University of Crete
Medical School

"Study of the antiviral properties of CD40L during lytic infection by Herpes Simplex Virus type 1 (HSV-1)"

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"The Molecular Basis of Human Disease" Graduate Program,
University of Crete, Medical School
2008-2010
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Πανεπιστήμιο Κρήτης
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"Μελέτη της αντιικής δράσης του CD40L κατά τη διάρκεια λυτικής λοίμωξης από τον ιό του απλού έρπητα τύπου 1 (HSV-1)"

Βιργινία-Μαρία Βλαχάβα

Μ.Π.Σ. "Κυτταρικής κ Γενετικής Αιτιολογίας, Διαγνωστικής κ Θεραπευτικής των Ασθενειών του Ανθρώπου"
Πανεπιστήμιο Κρήτης, Σχολή Επιστημών Υγείας,2008-2010
Επιτροπή Αξιολόγησης:
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Acknowledgments

I would like to thank my supervisor, Assistant Professor Dr. G. Sourvinos for the guidance and support as well as for providing the independence and framework for creative thinking and experimentation. I would also like to thank the Associate Professor A.G. Eliopoulos for his guidance as well as his lab members for their assistance in technical matters. Moreover, I would like to thank the members of the Virology lab and specifically, Harry Filippakis, George Siakallis, Antigoni Morou, Despoina Thiriou, Nektaria Goulidaki, and Giota Dimitropoulou not only for teaching me the virology techniques and assisting in my first steps in virology but also for their genuine support and for making time in the lab pleasurable. Last but not least I would like to thank my family for their support all those years.

Περίληψη

Ο ιός του απλού έρπητα τύπου 1 (HSV-1) είναι ένας νευροτροπικός, άλφα ερπητοϊός της οικογένειας *Herpesviridae*. Όπως και άλλοι ιοί, χρησιμοποιεί τους μηχανισμούς του κυττάρου ξενιστή ώστε να ενορχηστρώσει τον κύκλο της ζωής του. Γι το σκοπό αυτό, ακολουθεί ένα σαφώς οργανωμένο πρότυπο έκφρασης των ιϊκών γονιδίων που προσαρμόζεται ανάλογα με τις συνθήκες του περιβάλλοντος του ιού. Με αυτόν τον τρόπο ο ιός μπορεί να αποφεύγει την ανοσολογική απόκριση, να αναστέλλει την απόπτωση και επίσης να ρυθμίζει την τροποποίηση των ιστονών ώστε να μπορεί να μεταβεί από κατάσταση λυτικού κύκλου σε λανθάνουσα κατάσταση και αντίστροφα.

Διάφορες προσεγγίσεις έχουν πραγματοποιηθεί με στόχο την αποσαφήνιση της βιολογίας του απλού έρπητα και την ταυτοποίηση νέων στόχων για την θεραπεία του. Στα πλαίσια αυτά, έχουν μελετηθεί ο υποδοχέας CD40 καθώς και ο συνδέτης του CD40L αφού η σηματοδότησή τους είναι μείζονος σημασίας για το ανοσοποιητικό σύστημα. Πειράματα σε ποντίκια έχουν δείξει ότι απουσία του CD40L οδηγεί σε σοβαρή αποσταθεροποίηση της χυμικής ανοσίας. Παράλληλα, έχει δειχθεί ότι ο CD40L έχει και άμεση αντιϊκή δράση σε κύτταρα που εκφράζουν τον υποδοχέα του.

Προς αυτή την κατεύθυνση επιχειρήσαμε να διερευνήσουμε την επίδραση της σηματοδότησης του CD40 στην μόλυνση από τον ιό HSV-1 και πώς αυτή επηρεάζει την έκφραση των ιϊκών πρωτεϊνών κατά τη διάρκεια του λυτικού κύκλου. Πράγματι, παρατηρήθηκε μείωση του ιϊκού φορτίου έπειτα από επίδραση με CD40L σε κύτταρα που έφεραν τον υποδοχέα καθώς και κάποια μείωση της έκφρασης της πρώιμης πρωτεΐνης του ιού, ICP8.

Summary

Herpes simplex virus type 1 (HSV-1) is a neurotropic, alpha herpesvirus that belongs to the *Herpesviridae* family. Similar to other viruses, it utilizes the host cell machinery in order to orchestrate its life cycle. In order to do so, it follows a well organized pattern of viral gene expression which is modulated according to the environmental conditions. Thereby, HSV-1 can evade immune responses, apoptosis and also modulate histone modifications in order to switch from an active to a latent state and vice versa.

Several approaches have been made towards the elucidation of HSV-1 biology and to identification of targets for its treatment. In this context, CD40 and CD40 ligand have also been studied since their signaling is of major importance in the immune system. Experiments in mice have revealed severely compromised humoral immune responses in the absence of CD40L while a direct antiviral effect has been demonstrated in cells expressing CD40.

To that direction, we attempted to investigate the effect of CD40-CD40L signaling to HSV-1 infection and how this affects viral protein expression during lytic infection. We indeed, observed a reduction in viral yield upon treatment with CD40L as well as some decrease in the protein expression of the virus early protein ICP8.

Contents

1. Introduction

Herpes Simplex Virus type 1 (HSV-1) is a member of the alphaherpesvirus subfamily of the *Herpesviridae* family. It is a neurotropic virus characterized by a relatively short life cycle[1,7], a rapid cytopathic effect (CPE)[8] and an ability to establish latent infections in sensory ganglia[1]. Sensory neuron infection is the result of a primary infection of a mucosal epithelial surface usually during childhood or adolescence. The virus follows a lytic replicative cycle in the epithelia before entering neuronal cell bodies surrounding the infected area via retrograde transport[9].

HSV-1 worldwide seroprevalence rates have been found to fluctuate between 50-90%[10,11]. Its clinical manifestation varies from asymptomatic which is the most common, to severe neurological conditions such as encephalitis. Generally, HSV-1 infections display combinations of symptoms such as fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, edema, localized lymphadenopathy, anorexia and malaise while infection of the eye can cause keratoconjunctivitis[1]. Recent data have demonstrated that there is also a strong association between HSV-1 and Alzheimer's disease[12,13].

1.1 The Herpesviridae Family

Historically herpesviruses entered the *Herpesviridae* family on the basis of structure. For a virus to be classified as a herpesvirus, it needs to consist of an icosahedral capsid, a tegument and an envelope. The capsid is approximately 125nm in size; it is composed of 162 capsomeres with each one having a pore running down their axis. The capsid also contains the viral genome which is linear double stranded DNA. The tegument is an amorphous layer in the area between the capsid and the envelope. The viral envelope is the exterior part of a herpesvirus and contains viral glycoprotein spikes on its surface[1].

Until recently, a wide variety of viruses with hosts ranging from bivalves to humans where submitted in the *Herpesviridae* family on the basis of structure. Recently, the International Committee on Taxonomy of Viruses (ICTV), based on nucleotide sequence data introduced a higher taxonomic level of viruses the *Herpesvirales*[14]. Thereby, the former *Herpesviridae* family, now part of the *Herpesvirales* superfamily has been divided in three parts. The first one includes the herpesviruses of mammals, birds and reptiles and is the revised *Herpesviridae* family. The second one includes the fish and frog viruses and is called

the *Alloherpesviridae* family. And finally, the third one, called the Malacoherpesviridae family, contains a herpesvirus of bivalves.

Viruses belonging to the *Herpesviridae* family exhibit four specific biological properties. To begin with, a wide variety of enzymes related to nucleic acid metabolism, DNA synthesis and processing of proteins is defined by those viruses although each virus does not express the whole set of enzymes. Such enzymes are thymidine kinase, thymidilate synthase, dUTPase, ribonucleotide reductase, DNA polymerase, helicase, primase and various protein kinases. Secondly, in all of them, synthesis of the viral DNA and capsid assembly take place in the nucleus while the final form of the virion is completed in the cytoplasm. Another characteristic of the *Herpesviridae* family viruses is that production of infectious particles is closely associated with destruction of the host cell, otherwise known as a cytopathic effect. Finally, all of the known herpesviruses have the ability to remain latent in their host cells where their genomes take a circular form and only a small number of viral genes are expressed. Those genomes can be reactivated, produce infectious particles and consequently they can cause disease[1]. Regarding latency, it is neither a chronic nor an abortive infection. In reference to the first condition, there are no infectious viral progeny that can support a chronic infection while concerning the second condition the virus retains the ability to reactivate which is not the case in abortive infections.

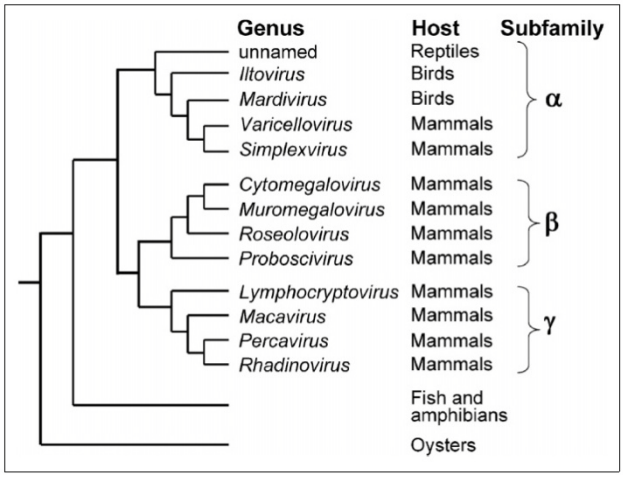

Figure 1.1: Major herpesvirus phylogenetic relationships and taxonomic subunits[1].

The *Herpesviridae* family is further divided in three subfamilies, the *Alphaherpesvirinae*, the *Betaherpesvirinae* and the *Gammaherpsvirinae*. Herpesviruses that have humans as their primary hosts span all three subfamilies of the *Herpesviridae* family.

Alphaherpesviruses are characterized by a variable host range, a short life cycle, a rapid spread in culture conditions, an intense cytopathic effect and an ability to establish latent infections mainly in sensory ganglia. Among others the *Alphaherpesvirinae* contain the human affecting genera *Simplexvirus* (HSV-1 or HHV1 and HSV-1 or HHV2) and *Varicellovirus* (VZV or HHV3).

Betaherpesviruses, in contrast to alphaherpesviruses are more restricted in their host range, they progress slowly in culture conditions and they have a long life cycle. Additionally,

carrier cultures are easily established. They also cause a distinctive enlargement of their host cells called cytomegalia. Betaherpesviruses establish latent infections as well but in contrast to alphaherpesviruses they do so in secretory glands, lymphoreticular cells, kidneys and other tissues. *Betaherpesvirianae* genera that contain human herpesviruses are the *Cytomegalovirus* genus (HCMV or HHV5) and the *Roseolovirus* genus (HHV6 and HHV7).

Gammaherpesviruses limit their host range to a family or order of organisms. They are lymphotropic viruses and they can all replicate in lymphoblastoid cells *in vitro*. Some can additionally cause lytic infection to epithelial cells and fibroblasts. However, gammaherpesviruses are usually specific for B or T lymphocytes. There are two genera containing human herpesviruses in this subfamily, the *Lymphocryptovirus* genus (EBV or HHV4) and the *Rhadinovirus* genus (Kaposi's sarcoma-associated herpesvirus or HHV8)[1,14].

In respect of evolution, the herpesviruses precise origins have not yet been deciphered. There is evidence though of an evolutionary relationship between herpesviruses and bacteriophages. Similarity has been detected between a terminase gene encoded by HSV-1 and a bacteriophage gene of related function. In addition, conserved unique protein folds consisting of three α-helices and two β-sheets have been found in the capsids of both herpesviruses and bacteriophages.

Within the Herpesviridae family, the alpha-, beta- and gammaherpesviruses have arisen from a common ancestor. Although there is apparently little genetic similarity between the three subfamilies the capsid structures are highly conserved. There is some controversy over the evolutionary succession of alpha-, beta- and gammaherpesviruses but it is well accepted that herpesviruses evolved in parallel with their hosts[15].

1.1.1. Herpes Simplex Virus type 1 (HSV-1 or HHV1)

1.1.1.1. HSV-1 structure

HSV-1 structure has been studied extensively. However recent cryo-electron tomography studies revealed a more detailed structure of HSV-1. HSV-1 virion ranges from 170nm to 200 nm in diameter with an average diameter of 186nm. Spikes protrude from its surface making its diameter approximately 225nm[7]. It consists of four main structural elements. An electron-opaque

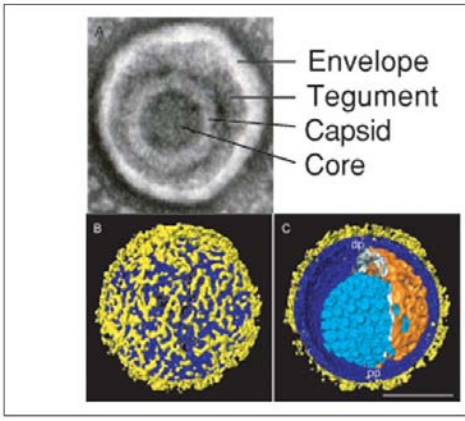

Figure 1.2: Structure of HSV-1 virion depicting the envelope, tegument, capsid and core[1,7].

core containing the viral DNA - a double stranded, 152Kbp molecule - the capsid, the tegument and the viral envelope (Fig. 1.2 and 1.3).

The capsid is an icosahedral structure. It consists of 162 capsomers, 150 hexons and 12 pentons[16]. Hexons and pentons consist of six and five VP5 molecules respectively. On the surface of the capsid six copies of VP26 molecule form a ring linked to the tip of each VP5 molecule on hexons[17]. The capsomers are interconnected via a complex comprising of two copies of VP23 protein and one molecule of VP19c protein[16,18,19].

The viral capsid is surrounded by the tegument, an amorphous structure containing at least 20 different proteins with both structural and functional roles[20,21]. It acts as a delivery compartment for proteins needed early in the course of infection. It has also been described that the tegument is not uniformly distributed around the capsid. On the contrary, the viral capsid is close to the envelope on one side (the proximal pole) and about 30-35nm away from the opposite site (the distal pole). The tegument is connected to the envelope by 4nm-wide linkers[7]. It carries no specific organization though some actin-like filaments up to 40nm long have been observed. Those filaments are either polymeric contents of the tegument or cellular filaments that have been incorporated in the virus.

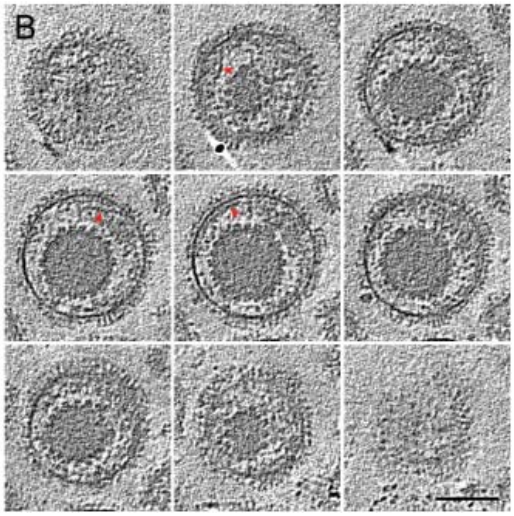

Figure 1.3: Gallery of parallel slices, 15.5nm apart and 5.2 nm thick, through the virion[7].

The outer layer of the virus, the envelope is comprised of a lipid bilayer of cellular origin and at least 11 viral glycoproteins embedded on it. Those proteins are fundamental for the host specific recognition, cell entry, cell-to-cell spread and immune evasion[20].

1.1.1.2.HSV-1 replication cycle.

1.1.1.2.1. Lytic HSV-1 Infection.

Herpes simplex virus type 1, as described earlier has the ability to establish both lytic and latent infections. The virus enters the epithelial cells through a rupture of the skin or through an oral mucosa[1] initiating a lytic infection. In specific, the virus initially binds to the cell surface, the viral envelope fuses with the cell membrane releasing the tegument-capsid structure in the cytoplasm from where it is transported to the host cell nucleus[22].

The initial event in HSV infection is binding of the virus on the host cell membrane by utilizing viral glycoproteins of the envelope. In detail gB and/or gC glycoproteins bind heparan sulfate of the proteoglycans on the host cell membrane [3,23]. Those interactions are crucial but not sufficient for viral entry. Following binding of the virus, interactions between viral glycoproteins and a variety of different receptors on the host cell surface lead to fusion of the viral envelope with the host cell membrane. The viral glycoproteins implicated in this process are gB and gD[3]. As for the host cell receptors that bind those viral ligands these include the herpes virus entry mediator (HVEM) a member of the TNF receptor family (TNFRSF14)[24,25], PILRα (paired immunoglobulin-like type 2 receptor-α) [26] nectins 1 and 2[27,28] and 3-O-sulphated heparan sulphate glycoproteins[23] of the host cell.

To sum up, HSV glycoproteins gB and gC mediate association of the virion with heparan sulphate on the host cell membrane. Subsequently gB binding of PILRα and gD binding of HVEM, nectin 1, nectin 2 or 3-O-sulphated heparan sulphate glycoproteins lead to membrane fusion facilitated by the viral glycoprotein heterodimer gH-gL[3,29].

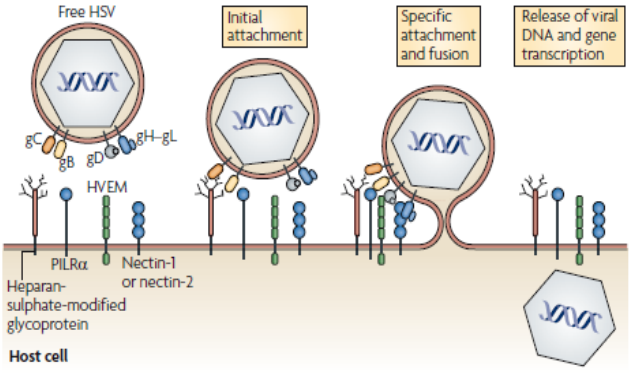

Figure 1.4: Herpes simplex virus entry receptors and ligands[3].

HSV can also enter the cell via the lipoprotein receptor LRP1. Specifically, viral glycoprotein gB has been found to carry highly homologous sequences to both the C-terminal fragment of beta-amyloid (APP)[30] and to APOE[31]. Alpha-2-macroglobulin (A2M) binds to APOE and APP as well. Both A2M and APOE are involved in beta - amyloid clearance. Thus, it becomes apparent that HSV, through its ability to bind to A2M, could utilize the beta – amyloid clearance pathway in order to gain entry to the cells[32] . Hence, it is expected that there might be a correlation between HSV-1 and Alzheimer's disease taking into consideration that one of the primary features of Alzheimer's disease is the extracellular concentration of Aβ amyloid, a neurotoxic peptide derived from APP cleavage[33,34]. Indeed it has been found that herpes virus affects APP processing[35] and that there is a prominent localization of HSV-1 DNA within plaques in the brains of Alzheimer's disease patients. Particularly 90% of the plaques contained viral DNA while 72% of the viral DNA was associated with plaques pinpointing HSV-1 as a significant etiological factor in Alzheimer's disease[13].

Following entry of the virus, the course of the deenveloped capsid-tegument structure towards the nucleus is enabled by the microtubular network[36]. Experiments with depolymerizing drugs showed that in both neuronal and retinal pigment epithelial cells, viral particles were prevented from moving from the periphery to the nucleus upon treatment. However, in various cell types polarity of the microtubule network diverges. While in fibroblasts and neuronal cells the minus ends are organized towards the nucleus, in retinal pigment epithelial cells the plus ends of the microtubules are located next to the nucleus. The above suggest that the deenveloped virus can utilize both dynein and kinesin superfamily members[22].

The deenveloped virus docks at the nuclear membrane of the cell and the viral DNA is released to the nucleus through pores on the capsid surface while empty capsids accumulate at the nuclear membrane[37]. The linear DNA of the virus circularizes upon entry and begins to transcribe the immediate early (IE) gene products, followed by the early (E) and finally the late (L) products (Fig. 1.5). All this process is accompanied by a reorganization of the host cell nucleus in compartments where the virus is replicated and new virions are assembled.

Initially, the circularized viral DNA localizes at sites near ND10 structures. ND10 which stands for Nuclear Domain/Dot 10 are sub-nuclear structures whose functions are not fully understood but it is well-established that they are modified during the cell cycle and are sensitive to stimuli such as heat shock and infection by a variety of viruses[2]. Whether this colocalization of ND10 structures and viral DNA is the outcome of the viral DNA being targeted to the ND10 structures or vice versa has not been clarified yet however there is evidence for both scenarios.

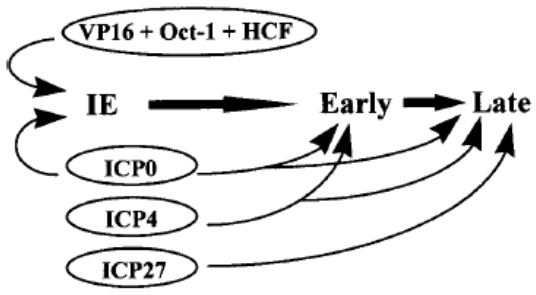

Figure 1. A simplified scheme of the sequence of viral gene expression during HSV-1 infection. VP16 present in the viral tegument activates IE gene expression when complexed with the cellular factors Oct-1 and HCF at specific sequence elements in the IE promoters. Subsequent production of ICP0 augments expression of all classes of viral genes. IE protein ICP4 is essential for the transcription of Early and Late promoters, and IE protein ICP27 is essential for efficient expression of Late gene products.

Figure 1.5: [2]

ND10 structures are subsequently disrupted by Infected Cell Protein 0 (ICP0), an immediate early viral protein. Viral DNA replication proteins along with viral DNA assemble near the ND10 structures to form pre-replicative sites which are converted to small replication compartments with the beginning of viral replication. As viral DNA replication proceeds, the replication compartments enlarge and coalesce filling that way the nucleus, causing the host chromatin to marginalize. Finally, the viral replication

compartments extend to the nuclear periphery after the nuclear lamina is disrupted and host chromatin is dispersed by the viral proteins UL31 and UL34 (Fig 1.6).

From a transcriptional point of view, an important molecule for the initiation of viral transcription is VP16, a transcriptional activator protein localized at the tegument of the virus that forms a complex with the cellular factor HCF (Host Cell Factor). The VP16-HCF complex transfers to the cell nucleus where it binds to the transcriptional factor OCT1 (octamer-binding protein 1) leading that way to the transcriptional activation of the immediate early regulatory genes of HSV-1 by tethering the VP16–HCF complex to IE gene

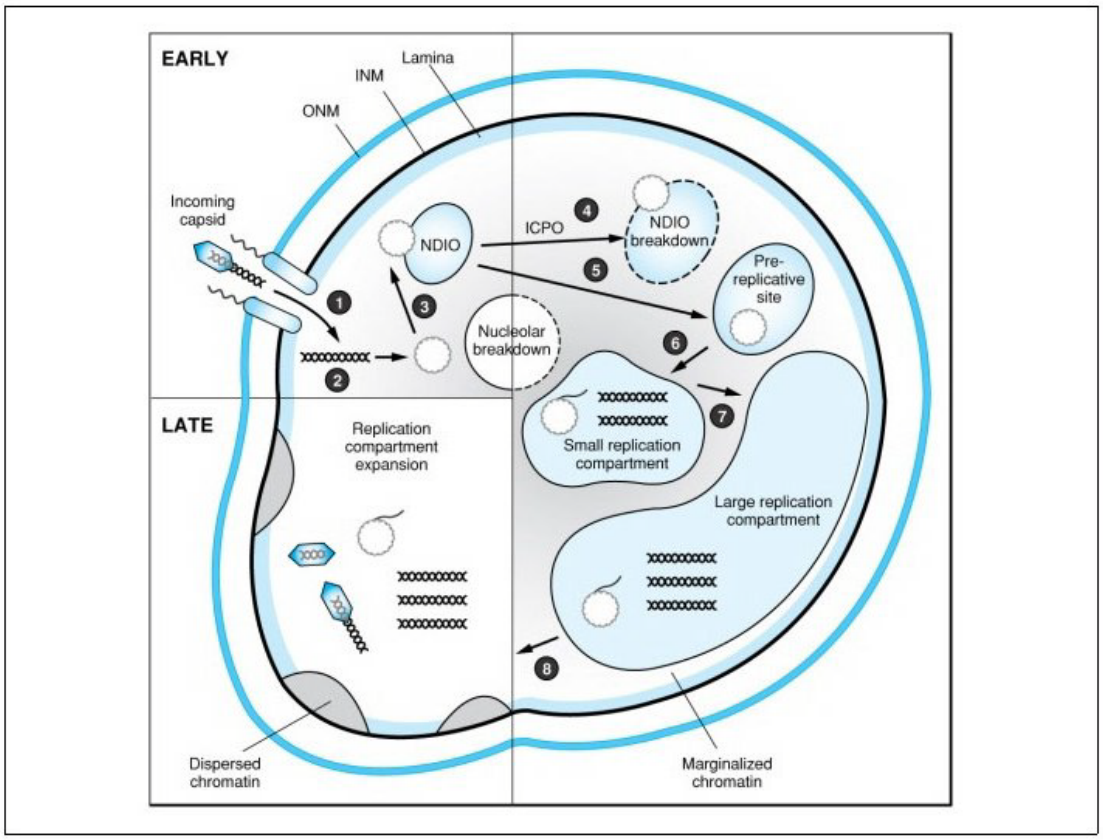

Figure 1.6: Remodeling of the host cell nucleus by HSV infection[1]

promoters and enabling the activator domain of VP16 to recruit transcription factors that stimulate IE gene transcription. IE gene products activate expression of the E gene products. ICP4 is required for all subsequent viral gene expression, most likely through its association with transcription factors and their adaptor proteins. Additionally, ICP4 along with ICP0 produce a synergistic activation of E gene transcription significantly enhanced as compared to activation observed by one of the two molecules alone[2]. ICP0 is also an immediate early protein and as reported above it has also been found to be associated with ND10 structures. It is a major regulatory molecule of HSV-1 that displays various functions while its requirement is cell-type dependent[38]. Although ICP0 does not directly bind DNA, its association with other proteins renders it capable of stimulating transcription from various viral promoters[39].

The E gene products are involved in viral DNA replication, after which the L genes are expressed. Activation of L gene expression requires DNA synthesis and at least 3 viral proteins: ICP4, ICP27 and ICP8. ICP27 stimulates transcription of L genes, cytoplasmic transport of viral mRNAs and translation of L mRNAs, but the mechanism that underlies these functions has not been fully defined. ICP27 also blocks IFNα induced accumulation of STAT-1 in the nucleus[40]. ICP8 is a single strand DNA binding protein which also serves as a recombinase that can carry out strand annealing reactions and it probably has a role in viral chromatin modulation[41].

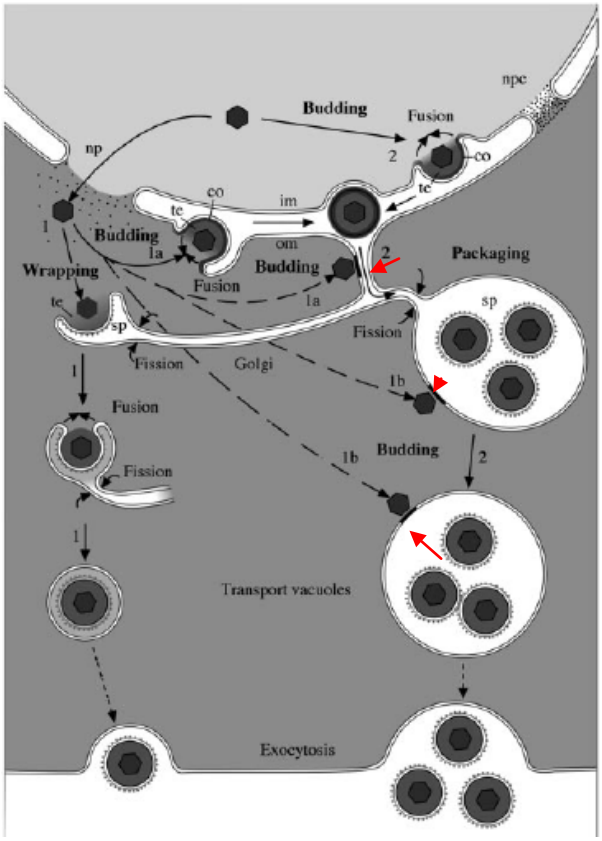

Figure 1.7 :Schematic drawing of the pathways of HSV-1 envelopment
[5], abb: np=nuclear pore, co=condensed im=inner nuclear membrane, om= outer nuclear membrane, npc= nuclear pore complex, sp= spikes, te=tegument

Once the components of the virion have been synthesized, the viral DNA gets packaged inside capsids in the nucleus. Following encapsidation, the virus acquires an envelope and leaves the cell. Recent findings by Leuzinger H. et al [5] demonstrated that HSV-1 employs two different pathways in order to do so. One is by cytoplasmic envelopment and the other is by nuclear envelopment (Fig. 1.7).

Through cytoplasmic envelopment (route 1 of Fig 1.7), the capsid leaves the nucleus through impaired nuclear pores. Nuclear pore impairment has been

shown by the translocation of Nup153 - a protein involved in anchoring the nuclear pore complex to the nuclear membrane, from the nuclear periphery into the cytoplasm upon infection [5]. Following nuclear egress, the capsid approaches the Golgi apparatus and buds into it leading to the acquisition of two membranes. The inner one becomes the viral envelope while the outer one becomes the vacuolar membrane that leads to the exocytosis of the virus. It is assumed that there might be "antifusion" proteins that prevent fusion of the envelope with the vacuolar membranes which is supported by an observation of the same group that an electron dense substance is thickening the membrane at the sites of budding (see red arrows at Fig 1.7). A candidate protein that is assumed to facilitate viral egress and down regulate undesirable fusions of the viral envelope with cellular membranes is glycoprotein K (gK)[42]. Apart from the Golgi apparatus, the capsid can also bud at the outer nuclear membrane (Fig 1.7, 1a), rough endoplasmic reticulum membranes (Fig 1.7, 1a), membranes of the dilated Golgi cisternae (Fig 1.7, 1b) and membranes of Golgi derived vacuoles that already contain virions (Fig 1.7, 1b).

As for nuclear envelopment (route 2 of Fig 1.7), capsids bud through the inner nuclear membrane and are transported from the perinuclear space to the Golgi cisternae by utilizing the rough endoplasmic reticulum membranes. At the Golgi, virions are packaged into transport vacuoles of various sizes and are released from the cell via exocytosis[5].

1.1.1.2.2. Latent HSV-1 Infection

Latency is a state characterized by a modification of viral transcription so that the virus can remain dormant for long periods of time and evade immune response. For a latent state to be established, the virus has to overcome some hindrance. At first, lytic gene expression should be silenced in order to avoid the cytopathic effects of lytic infection. In addition, host cell responses such as apoptosis and innate immunity must be blocked and lastly, the acquired immune response must be evaded so that the infected cell escapes clearance[4].

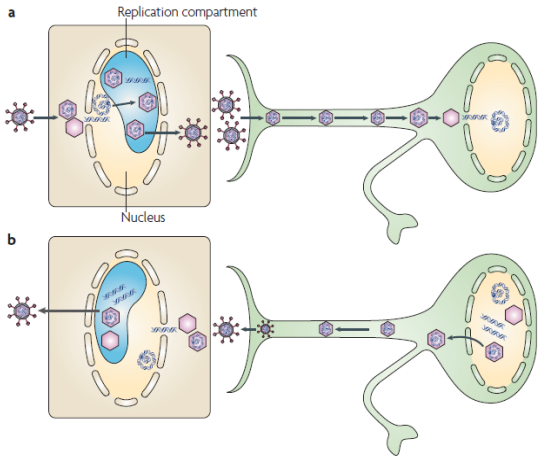

Figure 1.8: (a) Infection of neurons innervating infected epithelia and (b) reactivation of the virus and recurrent infection of the epithelial cells[4].

Latency is established in sensory neurons following an active infection of surrounding tissues. HSV-1 enters the nerve termini and reaches the neuronal cell nucleus by retrograde

transport (Fig 8). During latency, HSV-1 genome is maintained as a circular episome and viral gene expression is repressed with the only sequence transcribed being the LAT (latency associated transcript), a viral sequence which encodes for multiple transcripts and plays a central role in the silencing of lytic genes. The virus can be reactivated by stress stimuli but the actual molecular mechanism leading to this activation is not yet understood[39].

It has recently been proposed by D.M. Knipe and A. Cliffe[4] that the balance between lytic and latent infection is epigenetically regulated. Though HSV-1 genome is not associated with histones in the viral capsid[4,39] - only a few histones are present on the viral genome compared to those on cellular genes[43,44], LAT gene promotes formation of heterochromatin on lytic gene promoters and reduces euchromatin[45]. The model proposed is that in epithelial and other non-neuronal cells, viral proteins function towards reducing histone association with viral lytic gene promoters and promoting euchromatin histone modifications on those histones associated with viral DNA. Yet, in neuronal cells, to which LAT shows specificity, HSV-1 activity causes the acquisition of heterochromatin marks on viral lytic gene promoters so that lytic genes are silenced and a latent state can be established[4].

Another key molecule thought to be involved in the epigenetic regulation of HSV-1 state is ICP0. ICP0 forms a complex with an HDAC repressor complex, namely the RE1 silencing transcription factor corepressor to REST, (REST/CoREST)-HDAC repressor complex, dissociating HDAC1 from the latter[46,47] and possibly preventing the complex from regaining its repression activity[4] . In concordance are experiments demonstrating complementation of ICP0 defects by HDAC inhibitor trichostatin A (TSA)[48]. Conversely, another group demonstrated that there is no complementation of ICP0 null HSV-1 virus in normal human fibroblasts upon HDAC inhibition by TSA[49]. It should be highlighted though that in contrast to HDAC inhibitors that inflict overall changes in histone acetylation, ICP0 does not increase the acetylation of H4[50]. Thus it is not clear by which principle ICP0 and HDAC interaction leads to activation or silencing of viral genes.

There is also much controversy concerning miRNAs and their involvement in latent infection but no HSV-encoded miRNA has been shown to be expressed during latency[4].

Therefore, latency and the mechanisms that define it have not been yet elucidated.

1.1.1.3.Pathology and Epidemiology of HSV-1.

HSV-1 clinical manifestation though characterized by cold sores varies from asymptomatic, to severe neurological conditions such as encephalitis. Generally, HSV-1 infections display combinations of symptoms such as fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, edema, localized lymphadenopathy, anorexia and malaise while infection of the eye can cause keratoconjunctivitis. Recent data have demonstrated that there is also a strong association between HSV-1 and Alzheimer's disease[51].

HSV is a human virus and is transmitted only from infected humans to other humans during episodes of productive infection and by close personal contact. There is no seasonal variation of HSV infection frequency. The infection is rarely fatal and since this virus becomes latent and persists for the lifetime of the host, more than 50% of the world population has been infected[1].

Reactivation of the virus can be triggered by a variety of factors such as stress, ultraviolet light, fever, fatigue, sideropenia and impaired cell mediated immunity in the host [52]. However, in immunocompetent individuals, HSV infection is usually self-limiting. On the contrary, it causes significant morbidity and mortality in immunocompromised individuals who are in danger of acute herpetic disease. For neonates infected *in utero* or during delivery HSV infection can be lethal. In USA approximately 1500 newborns are infected each year and despite the availability of antiviral therapies, the mortality rate is still high. AIDS patients are also at high risk of severe herpetic disease and most drug resistant HSV strains derive from those patients. Some immunocompetent hosts also suffer from serious herpetic disease. Ocular infection is another serious condition. Recurrent herpes keratitis can cause corneal scarring and blindness. Moreover, central nervous system HSV infections or encephalitis can lead to death despite the antiviral therapies and even in those patients that manage to overcome encephalitis severe neurological conditions persist[4].

As mentioned above, it is estimated that more than 50% of the world population has been infected. Studies in more specific groups have shown some variability between populations yet the general tendency is that HSV-1 prevalence in non-high risk populations increases with age or plateaus after about 30 years of age with most acquisition taking place in childhood and adolescence. It has also been estimated that HSV-1 seroprevalence is lower at higher socioeconomic groups[52]. A USA based population study showed that HSV-1 seropositivity increased from 44% in young adults aged 12-19 years to 90% in adults over 70

years old. In contrast, HSV-1 seroprevalence decreased in women over 40 years old in Uganda, New Mexico and Turkey[10]. In European countries, namely Germany and Spain, 40-50% of adolescents aged 14-17 years have HSV-1 antibodies and the percentage reaches 90% by the fifth decade of life[52].

Concerning high risk populations, such as South Africa populations that exhibit higher risk sexual behaviors, HSV-1 seroprevalence is over 97% in all age groups. Among young, female, commercial sex workers aged 16-22 years in Mexico City, HSV-1 seroprevalence was over 93% (reviewed in [10]).

Nevertheless, worldwide epidemiology of HSV-1 is not static. There has been a decline in the age specific HSV-1 prevalence rates in industrialized middle class populations over the past years. For instance, pre-adolescent populations in the UK in 1953 exhibited a 63% HSV-1 seroprevalence which decreased to 23% in 1995 probably due to improved socioeconomic conditions[52].

To sum up, global HSV-1 prevalence is high regardless of the fluctuations in percentages and the aggravating factors which are latency and recurrent infections prevent annihilation of the disease. Those data point to the direction of discovering new more efficient drugs that target the central regulation machinery of HSV-1.

1.2 CD40 – CD40 ligand interactions

CD40 or TNFRSF5 is a member of the Tumour Necrosis Factor (TNF) Receptor superfamily. CD40 is a glycoprotein receptor that contains 4 TNFR – Cys repeats but no death domain as opposed to other TNF receptors. It appears in two isoforms, a single pass, type I membrane protein and a secreted one. It exists as a monomer of 48 kDa or as a homodimer. However, a variant form detected in the bladder carcinoma cell line Hu549 does not form homodimers. CD40 signals through the recruitment of adaptor proteins of the TNF receptor-associated factor (TRAF) family, namely TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6[53].

The receptor was first identified in 1986 as a surface marker of B lymphocytes that causes cell proliferation to activated B cells[54]. Nevertheless, CD40 is not only expressed on B cells. It is also expressed on monocytes, dendritic cells, endothelial cells, fibroblasts, neuronal cells, smooth muscle cells and thymic epithelial cells. Moreover, CD40 expression is detected at low levels in the basal proliferative compartment of human stratified squamous epithelium and normal ovarian and breast epithelial tissue. Apart from its normal expression patterns, CD40 is also expressed on malignant tissues. Thus, a variety of haematological malignancies such as non-Hodgkin's and Hodgkin's lymphomas, chronic lymphocytic leukaemia, multiple myeloma and acute myeloid leukaemia carry CD40. In addition, it is expressed at high levels in carcinomas of the nasopharynx, bladder, cervix and ovary and there are some studies showing expression in melanomas and soft tissue sarcomas (reviewed in [55]).

Lack of CD40 expression can also lead to serious conditions. CD40 mutations are the cause of an autosomal recessive disorder termed hyper-IgM immunodeficiency type 3 (HIGM3)[56]. This disorder is characterized by inability of B cells to undergo isotype switching and mount an antibody-specific immune response, in addition to lack of germinal center formation[53].

As for its counterpart, CD40 ligand (CD40L), it is also a TNF superfamily member (TNFSF5) and appears in two forms: a single pass, type II membrane protein, and a soluble, secreted form. The soluble form is derived from the membrane bound form via proteolytic cleavage[53]. CD40 forms homotrimers and transmits its signal as such. Trimeric CD40L leads to the trimerization of membrane bound CD40 as part of the signaling cascade of CD40/CD40L interaction[57].

CD40L is expressed on activated but not resting T lymphocytes and is primarily limited to $CD4^+$ T lymphocytes; yet, it is also encountered on the surface of a small portion of $CD8^+$ T cells. However, CD40L is not only detected on T lymphocytes. It has also been detected on B

lymphocytes and B cell lines as well as on primary mast cells and their cell lines, on basophils and eosinophils, NK cells, monocytes/macrophages, endothelial and smooth muscle cells, epithelial cells and platelets (reviewed in [58,59]) . Platelets also release secret large amounts of CD40L and are the main source of circulating soluble CD40L[60].

Lack of CD40L expression is the cause of an X-linked immunodeficiency syndrome named Hyper IGM type 1 (HIGM1) syndrome or XHIM. Patients with HIGM1 syndrome have defects in the CD40L gene which is located at the X chromosome. HIGM1 is characterized by a defect in the immunoglobulin isotype switching process leading to elevated IgM in the serum, and absence of all other isotypes. The disorder usually presents during infancy with recurrent upper and lower respiratory tract bacterial infections. Patients are prone to opportunistic infections such as *Pneumocystis jeroveci* pneumonia and intractable diarrhoea due to C*ryptosporidium parvum* infection[53]. Hematologic disorders are also common. Such conditions include neutropenia, thrombocytopenia, and anemia[61]. Despite the substitution of immunoglobulins intravenously, life expectancy is short with a death rate of about 10% before adolescence. The median survival of patients that have not undergone successful allogeneic bone marrow transplantation is less than 25 years[53,62].

As mentioned earlier, CD40 as well as CD40 ligand are expressed in a wide variety of cells, highlighting the importance of CD40/CD40L interactions for the immune system. CD40/CD40L interactions exert profound effects on DCs, B cells, endothelial cells and cells of haematopoietic and non-haematopoietic compartments. DCs rely on this interaction for the expression of co-stimulatory molecules on their surface, cross-presentation of antigens and cytokine production enabling consequently activation and differentiation of T cells. In addition, CD40 signalling of B cells induces germinal centre formation, immunoglobulin isotype switching, somatic hypermutation of the Ig in order to optimize affinity for the antigen, and lastly it is responsible for the generation of long lived plasma cells and memory B cells. Furthermore, CD40 signalling accounts for the survival of a variety of cell types such as germinal center B cells, DCs and endothelial cells under normal or inflammatory conditions[6]. Hence, one can conclude that CD40/CD40L interactions are of paramount significance for acquired immune responses.

CD40 signalling is substantiated via the collaboration of adapter protein molecules called TRAFs (TNF Receptor Associated Factors). After engagement of the ligand on the receptor various TRAF molecules bind to the cytoplasmic tail of CD40 initiating a signalling cascade. The signal transmitted is dependent on the specific TRAFs that will be drawn to the receptor as well as by the oligomerization of both TRAFs and CD40.

In specific, the initial event is binding of the trimerized CD40 ligand[53,63] on CD40. Engagement of CD40 by its ligand induces oligomerization of the former. Following receptor - ligand interaction, TRAF molecules are recruited to the cytoplasmic tail of CD40.

The TRAF family consists of 6 members named TRAF1 through TRAF6. They possess a highly conserved carboxyl domain, the TRAF domain, through which they interact with TNFR family members. In addition, all TRAFs apart from TRAF1 contain an amino-terminal ring finger domain followed by five zinc fingers and a coiled-coil domain. All this structure is described as the zinc-finger domain and its genetic ablation inhibits any downstream signalling hence highlighting its significance[6]. The coiled–coil domain in particular, mediates homo- and hetero- oligomerization[53]. Concerning CD40, TRAF1, TRAF2 and TRAF3 are drawn to binding sites with a PxQxT motif at the membrane distal domain of CD40's cytoplasmic tail, while TRAF6 is recruited at a membrane proximal binding site with a QxPxE motif[6]. Moreover, trimeric TRAF1, 2, 3 and 6 have been shown to interact with different affinities with trimeric CD40, namely TRAF2 > TRAF3 >> TRAF1 and TRAF6[64].

The most characterized signalling pathways activated via CD40 are the canonical and non-canonical or alternative NFκB pathway and TRAFs play various roles in order to orchestrate signaling towards those two different directions. The canonical NFkB pathway begins with the formation of the IKK complex (ie Inhibitor of KappaB (IkB) Kinase complex) which contains two catalytic subunits- IKKα and IKKβ, and a regulatory subunit called IKKγ or NEMO. This complex phosphorylates IkB, the inhibitor of NFkB leading to its proteasomal degradation and release of the NFkB subunits p50/RelA and p50/c-Rel. The heterodimers translocate to the nucleus and induce gene transcription. The non-canonical pathway begins with NIK (ie NFkB Inducing Kinase). NIK phosphorylates an "alternative IKK complex", a homodimer of IKKα. Activation of IKKα leads to the phosphorylation of p100 on the p100/RelB complex leading to ubiquitin/proteasome-dependent proteolysis of the precursor p100 which generates p52. The p52/RelB complex translocates to the nucleus inducing the expression of a different set of genes comparing to the canonical NFkB signaling[6,53].

Regarding TRAFs, TRAF1 and TRAF2 are required for the activation of the canonical NFkB pathway while TRAF2 is also a negative regulator of the alternative pathway. Additionally, TRAF1, whose binding site motif on CD40 overlaps with those of TRAF2 and TRAF3, is considered to be a regulator of CD40 signaling. TRAF1 regulates the recruitment and degradation of TRAF2; however, TRAF2 is also regulated by TRAF3. While TRAF1 can regulate the recruitment and degradation of TRAF2, TRAF3 that also regulates TRAF2 recruitment, additionally causes the accumulation of NIK in B cells. On the contrary, in

epithelial cells, overexpression of TRAF3 induces the canonical pathway. Therefore, this suggests that TRAF3 has different roles in different cell types and designates TRAF3 as another regulator for the selection between the canonical and the alternative pathway (Fig 1.10).

TRAF2 and TRAF3 are also implicated in a complex with cIAP (ie cellular Inhibitor of Apoptosis) 1 and 2, and NIK which regulates apoptosis in B cells[65]. When CD40L is not bound to the receptor, TRAF2 interacts with cIAP1/2 and in turn with TRAF3 and NIK. cIAP1/2 induces NIK degradation and antagonizes the alternative NFkB pathway leading to apoptosis of B cells. In contrast, when CD40L binds to CD40, the complex is destabilized and allows for accumulation of NIK which leads to activation of the non-canonical NFkB pathway and survival of B cells. TRAF2 and TRAF3 along with cIAP1/2 are recruited to the cytoplasmic domain of CD40, TRAF2 is degraded via its E3-ubiquitin ligase activity whereas TRAF3 degradation is dependent on cIAP1/2[6].

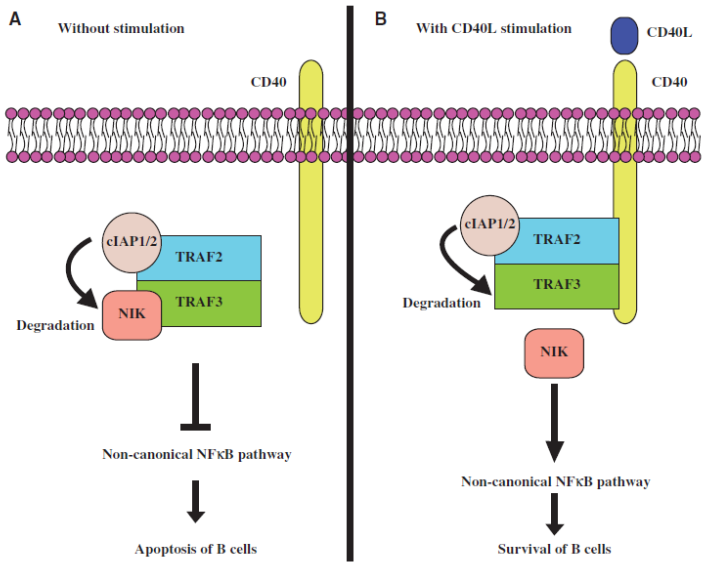

Figure 1.9:[6]

TRAF5 has been shown to form heterodimers with TRAF3 but its function is not fully understood. Silencing of TRAF5 in B cells treated with small interfering RNAs (siRNAs) caused ablation of both the canonical and non-canonical NFkB pathway[6] (Fig 1.10).

Last but not least, TRAF6 is involved in more than one pathways related to CD40. It activates the canonical NFkB pathway[66,67] as opposed to the alternative pathway and it is also involved in the activation of Jnk, p38 and Akt upon CD40L engagement[67]. In DCs, CD40L binding is followed by TRAF6 recruitment which forms a complex with Cbl-b –an E3 ubiquitin ligase, and PI3K. In B cells, CD40 requires c-Cbl for the same process. Cbl recruitment to TRAF6 is dependent on Src kinase activity and TRAF6 can enhance the phosphorylation of Cbl proteins by Src[68]. Additionally, in HeLa cells, it has been demonstrated that CD40 stimulation results in the activation of PI3K and use of PI3K inhibitors results in the abrogation of Akt phosphorylation[69]. Therefore, it is proposed that Cbl-b activates PI3K and induces Akt phosphorylation during CD40 signaling[6]. Moreover, another mechanism has

been identified with PI3K activating the mammalian target of rapamycin (mToR) which in turn induces the expression of anti-apoptotic proteins, namely caspase-8 and cFLIPp43[69]. Thus, CD40, probably through TRAF6, induces the activation of PI3K signaling that protects from apoptosis[6]. In B cells deficient in Cbl-b, another mechanism is observed. Cbl-b associates with TRAF2 upon CD40 ligation and inhibits its recruitment to CD40; lack of Cbl-b causes increased recruitment of TRAF2 to CD40 leading to activation of NFkB and Jnk pathways[70]. In addition, TRAF6 regulates CD40 signaling not only through directly binding to CD40 but also via its interaction with TRAF2 and it is proposed that this interaction between the two TRAFs is required for the recruitment of kinases responsible for activation of the NFkB, JNK, p38 and Akt pathways [67].

Lastly, apart from TRAFs, CD40 is also associated with Jak3 and has a proline rich binding site at the membrane proximal region of the cytoplasmic tail. In addition, in B lymphocytes, CD40 ligation induces phosphorylation of Jak3 and nuclear translocation of STAT3[71]. Another group performed studies on activated monocytes and resting B lymphocytes and demonstrated that though Jak3 is associated with CD40 in both cell populations, only in activated monocytes it induced activation of STA5a and translocation of the latter to the nucleus[72]. One can again appreciate the complexity of CD40 signaling which according to the cell type mobilizes different signaling molecules in order to mount a response.

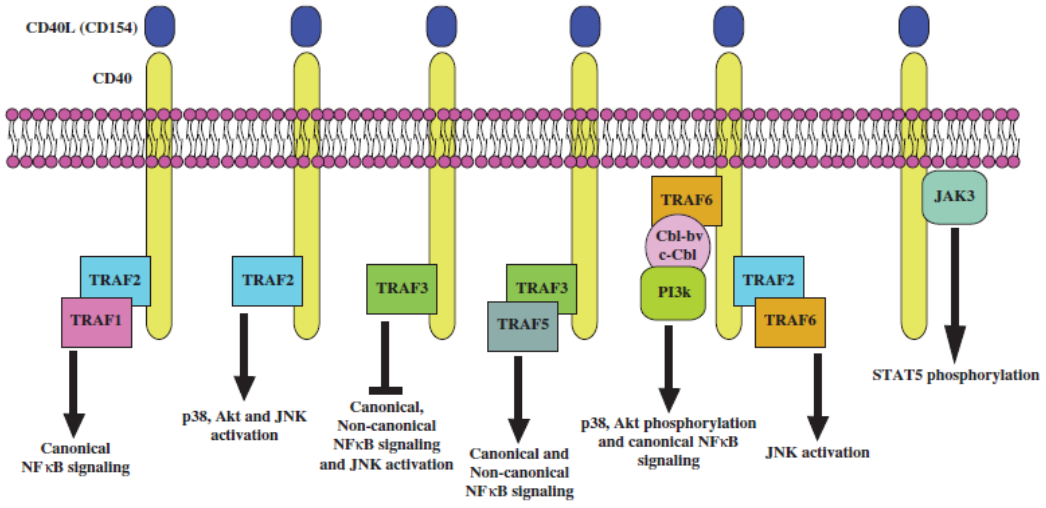

Figure 1.10: [6]

CD40/CD40L signaling has been studied meticulously for HIV (ie Human Immunodeficiency Virus) demonstrating about two decades ago that infection of $CD4^+$ T lymphocytes correlates with the expression of CD40 on their cell surface[73]. In addition, CD40L deficient mice have been shown to develop severely compromised humoral immune responses following infection with various viruses such as lymphocytic choriomeningitis virus (LCMV), Pichinde virus and vesicular stomatitis virus (VSV). However, moderate titers of IgM and IgG2a were produced by those mice in a $CD4^+$ T-cell independent fashion[74]. A more direct antiviral effect has been reported by Ruby *et al*, who showed that treatment with CD40L of L292 cells following HSV-1 infection inhibited HSV-1 replication[75].

Given the data cited above, we sought to verify a direct effect of CD40-CD40L activation in HSV-1 replication and propagation *in vitro* and to investigate the pathways implicated in CD40-CD40L dependent viral yield diminution.

2. Materials and Methods

The cells and viruses used for this study as well as the protocols for the propagation and titration of viruses and for protein expression analysis will be analyzed in this section.

2.1 Cell lines and viruses.

TRAF2 +/+ cells were used for the study of the effect of CD40-CD40L interaction on viral infection. These cells were established by Eliopoulos AG laboratory from mouse tongue fibroblasts of 1 week old TRAF2+/+ mice. The primary cells isolated were immortalized by simian virus 40 large T antigen to increase the transfection efficiency and allow the generation of stable lines transfected with human CD40 [67] in pcDNA3-CD40neo vector[76]. The medium required for traf2+/+ cells was DMEM (Dulbecco's Modified Eagle Medium, Biosera, UK) with 10% FBS (S1810, Biosera, UK), 100units/ml Penicillin/Streptomycin (Lonza, Switzerland, cat. No 09-757F) and 300μg/ml geneticin (G418).

BHK-21 cells[77] were used for propagation and titration of HSV-1. The medium required for cell culture was Glasgow MEM BHK 21 (Biosera, UK, cat. No L0221) supplemented with 10% New Born Calf Serum (Biosera, cat. No. NB-112), 10% Tryptose Phosphate Broth (Sigma-Aldrich, Germany, cat. No T8159) and 100units/ml Penicillin/Streptomycin (Lonza, Switzerland, cat. No 09-757F).

HSV-1 strain 17syn[78] was used as the wild type virus for all the experiments.

2.2 HSV-1 Virus Stock Preparation

HSV-1 was amplified in BHK-21 cells. Both the cell associated virus (CAV) and the cell released virus (CRV) were isolated. Upon extensive cytopathic effect (c.p.e.) BHK-21 infected cells were harvested. Supernatant and cells were separated by centrifugation at 2000 rpm for 10 minutes at 4^{o}C. The supernatant was kept separately for isolation of the CRV and the cells were further processed for isolation of the CAV.

For CAV isolation, the cells pelleted in the previous step were resuspended in 1ml serum-free DMEM and the suspension was subjected to 3 sonication cycles of 1 minute each at 50kHz intensity. Between the sonication cycles an 1 minute interval was allowed for the suspension to cool. It should be noted that the suspension should be kept on ice during the sonication process due to the intense heat generated by the sonication apparatus. Following sonication, the cell debris was pelleted by centrifugation at 2000rpm for 10 minutes at $+4^{0}$C.

The supernatant containing the virus was filtered in a 0.45μm filter (Sterile Acrodisc® Syringe Filters with Supor® Membrane, PALL Corporation, USA) and aliquots of the virus were made and stored at -80^{o}C.

For CRV isolation, the supernatant of the BHK-21 infected cells was centrifuged at 13,000 rpm for 2 hours at 4^{o}C. Following centrifugation the supernatant was discarded and the cell pellet was carefully resuspended in 1 ml serum-free DMEM. The virus was aliquoted and stored at -80^{o}C.

2.3 Plaque Assay

Plaque assay is a virology technique by which the viral titre can be estimated. For the titration of HSV-1, BHK-21 or Vero cells that are both susceptible to HSV-1 infection, were plated in a 6-well plate or 35mm dishes or alternatively in 60mm dishes. When cells were about 90% confluent, serial dilutions of the virus that was to be titrated were made, and the cells were infected. In specific, serial dilutions from 10^{-1} to 10^{-6} with cell-specific medium were made and each dilution was used in order to infect one well or plate of cells. Usually 100μl of the dilution were used for infection. It should be noted that the dilutions should be prepared on ice and that the medium covering the cells prior to infection should be reduced to minimum so that the virus is in close contact with the cells. Following infection, the plates containing the infected cells were transferred at 37^{o}C and were shaken every 10 minutes for 2 hours. Subsequently, the virus containing medium was removed and replaced with new medium containing 1/100 virus specific human IgG+ antibodies. The cells were finally incubated until plaques were formed in high dilutions.

Upon plaque formation, cells were fixed and stained so as to halt viral replication and count the plaques formed. For that reason, cells were carefully washed with cold PBS and fixed by the addition of methanol for 5 minutes. When methanol was removed, cells were allowed to air-dry and then stained by Giemsa. The cells were incubated with Giemsa solution for 10 minutes. Afterwards, Giemsa solution was rinsed carefully with tap water and cells were allowed to air-dry.

Following plaque assay and after Giemsa staining the plaques that developed during the plaque assay were counted and the viral titer was estimated by this formula:

$$x = \frac{\#plaques}{d * V}$$

Where X= the titer, d=dilution factor and V =the volume of diluted virus instilled on the cells.

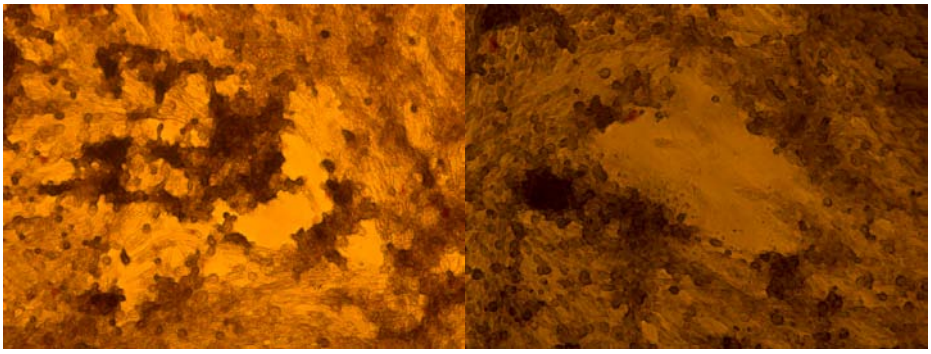

Figure 2.1: Plaques on BHK cells after fixation and Giemsa staining

2.4 Immunofluorescence

Immunofluorescence is a technique based on the use of fluorophores for visualization of various protein targets. In specific, secondary antibodies carrying these fluorophores make proteins visible through binding to specific for the protein primary antibodies.

In order to apply this technique cells were plated on cover slips placed inside wells of 24-well plates. Treatments on cells were maid according to the experiment taking place and the immunofluorescence protocol was used in order to visualize specific proteins. Before initiating the staining process, medium was removed; cells were gently washed with PBS and were subsequently fixed and permeabilized. In specific, 1x fixation solution (*Light Diagostics, CMV pp65 Antigenemia Immunofluorescence Assay, Cat.No. 5097, Millipore, USA*) was applied for 10 minutes and was subsequently removed. Following fixation, cells were again washed with PBS and they were permeabilized by addition of 1x permeabilization solution (*Cat.No. 5097, Millipore, USA)* for 10 minutes. After permeabilization cells were washed and in parallel blocked for non-specific signaling by a PBS-1%FBS wash. In succession, cells were stained with a primary antibody (table 2.1) diluted in PBS-1%FBS for 1 hour. The primary antibody was then removed and the cells were washed with PBS-1%FBS. After washing the cells the secondary antibody, also diluted in PBS-1%FBS, was added for 1 hour. Incubation has to take place in the dark because of the fluorophore's photosensitivity. When incubation with the secondary antibody was completed, the cells were washed and incubated with

10μg/μl DAPI solution (Invitrogen, USA, cat. No D1306) for 5 minutes. Cells were then washed and were allowed to air-dry. The dry cover slips were subsequently fixed on a slide with a drop of mounting solution (Ibidi, Germany, cat. No 50001) added between the cells and the slide to improve the optics of the sample. It should be noted that the mounting solution should be free of air bubbles and the cover slip should not be forced on the mounting solution but rather it should be left to diffuse evenly between the cells. Once the mounting solution has been evenly distributed the cover slip were sealed on the slide by the use of varnish. When the varnish was dry the stained cells were observed at a fluorescence microscope.

2.5 Protein isolation and determination of concentration.

In order to isolate proteins from infected and non-infected cells, cells were detached from culturing plates by incubation with PBS - 1Mm EDTA and proteins were extracted in suspension with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA, cat. No 78503) containing protease and phosphatase inhibitors (Thermo Scientific, USA, cat No 78415 and 78420 respectively) according to the manufacturer's protocol.

The protein extract concentration was determined with the Cayman Protein Determination Kit (Cayman Chemical Company, USA cat. No 704002).

2.6 Western Immunoblotting

Protein samples were analysed by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) on 0,75mm, 12%, polyacrylamide gels in a Mini PROTEAN Tetra Cell apparatus (BioRad Laboratories Ltd, U.K.). Electrophoresis was initiated at 80V and following entering of the proteins in the running gel, voltage was increased to 100V. The samples were subsequently transferred in 0,45μm nitrocellulose membrane (Thermo Scientific, USA, cat. No 88018) for 1 hour at 350mA in a Mini PROTEAN 3 Cell apparatus (Biorad Laboratories Ltd, U.K.) and the membrane was blocked by an 1h incubation with 5% non-fat milk(Regilait, France) diluted in TBST buffer. Next, the membrane was washed with TBST and incubated with various antibodies (Table 2.1) -depending on the protein to be detected, overnight at 4^{o}C. The membrane was washed with TBST to remove any remaining primary antibody and treated with the appropriate HRP conjugated secondary antibody (Table 2.1) for 1h at room temperature. After a wash in TBST, the membrane was developed by using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA, cat. No 34080) reagents

Luminol/Enhancer and Stable Peroxidase in a 1:1 ratio. The membrane was developed on radiography films (Fujifilm, Super-RX, Fujifilm Corporation, Tokyo) with a Kodak X-OMAT 1000 Processor (Kodak Int, USA).

Table 2. 1: Antibodies used in this study.

Primary Antibodies	Mouse Anti-Actin (Milipore, *Bilerrica, USA* cat. No MAB1501)
	Mouse Anti-β –Tubulin (Chemicon International, USA, cat. No MAB3408)
	Mouse Anti-HSV-1 ICP0 (Santa Cruz Biotechnology, U.S.A., cat. No sc-53070)
	Mouse Anti-HSV-1 ICP8 (Santa Cruz Biotechnology, U.S.A., cat. No sc-53329)
	Mouse Anti-HSV-1 gG Envelope Protein (Santa Cruz Biotechnology, U.S.A., cat. No sc-56984)
	Rabbit Anti-CD40 (Santa Cruz Biotechnology, U.S.A., cat. No sc-9096)
W.B. Secondary Antibodies	Goat Anti Mouse IgG (H&L) Peroxidase Conjugated Affinity Purified Antibody (Chemicon International, USA, cat. No AP124P)
	Goat Anti Rabbit IgG (H&L) Horseradish Peroxidase Conjugated Affinity Purified Antibody (Chemicon International, USA, cat. No AP132P)
I.F. Secondary Antibodies	Donkey Anti-Mouse Alexa Fluor 488 (Invitrogen, USA, cat. No A21202)
	Goat Anti-Rabbit Alexa Fluor 488 (Invitrogen, USA, cat. No A11008)

3. Results

3.1 Characteristics of the cells.

The cell lines used in this study were Monitored, providing valuable information for their treatment and the set up of the experiments. In specific, BHK21 cells replicate approximately every 14 hours, while traf2 +/+ have been found to replicate approximately every 22 hours. In addition, traf2 +/+ were tested for CD40 expression on their membrane by immunofluorescence (Fig. 3.1) and western blot analysis.

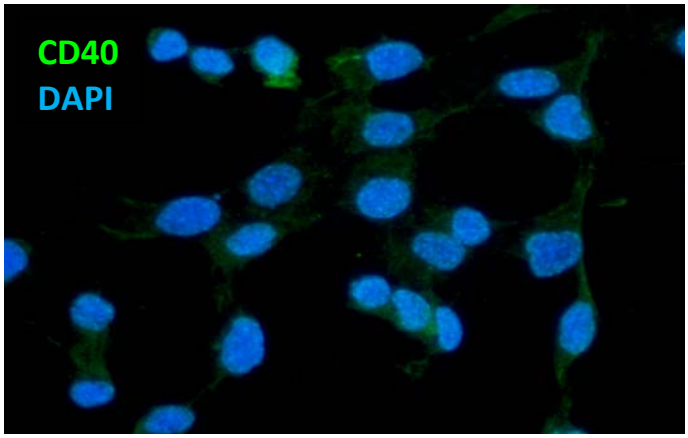

Figure 11: traf2 +/+ cells stained for CD40 (green =CD40, blue=nuclei stained with DAPI)

3.2 Reduction of viral titer with increasing hours of CD40L pre-treatment.

Traf2 +/+ cells were pre-treated with 0.5μg/ml CD40L (Bender MedSystems, cat. No BMS308/2) for 8 and 24 hours. Subsequently, the CD40L containing medium was removed and cells were infected with HSV-1 17syn at a multiplicity of infection (MOI[1]) 1. Supernatants were stored at 24 and 48 hours post infection; those were in turn used to infect BHK cells, in order to determine the titre of the virus collected from each condition.

With increasing hours of CD40L pre-treatment, the viral titre was found to decrease at supernatants collected 24h post infection, whereas at 48 hours post infection there was only a minor decrease of the viral titre at the pre-treated cells (Fig 3.2).

[1]MOI = Multiplicity of Infection and refers to the ration of infectious particles to infectious targets.

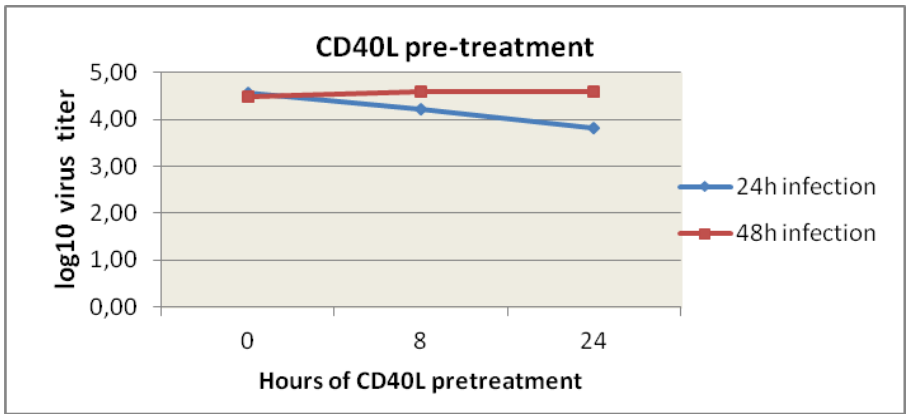

Figure 3.2: A decrease of viral titre is observed at 24h post infection with increased pre-treatment with CD40L whereas only a minor decrease is observed at 48h post infection.

3.3 Reduction of viral titer upon CD40L co-treatment.

Since increasing pre-treatment of cells with CD40L appeared to have an effect on the viral yield, we ventured to investigate the effect of CD40L treatment in parallel with HSV-1 infection. Traf2+/+ cells were treated with 0.5μg/ml CD40L and were at the same time infected with HSV-1 17syn at MOI 0.5. Supernatants were kept at 12, 24 and 30 hours post infection; those were in turn used to infect BHK cells, in order to estimate the titre of the virus collected from each condition.

In the presence of CD40L we observed an approximately 4-fold decrease in the viral yield at each time point. In addition, the rate at which the viral yield increases with time was greater for the untreated cells than for those treated with CD40L as one can see from the trend lines on Fig 3.3.

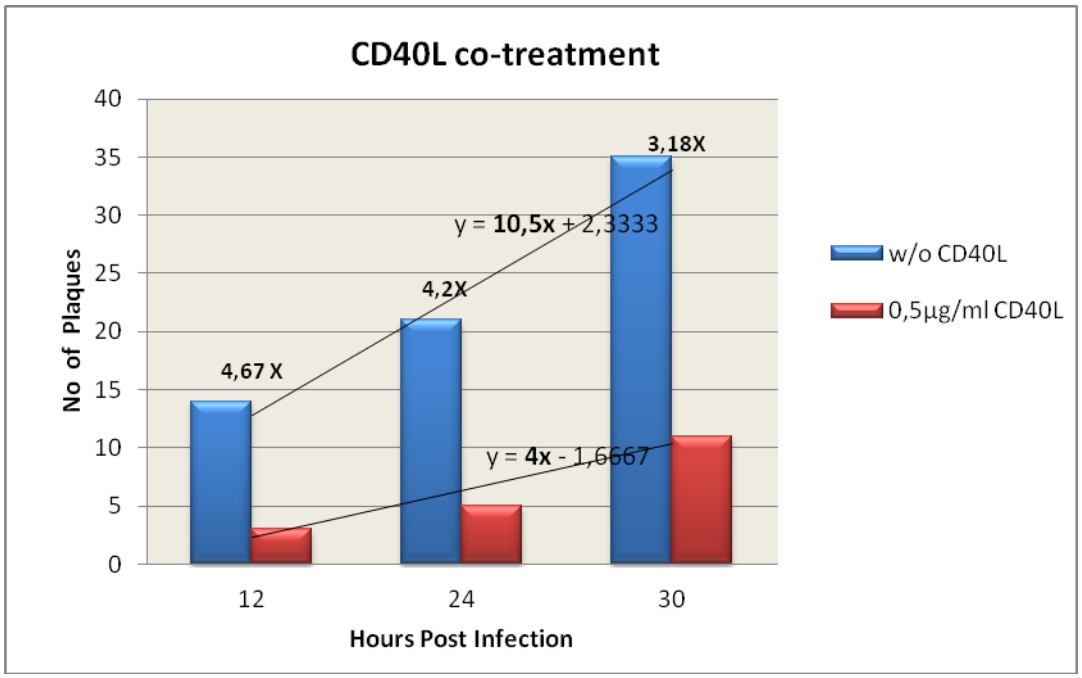

Figure 3.3: A decrease of viral titre is observed at each time point in comparison to the non-treated cells

At the protein level, a decrease in ICP8 is observed at 24 hours post infection while it is not clear what the case is for ICP0 (Fig 3.4).

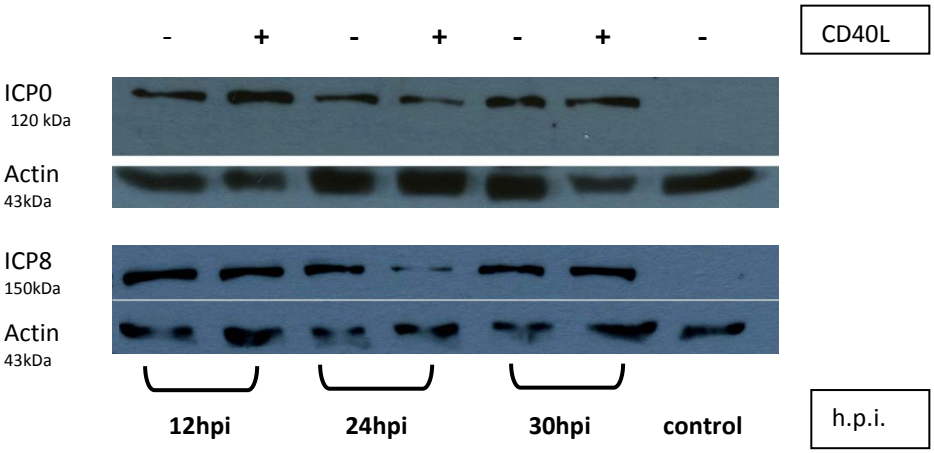

Figure 3.4: Western Immunoblotting for ICP0 and ICP8

4. Discussion

Ruby et al[75] in 1995 reported that CD40 ligand has potent antiviral activity and presented data for a direct antiviral effect of CD40L on HSV-1. In the present study we also report a potent, direct antiviral activity of CD40L on HSV-1. Our data show that in the presence of CD40L, viral yield decreases, particularly at the first replicative cycle of HSV-1, i.e. 18-22h post infection.

As presented in the results, for the CD40L pre-treatment experiment measurements were made at 24 and 48 hpi. Given that CD40 ligand is removed from the medium prior to HSV-1 infection, it can be appreciated that whichever antiviral effect CD40 ligand signalling might have, continuous signalling is required in order to sustain this effect for a prolonged period of time. Moreover, one can also infer that this is elicited during the first replication cycle of the virus that is, at the first 24 hours.

The observation that CD40L manifests its effect in the first 24 hours of viral infection and that increasing hours of pre-treatment leads to an increasing effect, led us to investigate the effect of CD40L in parallel with HSV-1 infection by treating cells with CD40L at the same time with infection. We demonstrated that way that the viral yield decreased 4-fold during co-treatment with CD40L and that the viral yield was mostly restricted in the first 24 hours. However, the viral yield remained also low at 30hpi since the viral progeny was low from the first replicative cycle of the virus. This is also depicted in mathematical terms via the rate of viral yield increase with time as compared between treated and untreated cells.

Since there was an indication that CD40L acts at the immediate early and early stages of HSV-1 life cycle we sought to identify any differences in the expression of the immediate early and early proteins ICP0 and ICP8 respectively. At the protein level however, the differences in the expression of those two proteins were not as major as expected. In particular, ICP8 appears to decrease in the presence of CD40L at 24 h.p.i. but it is not clear what the case is for ICP0 or gG, a late viral protein tested. Many repetitions were made in order to clarify the results but this was not achieved in the context of this thesis. In addition, traf2+/+ cells tended to lose CD40 expression as we observed by FACS measurements (data not shown) and this may be one of the reasons why we failed to produce replicable results. The above observations require further investigation and will be pursued.

It is intriguing though that the NFkB pathway, a pathway directly associated to CD40, is involved in HSV-1 infection in a virus-facilitating way. In specific, NFkB nuclear translocation

is induced in an HSV-1 dependent way leading to the prevention of apoptosis in the infected cell[78,79] which facilitates viral propagation. However, NFkB pathway acts in both a pro- and anti- apoptotic fashion according to the nature of the apoptotic stimulus as this has been demonstrated by various studies[80]. It has also been mentioned that CD40 orchestrates various signalling cascades depending on the TRAFs that bind on its cytoplasmic tail. It is thus interesting to speculate that CD40 can modulate the NFkB pathway so as to elicit an apoptotic instead of an anti-apoptotic effect which would lead to HSV-1 propagation arrest.

In conclusion, the identification of a new pathway implicated in HSV-1 life cycle such as that of CD40, would serve not only as a novel target for the treatment of HSV-1 infections but also towards understanding Herpes Simplex Virus type 1 biology.

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APPENDIX A (Western buffers)

Running buffer 10x (500ml)

Tris 0,25mM	15,15gr
Glycine 1,92mM	72,1gr
10% SDS	50ml
H_2O	Add up to 500ml
pH 8,3	

TBS-T 10x (500ml)

NaCl	40gr
Tris	12,1gr
Tween-20	5ml
H_2O	Add up to 500ml
pH 7,6	

Transfer buffer (1lt)

Running buffer 10x	100ml
MeOH	200ml
H_2O	700ml

Resolving Gel Buffer (RGB), 100ml

Tris base (1,5M)	18,15gr
SDS	0,4gr
pH 8,8	

Stacking Gel Buffer (SGB), 100ml

Tris (1M)	5,9gr
SDS	0,4gr
pH 6,8	

10% APS $(NH_4)_2SO_8$

Add 0,1gr $(NH_4)_2SO_8$ to 1ml of H_2O. (Store at 4°C for a week or make aliquots and keep them at -20°C.)

Sample Boiling Mix (3x)

SGB	1ml
20% SDS	1ml
Glycerol	1ml
14M β-mercaptoethanol	500µl
Bromophenol Blue Dye	Just enough to color the buffer.

Stripping buffer (500ml)

β-mercaptoethanol	3,5ml
2% SDS	10gr
Tris (62,5mM)	3,75gr
H_2O	Add up to 500ml
pH 6,7	

Running gel, 12%

Concentrated solution acrylamide* 30%	4ml
Resolving Gel Buffer (RGB)	2,5ml
Water for injection	3,3ml
10% SDS	100µl
TEMED (N,N,N',N'-tetramethylenediamine)	4µl
10% APS	100µl
Total volume	**10,004ml**

Stacking gel, 5%

Concentrated solution acrylamide 30%	850µl
Stacking Gel Buffer (SGB)	625µl
Water for injection	3,4ml
10% SDS	50µl
TEMED (N,N,N',N'-tetramethylenediamine)	5µl
10% APS	50µl
Total volume	4,980ml

APPENDIX B (Giemsa Staining)

10x Giemsa buffer	
KH_2PO_4	66,3gr
Na_2HPO_4	25,6gr
dH_2O	Add up to 1 Lt

Prepare 1x Giemsa buffer from concentrated 10x buffer by adding 10N NaOH as to adjust pH at 7.0