposed to HSV-1 inactivated by ultraviolet rays generated chemotactic monocyte protein 1 (MCP1/CCL2) in which it recruits activated macrophages in the longitudinal myenteric plexus muscles, the infiltrating macrophages play an essential role by inducing gastrointestinal (GI) dysmotility through generating reactive oxygen and nitrogen radical species which directly harms enteric neurons and results in developing GI dysmotility, the IG infection of mice resulted in apparent GI dysmotility in the following figure, when observing the percentage of probe retained in the stomach which is indicative of gastric emptying, It is clearly apparent that post infection as time passed gastric dysmotility was precipitated as more gastric residence was induced (Brun et al., 2018).

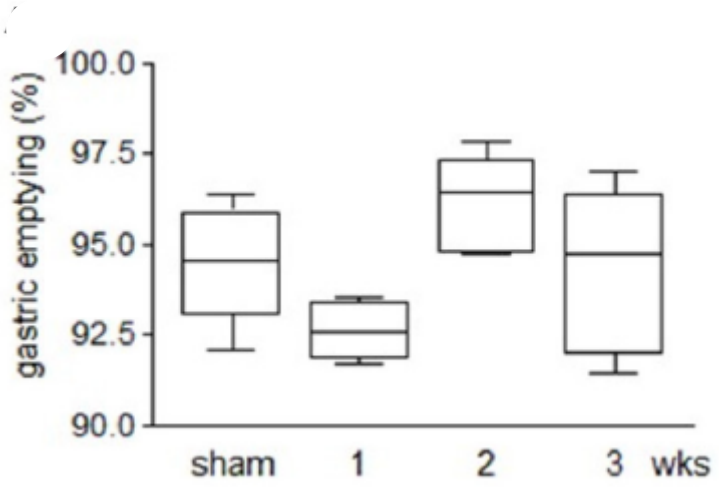


Figure (16) Gastric emptying calculated as percentage of probe retained in the stomach

The following figure represents the ileum of mice infected with the replication defective strain of HSV-1:

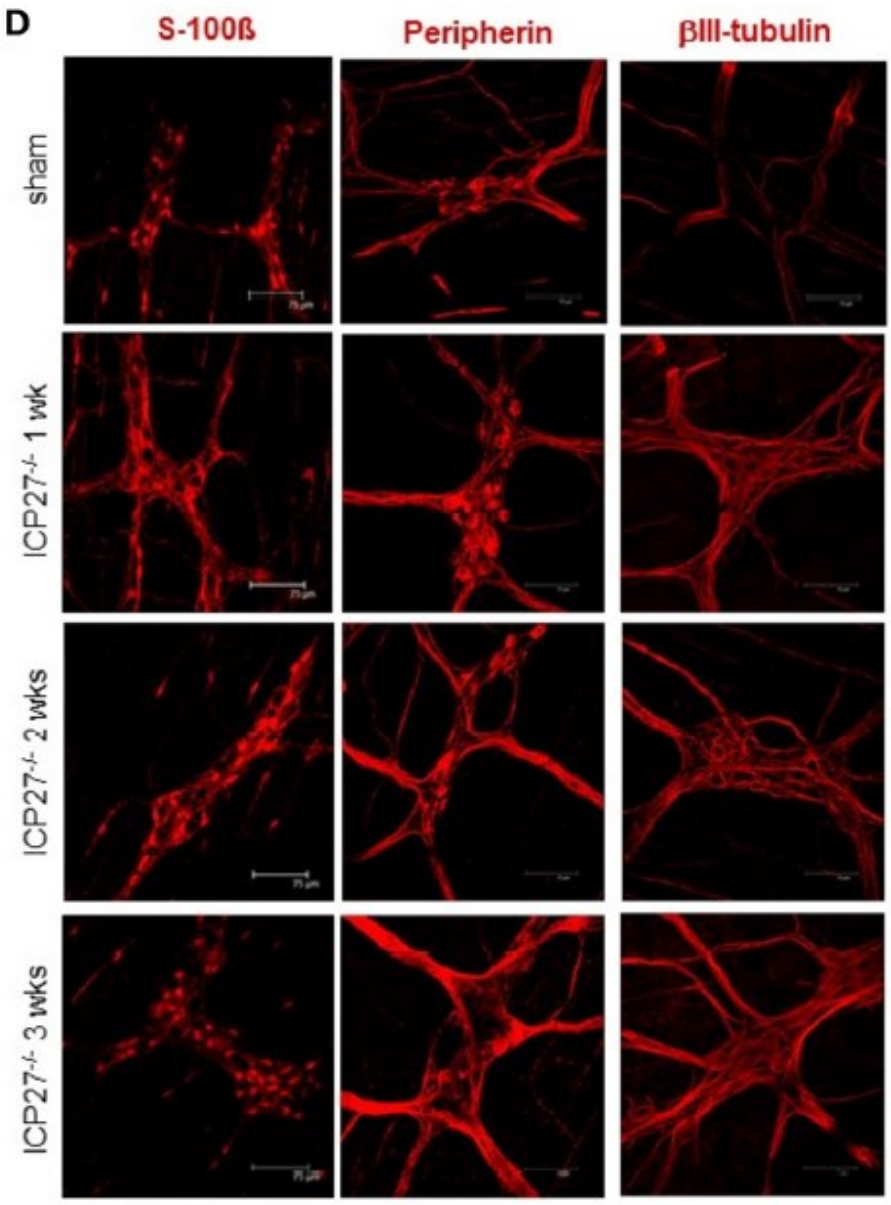


Figure (17) Infected (HSV-1 replication defective strain) versus sham mice ileum

It is visible through the mice ileum that there are apparent gastric anomalies induced post infection as time passes it precipitates into structural alterations which affect the functions of the gut leading to dysmotility and increased gastric residence time. This all confirms that HSV-1 induces the infiltration of macrophages which lead to this cascade of GI abnormalities thus, the mechanism is independent of HSV-1 viral replication. The host mediated immune response is the etiological cause of structural and functional anomalies observed in the mice (ENS) enteric nervous system (Brun et al., 2018).

Brun and colleagues animal model study of HSV-1 infecting the ENS reported that HSV-1 is recognized by the innate immune system as a consequence an inflammatory response is triggered that is recognized as a potential pathway of HSV-1 pathogenesis, the study involves the infection of the enteric nervous system with HSV-1 in wild type and toll like receptor 2 "TLR2" knock out (TLR^{KO}) mice, the study demonstrated abortive replication in the ENS of both wild type and knock out groups, additionally HSV-1 triggered the TLR2-MyD88 signaling pathway in the myenteric neurons which induced structural and functional alterations of the ENS, the GI dysmotility was less manifested in the knock out mice when compared to the wild type, HSV-1 led to the up regulation of monocyte chemoattractant protein 1 (CCL2) and also recruiting $CD11^{+}$ macrophages in the myenteric ganglia of wild type mice unlike the knock out, contrastingly the knock out mice exhibited dense infiltration of HSV-1 reactive $CD3^{+}$, $CD8^{+}$ and IFN^{+} lymphocytes in the myenteric plexus, assuring that the depletion of $CD3^{+}$ and $CD8^{+}$ by means of administrating anti-CD8 monoclonal antibody reduced the neuromuscular dysfunction of knock out mice infected with HSV-1, which confirms that the TLR2 is engaged in HSV-1 infection and mediates the production of CCL2 in infected neurons additionally, it coordinates the recruitment of macrophages and results in structural and functional alterations of the ENS (Brun et al., 2018).

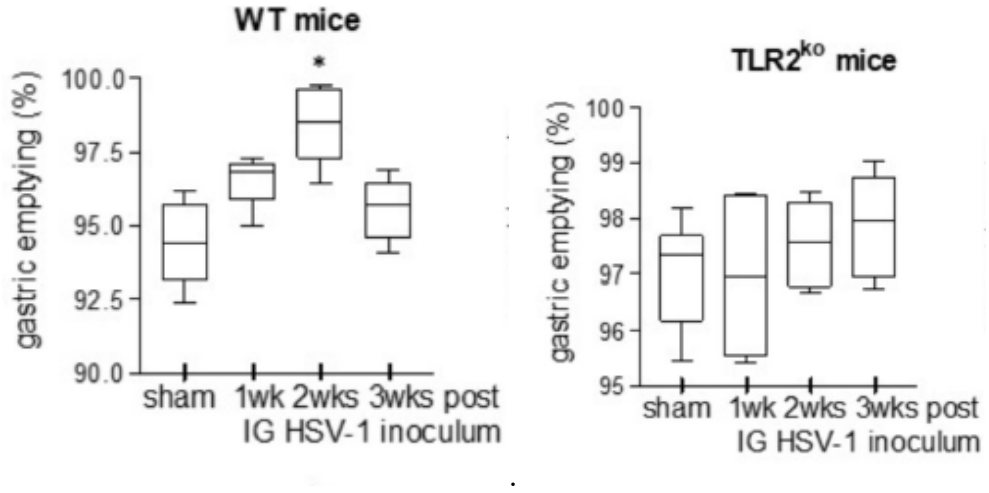


Figure (18) Gastric emptying of wild type versus TLR^{KO} mice respectively

The previous figure demonstrates gastric emptying rate as a percentage of retained probe. The significant change in GI dysmotility between the two groups is observed where the wild type exhibited signified dysmotility while the TLR^{KO} had mild GI dysmotility, moreover the following figure (Brun et al., 2018).

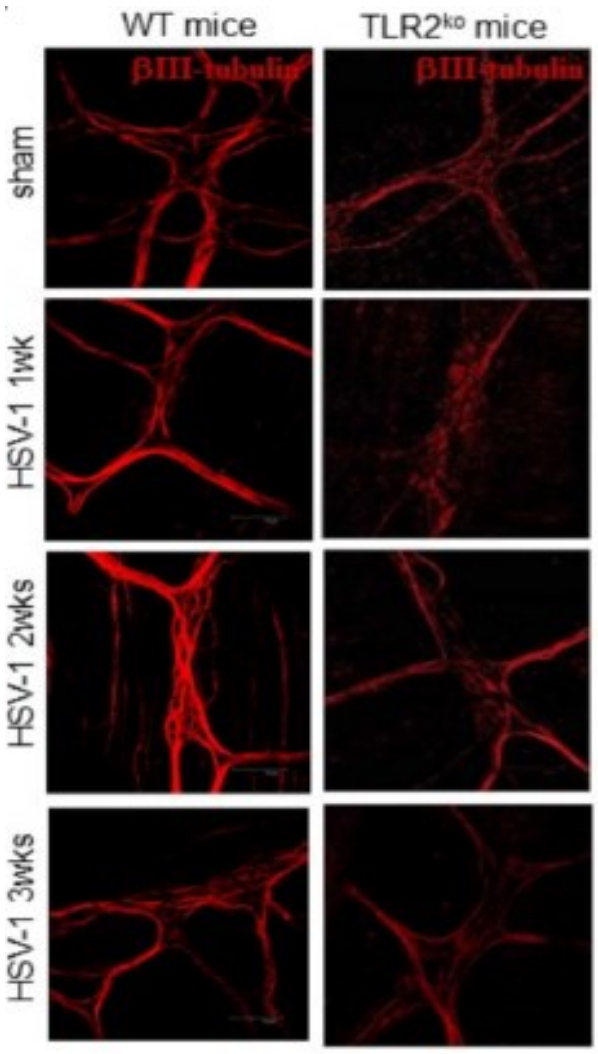


Figure (19) Wild type versus TLR^{KO} mice ileum

As apparent in the previous figure it shows the significant difference in structural modifications that are induced in wild type mice versus knock out as a result of the TLR pathway, the study confirmed the debilitating role of TLR responses in the enteric neurons during HSV-1 infection, the study generates a genuine model that is proof of TLR2 signaling on neuronal cells in which it plays an essential role in neuropathogenesis induced by HSV-1 (Brun et al., 2018).

1.3 Intestinal microbiota

Nearly after humans are born, a new microbial ecosystem is born too, the system resides in human's gastrointestinal tract. Even-though it is a universal and integral part of humans biological nature, the temporal progression of this process, the sources where the microbes that make up the ecosystem originate, the how and why it varies from one human to another in addition to how the composition of this ecosystem affects humans physiology, development and disease remains poorly understood and requires further investigation. The remarkably dense and diverse intestinal microbiota inhabiting humans has been recognized for nearly a century yet, we are only starting to appreciate and understand the variety of roles that these microbes possess in human health and development, identifying the composition of the microbiota is a critical step towards understanding its functions, the adult human body typically contains 10 times as much microbial cells as human cells, it is majorly due to the extremely high density of microbes in humans GI tract which typically contains 10^{11} to 10^{12} microbes per ml of luminal contents. The microbiota serves several important functions including protection from pathogens, stimulation of angiogenesis, nutrients processing and regulation of fat storage. We are still continuing to discover the new roles and relationships, in addition to several different pathological conditions that are either confirmed or suspected to be linked to the intestinal microbiota including inflammatory bowel disease, stomach cancer, mucosa associated lymphoma, necrotizing enterocolitis. The adult human colon microbiota contains is estimated to contain more than 400 bacterial species, members of the anaerobic genera include *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus* and *Faecalibacterium* have been identified to occupy a large portion of the human gut microbiota, although they do there is still individual differences and each adult's gut appears to have a unique microbial community contrastingly in infants the gastrointestinal microbiota is more variable in its composition and highly variable over time as in less stable (Palmer et al., 2007).

Recently we came to know that the gut microbiota plays a fundamental role in the development of the immune system of the hosts, which in turn shapes the structural and functional microbial communities of the gut microbiota, in germ free mice; the animals that are deficient in the gut microbiota exhibit an impaired immune development that is characterized by immature lymphoid tissues, diminished levels of antimicrobial peptides, decreased amount of intestinal lymphocytes and immunoglobulin IgA (Nishida et al., 2018).

A study reporting that dietary emulsifiers alter the composition of the intestinal microbiota and results in potentiation of intestinal inflammation, describing the central role of the intestinal microbiota in the pathogenesis and development of chronic inflammatory disease including inflammatory bowel disease and metabolic syndrome (Chassaing et al., 2017).

Moreover, there is a reported interplay between the intestinal microbiota and the brain, the intestinal microbiota consists of a large bacterial community that primarily resides lower gut and lives in symbiosis with the host, there is a bidirectional neurohumoral communication system that is referred to as the gut-brain axis which integrates the hosts gut and brain activities. The lower gastrointestinal tract consists of nearly one hundred trillion microorganisms in which most of them are bacteria, there are more than a 1000 identified bacterial species in the intestinal microbiota, they were detected by molecular based approaches, however the majority of the bacteria are strictly anaerobes they have not been detected yet, the microbiota encodes for 3.3 million non-redundant genes thus, exceeding humans genome encoding capacity by 150 folds, recently microbiota is linked with the development and functioning of the hosts brain, the intestinal microbiota and its influence on brain function has clinical origins including the observation reported when orally administering antibiotics to reverse encephalopathy in patients with decompensated liver disease, addedly there are psychiatric disorders that are frequently coexisting with gastrointestinal conditions such as irritable bowel syndrome (IBS) and all are associated with disturbances of the intestinal microbiota, thanks to animal model studies researchers are able to find the links between gut microbiota and neurological conditions such as multiple sclerosis, the gut-brain axis is a communication system that consists of hormonal, neural and immunological signaling between the gut and brain. It provides the intestinal microbiota and its metabolites a very essential role through which they can have access to the brain. This communication system is classified as a bidirectional system which allows the brain to affect the gastrointestinal functions including motility, mucin production and secretion, there are also immune functions that pertains the modulation of cytokine production by the cells of the immune system mucosa, emotional factors such as depression or stress may affect cases of inflammatory bowel disease including ulcerative colitis and Crohn's disease as well as IBS via the gut-brain axis, all of these conditions are also associated with dysbiosis, stress was observed to affect the integrity of the gut epithelium, motility, secretions and mucin production therefore, changing the habitat of resident bacteria and promotion of activity or compositional changes of the gut microbiota, the additive effect of stress is that it induces the release of catecholamines into the gut which affects the microbiota communities

via interferences of the interbacterial signaling and bacterial virulence gene expression (Collins, Surette and Berick, 2012).

1.3.1 Role of the microbiota in IBD

The pathogenesis of IBD is multifactorial and involves the interplay of genetics, environmental factors and immune dysregulation that result in apparent immune response and inflammation. The human gut microbiota serves as an important component of the pathogenesis of IBD namely Crohn's disease and ulcerative colitis in which the microbiota provides an antigenic stimuli via altered microbial compositions which enhance and promote microbiota imbalances in the host thus, leading to perturbed intestinal and immune homeostasis. There is an aggressive T cell mediated immune response to specific components of the intestinal microbiota in genetically predisposed individuals which leads to inflammation of the bowel in the case of Crohn's disease, where microbial dysbiosis is linked and associated with either the development or exacerbation of CD (Khanna and Raffals, 2017).

There is a marked decrease of bacteria in anti-inflammatory capacities and an increase of bacteria in inflammatory capacities, this is observed in patients suffering from IBD when comparing the condition to healthy individuals, the most consistent alterations include decreases in the diversity of the gut microbiota, lower abundance of *Firmicutes*, there are also reported increases regarding the abundance of *Proteobacteria* and *Bacteroidetes* however, decreases have been reported as well. *F. prausnitzii*, which is a member of the *Clostridium* cluster IV, has been noted to possess an anti-inflammatory effect via generating butyrate, the demonstration of decreased levels of *F. prausnitzii*, *Blautia faecis*, *Clostridium lavalense*, *Ruminococcus torques* and *Roseburia inulinivorans* in patients suffering from Crohn's disease when comparing them with healthy individuals, the amount of *F. prausnitzii* seems to be correlated with the risk of relapse of Crohn's ileal disease post-surgery. The defect of the colonization of *F. prausnitzii* was also observed in the case of ulcerative colitis patients during recovery and remission of *F. prausnitzii* population following relapse, it is associated with maintaining clinical remission, it has been shown in a 2008 study by Sokol et al. that the human peripheral blood mononuclear cells with *F. prausnitzii* lead to the stimulation and induction of the generation of interleukin 10 IL-10 and the inhibition of the generation of inflammatory cytokines such as IL-12 and IFNγ, moreover, the significant reduction of *Roseburia spp.* had been shown in the gut microbiota of healthy individuals that have a high genetic predisposition for IBD. Contrastingly there is a relative increase in proteobacteria majorly *E.coli* which was shown in CD patients, specifically on the mucosa associated microbiota when comparing it to fecal samples, the pro-inflammatory *E.coli* that is associated with CD is adherent invasive *E.coli* "AIEC", it was genuinely isolated from adult patients suffering from CD, it has been

reported that the amount of AIEC has increased in about 38% of the patients having active CD in comparison to 6% in healthy individuals, the increase of pathogenic bacteria that possesses the ability to adhere to the intestinal epithelium influences the permeability of the intestine, changes the diversity and composition of the gut microbiota, it induces the inflammatory responses via regulating the expression of the inflammatory genes, as a consequence leading to an induction of intestinal inflammation, addedly in situ analysis had shown an increased abundance of mucosa associated bacteria in IBD, this could be due to an increased amount of mucolytic bacteria such as *Ruminococcus torques* and *Runinococcus gnavas* in IBD patients, the generation of metabolites that are affected via the disruption of the gut microbiota is associated with the pathogenesis of IBD. There is a report of decreases in the concentration of short chain fatty acids "SCFAs" in IBD patients as a result of bacteria that produces butyrate such as *Clostridium* cluster IV, XIVa, XVIII and *F. prausnitzii*, the reduced levels of SCFAs influences the expansion and differentiation of Treg cells and the growth of epithelial cells, in which they play an essential role in the maintenance of intestinal homeostasis on the other hand, the amount of sulfate reducing bacteria such as *Desulfovibrio* is reported to be higher in IBD patients, this results in the generation of hydrogen sulfate which damages the epithelial cells of the intestine and induces inflammation of the mucosa, all of this data strongly indicates that the changes affecting the gut microbiota are associated with the pathogenesis of IBD (Nishida et al., 2018).

There are studies that report the gut microbiota in CD demonstrating an increased number of pathogenic microorganisms whereby the population of normal commensal phyla are diminished. In an interesting study, they pretreated the gut microbiota samples from patients with new onset CD in which it demonstrated an increased abundance of *Enterobacteriaceae*, *Pasteurellacaea*, *Veillonellaceae* and *Fusobacteriaceae* while a decreased abundance of *Erysipelotrichales*, *Bacteroidales* and *Clostridiales*. Rectal and ileal mucosal specimens revealed a demonstration of a decreased level of *Firmicutes* such as *Faecalibacterium prausnitzii* and an increased level of *Proteobacteria* such as *Escherichia coli*, *Veillonella*, *Haemophilus* and *Fusibacteria*. There are similarly previous studies that had shown an increase in mucosal *E. coli* in CD and a reduction in *Faecalibacterium prausnitzii*, in another study, it reported a decrease in populations of *Bacteroidetes* and *Firmicutes* in patients with CD whereby, the pathogenic bacteria such as *E. coli*, *Campylobacter* species and *Mycobacterium* species were increased, CD patients had been shown to have a greater number of mucosal

surface associated bacteria with higher adherence and invasion in comparison with healthy subjects (Khanna and Raffals, 2017).

1.3.2 Intestinal microbiota and viral infections

Recent research has immensely magnified our understanding of how gut microbiota influences the host's health, in the case of viral infections, viruses may influence the gut microbiota through substantial and intimate interactions, the integrity of the gut microbiota can be disturbed via invading viruses resulting in dysbiosis in the host (Li et al., 2019).

1.3.2.1 Rotavirus

Rotavirus infections are associated with decreased richness and diversity of the bacterial community in the microbiota, a study reports that the genera *E.coli* and *Clostridium* were affiliated with infected calves, the results indicated the abundance of *Proteobacteria* was apparent in Rotavirus infected calves as well, contrastingly there was an absence of *Bacteroidetes* in the infected calves. All of the following comparisons were held against healthy calves (Jang et al., 2019).

1.3.2.2 Influenza

Influenza viruses can alter the microbiota composition following infection, in which several studies demonstrated that the infection results in decreased colonization of healthy bacteria and increased abundance of potential pathogenic bacteria. A study reports that healthy *Prevotella spp.* and anaerobes were significantly decreased in infected patients, with which 8 potentially pathogenic bacteria were enriched including *H. influenzae*, *Moraxella catarrhalis*, *S. aureus*, *Corynebacterium propinquum*/*pseudodiphtheriticum* and *S. pneumoniae*. Consistently with another study showing *Prevotella* decreased in H1N1 infections. Although the dysbiosis is apparent in influenza infections several studies report that the dysbiosis of commensal microbiota is dependent on the strain of influenza virus (Li et al., 2019).

1.3.2.3 MERS-Cov

A study reports MERS-Cov as a causative agent of intestinal infection deeming the intestinal tract a susceptible part of the body to MERS-Cov replication, it further reports that the enteric infection causes progressive inflammation, epithelial degeneration and consequential respiratory infection moreover, MERS-Cov acts on the receptor DPP4 which is highly expressed in the intestinal tract thus, confirming the intestinal pathway of infection pathogenesis (Zhou et al., 2017).

1.3.2.4 SARS-Cov

A study reported in-vitro infection of SARS-Cov in epithelial cells, where they employed a total of 6 cultures two of which demonstrated highly permissive growth of SARS-Cov, the effect observed in the two cell lines is due to the presence of angiotensin converting enzyme 2 as the functional receptor hence, confirming the affinity of the virus to the receptor, moreover, there is a great abundance of ACE2 receptor in enterocytes of the small intestine which further explains the gastrointestinal manifestations exhibited in SARS-Cov infected patients (Cinatl et al., 2004).

ACE2 is characterized as an essential receptor for the expression of amino acid transporters in the gut and as mentioned previously, it is abundant in the gut, furthermore one study reports the possible mechanism of dysbiosis in which ACE2 is essential for the surface expression of the transporter B^0AT1 in the epithelium of the small intestine, the transporter is important for the uptake of tryptophan in which tryptophan levels regulate the mTOR pathway of antimicrobial peptide secretion thus, aberrant secretion during SARS-Cov influences the composition of the intestinal microbiota (Perlot and Penninger 2013).

1.3.2.5 SARS-Cov 2

SARS-Cov 2 (COVID-19) case reports plenty of patients exhibiting gastrointestinal abnormalities such as diarrhea, with a plethora of papers suggesting dysbiosis of the gut and the indication of probiotics to COVID-19 patients. ACE2 being highly expressed in the small intestine; SARS-Cov 2 is shown to have 10-20 times higher binding affinity for ACE2 than SARS-Cov suggesting higher potential for gut dysbiosis and GIT manifestations (D'Amico et al., 2020).

Furthermore, there is a suggested immunomodulatory pathway of gut microbiota in response to SARS-Cov2, in which under or over activity may lead to dysbiosis of the gut microbiota as represented in the figures below (Dhar and Mohanty, 2020).

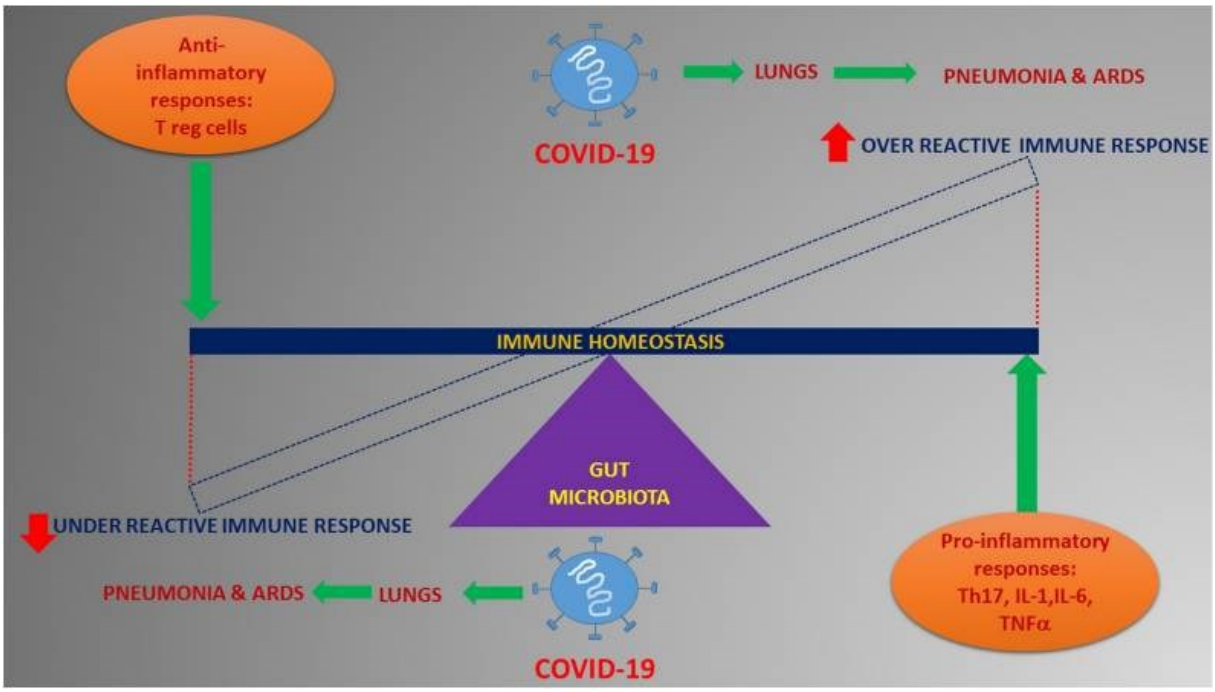


Figure (20) SARS-Cov2 and gut microbiota possible routes of dysbiosis

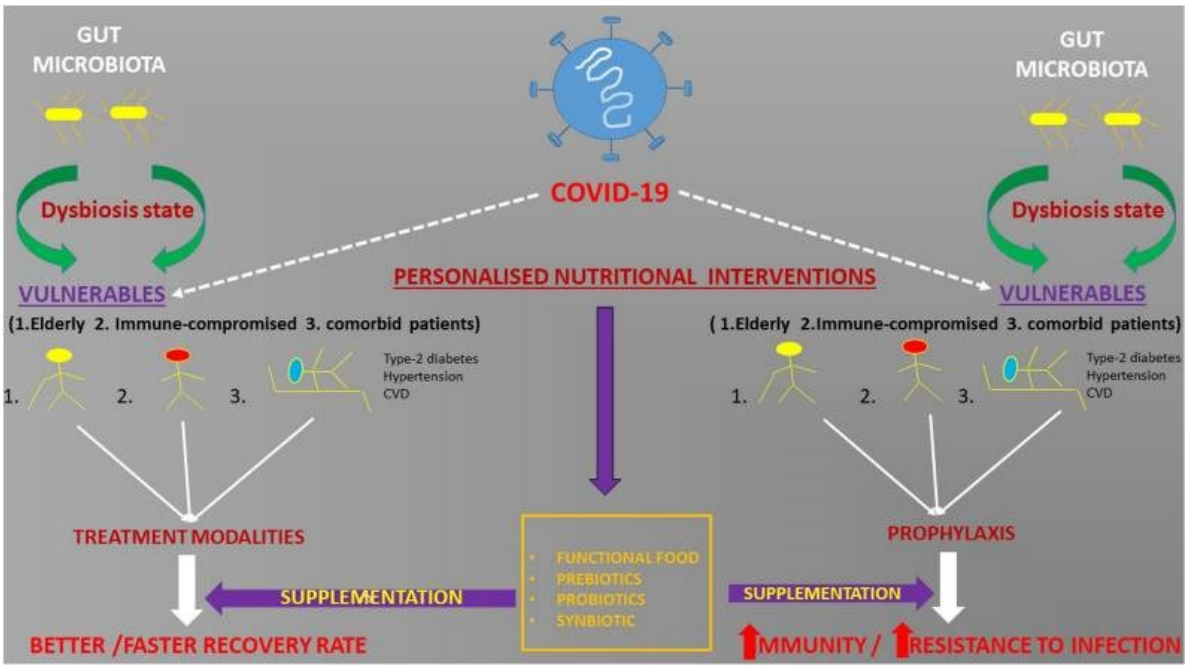


Figure (21) SARS-Cov2 induced dysbiosis and vulnerable groups

1.3.3 Gut microbiota and Alzheimer's disease

The incidence of neurological disorders is increasing worldwide, however their pathogenesis remains unclear. The bidirectional communication between the central nervous system and the gut's microbiota may play a role in the pathogenesis of neurological disorders such as Alzheimer's disease (AD). The CNS modulates gut functioning in response to psychological and physical stressors influencing secretion, motility as well as immune reactivity whereas alterations of the microbiota results in behavioral and neurochemical changes. Consequentially a terminology was proposed to describe this mechanism as microbiota-gut-brain axis (MGBA). The MGBA regulates the gastrointestinal tract and the CNS via the vagus nerve, hypothalamic-pituitary-adrenal axis and several cytokines. The intestinal mucosa, microbiota and immune cells are all components of the gut barrier against pathogens, if the integrity of the gut barrier is compromised leaky gut syndrome (LGS) occurs which leads to systemic and neuroinflammation thus, causing dysfunction in the cerebellum and hippocampus. Patients suffering from CNS disorders were found to have increased intestinal permeability and passage of harmful metabolites from the intestine to the blood which negatively influences the CNS. LGS is suggested to play a role in Alzheimer's disease pathogenesis via increasing the permeability of the blood brain barrier (BBB), in which pathogens are able to elicit an immune response that results in beta amyloid elevation and severe memory loss reported in mice (Grochowska et al., 2019). Alzheimer's disease patients are reported to have lower stool abundance of anti-inflammatory *E. rectale* and *B. fragilis*, also a higher abundance of pro-inflammatory *Escherichia spp.* and *shigella spp.* when compared to healthy controls. While *Lactobacillus spp.* and *Bifidobacterium spp.* are able to produce gamma aminobutyric acid (GABA) which may improve cognitive functioning of Alzheimer's disease patients when employed as probiotics. Postmortem studies of the Alzheimer's patients' brains revealed reduced GABA concentrations in many cortical and limbic regions including cingulate, amygdala and thalamus (Grochowska et al., 2019). A review by Fox and colleagues proposes the potential of the gut microbiota modulating AD pathogenesis, in which changes in the microbial composition across history suggests escalation of AD risk, as dysbiosis promotes immunoregulation dysfunction as a result of chronic inflammation and epithelial barrier permeability. Subsequently, pro-inflammatory agents and pathogens infiltrate the brain and promote AD pathogenesis (Fox et al. 2019).

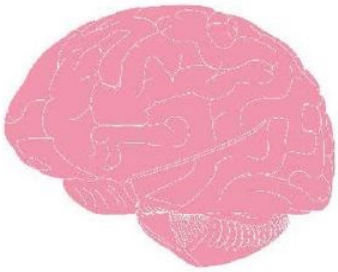
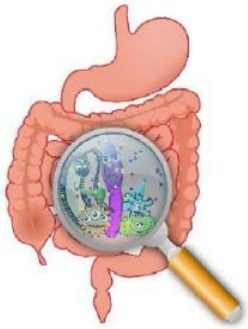
	Alzheimer' s Disease
 Factors affecting the function of Central Nervous System (diet, metabolites, hormones, immune modulators, which can be modified by gut microbiota)	⇓ Anti-inflammatory IL-10 in serum; ⇓ GABA- levels in CNS; ⇓ BDNF- involved in neuronal development, differentiation, synaptic plasticity in CNS; ⇓ DHA (ω-3 PUFA important for brain functioning); ⇓ EFA- mediate brain functions and structures; ⬆ Proinflammatory cytokines (IL-6, CXCL2, NLRP3, IL-1β); ⬆ Aβ in the cerebral cortex and other brain regions; ⬆ LPS- immune responses; ⬆ LGS- increased permeability of BBB, Aβ accumulation and inflammatory response; ⬆ High-fat/ high-calories diet; ⬆ Gluten, sugar- stimulate systemic inflammation;
 Gut microbiota disturbances	⇓ *E. rectale* ⇓ *B. fragilis* ⬆ *Escherichia* ⬆ *Shigella* ⬆ *Chlamydia pneumoniae* ⬆ Spirochaetes (*Borrelia burgdorferi, Treponema pallidum*) ⬆ *Helicobacter pylori*

Table (1) Factors affecting CNS and gut microbiota in Alzheimer's disease

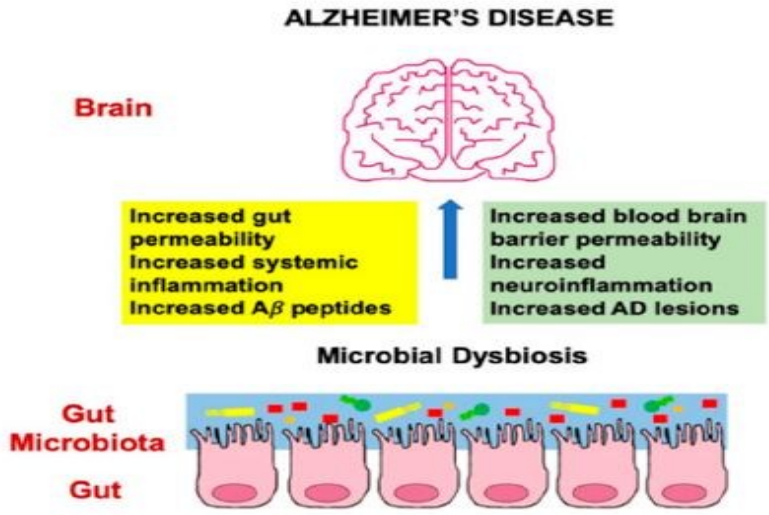


Figure (22) Relationship between the gut microbiota, brain and AD pathogenesis

1.4 Alzheimer's disease

Alzheimer's disease (AD) is the most common type of dementia, in which dementia is a wide term that is used to describe a decline in cognitive ability that is sufficiently severe to cause interference with daily life and activities. AD accounts for at least two-thirds of the dementia cases in patients that are 65 or older, it is characterized as a neurodegenerative disease that results in progressive gradual and disabling impairment of cognitive functions such as memory, language, comprehension, reasoning, attention and judgement. It is recognized as the sixth leading cause of death in the U.S, AD is a typical geriatric disease, the onset of the disease before the age of 65 is unusual and is observed in less than 10% of AD patients. The most common symptom exhibited by patients is selective short-term memory loss. AD is always progressive, and it eventually leads to severe cognitive decline, although there is no definitive identified cure for AD, there are other treatments that are employed to improve some of the symptoms exhibited by the patients (Kumar et al., 2020).

In 1906, Alois Alzheimer was first to describe the senile plaques and neurofibrillary tangles in the brain of the patient Auguste Deter (first AD patient to be diagnosed) who was suffering from progressive dementia, as we know call the condition Alzheimer's disease in recognition of his discovery, regardless of all of these early cases, the sensitive clinical criteria was established at the end of the 20th century, the sensitivity of the clinical diagnosis criteria is correct in most patients but there are instances where some patients are diagnosed with probable AD in which those patients may show different etiology of neuropathology (Zahn and Burns, 2017).

AD symptoms depend on the stage of the disease, which is further classified into preclinical, mild, moderate and late stage relying on the degree of cognitive impairment, the usual initial symptom is recent memory loss accompanied by spared long term memory. The short-term memory impairment is followed by impairment of judgement, problem solving, executive functioning, and the characteristic of disorganization and lack of motivation which leads to problems with abstract thinking and multitasking. In AD's early stages, executive functioning impairment may be mild, it is followed by language disorder and visuospatial skills impairment. The neuropsychiatric symptoms exhibited in AD include apathy, agitation, social withdrawal, disinhibition, psychosis and wandering which are common in mild to late stages of the disease, there are reported difficulties during AD including learned motor tasks (dyspraxia), sleep disturbances, olfactory distribution and extrapyramidal motor signs such as

dystonia, akathisia and parkinsonian symptoms at the late stages of the disease that is accompanied by incontinence, primitive reflexes and total dependence on care-providers or caregivers (Kumar et al., 2020). The following are highlights of typical AD cognitive and behavioral symptoms:

Recent memory impairment

The characteristic and lead symptom of typical AD is recent memory impairment, which involves patients not being able to remember important events including family gatherings that occurred days or weeks ago. The long term or old memories including childhood events are relatively spared in the initiative stages of the disease, recent memory impairment is associated with the degree of damage to the posterior cingulate cortex and medial temporal lobe which are influenced in the early stages of the typical AD (Zahn and Burns, 2017).

Behavioral symptoms

Ninety percent of AD patients develop behavioral disturbances during their course of illness, amongst these behaviors is aggression, agitation, delusions, hallucinations, sleep disturbances, apathy, depression, distractibility, wandering and aberrant motor behavior. AD behavioral symptoms usually increase with disease progression and their presence is related to worsening of prognosis, decrease in life quality and an increase in the chances of institutionalization. Depressive symptoms including sadness or anhedonia are quite common in AD and it could affect 80% of the cases while major depressive episodes could affect 50% of AD cases (Lleó and Blesa, 2017).

Impairment of language or speech

Language impairment with intact non-verbal memory and visuospatial skills is characteristic of fluent and non-fluent forms of progressive aphasia, the fluent form is referred to as semantic dementia due to patients losing their ability to comprehend the meaning of words not only that but also non-verbal materials including pictures. Both the fluent and non-fluent are classified as forms of degeneration of the frontotemporal lobar and they are usually confirmed via neuropathology, non-fluent patients are frequently diagnosed with AD accompanied by atypical distribution on neuropathology, patients suffering from progressive aphasia show intact delayed memory of geometric figures such as circles and that distinguishes them from patients of typical AD in neuropsychology (Zahn and Burns, 2017).

Visuospatial impairments

There is predominant visuospatial and apraxic difficulties due to atrophy of the occipital or parietal lobes (posterior or cortical atrophy) observed in some patients, in which most of these patients are AD-typical in neuropathology with atypical distribution (Zahn and Burns, 2017).

Etiology and pathophysiology

AD is a gradual and progressive neurodegenerative disease that is precipitated by the death of neuronal cells, it usually begins in the entorhinal cortex in the hippocampus, there is an identified role of genetics in both early and late onset of the disease, there are several risk factors that are related to AD in which advanced age is the uttermost important of these risk factors, followed by family history of dementia, depression, traumatic head injury, cardiovascular and cerebrovascular disease, smoking and also the presence of APOE e4 allele which is known to be a factor that increases the risk of AD. Anti-inflammatory drugs, regular aerobic exercise, getting higher education and the use of estrogen in women are all known to decrease the risk of AD. Possessing first degree relatives that have AD increases the risk of developing the condition by 10 to 30 percent, while that of an individual with two or more siblings with late onset AD increases one's chances by 3-fold when compared to the general population (Kumar et al., 2020).

Structural changes in the brain of AD Patients (DeKosky, 2001)

Neuron Loss	*Neuropil and Cellular Disruption*
• Large cortical neurons	• Neuritic plaques
• Amygdala	• Neurofibrillary tangles
• Hippocampus	• Synapse loss
• Entorhinal cortex	• Amyloid deposition
• Nucleus basalis of Meynert	• Oxidative stress
• Locus ceruleus	

Table (2) Structural changes in the brain of AD patients

Upon postmortem AD microscopic histopathological examination, the brain revealed the deposition of two protein aggregates parenchymal deposits of amyloid (Aβ) as plaques extracellularly, and intraneuronal deposits of tau protein fragments known as neurofibrillary tangles (NFT) that exceed the levels exhibited normally during ageing (Allen, 2017).

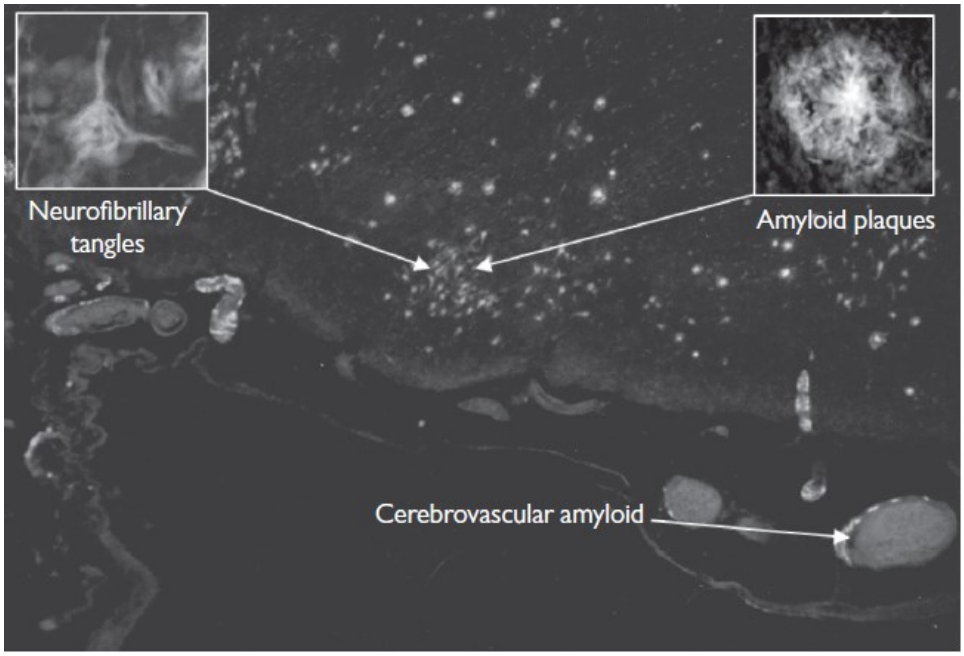


Figure (23) Microscopic examination of AD neuropathology in the temporal cortex

Plaques are spherical lesions under the microscope, in which they have a core of extracellular amyloid beta peptide that is surrounded by enlarged axonal endings, the beta amyloid peptide is derived from a transmembrane protein that is labelled as APP which stands for amyloid precursor protein, the beta amyloid peptide is cleaved from APP via the action of 3 proteases alpha α, beta β and gamma γ secretases (Kumar et al., 2020).

The consensus is that the initiative trigger of the disease process involves the generation of Aβ oligomers that over a period of time as a result of unmodulated cellular responses cause a variety of cellular stresses, a part of that process is related to the activation of specific kinases and abnormal phosphorylation of tau, one outcome of that process is the defective neuronal transport as well as loss of synaptic and neuronal cell communication. The pathological process that occurs during the onset and progression of AD is known to initiate 15 to 20 years before the symptoms start to appear (Allen, 2017).

1.4.1 Epidemiology

Alzheimer is a geriatric disease, in which its global prevalence is reported to be as high as 24 million and is projected to increase four times by 2050, health care cost estimates of AD is reported to be 172 billion per year in the U.S alone. In 2011 the U.S had an estimated 4.5 million people that are living with clinical AD aged 65 and beyond, the age related incidence increases significantly from less than 1 percent per year before the age of 65 to 6% per year after 85, the incidence rates of AD are slightly higher in women especially after the age of 85 (Kumar et al., 2020).

There is a growing proportion of elderly population (65+) in the world and projections point to the increase from 420 million in 2000 to nearly 1 billion by 2030, the worldwide ageing population is going to increase from 60 to exceed 70 percent. Therefore, an age dependent disorder such as AD will have a tremendous impact on healthcare, public health and social services throughout all countries in the world. The WHO reported an estimate of the overall prevalence of AD among people aged 60+ varied based on regions such as 2.1% in West Sub-Saharan Africa, 8.5% in Latin America, 5.57% in Asia-Pacific, 6.46% in North America U.S.A and 6.92% in Western Europe. In 2010 there were nearly 36 million people worldwide affected by dementia and the total number of individuals with dementia is projected to double every 20 years reaching 65.7 million by 2030 and 115.4 million by 2050, the global expenses allocated for dementia was 604 billion USD in 2010, this corresponds to about 1% of the global gross domestic product. In recent years there are reports that estimated the number of people living with dementia worldwide was 47.5 million with a 7.7 million novel case occurrence per year, it is extrapolated that the total number of cases with dementia will reach 75.6 million in 2030 and 135 million in 2050 hence, dementia or AD is becoming a global health priority. There are several meta-analyses and surveys that yielded similar patterns of age-specific prevalence of dementia across countries, in which age-specific prevalence of dementia almost doubles every 5 years after 65, approximately one in ten people aged 65-69 years is affected by dementia, whereas one third of people over the age of 85 may have dementia associated symptoms, AD and vascular dementia are two major subtypes of dementia that account for 50 to 70 percent and 15 to 25 percent of all dementia cases respectively, the distribution pattern of age specific prevalence of AD is similar to that of dementia. The incidence of AD almost doubles every 5 to 6 years from the age category 65 to 90, it is debatable as to whether the exponential increase in the risk of dementia will continue to increase in more advanced ages, there is an apparent decline in the incidence of AD among the extremely old in some studies which may be

contributable to survival effects, response rates and population nature moreover, several studies in Europe observed a higher AD incidence among women when compared to men especially at extremely old ages, whereas AD gender related occurrence differences fall short and are inconsistent in North America. Recent studies have supported evidence that suggest the incidence of dementia and AD might decline in high income countries (Qiu and Fratiglioni, 2017).

1.4.2 Pathophysiological mechanisms of AD

Under normal circumstances (Non-amyloidogenic pathway) the amyloid precursor protein (APP) is cleaved by either alpha or beta secretases which results in tiny fragments that are non-toxic to neurons contrastingly. The sequential cleavage by beta and gamma secretases yields 40 and 42 beta amyloids, in which the elevation of beta amyloid 42 results in aggregation of amyloid which causes neuronal toxicity, beta amyloid 42 results in preferential formation of aggregated fibrillary amyloid protein rather than the normal APP degradation. Moreover, APP is present on chromosome 21 which is one of the regions linked to familial AD (Kumar et al., 2020).

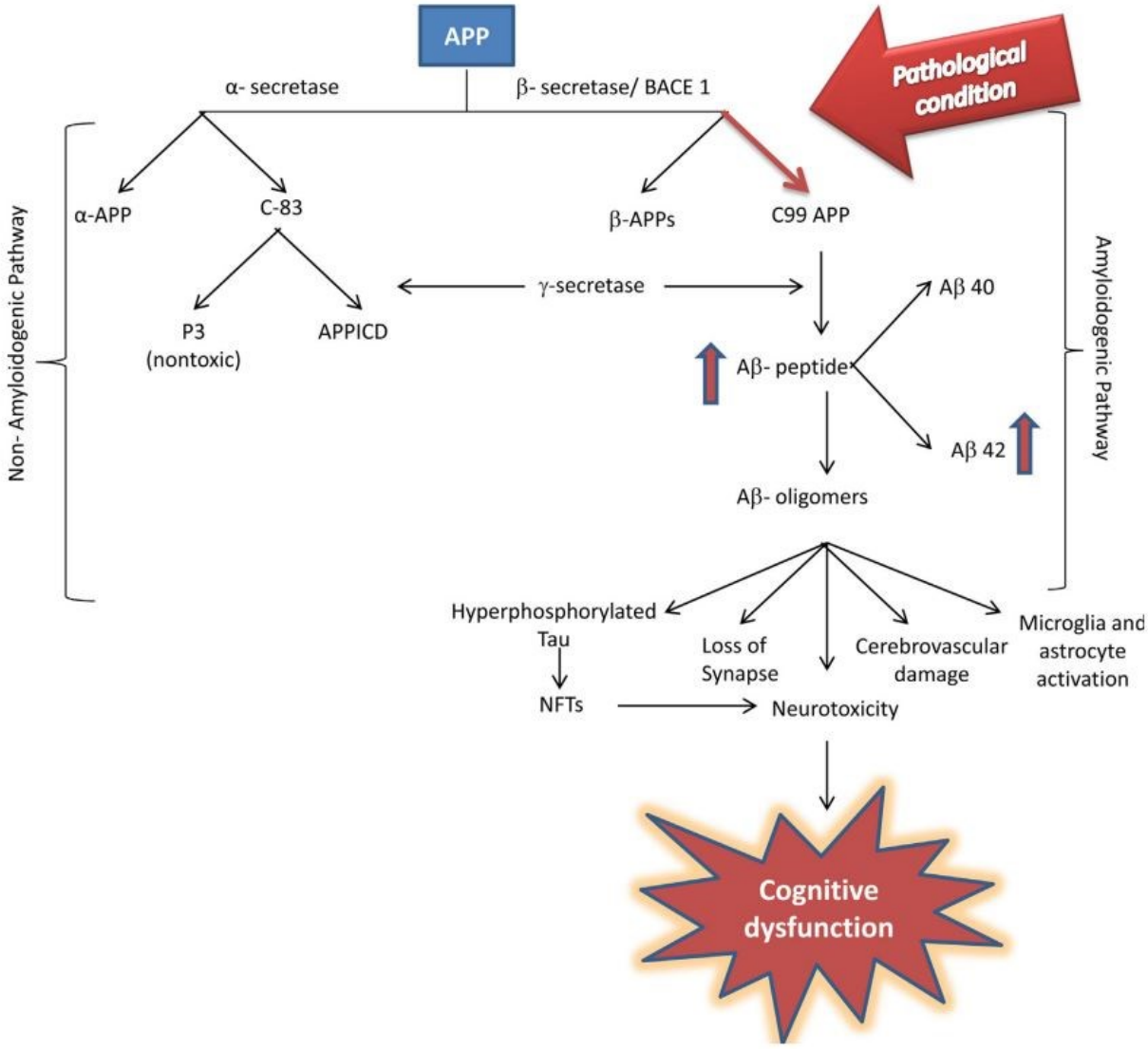


Figure (24) representation of APP processing pathways of neurodegeneration

Neurofibrillary tangles are fibrillary intracytoplasmic structures within the neurons that are composed of a protein referred to as tau, in which the primary function of tau is to stabilize axonal microtubules, microtubules are essential for intracellular transport, so tau protein keeps microtubule assembly held properly in order. In the case of AD as a result of aggregation of extracellular beta amyloid, there is hyperphosphorylation of tau which yields the formation of tau aggregates, tau aggregates form twisted paired helical filaments that are referred to as neurofibrillary tangles (NFTs) as represented in the aforementioned figure (Kumar et al., 2020).

The hallmark of the amyloidogenic pathway are senile plaques ad neurofibrillary tangles NFTs, in which cleavage of the integral neuronal cell membrane glycoprotein beta amyloid precursor protein (AβPP) via the enzyme beta secretase yields an N-terminal soluble fragment β-APPs into the extracellular space and an intramembranous C-terminal fragment protein C99 that is further cleaved by gamma secretase to form beta amyloid Aβ, meanwhile in the non-amyloidogenic pathway the cleavage of AβPP through α secretase releases a soluble protein α-APP into the extracellular space and an intramembranous C-terminal fragment C83 that is cleaved by γ secretase to form P3 and APP intracellular domain (APPICD), the two main isoforms of beta amyloid are 40 and 42, in which elevated levels of beta amyloid 42 (Aβ42) leads to aggregation and formation of insoluble oligomers and fibrillary configurations which yield senile plaque formation (Harris and Harris, 2018).

NFTs are composed of hyperphosphorylated tau proteins, NFTs are located within neurons, the tau proteins not only contribute to microtubule assembly but also stabilization which is essential for the cytoskeletal structure and axonal transport of vesicular and organelle structures, tau proteins are also essential for synaptic plasticity and synaptic function regulation, which under physiological conditions, the phosphorylation of tau proteins via kinases is a balanced process aided by dephosphorylation via phosphatases, this maintains the equilibrium that is required for interaction and binding of tau proteins to microtubules. However, when tau proteins are hyperphosphorylated, there are conformational changes that result in the formation of paired helical filaments (PHFs) and neurofibrillary tangles (NFTs) that result in microtubule destabilization, synaptic damage and neurodegeneration (Harris and Harris, 2018).

Microglial activation results in the generation and release of inflammatory cytokines including IL-1 beta, INF gamma and TNF alpha, which in turn causes stimulation of nearby astrocytes-neurons to produce more amounts of beta amyloid 42 oligomers, hence activation of more beta amyloid 42 generation and dispersal. The oligodendroglia (OLG) is also related to the neuron-astrocyte complex, in which the beta amyloid oligomers result in its destruction. Beta amyloid aggregates result in oxidative stress in which OLG is vulnerable due to their reduced glutathione content and high iron concentration, hence the impaired capability to scavenge oxygen radicals (Kumar et al., 2015).

APP genetic mutations may lead to increased generation and aggregation of beta amyloid peptide, two other genes involved in mutations include Presenilin 1 and 2 which result in the aggregation of beta amyloid via interference with gamma secretase processing, mutations in the previously mentioned three genes account to about 5% to 10% of all AD cases (Kumar et al., 2020).

1.4.3 The antimicrobial role of beta amyloid

Beta amyloid serving as part of the innate immune system was initially proposed by Bishop and Robinson, in which they called it the Bioflocculant hypothesis, they assumed that beta amyloid had aggregative properties making it ideal for surrounding and sequestering pathogens in the brain, through this process beta amyloid would limit the spread of pathogens and prep them for phagocytosis, they also noted that the positive charge of beta amyloid would be attracted to the negatively charged membrane of pathogens. A recent study by Pulze and colleagues identified a similar amyloid bioflocculant in humans, neutrophil extracellular traps (NETs) which is a decondensed chromatin network generated by immune cells in order to entrap microbial pathogens in which its structural backbone was found to be composed of amyloid fibrils. The positive charge of NET amyloid likely assists in pathogen targeting through providing the ability to distinguish between negatively charged pathogens and zwitterionic host membranes. Neutrophils, monocytes/macrophages, activated eosinophils and mast cells are able to secrete NETs which further suggests that amyloid bioflocculants could be an integral part of the human innated immunity. Many antimicrobial peptides and proteins (AMPs) possess an amyloid structure, the beta sheets of the amyloid can instantly be integrated into membranes leading to channel formation and death via disrupting ion dyshomeostasis. A number of amyloid AMPs are known in humans such as eosinophils which is a type of WBC in the innate immunity that utilizes amyloidogenic major basic protein-1 (MBP-1) in order to fight pathogens. If beta amyloid is indeed an AMP, it would be released from activated immune cells which has been demonstrated in multiple studies, cultures of microglial cells were found to express upregulated levels of beta amyloid upon exposure to bacterial LPS. Multiple studies had shown beta amyloid possessing antimicrobial activity against a variety of human pathogens, Soscia and colleagues were able to provide direct evidence of beta amyloid functions as an AMP, the minimum inhibitory concentration was determined for beta amyloid 40 and 42 against 12 common pathogens with IL37 being employed for comparison. Beta amyloid displayed antimicrobial activity against 8 of the 12 pathogens including Gram-positive and Gram-negative bacteria as well as yeast species (*Candida albicans*). Seven beta amyloid samples had potency equivalent to or exceeding that of the IL-37. Spitzer and colleagues had confirmed the antimicrobial properties of beta amyloid, in which following incubation of beta amyloid with bacterial and yeast species showed agglutination into large clusters and beta amyloid was found to be bound to the microbial surface. Electron microscopy of *enterococcus faecalis* treated with beta amyloid 42 showed that the bacteria acquired a dysmorphic shape

and accumulated a large amount of amorphic materials between the cells. Contrastingly human THP-1 cells treated with beta amyloid 42 did not yield any agglutination or toxicity. Spitzer was able to conclude that the capacity of beta amyloid 42 to selectively agglutinate pathogens could be a result of the heparin binding site of beta amyloid which has affinity for the mannan and glucan polysaccharides that are present in the cell walls of bacteria and fungi. Further studies had expanded to include the antimicrobial properties of beta amyloid against viruses in the human neuronal cells and against neurotropic viruses such herpes simplex-1 (HSV-1) infection, whereby beta amyloid was shown to be as effective as Acyclovir in the process of attenuation of the virus, it appears that beta amyloid interferes with HSV-1 fusogenic protein gB thus, prevents the virus from fusing to the plasma membrane and infecting host cells. Beta amyloid was also shown to protect against H3N2 and H1N1 strains of influenza A virus through triggering aggregation of viral particles and enhancement of phagocytosis via neutrophils and macrophages, all of the aforementioned evidence indeed confirms the potent and broad spectral antimicrobial properties of beta amyloid, the following table provides a summary of the antimicrobial properties of beta amyloid against a wide variety of pathogens (Gosztyla et al., 2018).

Pathogen	Pathogen type	Activity
Herpes simplex virus-1	Virus	↓ Infectivity, ↓ neuropathological responses in cell lines ↓ Viral replication
Influenza A	Virus	↓ Infectivity in cell lines, ↑ viral aggregation, ↑ immune phagocytosis
Enterococcus faecalis	Bacterium, gram-positive	↓ Growth in culture ↑ Aggregation ↑ Cellular dysmorphic shape
Escherichia coli	Bacterium, gram-negative	↓ Growth in culture ↑ Aggregation
Listeria monocytogenes	Bacterium, gram-positive	↓ Growth in culture ↑ Aggregation
Salmonella typhimurium	Bacterium, gram-negative	↑ Survival, ↓ infection progression, ↓ Bacterial load in Aβ overexpressing mice
Staphylococcus aureus	Bacterium, gram-positive	↓ Growth in culture ↑ Aggregation
Staphylococcus epidermidis	Bacterium, gram-positive	↓ Growth in culture
Streptococcus agalactiae	Bacterium, gram-positive	↓ Growth in culture
Streptococcus pneumoniae	Bacterium, gram-positive	↓ Growth in culture
Candida albicans	Fungus	↑ Survival in Aβ expressing nematodes ↑ Survival, ↓ fungal load in Aβ overexpressing cell lines ↓ Growth in culture ↑ Aggregation

Table (3) The antimicrobial properties of beta amyloid (Aβ)

1.4.4 The role of microbial insults in AD pathogenesis

The determination of pathogenic DNA within the beta amyloid plaques provided support to the theory that beta amyloid may aggregate in response to infectious agents in the brain. A study by Wozniak reported in-situ PCR detection of HSV-1 in the brain tissues of 6 AD patients, they all tested positive for HSV-1 infection. In the brains of AD patients 90% of beta amyloid plaques contained HSV-1 DNA and 80% of healthy brain plaques were associated with HSV-1 DNA, suggesting that HSV-1 may result in higher than normal beta amyloid deposition, which could be either due to overproduction of beta amyloid or reduced clearance rate. Moreover, another study by Miklossy reported 3 AD brains infected with *Borrelia Burgdorferi* showing beta amyloid plaques colocalized with bacterial antigens and DNA, this confirmed that in cases of infection a large proportion of beta amyloid plaques were found to contain viral or bacterial DNA. This association is further supported through the evident process where the infectious pathogen stimulates the generation of beta amyloid. HSV-1 encephalitis in human subjects is found to be associated with reduced levels of beta amyloid 42 in the CSF in comparison to healthy controls, a similar reduction is observed in AD patients and it serves as an indirect indication that beta amyloid 42 deposition is enhanced in the brain. HSV-1 infection in humans and rats is shown to activate the amyloidogenic pathway of AβPP processing while inhibiting beta amyloid degradation which leads to intracellular accumulation of beta amyloid. In autopsy studies of brain tissues infected with HIV; it was shown that approximately half of the frontal cortices were found to contain beta amyloid plaques and intraneuronal inclusions of beta amyloid, also in cell cultures of HIV infected human brains, neuroblastoma cells were showing increased rates of beta amyloid generation and secretion thus; confirming that HIV plays a role in upregulating the expression of beta amyloid. Bacteria are also able to induce the deposition of beta amyloid such as *Chlamydia pneumoniae* in which immunohistochemical analysis of infected mice revealed that the olfactory bulb and cerebrum had deposits of beta amyloid. Furthermore, there is the common oral bacterium *Porphyromonas gingivalis* which is linked to beta amyloid deposition in which oral administration of the bacteria in mice showed significant increase in the levels of LPS in the brain indicating the spread of the infection to the CNS. The infected mice had revealed an increased load of beta amyloid plaques in the hippocampus and high concentrations of both beta amyloid 40 and 42 in the cortex as well as the hippocampus. In mice neuronal cell cultures, the exposure of LPS from *P. gingivalis* resulted in an increase in the secretion of beta amyloid 40 and 42 in a concentration dependent

manner. The following table summarizes different pathogens and their roles in beta amyloid deposition and AD pathogenesis (Gosztyla et al., 2018).

Pathogen	Pathogen type	Effects
Herpes simplex virus-1	Virus	↑Aβ deposition ↑AβPP phosphorylation ↑Amyloidogenic AβPP processing ↓ Non-amyloidogenic AβPP processing ↓ Aβ degradation ↓ CSF $Aβ_{42}$
Herpes simplex virus-2	Virus	↑Aβ intracellular accumulation ↓ Non-amyloidogenic AβPP processing, ↓ Aβ degradation
Pseudorabies virus (suid herpesvirus-1)	Virus	↑ Insoluble Aβ
HIV-1	Virus	↑Aβ deposition ↑AβPP expression ↑Amyloidogenic AβPP processing ↓ Aβ degradation ↓ CSF $Aβ_{42}$
Chlamydia pneumoniae	Bacterium	↑Aβ deposition
Treponema palladium	Bacterium	↑Aβ deposition
Borrelia burgdorferi	Bacterium	↑Aβ deposition ↑AβPP production
Porphyromonas gingivalis	Bacterium	↑Aβ deposition

Table (4) Role of different pathogens in beta amyloid deposition and AD pathogenesis

1.4.4.1 Neurotropic viruses in AD pathogenesis

Neurotropic viruses have the capacity to enter the CNS via peripheral axons and the bloodstream, they have evolved methods to avoid and traverse the blood brain barrier (BBB) to be able to enter and infect the CNS, the impact of viral proteins on the neurons and glia through autophagy, progenitor cell proliferation, migration and maturation all result in initiating or exacerbating neurodegenerative diseases onset and progression (Hogestyn et al., 2018).

1.4.4.2 HSV-1 and AD

In 1991, Dr. Itzhaki's lab were the first to discover HSV-1 DNA high proportional presence in the brains of both AD patients and normal elderly subjects. Later in 1997, Itzhaki and colleagues found that HSV-1 DNA in the brains of carriers of apolipoprotein E-Ɛ4 allele conferred high risk for developing AD, which was also confirmed by Itabashi and colleagues in another study. It was proposed that the reactivation of HSV in the brain is recurrent thus, damage accumulates with eventual gradual development of AD. The next discoveries provided direct links between HSV-1 and damage in AD brain, through multiple studies by Itzhaki and other groups, it was shown that beta amyloid and phosphorylated tau were greatly increased in HSV-1 infected cell cultures. Further investigations by Wozniak and colleagues in 2009, on the proximity of HSV-1 to beta amyloid plaques revealed a striking co-localization where 90% of plaques contained HSV-1 DNA and in AD brains 72% of the viral DNA was associated with plaques. Summation of the findings alongside beta amyloid accumulation after infection suggests that HSV-1 causes the formation of toxic beta amyloid plaques (Fülöp et al., 2018).

Moreover, a study by Piacentini and colleagues reports a mechanism of HSV-1 induction of APP cleavage, in which HSV-1 binds to the heparan sulfate proteoaminoglycans (HSPGs) that are expressed on the plasma membrane of neurons, binding induces Ca^{2+} signals that triggers APP phosphorylation at Thr668 position which in turn increases beta and gamma secretases activity, as a consequence, there is an elevated generation of beta amyloid that aggregates forming oligomers, the C-terminal APP fragment APPICD or AICD is created via cleavage of gamma secretase and is phosphorylated at Thr668 position, it is further internalized into the nucleus where it may modulate genetic transcription (Piacentini et al., 2014).

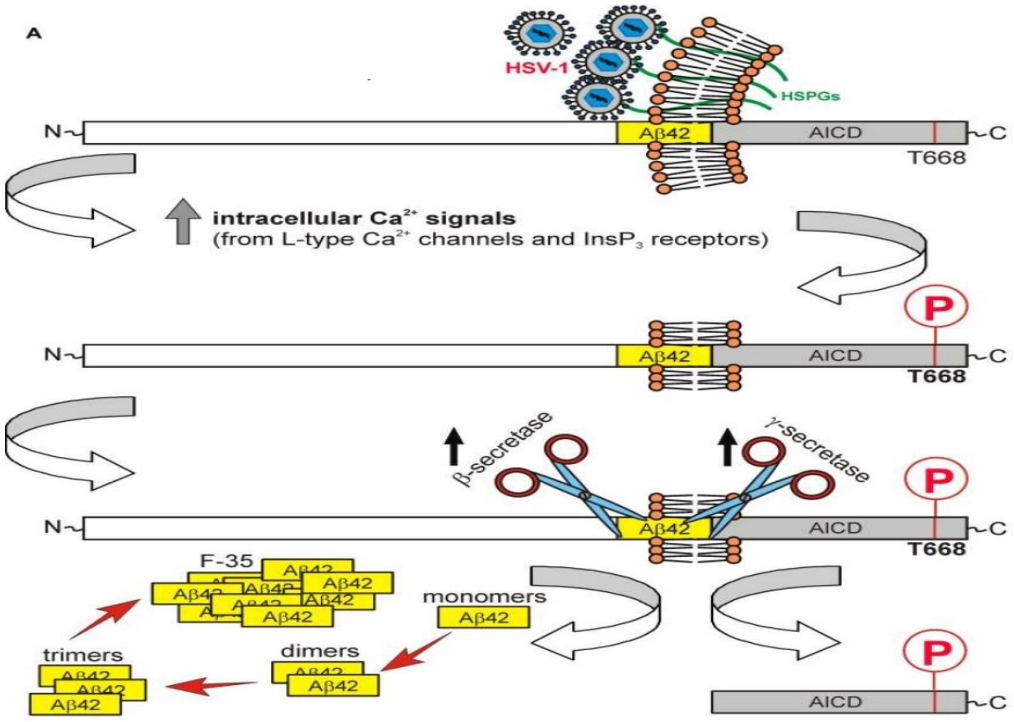


Figure (25) HSV-1 induces APP cleavage

Another important discovery was a study by Piacentini and colleagues in 2015, where it was demonstrated that HSV-1 infection reduces the expression of presynaptic proteins synapsin-1 and synaptophysin, decreases synaptic transmission; the inhibitory effects on synaptic function were dependent on the glycogen synthase kinase 3 (GSK-3) activation and consequential intraneuronal accumulation of beta amyloid. It also revealed that in the cortical neurons of mice HSV-1 induced Ca^{2+} dependent GSK-3 was critical for the HSV-1 dependent phosphorylation of APPICD at Thr668 position and consequentially intraneuronal accumulation of beta amyloid Aβ (Piacentini et al., 2015).

An earlier study in 2009 was conducted in-vitro on cell cultures in which Wozniak and colleagues were able to deduce that HSV-1 induced tau phosphorylation at several sites Threonine 212, Serine 202, 396 and 404 via GSK-3 while phosphorylation at Serine 214 via protein kinase A (PKA), the concentration of abnormal tau phosphorylation was immensely great in HSV-1 infected cells in comparison to non-infected cells (Wozniak et al., 2009).

HSV-1 alters the production and degradation of beta amyloid, in which the amyloid precursor protein (APP) is produced in the endoplasmic reticulum and Golgi system, APP is located on the surfaces of both plasma membranes and endosomes, after cleavage of APP with secretases it yields beta amyloid, under normal conditions (uninfected) in which beta amyloid protein and aggregates can be broken down via autophagy process, where aggregates are integrated into autophagosomes and fuse with lysosomal compartments with which they are degraded by lysosomal hydrolases. During HSV-1 infection the viral capsids alter the distribution of APP, it also increases the expression of secretases which in turn promotes the production of beta amyloid, ultimately HSV-1 inhibits the autophagy processing of beta amyloid as a consequence intracellular beta amyloid oligomers accumulate within autophagosomes and endosomes, as represented in the following figure (Hogestyn et al., 2018).

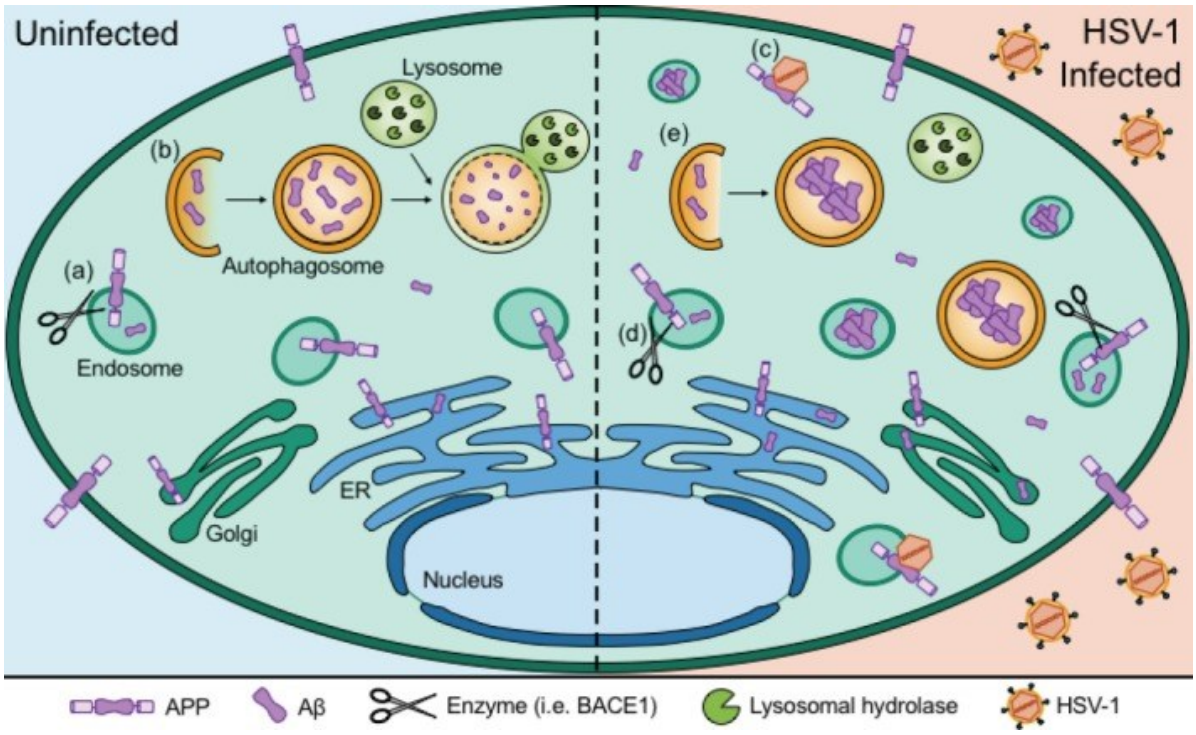


Figure (26) HSV-1 alters degradation and production of beta amyloid

1.4.4.3 SARS-Cov2 “COVID-19” in AD pathogenesis? (Novel hypothesis)

As characterized in studies SARS-Cov2 acts on the ACE2 receptor, and there is an evident distribution of ACE2 in the brain, the receptors were detected in the glial cells and neurons, previous studies reported the ability of SARS-Cov to cause neuronal death in mice by brain invasion via the nose’s olfactory epithelium (Netland et al., 2008). SARS-Cov2 was shown to possess ACE2 binding affinity that is 10 to 20 times higher than that of SARS-Cov hence, contributes to the neurotropic potential of SARS-Cov2. Addedly COVID-19 patient case reports show complaints of neurological manifestations thus, confirming the rationale of the neurotropic potential of SARS-Cov2 (COVID-19). Autopsies of COVID-19 patients could use detailed neurological investigations in order to shed light into the role played by the novel SARS-Cov2 (Baig et al., 2020). Following this recent significant development, I would propose a novel hypothesis that SARS-Cov2 (COVID-19) could play a role as a novel neurotropic virus in the pathogenesis of neurodegenerative diseases such as AD perhaps through the beta amyloid cascade. There is a lot to investigate and such studies would require a lot of resources and funding therefore, it will not be a subject of investigation or experimentation in this study.

1.4.5 The ENS: a link between microbial insults and AD

The enteric nervous system (ENS) is regarded as a displaced part of the CNS that retains communication via the sympathetic and parasympathetic afferent and efferent neurons, the part of the CNS that is connected to the ENS is now labeled as the central autonomic neural network, together with these connections the ENS provides neural control of all the functions of the GIT. Within the ENS, the nerve cell bodies are categorized into small ganglia that are by bundles of nerve processes that makeup two major networks or plexuses referred to as the myenteric or (Auerbach's) plexus and the submucous or (Meissner's) plexus. The myenteric plexus is located between the longitudinal and circular layers of muscles and it extends throughout the entire length of the gut while the submucous plexus lies in the submucosa between the circular muscle layer and the muscularis mucosa. The enteric ganglion cells and nerve fiber tracts are supported by plenty of glial cells; which are nucleated satellite cells around nerve cell bodies of the enteric ganglia and are generally referred to as Schwann cells (Goyal and Hirano, 1996; Furness, 2006).

The ENS has several roles including determining patterns of movement of the GIT, regulating fluid movement, controlling gastric acid secretion, altering local blood flow and nutrient handling, interacting with immune and endocrine systems of the gut in addition to roles alongside glial cells which include maintenance of the integrity of epithelial barriers between the cells and tissues within the gut and gut lumen (Furness, 2012).

The gut brain axis (GBA) is a bidirectional communicative network between the central and enteric nervous system, the signaling interaction between gut microbiota and GBA is evident through a plethora of studies, one that addresses clinical practice is the evidence of microbiota-GBA interactions that initiates from the association of dysbiosis with CNS disorders and functional GIT disorders, prime examples would be inflammatory and irritable bowel syndromes, in which associatively dysbiosis and disruption of microbiota-gut-brain axis (MGBA) occurs (Carabotti et al., 2015).

1.4.5.1 Extra-CNS origin of neurodegenerative disorders

New investigations have revealed that activated enteric glial cells (EGCs), astrocyte like cells of the enteric nervous system (ENS) represent a possible extra-CNS trigger point of neuro-degenerative processes in impaired intestinal permeability conditions. The shift from neuron centric to glial centric view is promising in which glial cells possess the machinery to generate

pro-inflammatory signaling molecules, cytokines, prostaglandins, chemokines and other cytotoxic mediators that regulate hypertrophy and proliferation. There is aberrant production of these mediators under persistent CNS stress conditions thus, provides detrimental signals that result in both microglia and astrocytes switch-on towards a pro-inflammatory phenotype. Consequentially a neuroinflammatory response that often precipitates neurodegeneration. The pathological glial activation known as reactive gliosis results in a wide range of alterations including the activation of Toll-like receptors (TLRs) and Receptor Advanced Glycation End-products (RAGE). Altered expression and release of multiple glia associated proteins and enzymes such as glial fibrillary acid protein (GFAP), S100B, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), vimentin and metalloproteinase (MMPs) as well as the activation of pro-inflammatory pathways including NF-kB and JAK/Stat. This cascade of events determines the release of pro-inflammatory cytokines such as tumor necrosis factor α, interleukin 1β and interleukin 6 thus, the neuroinflammatory loop is established, under these conditions the initial inflammatory process is amplified resulting in irreversible damage that is responsible for neurodegeneration as well as lack of efficacy that is observed currently in neuroprotective drugs (Seguella et al., 2019).

For many years, it was wrongly thought and concluded that the etiology of neurodegenerative disorders is limited to neural changes that occur exclusively in the CNS. Moreover, recently with the MGBA being a factor that deeply affects CNS health via a complex interaction, where the gut homeostasis is altered after injury and inflammation caused by pathogens for instance, resulting in leaky gut syndrome that as a consequence increases permeability and hence, translocate pathogens as well as their harmful components (LPS, peptidoglycan, flagellin, prion proteins, viruses and neurotoxic agents such as toxins) from the intestine to tissues, thereby reaching the nerve portion of the gut, the ENS. As a consequential result, a neuroinflammatory response exacerbates the mucosal barrier disruption which promotes neuropathy development and GI dysmotility. The enteric glial cells (EGCs) are the priming missing link that connect the gut neuroinflammation and CNS neuropathology. The ENS was found to be compromised in transgenic murine models of AD revealing enteric neuron loss as well as beta amyloid deposition. As a result localized neuroinflammation and GI dysmotility detection, it is suggested that the ENS is affected in AD, moreover, rat myenteric plexus revealed increased deposition of hyperphosphorylated tau, **further investigations are needed** to clarify the potential multicentric nature of AD and beta amyloid spreading from the gut to the CNS. The

appearance of neuropathological markers in the ENS emphasizes the extra-CNS origin of neurodegenerative diseases (Seguella et al., 2019).

In the case of microbiological pathogens such as bacteria, viruses and toxins, when they pass into the gut, as a consequence of the body's exposure to pathogens, elicitation of an immune response driven by the MGBA results in the generation of neuronal beta amyloid (anti-microbial), the sequence of events displayed in several studies in mice mostly about bacteria and bacterial LPS indicate the deposition of beta amyloid in the brain and gut of mice in addition to evidently enhanced microgliosis, astrogliosis with increased expression of TLR2, IL-6 and TNF which would further clarify the microbiota associated inflammation. Gut inflammation induced by dysbiosis is directly associated with gut barrier dysfunction and increased intestinal permeability leaky gut syndrome (LGS) which is incriminated in the cascade of events that take place in the ENS and contribute to the process of neurodegeneration (Kowalski and Mulak, 2019).

In light of the EGCs changes that occur as a result of the switch-on activated phenotype in leaky gut syndrome, it would be of predictive benefit to sample EGCs to detect neuropathological conditions thus, anticipating their symptomatic manifestation (Seguella et al., 2019).

There is an apparent lack of data regarding the effects that viruses induce through the beta amyloid pathway in the ENS specifically HSV-1, which is a major neurotropic pathogen increasingly linked with neurodegeneration and AD pathogenesis.

1.4.5.2 AD starts in the gut (Novel hypothesis)

As a result of recent developments and research, a novel hypothesis is proposed in which Alzheimer's disease is not only a CNS disease, as implied earlier there is an extra-CNS origin as the neuroinflammatory cascade of events has been identified with EGCs being a link between the ENS and the CNS as well as investigations of beta amyloid in the enteric nervous system of mice infected with pathogens such as bacteria, in which the antimicrobial activity of beta amyloid interplays in this mechanism via the immune response cascade, the MGBA cross talk initiating the cascade of events of neuroinflammation and deposition of beta amyloid incriminates the extra-CNS origin of AD, in which the gut is considered as the place where AD pathology initiates, This is a promising hypothesis but of a bigger project that requires longer time and much more resources.

Therefore, looking to the importance of the beta amyloid cascade, my internal study will be focused on investigating HSV-1 effects on this cascade of beta amyloid deposition in the ENS.

1.4.5.3 Novel AD treatment strategy

As support to the previous hypothesis of extra-CNS AD, there are several studies that potentially reveal the consequences of inhibiting reactive gliosis and restoration of astrocyte functions as a fascinating and promising technique towards the promotion of neuroprotection and halting the progression of a variety of neurodegenerative disorders. Therefore, directing the therapies toward the peripheral management of neurodegeneration. (Seguella et al., 2019).

Chapter 2

Aims of the study

HSV-1 is a neurotropic virus that is capable of infecting a variety of cells such as epithelial and neuronal cells and it also has the potential to reactivate once it generates a latent infection in the central nervous system neurons. There are increasing evidence that HSV-1 also infects the cells of the enteric nervous system triggering an inflammatory response which could contribute to intestinal dysmotility and neuronal degeneration. Among the intestinal neurodegenerative disorder, deposition of beta amyloid has been described in aging and transgenic mice following important, microbiological-driven inflammatory insults. As the alterations of the gut microbiota greatly contribute to the local inflammatory events, this study aims at investigating the role of HSV-1 infection of the enteric nervous system in the beta amyloid deposition during gut dysbiosis.

Chapter 3

Materials and methods

3.1 Materials

HSV-1 SC16 strain, American Type Culture Collection (ATCC), VA, U.S.A

Vero cells, American Type Culture Collection (ATCC), VA, U.S.A

Dulbecco's Modified Eagle's Medium (DMEM), Gibco, UK

Fetal bovine serum, Gibco, UK

Penicillin/Streptomycin, Gibco, UK

Wild type (WT) C57BL/6J mice (8-10 weeks old), Envigo Laboratories, Udine, Italy

Neomycin, Sigma-Aldrich, Italy

Vancomycin, Sigma-Aldrich, Italy

Ampicillin, Sigma-Aldrich, Italy

Metronidazole, Sigma-Aldrich, Italy

Liquid Nitrogen, Sigma-Aldrich, Italy

Collagenase Type II, Sigma-Aldrich, Italy

DNA lysis buffer, Sigma-Aldrich, Italy

Sucrose, Sigma-Aldrich, Italy

Sodium Chloride, Sigma-Aldrich, Italy

Magnesium Chloride, Sigma-Aldrich, Italy

Krebs Solution, Sigma-Aldrich, Italy

Phenol/Chloroform, Sigma-Aldrich, Italy

Sodium Dodecyl Sulfate (SDS), Sigma-Aldrich, Italy

Bovine Serum Albumin, Sigma-Aldrich, Italy

Proteinase K, Sigma-Aldrich, Italy

Laminin, Sigma-Aldrich, Italy

Poly-D-lysine, Sigma-Aldrich, Italy

Neurobasal A media, Bio-Legend, Italy

Nerve Growth Factor, Bio-Legend, Italy

Ethidium Bromide, Sigma-Aldrich, Italy

Phosphate Buffer Saline (PBS), Sigma-Aldrich, Italy

DNase I treatment, Promega, Italy

Dispase, Sigma-Aldrich, Italy

DNase I, Sigma-Aldrich, Italy

Protease Inhibitors (Phenymethylsulfonyl fluoride, aprotinin and leupeptin), Sigma-Aldrich, Italy

Ethanol, Sigma-Aldrich, Italy

Xylene, Sigma-Aldrich, Italy

Formalin, Sigma-Aldrich, Italy

Universal Blocking Solution, Lab Vision Corporation, CA, U.S.A

Paraffin, Sigma-Aldrich, Italy

Sodium Citrate Buffer, Sigma-Aldrich, Italy

3,3' Diaminobenzidine Tetrahydrochloride (DAB), Sigma-Aldrich, Italy

Hydrogen Peroxide, Sigma-Aldrich, Italy

Anti-$CD3^+$ Mouse Monoclonal Antibody, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A

Goat Anti-mouse Antibody, Dako Corporation, Glostrup, Denmark

Fixation/Permeabilization Buffer, eBioScience, Italy

2',7' Dichlorodihydrofluorescein Diacetate (H_2DCFDA), Molecular Probes, Italy

4,5 Diaminofluorescein Diacetate (DAF-FM), Molecular Probes, Italy

Mouse Monoclonal Antibody Against Recombinant APP_{695}, Boehringer-Mannhein, Germany

Sheep Anti-mouse IgG, Ammershan, UK

Sodium Pyruvate, Gibco, UK

Forskolin, Gibco, UK

Bovine Pituitary Extract (BPE), Gibco, UK

Anti-mouse Ty1.1 Monoclonal Antibody, Sigma-Aldrich, Italy

Blocking Buffer, Sigma-Aldrich, Italy

Permeabilization Buffer, Sigma-Aldrich, Italy

Rabbit Anti-human GFAP, Sigma-Aldrich, Italy

Alexa Fluor 647 Goat Anti-rabbit, Invitrogen, Milan, Italy

Mouse Monoclonal anti-S100B, AbCam, U.S.A

Alexa Fluor 488 goat anti-mouse, AbCam, U.S.A

3.2 Equipment

Catheter (24 gauge, 9-cm), Sigma-Aldrich, Italy

Syringes, Sigma-Aldrich, Italy

Lab racks, Sigma-Aldrich, Italy

Eppendorf Tubes, Sigma-Aldrich, Italy

Eppendorf Pipettes, Sigma-Aldrich, Italy

Stainless Steel Feeding Tube, Sigma-Aldrich, Italy

Dissection Scalpels, Sigma-Aldrich, Italy

Dissection Scissors, Sigma-Aldrich, Italy

Light Microscope, Sigma-Aldrich, Italy

ABI Prism 7700 Sequence Detection System, PerkinElmer, Monza, Italy

TaqMan Universal PCR Master Mix, Applied Biosystems, Monza, Italy

Tissue DNA Extraction Kit, Millipore, Milan, Italy

Enzyme-linked Immunosorbent Assay, BioSource, Milan, Italy

Retsch MM300 mixer, Promega, Italy

SV total RNA isolation system, Promega, Italy

Dako Envision+ System-HRP labeled Polymer Detection, Dako, CA, U.S.A

Leica DM4500B microscope, Leica Microsystems, UK

Mouse/Rat HSV-1 IgG ELISA, Calbiotech, CA, U.S.A

Cell Strainer, Sigma-Aldrich, Italy

Ficoll-Hypaque Density Gradient Centrifuge, Sigma-Aldrich, Italy

BD FACSCanto Flow Cytometer, BD Bioscience, Italy

Enzyme-linked Immunosorbent Assay (ELISA), Biosource, Milan, Italy

Microplate Reader, Sunrise, Tecan, Switzerland

Nikon Eclipse E600 Microscope, Nikon, Italy

Fecal Collection Kits, Sigma-Aldrich, Italy

FastDNA SPIN Kit, MP-Biomedicals, U.S.A

QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany

NanoDrop Spectrophotometer, Sigma-Aldrich, Italy

AMPure XP Bead Kits, Beckman Coulter, U.S.A

454 Kapa Library Quantification Kit, KAPA Biosystems, U.S.A

3.3 Methodology

3.3.1 Preparation of viral stocks

The HSV-1 strain SC16 is propagated on Vero cells, in which Vero cells are maintained in DMEM that is supplemented by 10% heat inactivated fetal bovine serum and 1% of penicillin/streptomycin under the following ambient conditions of 37 °C and 5% CO_2. The HSV-1 stocks are prepared in DMEM 2% fetal bovine serum and titrated on the Vero cells through utilizing the standard plaque technique, in which the viral stocks are adjusted to the following plaque forming units 1 X 10^8 (PFU)/mL (Brun et al., 2018).

3.3.2 HSV-1 infection of the ENS

3.3.2.1 Animal infection model

The wildtype (8-10 weeks old) C57BL/6J mice are kept at ambient controlled temperature of 22 ± 2 °C with 12 hrs. of light/dark cycle and *ad libitum* supply of standard rodent food and tap water. A week post acclimation, the mice are infected via intranasal instillation of 10^2 PFU (final volume 10µl) of HSV-1 SC16 strain. Four weeks post instillation, the mice are inoculated with 1 x 10^7 PFU of HSV-1 SC16 strain through intragastrical gavage (IG) via a 24 gauge, 9-cm catheter for test group infection, while for sham infection (control group) an equal volume of Vero cell lysate is employed for inoculation. The Two-step HSV-1 inoculation protocol is adopted to prime the host immune system to the virus and prevent the high mortality rate exhibited by the mice following inoculation with 1 x 10^7 PFU of HSV-1 (Brun et al. 2010). Post IG inoculation, the mice are observed on a daily basis for signs of pathology, afterwards the mice are sacrificed 1, 2 or 3 weeks later, in which the sham infected mice are also sacrificed at matching times. As the sham results are comparable, the data is pooled together and reported as a single sham group (Brun et al., 2018). Moreover, in order to detect the etiological factor behind dysbiosis, the mice intestinal microbiota is depleted to yield a reduction in gut microbiota through the following protocol, where freshly prepared antibiotic solution that is composed of 100mg/kg neomycin, 50mg/kg vancomycin, 100mg/kg ampicillin and 100 mg/kg metronidazole, which is administered daily for 14 days through a 100µl gavage every 12 hours via a stainless- steel feeding tube (Brun et al. 2013). All of the experimental protocols are approved by the Animal Care and Use Committee of the University of Padova which is under licensure from the Italian Ministry of Health, following the guidelines of the National and European handling and use of experimental animals (Brun et al., 2018).

3.3.2.2 Infection assessment

In order to assess the infection, we need to undergo a polymerase chain reaction (PCR) for the detection of viral genes.

Oligonucleotides	Sequences	Tm °C
tk	Fw 5′-tagcccggccgtgtgaca-3′ Rv 5′-cataccggaacgcaccacacaa	60
ICP0	Fw 5′-ggtgtacctgatagtgggcg-3′ Rv 5′-gctgattgcccgtccagata-3′	60
ICP4	Fw 5′-atgacggggacgagtacgac-3′ Rv 5′-acgacgaggacgaagaggat-3’	56
VP16	Fw 5′-tgcgggagctaaaccacatt-3′ Rv 5′-tccaacttcgcccgaatcaa-3′	60
gB	Fw 5′-ggctccttccgattctcc-3′ Rv 5′-ggtactcggtcaggttggtg-3′	60
gC	Fw 5′-ccaaacccaagaacaacacc-3′ Rv 5′-tgttcgtcaggacctcctct-3′	60
gD	Fw 5′-gccaggttgggggccgtgat-3′ Rv 5′-acctgcggctcgtgaagata-3′	60
LATs	Fw 5′-gacagcaaaacaataaggg-3′ Rv 5′-acgagggaaaacaataaggg-3′	60
Rn18S	Fw 5′-tcaagaacgaaagtcggagg-3′ Rv 5′-ggacatctaagggcatca-3′	60

Table (5) PCR primers of viral genes

HSV-1 infection is evaluated in the contents of the ileum, where IG inoculated mice of both HSV-1 SC16 and sham groups are sacrificed as mentioned previously. The gut is dissected carefully, and the ileum contents are collected through 5 ml sterile PBS flushing. The samples are vigorously vortexed, centrifuged at 4 °C, 3000 rpm for 10 minutes and an equal volume of clarified supernatant is serially diluted and incubated with Vero cell monolayers. Vero cell cultures are monitored for up to 72 hours in order to observe the cytopathic effects. The cells are fixed and stained with crystal violet and the number of plaques is determined. The data is documented as the percentage of the number of plaques yielded in Vero cells that are incubated

with HSV-1 SC16 (1 x 10^7 PFU). HSV-1 presence is evaluated and confirmed via PCR assay of the viral DNA extracted from Vero cells (Brun et al., 2018).

3.3.2.2.1 Dissection of longitudinal muscle myenteric plexus

Dissection of the longitudinal muscle myenteric plexus (LMMP) following sacrifice, in which the abdomen is opened through a midline incision where the small intestine is excised and removed aseptically. Then it is placed in oxygenated Krebs solution (126mM Nacl, 25mM $NaHCO_3$, 2.5 mM $CaCl_2$, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, pH 7.2). The tissues are cut into pieces of 1cm length. LMMP is peeled off and placed in a sterile ice-cold Krebs solution for further assays and nucleic acid extraction. The samples are immediately snap frozen with liquid nitrogen or subjected to enzymatic digestion to yield single cell suspensions (Brun et al., 2018).

3.3.2.2.2 Enteric neurons isolation and culturing

Freshly obtained LMMP is rinsed 3 times in Krebs solution, furthermore, minced with scissors and digested in 1.3 mg/ml collagenase type II and 0.3 mg/ml bovine serum albumin for 15 minutes at 37 °C. The cells are co-cultured with Vero cells or seeded on laminin and poly-D-lysine coated coverslips in Neurobasal A media that contains B-27 supplement, 1% fetal bovine serum, 10 ng/ml nerve growth factor (NGF) and penicillin/streptomycin. Half of the cell media is replaced every 2 days with fresh complete growth media. At the fifth culture day, the neurons are incubated with HSV-1 for 36 hours and then fixed for 20 minutes in 4% paraformaldehyde (PFA) at room temperature and is subjected to immunocytochemistry. An alternative to immunocytochemistry, is challenging the enteric neurons with HSV-1 inactivated through exposure to 20 joules of UV light for 10 minutes at maximal power output (Brun et al., 2018).

3.3.2.2.3 Nucleic acid extraction and assay

In order to extract the DNA from tissue specimens, enteric ganglia and Vero cells, all specimens are homogenized in digestion buffer (DNA lysis buffer: 1.25% wt/vol sucrose, 0.3% vol/vol NP-40, 10 mmol/L NaCl, 3 mmol/L MgCl2, 20 mmol/L Tris-HCl, pH 7.4) after that, incubation at 56 °C in 10% sodium dodecyl sulfate and 200 µg/ml proteinase K. The DNA is further purified through phenol/chloroform extraction and ethanol for precipitation, in which the DNA is extracted via Tissue DNA Extraction Kit. The LMMP is homogenized via Retsch MM300 mixer and the RNA is extracted via SV total RNA isolation system, according to the manufacturers protocol. The contaminating DNA is removed via DNase I treatment. The

complementary DNA (cDNA) is generated and synthesized via utilizing 1 µg of RNA as a template. Afterwards, the PCR experiment is performed via an ABI Prism 7700 Sequence Detection System on 200 ng of DNA in the presence of TaqMan Universal PCR Master Mix, with primers of HSV-1 thymidine kinase and internally annealing probes (Brun et al., 2010; Brun et al., 2018).

3.3.2.2.4 Histopathological evaluation

The mice being sacrificed at a specified time, we are able to obtain specimens of the ileum 5-10 cm long segments (initiating at the ileocecal valve). The specimens are fixed in 10% neutral formalin buffer for 24 hours, embedded in paraffin and sectioned at 5 µm thickness. All the representative sections per mouse are subjected to Hematoxylin and Eosin staining (H&E), with a minimum of 10 independent fields per mouse are examined via Leica microscope that is equipped with a digital camera (Brun et al., 2018).

3.3.2.2.5 Immunohistochemistry (IHC)

The assay is conducted with antibodies against viral proteins, the specimens are obtained from the paraffin embedded samples of the ileum, in which the samples are cut, deparaffinized, rehydrated (xylene 5 min; ethanol 100%, 95%, 70%, 1 min each) and treated with 10 mmol/L sodium citrate buffer pH 6.0 at 90 °C for 20 minutes. In order to block the endogenous peroxidase activity, the samples are exposed to 10% hydrogen peroxide (H_2O_2) and treated with citrate buffer at pH 9 in order to retrieve the antigen. Afterwards, the tissues sections are incubated with a universal blocking solution and exposed to primary antibodies against viral proteins such as anti-$CD3^+$ mouse monoclonal antibody for an hour at 22 °C. Then, the tissues are washed with PBS and exposed to secondary antibodies such as (goat anti-mouse antibody) for 30 minutes. Consequently, the immune-complexes are visualized using Dako Envision+ System-HRP labeled Polymer Detection and 3,3’ diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate. The sections are counterstained, cover-slipped and observed via Leica DM4500B microscope. And as a negative control, employing either isotype matched antibody of inappropriate specificity or omitting the primary antibody (Brun et al., 2010; Brun et al., 2018).

3.3.2.2.6 Serological assay

Blood samples of 1 ml are withdrawn from sham, IN and IG HSV-1 infected mice, in which they are incubated for 1 hour at room temperature to allow for clotting. The serum is further

separated via centrifugation for 10 minutes at 2000 rpm and stored at 20 °C until utilization for the determination of the presence of anti-HSV-1 IgG via commercially available ELISA with the manufacturer's protocol followed throughout the operation (Brun et al., 2018).

3.3.2.3 Inflammatory reaction assessment

After the sacrifice, dissection, and isolation of the LMMP as mentioned previously, we are able to run several assays to evaluate the inflammatory response.

3.3.2.3.1 Mononuclear cells isolation

In order to isolate lymphocytes, the spleens are dissociated by forcing the tissue through a 100 µm metal mesh. The technique utilized to obtain lymphocytes and macrophages from the LMMP involves cutting freshly isolated ileum into small pieces and peeling off the LMMP. Afterwards, the LMMP is finely minced via scissors and dissociated through a 10-minute incubation at 30 °C with 10 mg/ml collagenase type II from *Clostridium histolyticum*, 60 µg/ml dispase, 10 µg/ml DNase I. The tissue debris is filtered through a cell strainer. The cells are collected (900 x g for 5 minutes) then purified by the Ficoll-Hypaque density gradient centrifuge and immediately stained for flow cytometry assay or otherwise cultured at 37 °C for 16 hours with or without UV-inactivated HSV-1 (Brun et al., 2018).

3.3.2.3.2 Flow-cytometry assay of immune cells

10^6/ml of macrophages and lymphocytes are freshly dissociated and incubated for 20 minutes in PBS that contains 2% w/v bovine serum albumin, furthermore, it is stained at 4 °C for 30 minutes with the proper antibodies against markers expressed in macrophages and lymphocytes to evaluate the inflammatory cellular infiltration, as for intracellular cytokine staining, the cells are subsequently incubated at room temperature for 30 minutes in a fixation/permeabilization buffer that contains the correlating antibody. The cells are then washed, and the fluorescence generated is recorded via BD FACSCanto Flow Cytometer. The results are analyzed through the software program WinMDI 2.9 which stands for "Windows Multiple Interface for Flow Cytometry". Afterwards, a comparison of the results to conclude the difference between the HSV-1 infected mice versus sham mice (Brun et al., 2018).

In order to detect free radicals intracellularly, 10^6/ml of macrophages freshly dissociated as mentioned previously are loaded at 37 °C for 30 minutes with 10 µM of 2',7' dichlorodihydrofluorescein diacetate (H_2DCFDA) for the detection of intracellular ROS (reactive oxygen species), also 5 µM 4,5 Diaminofluorescein Diacetate (DAF-FM) is utilized

to reveal intracellular NO (Nitric Oxide). At the incubations end, the cells are washed twice, stained with the correlating antibodies, and analyzed via flow cytometry (Brun et al., 2018).

3.3.2.3.3 ELISA and quantitative RT-PCR assay

LMMPs are homogenized in PBS 1:10 wt/vol that is supplemented with protease inhibitors (1 mmol/L Phenymethylsulfonyl fluoride, 10 µg/ml aprotinin and 10 µg/ml leupeptin) and centrifuged at 10,000 X g, 4 °C for 10 minutes. The clarified supernatant is evaluated for pro-inflammatory cytokines such as interleukin-2 IL-2, interferon-gamma IFN-γ, tumor necrosis factor-α, CCL2, CXCL9 and CXCL11 levels which is all quantified via quantitative RT-PCR and enzyme-linked immunosorbent assay (ELISA) using commercially available kits conducted following the manufacturer's protocol. Optical densities are registered at 450nm via a microplate reader. The assay's sensitivity is 15 pg/ml. The data is normalized through total protein content measured using bicinchoninic acid method (Brun et al., 2010; Brun et al., 2018).

Oligonucleotides	Sequences	Tm °C
Cxcl11	Fw 5′-cagctgctcaaggcttcctta-3′ Rv 5′-ctttgtcgcagccgttactc-3′	60
Ccl2	Fw 5′-gcctgctgttcacagttgc-3′ Rv 5′-caggtgagtggggcgtta-3′	60
Cxcl9	Fw 5′-tcggacttcactccaacacag-3′ Rv 5′-agggttcctcgaactccacac-3′	60
Ccr2	Fw 5′-acctgtaaatgccatgcaagt-3′ Rv 5'-tgtcttccatttcctttgatttg-3′	60

Table (6) PCR primers of inflammatory markers

3.3.3 Beta amyloid deposition and EGCs Assay

Beta amyloid is evaluated in the enteric specimens through immunohistochemistry, furthermore, evaluation, isolation, and culturing of enteric neurons.

3.3.3.1 Histological evaluation of β-amyloid

The intestine specimens are collected and immersed in 4% PFA for 24 hours, cryoprotected through two successive 30% sucrose changes. The samples are serially cryosectioned to 10µm, stained with H&E and Alcian blue stains. The images are taken with Leica Microscope and digital camera system (Puig et al., 2015).

3.3.3.2 Immunohistochemistry (IHC) of Aβ

Specimens are obtained from the gut sections serially with 10-mm thickness, mounted on gelatin coated slides and processed for indirect peroxidase-antiperoxidase immunohistochemistry. After blocking the endogenous peroxidase activity and non-specific labeling, the sections are incubated at 4 °C in a humid chamber overnight with mouse monoclonal antibody against recombinant APP_{695}, it recognizes an epitope of the N- terminus region of all APP isoforms and related molecules. Afterwards, washing twice with 0.1 M PBS at pH 7.6, then the sections are incubated with peroxidase-labeled sheep anti-mouse IgG at room temperature for an hour in a humid chamber. Finally, the sections are washed with PBS and treated with 0.05% 3,3' diamino-benzidine tetrahydrochloride (DAB)/hydrogen peroxide 0.01% for 5-10 minutes. All the antibodies employed are effective in formaldehyde-fixed paraffin embedded sections according to the manufacturer's notice. As for the sham, the sections are processed in the same fashion where antigen absorbed mouse sera is employed instead of primary antibodies (Cabal et al., 1995).

A recent and alternative approach involves obtaining fixed ileal specimens that are serially cryosectioned into 10 µm thickness and immunostaining the specimens with anti-AβPP, Aβ, CD68, COX-2, occludin, phospo-tau (PHF-1) and CD36. The samples are first rinsed in xylene 3 times for 5 minutes each, then rehydration follows with 2 absolute alcohol changes for 5 minutes, afterwards 95%, 80%, 70% alcohol for 5 minutes each, this is followed by rinsing in distilled water for 5 minutes in which the antigens are retrieved in boiling Tris-EDTA for 20 minutes at pH 9. The antibody binding is visualized via Vector VIP chromogen and the images are taken through Leica microscope digital camera system, then the figures are made via Adobe Photoshop software program (Puig et al., 2015).

3.3.3.3 Western blot of Aβ

The intestine specimens are collected, rinsed to remove lumen contents, flash frozen, pulverized and lysed through ice cold radioimmunoprecipitation assay "RIPA" buffer with protease inhibitors and DNase I to remove the insoluble matter, the cellular lysates are then sonicated and centrifuged for 10 minutes at 14,000 rpm and 4 °C. The Bradford method is employed for quantifying proteins. The proteins are resolved in SDS-PAGE and transferred to polyvinylidene difluoride membranes "PVDF" for western blotting via anti-AβPP, BACE, occludin, COX-2, CD68, CD36, and actin as the loading control antibody. The antibody binding is detected via chemiluminescence. Western blots are quantified through the Adobe Photoshop Software (Puig et al., 2015).

3.3.3.4 Enteric glial cell (EGCs) isolation and culturing

In order to isolate enteric glial cells, the LMMP cell suspensions are seeded onto 6-well culture plates, they are cultured in DMEM supplemented with 20% fetal bovine serum, 1mM sodium pyruvate, 1 µM forskolin, 10 µM bovine pituitary extract (BPE), 100 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin. The contaminating fibroblasts are removed by incubating primary cultures with anti-mouse Ty1.1 monoclonal antibody in the presence of complement. The purity of the EGCs is evaluated via positive immunoreactivity for glial fibrillar protein (GFAP) and the absence of Willebrand factor, Thy1.1 and alpha-SMA expression (Brun et al., 2015).

3.3.3.5 Flow cytometry of GFAP

The expression levels of GFAP in EGCs correlates to the functional state of EGCs hence, the measurement of GFAP levels in HSV-1 infected mice versus sham will be representative of differentiation, inflammation and injury therefore, the activated switched-on phenotype of EGCs (Ochoa-Cortes et al., 2016).

After counting EGCs, the intracellular GFAP staining is conducted by permeabilizing the cells in PBS that contains permeabilization buffer (5% FCS, 0.2% Triton X-100 and 0.5% glycine) at 4 °C for 20 minutes. The cells are incubated overnight at 4 °C with 0.1 ml of rabbit anti-human GFAP in the permeabilization buffer. Afterwards, the cells are washed with PBS 3 times and exposed to 0.1 ml of the secondary antibody Alexa Fluor 647 goat anti-rabbit in the permeabilization buffer for 45 minutes at room temperature, after washing the stained cells are suspended in the permeabilization buffer for FACS analysis (Cirillo et al., 2011). The GFAP results of infected specimens versus sham is then compared.

3.3.3.6 Immunocytochemistry (ICC) of S100B

Measurement of the S100B concentrations is conducted in both HSV-1 infected versus sham, in which S100B nanomolar concentrations regulate microenvironmental homeostasis whereas in micromolar it is correlated with a pathologic and inflammatory status (Ochoa-Cortes et al., 2016).

EGCs are fixed at room temperature for 30 minutes in 4% PFA. The cells are further processed for blocking non-specific binding sites with the blocking buffer (0.1 M PBS, Triton X and 4% goat serum) at room temperature for 2 hours. Then the cells are exposed to S100B primary antibody such as mouse monoclonal anti-S100B. After incubation, the cells are rinsed with PBS and incubated at room temperature for 1 hour with the secondary antibody Alexa Fluor 488 goat anti-mouse diluted in blocking buffer. The fluorescence is visualized via Nikon Eclipse E600 microscope (Cirillo et al., 2011). The S100B results of infected specimens versus sham is then compared.

3.3.4 Gut dysbiosis assay

3.3.4.1 Fecal pellet collection

Fecal pellets are collected to assay for gut dysbiosis, in which the collection of specimens from HSV-1 infected and sham mice is conducted. The fecal pellet samples are collected via fecal collection kits according to the manufacturer's protocol from each mouse and immediately stored at -80 °C until use (Brun et al., 2013; Raimondi et al., 2019).

3.3.4.2 DNA extraction

The DNA is extracted from fecal samples via FastDNA SPIN Kit employed for feces. In which the samples are suspended in water or isotonic saline solution, then centrifuged 14,000 x g for 5-10 minutes to obtain a sufficient volume. The samples are resuspended again in water or isotonic saline to yield a maximum suspension volume of 200 µl. According to the kit the proper cell lysis solution is employed to extract bacterial DNA. Afterwards, the samples are homogenized, centrifuged, then the supernatants are transferred to microcentrifuge tubes with an equal volume of binding matrix and inverted to mix, incubated with gentle agitation at room temperature for 5 minutes on a rotator. Then the samples are centrifuged 14,000 x g for 10 seconds and the supernatant is discarded. Then the addition of SEWS-M (in the kit) and the samples are gently resuspended, transferred to the SPIN filter and centrifuged 14,000 x g for 1 minute, the contents in the catch tube are discarded, replaced and the samples are centrifuged

with a new catch tube same protocol of 14,000 x g for 1 minute. The DNA is eluted through gently resuspending the samples above the SPIN filter, then incubated at 55 °C for 5 minutes in a heat block or water bath. Finally, the samples are centrifuged 14,000 x g for 1 minute to bring the eluted DNA into the clean catch tube. The DNA was also extracted from a subset of identical samples via QIAamp DNA Stool Mini Kit according to the manufacturer's protocol. The DNA quality is checked on 1% agarose gel TAE 1X and quantified with a NanoDrop spectrophoto-meter (Raimondi et al., 2019).

3.3.4.3 PCR and 16S rRNA analysis

A problem persists that the bacterial DNA obtained is both from the fecal samples as well as from the host's intestinal epithelial shedding. Therefore, in order to confirm the bacterial DNA, we need to amplify the samples DNA by PCR using 16S degenerated oligonucleotides (Fw 5'-ggaggcagcagtggggaata-3', Rv 5'-tgacgggcggtgtgtacaag-3' with Tm of 54 °C) and analyze the DNA sequences which is done via an external company such as Qiagen (Brun et al., 2013).

The PCR products are electrophoresed, cleaned via AMPure XP bead kits and quantified by quantitative PCR utilizing 454 Kapa Library Quantification Kit (Raimondi et al., 2019). Afterwards, the samples are sent to Qiagen for DNA sequencing.

3.3.5 Statistics

The results are provided as a mean ± "SEM" standard error of the mean and the statistical differences are evaluated by one-way ANOVA analysis followed by Bonferroni multicomparison *post-hoc* tests. The *P*-value of 0.05 or less is considered statistically significant. The statistical analysis is performed via GraphPad Prism 3.03 Software program (Brun et al., 2018).

3.3.6 Budget and Gantt tables

Based on the availability of most of the materials and equipment in Padova University's labs, accordingly, only the consumables that are lacking were estimated and the rough estimation of the project's expenses is provided in the table next page. The budget is reached after careful consideration of the variety of experimental aspects and price variability.

Experimental Expenses	
Consumables (Animals, kits, antibodies, single use lab ware, culture media and supplements etc..)	25,000€
Equipment	0
Computers	0
External services (Illumina platform analysis, DNA sequencing etc.)	20,000€
Travel expenses	0
Patents	0
Conferences and seminars	0
Others	0
Overheads	4,500€
Total	49,500€
Budget Requested: 49,500€	

Table (7) Experimental budget

Gantt table															
Weeks **Thesis Project**	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29
Viral Stocks Preparation															
Animal Infection and Incubation															
Infection Assessment															
Evaluation of Inflammatory Reaction															
Beta Amyloid and EGCs Assay															
Gut Dysbiosis Assay															
Statistics and Data interpretation															
Thesis Submission															

Table (8) Gantt thesis project timeline

Chapter 4

Results and discussion

4.1 HSV-1 infection assessment

HSV-1 is detectable in the brain of IN infected rats, HSV-1 tk DNA is detected in the brain and isolated at 1,2,4,6, and 10 weeks after IG HSV-1 administration. HSV-1 tk DNA is progressively reduced in the myenteric ganglia during the experimental period even-though it is detectable 10 weeks after. In the following figure as visible, HSV-1 tk DNA levels exhibit a transient increase in the brain at the second week. Contrastingly, it is undetectable in the nasal mucosa, lung, liver, spleen, and skeletal muscle of infected mice. After the exposure to HSV-1, it can either initiate an acute virulent infection via early and late viral genes expression or establishing a latent infection in the neurons via the generation of noncoding viral transcripts that are referred to as LATs. The nature of the infection with HSV-1 is established by real-time PCR analysis, where HSV-1 LAT specific transcripts are detected in the mice brains after IN inoculation as well as in the brains and enteric nervous system of up to 10 weeks post IG administration. Early genes ICP4 and tk are detected in the MG only 1 and 2 weeks after IG inoculation whereas they are undetectable in the latter samples. The previous studies by Brun and colleagues report lack of late genes such as gD and VP16 which is correlated with the absence of structural virion proteins that result in abortive viral replication without the production of infectious viral progeny. Additionally, the anti-HSV-1 gD antibody is negative at all time points which supports the abortive viral replication consensus as shown in the following figure where RNA was purified from the (1) brain, (2) MG, (3) nasal mucosa and (4) liver while C+ represents a positive control, which is displayed after IN or IN and IG 1,2,4,6 and 10 weeks administration. As a verification procedure from previous literature, HSV-1 DNA can be detected in freshly isolated MG and is indeed confirmed to be present in the ENS via in situ X-gal staining which is performed on ileal whole mount preparations according to Brun and colleagues. HSV-1 is infective in the myenteric ganglia as latent HSV-1 in neurons can be reactivated at a later time, in which co-incubation of brain and ileal samples of sham and HSV-1 infected animals with Vero cells confirmed the presence of HSV-1 in Vero cells via PCR analysis (Brun et al., 2010).

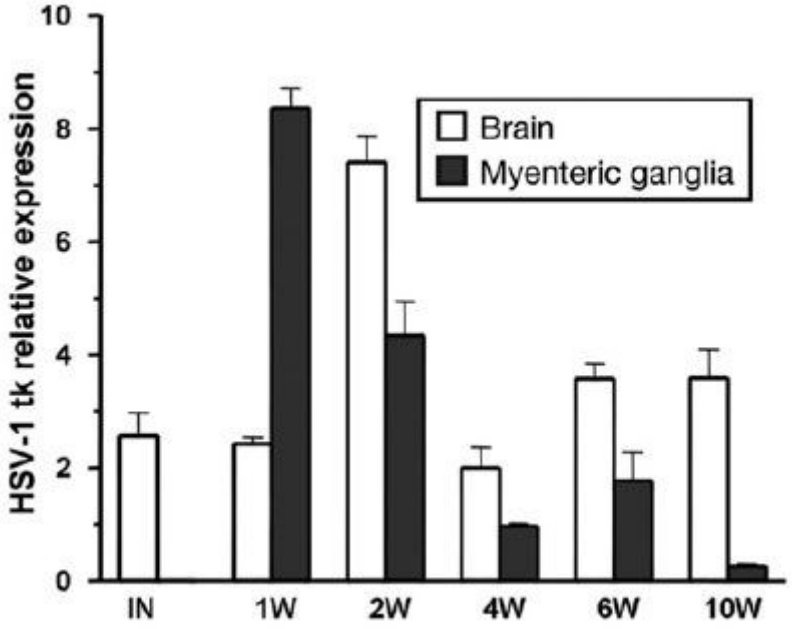


Figure (27) HSV-1 tk levels in the brain and MG via qPCR

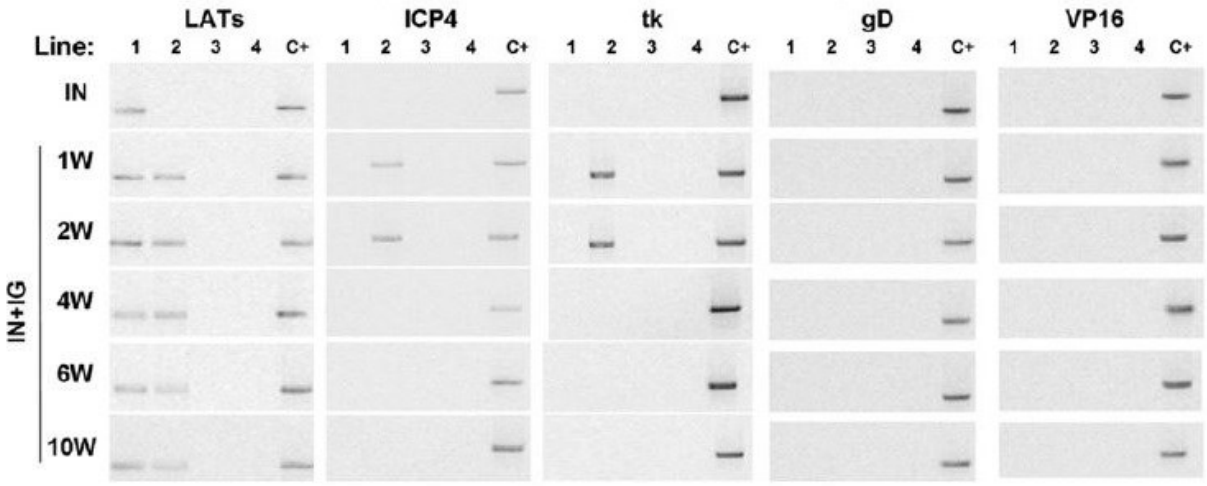


Figure (28) HSV-1 LATs, ICP4, tk. gD and VP16 via semi-qPCR

As mentioned in previous studies of Brun and colleagues (2010), it is reported that HSV-1 establishes a latent infection in the MG of rats. It is confirmed that following IN inoculation of HSV-1, it has been observed that latency-associated and early viral gene transcripts in ileal neurons with no expression of late viral gene thus, the HSV-1 abortive replication consensus is reached. Although there is ease in studying rat neurons, yet there are many limitations including species-specific reagents etc. The utilization of mice as animal models of HSV-1 infection of the ENS is justified under the following umbrella to reduce difficulties in conducting experiments. Brun and colleagues in 2018 reported the ability of HSV-1 to retain infectivity following intragastrical inoculation. Indeed, mice were inoculated with HSV-1 SC16 strain (IG) or an equal volume of Vero cell lysate (sham infection) and ileum content was recovered, vigorously mixed and centrifuged. In which the supernatants are incubated on Vero cell monolayers. As observed in the following figure, the contents of the ileum are collected 15- and 45-minutes post IG HSV-1 SC16 (1 x 10^7 PFU), intestinal contents are co-cultured on Vero cells and plaques are counted, the data is represented as a mean ± SEM of 3 separate experiments. The viral inoculation shows 1% and 10% of injected PFU via a classical titration assay (Brun et al., 2018).

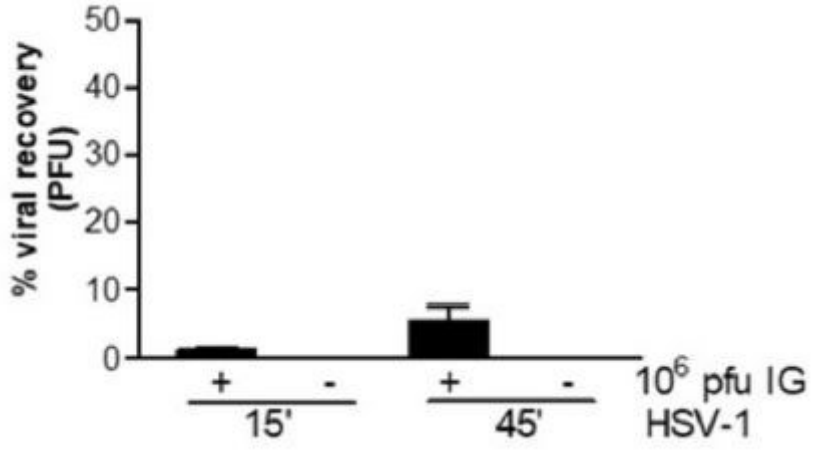


Figure (29) HSV-1 viral recovery from ileal specimens

The presence of HSV-1 I Vero cells is confirmed via semi-qPCR assay. The samples are obtained from mice that are subjected to sham infection in which they did not produce cytopathic effects on Vero cells, as visible in the following figure which confirms the presence of HSV-1 in IG infected animals versus sham group. The figure represents the total DNA that is isolated from Vero cells that are exposed to ileal contents from IG HSV-1 infected and sham

up to 72 hours, in which the DNA is subjected to PCR using the tk HSV-1 specific primers (Brun et al., 2018).

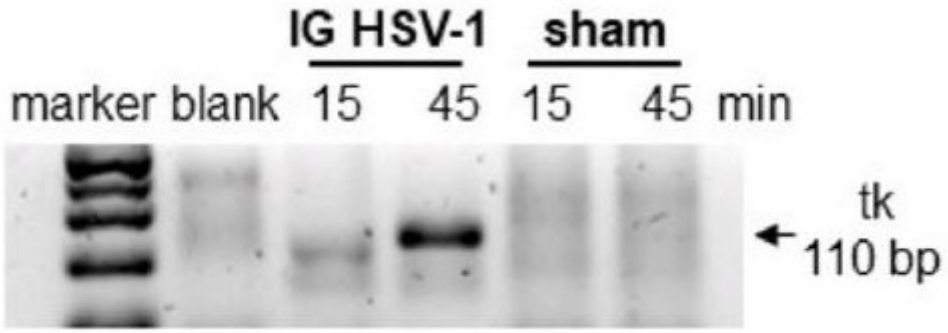


Figure (30) HSV-1 total DNA via PCR using tk primers

According to Brun and colleagues, they were able to demonstrate HSV-1 SC16 strain's ability to infect murine enteric neurons, as they cultured primary murine enteric neurons infected with HSV-1 SC16 in which the cells were fixed for 36 hours and later stained with anti-ICP-27 antibody, where it is evident that the immunoreactivity in beta III tubulin is positive for enteric neurons and therefore, confirming the ability of HSV-1 to infect and replicate in mice enteric neurons as visible in the following figure comparing HSV-1 IG infected vs sham. The specimens are visualized via confocal microscopy (Brun et al., 2018).

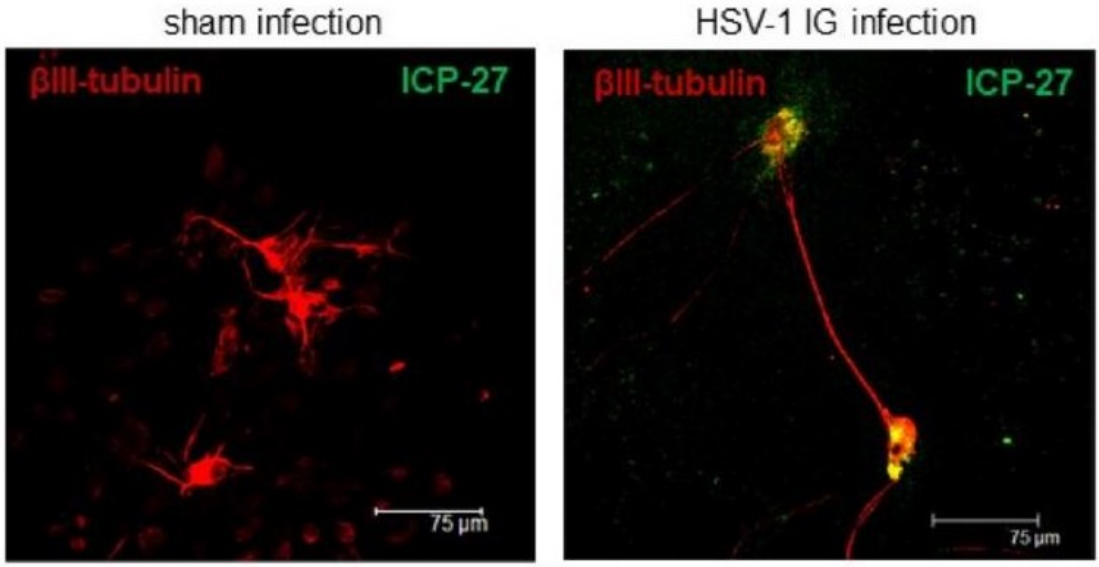


Figure (31) Enteric neurons probed with anti-β-III tubulin and anti-ICP-27

In order to determine the nature of HSV-1 infection in the myenteric plexus, the total RNA isolated from the LMMP preparations of IG infected mice. HSV-1 LATs and the early gene's ICP4 mRNA transcripts are detected in variable extents in the LMMP after IG administered animals at 1,2 and 3 weeks however, they are undetectable in mice inoculated with Vero cell lysate as in sham mice. HSV-1 tk mRNA is evident in the LMMP at only 2- and 3-weeks post IG inoculation as visible in the following figure. Expression of late genes (VP16, gB and gC) are not observed therefore, the conclusion is that HSV-1 persistently infects the mice myenteric plexus and carries an abortive viral replication similar to the data obtained in Brun and colleagues' study in 2010. The following figure shows the total RNA purified from the LMMP of LATs and early genes ICP4, tk that are evaluated via semi-quantitative PCR. The sham mice are IG inoculated with Vero cell lysate as mentioned previously while the Rn18S serves as an extraction and loading control. (Brun et al., 2018).

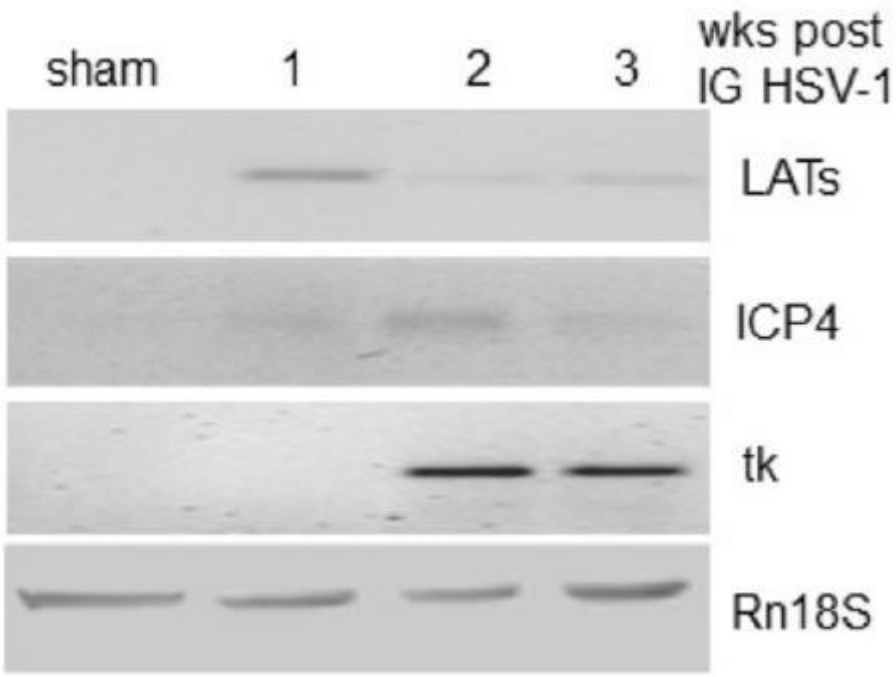


Figure (32) HSV-1 LATs, ICP4 and tk total RNA from the LMMP via semi-qPCR

4.2 HSV-1 inflammatory reaction assessment

4.2.1 Adaptive immune system assessment

The histological examination of HSV-1 infected mice shows conserved architecture in the small intestine, the following figure shows 1,2- and 3-weeks post IG infection with HSV-1 SC16 strain versus sham. The distal ileum is removed and fixed in neutral buffer formalin, then embedded in paraffin, and subjected to H&E staining. The left panels scale bars are 40µm while the right panels are 20 µm (Brun et al., 2018).

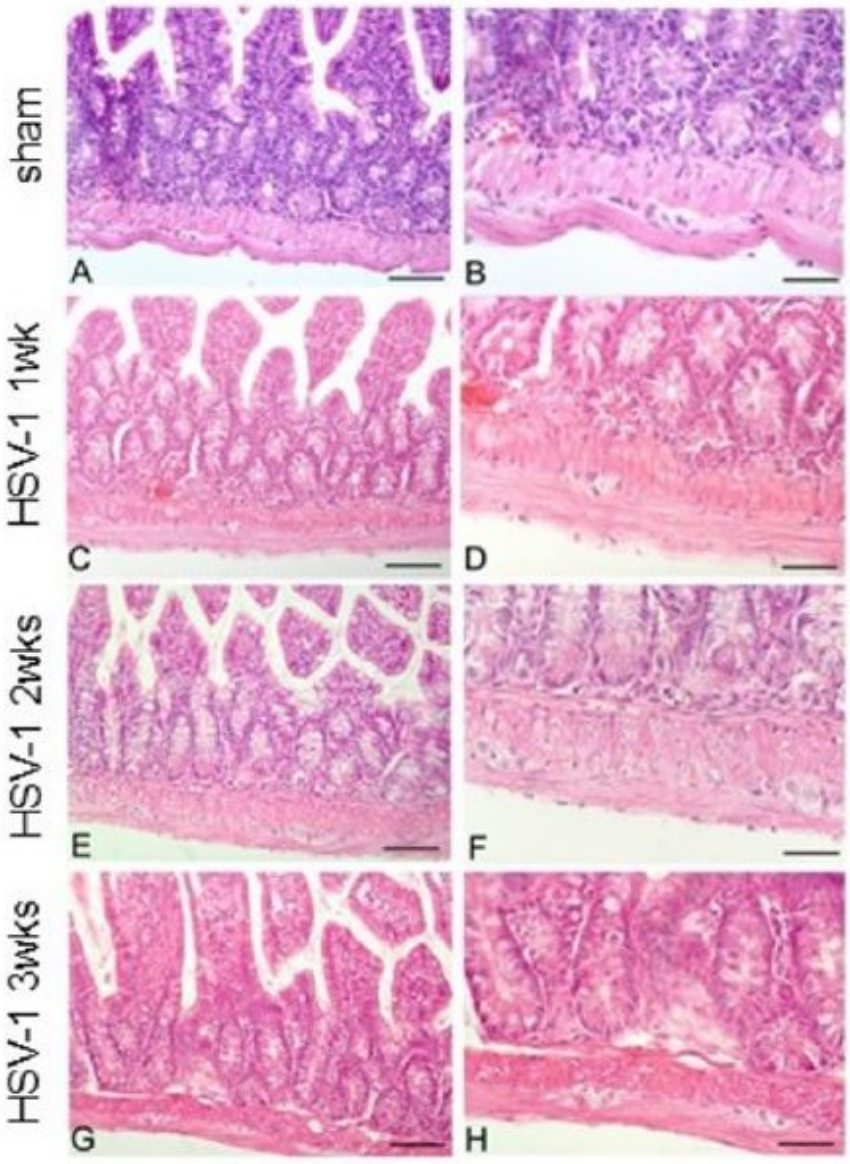


Figure (33) Distal ileum of infected versus sham mice (H&E)

The following figure represents ileal sections obtained from HSV-1 SC16 infected mice (1,2 and 3 weeks) versus sham which are subjected to immunohistochemical assay for CD3, in which the scale bars are 40 µm, the immunohistochemical assay shows evident elevated CD3 in infected mice in comparison to sham and an exponential elevation as a unit of time (Brun et al., 2018).

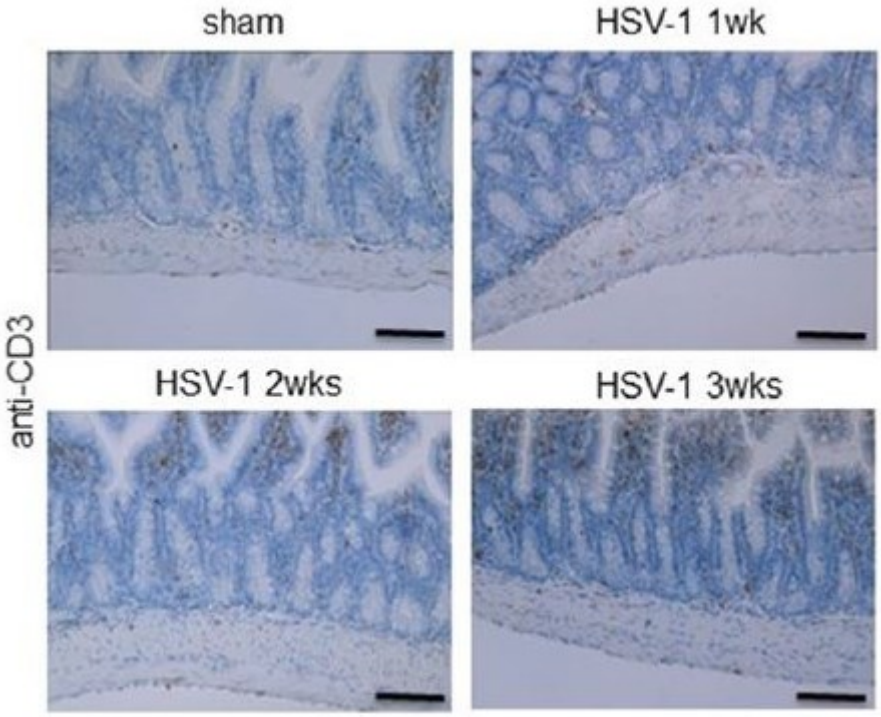


Figure (34) Ileal sections of infected versus sham by immunohistochemistry for CD3

Four weeks post HSV-1 inoculation, the circulating IgG and HSV-1 reactive spleen $CD3^+$ lymphocytes are evident. In the LMMP the distribution and percentage of $CD3^+$ cells of infected mice to sham seem to be increasing as unit of time throughout up until the second week, where it slightly decreases by the third week conversely, the ratio of $CD3^+$ $CD4^+$: $CD3^+$ $CD8^+$ initiates a slightly weaker signal in comparison to sham in the first week, followed by an exponential growth as a unit of time throughout the three weeks, as visible in the following figure of freshly collected LMMP that is digested and the yielded cell suspensions are labeled with anti-CD3 antibody and analyzed by flow cytometry, where $CD3^+$ cells are expressed as the percentage of 10^5 collected events (Brun et al., 2018).

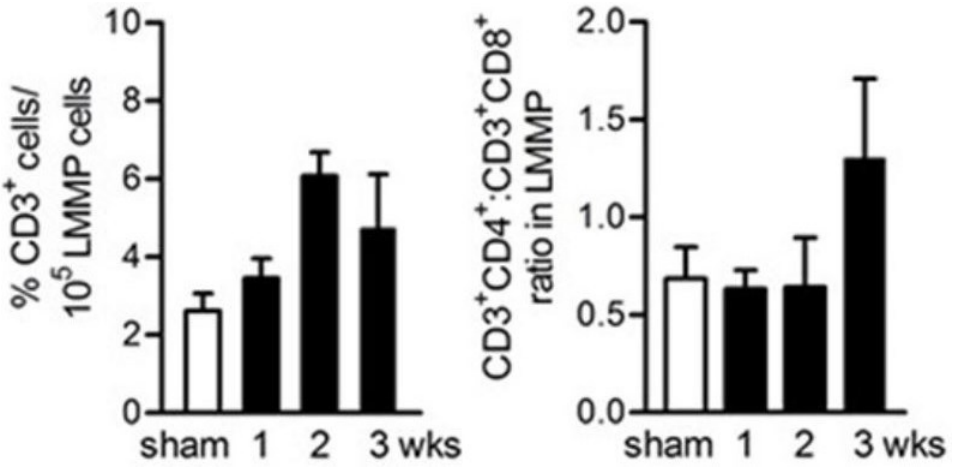


Figure (35) LMMP cell suspensions of immune cells via flow cytometry

Further flow cytometry assays of cell suspensions obtained from LMMP which were incubated for 16 hours in presence and absence of UV-inactivated HSV-1 SC16 strain, in which the cells are collected and labeled with anti-CD3, anti-CD8 and anti-IFNγ. The antibodies are analyzed by flow cytometry in 5 x 10^4 collected events. The following figure shows pulsed versus non pulsed of HSV-1 infected and sham mice, where there is a marked increase in the pulsed section in comparison to non-pulsed in sham, while in infected specimens the non-pulsed overshadows the pulsed across all weeks, nevertheless there is fluctuation or variability in the infected pulsed signals over time. The non-pulsed section also exhibits an increase over time (Brun et al., 2018).

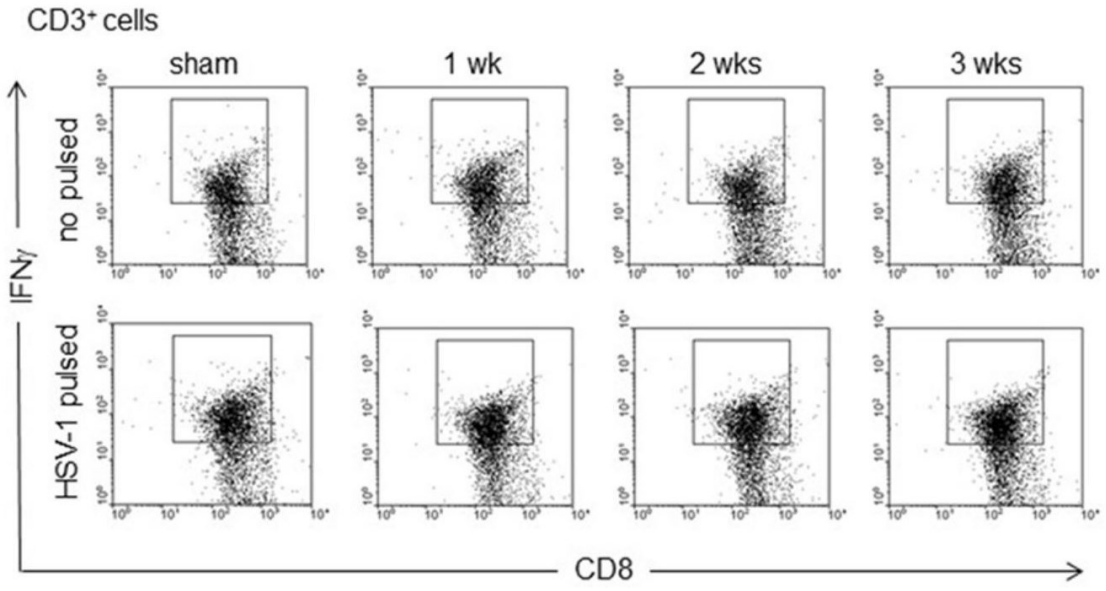


Figure (36) LMMP (anti-CD3, anti-CD8 and anti-IFNγ) cell suspensions via flow cytometry

The following figure represents flow cytometry of LMMP cell suspensions that are incubated for 16 hours in presence and absence of UV-inactivated HSV-1 SC16 strain, same protocol as previously mentioned, in which the cells are collected and labeled with anti-CD3, anti-CD8 and anti-IL4. The figure shows a slight decline in the signal across the pulsed section when comparing sham with infected specimens while the infected may show an increasing signal especially after the second week, it also overshadows the non-pulsed section from all across the sham and infected mice. Meanwhile, in the non-pulsed section the results look the same across all the specimens, over time as well (Brun et al., 2018).

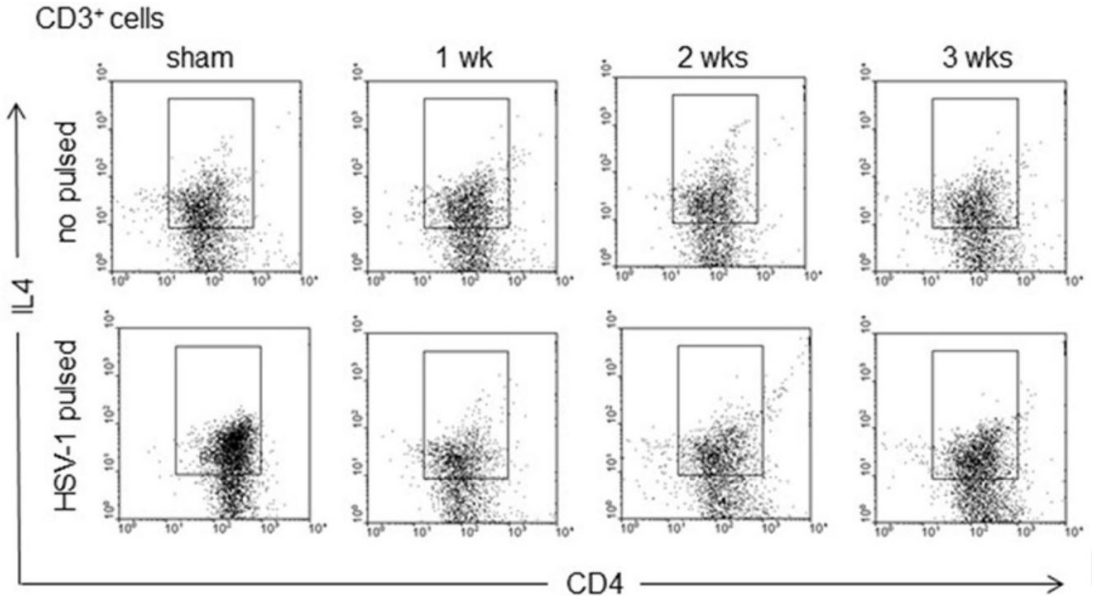


Figure (37) LMMP (anti-CD3, anti-CD8 and anti-IL4) cell suspensions via flow cytometry

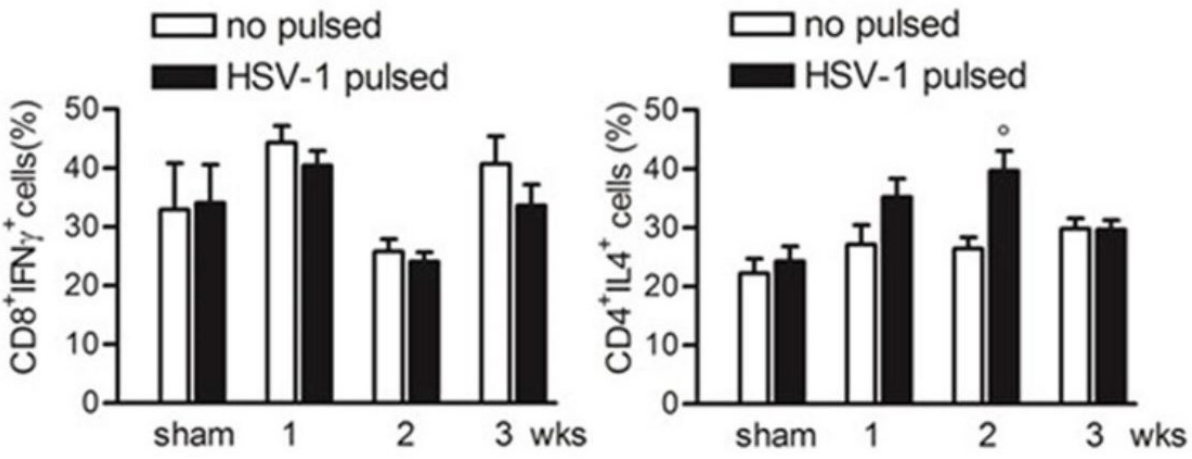


Figure (38) Percentages of fluorescence reported in the two previous figures

4.2.2 Innate immune system assessment

The innate immunity as cells that control the early phases of infection are studied by flow cytometry during HSV-1 infection on cells that are isolated from the LMMP and characterized as $CD11b^{+}F4/80^{+}$ cells. The cells are barely detectable in the LMMP of sham mice. While in the SC16 HSV-1 IG inoculated specimens show a significant increase in the first week in $CD11b^{+}F4/80^{+}$ macrophages as observable in the two following figures of freshly collected LMMP that is digested yielding cell suspensions that are labeled with anti- CD11b and anti-F4/80 antibodies and analyzed via flow cytometry (Brun et al., 2018).

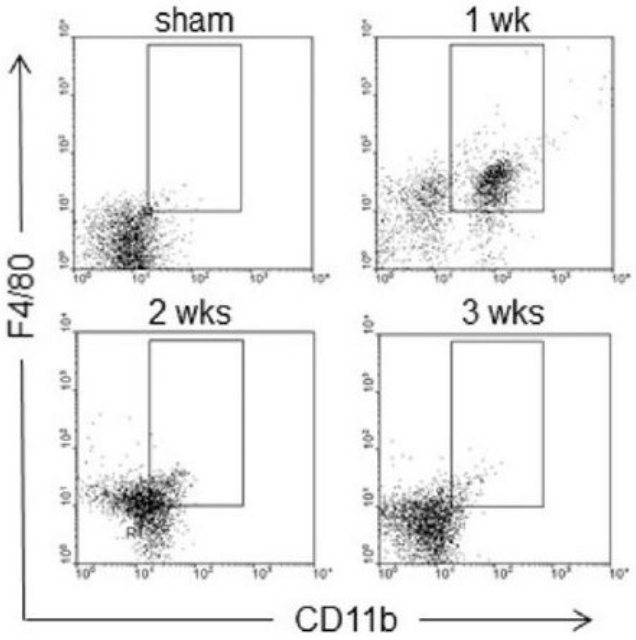


Figure (39) $CD11b^{+}F4/80^{+}$ macrophages via flow cytometry

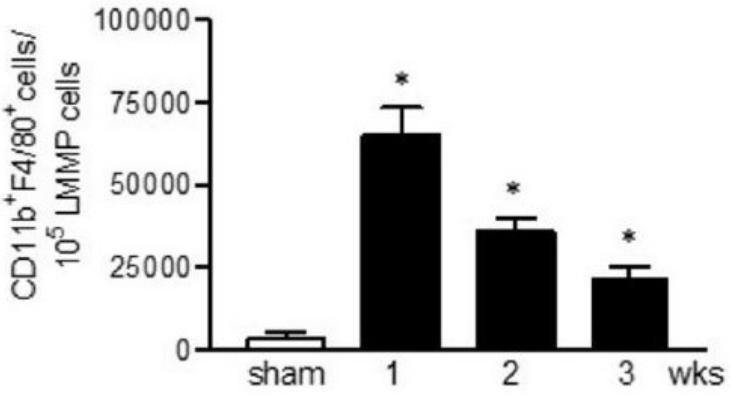


Figure (40) $CD11b^{+}F4/80^{+}$ macrophages via flow cytometry

The immunohistochemical assay of ileal sections show a contiguous pattern of $CD11b^{+}$ cells in the myenteric ganglia after IG viral inoculation as visible in the two following figures (Brun et al., 2018).

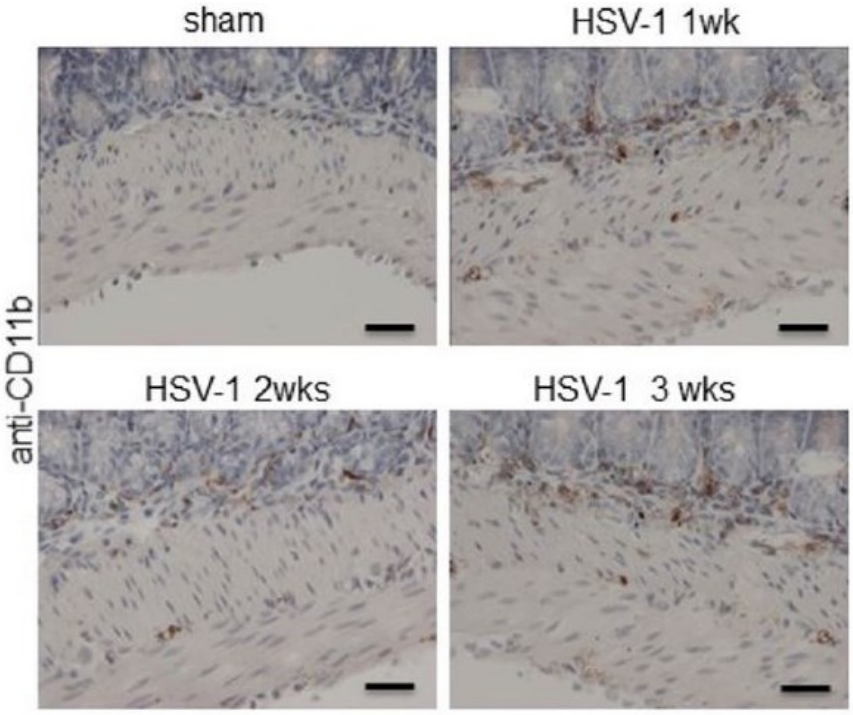


Figure (41) Ileal sections of CD11b by immunohistochemistry

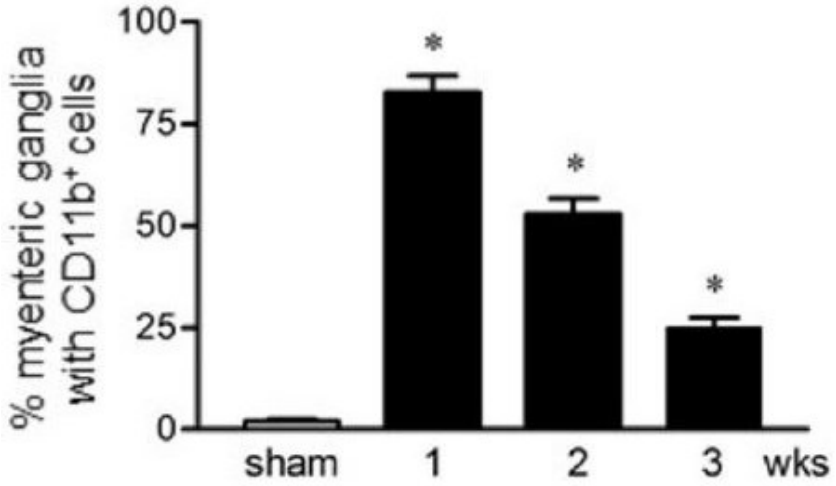


Figure (42) Percentages of myenteric ganglia with CD11b cells from the ileal sections (previous figure)

The $CD11b^+F4/80^+$ macrophages that infiltrated the LMMP of HSV-1 infected mice exhibited an activated phenotype as they produced free radicals, two of which are the reactive oxygen species (ROS) and nitric oxide (NO), as visible in the following figure of cells obtained from LMMP and are incubated for 30 minutes at 37 °C with H_2DCFDA (probe) which detects ROS or DAF-FM (probe) which detects NO. The cells are labeled with anti-CD11b and anti-F4/80 antibodies. The mean fluorescent intensity (MFI) is detected by flow cytometry. ROS fluorescence shows a significantly high result when compared to sham while being highest in the second week. NO exhibits similar overall results to ROS (Brun et al., 2018)

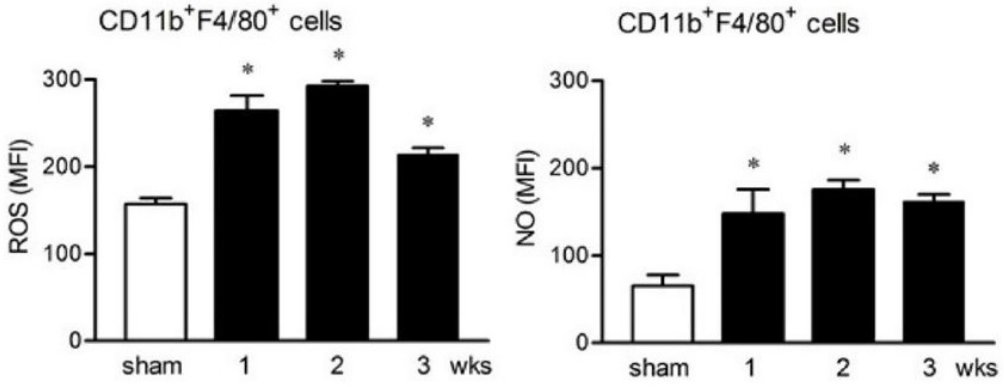


Figure (43) ROS and NO mean fluorescent intensity via flow cytometry

The monocyte chemoattractant protein 1 (MCP-1/CCL2) and its corresponding receptor CCR2 are involved in macrophage dependent tissue damage. Therefore, the investigation of CCL2/CCR2 is necessary to map the pathway of inflammatory insults to the ENS. CCL2 are found to be in levels that are significantly increased in the LMMP post HSV-1 IG inoculation which is determined via ELISA and qPCR as visible in the following figure (Brun et al., 2018).

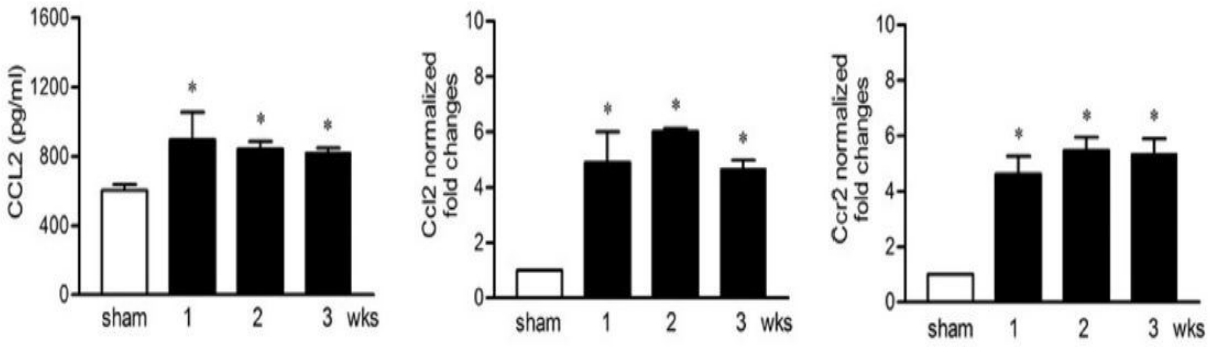


Figure (44) CCL2 levels by ELISA, CCL2 and Ccr2 mRNA by qPCR respectively

As visible in the previous figure the CCL2 levels detected by ELISA show an induction in HSV-1 infection priming in the first week followed by contiguous pattern per time. While that of CCL2 and Ccr2 mRNA levels detected by quantitative RT-PCR are induced by HSV-1 infection. In which CCL2 mRNA levels prime at the second week with obvious significant induction, meanwhile Ccr2 mRNA levels also peak in the second week and remain similar across the third week. The immunohistochemical assay on ileal sections shows CCL2 positive cells that are located only in the mucosa of sham mice whereas one-week post HSV-1 infection show CCL2 positive cells detected in the myenteric ganglia, as visible in the following figure. (Brun et al., 2018).

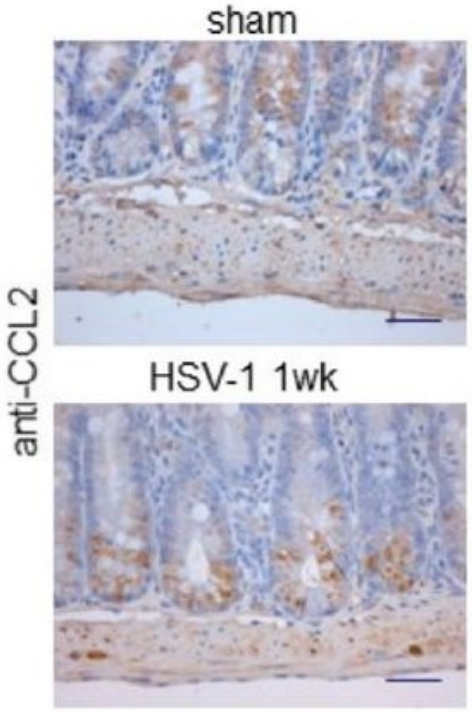


Figure (45) Immunohistochemistry on Ileal sections for CCL2

According to Brun and colleagues in 2018, exposure of enteric neurons for 16 hours to HSV-1 antigens resulted in increased levels of CCL2 via immunostaining, therefore the utilization of CCL2 for assay is important for mapping the inflammatory response against HSV-1. The following results indicate the CCL2 implication in the recruitment and activation of macrophages and reactive species as the apparent cause of ENS alterations, intestinal dysmotility1 (Brun et al., 2018).

ELISA assay of HSV-1 infected animals displays significantly elevated levels of IL2, TNFα and IFNγ in the LMMP at weeks 1 and 6, the increased levels exhibited highlight the immune response and inflammatory reaction against HSV-1 (Brun et al., 2010).

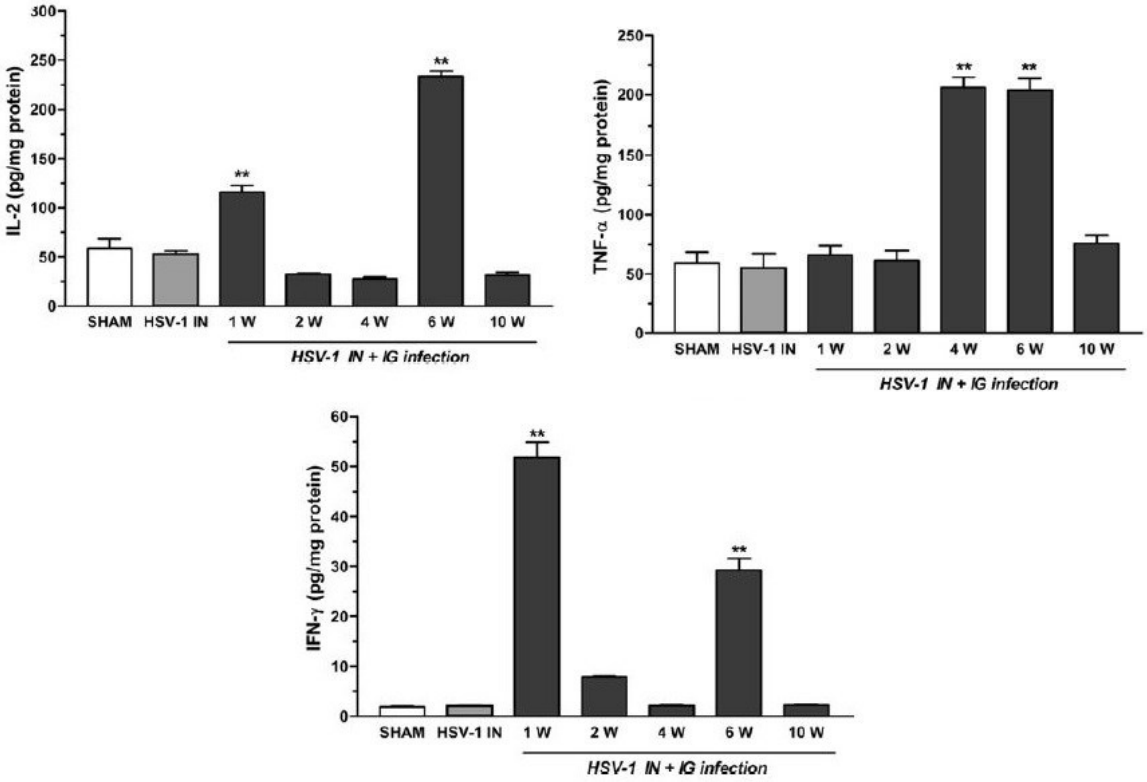


Figure (46) Inflammatory markers (IL2, TNFα and IFNγ) by ELISA

4.3 Beta amyloid deposition and EGCs assessment

4.3.1 Expected beta amyloid results (Simulations)

Histological intestinal specimens of HSV-1 SC16 infected mice are expected to show altered AβPP in comparison with sham infected animals. To demonstrate the hypothesis, the ileal samples of sham and HSV-1 SC16 infected mice are fixed in 4% PFA, serially sectioned at 10μm, and stained with the routine stain H&E and Alcian blue staining the β amyloid deposits. As visible in the following figure, there is an apparent histopathological nature when comparing sham on the left to HSV-1 SC16 infected mice on the right, in which the HSV-1 SC16 is simulated by mice induced with Alzheimer's to characterize the expected result (Puig et al., 2015).

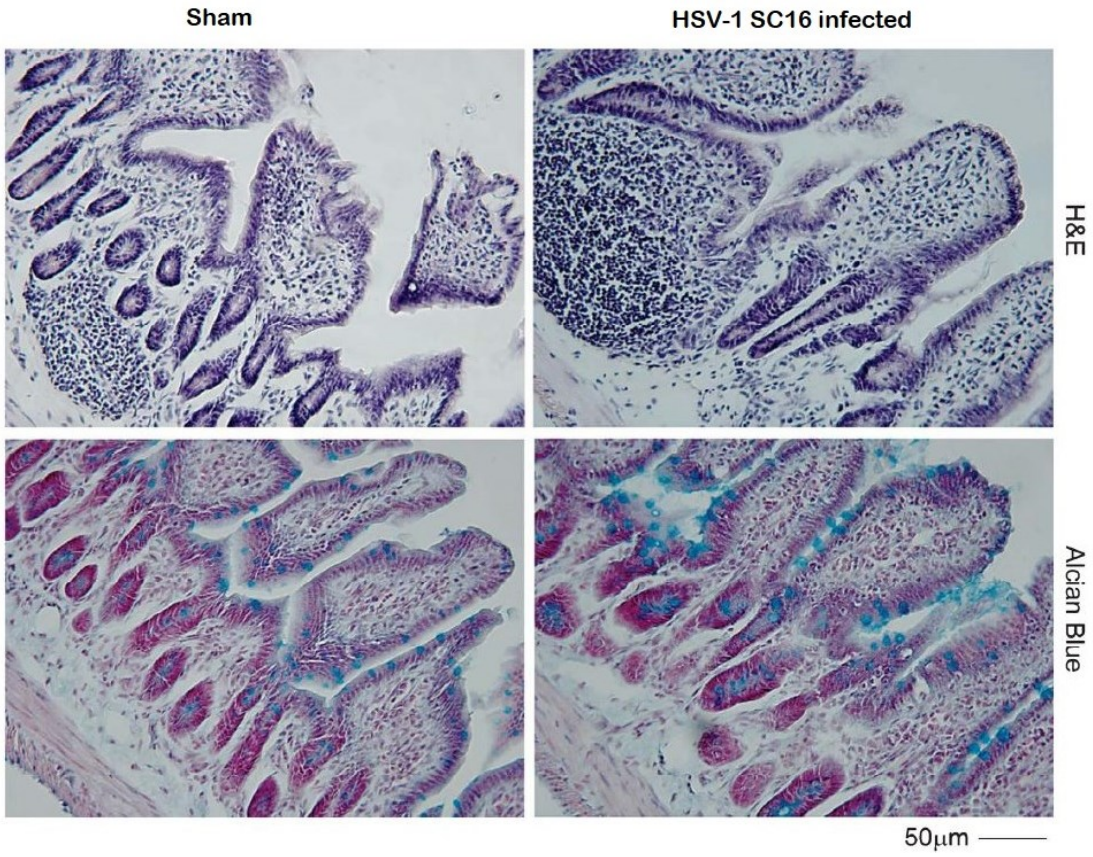


Figure (47) Sham versus HSV-1 SC16 infected histological simulation

The immunodetection of beta amyloid precursor protein in HSV-1 SC16 infected mice via mouse monoclonal antibody in the jejune and ileum is expected, in which the proteins with molecular masses of roughly 97-115 kDa correspond to the full length of the membrane bound beta amyloid precursor protein isoforms. As observed in the following figure the

molecular mass standards are indicated in kDa, where lane 1 is the jejune while 2 is the ileum (Cabal et al., 1995).

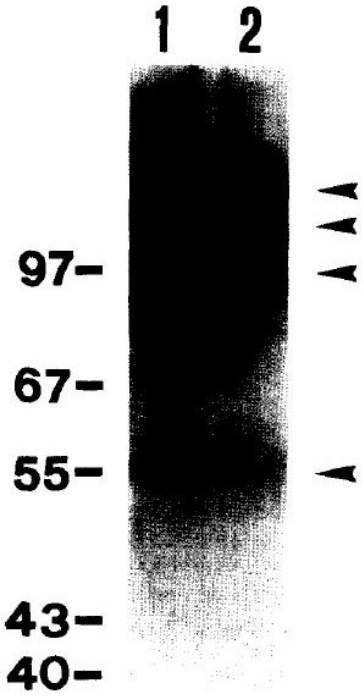


Figure (48) AβPP immunodetection in the jejune and ileum

A western blot can also be performed to confirm the detection of AβPP in HSV-1 SC16 infected mice versus sham, where a significantly increased level of AβPP is expected in specimens of HSV-1 SC16 when compared to sham. After that the antibody binding is visualized, we are able to normalize the data and quantify it in the form of a plot that represents sham versus HSV-1 SC16 infected mice, the following figure is an example from simulations of sham versus mice induced with Alzheimer's (Puig et al., 2015).

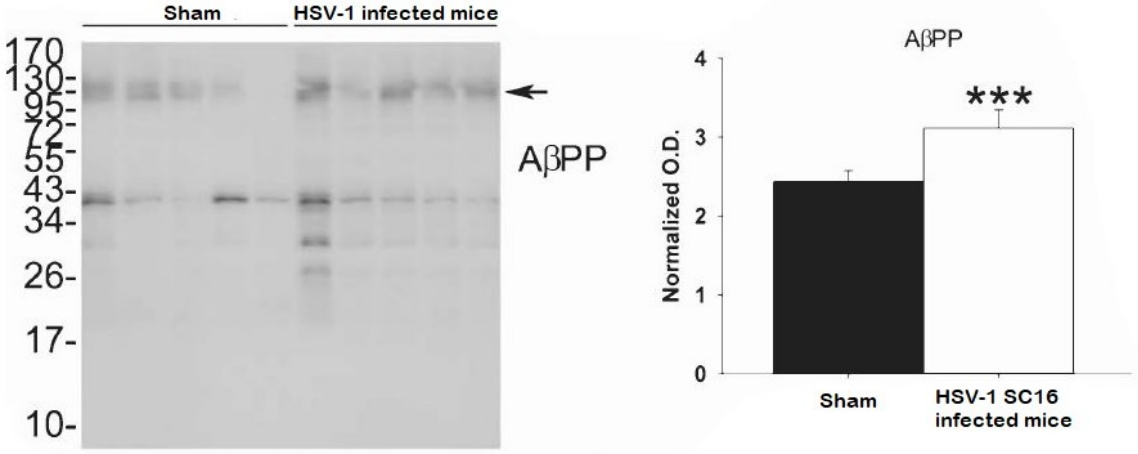


Figure (49) AβPP immunodetection and quantification

Based on previous studies by Piacentini and colleagues in 2011, 2014 and 2015, we are able to visualize the beta amyloid intracellular signals in the CNS and clarify that HSV-1 induces intracellular accumulation of AβPP fragments. In the following figure on the right it shows intracellular accumulation of beta amyloid 42 in HSV-1 infected neurons, the visualization is performed through confocal microscopy (Piacentini et al., 2014).

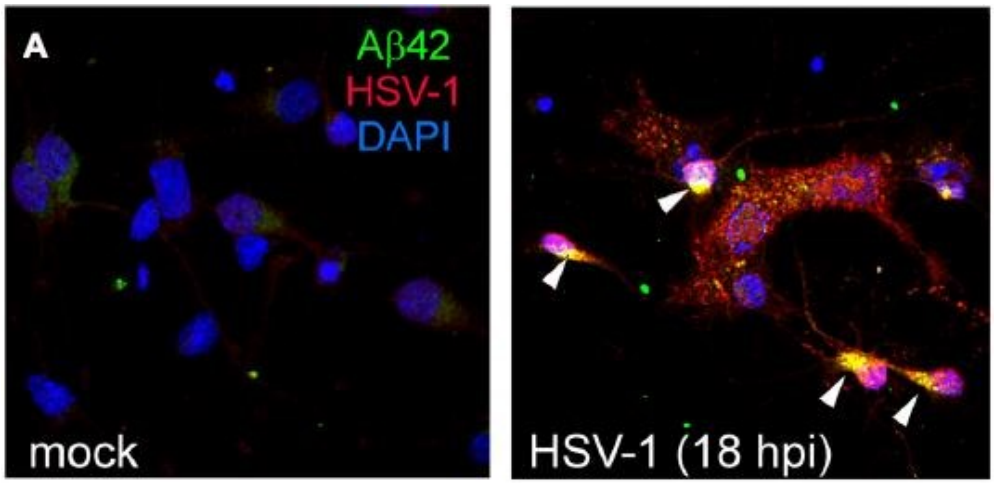


Figure (50) Aβ visualization in HSV-1 infected neurons via immunocytochemistry

The previous figure is an example of the detection process that is employed for the detection and visualization of beta amyloid, which is expected in the HSV-1 mice model that is utilized for this experiment. The literature provides a promising optimistic view on the detection of beta amyloid. Moreover, there is a study by Puig and colleagues in 2015, that confirms the presence of elevated beta amyloid in the intestine and fecal pellets of mice with Alzheimer's when compared with wild type, which further clarifies the potential possibilities of beta amyloid detection in HSV-1 SC16 mice model (Puig et al., 2015).

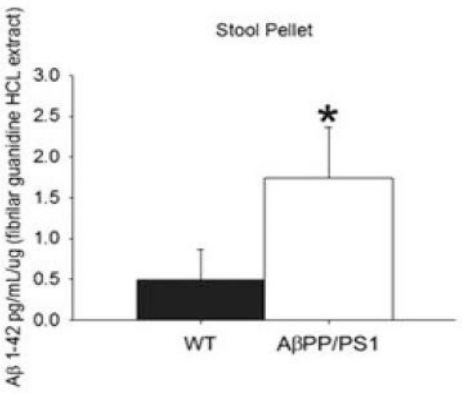


Figure (51) Aβ in stool pellets

4.3.2 EGCs assessment

4.3.2.1 GFAP signal (Simulation)

As mentioned previously in the materials and methods section, the assessment of EGCs for GFAP is an important indicator of activated as in switched-on phenotype of EGCs differentiation, inflammation and injury (Ochoa-Cortes et al., 2016). The GFAP signals are expected to be intense in EGCs of HSV-1 infected mice in comparison to sham. As witnessed in literature the markers of inflammation are present and in high levels which provides support for the expectations. The following figure is an example of the detection and quantification of GFAP in EGCs (Cirillo et al., 2011).

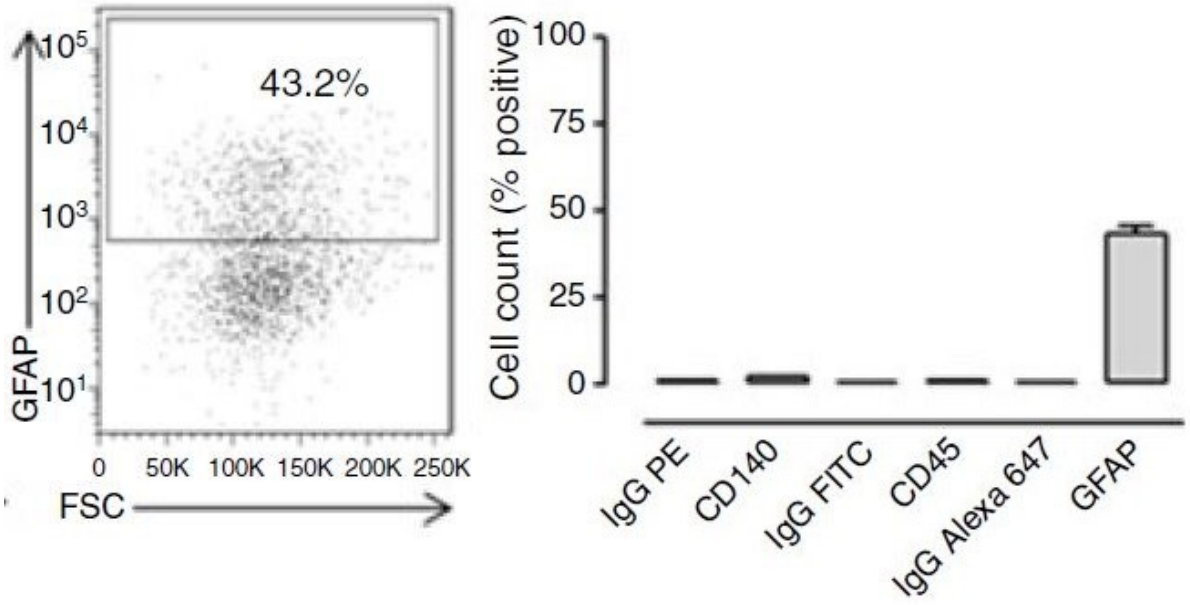


Figure (52) GFAP detection and quantification in EGCs via FACS

4.3.2.2 S100B detection

The immunoreactivity of the glial marker S100B, being a calcium modulated protein implicated in extra and intracellular regulatory activities in EGCs as well as a marker of pathological and inflammatory status, is found to be increased mainly at the second and third week after IG HSV-1 inoculation thus, indicating the activation of enteric glial cells to the switched on phenotype, as visible in the following figure, the distal ileum samples are fixed to obtain whole mount preparation and assayed via immunofluorescence (Brun et al., 2018).

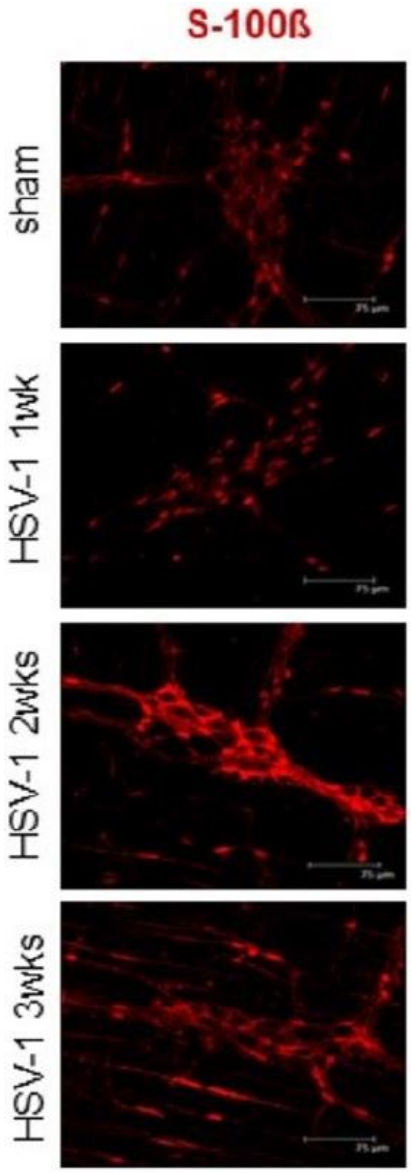


Figure (53) S100B detection via immunofluorescence

Mice post HSV-1 SC16 IG inoculation show the enteric nervous system underwent a variety of plastic changes with apparent S100B expression (Brun et al., 2018).

Based on previous studies, the EGCs are activated by pro-inflammatory cytokines and stimuli in that sense of significant presence and detection of elevated inflammatory mediators, cytokines, beta amyloid deposition and markers, with HSV-1 being the stimuli in this study. Therefore, the EGCs are bound to generate inflammatory mediators themselves with detectable elevated levels of GFAP & S100B. The EGCs are able to switch to the activated phenotype referred to as microgliosis and astrogliosis that precipitate the cascade of ENS insults with alterations generating gastric dysmotility, inflammatory anomalies and beta amyloid deposition. The inflammatory anomalies that are brought about are correlated with and observed in IBD patients (Cirillo et al., 2011; Brun et al., 2018).

4.4 Gut dysbiosis assessment

4.4.1 Post antibiotic treatment (Simulation)

Treating the mice with the antibiotics mentioned in the materials and methods section, yields a reduction in gut microbiota that is necessary for detrimental assay of etiological factors behind gut dysbiosis, as visible in the following figure, it shows the expected phyla before treatment (BF), after treatment (AF) with global antibiotics and after treatment with beta lactam and fluoroquinolone antibiotics (Panda et al., 2014).

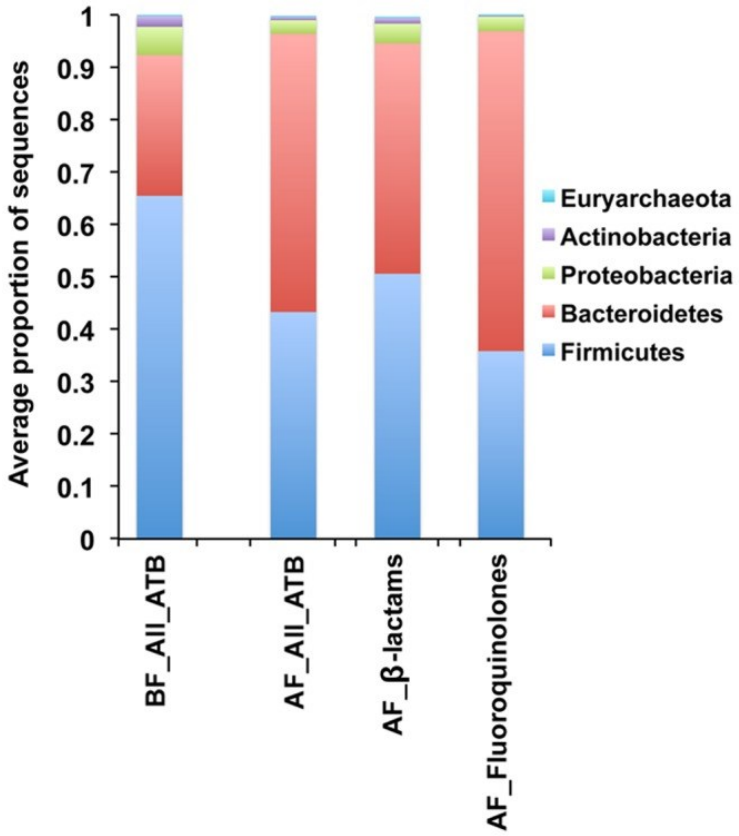


Figure (54) Expected phyla before and after antibiotic treatment

4.4.2 Post HSV-1 infection (Simulation)

The expected gut microbiota dysbiosis that takes place post infection is contributed to both HSV-1 infection and inflammatory infiltrates, in a study conducted by Ramakrishna and colleagues in 2019, shows that HSV-1 infection results in dysbiosis as well as immunoglobulins yield an apparent dysbiosis. Where *Bacteroidetes* and Anti-inflammatory *Clostridia* (AIC) are found to be reduced in mice infected with HSV-1. There is also a significant reduction in taxa associated with protection against gut barrier dysfunction. A prime example are *Clostridium aerotolerans* and other clostridial species such as *Clostridium XIVa* which ferment carbohydrates in the gut and result in the generation of SCFAs that are important for maintaining gut barrier integrity as well as anti-inflammatory properties, are found to be reduced in HSV-1 infected mice. The following figures represent the gut microbiota profiles of male (M) mice infected with HSV-1 versus non-HSV infected mice (Ramakrishna., 2019).

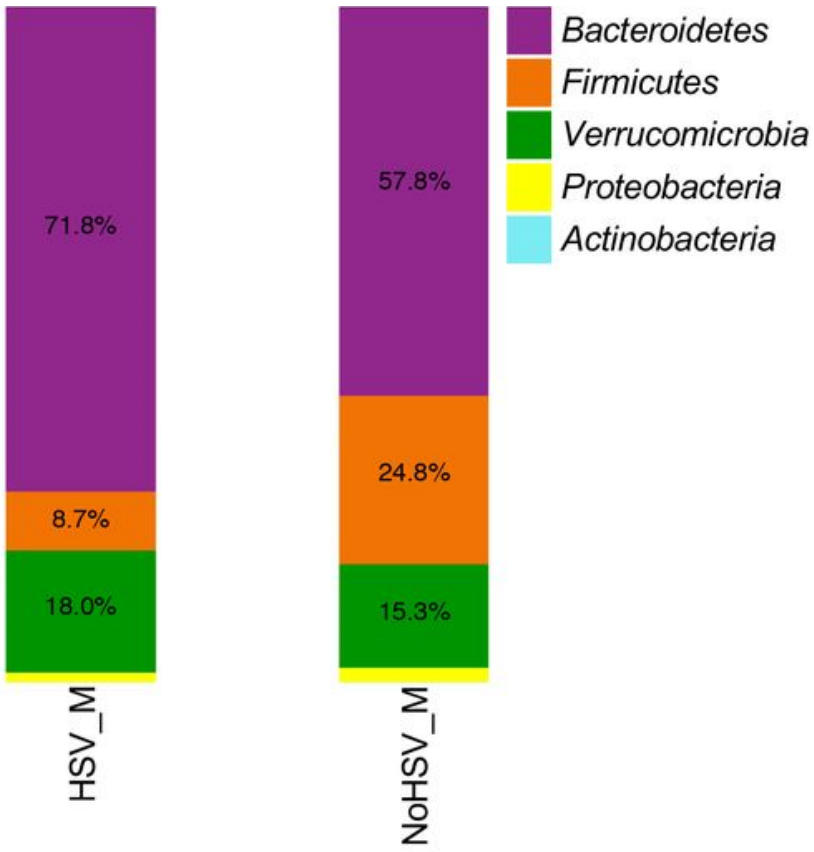


Figure (55) Expected phyla in HSV-1 infected mice

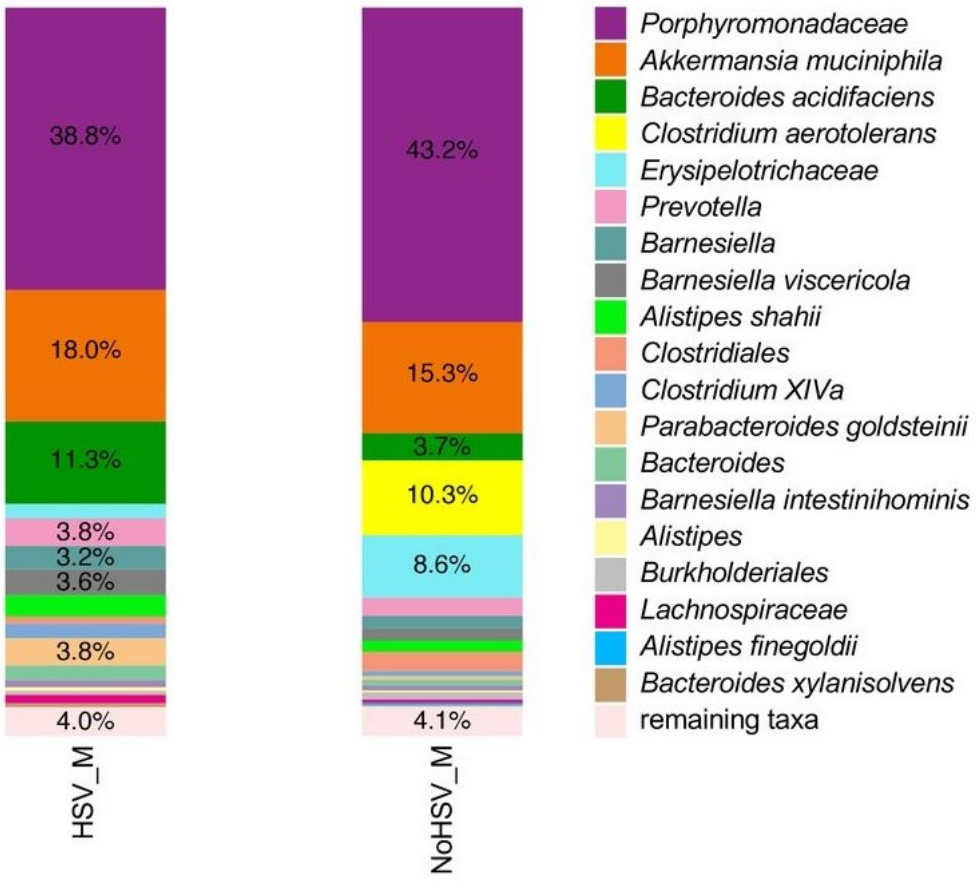


Figure (56) Expected phyla in HSV-1 infected mice

The apparent dysbiosis exhibited by HSV-1 infected mice in a variety of bacterial species that are necessary for maintaining gut barrier integrity, preventing gut dysfunction as well as possessing anti-inflammatory properties is consistent with the cascade of HSV-1 stimulation of immune responses, inflammatory infiltration, gut barrier dysfunction and dysmotility precipitated by EGCs and the beta amyloid deposition is a consequence of microgliosis and astrogliosis generated by the activated phenotype of EGCs.

4.5 Highlights from compiling the data

The apparent consensus is that functional GI disorders such as Inflammatory bowel diseases are either directly or indirectly attributed to alterations of the ENS. Based on the previous studies and reports implicating intestinal dysmotility and degenerative neuropathy in patients suffering from IBDs. Studies by Brun and colleagues were crucial to provide a picture on the ENS alterations that occurs secondary to HSV-1 infection and primary to HSV-1 induced immune response. The neurotropic potential of HSV-1 eludes the gastric barrier, reaches the gut lumen and persists in the enteric neurons. The recruitment and activation of macrophages via CCL2 is a potential pathway that is responsible for neuromuscular dysfunction (Brun et al., 2018).

As we recognize and observe the anti-microbial role of beta amyloid, evidence suggests that the pathway is correlated with deposition in both the CNS and ENS. Piacentini and colleagues displayed the neurotropic pathway of HSV-1 beta amyloid deposition. Furthermore, the enteric glial cells are the key modulators of the MGBA, essentially responsible for the inflammatory and neurodegenerative mechanism driven by a stimulus.

In this study HSV-1 is the stimulus in question and based on the results observed the inflammatory pathway is suggestive of an innate immune response that is primarily implicated in the intestinal dysmotility (observed in Brun and colleagues studies) and an adaptive immune response that is stimulated as well however, secondarily implicated in alterations of the ENS. The EGCs markers are detected in an upregulated fashion indicating the potential activation or switched on phenotype of EGCs that drives the mechanism of neurodegeneration and beta amyloid deposition. Simulations of beta amyloid supported by literature reviews provide reasonably strong evidence that is necessary to yield an optimistic view experimentally. Looking towards the gut dysbiosis assay in a simulatory fashion proved to be pretty useful and coherent with the data expected from the assay as the HSV-1 infected mice exhibited a reduction in anti-inflammatory as well as gastro protective flora, indicating the consistent presence of pathology manifested by inflammation and neurodegeneration driven by the immune response to the stimulus (HSV-1) under investigation. Finally, the sequence of events originally hypothesized is coherent with the results obtained. The HSV-1 infection of the ENS is indeed a stimulus that contributes to inflammation, intestinal dysmotility with implications in IBD patients, as well as neurodegeneration and most importantly EGCs contribution being the mediator of the mechanism of neurodegeneration and AβPP deposition.

Chapter 5

Conclusion

This study demonstrates the effects of HSV-1 infection of the ENS during gut dysbiosis, in which following orogastric inoculation of HSV-1, it reaches and infects the enteric neurons. As a consequence, an innate immune response through a characterized CCL2/CCR2 pathway that results in recruitment of inflammatory macrophages, release of reactive oxygen species that causes GI dysmotility. There is also an upregulation observed in the adaptive immune system. The climax is reached through the detection of EGCs markers with an indicative upregulation that is suggestive for the switched-on phenotype that contributes to neurodegeneration and beta amyloid deposition. Beta amyloid detection in the ENS is simulated as well as gut dysbiosis assay through fecal pellets and are both supplementary to the investigation, in addition to aiding to define an expected result and support an optimistic view. Finally, results point out the role that neurotropic viruses such as HSV-1 may play upon infecting the ENS during gut dysbiosis.

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