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THE INTERPLAY BETWEEN THE HUMAN HERPES SIMPLEX VIRUS TYPE 1 AND THE CASPASE-8 AND NF-KB SIGNALLING PATHWAYS

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ABSTRACT

Herpes simplex virus-1 (HSV-1) infects epithelial cells and establishes a lifelong latency in neuronal ganglia after primary infection. The ability of HSV-1 to replicate and reactivate from latency is due to several virulence factors by which the virus can evade the host antiviral response and persist for a lifetime. Indeed, despite many vaccines have been developed, the restriction of the infection is made difficult due to the high latency potential of the virus. Moreover, the effectiveness of antiviral therapies is limited by the development of antiviral resistance who is most common in immunocompromised individuals or patients with chronic and/or progressive infections or which have been exposed to prolonged antiviral therapies.

In this scenario, a comprehensive understanding of the interaction between the virus and the host immune system as well as the characterization of novel antiviral drugs is of crucial importance. Therefore, the research activity carried out during the PhD program was focused on the study of the immune response pathways mediated by HSV-1 and in parallel on the biological characterization of the antiviral activity of various compounds from natural sources.

HSV-1 is known to modulate several intracellular signaling pathways and transcription factors, including the FADD/caspase-8 death-signalling pathways and the nuclear factor-κB (NF-κB) pathway. Caspase-8 is the initiator caspase of the extrinsic apoptosis pathway in mammals. Classically, in response to viral infection, host cells undergo apoptosis to promote the elimination of infected cells and limit the release of progeny virus. Besides its role in apoptosis signalling, several reports showed that, under certain conditions, caspase-8 might direct the signalling toward cell death or pro-survival pathways. However, viral infection also stimulates an NFκB-dependent response which is essential to promote the expression of various anti-apoptotic genes. The balance between pro-apoptotic and pro-survival pathways strongly influences virus-host interaction and viral pathogenesis. Moreover, several host cellular microRNAs (miRNAs) have been reported to manipulate the NFκB-signaling pathway and apoptosis response upon viral infection. On the other side, the viral encoded Us11 protein is known to interfere with the host response to HSV-1 infection that leads to apoptosis. It has been previously shown that Us11 expression increases cell survival and protect the cells against heat and staurosporine-induced apoptosis.

Therefore, the aim of the present work was to investigate the role of Us11 in apoptosis signalling and its interaction with caspase-8 during HSV-1 replication, as well as the contribution of miRNA-146a as regulatory factor of the NF-κB pathway during HSV-1 replication. Moreover, the antiviral activity of polyphenol-rich extracts from pistachios kernel (*Pistacia vera*, L.) and almond skin (*Prunus dulcis*) was assessed against HSV-1. Indeed, despite the host immune response are usually effective against HSV-1 infections, the use of antiviral drugs is often required for the clearance of the virus. Furthermore, the increasing phenomena of drug-resistance represent a serious public health problem, and thus the discovery of novel antiviral therapies is of fundamental importance. The search for new safe and effective drugs has recently led to the discovery of molecules from natural sources for providing alternative therapeutic solutions to conventional drugs. Natural products are widely used globally for both preventive and therapeutic purposes. In particular, the natural skin of the almond and extracts from pistachios kernels represents a rich source of phenolic compounds, with important health beneficial properties. Therefore, the use of natural products such as flavonoids and polyphenols as antiviral drugs could represent an excellent tool for the development of topical drugs for the treatment of HSV-1 infections.

Chapter I

Biology of herpes simplex virus type 1 (HSV-1)

Herpes simplex type 1 (HSV-1) is a DNA virus belonging to the *Alphaherpesvirinae* family. HSV-1 is usually transmitted from infected to susceptible individuals following close human mucosal contact via transferring of infected-salivary secretions. HSV-1 pathogenicity is associated with various clinical manifestations, including orofacial lesions, known as “cold sores”, keratitis and conjunctivitis. Although HSV-related diseases are not fatal, in immunocompromised individuals, such as transplanted or AIDS patients and newborns, disseminated HSV infections are responsible for several illnesses including blindness and encephalitis which result in significant morbidity and mortality (Boehmer and Nimonkar, 2003).

Primary HSV-1 infection occurs in epithelial cells of the oral, nasal or ocular mucosa. Productive replication in epithelial cells generates lesions evolving from vesicles to superficial erythematous ulcerations, a characteristic cytopathic effect that lead to the shedding of the virus in the site of infection which persists up to 2 weeks (Arvin et al., 2007). Lifelong infection in the host by HSV is due to its ability to infect sensory neurons mainly those enervating infected tissues in the skin as well as oral and ocular mucosa. Thus, once the nerve termini are invading, the virions are transported in a retrograde manner from the initial site of infection to the axon of these cells. Sporadic HSV reactivation is achieved through anterograde movements of the infectious particles from these cells to infect other neurons that innervate the brain or to infect epithelial cells located near the initial site of infection where lytic replication ensues. The viral genome largely remains inactive during latency, except for the expression of the latency-associated transcript (LAT), a non-protein coding RNA that repress viral gene expression (Nicoll et al., 2016). Reactivation may occur spontaneously or in response to various stimuli including stress, cell cycle progression or immune pressure and results in increased expression of lytic gene and production of infectious virions (Suzich and Cliffe, 2018).

1.1. HSV-1: architecture, genomic organization and viral life cycle

The HSV virion particles have a spherical shape of 150-225 nm and it is made of three different elements: (i) an icosahedral capsid containing the DNA core, (ii) a proteinaceous layer called tegument surrounding the capsid and (iii) a lipid envelope exhibiting glycoproteins (Fig. 1). HSV-1 particles contain more than 30 different virion polypeptides (VP) named with serial numbers based on gel migration (e.g. VP1/2) or based on the open reading frame (ORF) encoding them (e.g., UL8), or as infected cell proteins (ICPs) (Knipe et al., 2002).

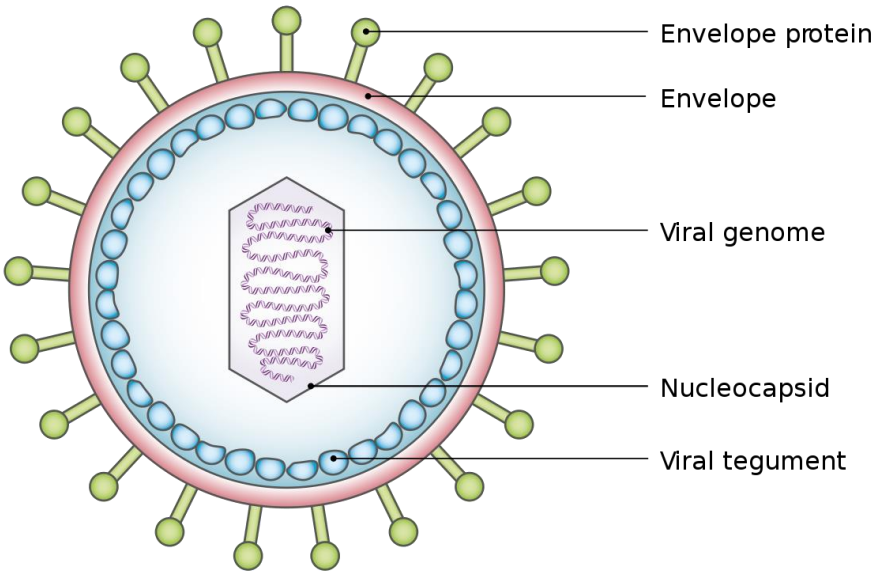

Figure 1. HSV-1 virion structure. The HSV-1 virion consists of the envelope, the tegument and the nucleocapsid. The envelope is constituted by a lipid layer derived from the host cell membranes and contains viral glycoprotein spikes on its surface. The viral genome is surrounded by the viral tegument proteins and formed the nucleocapsid.

Viral surface glycoproteins mediate the attachment and the penetration of the virus into host cells and they also elicit host immune responses to the virus. The tegument proteins are required early during the infection process. Among them, the transactivator protein VP16, also known as a trans-inducing factor or αTIF, and the virion host shutoff (VHS) protein, are necessary for productive infection (Taddeo et al., 2007). The capsid shell contains four different proteins, VP5, VP19C, VP23 and VP26. The capsid contains also the UL6 protein and the VP24 protease which are required for DNA packaging and

encapsidation, respectively (Cardone et al., 2012). The HSV-1 DNA consists of a single copy of a linear dsDNA of approximately 152 kilo base pair. The genome is arranged into two unique sequences designated as UL (unique long) and US (unique short), which are flanked by inverted repeat elements giving rise to different isoforms (Boehmer and Nimonkar, 2003) (Fig. 2).

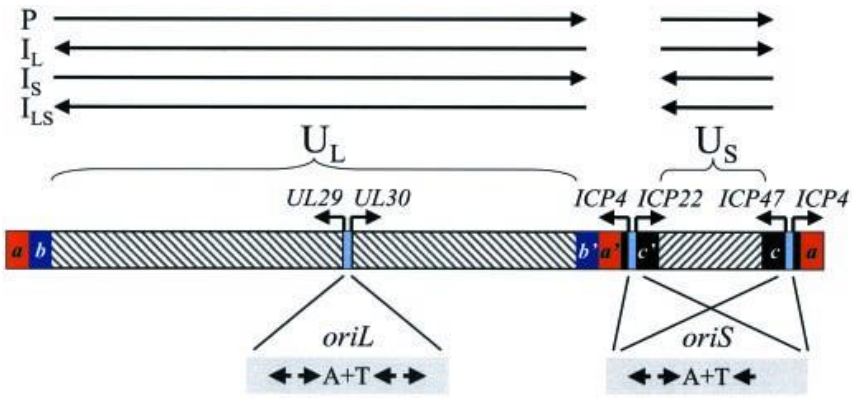

Figure 2. Schematic representation of HSV-1 genome and its isoforms by Boehmer and Nimonkar, 2003. The HSV-1 genome is arranged into two unique sequences designated as U_L (unique long) and U_S (unique short), which invert relative to each other by intramolecular recombination. The unique sequences are flanked by inverted repeat elements, designates as *ab* and *b'a'* and *a'c'* and *ca* which give rise to various isoforms defined as P (prototype), I_L (inversion of the L region), I_S (inversion of the S region) and I_{LS} (inversion of both the L and the S regions).

HSV entry is a cell-type-dependent mechanism, which occurred through plasma membranes fusion or endocytosis mechanisms. After the entry step, the capsid and the tegument proteins are transported through the cytosol and the viral DNA is released into the nucleus where the viral genes were transcribed through a sequential cascade (Ojala et al., 2000). The entry of HSV-1 into the host cells involves the interaction between at least four glycoproteins (gB, gC, gD, gH and gL) embedded in the envelope with specific cell surface receptors (Gianni et al., 2010). To date three alternative gD receptors have been identified and include: (i) the herpesvirus entry mediator (HVEM) also known as HveA, a member of the TNFR family; (ii) two intercellular adhesion molecules nectine-1and nectine-2; (iii) a modified heparin sulphate receptor 3-O-sulfated heparan sulfate (3-OS HS).

After the entry, several tegument proteins, such as VP26, VP1-2, and UL37 remain associated with the nucleocapsid and promote the capsid trafficking to the nuclear pore complex (NPC), along with the tubulin microtubules network and dynein/dynactin motor protein complex. The capsid binds to the nuclear membrane through a mechanism

mediated by importin β and the viral genome is released through the U_L6 portal protein (Ojala et al., 2000). Once in the nucleus, the linear genome is converted into covalently closed "endless" circular form (Strang and Stow, 2005). Viral transcription is regulated by both viral and cellular proteins and occurred in a well-orchestrated cascade, starting with the immediate early (IE) genes, followed by the early (E) genes, and finally the late (L) genes group. All viral transcripts are produced by the host cell RNA polymerase II (RNAPII) and are 5' capped and 3' polyadenylated.

Transcriptional activation is initiated by the tegument protein VP16, which enhance the recruitment of cellular transcription factors and promote and the transcription of IE genes (LaBoissière and O'Hare, 2000). The IE proteins ICP0, ICP4, ICP22, ICP27, and ICP4 are required to activate the transcription of E and L genes. In addition, ICP27 is also involved in the inhibition of cellular gene expression (Rice et al., 1989). The E genes include ICP8, an ssDNA binding protein, scavenger enzymes, such as the viral thymidine kinase and ribonucleotide reductase, as well as the viral DNA polymerase and DNA helicase (McNamee et al., 2000). The L genes encode structural proteins necessary for the assembly of infectious progeny virions, which traffic out of the nucleus to the Golgi for envelopment and egress out of the infected cell.

Transition to E gene transcription initiates replication of the HSV-1 genome. Although different tropism, the mechanisms of DNA replication are largely conserved during productive infection among the members of the *Herpesviridae*. In addition, many of the replication proteins encoded by HSV-1 represent functional analogues of the eukaryotic DNA replication machinery (Weller and Coen, 2012). Viral DNA synthesis replication initiates via binding of the U_L9 homodimers to one of the three viral origins of replication. U_L9 together with ICP8 bind to A+T- rich region, induces the unwind of the viral DNA and recruits the helicase/primase complex, which establishes a bi-directional replication fork, and the DNA polymerase which replicates the viral DNA (Aslani et al., 2002; He and Lehman, 2001). Replication of the viral genome proceeds by a theta mechanism and switches to a rolling circle mode, which leads to the formation of head-to-tail concatemers. Viral capsids accumulate and assemble in the infected cell nucleus and viral DNA is then inserted by an energy-dependent process. Tegumentation involves Golgi-derived vesicles, which contain the membrane glycoproteins. Vesicles are then transported to the cellular membrane where they fuse, and the mature enveloped viruses are released (Homa and Brown, 1997).

1.2. Immunity to HSV infections

The innate immune system contributes to the control of HSV-1 infection. Natural killer (NK), macrophages, dendritic cells (DCs) and monocytes have an important role in the restriction of virus infection by producing chemokines, interferons (IFNs) and other cytokines. The type I and type II interferons (IFNα/β and IFN-γ) are the major component of the innate immunity against viral infections and exert its activities through the induction of IFN-stimulated genes (ISGs) and activation of the JAK/STAT pathway. These genes encode the double-stranded RNA-activated protein kinase (PKR) and the 2'-5'' oligoadenylate synthetases (OAS) that actively participate in inhibiting viral replication by different mechanisms (Levy, 2001). PKR possess an amino-terminal regulatory domain and is activated by double-stranded RNA. PKR has a dsRNA binding activity and a carboxy-terminal catalytic domain responsible for its serine/threonine kinase activity. Upon binding to dsRNA generated during HSV-1 infection, autophosphorylated PKR forms dimers that phosphorylate the α subunit of the translation elongation factor eIF-2α and IκBα. Phosphorylation of eIF-2α results in its inactivation, leading to a general shutoff of protein synthesis and activation of host immune response including the FADD/caspase-8 death-signalling pathways and autophagy response (Balachandran et al., 2000; Cassady and Gross, 2002; Khoo et al., 2002).

Apoptosis is considered an immunological silent process, that can be initiated by extracellular ligands (extrinsic pathway), intracellular signals (intrinsic pathway) or a combination of these stimuli. Apoptosis signalling pathway culminates in activation of apical caspase-like caspase-8, which in turn activates the downstream caspase-3 and -7 to initiate an orchestrated cellular breakdown which results in the elimination of infected cells and/or degradation of viral components. Classically, the activation of apoptosis during HSV infection is considered an important virus-host interaction process. Moreover, the modulation of apoptosis by HSV is a multicomponent cell-dependent process that involves a wide variety of virus and host cell factors and plays an important role in the development of herpetic disease. Indeed, HSV-1 is able to induce and subsequently prevent the activation of apoptosis in epithelial cells during productive infection (Nguyen and Blaho, 2006). Additionally, immune cells such as natural killer cells (NK cells), macrophages and monocytes are more susceptible to HSV-1 induced apoptosis. Particularly, a "fratricide" mechanism has been observed during HSV infection in T cells, which showed increased expression of death ligands in their surface and induce

the apoptosis of neighbour T cells (Raftery et al., 1999). The fact that HSV-1 differentially modulates the apoptosis response in epithelial and immune cells may interfere with the host antiviral response and allow viral replication to occur in epithelial cells.

1.3. Immune evasion strategies

The ability of HSV-1 to replicate and reactivate from latency is due to several virulence factors by which the virus can evade the host antiviral response. Similar to other intracellular pathogens, HSV-1 modulates cellular antiviral defences to promote viral propagation (You et al., 2017). HSV counteracts the IFN-signaling pathways and PKR-mediated immune response through several viral products.

The Us11 and ICP34.5 gene products are required to inhibit PKR activity. The late protein ICP34.5 counteracts PKR function by reversing the PKR-mediated phosphorylation of eIF2α through the recruitment of the cellular protein phosphatase 1α (Chou et al., 1995; He et al., 1997, p. 5). Moreover, the Us11 protein act as an RNA-binding protein and inhibits PKR by binding to dsRNA and/or PKR (Peters et al., 2002; Poppers et al., 2000). ICP0 enhanced the resistance to IFN-α/β and inhibits the activation of ISGs (Eidson et al., 2002; Mossman et al., 2000) while ICP27 inhibits the IFN signalling and the activation and translocation of STAT1 to the nucleus (Johnson et al., 2008).

HSV encodes several anti-apoptotic proteins to counteract the apoptotic signalling pathway. Among others, the ICP6 protein (also known as R1 protein) is a well-defined apoptosis inhibitor. ICP6 is able to prevent the apoptotic response through its interaction with caspase-8 and RIPK1, which are two key proteins of the apoptotic pathway (F. Dufour et al., 2011; Florent Dufour et al., 2011; Guo et al., 2015). The serine/threonine kinase Us3 inhibits the UV-induced apoptosis as well as promote the phosphorylation of pro-apoptotic Bcl-2 family members Bad and Bid, which results in blocking mitochondrial apoptosis (Cartier et al., 2003; Munger et al., 2001; Munger and Roizman, 2001). Moreover, the expression of latency associated transcript (LAT) showed a protective effect against apoptosis in neuronal cells (Branco and Fraser, 2005; Henderson et al., 2002).

1.4. Antiviral therapies

HSV-1 infections are treated with antiviral drugs targeting viral DNA replication. To date, three classes of drugs were approved for the treatment of HSV infections, which are (i) acyclic guanosine analogues, such as Acyclovir, Ganciclovir and Famciclovir; (ii) acyclic nucleotide analogues such as Cidofovir; (iii) pyrophosphate analogues such as Foscarnet, (Arvin et al., 2007).

The mechanism of action of nucleoside analogues is similar. These drugs are preferentially phosphorylated in infected cells by the virus-encoded thymidine kinase (TK). Subsequently, host cell kinase converts the monophosphate acyclovir in acyclovir triphosphate which competes with deoxyguanosine triphosphate as a substrate for viral DNA polymerase. Since the nucleoside analogues lacks the 3'-hydroxyl group, their incorporation into the DNA chain results in blocking of viral DNA synthesis (Reardon, 1989). Valaciclovir and Famciclovir are two prodrugs that are rapidly converted to acyclic nucleoside analogue after oral administration, while Cidofovir is activated by unique phosphorylation and it is used against acyclovir- and foscarnet-resistant HSV isolates (Safrin et al., 1999).

Foscarnet, also known as phosphonoformic acid (PFA), is the analogue of inorganic pyrophosphate. It acts as a non-competitive inhibitor of viral DNA polymerases by interacting with the pyrophosphate binding site and preventing the cleavage of pyrophosphate on deoxynucleotide (Wagstaff and Bryson, 1994). Because Foscarned does not require phosphorylation to become active, it can be preferentially used against virus strain with a mutant or deficient thymidine kinase.

Indeed, the prolonged exposure to these antiviral drugs, specially ACV and its derivatives lead to drug resistance phenomena with a great increase in immunocompromised individuals (Jiang et al., 2016). HSV resistance to ACV is due to decreased production or deficiency in viral TK activity and/or mutant TK protein or DNA polymerase with altered substrate specificity. Moreover, ACV resistant strains are also not susceptible to other TK-dependent antiviral drugs (Morfin and Thouvenot, 2003). HSV-1 resistance is a serious public health problem and thus the development or discovery of novel antiviral molecules is of crucial importance.

Chapter II

Interaction between the viral protein Us11 and caspase-8 and its implication for viral replication.

Caspase 8 is the key enzyme of apoptosis cell death, a physiological process in which the unwanted, damaged or infected cells are removed from a multicellular organism. HSV-1 it is known to efficiently modulate apoptosis to promote its replication through the expression of several virulence factors. One of them is the tegument protein Us11, which is known to counteract the heat and staurosporine-induced apoptosis as well as the autophagy response. In the first part of this research project, the interaction between the viral protein Us11 and caspase-8 was investigated.

2.1. The HSV-1 Us11 tegument protein

The viral protein Us11 is a true late (γ_2) gene product which possess multiple functions (Johnson et al., 1986; Tohme et al., 2011). As a tegument protein, Us11 associates with the new capsids and is delivered into newly infected cells before to viral genes expression. However, despite its multiple functions, Us11 is not essential for viral replication *in vitro*.

2.1.1. The structure of Us11 protein

Us11 is a small basic phosphoprotein, expressed late during HSV infection, which is phosphorylated on serine residues and exhibit double-stranded RNA (dsRNA) binding proprieties. The Us11 protein is packaged into the virion as a tegument protein; the protein is delivered into infected cells after infection and accumulates in both nuclei and cytoplasmic fractions (Johnson et al., 1986; MacLean et al., 1987).

Us11 can be phosphorylated in its serine residues either by viral or cellular kinases. It has been supposed that the phosphorylation of Us11 may have a key role on post-transcriptional regulation of gene expression (Simonin et al., 1995). The predicted molecular weight of Us11 is 18 kDa approximately. However, in sodium dodecyl sulfate (SDS)-polyacrylamide gels Us11 protein migrate as a doublet of 21-23 kDa (Roller and Roizman, 1991).

2.1.2. The role of Us11 in immune escape mechanisms

The main identified function of Us11 is to counteracts the antiviral defense mechanisms by precluding the host protein shutoff. Particularly, Us11 acts as an inhibitor of cellular pattern recognition receptor (PRR)-mediated pathways that lead to the shutoff of protein synthesis, thus maintaining protein translation late in infection. Due to its dsRNA scavenging activity, Us11 prevents IFN-β production and interferon-stimulated genes (ISG) transcription via direct interaction with RIG-I and MDA-5 (Xing et al., 2012). Besides, Us11 represses the 2'-to-5′ (2'-5′) oligoadenylate synthetase (OAS), another cellular protein critical for antiviral host defence (Sànchez and Mohr, 2007). Finally, by interacting with the protein kinase-R (PKR), Us11 could interfere with PKR-mediated host immune responses, including the FADD/caspase-8 death-signalling pathways and autophagy response (Balachandran et al., 2000; Cassady and Gross, 2002; Khoo et al., 2002). Indeed, Us11 has been shown to protect HeLa cells from heat and staurosporine induced apoptosis as well as to inhibits autophagy through its interaction with PKR (Javouhey et al., 2008; Lussignol et al., 2013).

2.2. Caspase-8 structure, activation and cleavage

Caspase-8 belongs to the family of caspases which comprise several intracellular proteases synthetized as inactive zymogens into the cytosol. Caspases proteins contain a conserved catalytic domain consisting of a Cys side chain, which catalyzes peptide bond cleavage with specificity for protein substrates containing Asp residues. Among the apoptotic caspases group, caspase 8, -9, -10 belong to the "initiator" caspases subgroup, which activates "effector" caspases such as caspase-3, -6, -7 to execute apoptosis (Thornberry, 1998). The activation of the effector caspase is due to the intrachain cleavage, mediated by their upstream initiators, which results in enhancing the catalytic activity of the effector caspase by several orders. For the initiator caspases, the definition of activation is quite different from those of the effector caspases. Caspase-8 inert monomers require homodimerization for their activation, while maturation events involve autoproteolytic cleavage by caspase-8 itself. Both the dimerization and cleavage are mutually critical to each other for attaining a fully active caspase-8.

The human caspase-8 protein is a cysteine-dependent aspartate protease encoded by the CASP8 gene located on chromosome 2q33-34. It has been reported that alternative splicing of CASP8 mRNA generates eight different isoforms, caspase-8 a-h (Fig. 3). The predominantly expressed and easily detectable in cells of different origin are caspase-8a

(55kDa) and caspase-8b (54kDa), which only differ for an additional 15 amino acids in the linker between the prodomain and catalytic domains (Scaffidi et al., 1997). Caspase-8a is considered the canonical isoform and its sequence is mostly used as a reference. Procaspase-8 molecules are synthesized as single-chain inactive zymogens in the cytosol. The structure of procaspase-8 consists of a long prodomain containing two protein-protein interaction motifs defined as death effector domains (DED1 and DED2) of 180 amino acids and a C-terminal catalytic domain of ~260 amino acids composed of a ~20kDa large α subunit (p20/p18) containing the active site cysteine and a ~10kDa small β subunit (p12/p10) containing the substrate binding region (Pop and Salvesen, 2009).

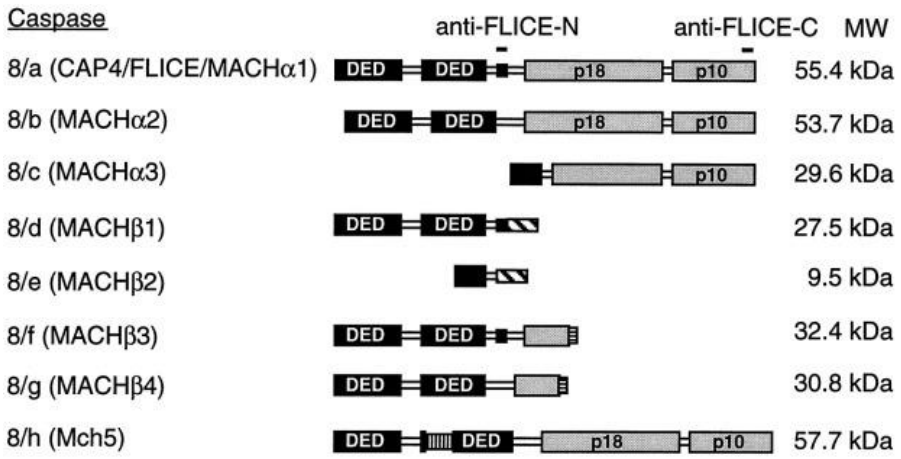

Figure 3. Caspase-8 isoforms by Scaffidi et al., 1997. The prodomain containing the death effector domains (DEDs) is indicated with black color and the active subunits p18 and p10 are in grey color. Hatched boxes show domains that differ from the sequence of caspase-8/a.

Dimerization of procaspase-8 molecules occurs at an oligomeric activation platform known as DISC (Death-Inducing Signaling Complex) subsequent to an apoptotic signal. Upon dimerization, procaspase-8 acquires enzymatic competency and specifically processes one another. However, maturation events are required for full activation of procaspase-8 as only mature caspase-8 can cleave effector caspases and other substrates (Chang, 2003).

Maturation events of procaspase-8a/b (55/53 kDa) involve autoproteolytic processing at conserved internal aspartic residues between the enzymatic subdomains or between the prodomain and enzymatic domain. The cleavage between the enzymatic subdomains at Asp374 (D374) and Asp 348 (D348) results in the separation of the large and small enzymatic subunits and thus generates two cleavage intermediates p43/p41

(43/41 kDa) and p10 (12 kDa). The cleavage between the prodomain and enzymatic domain at Asp 220 (D220) and Asp216 (D216) generates the fragments p26/p24 (26/24k Da) and p30 (30 kDa). Afterwards, additional cleavage of either p43 or p30 generates the fragments p18 and p10. Two cleavage products, p43 and p18, can cleave downstream substrates of caspase-8. The cleavage activity of the membrane-bound p43 is restricted to the plasma membrane while the subunits p18 and p10 form a heterotetramer $p18_2$-$p10_2$, which dissociate from the DISC and enters the cytosol as active caspase-8 (Fig. 4) (Kallenberger et al., 2014).

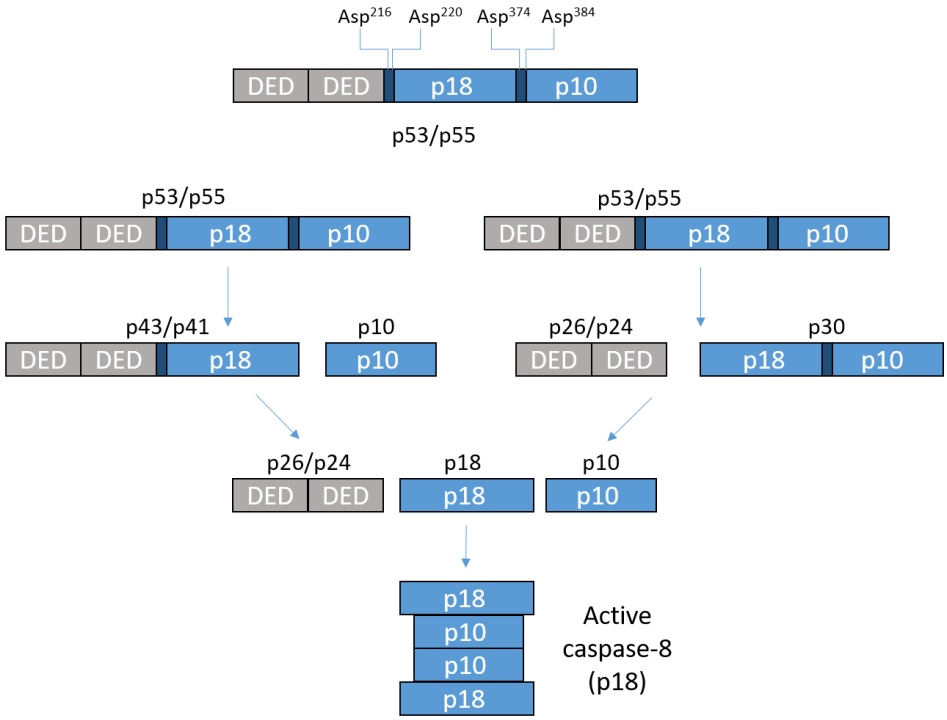

Figure 4. Cleavage site in procaspase-8 enzyme. Schematic representation of the cleavage sites and subdomains in procaspase-8 showing the two pathways through which the final products p26/p24, p18, and p10 can be generated. Cleavage at Asp374 (D374) and Asp 348 (D348) generates two cleavage intermediates p43/p41 and p10. Cleavage between Asp 220 (D220) and Asp216 (D216) generates the fragments p26/p24 and p30. Further cleavage of either p43/41 or p30 generates the p18 and p10.

2.2.1. Caspase-8 activity modulation by FLIP proteins

The cellular FLICE-like inhibitory proteins (c-FLIP) are the main apoptosis-regulatory proteins, which can influence caspase-8 activity. Similar to caspase-8, FLIP proteins contain two DED domains in their N-terminal domain and act as a catalytically inactive homologue of caspase-8 by competing with caspase-8 for the recruitment to the DISC. Moreover, FLIP proteins are able to heterodimerize with caspase-8 thus impinging on its activation and functionality depending on the specific context and on their level of expression (Peter, 2004). Human FLIP proteins are expressed in two splice forms c-FLIPS (short form, 27 kDa) and c-FLIPL (long-form, 55 kDa). $FLIP_S$ compete with caspase-8 monomers for the DED-mediated recruitment to the DISC and block activation of procaspase-8 to prevents apoptosis. Conversely, $FLIP_L$ forms heterodimers with caspase-8 monomers and altered its substrate specificity. Procaspase-8-c$FLIP_L$ heterodimerization generates p43-FLIP and p22-FLIP but does not results in processed procaspase-8, leading to activation of cell survival pathways (Tsuchiya et al., 2015).

2.2.2. Post-translational ubiquitination alters caspase-8 activity

The activation of caspase-8 downstream death receptors is also regulated by ubiquitination. Death receptor stimulation induces polyubiquitination of caspase-8 within the small subunit (p10), through the recruitment of a cullin3 (CUL3)-based E3 ligase in the DISC platform. The ubiquitin-binding protein p62/sequestosome-1 promote the aggregation of CUL3-polyubiquitinated caspase-8 leading to processing and full activation of caspase-8 heterotetramers (Jin et al., 2009). Conversely, polyubiquitination of caspase-8 in the large catalytic domain (p18), mediated by TRAF2 (TNF Receptor Associated Factor 2) ubiquitin ligase, promotes the proteasomal degradation of the protein upon autoprocessing and cytoplasmic translocation. The proteasome degradation of caspase-8 downregulates apoptosis response (Gonzalvez et al., 2012).

2.2.3. Phosphorylation of caspase-8 by cellular kinases

Phosphorylation events are crucial for modulating caspase-8 activity. The phosphorylation is mediated by the cellular tyrosine-protein kinases, also known as Src kinases, which phosphorylates caspase-8 on Tyr380, in the linker region between the large and the small catalytic subunits. Phosphorylation at Tyr380 blocks the cleavage to p43 and subsequent inhibits DR-mediated apoptosis (Cursi et al., 2006). Consistently, the

tyrosine phosphorylation drives the interaction of caspase-8 with several SH2 (Src Homology 2) domain containing proteins, including p85α subunit of phosphatidylinositol 3-kinase (PI3K), thus promoting the recruitment of caspase-8 in a multiprotein complex which are involved in cell adhesion, migration and cytoskeletal remodelling (Keller et al., 2018). Recently, it has been reported that Src kinase activity is aberrantly upregulated in many tumors consistently with the phosphorylation of caspase-8. Indeed, it has been established that phosphorylation on Tyr380 not only downregulates apoptotic signalling but also promotes new tumorigenic functions of caspase-8 including its ability to enhance cell adhesion, migration and *in vitro* neoplastic transformation (Fianco et al., 2016; Finlay and Vuori, 2007) (Fig. 5). In addition to Tyr380, other tyrosine residues, including Tyr334 and Tyr448 has been reported to be phosphorylated. However, the significance of these modifications is still unknown. Other phosphorylation site includes Ser347 and Ser387 which are operated by the serine/threonine mitogen-activated protein kinase p38-MAPK and the cyclin-dependent kinase 1 (CDK1), respectively. These phosphorylation events also impair caspase-8 activity and block CD95L-induced apoptotic signalling (Alvarado-Kristensson et al., 2004; Matthess et al., 2010).

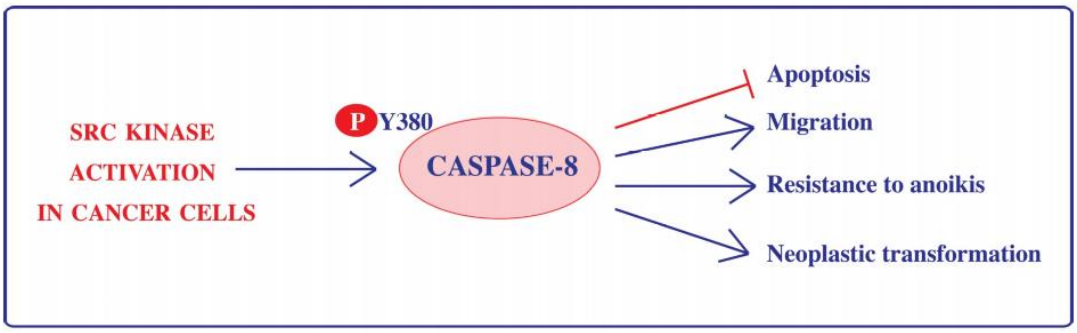

Figure 5. Phosphorylation on Tyr380 affect the functional role of caspase-8 by Fianco et al., 2018. Src kinase-dependent phosphorilation of caspase-8 on Tyr380 impairs the apoptotic function of caspase-8 and therefore promote the recruitment of caspase-8 in cytoskeletal remodeling, cell adhesion and migration, neoplastic transformation and resistance to anoikis.

2.3. Caspase-8 regulates programmed cell death pathways

Apoptosis, autophagy and necroptosis are three types of programmed cell death that are crucial for animal development, tissue homeostasis and viral pathogenesis. Caspase-8 exerts a key role in the crosstalk between apoptosis and autophagy as well as in the physiological suppression of necroptosis.

The extrinsic apoptosis pathway is initiated by ligation of death receptors (DRs), a subset of the TNF receptor (TNFR) superfamily, by their cognate ligands, which can also induce necroptosis. Thus, it has become evident that extrinsic apoptotic and necroptosis pathways are tightly interconnected one to another and are regulated by multiple proteins interactions (Tummers and Green, 2017). Unlike apoptosis, autophagy promotes cell survival by providing the turnover of intracellular constituents through their lysosomal degradation. However, under basal conditions, apoptosis and autophagy are cross-regulated and caspase-8 has been found to interact with autophagy-related proteins at molecular level to modulate the autophagy cascade (Wu et al., 2014).

2.3.1. The apoptotic cell death pathway

Apoptosis is a highly regulated process required for tissue development and maintenance of cellular homoeostasis in mammals. In the context of host-pathogen interactions, apoptosis is an important mechanism of immune defence by inducing the premature death of infected cell and limiting the replication and dissemination of the pathogens, while concomitantly alerting neighbouring cells.

The extrinsic or “death receptor-mediated” apoptotic pathway is initiated by extracellular ligands and culminate in the formation of the death receptor complex or ripoptosome platform in which caspase-8 became activated (Locksley et al., 2001). Alternatively, intracellular death signals, such as cytotoxic stress, internal or external DNA damage (UV or γ-radiation) and growth factor deficiency can trigger the intrinsic or “mitochondria-dependent” apoptosis pathway which is tightly regulated by the pro- and anti-apoptotic members of the BCL-2 protein family (Delbridge et al., 2016). In type I cells, the activation of caspase-8 alone is sufficient to activate procaspase-3 in response to receptor-mediated apoptotic stimuli. Cells in which the intrinsic apoptotic pathway is required to enhance the apoptotic signal are termed type II cells.

From the early stage of apoptosis, several changes occur in cells. Small protrusions became evident from the plasma membrane, and this phenomenon is defined

as “membrane blebbing”. Chromosomal DNA condensates and the nucleus is disintegrated into small fragments. Dying cells detach from the adjacent cells and condense into apoptotic bodies surrounded by the cellular membrane, which will be phagocytized by macrophages. Apoptotic cell death prevents the release of the intracellular components to avoid the immune response from occurring (Häcker, 2000).

2.3.2. TRAIL and Fas-mediated apoptosis

In the extrinsic apoptotic pathway, the activation of caspase-8 is triggered by the binding of extracellular death ligands, such as CD95L (also known as Fas ligand), TRAIL and TNF-α, with the death receptors (DRs) of the TNF receptor superfamily like FAS (APO-I/CD95), TRAIL-R (TRAIL-RI/DR4 and TRAIL-RII/DR5) and TNFR1, respectively. Among the DRs, the FAS receptor is the best studied one. FAS receptors possess an extracellular, transmembrane and cytoplasmic component (Guicciardi and Gores, 2009). The role of FAS and TRAILR is to drive caspase activation and apoptosis, while TNFR1 signalling is mainly linked to NF-kB activation and inflammatory gene transcription. Upon stimulation of DR4-DR5 (TRAIL) or FAS (FasL) receptors, the adaptor protein Fas Associated Death Domain (FADD) is recruited through the interactions between the death domains (DDs). Subsequently, FADD recruits procaspase-8 via homotypic interactions between their DEDs thus enforcing a local increase in caspase-8 concentration in the DISC and generating activity presumably by proximity-induced dimerization (Shen et al., 2018). The active caspase-8 operates the cleavage of the downstream molecule caspase-3 which in turn cleave the poly (ADP-ribose) polymerase (PARP) to initiate cell apoptosis.

Alternatively, caspase 8 could amplify apoptosis signalling by cleavage of Bcl-2 homology-3 (BH3)-only protein Bid to its truncated form tBid. The translocation of tBid to the outer mitochondrial membrane promote oligomerization of BAX and BAK is responsible for the mitochondrial outer membranes permeabilization (MOMP) and the release of pro-apoptotic proteins, such as cytochrome c, a second mitochondria-derived activator of caspase (SMAC) and apoptosis-inducing factor (AIF), from the intermembrane space into the cytosol (Wang and Tjandra, 2013). In the cytoplasm, the cytochrome c together with the apoptotic protease-activating factor 1 (Apaf-1) and caspase-9 forms a multi-protein complex defined as apoptosome, which serves as a platform for caspase-9 processing and activation. After activation, caspase-9

subsequently cleaves and activates downstream caspases -3 and -7, to orchestrate apoptotic cell death (Zou et al., 2003).

2.3.3. TNFR1 signalling, ripoptosome formation and necroptosis

Death receptor signal cascade not only culminates in caspase-8-mediated apoptosis but also results in TNFR1 induced pro-survival responses through activation of NF-kB or MAPK pathways (Micheau and Tschopp, 2003). TNF is expressed predominantly on the plasma membrane of activated macrophages and T lymphocytes (Sedger and McDermott, 2014).

The binding of TNF to TNFR1 induces the recruitment of TNFR1-associated death domain protein (TRADD) and receptor interacting protein kinase 1 (RIPK1) to the TNF receptor signalling complex known as TNFRSC or Complex I. TRADD interacts with TNF receptor-associated factor 2 (TRAF2), thus allowing the recruitment of the cellular inhibitor of apoptosis proteins (cIAP1/cIAP2) and the hetero-trimeric linear ubiquitin chain assembly complex (LUBAC). LUBAC, in turn, ubiquitylate NEMO and RIPK1 to promote TAK1- and IKK-dependent transcriptional activation of canonical NF-kB and transcription of inflammatory cytokine (TNF and IL-6) and pro-survival antiapoptotic proteins (c-FLIP and cIAPs) (Iwai et al., 2014). The ubiquitination of RIPK1 as well as its phosphorylation operated by IKKα, IKKβ and NEMO, prevents the association of RIPK1 with procaspase-8. Dysregulation of these regulatory mechanisms provokes the dissociation of complex I. Subsequently, de-ubiquitinated RIPK1 associate with FADD-procaspase-8 and form the complex IIa, also known as “ripoptosome”. RIPK1 then recruits RIPK3 through RIP homotypic interaction motifs (RHIM). The formation of this secondary complex is independent from receptor signaling and drive caspase-8 mediated apoptosis or caspase-indipendent necroptosis. In the first case, caspase-8 cleaves and inactivate both RIPK1 and RIPK3 and induces the disassembly of ripoptosome thus promoting apoptosis response (Feng et al., 2007).

Alternatively, when caspase-8 forms heterodimers with c-FLIP, caspase-8 activity towards its apoptotic substrate procaspase-3 and Bid is reduced and, although ripoptosoma is disassembled, apoptosis response is not triggered (Schilling et al., 2014). Consistently, when caspase-8 activity is compromised, by either high expression of FLIP or pharmacological inhibitors like zVAD-fmk or down-regulation of FADD expression, TNF stimulation culminates in necroptosis pathway through the formation of complex II-

b or “necroptosome”. Phosphorylation of RIPK1 and RIPK3 result in the oligomerization and activation of RIPK3, which in turn recruits, phosphorylate and activate the Mixed Lineage Kinase domain-Like (MLKL) protein (Sun et al., 2012). The active MLKL protein migrates to the plasma membrane and cause necroptotic cell death through membrane pore formation and the release of inflammatory host-derived factors and damage associated molecular pattern (DAMPs) (Cai et al., 2014).

2.3.4. Caspase-8 suppresses necroptosis

Necroptosis is a type of programmed cell death with an important role in various pathological processes, such as viral and bacterial infections, cancer, neurodegeneration and inflammatory diseases. Necroptosis can be induced by various stimuli, including lipopolysaccharide (LPS), TNFα and interferon γ (IFN-γ) that engage the Toll-like receptors (TLRs), TNF receptor family members or interferon receptors (IFNRs). Unlike apoptosis, necrosis is a caspase-independent process that is controlled by specific intrinsic programs and requires the assembly of a RIP homotypic interaction motif (RHIM)-dependent signalling complex of RIPK1 and RIPK3 (Fig. 6). The morphological features of necroptosis are cellular swelling, loss of cell membrane integrity cytoplasmic leakage, the release of proinflammatory agents and tissue damage (Cao and Mu, 2021). Caspase-8 exerts a key role in the physiological suppression of necroptosis. Indeed, inhibition of caspase-8 activity by pathogens/mammalian inhibitors or chemical inhibitors promote necroptosis downstream to DRs or TLRs stimulation. Genetic loss of the apoptotic machinery that is the caspase 8 gene, the FADD gene or the cFLIP gene (also known as CFLAR), results in embryonic lethality of mice knockout model. Interestingly, the phenotype of death in caspase 8 knockout mice is completely rescued by co-deletion of necroptotic machinery RIPK3 and MLKL as well. As a consequence, *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and *Mlkl*$^{-/-}$*Casp8*$^{-/-}$ as well as in *Fadd*$^{-/-}$ *Ripk1*$^{-/-}$ mice are healthy and viable (Kaiser et al., 2011). This evidence clearly confirms the peculiarity of caspase-8-FADD-cFLIP complexes, which regulates the choice between caspase 8-mediated apoptosis and the induction of programmed necrosis.

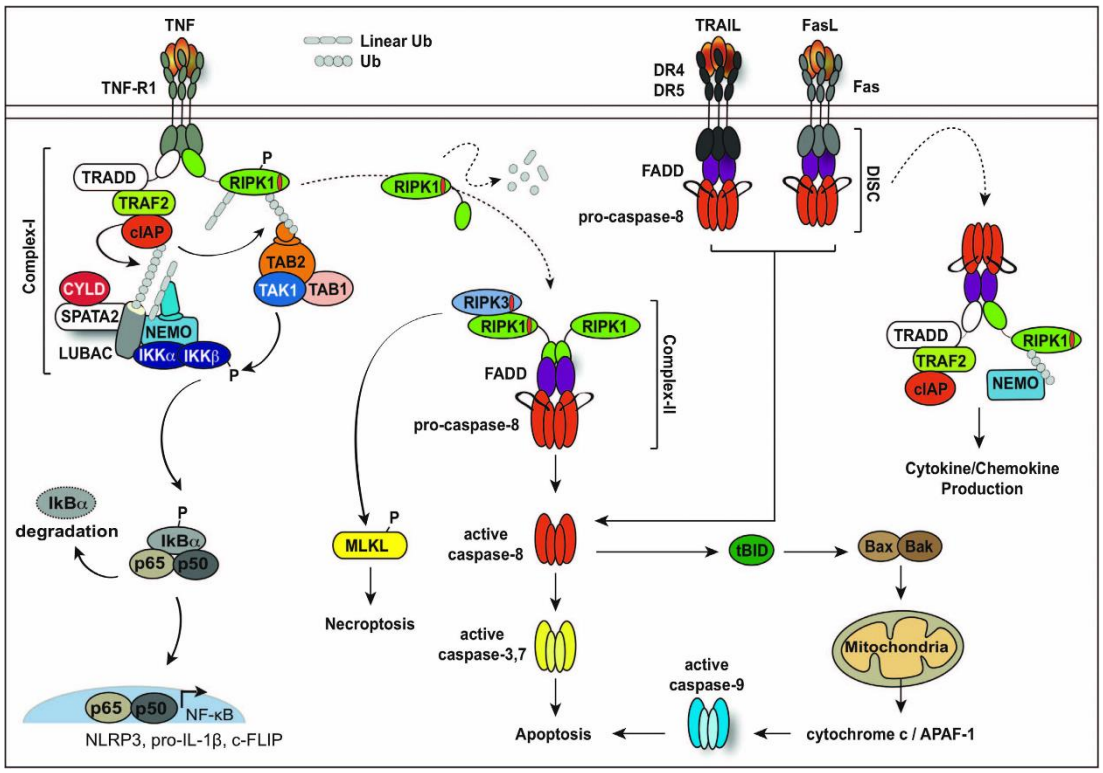

Figure 6. Caspase 8 regulate apoptosis and necroptosis pathway by Feltham et al., 2017. Schematic depicting the activation of the death receptor signalling pathways upon ligation of TNF-TNFR1, FasL-Fas and TRAIL-DR4/DR5. The stimulation of TNF-R1 with TNF induces the recruitment of the adaptor proteins TRADD and RIPK1. TRADD interacts with TRAF2 and recruits cIAP and LUBAC proteins which ubiquitylate RIPK1 and thus preventing the association of RIPK1 with caspase-8. Dysregulation of either the ubiquitylation or phosphorylation of RIPK1 causes the dismantling of Complex-I and stimulates the formation and activation of complex-II, which has the ability to drive apoptosis or necroptosis (when caspase activity is inhibited).

2.3.5. Caspase-8 regulating the crosstalk between apoptosis and autophagy

It has been extensively reported that caspase-8 acts as a switch point between autophagy and apoptosis, exerting an important role in cell-fate determination (Yu, 2004) (Fig. 7). Autophagy is a catabolic process tightly controlled by various autophagy related (Atg) proteins that drive the formation of autophagosome by which cytoplasmic components are degraded (Tanida, 2011). Beclin-1 act as a platform for autophagosome formation by binding several cofactors while the adaptor molecule p62 shuttles ubiquitinated proteins to autophagosomes (Furuya et al., 2005; Liu et al., 2016). Emerging evidence revealed a complex counter-regulation between autophagy and apoptosis at the molecular level, in which caspase-8 plays an important role. Particularly, activated caspase-8 can directly cleave autophagy proteins to promote a negative feedback loop and shutdown the autophagy response. In turn, autophagy proteins can regulate the activity of caspase-8 thus influencing apoptotic cascades.

Interestingly, it has been reported that the autophagosomes are required for intracellular DISC formation which is essential for caspase-8 self-cleavage and apoptosis activity (Young et al., 2012). Moreover, the adapter protein p62 may have an important role in facilitating the recruitment of caspase-8 to the autophagosomal membrane as the autophagy inhibition upregulates p62 and promotes caspase-8-dependent apoptosis (Huang et al., 2013). Conversely, autophagic degradation of caspase-8 enzyme can retain caspase-8-induced apoptosis (Hou et al., 2010). Besides, it has been demonstrated that caspase-8 mediates the cleavage and inactivation of Atg3, a critical regulatory component for autophagosome formation (Oral et al., 2012), and Beclin-1 thus downregulating autophagy and enhancing apoptosis (Wirawan et al., 2010). Data from in vitro studies demonstrated that caspase 8 is recruited during T cells clonal expansion, through interaction with FADD-Atg5-Atg12 complexes. Indeed, autophagy is required during T cell proliferation and FADD-caspase8 activity is necessary to prevent hyperactive autophagy and prevent RIPK1-dependent necroptotic cell death (Bell et al., 2008).

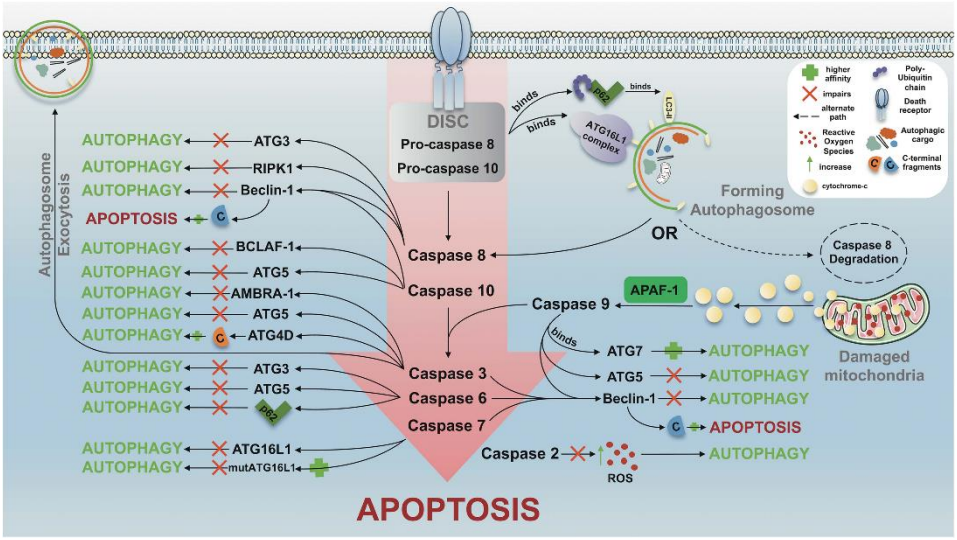

Figure 7. Caspase-8 regulates apoptosis and autophagy by Tsapras and Nezis, 2017. Procaspase-8 could be activated in autophagosome through the interaction with the ATG16L1-complex and its consequent degradation by lysosomal hydrolases upon autolysosome formation. Moreover, many ATGs proteins are recognized and cleaved by caspases. These interactions result in abrogation of the autophagic function of these proteins and the balance shifts to an apoptotic response.

2.4. Pro-survival function of caspase-8

The mechanism of caspase-8 activation is tightly regulated through multiple mechanisms that have been only partially elucidated. Thus, different activation pathways could trigger caspase-8 enzymatic activity and/or may promote the switch between its apoptotic and non-apoptotic functions. Indeed, increasing literature studies indicate that caspase-8 exert several non-apoptotic functions, including promotion of cell adhesion, embryonic development, monocyte differentiation, T and B cell proliferation, activation of NF-kB and tumorigenesis (Fianco et al., 2018; Shalini et al., 2015; Kang et al., 2004; Sordet et al., 2002). Significant understanding of the developmental role of caspase-8 came from studies on caspases knockout mice. The phenotype of caspase-8 knockout was dramatic as the knockout mice die *in utero* at around day 11 of gestation as a result of defective cardiac development and hematopoietic progenitor deficiency (Varfolomeev et al., 1998). In the context of carcinogenesis, caspase-8 expression is often lost, consistently with apoptotic signalling. However, in several tumors, including glioblastoma, caspase-8 activity is retained but does not culminate in apoptotic response while promoting tumorigenesis, through NF-kB dependent expression of cytokines (Fianco et al., 2017).

2.4.1. Caspase-8 in embryonic development

Several reports showed that caspase-8 protease activity is necessary for tissue formation during embryonic development. Caspase-8 knockout mice showed a phenotype of embryonical death due to developmental defects in the yolk sac vascular system, as well as hyperemia in blood vessels, liver erythrocytosis and impaired heart muscle and neuronal tube development (Sakamaki et al., 2002; Varfolomeev et al., 1998). Moreover, studies from a partial knockout of caspase-8, in which only the DED domain was expressed, revealed that the neural and heart defects are a secondary effect caused by the impairment of angiogenesis of the yolk sac (Sakamaki et al., 2002). This evidence was confirmed in another study, by using mice with a cell specific deletion of caspase-8 in endothelial cells generated by the Cre/loxP recombination system. Indeed, deletion of caspase-8 in endothelial cells resulted in the impaired formation of yolk sac vasculature, circulatory failure and embryonal death as reported for caspase-8 full knockout mice (Kang et al., 2004). Consistently, it has been shown that selective impairment of caspase-8 expression in T-cells leads to immunodeficiency in mice and humans as well (Chun et al., 2002; Salmena, 2003). Unlike mice, the phenotype seen in caspase-8 deficient humans

is mainly restricted to defect in the immune system. In humans, postnatal survival of individuals with inherited caspase-8 deficiency may be due to the function of caspase-10, which is a paralogue of caspase-8 in humans while mice do not have an orthologue of caspase-10. This evidence suggests that caspase-8 activation at the DISC is essential not only for cell death but also in embryonic development (Yeh, 1998; Yeh et al., 2000).

2.4.2. Caspase 8 in cancer

Disturbance of caspase-8 expression or function, through genetic, epigenetic and posttranslational changes, can confer resistance to cell death thus promoting carcinogenesis and tumor progression. Indeed, a characteristic hallmark of human cancer is its inability to undergo apoptosis in response to apoptotic stimuli. In some cancer, including gastric, colorectal and head carcinoma caspase-8 mutation have been identified, although with low frequency mutated caspase-8 block the signal transduction via the death receptor pathway by preventing the recruitment of wild-type caspase-8 to the death receptors upon their stimulation (Chun et al., 2002; Kim et al., 2003; Mandruzzato et al., 1997). Moreover, it has been reported that caspase-8 inactivation in neuroblastoma is mostly due to DNA methylation and gene deletion as well as to allelic imbalance on chromosome 2q (Takita et al., 2001; Teitz et al., 2000). Hypermethylation of regulatory sequences of the caspase-8 gene has been associated with a variety of cancer such as neuroblastoma, glioblastoma, retinoblastoma and lung carcinoma (Fulda et al., 2001; Harada et al., 2002; Hopkins-Donaldson et al., 2003, 2000). Caspase-8 function can also be impaired by a dominant-negative variant, the caspase-8 long (caspase-8L) isoform, which is generated by an alternative splicing process of the caspase-8 gene. Caspase-8L is proteolytically inactive since it contains only the two DED repeats and lacks the protease domain. Thus, it can be recruited into the DISC upon stimulation of death receptors and compete with the wild-type caspase-8, while remaining inactive and blocking the apoptotic signal transduction (Himeji et al., 2002; Miller et al., 2006; Mohr et al., 2005). As described above, phosphorylation of caspase-8 on Tyr380 by Src kinases promotes tumorigenic functions of caspase-8 including modulation of cell adhesion, migration and *in vitro* neoplastic transformation. Caspase-8 phosphorylation on Tyr380 has been associated with neoplastic transformation and resistance to anoikis in U87MG and U251MG glioblastoma cell lines (Fianco et al., 2016). Furthermore, it has been demonstrated that caspase-8 expression level correlated with the worst prognosis in

glioblastoma by sustaining NF-kB activation, cytokine secretion, neoangiogenesis and tumor growth (Fianco et al., 2017). Overall, these observations suggest that the protease activity of caspase-8 maybe switch off and its function rewired to sustain tumor growth and resistance to chemotherapy. (Stupack, 2013).

2.4.3. Caspase 8 is required for monocytes-to-macrophages differentiation

The activity of caspase-8 has been specifically related to the differentiation of monocytes into macrophages. This activation is specific to macrophages maturation as it is not detected during the differentiation of monocytes into dendritic cells. Circulating monocytes are secreted daily and they can infiltrate mucosal or inflamed tissue and differentiate into either macrophages or dendritic cells, while the excess monocytes are deleted through a process that may involve the Fas-dependent apoptotic pathway (Brown et al., 2004; Kiener et al., 1997).

Deletion of caspase 8 genes in myeloid cells suggest a critical role for caspase-8 during the differentiation process. (Kang et al., 2004). Consistently, limited caspase-8 activity has been reported in human peripheral blood monocytes stimulated with the macrophage colony-stimulating factor (M-CSF). However, caspase-8 activation does not lead to apoptotic cell death and interestingly, the exposure of monocytes to the broad-spectrum inhibitor benzyloxycarbonyl-Val-Ala-(DL)-Asp-fluoromethylketone (z-VAD-fmk) or the p35 baculovirus inhibitory protein inhibited the differentiation process (Sordet et al., 2002). Several studies demonstrated that caspase 8 activity modulates the cleavage of specific proteins involved in cytoskeleton rearrangements, changes in cell adhesion and transcriptional regulation required for the differentiation of monocytes into macrophages (Solier et al., 2017).

During *in vitro* macrophage maturation, M-CSF interacts with its receptor at the surface of peripheral blood monocytes and induces the activation of the phosphatidylinositol-3-kinase and the kinase AKT as well as the formation of a molecular platform which includes the adaptor protein FADD, RIPK1, FLIP and procaspase-8 (Jacquel et al., 2009; Rébé et al., 2007). Caspase-8 activation in this platform does not require interaction with death receptors and active caspase-8 drive the proteolytic cleavage of specific proteins mainly related to cytoskeletal reorganization. Several targets in M-CSF-treated monocytes were identified including nucleophosmin (NPM1), the plasminogen activator inhibitor-2 (PAI-2), the serine/threonine-protein kinase PAK-

2 (p21-activated kinase 2), α-tubulin, β-actine, vinculin-α and several others (Cathelin et al., 2006). The cleavage of RIPK1 mediated by caspase-8 prevents sustained NF-kB activation, which in turn favour the macrophages differentiation process (Rébé et al., 2007). By contrast, unlike downstream effector caspase, such as caspase-3 and 7 are cleaved, many other apoptotic targets are not cleaved, no phosphatidylserine molecules are exposed on the cell surface and no DNA fragmentation is detected (Cathelin et al., 2006; Sordet et al., 2002). The fact that during monocyte differentiation some apoptotic-related proteins such as the poly ADP-ribose polymerase and lamin B, both targets of caspase-3, are protected from processing, suggest that a selective cleavage of substrates is an important regulatory mechanism between differentiation and apoptosis. Another pathway triggered by the M-CSF in monocytes is autophagy (Jacquel et al., 2012a, 2012b; Obba et al., 2015). Indeed, both ATG5 and p62, keys protein in autophagy, were found to co-localize with caspase-8, FADD, FLIP and RIPK1, suggesting that the induction of autophagy occur in the same subcellular compartment required for activation of caspase-8 during monocytes differentiation (Jacquel et al., 2012b). Overall, due to the importance of caspase-8 in the monocyte differentiation process, the understanding of molecular mechanism behind its activation and the identification of its substrates could be an interesting target for human malignancies that affect monocytes population, including chronic myelomonocytic leukaemia.

2.4.4. Caspase-8 is a key regulator of T cell activation

In the immune system, apoptosis is required for the homeostatic balance of lymphocytes number to allows a proper immune response and avoid autoimmunity. The lymphocyte apoptosis is triggered by the Fas-dependent pathway. It has been reported that heterozygous mutations on Fas, FasL and caspase-8 genes caused pleiotropic defects in lymphocyte activation and lead to immunodeficiency in humans (Chun et al., 2002; Fisher et al., 1995; Wu et al., 1996). A prominent feature of human homozygous caspase-8 deficiency is defective lymphocyte apoptosis during the negative selection of autoreactive double-positive (CD4+/CD8+) T cells resulting in a rare autoimmune lymphoproliferative syndrome (ALPS) characterized by lymphadenopathy, splenomegaly and autoimmune phenomena. Besides, defects in the activation of T and B lymphocytes and NK cells due to caspase-8 deficiency was found to be accompanied with severe infection by mucocutaneous herpes simplex virus and weak response to

immunization (Chun et al., 2002; Rager-Zisman et al., 1987). However, the combined T, B, and NK cell immunodeficiency is not seen in ALPS patients with Fas, Fas ligand, or caspase-10 mutations.

During T cell proliferation, caspase-8 is activated thought a pathway involves T cell receptors (TCR), upon their stimulation with CD3. Caspase-8 activity results in selective substrate specificity and the pharmacological inhibition of caspase-8 with the caspase inhibitors z-VADfmk or z-IETD-fmk decreased T cell proliferation and interleukin-2 (IL-2) production (Alam et al., 1999; Kennedy et al., 1999). Additionally, not only caspase-8 but also deficiency of two other members of the DISC complex, FADD and FLIP, both showed a very similar phenotype (Newton, 1998; Walsh et al., 1998; Yeh et al., 2000; Zhang et al., 2008).

The molecular mechanism of caspase-8 inhibition and immunodeficiency is due to the ability of caspase-8 expression to promote NF-kB nuclear translocation, an essential cellular factor in T cell activation. Indeed, several evidence demonstrated that caspase-8 activation is required for NF-kB activation, downstream to TNFR, through its N-terminal prodomain, without the requirement of enzymatic activity (Chaudhary et al., 2000; Hu et al., 2000). Consistently, caspase-8 deficiency abolishes activation of NF-kB upon stimulation by antigen receptors in humans and mice as well. Upon its activation, caspase 8 associate with the T cell signalling molecules B cell leukaemia/lymphoma 10 (Bcl10) and the paracaspase mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1), thus recruiting the IκB kinase (IKK) to the TCR signalling platform, resulting in NF-kB activation (Su, 2005). Additionally, it was further showed that, upon TCR stimulation, the ubiquitin ligase TRAF6 interact with active caspase-8 and facilitates its translocation to the lipid raft where NF-kB activating substrates are cleaved and apoptosis inducing substrates remain unprocessed (Bidère et al., 2006). Thus, the phenotype of caspase-8 knockout T cells can be explained by a diminished NF-kB activation after TCR stimulation. Since NF-kB is a known antagonist of apoptosis, caspase-8 has a critical role in regulating the balance between cell survival and apoptosis during the clonal expansion of T cells. Additionally, several reports indicate that the lack of caspase-8 drives from an apoptotic towards a necrotic like autophagic cell death (Vercammen et al., 1998; Yu, 2004). Moreover, a FADD/caspase-8-signaling axis was found to promotes T cell cycle progression through sustained S6K activity which contributes to the S-phase entry during the cell cycle (Arechiga et al., 2007).

2.5. Aim of the research

In response to viral infection, host cells are susceptible to apoptosis, which ensure the degradation of viral factors with consequent elimination of infected cells in order to limit the release of progeny virus. Among other, herpes simplex virus type 1 (HSV-1) modulate the apoptosis pathways to subvert cellular antiviral defences and promote viral propagation (You et al., 2017).

Infection of human epithelial cells with the wild-type HSV-1 leads to both the induction and prevention of apoptosis in a specific "prevention windows" kinetic model. HSV-1 can induce apoptosis immediately after the virus entry to the host cell probably due to viral transcription or mRNA processing event. However, the expression of specific HSV-1 proteins synthesized after 3h but before 6h post-infection (prevention windows) act to prevent the process of cell death (Aubert and Blaho, 1999). Indeed, it has been reported that the prevention of cell death during HSV-1 infection is dependent on anti-apoptotic viral factors, including the immediate-early genes ICP4, ICP27 and ICP22 (Aubert et al., 1999; Aubert and Blaho, 1999, p. 4; Leopardi and Roizman, 1996), the glycoproteins gD and gJ (Zhou et al., 2000), the protein kinase Us3 (Leopardi et al., 1997), the latency-associated transcript (LAT) (Perng, 2000) and the ribonucleotide reductase large subunit (R1) (F. Dufour et al., 2011; Florent Dufour et al., 2011). Additionally, apoptosis prevention in a human cell is also modulated differently by different virus strains such as HSV-1 (KOS1.1) and HSV-1 (F) (Aubert et al., 1999). Interestingly, HSV-1 also prevents apoptosis induced by external stimuli (e.g. cycloheximide, staurosporine, osmotic shock, TNF or anti-Fas antibody) by the same mechanism that blocks virus-induced apoptosis (Adachi and Koyama, 1997; Aubert et al., 1999; Galvan and Roizman, 1998; Koyama and Miwa, 1997). Unlike permissive epithelial cells, in which HSV-1 prevent apoptosis, immune cells, including human monocytes, dendritic cells and T lymphocytes are more sensitive to HSV-1-induced apoptosis (Bosnjak et al., 2005; Iannello et al., 2011; Ito et al., 1997; Kather et al., 2010; Mastino et al., 1997). In response to viral infection these cells represent an important cellular component of the immune system. However, the mechanism of this virus-induced apoptosis in monocytes is not fully understood as well as the biological implication for viral replication and antiviral immunity.

The viral protein Us11 is known to interfere with the cellular response that can lead to apoptosis by interacting with the cellular kinase PKR (Balachandran et al., 2000;

Cassady and Gross, 2002; Khoo et al., 2002). Therefore, the aim of the present research was focused on the role of Us11 in the apoptosis signalling pathway during HSV-1 replication. Among other caspases, caspase-8 is considered a key enzyme for apoptosis cell death, and thus the interaction between Us11 and caspase-8 as well as the role of caspase-8 in HSV-1 infection was deeply explored.

The study was focused on three main aims: (i) verify the impact of Us11 deletion on canonical caspase-8 functions related to apoptosis response; (ii) verify the physical interaction of Us11 and caspase-8 in the contest of viral infection as well as in a cell-free in vitro system (iii) investigate the role of caspase-8 on HSV replication. Given their appreciable apoptotic response, the acute leukaemia THP-1 cell lines were used as a cellular model for these investigations. Besides, 293T cells were employed to perform a transfection experiment and a caspase-8 deficient HEp-2 cell line ($CASP8^{-/-}$) were used to study the role of caspase-8 in the HSV-1 replication process. A combinatory approach of HSV-1 mutant (R3630-ΔUs11/ΔUs12) and recombinant (HSV-1-VP26GFP) viruses as well as Us11- and Us12-encoding plasmid were employed to investigate the ability of Us11 to interact with caspase-8 in immune cells, as well as in non-immune epithelial cells, which are the fully permissive model for HSV-1 infection. Moreover, GST-Us11 and GST-caspase-8 recombinant proteins were produced and used to verify the interaction of these proteins in a cell-free system as well.

2.6. Materials and Methods

Cell culture

VERO (African green monkey kidney), 293T (human embryonic kidney), HEp-2 (human HeLa contaminant carcinoma), THP-1 (human acute monocytic leukemia) cells were all originally obtained from ATCC (https://www.atcc.org/). VERO cells were cultured in DMEM medium (Corning) supplemented with 6% fetal bovine serum (FBS, Euroclone). 293T cells were cultured in DMEM supplemented with 10% FBS. HEp-2 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS. THP-1 cells were cultured in RPMI-1640 medium (Corning) supplemented with 10% FBS (Euroclone), 4,5g/L D-glucose (Sigma-Aldrich), 1mM Sodium Pyruvate (Sigma-Aldrich), 10 mM Hepes buffer (Sigma-Aldrich). All culture media were supplemented with mixture of 100 I.U./ml penicillin and 100 µg/ml streptomycin (Lonza, Belgium). All cell lines were incubated at 37° C whit 5% CO_2.

Viruses

The wild type herpes simplex virus type 1 (HSV-1) and the recombinant R3630 were kindly provided by Professor Bernard Roizman (University of Chicago). HSV-1 (F) is the prototype HSV-1 strain F, whereas the recombinant R3630 virus is lacking the genes Us11 and Us12. HSV-1-VP26GFP virus, expressing a GFP tagged capsid protein VP26 was described previously (Siracusano et al., 2016). Viral stocks were propagated and then titrated in VERO cells. For experimental infection HSV-1, R3630 and HSV-1-VP26GFP diluted in medium or medium alone (mock-infected) were adsorbed onto cells for 1 h at 37 °C in 5% CO_2 with gentle shaking, at different multiplicity of infection (MOI). The inoculum was then removed and replaced with fresh medium, cells were incubated at 37°C in 5% CO_2 and collected at the indicated times post infection (p.i.) to perform experiments. The MOI used for experimental infection was MOI 10 for HEp-2 and 293T cells and MOI 50 for THP-1 cells.

Protein extraction and immunoblot analysis

Cell pellets were collected at the indicated time after infection or transfection, washed in 1X phosphate-buffered saline (PBS) and lysed with cell lysis buffer (Cell Signaling Technology). An equal amount of protein extracts was subjected to Sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad Life Science Research, Hercules, CA), blocked and reacted with primary antibody and appropriate secondary antibody, followed by chemiluminescent detection. Quantitative densitometry analysis of immunoblot band intensities was performed by using the TINA software (version 2.10, Raytest, Straubenhardt, Germany).

Antibodies and reagents

Caspase-8 (human) monoclonal antibody (12F5; ALX-804-242) directed against the p18 subunit was purchased from Enzo Life Sciences. Monoclonal anti-US11 and anti-ICP8 were provided by professor Bernad Roizman. Anti-GAPDH (sc-32233), anti-HSV-1 UL42 (sc-53333) and goat anti-mouse IgG, F(ab')2-PE (sc-3798) were purchased from Santa Cruz Biotechnology. Anti-caspase 3 (#9662), anti-PARP (#9542) and anti-Rab5 (#3547) were provided from Cell Signaling Technology. Anti-LAMP1 (ab24170) and secondary HRP-conjugated anti-mouse IgGVeriBlot for IP were from Abcam. Secondary HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Millipore. The z-IETD-FMK caspase-8 inhibitor (ab141382) was purchased from Abcam.

Immunofluorescence assays

For immunofluorescence analysis cells were layered on polylysinated slides, fixed in 4% paraformaldehyde (PFA 4%) in PBS 1X for 15 min and permeabilized with 0.1% Triton X-100 in PBS 1X. Cells were washed three times with PBS 1X and incubated with the primary antibody for 1 h at 37 °C, followed by incubation with the phycoerythrin (PE)-conjugated anti-rabbit antibody for 1h at 37°C. Nuclei were stained with Hoechst 2,5 μg/mL. Samples were analyzed on a fluorescence microscope.

Immunoprecipitation

THP-1 cells were infected or mock-infected with HSV-1 at MOI 50, collected at 18h p.i. and lysed with cold lysis buffer (20 mM Tris-HCl pH 8, 1 mM EDTA, 200 mM NaCl, 1% Nonidet P-40, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM NaF, 0.1 μg/mL Protease Inhibitors). The supernatants were collected and precleared with 50% of protein-A slurry for 18 h. Immunoprecipitations were performed with 5 μL of the anti-Us11 monoclonal

antibody pre-adsorbed on protein A-Sepharose beads (Amersham Pharmacia Biotech AB) for 2 h at 4 °C. After overnight incubation, complexate-beads were resolved by SDS–PAGE and transferred to nitrocellulose membranes (Biorad). Immunoblotting was performed by using anti-Caspase 8 antibody (12F5 Enzo) and secondary antibodies specific for IP (Abcam).

Construction of recombinant Baculoviruses

Full length caspase-8 isoform *a* was cloned into the pAcGHLT-A baculovirus transfer vector (PharMingen) derived from pAcG1 vector and containing a 6xHis tag and a glutathione S-transferase (GST) tag upstream of the MCS (multiple cloning site). An *NdeI*/*Not*I fragment containing the coding sequence of the full-lenght caspase 8 was amplified by PCR from a cDNA template from THP-1 cells by using the following primers: Fw-*NdeI*-5'-ggcatatgcatggacttcagcagaaatctttatgatattg-3', Rev-Casp8-*NotI*-5'-ttgcggccgctcaatcagaagggaacagaagtttttttc-3'. The recombinant plasmid was generated by inserting the *NdeI/NotI* fragment containing the Caspase 8 coding sequence into the *NdeI/NotI* -digested plasmid pAcGHLT-A. The Caspase 8-pAcGHLT-A plasmid sequence was analyzed after cloning. The recombinant GST-Caspase8 baculovirus was generated by cotransfection of Sf9 insect cells with the Caspase8-pAcGHLT-A transfer plasmids along with baculoGold DNA (PharMingen), according to the manufacturer's instructions and with the aid of the Mirus TransIT®-2020 Transfection Reagent reagent. The Us11 coding sequence from Us11-pRB5850 (Sciortino et al., 2002) was digested with EcoRI/BglII restriction enzyme and subcloned into pAcGHLT-A baculovirus transfer vector. The recombinant GST-Us11 baculovirus was generated by cotransfection of Sf9 insect cells with the Us11-pAcGHLT-A transfer plasmids along with baculoGold DNA (PharMingen), according to the manufacturer's instructions. All the recombinant baculoviruses were amplified in Sf9 cells and the expression of the recombinant proteins was verified by western blot analysis of the GST-tagged proteins.

Purification of GST-Caspase 8 proteins

The recombinant GST-Caspase 8 protein was produced by infecting Sf9 cell with the recombinant baculovirus and incubating the cells for 3 days at 27°C. The cells were then collected through centrifugation at 1500 rpm for 5 min at 4°C. The cells pellet was lysed in ice-cold Insect Cell Lysis Buffer (Cat. No. 21425A) containing reconstituted

Protease Inhibitor Cocktail (Cat. No. 21426Z) for 45 min on ice. The lysate was cleared from cellular debris by centrifugation at 14000 rpm for 1h at 4°C. The clarified lysate, which contain the recombinant protein, was then incubated with pre-washed GST-agarose beads for 18h at 4°C (10:1 ratio insect cell lysate: GST-agarose beads). After the incubation time the slurry beads were washed three times with 5 bead volumes of PBS 1X for 20 min at 4°C and centrifuged at 1500 rpm for 5 min to sediment the matrix. from SDS–page was performed to determine the binding capacity of the glutathione beads using the supernatant fractions as a control and to quantify the amount of GST fusion protein that bound to the matrix by Coomassie blue-staining of the poliacrilammide gel.

Caspase 8 in vitro cleavage assay

In vitro Caspase 8 cleavage assay was performed in a cell-free system using Caspase 8 assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol). GST-Us11 recombinant protein was incubated with GST-Caspase 8 recombinant protein in Caspase 8 assay buffer for different time from 45 min up to 3h. After the incubation time the activation of Caspase 8 was verified by western blot analysis.

Transient transfection

The cells were transiently transfected with pUs11 and pUs12 plasmids, separately. The Us11 and Us12 plasmids were constructed as described previously (Sciortino et al., 2002). pcDNA 3.1 (Invitrogen) was used as a transfection control. Briefly, 24h prior transfection a total of 3×10^6 cells were seeded in 6-well plates in DMEM medium (Lonza) supplemented with 10% FBS (Euroclone). 1.5 μg of total DNA representing the plasmids above was incubated with Lipofectamine Reagent Plus (Invitrogen) and in OptiMEM medium (Gibco) according to the manufacturer's instructions. The DNA-Lipofectamine mixture was then added to cultured cells and incubated for 4h at 37°C. The medium was then replaced with OptiMEM supplemented with 10% FBS and the cells were incubated for 72h at 37°C under 5% CO_2. The cells were then collected and processed for western blot analysis.

Quantification and Statistical Analysis

Data are expressed as results of the mean ± SD of three independent experiments. For data analysis, the Graphpad Prism 6 software (GraphPad Software, San Diego, CA, USA) was used. Student`s t-test and One-way ANOVA were used for statistical analysis to compare different conditions. The asterisks (*, ** and ***) indicate the significance of *p*-values less than 0.05, 0.01 and 0.001, respectively. Immunofluorescence images were acquired using Leica SP5 microscope. Quantitative densitometry analysis of immunoblot band intensities was performed by using the TINA software (version 2.10, Raytest, Straubenhardt, Germany).

2.7. Results

2.7.1. The viral protein Us11 promotes a p18 cleavage of caspase-8.

The viral encoded Us11 is a true late (γ2) protein which is not essential for HSV-1 replication *in vitro*. To assess its role in caspase-8 activation and cleavage a recombinant R3630 (ΔUs11/ΔUs12) virus lacking the Us11 and Us12 genes was used. The human acute monocytic leukemia cell line (THP-1) was used as a model for human monocytes.

The western blot analysis were then performed using THP-1 cells infected or not with the wild-type HSV-1 and the recombinant R3630 viruses (50 PFU/cell). To detect the active p43/41 and p18 cleavage products a specific antibody directed to the p18 fragment (ALX-804-242-12F5) was used. The canonical "two-step model" activation of caspase-8 requires a first cleavage step between the enzymatic subdomains p18 and p10, generating the p43/p41 and p12 cleavage intermediates, and a second cleavage of the p43/p41 generating the active enzyme subunits p18, p10 and prodomain p26/p24 (Fig. 8A).

Results from a time-course infection in THP-1 cells (1h, 3h, 6h, 24h and 48h post infection) showed a different cleavage pattern of caspase-8 in absence of Us11 protein (Fig. 8B). Particularly, the cleaved form p43/p41 was detected in both HSV-infected and R3630-infected cells compared to the uninfected cells at late stage of infection (24 and 48 hpi). Interestingly, the fully-active p18 cleavage product was only detected in HSV-infected cells (24 and 48h p.i.), but not in R3630 infected cells, suggesting that the lack of both Us11 and Us12 viral proteins could affect the second cleavage step (Fig. 8B).

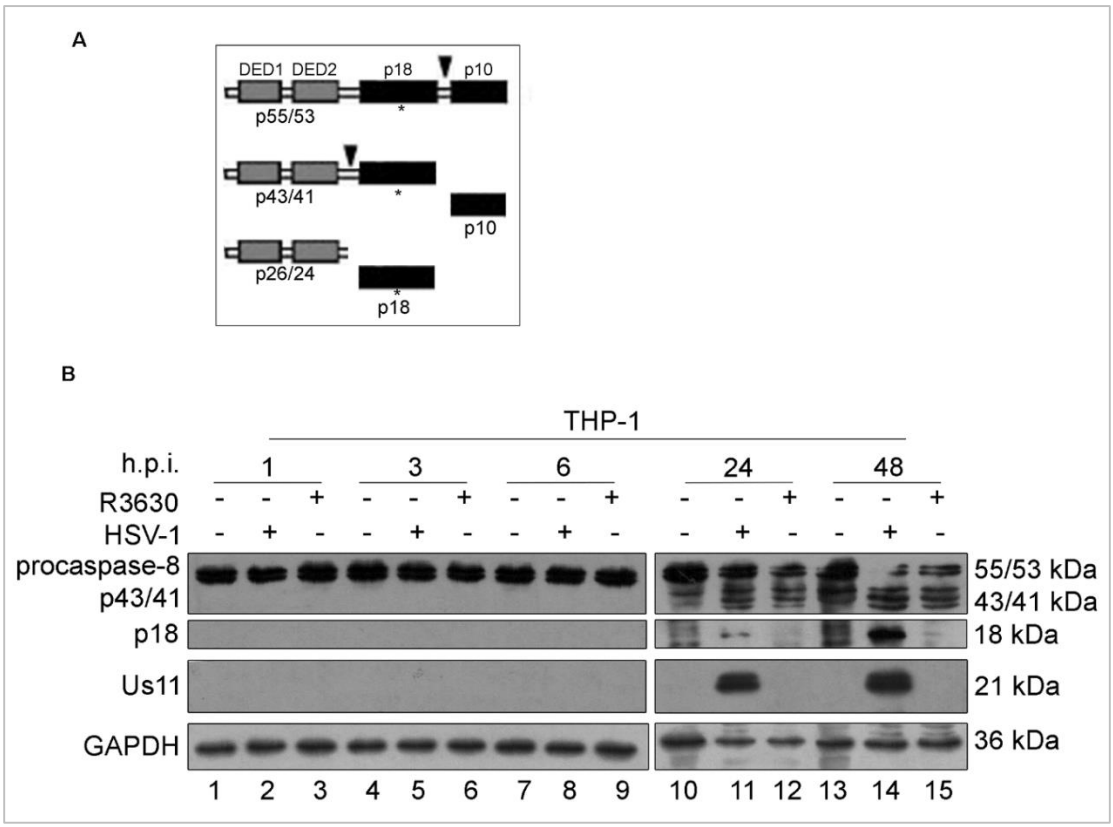

Figure 8. Effect of Us11 deletion on caspase-8 cleavage. Schematic representation of the "two-step" model proposed for cleavage and activation of caspase-8 (A); a time course analysis were performed using THP-1 cell infected or not with HSV-1 and R3630 viruses at MOI 50 (B). The full-length (p55/53) and cleaved form (p43/41 and p18) of caspase-8 was detected by using a specific antibody directed to the p18 subunit (ALX-804-242-12F5). GAPDH was used as loading control.

Given the fact that R3630 virus carries a deletion in both Us11 and Us12 genes, transfection experiments were also included to verify the pattern of caspase 8 cleavage observed in R3630 infected cells. Transfection experiments carried out in 293T cells, demonstrated that the overexpression of Us11 but not Us12, specifically induces p18 accumulation in transfected cells (Fig. 9). Interestingly, the p43/41 fragment was not detected in transfected cells, as observed instead in THP-1 cells infected with HSV-1 and R3630 (Fig. 9).

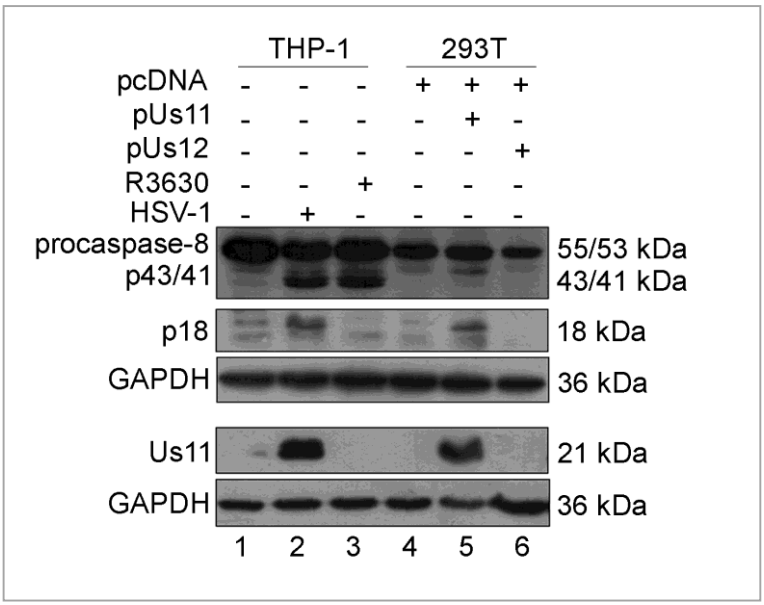

Figure 9. Cleavage pattern of caspase-8 induced by infection with HSV-1 and overexpression of the Us11 viral protein. Western blot analysis of caspase-8 cleavages was performed using both THP-1 cells infected or not with HSV-1 or R3630 (50 PFU/cell) for 24h and 293T cells transfected with pUs11 and pUs12 plasmids and collected at 72h post-transfection. The full-length (p55/53) and cleaved form (p43/41 and p18) of caspase-8 were detected by using a specific antibody directed to the p18 subunit (ALX-804-242-12F5). GAPDH was used as a loading control**.**

These data were confirmed by further experiments in which the accumulation of γ2 proteins, including Us11, was inhibited by phosphonoacetic acid (PAA) treatment during infection (Fig 10A). The obtained results showed that Us11-mediated accumulation of p18 was abrogated in HSV-infected and PAA-treated cells compared to non-treated control cells. Conversely, the p43/41 fragment was detected in HSV-infected cells treated or not with PAA (Fig 10A). This evidence indicated that during infection, even in absence of Us11, the first cleavage step of caspase 8 occurs and generates the p43/41 fragment. Besides, in both infected or transfected cells, Us11 promotes an accumulation of p18, which does not require p43/41 fragment. These results were clearly confirmed by using a synthetic and cell permeable caspase-8 inhibitor z-IETD-fmk, which inhibits the canonical cleavage and particularly the first cleavage step. As shown in Figure 10B, this alternative cleavage of caspase-8 required viral Us11 protein and was not inhibited by z-IETD treatment (Fig. 10B).

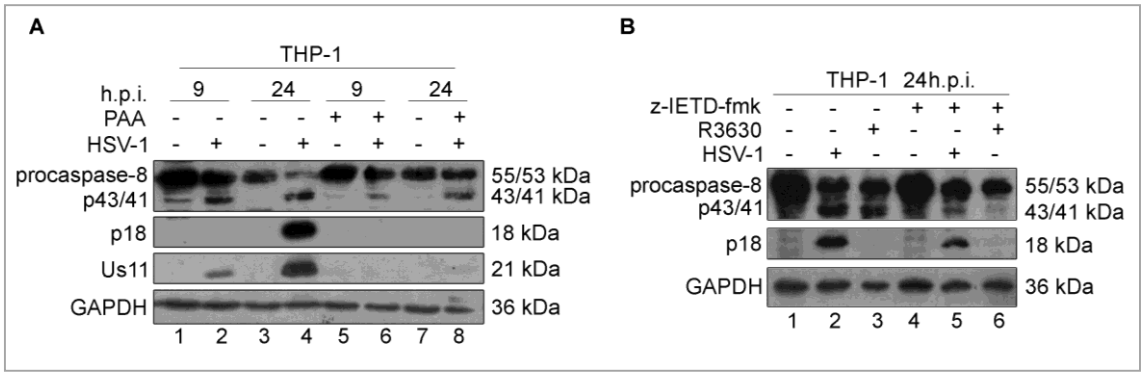

Figure 10. Effect of the PAA and z-IETD-fmk treatment on caspase-8 cleavage during HSV-1 infection. Western blot analyses of caspase-8 from: (A) THP-1 cells infected with HSV-1 (50 PFU/cell) and treated or not with PAA (300 µg/mL); (B) THP-1 cells infected with HSV-1 or R3630 (50 PFU/cell) and treated or not with the caspase-8 inhibitor z-IETD-fmk (100 µM). The full-length (p55/53) and cleaved form (p43/41 and p18) of caspase-8 was detected by using a specific antibody directed to the p18 subunit (ALX-804-242-12F5). GAPDH was used as loading control.

Additionally, fluorescence microscope analyses were performed to verify wheter the accumulation of p18 fragmentation occurred in actively replicating cells. Indeed, HSV-1 infection in THP-1 cells gives rise to a population of cells that get infected and replicate the viral genome while the others are resistant to HSV-1 infection. This is not surprising due to the fact that HSV-1 infects several cell types *in vitro* with different degrees of permisiveness and immune cells, including monocytic cells, macrophages,

dendritic cells and T lymphocytes are less permissive to HSV-1 infection and viral replication than other cell types, such as epithelial cells. Moreover, macrophage-like cells are more susceptible to HSV infection compared to freshly isolated or non-activated monocytic cells (Albers et al., 1989; Tenney and Morahan, 1991, p. 937). In addition, unlike permissive epithelial cells, immune cells are more resistant to HSV-1-mediated suppression of apoptosis pathway (Bosnjak et al., 2005; Iannello et al., 2011; Ito et al., 1997; Kather et al., 2010; Mastino et al., 1997). The fact that HSV-1 differentially modulates the apoptosis response as well as the infection and survival in epithelial (permissive) and immune (semi-permissive) cells could represent an important mechanism for immune escape and virus dissemination.

To this purpose, THP-1 cells were infected or not with a recombinant virus expressing the viral capsid VP26 tagged with GFP (HSV-1-VP26GFP). The samples were collected at different times p.i. and immunofluorescence staining was performed to detect the active p18 fragment of caspase-8. As shown in Figure 11, a greater increase of p18 accumulation was observed consistently with the increase of VP26-GFP-dots in THP-1 cells over the time of viral replication, indicating that p18 accumulation occurs during active HSV-replication (Fig. 11A). Indeed, at least 50% of infected cells (GFP+) cells were positive for p18 as well (GFP+/caspase8+) (Fig.11B). Uninfected cells only showed mainly a cytoplasmatic localization of the fluorescence corresponding to the basal level of inactive caspase-8.

Moreover, to confirm this data double staining of THP-1 cells infected with HSV-1 was performed against the active p18 fragment and the late gen product *vhs*. As showed in Figure 11C, the co-localization of p18 and *vhs* observed in HSV-infected cells clearly confirm that the accumulation of p18 fragment occurred in actively replicating cells.

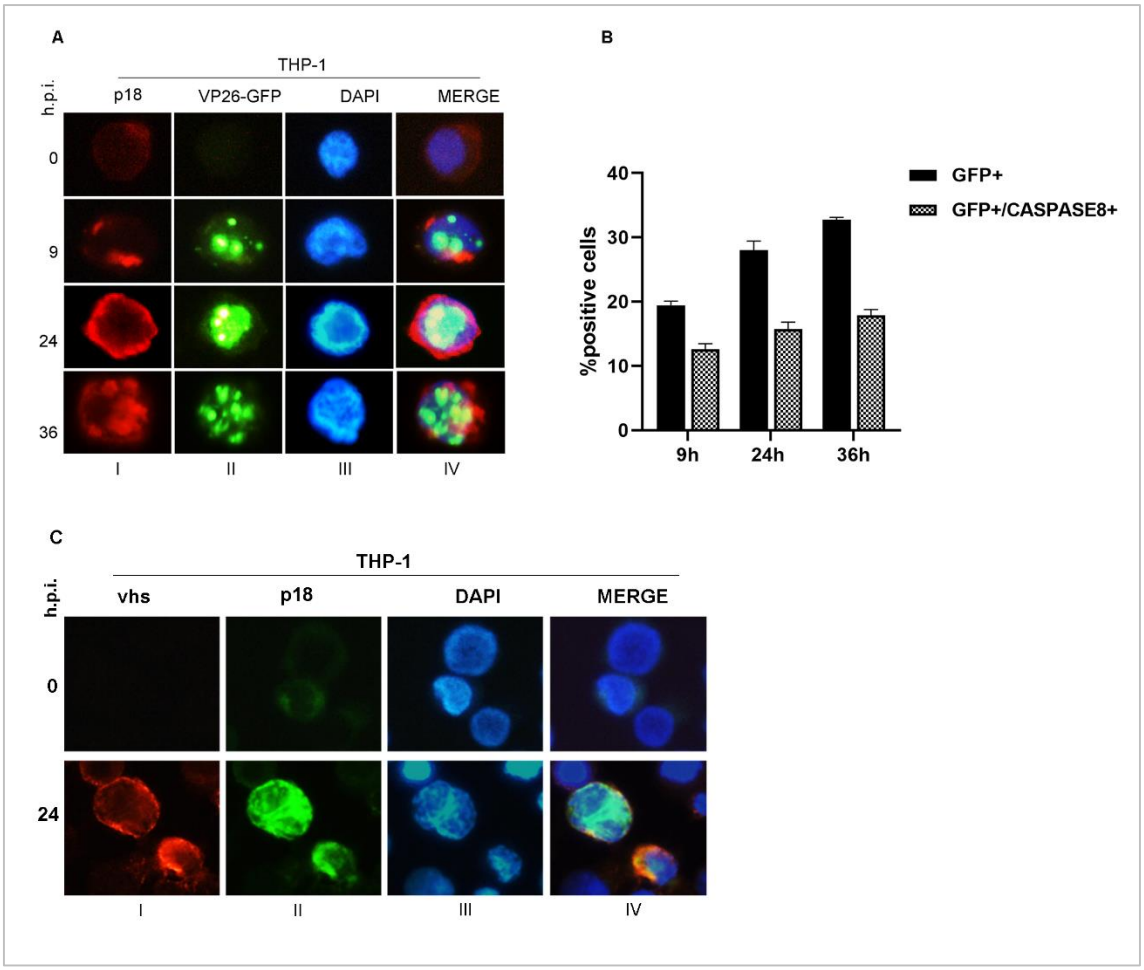

Figure 11. Fluorescence microscope analysis of p18 fragment in THP-1 infected with HSV-1-VP26GFP and co-localization of p18 and the late gene product *vhs*. (A) THP-1 cells infected or not with HSV-1-VP26GFP were collected at 9h, 24h and 36h p.i. and stained with anti-p18 antibody in red (I). The green dots are representative of autofluorescent VP26GFP protein (II). Hoechst was used to stain the nuclei (III); the IV column represent the merged images (B) Quantization of infected cells (GFP+) cells positive as well as infected cells (GFP+) cells positive for p18 (GFP+/caspase8 +); (C) THP-1 cells were infected or not with HSV-1, collected 24h p.i. and stained with both anti-p18 (II) and anti-*vhs* antibodies (I). Hoechst was used to stain the nuclei (III); the IV column represent the merged images.

2.7.2. Us11 binds to p18 fragment and induces its accumulation in a cell-free system.

Results obtained in Figure 9 and Figure 10 clearly demonstrated that Us11 promote a p18 cleavage of caspase-8. To verify whether the p18 cleavage was mediated by the interaction with the viral protein Us11, the physical interaction between Us11 and caspase-8 proteins was verified through an immunoprecipitation assay. THP-1 cells were infected or not with 50 PFU/cells of HSV-1, lysed after 18 h.p.i. and then incubated overnight at 4 °C with 5 µl of Us11 antibody pre-adsorbed on protein-A Sepharose Beads. After overnight incubation complexate-beads were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoprecipitation assay clearly showed that Us11 physically interacts and precipitate the p18 fragment of caspase-8 (Fig. 12).

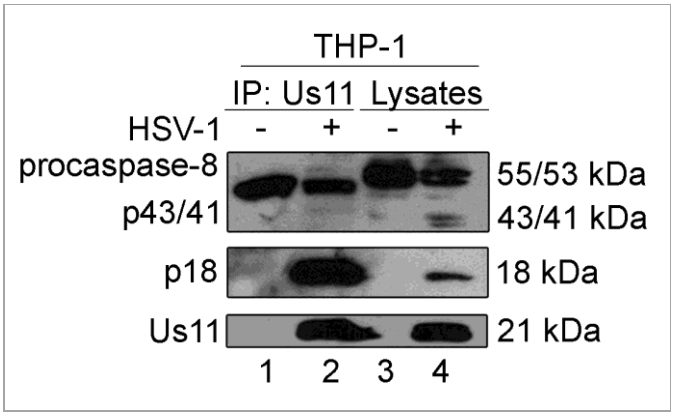

Figure 12. Immunoprecipitation assay. THP-1 cells were infected or not with of HSV-1 (MOI 50), lysed and then incubated overnight at 4 °C with 5µl of Us11 antibody pre-adsorbed on protein-A Sepharose Beads. After overnight incubation with the extracts, complexate-beads were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The full-length (p55/53) and cleaved form (p43/41 and p18) of caspase-8 was detected by using a specific antibody directed to the p18 subunit (ALX-804-242-12F5).

Lastly, to verify whether the viral protein Us11 was responsible for p18 cleavage of caspase-8, a cell-free caspase-8 cleavage assay was performed by using GST-Us11 and GST-caspase-8 recombinant proteins. GST-Us11 and GST-caspase8 recombinant proteins were produced by using the Baculovirus Expression Vector System (BEVS) technology. The recombinant GST-Us11 and GST-Caspase8 baculoviruses were generated separately by cotransfection of Sf9 insect cells with the Us11-pAcGHLT-A and Caspase8-pAcGHLT-A transfer plasmids along with baculoGold DNA. The recombinant baculoviruses were then amplified in Sf9 cells and the expression of the recombinant

proteins was verified by SDS-page and Coomassie blue-staining in a polyacrylamide gel. Caspase-8 cleavage assay was performed by incubating GST-Us11 and GST-caspase-8 recombinant proteins in caspase 8 assay buffer. The assay was performed by incubating GST-Caspase-8 with three different concentrations of GST-Us11 for 1h or for different times from 45 min up to 3h. After the incubation time, the proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed for caspase 8 and Us11. The results indicated that the presence of GST-Us11 protein increases the accumulation of p18 fragment from GST-caspase-8 in a cell-free system (Fig. 13). Particularly, the p18 fragment accumulates in a dose- and time dependent-manner in GST-Us11-incubated samples compared to GST-caspase-8 alone as indicated in Figures 13C and 13D.

Taken together these finding support the hypothesis that Us11 exert a critical role for the cleavage of caspase-8. Indeed, Us11 is able to interact with caspase-8 and promote its cleavage to the p18 active form not only *in vitro* but also in a cell-free system. Moreover, it is presumable that upon their interaction, Us11 and the active form p18 remain physically associated as suggested from the immunoprecipitation analysis.

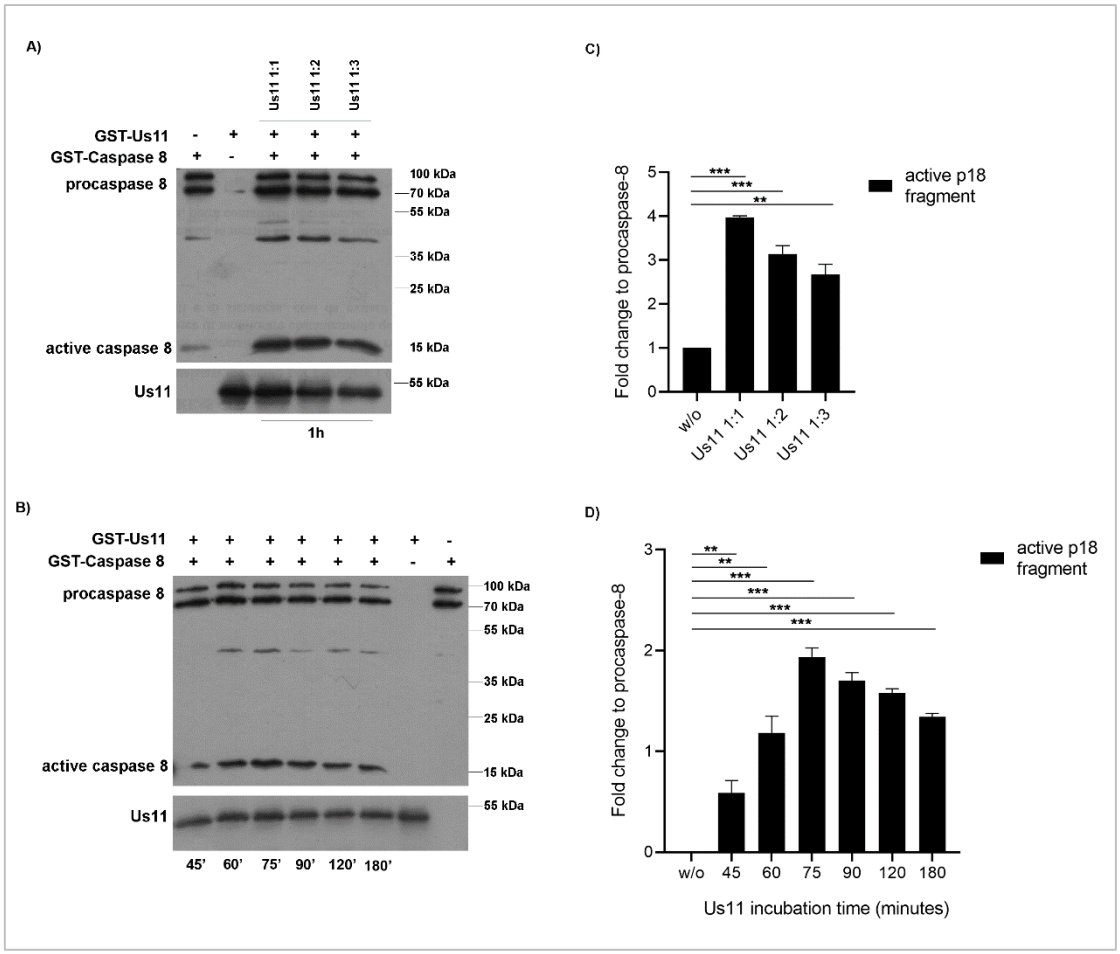

Figure 13. Caspase-8 cleavage assay. (A) GST-Caspase 8 recombinant protein was incubated with a serial dilution of GST-Us11 recombinant protein (1:1, 1:2, 1:3) in caspase-8 assay buffer as described in material and methods. After 1h incubation time, the activation of caspase 8 was verified by SDS-PAGE. The membranes were probed with antibodies directed to caspase-8 and Us11 proteins. (B) GST-Caspase 8 recombinant protein was incubated with GST-Us11 recombinant protein (1:1) in caspase-8 assay buffer for a different time from 45 min up to 3h. After the incubation time, the activation of caspase 8 was verified by SDS-PAGE. The membranes were probed with polyclonal antibody directed to caspase-8 and Us11; (C) and (D) The graphs represent the relative fold change of p18 band intensity over the procaspase-8 band intensity.

2.7.3. Us11-dependent p18 accumulation does not trigger the apoptosis pathway.

To verify whether the p18 cleavage of caspase-8 observed in HSV-infected and Us11-trasfected cells modulates the cell death signaling, we investigate the apoptosis activation throught the detection of caspase-3 (21kDa) and PARP (118 kDa) proteins. A time-course analysis of THP-1 infected cells indicates that in both wild-type HSV-1 and R3630 infected cells the cleavage of caspase-3 and PARP occurred at late time point post infection (24 and 48 h.p.i) confirming the activation of apoptotic pathway (Fig. 14A). However, these results showed that the different cleavage pattern of caspase-8 observed between the HSV and R3630 infected cells does not result in a different cleavage of apoptosis markers such as caspase-3 and PARP. Indeed, by preventing the canonical caspase-8 cleavage with the z-IETD-fmk inhibitor, we found that cleaved caspase 3 (19-17 kDa) and cleaved PARP (89 kDa) accumulation were reduced by z-IETD-fmk treatment in both HSV and R3630 infected cells (Fig. 14B). This evidence confirmed that the canonical cleavage of procaspase-8, induced during HSV-1 and R3630 infection, results in apoptosis induction as demonstrated by p43/41-dependent PARP and caspase-3 cleavage. Conversely, the accumulation of p18 which is dependent on Us11 expression was not related to apoptosis pathway activation. Indeed, overexpression of Us11 in non-infected cells does not result in caspase-3 and PARP cleavage (Fig. 14C).

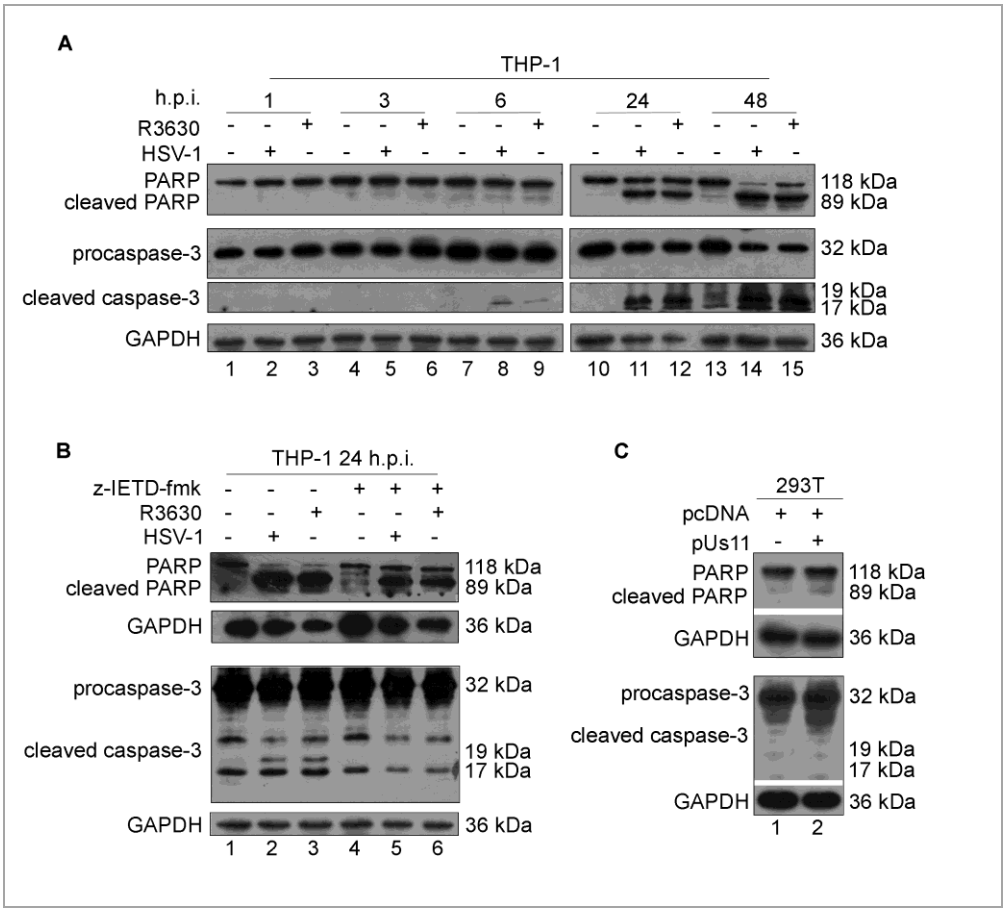

Figure 14. Detection of apoptotic markers. Western blot analysis of PARP and caspase-3 from: (A) THP-1 cells infected with HSV-1 or R3630 (50 PFU/cell) and collected at 1,3,6,24 and 48 h.p.i; (B) THP-1 cells infected with HSV-1 or R3630 (50 PFU/cell) and treated or not with the caspase-8 inhibitor z-IETD-fmk (100 µM); (C) THP-1 cells transfected with pUs11 and collected 72h post transfection. The membranes were probed with an antibody directed to caspase-3 and PARP. GAPDH was used as a loading control.

Thus it was subsequently verified whether the p18-cleavage, not related to apoptosis signalling, occurs in non-immune permissive epithelial cells which are known to counteract the apoptotic response follow HSV-1 infection. To this purpose, 293T and HEp-2 cell lines were infected or not with 10 PFU/cell of HSV-1. The cells samples were subjected to analysis for the detection of caspase-8 and apoptosis markers cleavage. As shown in Figures 15A and 15B, p18 accumulation was observed in both 293T and HEp-2 infected cells (24h.p.i.), while neither p43/41 nor cleaved PARP and cleaved caspase-3 were detected. Overall, results obtained from these experiments clearly demonstrated that Us11-dependent accumulation of p18 does not enhance apoptosis response during HSV-1 infection either in immune cells as well as in non-immune cells.

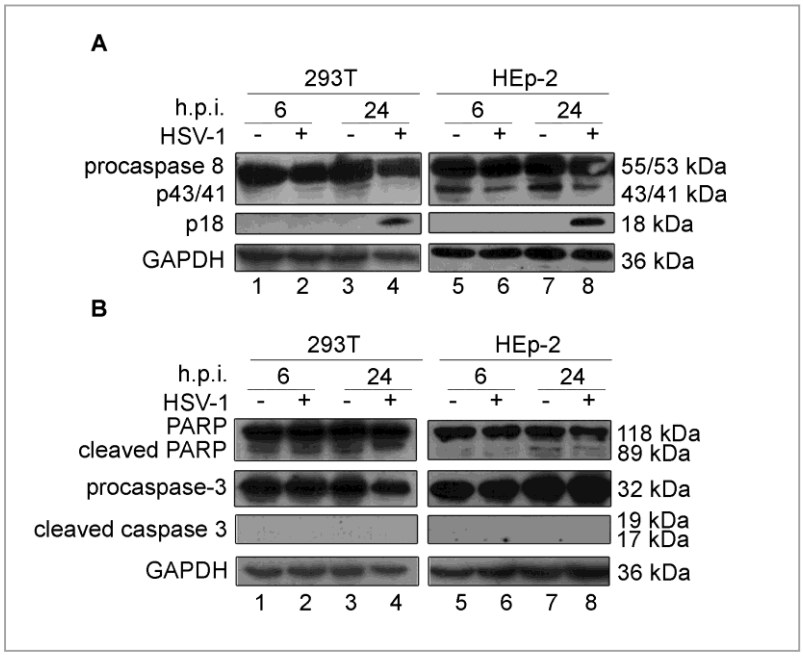

Figure 15.Western blot analysis of caspase-8 and apoptosis marker in non-immune cells. 293T and HEp-2 cell lines were infected or not with HSV-1 (10 PFU/cell) for 1h at 37°C and harvested at 6 and 24h p.i. for proteins extraction. The membranes were probed with A) antibody directed to caspase-8 and B) antibodies directed to caspase-3 and PARP. GAPDH was used as a loading control.

2.7.4. Biological effect of caspase-8 during HSV-1 replication.

To further investigate the role of Us11-mediated p18 cleavage on HSV-1 replication, caspase-8 deficient HEp-2 cells (CASP8$^{-/-}$) were used. Firstly, the virus yield and cytopathic effect were evaluated in both caspase-8 deficient CASP8-\- and the wild type CASP8$^{+\backslash+}$ cell lines. The HSV-1 infection displayed a different phenotype between the two cell lines as showed in Figure 16A. At 9h and 24h post-infection (MOI 10), the characteristic cytopathic effect (round cells/detached cells), due to HSV-1 replication, was detected in the wild type cells expressing caspase-8 (CASP8+\+), while the CASP8$^{-\backslash-}$ cells showed a less evident cytopathic effect, confirmed by total viral particle titration (extracellular and intracellular viral particles) (Fig. 16B).

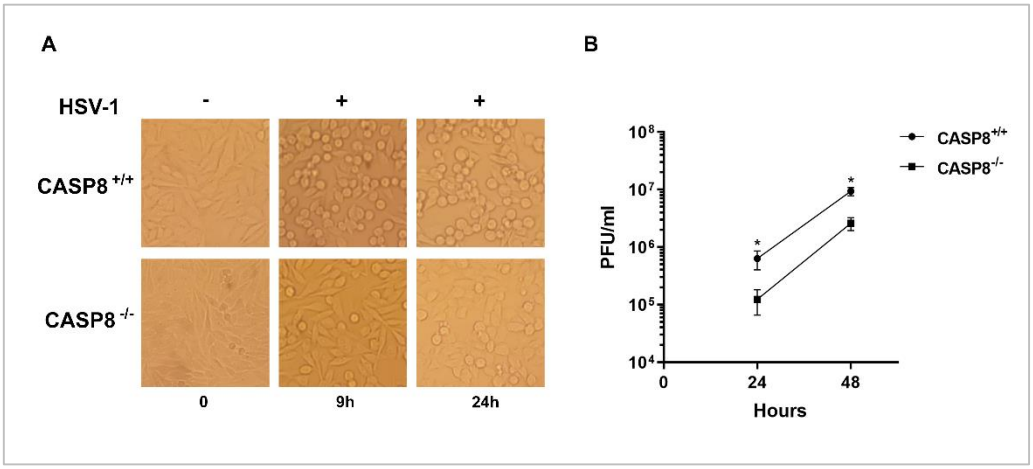

Figure 16. Comparison of HSV-1 cytopathic effect between CASP8 $^{+\backslash+}$ and CASP8 $^{-\backslash-}$ cells. Caspase-8 deficient (CASP8$^{-\backslash-}$) and the wild type (CASP8$^{+\backslash+}$) cell lines were infected with HSV-1 (10 PFU/cell) and (A) the cytopathic effect was observed under an inverted light microscopy at 9h and 24 h.p.i; (B) Samples were collected at 24 and 48 h.p.i and the virus yield was evaluated by total viral particle titration.

To better investigate the different phenotypes, a MOI-dependent infection was performed in CASP8$^{+/+}$ and CASP8$^{-/-}$ cell lines. Samples were collected and titration of extracellular viral particles (EV) and intracellular viral particles (IV) was performed on VERO cells. The results showed in Figure 17 indicate that at MOI 1 no difference was observed for intracellular virus titration between the two different cell lines, while a great increase of the released virus was observed in CASP8$^{+/+}$ rather than CASP8$^{-/-}$. Consistently, when MOI 10 was used an increase of either intracellular and extracellular virus titer was observed again in CASP8$^{+/+}$ cell lines. These results indicate that caspase-8 protein could be essential during the late phase of HSV-1 replication, leading to hypothesize a caspase-8 contribution during the viral egress, given the fact that the higher

difference between CASP8$^{+/+}$ and CASP8$^{-/-}$ cells lines were observed in the released virus particles.

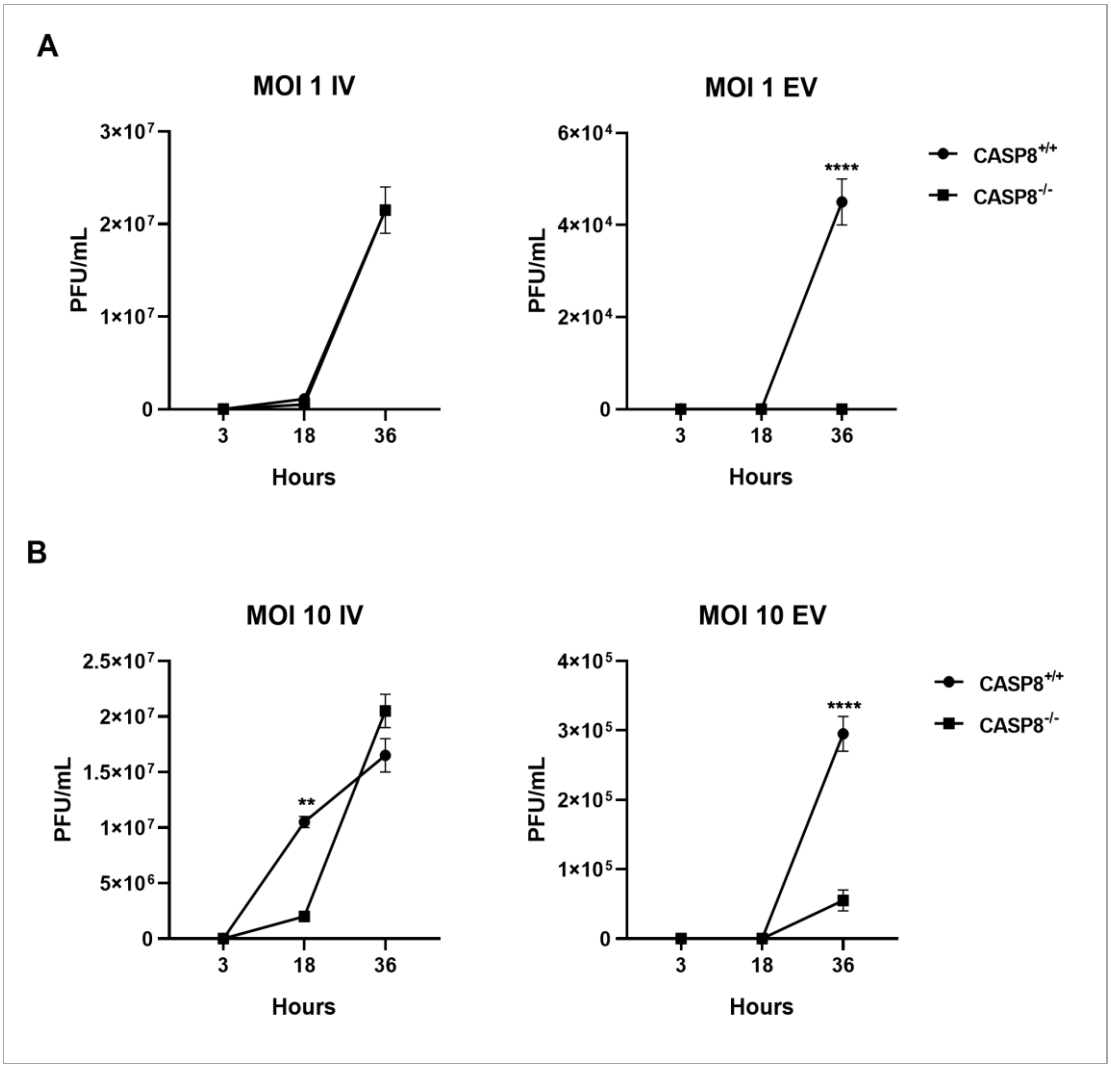

Figure 17. Intracellular (IV) and extracellular (EV) virus yield from CASP8$^{+\backslash+}$ and CASP8$^{-\backslash-}$ HSV-1 infected cells. Caspase-8 deficient (CASP8$^{-\backslash-}$) and the wild type (CASP8$^{+\backslash+}$) cell lines were infected with HSV-1 (1 PFU/cell and 10 PFU/cell) and collected at 3, 18 and 36 h. p.i. Titration of extracellular viral particles (EV) and intracellular viral particles (IV) was performed on VERO cells.

Subsequently, the analysis of viral protein expression was also included. CASP8$^{+/+}$ and CASP8$^{-/-}$ cells were mock infected or infected with 10 PFU/cells of HSV-1. The cells were collected at 3, 6 and 18h p.i. to perform western blot analysis to detect the viral protein ICP8 (β), UL42 (β) and Us11 (γ_2). The results showed a different accumulation of the viral proteins in CASP8$^{-/-}$ cells if compared to CASP8$^{+/+}$ (Fig. 18).

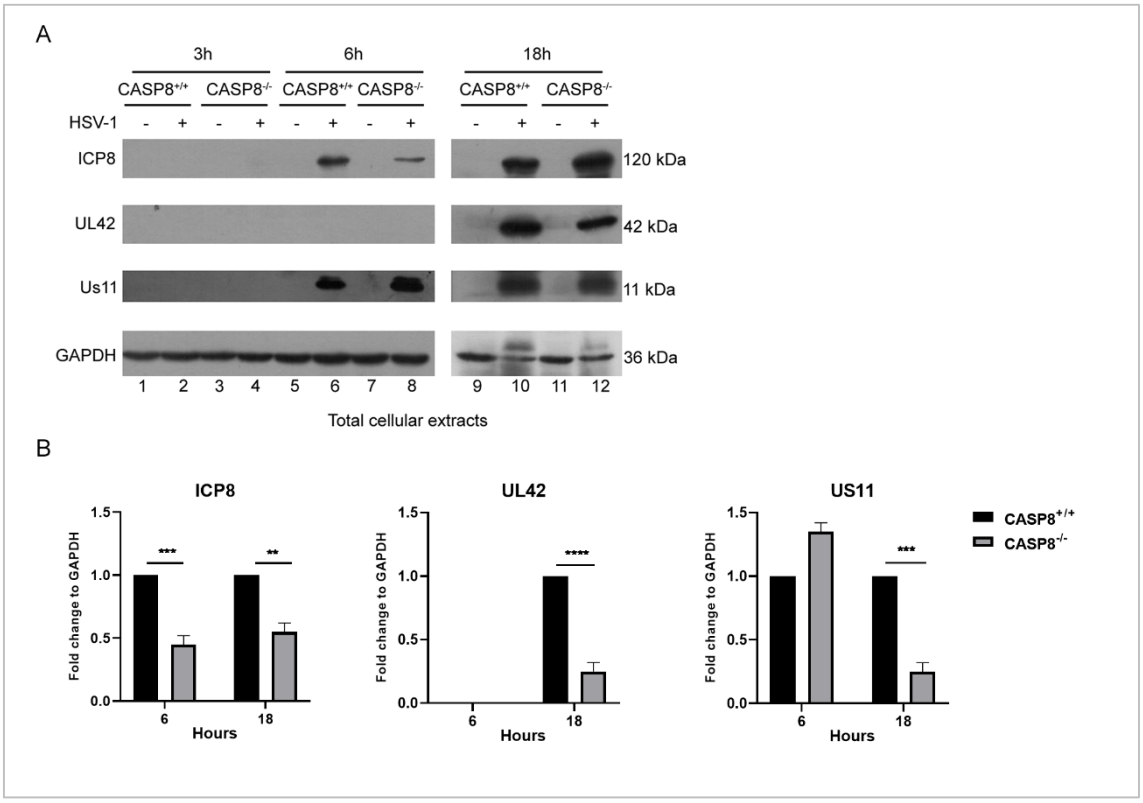

Figure 18. Analysis of HSV-1 proteins accumulation. CASP8$^{+/+}$ and CASP8$^{-/-}$ cells were mock infected or infected with 10 PFU/cells of HSV-1. The cells were collected at 3, 6 and 18h p.i. to perform western blot analysis. (A) The proteins were resolved by SDS-PAGE and the membranes were probed with antibodies directed against the viral protein ICP8 (β), UL42 (β) and Us11 (γ2). GAPDH was used as a loading control (B). Quantitative densitometry analysis of immunoblot band intensities was performed with the TINA software (version 2.10, Raytest, Straubenhardt, Germany) and it is expressed as the fold change over the housekeeping gene GAPDH**.**

Analysis of cytoplasmic and nuclear fractions confirm the different distribution of the viral proteins in CASP8$^{-/-}$ cells compared to CASP8$^{+/+}$ (Figure 19). Furthermore, the quantization of viral DNA demonstrated that in CASP8$^{+/+}$ cell the accumulation of viral DNA was higher compared to CASP8$^{-/-}$ cells (Fig. 20).

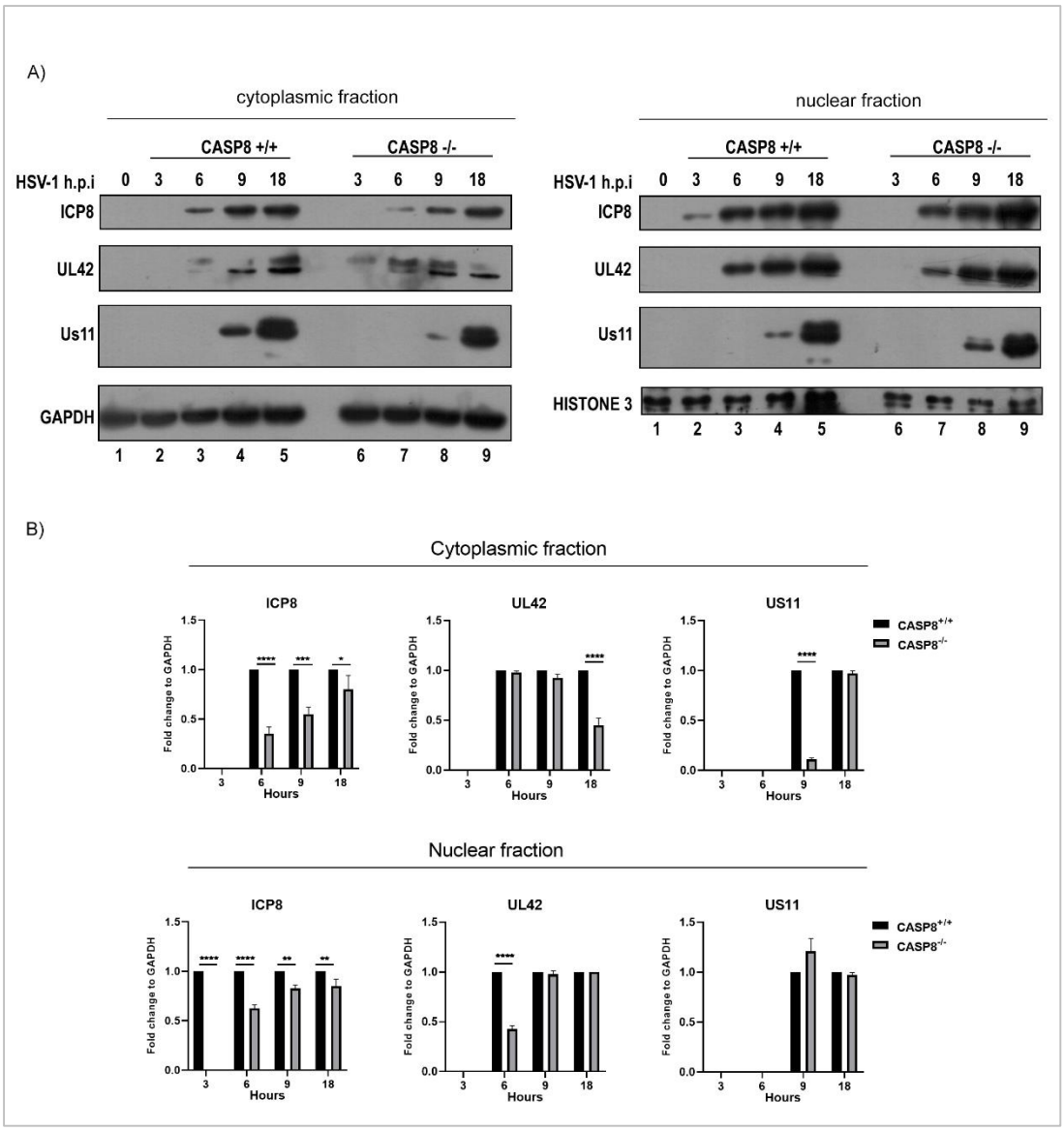

Figure 19. Analysis of HSV-1 proteins accumulation in cytoplasmic and nuclear fraction. CASP8$^{+/+}$ and CASP8$^{-/-}$ cells were mock infected or infected with 10 PFU/cells of HSV-1. The cells were collected at 3, 6, 9 and 18h p.i. to perform western blot analysis. (A) Equal amount of cytoplasmic and nuclear proteins were resolved by SDS-PAGE and the membranes were probed with antibodies directed against the viral protein ICP8 (β), UL42 (β) and Us11 (γ2). GAPDH and Histone 3 were used as a loading control (B) Quantitative densitometry analysis of immunoblot band intensities was performed with the TINA software (version 2.10, Raytest, Straubenhardt, Germany) and it is expressed as the fold change over the appropriate housekeeping genes.

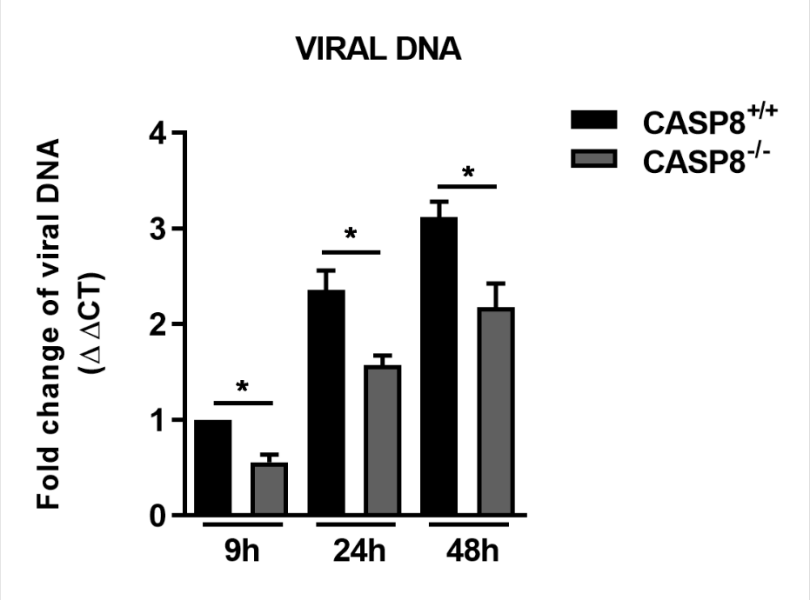

Figure 20. Analysis of viral DNA accumulation. CASP8$^{+/+}$ and CASP8$^{-/-}$ cells were mock infected or infected with 10 PFU/cells of HSV-1. The cells were collected at 9, 24 and 48h p.i. to perform viral DNA extraction and qPCR. Relative quantization of viral DNA was performed by using specific HSV-1 TaqMan probe and analyzed by the comparative Ct method (ΔΔCt).

2.7.5. Involvement of caspase-8 in Rab5 and Lamp1 protein expression following HSV-1 infection.

The previous results demonstrated that HSV-1 replication is reduced or delayed in caspase-8 knockout compared to the wild-type cells, suggesting that caspase-8 could affect the spread of the virus.

HSV-1 uses several mechanisms to spread from infected to uninfected cells (Agelidis and Shukla, 2015). Production of secreted vesicles by HSV-1-infected cells has been previously reported (McLauchlan et al., 1992). Recent studies suggested that HSV-1 might limit the spread of infection from cell-to-cell in order to control its virulence and facilitate the dissemination between individuals (Deschamps and Kalamvoki, 2018; Kalamvoki et al., 2014; Kalamvoki and Deschamps, 2016). On the other hand, previous studies indicated that Rabs GTPase proteins play an important role in HSV-1 assembly and release (Raza et al., 2018).

Based on this evidence, immunofluorescence analysis was employed to explore the impact of caspase-8 deletion on Rab5 and LAMP-1 (lysosome-associated membrane protein), which are two key proteins for HSV-1 envelopment and egress (Raza et al., 2018). To this purpose, $CASP8^{+/+}$ and $CASP8^{-/-}$ cells were infected with a recombinant virus expressing the viral capsid VP26 tagged with GFP (HSV-1-VP26GFP). The samples were collected at different times p.i. and immunofluorescence staining was performed to detect the LAMP-1 and Rab5 proteins. As showed in Figure 21, a greater increase of both LAMP-1 and Rab-5 fluorescence signal was observed over the time of viral replication. However, $CASP8^{-/-}$ at 18 h p.i. cells showed a higher accumulation of LAMP-1 and Rab5 in a dot like structure compared to $CASP8^{-/-}$ cells, which showed a more diffuse fluorescence. Therefore, it is possible to assume that the lack of caspase-8 could affect the expression and accumulation of two key proteins involved in the secondary envelopment and egress of HSV-1 viral particles. These results definite a possible regulatory role of caspase-8 during viral replication by promoting viral replication and spread, rather than a canonical role in the apoptotic cascade induced by HSV-1.

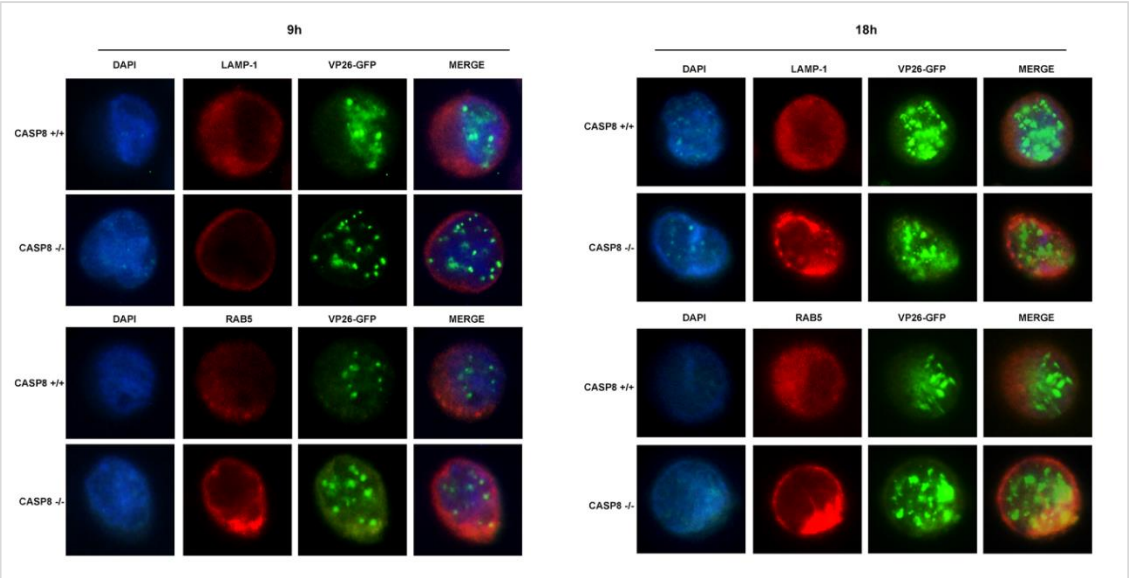

Figure 21. Immunofluorescence analysis of CASP8$^{-/-}$ and CASP8$^{+/+}$ cells infected with a HSV-1-VP26GFP and probed for LAMP-1 and Rab5. CASP8$^{+/+}$ and CASP8$^{-/-}$ cells were mock infected or infected with 10 PFU/cells of HSV-1-VP26GFP and collected at 9 and 18h p.i. to perform immunofluorescence analysis. The cells were stained with specific antibodies against LAMP-1 and Rab5 (in red). The green is representative of auto-fluorescent VP26GFP viral protein and nuclei were stained with Hoechst (in blue).

2.8. Discussion

Immune cells represent the first line of defence against viral pathogens and apoptosis is an innate immune response activated in order to restrict viral replication. On the other hand, HSV-1 has evolved several strategies to subvert immune antiviral response and modulates cell death differentially in non-immune and immune cells through the production of several virulence factors. It has been reported that immune cells such as lymphocytes, monocyte/macrophages and dendritic cells, sustain a low-productive infection characterized by induction of apoptosis as a cytopathic effect. However, the molecular mechanisms that control the restriction of HSV-1 replication in immune cells are not completely understood. Recently, it has been demonstrated that HSV-1 induces the early activation of autophagy in human monocytic THP-1 cells, most likely promoting viral internalization. Conversely, at late stage of infection autophagy is inhibited or stabilized to support efficient viral replication (Siracusano et al., 2016).

The viral encoded Us11 late protein has been shown to protect HeLa cells from heat- and staurosporine-induced apoptosis as well as to counteract the host's autophagy response (Javouhey et al., 2008; Lussignol et al., 2013). Accordingly, the role of Us11 protein in HSV-induced apoptosis was explored in a monocytoid cell system such as THP-1 cells, which are sensitive to apoptosis signalling (Iannello et al., 2011). Besides, given the importance of caspase-8 as a key enzyme of apoptotic cell death, the interaction between Us11 and caspase-8 was also investigated.

Caspases-8 is the initiator caspase of extrinsic or death receptor-mediated apoptotic cell death in mammals. Besides its roles in apoptosis, caspase-8 is also implicated in other cell death process like anoikis, necroptosis and autophagy and acts as a switch point between autophagy and apoptosis, exerting an important role in cell-fate determination and immune response.

In a “two-step model”, proposed for caspase-8 autoproteolytic processing and activation, the first cleavage step occurs between the enzymatic subdomains p18 and p10 and thus generates two cleavage intermediates p43/p41 and p12. The second cleavage step of p43/p41 generates the active enzyme subunits p18, p10 and prodomain p26/p24 (Kallenberger et al., 2014).

The results obtained from this research work demonstrate that Us11 is able to induce directly an “alternative” cleavage of caspase-8 which does not require the first cleavage step and result in the release of p18 fragment only. Interestingly, an alternative

cleavage model has been described by Hoffmann and colleagues (Hoffmann et al., 2009) in B-lymphoblastoid cell lines SKW6.4, Raji, and BJAB and the T-cell lines CEM and Jurkat 16, upon CD95 stimulation. Accordingly, to their finding, the first cleavage can also occur between the prodomain and the large enzymatic subunit p18, thus generating the p26/p24 and p30 product. Afterwards, additional cleavage of p30 by active caspases (caspase-8 and -9 but not by caspase-3) results in the release of p18 and p10. Furthermore, p30 can sensitize cells toward death receptor-induced apoptosis. Thus, different activation pathways could trigger caspase-8 enzymatic activity and/or may promote the switch between its apoptotic and non-apoptotic functions.

The cleavage of caspase-8 mediated by the Us11 protein was observed not only in monocytes but also in epithelial cells and was not related to a downstream modulation of the apoptosis pathway. Furthermore, in caspase-8 knockout cell line, the accumulation of viral protein and viral DNA, as well as the virus yield, were affected compared to wild-type cells expressing caspase-8. This evidence suggested that the lack of caspase-8 could disturb the late events associated with the replication cycle of the virus. The immunofluorescence analysis used to explore the impact of caspase-8 deletion on Rab5 and LAMP-1 (lysosome-associated membrane protein), which are two key proteins for HSV-1 envelopment and egress, displayed different phenotypes. Indeed, a differential distribution of fluorescence signaling for both LAMP-1 and Rab5 protein observed in caspase-8 knockout cells compared to the wild-type cells, suggest that caspase-8 could exert a key role for maturation of HSV-1 viral particles. Taken together the results obtained in the present study demonstrated for the first time that the viral protein Us11 is able to interact and modulate the cleavage of caspase-8 through a “non-canonical” mechanism not related to apoptosis response.

Interestingly, increasing literature studies indicate that caspase-8 exert several non-apoptotic functions, including promotion of cell adhesion, embryonic development, monocyte differentiation, T and B cell proliferation, activation of NF-kB and tumorigenesis (Fianco et al., 2018; Shalini et al., 2015). Caspase-8 expression is retained in many tumors suggesting that its apoptotic activity may be switch off and its function rewired to sustain tumor growth (Stupack, 2013). Consistently, it has been shown that selective impairment of caspase-8 expression in T-cells leads to immunodeficiency in mice and humans as well (Chun et al., 2002; Salmena, 2003). Several studies have demonstrated that caspase 3 and 8 activities in monocytes modulate the delicate balance

between apoptosis and myeloid differentiation following the treatment with the macrophage colony-stimulating factor (M-CSF) (Kang et al., 2004; Sordet et al., 2002). Interestingly, activation of caspases in this condition does not lead to apoptosis but may regulate the cleavage of specific proteins involved in the differentiation process which require cytoskeleton rearrangements, differential transcriptional regulation and changes in cell adhesion. Interestingly data have demonstrated that another herpesvirus, the human cytomegalovirus (HCMV) tightly modulate the caspase 3 and to a lesser extent the caspase-8 activity to promote myeloid differentiation, a key process in the viral dissemination and persistence strategy (Chan et al., 2012).

In conclusion, these findings support the hypothesis that caspase-8 could promote the viral replication and the spread of the virus with a potential implication in the envelopment of HSV-1 viral particles and maturation events.

Chapter III

Contribution of miRNA-146a as regulatory factor of the NF-κB pathway during HSV-1/EGFP replication.

The transcriptional factor NF-κB is considered an attractive target for viral pathogens and it plays an important role in innate immunity, cell proliferation, inflammation and cancer development (Gilmore, 2006) . The role of NF-κB as an immune response regulator during HSV-1 infection has been extensively explored. However, the molecular mechanisms by which NF-κB is modulated during HSV-1 infection is not fully understood. Several reports have demonstrated that the NF-κB pathway is modulated by non-coding RNA, including microRNA (miRNAs), which act as posttranscriptional regulators of gene expression (Boldin and Baltimore, 2012). Therefore, in the second part of the thesis, the interplay between NF-κB pathway and miRNAs during HSV-1 infection was explored.

3.1. The NF-κB pathway

The NF-κB signalling pathway has been considered a pro-inflammatory signalling pathway. The activation of NF-κB is triggered by proinflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα). The NF-κB family proteins are composed of two subfamilies: the "NF-κB" proteins and the "Rel" proteins. The members of the NF-κB subfamily, p105 and p100 are distinguished by their long C-terminal domains while the Rel subfamily includes RelA, also known as p65, RelB and c-Rel, which contains a C-terminal transactivation domain. The activity of NF-κB is regulated by inhibitory proteins IκBα, IκBβ, IκBγ and IκBε that possess different affinities for NF-κB dimers and are regulated by phosphorylation and proteolysis. The activation of NF-κB involves the recruitment of the IκB kinase (IKK) complex, which consists of catalytic kinase subunits (IKKα and/or IKKβ) and a scaffold NF-κB essential modulator (NEMO). The IκB kinase (IKK) complex induces the degradation of the IκB inhibitors, which promote the translocation of the NF-κB dimers to the nucleus and thus the transcription of specific target genes (Hoffmann et al., 2006).

3.1.1 Modulation of NF-κB pathway by HSV-1

Several reports demonstrated that NF-κB is activated during HSV-1 infection due to the interaction of several structural proteins such as gD, gH/gL and UL37 with particular cells surface receptors. It has been demonstrating that UV-inactivated HSV-1 or purified gD stimulates the activation of NF-κB in monocytes through the interaction with HVEM receptor. Moreover, it has been reported that ICP27 protein and ICP0, two important α gene product, are essential for NF-κB activation and translocation to the nucleus (Diao et al., 2005; Hargett et al., 2006). Besides, NF-κB bind to ICP0 promoter and trigger the ICP0 mRNA transcription during viral replication (Amici et al., 2006). In addition, it has been shown that the HSV-1 DNA polymerase processivity factor UL42 bind to p65 and p50 and reduced the TNF-α mediated translocation to the nucleus (Xu et al., 2017; Zhang et al., 2013). Recent report suggests that NF-κB activation in U937 cells results in limited apoptosis response during HSV-1 replication (Marino-Merlo et al., 2016) .

3.2. Role of miR-146 in immune response

MicroRNAs (miRNAs) are small non-coding RNA molecules, playing a key role in RNA silencing by regulating gene expression at the post-transcriptional level (Makeyev and Maniatis, 2008). miRNAs have been identified as key modulators of several biological processes, such as cell differentiation, cancer, immune response and viral infections.

3.2.1. Biogenesis of miRNAs

The miRNAs biogenesis in mammals involves the formation of the long primary transcripts (pri-miRNAs) from both exons and introns of coding and noncoding genes by either RNA polymerase II or RNA polymerase III into the nucleus. After intranuclear transcription, pri-miRNAs are cleaved in small products, by a multiprotein complex called the “microprocessor complex”, which include the Drosha (Intranuclear RNase II) and Pasha (also known as DGCR8, dsRNA binding domain protein) proteins (Denli et al., 2004). The resulting pre-miRNA possess two-nucleotide overhang at the 3’ terminal end which bound to exportin-5 and induces the pre-miRNA translocation from the nucleus to the cytoplasm (Güttler and Görlich, 2011). Once in the cytoplasm, a second round of pre-miRNAs processing is catalyzed by the Dicer enzyme. This cleavage

produces a 22 bp mature miRNA duplex which is incorporated into the ribonucleoprotein complex RISC, bind to mRNAs target inducing their degradation or repress their translation.

The binding between the mature miRNAs and the mRNAs is due to miRNA recognition elements (MRE) located in the 3' UTR of target mRNAs. The consequence of miRNA/mRNA base pairing is the target degradation or slicing, which leads to a decrease in both mRNA and protein levels (Fabian et al., 2010).

3.2.2. miR-146

The miR-146 family consists of two family members, miR-146a and miR-146b that are located on chromosomes 5 and 10, respectively. The mature sequences for miR-146a and miR-146b differ by only two nucleotides. In some cell types, such as monocytes and macrophages, these miRNAs have similar functions, while in other types of cells, such as regulatory T cells, only miR-146a is highly expressed (Rusca and Monticelli, 2011).

The miR-146a acts as negative feedback to prevent the overstimulation of inflammation and immune response. It has been reported to regulate the NF-κB pathway and immune response by targeting the IL-1 receptor associated kinase 1 (IRAK1) and the receptor-associated factor 6 (TRAF6), two key adapter molecules downstream to cytokine and TLR signalling (Taganov et al., 2006). Thus, many viruses are able to regulate the expression of miR-146a in order to manipulate the NF-κB pathway and promote their survival in the host cells and viral replication. The human cytomegalovirus (HCMV) and the vesicular stomatitis virus (VSV) suppresses the type I IFN response through the upregulation of miR-146a (Hou et al., 2009; Wu et al., n.d.). Besides, HSV-1 induces the upregulation of miR-146a in neuronal cells in the late stage of infection (Hill et al., 2009).

3.3. Aim of the study

Previous data obtained from several groups clearly demonstrated the activation of NF-κB in monocytic cells infected with HSV-1 (Gianni et al., 2013; Leoni et al., 2012; Liu et al., 2008; Sciortino et al., 2008; Teresa Sciortino et al., 2007). A recombinant HSV-1/EGFP virus, expressing the enhanced green fluorescent protein (EGFP), was generated and used in our laboratory to monitor the recruitment of NF-κB during HSV-1 infection (Venuti et al., 2019). The recombinant virus was produced by using the HSV-1 (F) bacterial artificial chromosome (BAC-HSV-1) with the transfer plasmid pRB5708 and it showed the same biological and growth properties of the wild-type HSV-1 as described previously (Venuti et al., 2019). Therefore, by using the EGFP gene as a reporter gene, it was possible to identify the cellular complex related to NF-κB signalling during HSV-1 infection in THP-1 cells. Particularly, it has been demonstrated that: (i) HSV-1/EGFP activates the transcriptional factor NF-κB in a time-dependent manner in THP-1 cells; (ii) the phosphorylation status of p65 protein correlates with an actively replicating virus at single-cell level; (iii) HSV-1/EGFP induces the accumulation of phospho-p50/p65 complex in both cytoplasmic and nuclear fractions of infected THP-1 cells.

Based on this previous data, the aim of this second part of the thesis was focused on the analysis of molecular signalling downstream to NF-κB triggered by HSV-1. Given the importance of cellular miRNAs in the immune function and antiviral defence mechanisms, the correlation between HSV-1, NF-κB and miR-146 was mainly explored in human monocytic THP-1 cells. Indeed, several reports support the role of miR-146a in autoimmune diseases, cancer development and viral pathogenesis. Moreover, in response to microbial infection, miR-146a regulate the NF-κB activation through a negative feedback which involves the IL-1 receptor associated kinase 1 (IRAK1) and the receptor-associated factor 6 (TRAF6) (Taganov et al., 2006).

Therefore, the expression of miR-146 and its predicted target IRAK1 was investigated upon HSV-1 infection in both wild-type and DN IκBα THP-1 cells, which express a dominant-negative mutant IκBα. Additionally, the overexpression of exogenous miR-146a was performed in THP-1 cells during infection with HSV-1, in order to explore its role in the biology of HSV-1.

3.4. Materials and Methods

Cell cultures

The acute monocytic leukemia (THP-1) cells were maintained in RPMI-1640 medium (Corning) supplemented with 10% FBS (Euroclone), 4,5g/L D-glucose (Sigma-Aldrich), 1mM Sodium Pyruvate (Sigma-Aldrich), 10 mM Hepes buffer (Sigma-Aldrich). Dominant negative (DN) IκBα THP-1 cells, stably transfected with a dominant negative mutant IκBα, were maintained under selection with 400 μg/ml of Geneticin (Gibco). HEp-2 (human HeLa contaminant carcinoma) and VERO (African green monkey kidney) cells were cultured in DMEM medium (Corning) supplemented with 10% and 6% of FBS (Euroclone), respectively. The cell culture media were supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. All cell lines were incubated at 37° C whit 5% CO_2.

Viruses

The wild-type HSV-1 strain F was kindly provided by Prof. Bernard Roizman (University of Chicago). The mutant HSV-1/EGFP mutant virus expressing the enhanced green fluorescence protein (EGFP) was constructed by using bacterial artificial chromosome (BAC-HSV-1) as described previously (Venuti et al., 2019). HSV-1 and HSV-1/EGFP were both propagated and titred in VERO cells. The virus stocks were stored at -80° C. For experimental infections, the virus inoculum was diluted in RPMI and adsorbed to the cells for 1h at 37°C. The virus was then diluted with fresh medium and the cells were collected at different times post infection (p.i.) to perform several analyses.

RNA extraction, reverse transcription and real-time PCR analysis

Total RNA was extracted using TRIzol® (Life Technologies) and reverse transcribed with Revert Aid H Minus M-MLV Reverse transcriptase (Thermo Fisher Scientific) at 42 °C for 60 min, followed by 90 °C for 5 min. Quantitative real-time PCR was performed in a Cepheid Smart Cycler II System (Cepheid Europe, France) by using maxima SYBR Green probe (Thermo Fisher Scientific), under the following conditions: 95 °C for 10 min, 35 cycles at 95 °C for 30 sec/60 °C for 30 sec/ 72 °C for 45 sec. The

cDNA copy numbers were normalized to GAPDH gene housekeeping. Each analysis includes a negative control.

The pairs of foreword and reverse primers used were the follow:

- IRAK1 Forw-5′ gctggctactgtgctcagaac; IRAK1 Rev-5′ cagcctctcatccagaaggac;
- EGFP Forw-5′gagctgaagggcatcgactt; EGFP Rev-5′ ctcaggtagtggttgtcggg;
- p50-Forw-5’cgtggtgcggctcatgtttac; p50-Rev-5’tttcaagttggatgcattggg;
- p65-Forw-5’tcagtgagcccatggaattcc; p65-Rev-5’cacagcaatgcgtcgaggtg.
- GAPDH Forw-5′ gagaaggctggggctcat, GAPDH Rev-5′ tgctgatgatcttgaggctg.

miRNA analysis

Total RNA was purified with the RNAeasy mini kit (Qiagen) and reverse transcription was carried out with TaqMan™ Advanced miRNA cDNA Synthesis Kit (Termo Fisher Scientific). The detection of miR-146a was performed using the hsa-miR-146a-5p probe (478399 mir) and TaqMan Fast Advanced Master on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) under the following conditions: 20 s at 95 °C, 40 cycles of 3 s at 95 °C and 30 s at 60 °C. miR-let7a and miR-361 were used as endogenous control.

Statistical analysis

Data are expressed as a results of three independent experiments. Student’s t-test and analysis of variance (ANOVA) were used for statistical analysis with the Graphpad Prism 6 software (GraphPad Software, San Diego, CA, USA). The asterisks (*, ** and ***) indicate the significance of *p*-values less than 0.05, 0.01 and 0.001, respectively.

3.5. Results

3.5.1. NF-κB-dependent induction of miR-146a following HSV-1 replication in THP-1 cells.

The miR-146a and miR-146b are the two members of the miR-146 family. They are located on different chromosomes and, in many cases, possess differential functions (Paterson and Kriegel, 2017). Thus, the analysis of both miR-146a and miR-146b was performed to verify their expression levels in THP-1 cells during HSV-1 replication. THP-1 cells were uninfected (mock) or infected with HSV-1 at MOI 10 and MOI 50 and collected at several times point post infection (3h, 18h, 24h and 48h) to perform qPCR miRNAs analysis as described in Material and Methods. The results shown in Figure 22 indicate that the infection with HSV-1 significantly induces an increased expression of miR-146a/b at the late stage of viral replication (24-48h) in a time and MOI-dependent manner. Additionally, miR-146a was highly expressed compared to the miR-146b in HSV-1 infected cells.

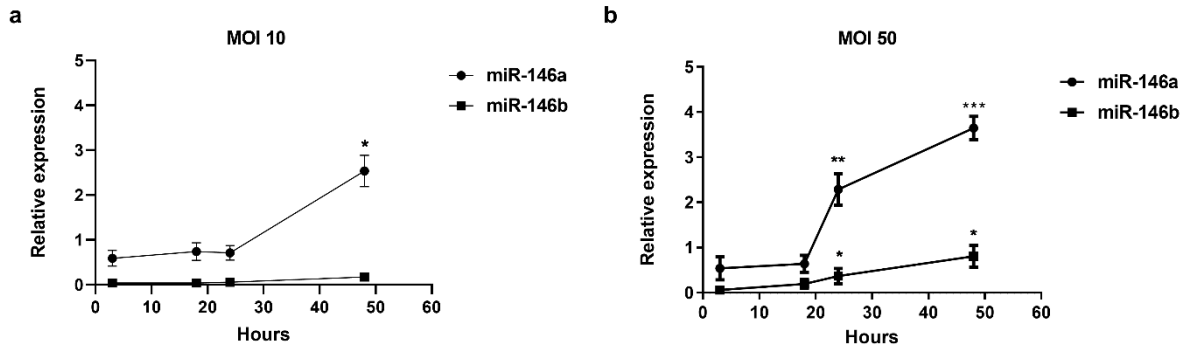

Figure 22. Linear regression of miR-146a and miR-146b qPCR analysis. THP-1 cells were infected or not with HSV-1 at MOI 10 and MOI 50 and collected at 3h, 18, 24 and 48h p.i. to perform qPCR analysis of miR-146a and miR-146b.

In addition, qPCR analysis performed in epithelial cells indicated that the transcriptional level of miR-146a also differs based on the cell types. A permissive HEp-2 cell line was exposed to HSV-1 (MOI 10) for 24h and 48h and then the analysis of miR-146a was performed. THP-1 cells infected with HSV-1 (MOI 50) and collected at 24h were used as a positive control. The results shown in Figure 23 indicated that HSV-1 induces the upregulation of miR-146a specifically in monocytes THP-1 but not in

epithelial non-immune cells model such as HEp-2 cells, which are more susceptible and permissive to HSV-1 infection compared to monocytes and other immune cells.

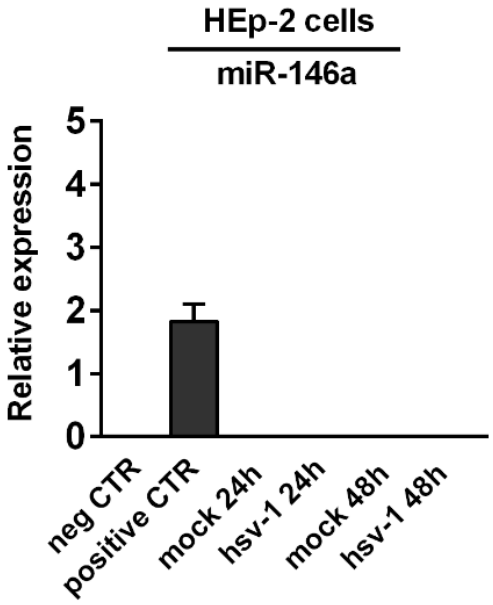

Figure 23. Analysis of miR-146a expression in a permissive HEp-2 cell line after exposure to HSV-1 by Venuti et al., 2019. HEp-2 cells infected or not with HSV-1 at MOI 10, were collected at 24h p.i. and qPCR analysis was performed. THP-1 cells were used as a positive control. Results are the mean ± SD of three independent experiments.

Next, the miR146a expression levels was investigated in both wild-type and DN IκBα THP-1 cells, which express a dominant negative IκBα, in order to verify the correlation with the NF-κB pathway. To this purpose, THP-1 and DN IκBα THP-1 cells were infected or not with HSV-1 at MOI 50 and collected at various time point p.i. to verify the expression levels of miR-146a by qPCR analysis. As shown in Figure 24a, a significant induction of miR-146a was confirmed for THP-1 cells upon HSV-1 infection, with a peak observed at 48h p.i., indicating that the miR-146a increase strictly correlated with the HSV-1 replication process. Conversely, the DN IκBα THP-1 cells were not susceptible to miR-146a increase following HSV-1 infection. These data suggested that the lack of NF-κB pathway could interfere with the upregulation of miR-146a mediated by HSV-1 in monocytic cells.

Besides the transcriptional level of the miR-146a target IRAK1 was verified in both THP-1 and DN IκBα THP-1. In wild type THP-1 cells, a relevant increase of IRAK mRNA levels was found at 3h p.i. (early phase) in infected cells compared to uninfected cells, while later in infection the down-modulation of IRAK1 was observed and correlates

with the increasing expression of miR-146a (Figure 24b). Conversely, in DN IκBα THP-1 cells the mRNA levels of IRAK1 were similar in infected or uninfected cells (Figure 24b). To note, the upregulation of miR-146a levels in THP-1 cells at early stages of viral infection (60' p.i.) does not lead to downregulation of its target IRAK1, as observed during the late stage, suggesting that other cellular and/or viral factors could have a regulatory function on this pathway (Figure 24c).

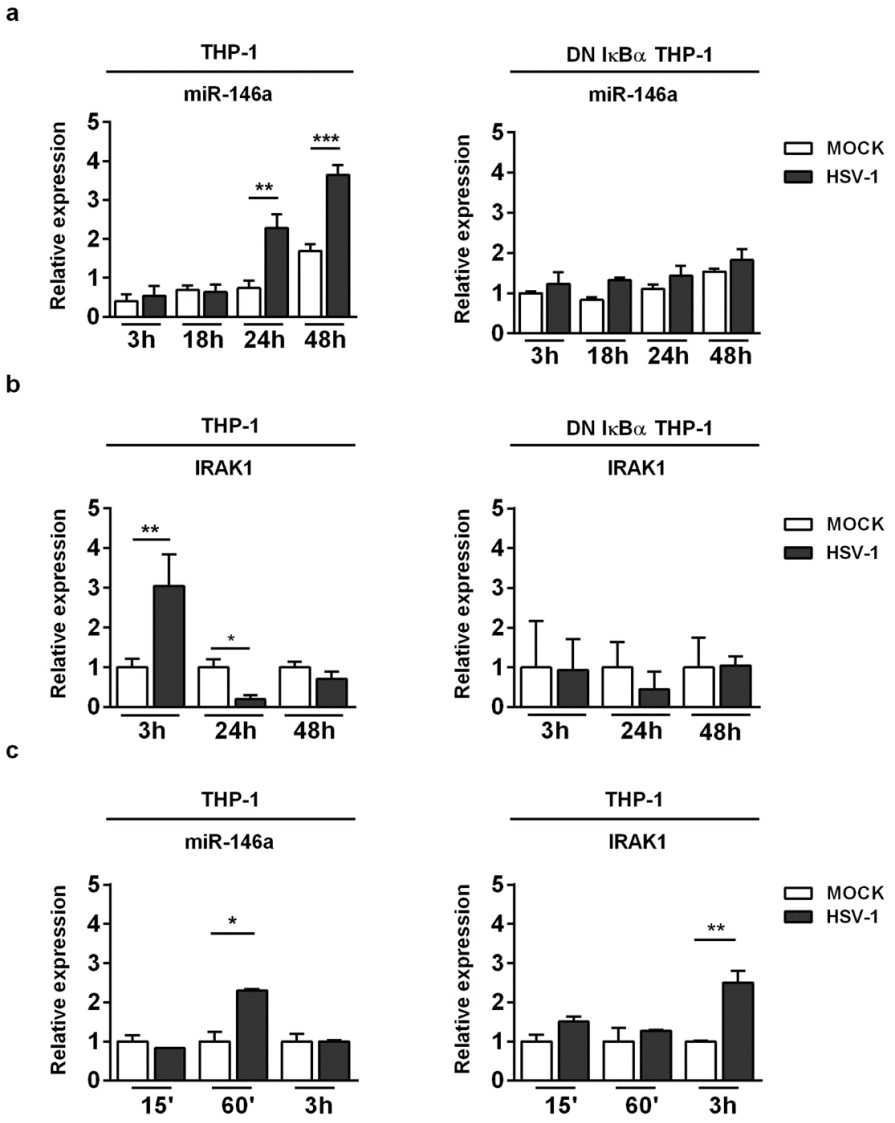

Figure 24. NF-κB-dependent activation of miR-146a in HSV-1-infected monocytic leukemia cell line by Venuti et al., 2019. (a) THP-1 and DN IκBα THP-1 cells were infected with HSV-1 at MOI 50 and collected at the indicated time p.i. MiR-146a expression level in HSV-1 infected THP-1 cells compared to DN IκBα THP-1 infected cells; (b) Analysis of miR-146a-target IRAK1 expression level in THP-1 and DN IκBα THP-1 cells infected or mock infected with HSV-1. (c) Expression pattern of miR-146a and IRAK1in THP-1 cells at early stages of infection. THP-1 cells were mock infected or infected with HSV-1 at MOI 50 PFU/cell and collected at 15′, 60′ and 3 h p.i. and qPCR analysis were performed. Mean ± standard error of the mean (SEM) is indicated. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

To further investigate if the late downregulation of IRAK1 observed in THP-1 cells would have affecting the NF-κB pathway, the transcriptional levels of p50 and p65 were also analyzed during HSV-1 infection. THP-1 cells were mock infected or infected with HSV-1 at MOI 50 and collected at 24h p.i. As showed in Figure 25, neither activation nor deregulation was found in HSV infected cells compared to uninfected cells indicating that the overexpression of miR146a later during HSV-1 infection, which results in downregulation of IRAK1, could negatively regulate the NF-κB signaling pathway, and thus the expression of p50 and p65 mRNA levels.

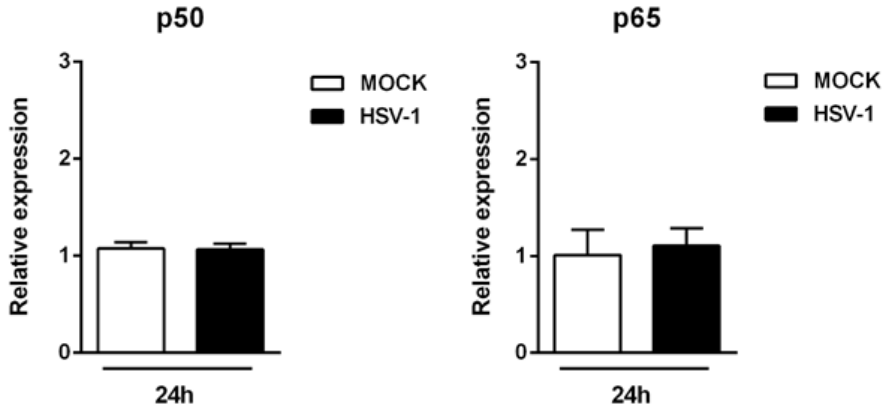

Figure 25. Analysis of p50 and p65 mRNA levels in THP-1 cell after exposure to HSV-1 by Venuti et al., 2019. THP-1 cells were mock infected or infected with HSV-1 at MOI 50 and collected at 24h p.i. qPCR analysis of p50 and p65 was performed by using the following primers: p50-Forw-5'cgtggtgcggctcatgtttac; p50-Rev-5'tttcaagttggatgcattggg; p65-Forw-5'tcagtgagcccatggaattcc; p65-Rev-5'cacagcaatgcgtcgaggtg. Results are the mean ± SD of three independent experiments.

3.5.2. Role of miR-146a in the biology of HSV-1.

To study the biological effect of miR-146a on HSV-1 replication, overexpression of exogenous miR-146a was performed in THP-1 cells prior to infect them with the virus. The recombinant virus HSV-1/EGFP virus, expressing the enhanced green fluorescent protein (EGFP), was used in this analysis and the expression of the reporter gene EGFP was used to evaluate the contribution of miR-146a in regulating HSV-1 gene expression.

qPCR analysis and virus yield quantification as well as viral DNA quantification were performed from THP-1 cells nucleofected with miR-146a mimic or miRNA mimic negative control and then infected with HSV-1/EGFP at MOI 50 at 24 and 48h p.i. As

shown in Figure 26, the overexpression of miR-146a induces a significant decrease of IRAK1 mRNA levels and a relevant increase of EGFP expression in HSV-1 infected cells compared to the control cells (Fig. 26b, 26c). Moreover, the virus yield as well as the accumulation of viral DNA increased significantly in THP-1 cells upon the overexpression of miR-146a (Fig. 26d, 26e). Taken together these results clearly confirmed that the miR-146a suppress the NF-κB signaling pathway, through the downregulation of IRAK1, thus promoting the HSV-1 replication in THP-1 cells.

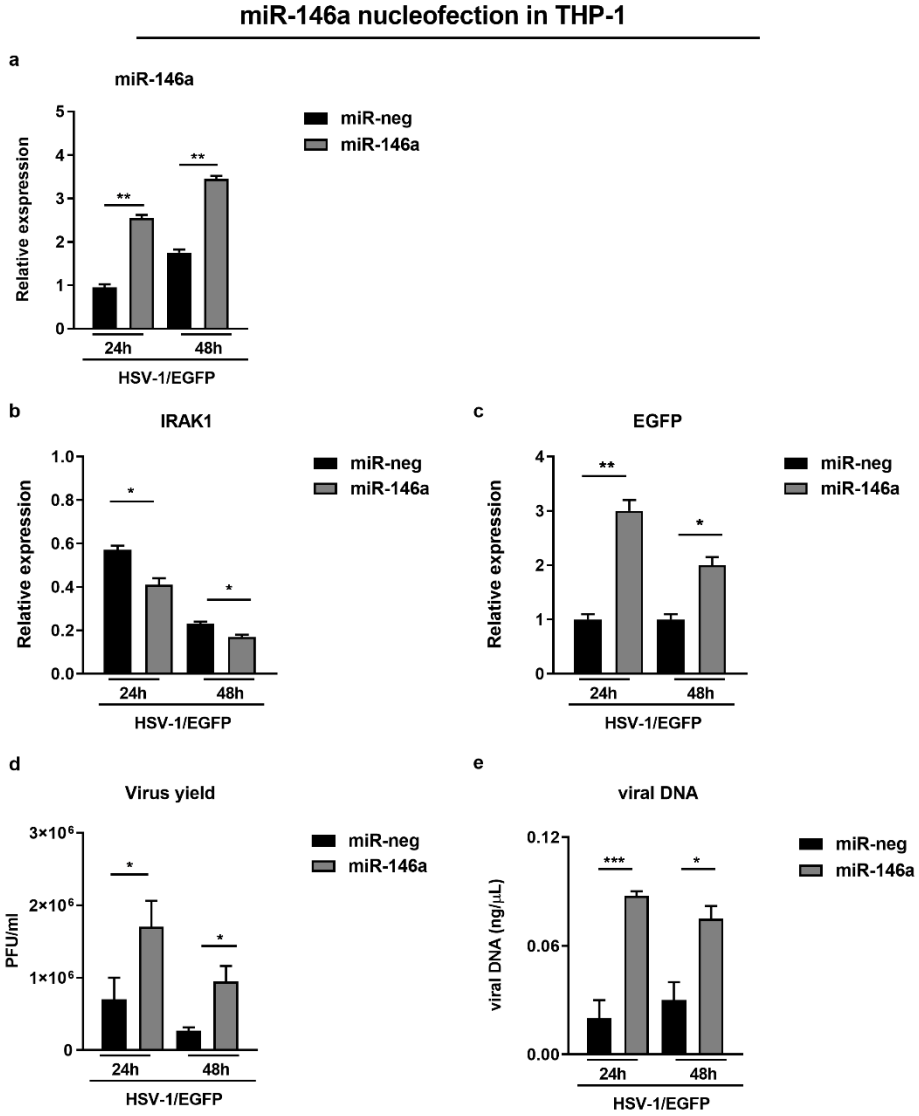

Figure 26. Role of miR-146a in the biology of HSV-1 by Venuti et al., 2019. THP-1 cells were nucleofected with 300 nM of miR-146a mimic or miRNA mimic negative control, followed by infection with HSV-1\EGFP at MOI 50. The cells were collected at 24h and 48h p.i. to perform qPCR analysis and titration. (a) relative expression of miR-146a;(b, c) mRNA expression levels of IRAK1 and EGFP; (d) virus yield quantified by standard plaque forming assay; (e) viral DNA quantified from Ct using the standard curve method and expressed as concentration of ng of DNA for µl. Mean ± standard error of the mean (SEM) is indicated. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

3.6. Discussion

The activation of the innate immune response against viral pathogens is a highly orchestrated process that involves various host cells and viral factors. The viral immunopathogenesis is a multistage process that involves a balance between the activation and inhibition of NF-κB. HSV-1 encodes multiple NF-κB regulators, including ICP0 (Amici et al., 2006; Diao et al., 2005), ICP27 , UL24 (Xu et al., 2017) and UL42 (Zhang et al., 2013, p. 42), which impact directly or indirectly on NF-κB signalling by different mechanisms. However, the intrinsic role of NF-κB in innate immune responses against the HSV-1 is not fully understood.

During the last years, it has been reported that small non-coding RNAs, such as miRNAs, act as posttranscriptional regulators of gene expression and, at the same time, modulate the NF-κB response. The discovery of epigenetics and non-coding RNAs has rapidly established miRNAs as crucial components of the signalling networks that regulate the inflammatory process (Boldin and Baltimore, 2012). Recent findings on the role of miRNAs in the NF-κB signalling cascade indicated that miR-146a is critical for regulating the immune response (Rebane et al., 2014). These findings led many researchers to explore the correlations between miRNAs and immune response against viruses. There is evidence that miR-146a, upregulated during viral infection, suppressed the NF-κB pathway by down-regulating the adaptor proteins IRAK1 and TRAF6 (Hou et al., 2009). Interestingly, the processing of pri-miR-146a into mature miR-146a is also under the control of pro-inflammatory signalling (Boldin and Baltimore, 2012). Viral infection also stimulates miR-146a expression in an NF-κB-dependent manner (Hou et al., 2009).

In the present study, the correlation between HSV-1, NF-κB and miR-146 was mainly explored in human monocytic THP-1 cells. Results obtained from this research clearly indicate an additional mechanism by which HSV-1 can regulate the innate immune response through NF-κB by recruiting miR-146a. The functional consequence of miR-146a activation mediated by HSV-1 was the transcriptional upregulation of the putative targets such as the interleukin-1 receptor-associate kinase 1 (IRAK1). The adapter molecule IRAK1 is known to operate downstream of a number of immune receptors and control the activation of NF-κB. Recent data on human cytomegalovirus (HCMV) showed that the promotion of viral replication is a consequence of upregulation of miR-146a during HCMV viral replication (Chan et al., 2012). In THP-1 cells model,

the overexpression of miR-146a, following viral infection, significantly decreases the transcriptional levels of IRAK1. As a consequence, miR-146a overexpression promotes the accumulation of viral DNA and enhance both the expression of the Tag protein EGFP and HSV yield. Based on these observations, miR-146a can act as a negative regulator of NF-κB pathway through the suppression of IRAK1.

The miRNA-146a upregulation mediated by HSV-1 can be an additional mechanism, enrolled by the virus, to avoid the uncontrolled immune response during the viral invasion, which helps its survival in host cells. Not surprisingly, many research groups have discovered a broad of interesting proteins able to inhibit NF-κB activation and control the onset of inflammation (Liew et al., 2005). Inappropriate activation of inflammatory processes is harmful to an organism and can lead to the development of disease. Thus, multiple molecular mechanisms may keep this process under tight control and allow the suppression of the inflammatory response.

Chapter IV

Biological characterization and antiviral activity of polyphenol-rich extracts against HSV-1

The search for novel safe and effective drugs has recently led to the discovery of several molecules of natural and synthetic origin as alternative therapeutic solutions to conventional drugs. The standard antiviral treatments against the herpes simplex virus type I (HSV-1) are mainly based on the guanosine analogues such as acyclovir which target the viral DNA polymerase and inhibits the viral genome replication. However, drug-resistant strains of HSV frequently develop following therapeutic treatment with conventional antiviral drugs and the lack of a fully effective vaccine against herpes viruses represents a serious public health problem (Jiang et al., 2016). Therefore, the discovery of novel anti-HSV drugs deserves great effort.

4.1. Natural product as antivirals drugs against HSV-1.

A wide range of studies have shown the beneficial biological properties of bioactive compounds derived from natural sources, including leaves and seeds extracts, essential oils and secondary metabolites (Kandlakunta et al., 2015).

Among others, polyphenols possess a great antiviral potential. Indeed, several phenolic compound inhibits the viral attachment by preventing the binding between the HSV-1 glicoproteins's and the glycosaminoglycan (GAG) receptors on the cell surface (El-Toumy et al., 2018). The antiviral effect of quercetin was assessed against HSV-1, HSV-2 and acyclovir-resistant HSV-1 (Hung et al., 2015). Indeed, Hung and colleagues demonstrated that quercetin blocks the viral entry into the cells and the NF-kB pathway activation. Similarly, a significative anti-binding effect of almond (*Prunus dulcis*) skin extracts was demonstrated against HSV-1 (Bisignano et al., 2017).

Another potential antiviral agent against HSV-1 is represented by curcumin. The antiviral activity of curcumin and its derivatives gallium-curcumin and Cu-curcumin is exerted through the suppression of immediate-early gene expression and viral replication (Kutluay et al., 2008; Zandi et al., 2010). Polyphenols-rich extracts from the leaves of *Morus alba* and *Aloe vera* showed a significant antiviral effect against HSV-1 (El-Toumy et al., 2018; Rezazadeh et al., 2016). Other natural products have been shown to target

host cellular factors. Homoharringtonine from *Cephalotaxaceae* induces the degradation of the eukaryotic translation initiation factor-4E (eIF4E), thus inhibiting the protein translation (Dong et al., 2018; Gu et al., 2015).

4.2. Aim of the research

In recent years several reports indicated that phytochemicals present in almond and pistachios are responsible for antioxidant beneficial effects associated with their diet assumption. The aim of this research was to explore the antiviral activity of polyphenol-rich extracts from almond (*Prunus dulcis* L.) skins and pistachios (*Pistacia vera* L.) kernels against HSV-1.

The antimicrobial potential of polyphenols-rich extracts from almond skins has been previously demonstrated against both HSV-1 and HSV-2 (Arena et al., 2015; Bisignano et al., 2017). The most represented flavonoids in almond skins were (+)-catechin, (-)-epicatechin, kaempferol and isorhamnetin (3-*O*-rutinoside and 3-*O*-glucoside) (Mandalari et al., 2010; Milbury et al., 2006). Therefore, the purpose of this work was to assess the antiherpetic effect of a representative mix of polyphenols identified in the almond skin extracts (NS MIX). Moreover, the antibacterial, antifungal and antiviral activities of lipophilic extracts from kernel and pistachios seeds (*Pistacia vera,* L.*)* have been assessed (Bisignano et al., 2013; La Camera et al., 2018; Özçelik et al., 2005). Thus, the anti-HSV-1 effect of polyphenol-rich extracts from natural shelled (NPRE) pistachios kernels (*Pistacia vera* L.) and a mixture of representative polyphenolic compound from pistachios kernels (NP MIX) was also investigated.

4.3. Materials and Methods

Cell cultures and viruses

African green monkey kidney (VERO) cells were cultured in DMEM medium (Corning) supplemented with 6% of FBS (Euroclone), 100 U/ml penicillin and 100 mg/ml streptomycin and maintained at 37° C whit 5% CO_2. The wild-type HSV-1 was a gift from Professor Bernard Roizman (University of Chicago) and the HSV-1-VP26GFP virus, expressing a GFP tagged capsid protein VP26, was described previously (Siracusano et al., 2016). The virus stocks were propagated in VERO cells. Cell-free supernatant containing the viral suspension was stored at -80 °C.

Plant materials

Natural almonds (NS) were provided by the Almond Board of California. A mixture of polyphenols extract from almond skin (NS MIX) was prepared as previously reported (Bisignano et al., 2017). Briefly, catechin, naringenin-7-O-glucoside, kaempferol-3-O-glucoside, epicatechin, isorhamnetin-3-O-rutinoside and isorhamnetin-3-O-glucoside were acquired from Extrasynthese (Genay, France) and mixed (28:20:17:15.5:10:9.5 w/w). American natural shelled (NP) pistachio kernels were provided by Di Bartolo S.r.l., Calatabiano (Italy). Polyphenol-rich extracts (NPRE) and a mix of pure polyphenol compounds (NP MIX) were obtained from NP as previously reported (La Camera et al., 2018; Mandalari et al., 2013; Musarra-Pizzo et al., 2019). Briefly, for the preparation of the NP MIX, catechin, eriodictyol-7-*O*-glucoside, gallic acid, protocatechuic acid, caffeic acid, rutin, and isoquercetin were acquired from Extrasynthese (Genay, France) and mixed (30:20:15:15:11:5:4, w/w). The compounds were dissolved in DMSO (stock solution 100 mg/mL) and properly diluted in order to obtain a final DMSO concentration ≤0.01%.

Cytotoxicity assay

VERO cells were seeded in a 96-well plate ($2*10^4$ cells/well). The next day the cells were treated with various dilution of natural and synthetic extracts: NS MIX (0.1-1.6 mg/mL); NPRE (0.4-1.4 mg/mL); NP MIX (0.1 mg/mL). A DMSO control was included for each dilution. After 72h the cell viability was measured by monitoring the intracellular ATP concentration by using the ViaLight plus kit (Lonza, Basel,

Switzerland) according to the manufacturer's instructions. Briefly, the cells were the cells were lysed for at least 10 min and the ATP monitoring reagent plus (AMR plus) was added to each well and incubated for 2 min at room temperature (RT). The luminescence value was measured in a GloMax Multi Microplate Luminometer (Promega Corporation, Madison, WI, USA) and converted to the cell proliferation index (%) using the following equation:

$$\text{Cell viability } \% = (A - B / C - B)\ \%;$$

A: average of treated sample; B: background luminescence; C: average of untreated samples.

Plaque Reduction Assay

Plaque reduction assay was carried out on VERO cells in a 24-well plates. Approssimatively $2*10^5$ cells/well were seeded in 24-well plate, the virus was diluted to yield 60 plaques/100 µL. the infection was carried out for 1h at 37°C by gently shaking. The viral inoculum was then removed and the cells were covered with medium containing 0.8% methylcellulose in the presence of the different compound at various concentrations. After 72h the cells were stained with crystal violet and the plaques were detected and counted with an inverted microscope (Leica DMIL, Nuβloch, Germany).

Binding Assay

The binding assay was performed by using the HSV-1-VP26GFP virus. The viral suspension (MOI 1) was incubated with the NPRE (0.8mg/ml) and the NP MIX (0.1 mg/mL) for 1h at 4°C. The viral infection was performed at 4 °C for 1h with gently shaking, in order to allow the binding of the virus to the cell receptors, but not the entry (Bisignano et al., 2017). The cells were washed with cold PBS to remove the unbound viruses' particles prior to add fresh media. After 24h post infection (p.i.) the expression of the VP26GFP protein was analysed by (i) detection of the auto-fluorescence of VP26-tagged protein and (ii) western blot analysis.

Fluorescent microscopy analysis

The samples were layered on polylysinated slides, fixed with 4% paraformaldehyde (PFA 4%), washed three times with PBS 1X and stained with Hoechst 33342. Samples were analysed on a fluorescence microscope (Leitz, Wetzlar, Germany).

Protein extraction and western blot.

Cell pellets were collected, washed in 1X phosphate-buffered saline (PBS) and lysed with cell lysis buffer (Cell Signaling Technology). An equal amount of protein extracts was subjected to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad Life Science Research, Hercules, CA), blocked and reacted with primary antibody and appropriate secondary antibody, followed by chemiluminescent detection. Quantitative densitometry analysis of immunoblot band intensities was performed by using the TINA software (version 2.10, Raytest, Straubenhardt, Germany).

Antibodies

Monoclonal anti-US11 and anti-ICP8 were provided by professor Bernad Roizman. Anti-GAPDH (sc-32233), anti-HSV-1 UL42 (sc-53333) and anti-GFP (sc-9996) were purchased from Santa Cruz Biotechnology. Anti-β-actin (ab8226) was provided from Abcam. Secondary HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Millipore.

Viral DNA extraction and qPCR

The cell pellets were lysed using TRIzol® (Life Technologies, CA, United States), according to the manufacturer's instruction. The DNA solutions were extracted with phenol-chloroform and precipitated from the interphase and organic phase with 100% ethanol. The DNA pellet was washed twice with 0.1 M sodium citrate in 10% ethanol and dissolved with 8 mM NaOH. The concentration of DNA was determined by fluorometer analysis with the Qubit dsDNA HS (High Sensitivity) Assay Kit according with the manufacturer's instruction. Quantitative Real-Time PCR was performed in a Cepheid SmartCycler II System (Cepheid Europe, Maurens-Scopont, France), using specific TaqMan probe. Total cellular DNA (1µg) was mixed with 0.5µM of each forward and reverse primers, 1µM of TaqMan probe, 1 µM of dNTP mix, NH4 reaction buffer 1X, 2mM of MgCl2, and 5U/µL of thermostable DNA polymerase BIOTAQTM (BIO-21040 Bioline) in a total volume of 25µL. The oligonucleotide primer pairs were as follows: HSV-1 Fw 5'-catcaccgacccggagagggac; HSV-1 Rev 5'gggccaggcgcttgttggtgta, HSV-1

TaqMan probe 5'-6FAM-ccgccgaactgagcagacacccgcgc-TAMRA, (6FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). The amplification was performed following specific steps (10 min at 95 °C, 30 s at 95 °C for 40 cycles, 30 s at 55 °C, and 30 s at 72 °C, 5 min at 72 °C) and a negative sample was used as amplification control for each run. The relative quantitation of HSV-1 DNA was generated by comparative Ct method using GAPDH as housekeeping gene.

Statistical analysis

The data were analysed as the means of triplicates ± SD for each dilution from three biological independent experiments. The Graphpad Prism 6 software (GraphPad Software, San Diego, CA, USA) was used for data analysis. Student`s t-test and one-way ANOVA were used to compare different conditions. The asterisks (*, ** and ***) indicate the significance of *p*-values less than 0.05, 0.01 and 0.001, respectively. The 50% cytotoxic concentration (CC_{50}) and half maximal effective concentration (EC_{50}) were calculated from concentration-effect curves by using non-linear regression analysis.

4.4. Results

4.4.1. Antiviral activity of polyphenols-rich extracts from natural almond skins.

The anti-HSV activity of polyphenols-rich extracts from almond skins from *Prunus dulcis* was previously demonstrated on VERO cells (Bisignano et al., 2017). Therefore, a mix of representative polyphenols identified in the natural almond skin (NS) extracts were prepared as reported in the Methods section and tested against HSV-1. The polyphenols compounds used to prepare the NS MIX are indicated in Table 1.

Table 1. Polyphenolic compounds used to prepare the NS MIX.

Compound	Polyphenols components
NS MIX	Catechin Naringenin-7-O-glucoside Kaempferol-3-O-glucoside Epicatechin Isorhamnetin-3-O-rutinoside Isorhamnetin-3-O-glucoside

The cytotoxic effect of the NS MIX was evaluated on VERO cells, which are the permissive cellular model for HSV-1 replication. Thus, VERO cells were treated or not with the NS MIX at various concentration (0.1-1.6mg/ml) and the cell viability was measured by using a luminescence assay as described in Materials and Methods. As shown in Figure 27, no cytotoxic effect was found at concentrations below 0.4 mg/mL and the half maximum cytotoxic concentration (CC_{50}) was 0.604 mg/ml (Fig. 27).

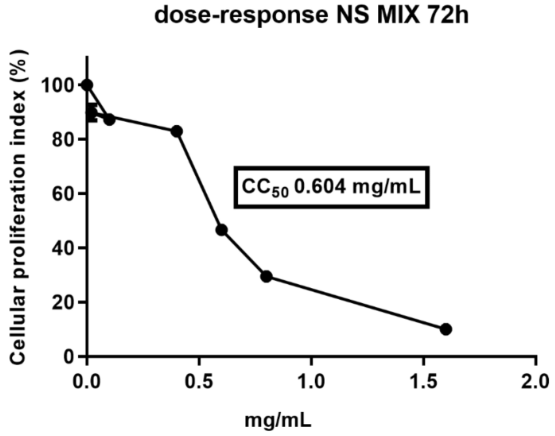

Figure 27. Viability of VERO cells treated with NS MIX by Musarra-Pizzo et al., 2019. VERO cells were incubated with 1.6, 0.8, 0.6, 0.4, 0.2, and 0.1 mg/mL of NS MIX for 72 h. DMSO was used as a negative control. Cells were then collected and their viability was determined using the ViaLight™ plus cell proliferation and cytotoxicity bioassay kit (Lonza Group Ltd, Basel, Switzerland). The luminescence value was converted into cellular proliferation index as described in Materials and Methods. Results represent the mean of three biological independent experiments ± SD.

To evaluate the antiviral effect of the mix on HSV-1, a plaque reduction assay was performed following the treatment with three non-cytotoxic concentrations (0.4, 0.2 and 0.1 mg/mL) of NS MIX. The results showed a significant reduction in viral titer at the concentration of 0.4mg/mL (** p <0.01) (Fig. 28A). No antiviral effect was found at the lower concentrations (0.2 and 0.1 mg / mL). In addition, a reduced plaque size morphology was also observed in a dose-dependent manner (Fig. 28B). Acyclovir was used as a positive control (Fig. 28C).

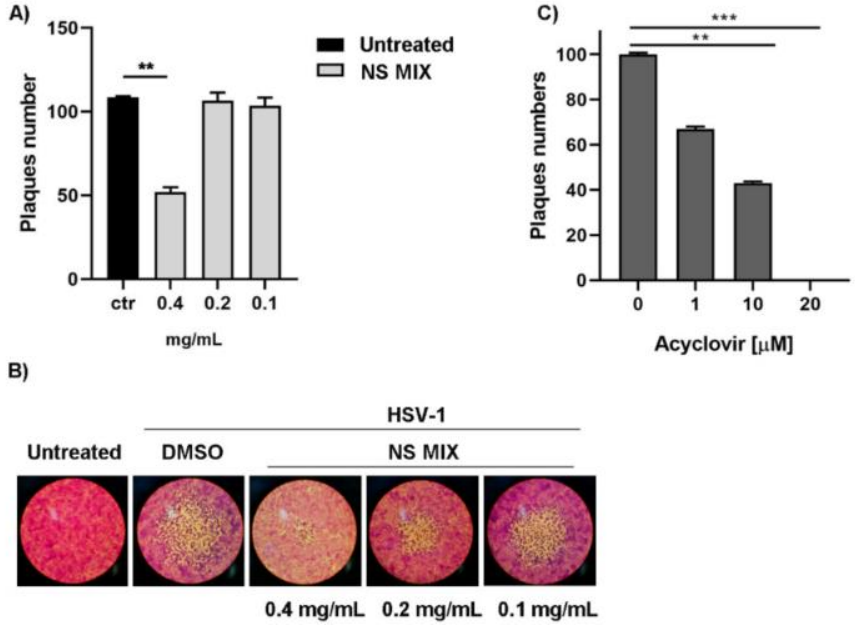

Figure 28. Antiviral activity of NS MIX by Musarra-Pizzo et al., 2019. VERO cells were pre-treated with 0.4, 0.2, and 0.1 mg/mL of NS MIX for 1 h and the cells were then infected with HSV-1 for 1 h at 37 °C under gentle shaking. After incubation, the cells were covered with medium containing 0.8% methylcellulose in the presence of NS MIX at 0.4, 0.2, and 0.1 mg/mL, separately. After three days the cells were fixed and stained with crystal violet and the plaques were visualized with an inverted microscope. (A) Plaque reduction assay following the NS MIX treatment. (B) Plaque morphological change due to the NS MIX treatment. Results are the mean of three biological independent experiments ± SD for each dilution. (C) Antiviral activity of acyclovir was tested in Vero cells infected with HSV-1 and incubated with medium containing 0.8% methylcellulose in the presence of acyclovir at 1, 10, and 20 μM. The cells were stained with crystal violet for plaque counting and detection. Data are expressed as a mean (± SD) of at least three experiments and asterisks (**, ***) indicate the significance of p-values less than 0.01, and 0.001, respectively.

Next, the expression of viral DNA and viral proteins was evaluated following the treatment with the 0.4 mg/mL of the NS MIX. As shown in Figure 29A, the treatment with the NS MIX clearly reduced the cytopathic effect induced by HSV-1 infection. Furthermore, a reduced accumulation of the viral proteins ICP0, UL42 and Us11 (Fig. 29B) and viral DNA (* p <0.05) (Fig. 29C) was also observed in HSV-infected and treated cells compared to non-treated infected cells.

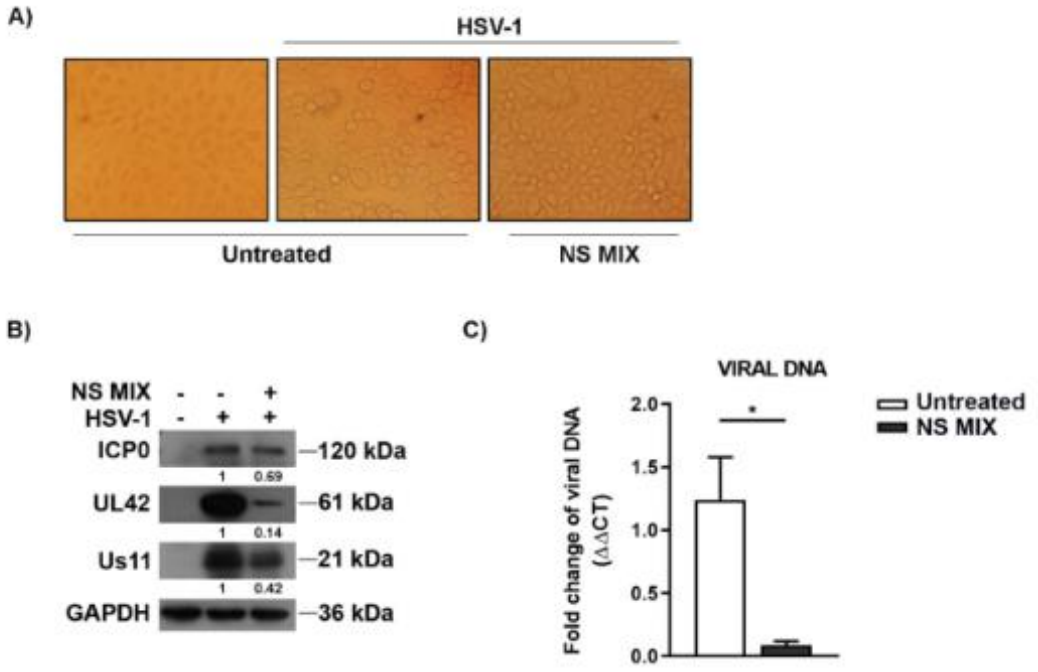

Figure 29. NS MIX affected the expression of viral antigens and HSV-1 replication by Musarra-Pizzo et al., 2019. (A) Normal phase contrast inverted micrographs of VERO cells treated with 0.4 mg/mL of NS MIX. The cells were pre-treated with NS MIX for 1 h at 37 °C, then cells either mock infected or infected with HSV-1 at multiplicity of infection (MOI) of 1 for 1 h and incubated in presence of 0.4 mg/mL of the NS MIX for 24 h. (B) Immunoblot analysis was performed to detect α (ICP0), β (UL42), and γ (US11) viral proteins. GAPDH protein was used as loading control. Band density indicated in the figure was determined with the TINA program (version 2.10, Raytest, Straubenhardt, Germany) and expressed as the fold change over the housekeeping gene GAPDH. (C) Relative quantization of viral DNA was performed using real-time quantitative PCR and analyzed by the comparative Ct method (ΔΔCt). Data are expressed as a mean (± SD) of at least three experiments and asterisk (*) indicate the significance of p-values less than 0.05.

4.4.2. Antiviral activity of polyphenols-rich extracts from natural shelled pistachios kernels (NPRE)

The antimicrobial properties of polyphenol-rich extracts from pistachios (*Pistacia vera*, L.) have been reported by several studies. Particularly, their bactericidal action has been demonstrated against *Listeria monocytogenes*, *Staphylococcus aureus* and MRSA (*Staphylococcus aureus* methicillin-resistent) (Bisignano et al., 2013; La Camera et al., 2018). Furthermore, the antiviral activity of lipophilic extracts obtained from different parts of *Pistacia vera* has also been demonstrated against HSV-1 (Özçelik et al., 2005). The effect of polyphenol-rich extracts derived from raw shelled pistachios (NPRE) (*Pistacia vera*, L.) was investigated against HSV-1. To evaluate the cytotoxic effects of pistachios extracts, a cell viability assay was performed on VERO cells treated with

NPRE at different concentrations (0.4-1.4 mg/mL) for 72h. The cell proliferation index (%) was calculated by measuring the intracellular production of ATP and the half maximum cytotoxic concentration (CC_{50}) was 1.2 mg/mL (Fig. 30).

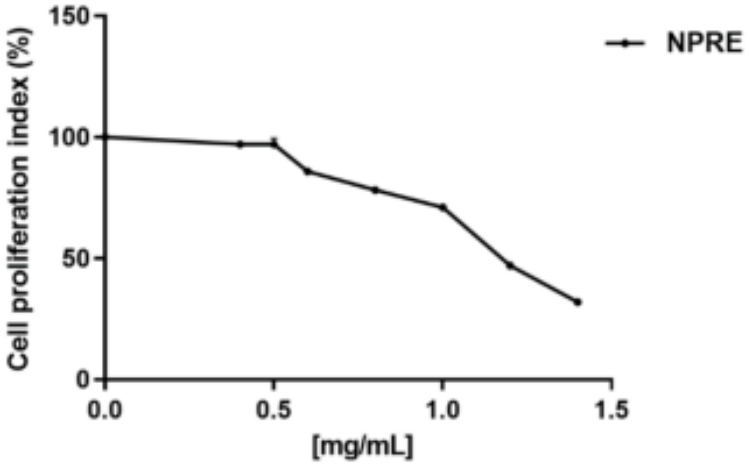

Figure 30. Cell viability of VERO cells treated with increasing concentration of polyphenol-rich extracts from natural shelled pistachios kernels (NPRE) by Musarra-Pizzo et al., 2020. The cell proliferation index (%) was calculated by means of cellular ATP level measured after 72 h treatment. The values were expressed as percentages of treated vs. control cells (DMSO). Each value is the mean ± standard deviation (SD) of three experiments.

The antiviral effect of NPRE was determined by: (i) plaque reduction assay, (ii) viral protein expression analysis, (iii) viral DNA quantization. VERO cells were infected and treated with various concentration of NPRE (0.1-0.8 mg/mL) for 24 h and then collected to perform the analisis.

The results of plaques assay showed that the treatment with NPRE resulted in a dose-dependent antiviral effect. Particularly, by using the maximum non-cytotoxic concentration of NPRE (0.8 mg/ml), plaques formation was totally reduced (100%) as observed with Acyclovir (20μM) (Fig. 31). The half maximal effective concentration (EC_{50}) was 0.4mg/mL and the selectivity index (SI) value was 3.

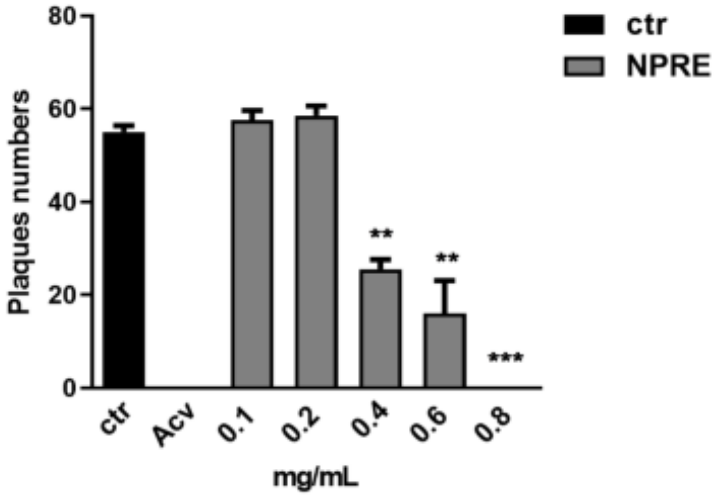

Figure 31. Effects of NPRE on HSV-1 replication by plaque reduction assay by Musarra-Pizzo et al., 2020. VERO cells were infected with HSV-1 (50PFU/100 µL) for 1 h and then treated with NPRE (0.1, 0.2, 0.4, 0.6, 0.8 mg/mL). The DMSO was used in the control samples and acyclovir (20 µM) was used as positive control. Data are expressed as a mean (± SD) of at least three experiments, and asterisks (**, and ***) indicate the significance of p-values less than 0.01 and 0.001, respectively.

Western blot analysis showed that the treatment with NPRE reduced the accumulation of viral proteins ICP8, UL42 and Us11 in infected and treated cells compared to the relative controls. The reduction was greater at the highest concentration used (0.8 mg / ml) confirming the dose-dependent effect of NPRE (Fig. 32).

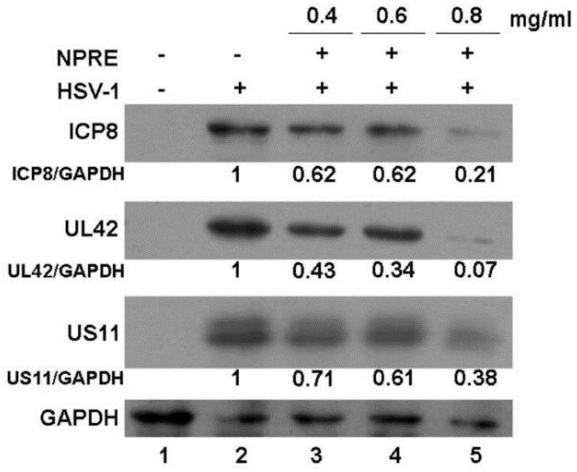

Figure 32. Analysis of viral protein expression by western blot analysis by Musarra-Pizzo et al., 2020. VERO cells were infected with HSV-1 at MOI 1 and then treated with NPRE (0.4, 0.6, 0.8 mg/mL). The cells were then collected to perform western blot analysis.

In order to verify whether the decrease of viral protein accumulation in infected and treated cells led to a decrease of viral DNA synthesis, qPCR analysis were performed using a specific TaqMan probe for HSV-1. As shown in Figure 33, viral DNA accumulation was significantly reduced in infected and treated cells compared to untreated infected cells, suggesting that NPRE treatment may also interfere with the viral DNA synthesis process leading to a reduced amount of viral DNA.

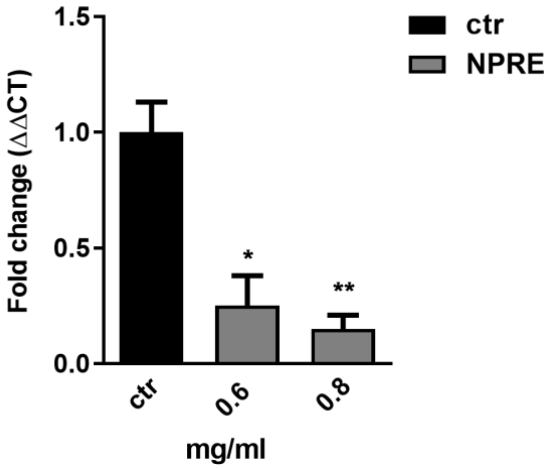

Figure 33. Relative quantization of viral DNA with specific HSV-1 TaqMan probe in real-time PCR by Musarra-Pizzo et al., 2020. DMSO was used in the infected control samples. Data are expressed as a mean (± SD) of at least three experiments and asterisks (* and **) indicate the significance of p-values less than 0.05 and 0.01, respectively.

Subsequently, a binding inhibition assay was performed in order to investigate the effect of pistachios extracts on virus attachment. Therefore, a recombinant virus HSV-1-VP26GFP, expressing a GFP fluorescent VP26 protein was used to monitor the infection through the detection of the autofluorescent tag. Thus, the viral inoculum was treated or not with the NPRE and a mixture of representative polyphenolic compound (NP MIX) separately, prior to infect the cells. The NP MIX was prepared as reported in the Methods section and the polyphenolic compounds are indicated in Table 2.

Table 2. Polyphenolic compounds used to prepare the NP MIX.

Compound	Polyphenols components
NP MIX	Catechin Eriodictyol-7-O-glucoside Gallic acid Protocatechuic acid Caffeic acid Rutin Isoquercetin

The infection was carried out at 4°C as described in Material and Methods. The results showed that NPRE treatment does not inhibits the virus attachment (data not showed). However, the treatment with NP MIX (0.1 mg/ml) results in a 50% decrease of the VP26-GFP autofluorescence (Fig. 34a, 34b) and a decrease of VP26-GFP protein expression compared to the control (Fig. 34c). However, the NP MIX did not show good tolerability due to the significant cytotoxic effect which resulted in a 40% reduction in cell proliferation index compared to untreated cells (data not shown). Further investigation should be performed in order to evaluate the cellular tolerability of NP MIX as well as its antiviral activity.

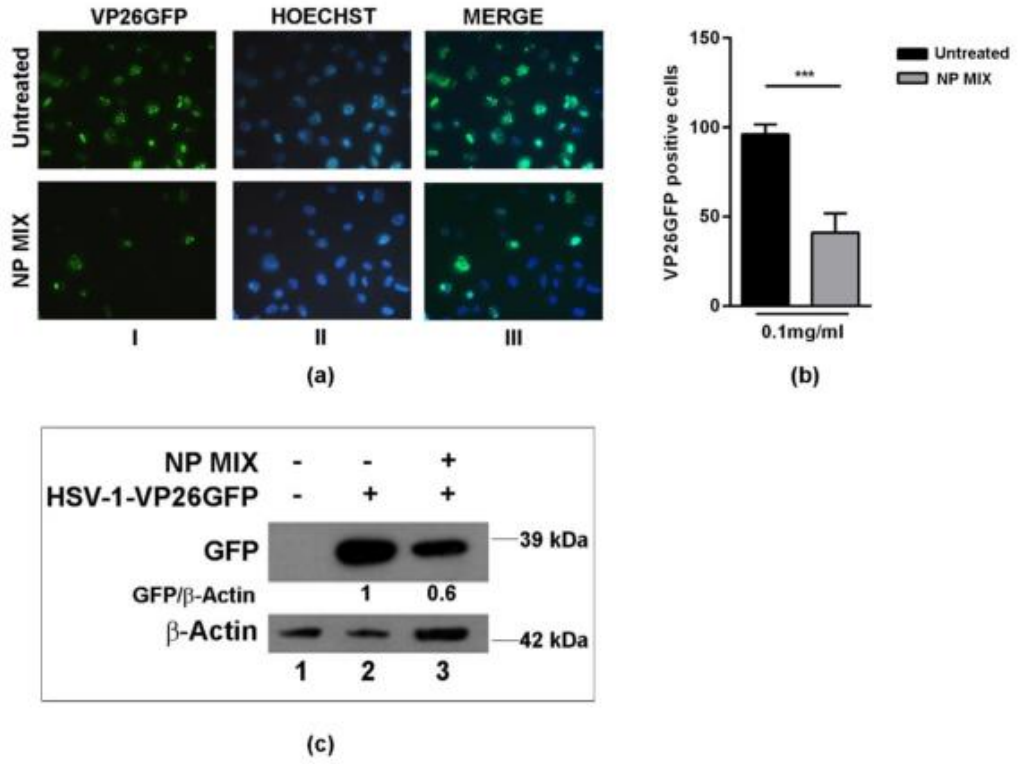

Figure 34. Effect of the NP MIX treatment on HSV-1 replication. VERO cells were either infected or mock-infected with HSV-1-VP26GFP at multiplicity of infection (MOI) 1, as described in the Materials and Methods section. Then, the cells were analysed at 24 h post infection (p.i.): (a) fluorescent images showed the green dots representing the VP26 GFP viral antigen localization (I) Hoechst (blue) was used to stain the nuclei (II) and the merged images are shown in column III; (b) the graph is indicative of the percentage of VP26 GFP-positive cells; (c) Western blot analysis of VP26 GFP-tagged protein. Data are expressed as a mean (± SD) of at least three experiments, and asterisks (***) indicate the significance of p-values less than 0.001.

4.5. Discussion

Acyclovir (ACV) and related nucleoside analogues such as valacyclovir (VCV), famciclovir, ganciclovir and foscarnet, are the standard therapeutic treatment for HSV infections. These antiviral drugs target the HSV-encoded thymidine kinase (TK) and the viral DNA polymerase, thus blocking the viral DNA replication (Whitley, 2006). However, recurrent HSV infection and prolonged pharmacological treatment result in increasing appearance of drug-resistant HSV strains which carried out genetic mutations on HSV-DNA polymerase or thymidine kinase as well as reduced activity of these enzymes (Chakrabarti et al., 2000; Chen et al., 2000; Levin et al., 2004; Piret and Boivin, 2011). Therefore, since no prophylactic vaccine has been developed against HSV, the search for novel antiviral therapies is of fundamental importance, especially for immunocompromised or HIV-infected individuals. Indeed, HSV-1 become a potential risk factor for HIV infection due to ongoing recombination between HSV-1 and HSV-2 in humans which results in increasing genital herpes due to HSV-1 (Casto et al., 2019; Roberts et al., 2003).

Over the past years, plant-derived compounds have been shown to possess great potential in therapeutic treatment as alternative antiviral agents. Indeed, crude extracts, as well as pure compounds isolated from plants, have been studied for their health beneficial properties. Particularly, studies on the structure-activity relationships provide essential information for the design and synthesis of novel anti-viral agents as well as for the discovery of novel therapeutic targets. Indeed, although nucleoside analogues only inhibit viral DNA replication enzymes, natural compounds could interfere with multiple stages of the virus life cycle, expanding the spectrum of antiviral activity against HSV infections. Natural products such as crude extracts and phenolic compounds are known for their broad spectrum antimicrobial and antioxidant activity (Schnitzler et al., 2010; Son et al., 2013). Particularly, the skin of almonds and kernels from pistachios are a rich source of phenolic compounds which possess beneficial properties for human health. In addition, thanks to their antimicrobial and antiviral potential *in vitro*, the study of polyphenols is receiving a lot of attention from the pharmaceutical and healthcare industries.

Therefore, the purpose of this research was to explore the antiviral activity of a mixture of polyphenols from almond skin (*Prunus dulcis*) and pistachios kernels (*Pistacia*

vera, L.) in order to identify novel molecular target and therapeutic approaches for the treatment of herpetic infections.

The data obtained showed that the treatment with a mixture of polyphenolic compounds from almond skin (NS MIX) induces a significant reduction in the viral titer and accumulation of viral proteins and viral DNA. However, the individual components did not show any antiviral activity *in vitro*. Furthermore, a drastic decrease in viral infectivity was detected when the treatment with the mixture was performed directly on the cell monolayer but not on the viral suspension. On the other hand, the pre-treatment of the monolayers with the mixture induced a reduction in the accumulation of early and immediately early viral proteins, such as ICP0 or UL42, as well as viral DNA. According to these data and previous literature data, it is possible to hypothesize that the polyphenol mixture interferes with the HSV-1 gene expression of HSV-1 in the early or late stages of viral replication (Hirabayashi et al., 1991; Ren et al., 2010).

Moreover, results obtained from the present study showed that polyphenol-rich extracts derived from shelled pistachios (NPRE) have a significant antiviral effect against HSV-1. In particular, the antiviral activity of NPRE was exert in a concentration-dependent manner and by using the maximum no cytotoxic concentration (0.8 mg/ml), the viral infection and replication were totally inhibited. Furthermore, these results showed that the antiviral effect was higher when NPRE was added immediately after the binding step, suggesting a possible effect of the extract on protein expression and viral DNA synthesis. Finally, the antiviral action of a mixture of representative polyphenolic components (NP MIX) present in the NPRE was evaluated. The data obtained showed a reduction in the expression of the viral protein VP26-GFP in NP MIX treated and infected samples compared to the control cells. However, given the low cellular tolerability of pure polyphenols compared to the crude extracts, additional analyses are required to optimize the phenolic composition of the mix and characterize their biological and antiviral action. Indeed, the data obtained from this study suggested that the NPRE mechanism of action differs from those of the mixture. This is probably due to a potential synergistic effect between the polyphenols and other components of the crude extracts which results in a more effective antiviral activity.

However, from the data obtained so far, it is not clear whether the molecular mechanisms underlying the antiviral action target cellular or viral structures. Therefore, further studies will be needed to understand the molecular mechanisms underlying the

antimicrobial activity of polyphenols. These studies are fundamental to provide new tools in the formulation of innovative antiviral agents and topical drugs for the treatment of HSV-1 infections as well as to fight the emergence of antiviral drug resistance.

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