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VIRAL AND CELLULAR MODULATION OF VIRUS-INDUCED APOPTOSIS IN EXPERIMENTAL INFECTION MODELS

by

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To my family

ABSTRACT

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Viral and cellular modulation of virus-induced apoptosis in experimental infection models

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The aim of this study was to investigate herpes simplex virus type 1 (HSV-1)- and measles virus (MV)-induced cell death. HSV-1 with deletion in genes encoding infected cell protein (ICP)4 and protein kinase Us3 (d120) induced apoptosis and cathepsin activation in epithelial (HEp-2) and monocytic (U937) cells. Inhibition of cathepsin activity decreased the amount of d120-induced apoptosis indicating that d120-induced apoptosis could be cathepsin-mediated. Also, HSV-1 infection increased caspase activation suggesting that d120-induced apoptosis is probably caspase-mediated. Cystatin treatment decreased the activity of cathepsins and the replication of HSV-1 indicating that cathepsins contribute to HSV-1 infection. Interestingly, d120 induced also necroptosis in monocytic cells. This is the first report on necroptosis in HSV-1-infected cells.

MV induced apoptosis in uninfected bystander T lymphocytes, probably via interaction of MV-infected monocytes with uninfected lymphocytes. The expression of death receptor Fas was clearly increased on the surface of lymphocytes. The number of apoptotic cells and the activation of cathepsins and caspases were increased in MV-infected U937 cells suggesting that MV-induced apoptosis could be cathepsin- and caspase-mediated. Cystatin treatment inhibited cathepsin activities but not MV-induced apoptosis.

Besides HSV-1-induced apoptosis, innate immune responses were studied in HSV-1-infection. HSV-1 viruses with either ICP4 and Us3, or Us3 deletion only, increased the expression of Toll-like receptor (TLR)3 and stimulated its downstream pathways leading to increased expression of type I interferon gene and to functional interferons. These findings suggest that besides controlling apoptosis, HSV-1 ICP4 and Us3 genes are involved in the control of TLR3 response in infected cell.

Keywords: herpes simplex virus type 1 (HSV-1), measles virus (MV), apoptosis, necroptosis, cathepsin, cystatin, caspase-3 and -8, innate immunity, Toll-like receptor (TLR)

TIIVISTELMÄ

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Viruksen aiheuttama solukuolema kokeellisissa infektiomalleissa
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Tämän työn tavoitteena oli tutkia herpes simplex virus tyypin 1 (HSV-1) ja tuhkarokkoviruksen (MV) aiheuttamaa solukuolemaa. HSV-1, jolta on poistettu infektoituneen solun proteiini (ICP)4:ää ja proteiinikinaasi Us3:a koodaavat geenit (d120), aiheutti apoptoosia ja katepsiinien aktivaatiota epiteeli- (HEp-2) ja monosyyttisoluissa (U937). Kun katepsiinien aktiivisuus estettiin, d120-viruksen aiheuttaman apoptoosin määrä väheni osoituksena katepsiinien mahdollisesta osuudesta d120-viruksen aiheuttaman apoptoosin välittäjinä. HSV-1-infektio lisäsi myös kaspaasien aktivaatiota, joten d120-viruksen aiheuttama apoptoosi saattaa olla kaspaasien välittämää. Kystatiinikäsittely vähensi katepsiinien aktiivisuutta ja HSV-1:n lisääntymistä osoituksena katepsiinien osallisuudesta HSV-1-infektioon. Mielenkiintoinen havainto oli myös se, että d120 aiheutti nekroptoosia monosyyttisoluissa. Tämä on ensimmäinen kerta, kun HSV-1:n on osoitettu aiheuttavan nekroptoosia.

MV aiheutti apoptoosia infektoitumattomissa T-lymfosyyteissä, todennäköisesti MV-infektoituneiden monosyyttien ja infektoitumattomien lymfosyyttien vuorovaikutuksen kautta. MV-infektio lisäsi selvästi solukuolemareseptorin Fas:n ilmentymistä lymfosyyttien pinnalla. Apoptoottisten solujen määrä sekä katepsiinien ja kaspaasien aktivaatio lisääntyi MV-infektion seurauksena, mikä saattaa tarkoittaa sitä, että MV:n aiheuttama apoptoosi on katepsiini- ja/tai kaspaasi-välitteistä. Kystatiinikäsittely vähensi katepsiinien aktiivisuutta, mutta ei estänyt MV:n aiheuttamaa apoptoosia.

Viruksen aiheuttaman apoptoosin lisäksi työssä tutkittiin luonnollisen immuniteetin vastetta HSV-1-infektiossa. HSV-1-virukset, joilta oli poistettu joko ICP4- ja Us3- tai pelkkä Us3-geeni(t) lisäsivät Tollin kaltaisen reseptorin (TLR)3:n ilmentymistä sekä TLR3-signaalivälitysketjun stimuloitumista johtaen tyypin I interferonien ilmentymisen ja toiminnallisten interferonien määrän lisääntymiseen. Tulosten mukaan apoptoosin säätelyn lisäksi HSV-1:n ICP4- ja Us3-geenit osallistuvat TLR3-vasteen säätelyyn infektoituneissa soluissa.

Avainsanat: herpes simplex –virus tyyppi 1 (HSV-1), tuhkarokkovirus (MV), apoptoosi, nekroptoosi, katepsiini, kystatiini, kaspaasi-3 ja -8, luonnollinen immuniteetti, Tollin kaltainen reseptori (TLR)

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ABBREVIATIONS

ADAR	RNA-specific adenosine deaminase
AIF	apoptosis-inducing factor
BBD	Beclin 1 binding domain
B cell	B lymphocyte
CNS	central nervous system
CoREST	corepressor of REST
DC, pDC	dendritic cell, plasmacytoid dendritic cell
DISC	death-inducing signaling complex
EMMPRIN	extracellular matrix metalloproteinase inducer (CD147)
FADD	Fas-associated death domain
FCS	fetal calf serum
HDAC	histone deacetylase
HRP	horseradish peroxidase
HSV-1	herpes simplex virus type 1
IAP	inhibitor of apoptosis protein
IFN	interferon
IL	interleukin
IPC	interferon-producing cell
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
LMP	lysosomal membrane permeabilization
mda-5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
moi	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
mTOR	mammalian target of rapamycin
MV	measles virus
MxA	myxovirus resistance protein A
MyD88	myeloid differentiation factor 88
ND-10	nuclear domain 10
Nec-1	necrostatin 1, inhibitor of necroptosis
NF-κB	nuclear factor–κB
NLR	nod-like receptor
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PFU	plaque forming unit
PHA	phytohemagglutinin
PKR	RNA-dependent protein kinase
PML	promyelocytic leukemia protein
PRR	pattern recognition receptor
REST	RE-1 silencing transcription factor
RIG-I	retinoic acid inducing gene I
RIP1	receptor-interacting protein 1
RLR	retinoic acid inducing gene I-like receptors

ROS	reactive oxygen species
Smac/Diablo	second mitochondria-derived activator of caspases/direct IAP binding protein with low PI
SLAM	signaling lymphocyte activating molecule
SSPE	subacute sclerosing panencephalitis
T cell	T lymphocyte
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRIF	TIR domain-containing adaptor protein inducing interferon-β
vhs	virion host shut-off
XIAP	X-linked inhibitor of apoptosis protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I to IV), and on supplementary data.

I Vuorinen T, Peri P, and Vainionpää R (2003). Measles virus induces apoptosis in uninfected bystander T cells and leads to granzyme B and caspase activation in peripheral blood mononuclear cell cultures. European Journal of Clinical Investigation 33(5)434-42.

II Peri P, Hukkanen V, Nuutila K, Saukko P, Abrahamson M, and Vuorinen T (2007): The cysteine protease inhibitors cystatins inhibit herpes simplex virus type 1-induced apoptosis and virus yield in HEp-2 cells. Journal of General Virology, Aug;88(Pt 8):2101-5.

III Peri P, Mattila RK, Kantola H, Broberg E, Karttunen HS, Waris M, Vuorinen T, and Hukkanen V (2008): Herpes simplex virus type 1 Us3 gene deletion influences Toll-like receptor responses in cultured monocytic cells. Virology Journal, 2008 Nov 21;5:140.

IV Peri P, Nuutila K, Vuorinen T, Saukko P, and Hukkanen V (2010): Cathepsins are involved in virus-induced cell death in ICP4 and Us3 deletion mutant herpes simplex virus type 1-infected monocytic cells. Journal of General Virology, in Press. Published September 29, 2010 as doi:10.1099/vir.0.025080-0

1 INTRODUCTION

Apoptosis is a tightly controlled process of programmed cell death, which regulates homeostasis and tissue development. In addition, apoptosis plays a protective role eliminating cells that are no longer useful or cells infected by pathogens. Apoptosis is characterized by chromatin condensation, DNA fragmentation, phosphatidylserine exposure, plasma membrane blebbing, cell shrinkage, formation of apoptotic bodies and activation of caspase cascade. Apoptotic caspases are involved in both the initial signaling events and downstream proteolytic cleavages. Other important factors in the regulation of apoptosis are the Bcl-2 family members, which controls mitochondrial integrity.

Viruses have developed various strategies either to prevent or actively use apoptosis in virus infections. Herpes simplex virus type 1 (HSV-1) is a common human pathogen, which causes orolabial infections, keratitis and encephalitis. HSV-1 induces apoptosis in the host cell. The extent of HSV-1-induced apoptosis is cell type-dependent, indicating that HSV-1-induced apoptosis is regulated by different cellular factors. However, to delay the apoptotic dying of the host cell until HSV-1 has replicated, HSV-1 have developed several strategies to block the HSV-1-induced apoptosis at multiple steps of infection. Hence, in HSV-1-infected cell, there is a complex balance between pro-apoptotic and anti-apoptotic factors.

Measles virus (MV) causes a typical childhood infection that spreads through respiratory route and causes rash. MV infection is associated with transient but strong immunosuppression that contributes to secondary infections and mortality. The mechanisms of immunosuppression are not yet fully understood. Since MV induces apoptosis in several cell types, apoptosis has been suggested to play role in the MV-induced immunosuppression.

Cysteine cathepsins and their inhibitors cystatins are widely distributed in the body. They have an important role in regulating cell survival and death. In addition, cathepsins and cystatins are involved in virus replication. In this thesis, the mechanisms of HSV-1- and MV-induced cell death and the roles of cathepsins and cystatins in the regulation of virus-induced apoptosis and in virus replication were studied. Also, modulation of innate immune response by HSV-1 infections was investigated.

2 REVIEW OF LITERATURE

2.1 Herpes simplex virus type 1

2.1.1 Structure and genome

Herpes simplex virus type 1 (HSV-1) is a neurotropic herpesvirus that causes a variety of infections in humans (Whitley & Roizman, 2001). HSV-1 belongs to the Alphaherpesvirus group in the virus family *Herpesviridae*. As other herpesviruses, HSV-1 is a large DNA virus expressing a large number of enzymes involved in metabolism of nucleic acid, DNA synthesis and processing of proteins. The viral DNA synthesis and capsid assembly of HSV-1 takes place in the host cell nucleus. Productive viral infection is accompanied by inevitable cell destruction. After lytic infection HSV-1 remains latent in the neurons of its host for life and can be reactivated to cause lesions at or near the initial site of infection (Roizman *et al.*, 2007).

The HSV-1 virion is composed of (a) a core containing the viral DNA, (b) an icosahedral capsid surrounding the core, (c) an amorphous protein layer called the tegument and (d) an outer lipid envelope with glycoproteins (Figure 1). The core contains 152 261 basepairs long double-stranded linear DNA genome (strain HSV-1 17+). The capsid is composed of 162 capsomers arranged in an icosahedral symmetry. Four viral proteins (VP5, VP26, VP23 and VP19C) compose the outer shell of the nucleocapsid. Tegument is mainly unstructured and it is composed of at least 20 viral proteins. The most important viral proteins associated with the tegument are the VP16 (or αTIF) virion transactivator protein, the virion host shut-off (*vhs*) protein and a very large protein (VP1-2), which may be involved in the DNA release at the nuclear pore during viral entry. The lipid envelope consists of approximately 11 different viral glycoproteins randomly distributed on the surface of the virion (Grünewald *et al.*, 2003; Roizman *et al.*, 2007). Some glycoproteins may exert their main function within the infected cells.

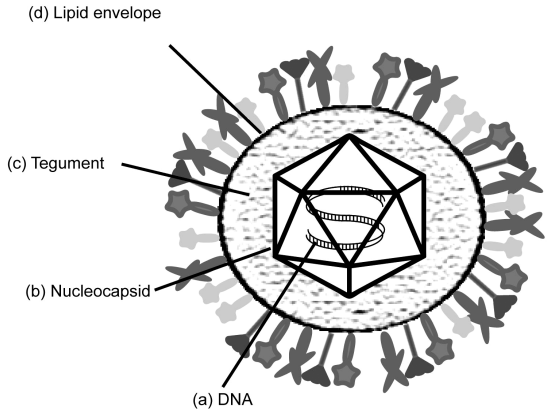

Figure1. Schematic structure of herpes simplex virus type 1 virion.

The HSV-1 genome consist of two covalently linked components, called L (long) and S (short), which consist of unique sequences (U_L and U_S) bracketed by inverted repeats. The repeats are designated as ab and b´a´ for the L component and a´c´ and ca for the S component (Figure 2). The HSV-1 genome encodes approximately 90 transcriptional units, of which at least 84 are translated into proteins. The HSV-1 genes are divided into three general kinetic classes: the α or immediate early genes regulate viral replication, the β or early genes are involved in the viral DNA synthesis and packaging, and the γ or late genes encode the virion proteins (Honess & Roizman, 1974).

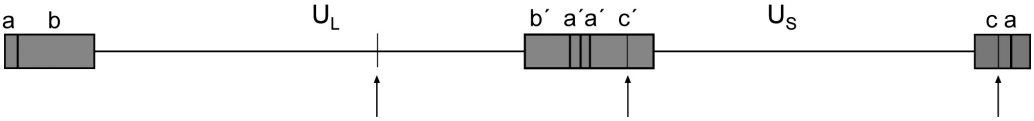

Figure 2. Schematic representation of the arrangement of DNA sequences in the HSV genome. Lines represent the unique sequences (U_L, U_S) and boxes the inverted repeats. The arrows show the location of the origins of replication (*ori*-sequences, one ori_L and two ori_S).

2.1.2 Replication

HSV-1 replicates in a variety of cell types during acute infection with a replication cycle approximately 18 hours, reviewed in Roizman *et al.* (2007) and Hukkanen *et al.* (2010). The replication is initiated when the glycoproteins gC and gB of the HSV-1 virion envelope bind to cell surface heparin sulphate proteoglycan (Herold *et al.*, 1991; Shieh *et al.*, 1992; WuDunn & Spear, 1989). Next, the glycoprotein gD interacts with a specific receptor, such as nectin-1 and -2, herpes virus entry mediator (HVEM), or 3-O-sulphated heparan sulphate (Cocchi *et al.*, 2000; Geraghty *et al.*, 1998; Montgomery *et al.*, 1996; Shukla *et al.*, 1999; Warner *et al.*, 1998). The glycoproteins gD, gB, gH and gL enable the fusion of the HSV-1 envelope with the plasma membrane (Figure 3, step 1), as reviewed in Campadelli-Fiume *et al.* (2007) and Spear & Longnecker (2003). The capsid and tegument are released into the cytoplasm and transported along the microtubular network to the nuclear pore complex, where the viral DNA is released into the nucleus (Kristensson *et al.*, 1986; Sodeik *et al.*, 1997; Topp *et al.*, 1994) (Figure 3, step 2). The virion host shut-off protein (U_L41 gene product, *vhs*) degrades the host mRNA and shuts off the host protein synthesis facilitating the transition from cellular to viral gene expression (Feng *et al.*, 2005) (Figure 3, step 3). Tegument protein VP16 locates to the nucleus (Batterson & Roizman, 1983; Mackem & Roizman, 1982; Post *et al.*, 1981) and, together with cellular transcription factors Oct-1 and C1, activates the α gene expression (Campbell *et al.*, 1984; Kristie & Sharp, 1990; McKnight *et al.*, 1987; Pellett *et al.*, 1985; Post *et al.*, 1981; Vogel & Kristie, 2000; Wilson *et al.*, 1993) (Figure 3, step 4).

The viral DNA circularizes (Garber *et al.*, 1993; Poffenberger & Roizman, 1985) and the viral DNA is transcribed by host RNA polymerase II (pol II) (Alwine *et al.*, 1974; Costanzo *et al.*, 1977) in co-operation with viral factors at all stages of the infection (Figure 3, step 5). The transcription process is tightly co-ordinated and regulated in a cascade fashion (Honess & Roizman, 1974). Shortly after infection, approximately 2 to 4 hours post infection, the α genes are expressed (Figure 3, step 6). The α proteins are

produced in the cytoplasm as well as the other viral proteins. After translation, five of the six α proteins (infected cell proteins (ICP) 0, ICP4, ICP22, ICP27 and U_S1.5) are transported back to nucleus and used as transactivators of β genes (Ogle & Roizman, 1999; Sacks *et al.*, 1985; Sacks & Schaffer, 1987) (Figure 3, step 7). The β genes are expressed approximately 4 to 8 hours post infection (Honess & Roizman, 1974). Functional α protein ICP4 is required for maximal levels of all post α gene expression (Clements *et al.*, 1977; Knipe *et al.*, 1978; Preston, 1979; Watson & Clements, 1980). The α protein ICP27 is required for expression of some β genes (Sacks *et al.*, 1985). The translated β proteins promote the replication of viral DNA and the expression of γ genes (Figure 3, step 8). The viral DNA synthesis enhances the γ gene expression (Godowski & Knipe, 1986; Honess & Watson, 1974) (Figure 3, step 9). The replication of viral DNA is not essential for the expression of γ_1 genes, encoding i.e. gB, gD and $\gamma_1$34.5, but for the expression of the γ_2 genes, encoding i.e. gC, U_S9 and U_S11, the replication of the viral DNA must be ongoing (Conley *et al.*, 1981; Costa *et al.*, 1981). The γ proteins, such as the capsid protein VP5 and glycoproteins, participate in assembling the capsid in the nucleus and modifying the membranes for virion formation (Church & Wilson, 1997; Newcomb *et al.*, 1996; Newcomb *et al.*, 1993; Thomsen *et al.*, 1995; Trus *et al.*, 1996) (Figure 3, step 10). The viral DNA is packaged into the pre-assembled capsid (Newcomb *et al.*, 1996) (Figure 3, step 11). The filled capsid or nucleocapsid matures into a virion and buds through the inner lamella of the nuclear membrane. The transition of the virion from the space between the inner and outer nuclear membranes to the subcellular space is not yet fully defined. There is evidence that HSV-1 nucleocapsids enter the perinuclear space by budding through the inner nuclear membrane. The enveloped nucleocapsids thereafter fuse with the outer nuclear membrane to enter the cytoplasm (Campadelli-Fiume & Roizman, 2006; Leuzinger *et al.*, 2005; Sagou *et al.*, 2010). The assembly and maturation of HSV-1 virions involves likely secondary envelopment outside the nucleus, as reviewed in Mettenleiter *et al.* (2006). In the cytoplasm, virion is transported to the Golgi apparatus (Stackpole, 1969). The virion envelope is processed either at the Golgi membrane or at the inner nuclear membrane. The progeny virus is released from the host cell by exocytosis (Mettenleiter, 2002) (Figure 3, step 12). The virus is also able to spread laterally among epithelial cells by using its gE and gI glycoproteins (Dingwell *et al.*, 1994).

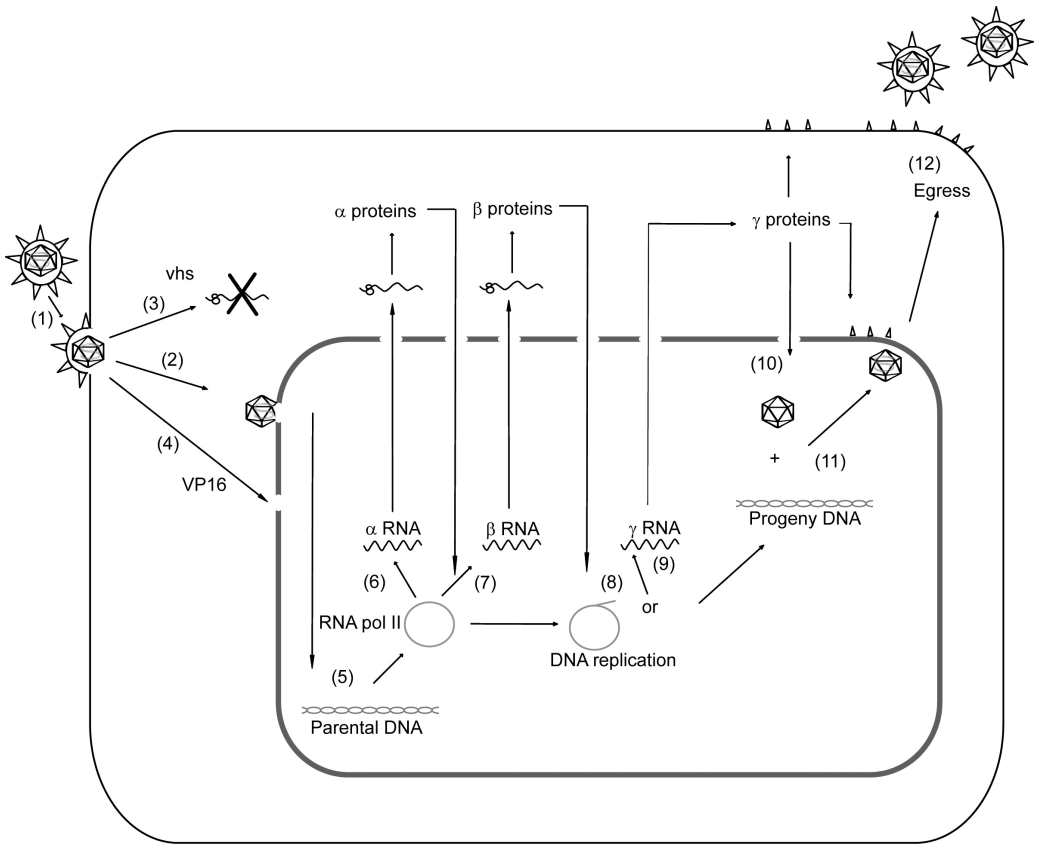

Figure 3. Replication cycle of HSV-1. Modified from Roizman *et al.* (2007). See text for details.

2.1.3 Pathogenesis

HSV-1 is able to infect several types of cells. Epithelial cells, usually in oral mucosa, are the primary site of replication for HSV-1. Progeny virus infects neighbouring epithelial cells leading to blister formation. Next, the virus invades the underlying sensory neurons and travels by retrograde axonal transport to the corresponding sensory ganglia, usually to the trigeminal ganglia. In the ganglia, HSV-1 remains latent for lifetime of the neuron and can reactivate to cause lesions at or near the initial site of infection. During reactivation, HSV-1 can invade the central nervous system (CNS), but predominantly HSV-1 travels back to the epithelial cells and causes blisters (Cushing, 1904; Goodpasture, 1929; Roizman *et al.*, 2007).

Effects on the host cell

In HSV-1-infected cell, several cytopathic changes are observed, reviewed in Arduino & Porter (2008). The nucleolus becomes enlarged and fragmented, and chromosomes become condensed and marginalized (Simpson-Holley *et al.*, 2004). Aggregation of replication proteins, progeny viral DNA and nucleocapsid components accumulate in the nucleus forming “replication compartments”. HSV-1 also modifies and degrades nuclear domain 10 (ND-10) structures (promyelocytic leukemia protein (PML bodies)

(Everett *et al.*, 2006; Lopez *et al.*, 2002). In addition, HSV-1 alters folding of the intracellular membranes and in some cells the Golgi vesicle becomes dispersed and fragmented. Viral glycoproteins are expressed in cytoplasm and plasma membranes and the antigenic specificity of the cell is changed. HSV-1-infected cells are ballooned and clumped together. Also, HSV-1 causes re-arrangement of microtubules to form bundles around the nucleus for the cytoplasmic transport of the progeny virus (Ward *et al.*, 1998).

In the productive HSV-1 infection the host cell eventually dies. To prevent host cell responses and the dying of the host cell before HSV-1 has replicated, HSV-1 is able to shut off the cellular functions. HSV-1 degrades host mRNAs and proteins and turns off mRNA transcription. In addition, HSV-1 uses cellular proteins for its own purposes. Both host mRNA and viral RNA transcripts are degraded by the viral U_L41 gene product, *vhs* protein, which is delivered to infected cells as a tegument protein (Feng *et al.*, 2005). Along with the progression of infection, *vhs* complexes with the other tegument protein VP16, resulting in decline of the degradation activity of *vhs* and increase in the half-lives of viral mRNA (Strand & Leib, 2004). To reduce the transcription of cellular genes and to favor transcription of the viral genes, HSV-1 is able to inhibit RNA splicing by the U_L54 gene product, ICP27 (Sandri-Goldin, 2008). The inhibition of RNA splicing has a lesser effect on HSV-1 mRNA, because only a few HSV-1 transcripts are spliced. Three of the four spliced HSV-1 transcripts (ICP0, ICP22 and ICP47) are expressed and spliced before ICP27-induced block. Later in infection the blocking function of ICP27 is repressed, which allows the splicing of the fourth HSV-1 transcript (U_L15). In addition to shutting off the *de novo* synthesis of cellular proteins, HSV-1 selectively degrades and stabilizes host proteins. In HSV-1-infected cells, cellular cyclins and cell cycle kinases, transcription initiation factors and translation factors are affected (Advani *et al.*, 2000a; Advani *et al.*, 2000b; Kawaguchi *et al.*, 1997). In addition, HSV-1 is able to inhibit virus-induced cell death (See: 2.3.2 Herpes simplex virus type 1-induced programmed cell death).

Neurovirulence

The ability of HSV-1 to invade the CNS and infect and replicate in the neurons is called neurovirulence. Several genes of HSV-1 affects the neurovirulence, but especially the "neurovirulence gene" $\gamma_1$34.5 is required for the HSV-1 ability to both invade and replicate in CNS (Chou *et al.*, 1990). The late gene $\gamma_1$34.5 encodes for the ICP34.5 protein, which blocks the effects of host RNA-dependent protein kinase (PKR). During HSV-1 infection viral dsRNA activates PKR, which phosphorylates the translation cellular factor eIF-2α. The phosphorylation of eIF-2α then shuts off the cellular protein synthesis. However, this total shut off of cellular protein synthesis in neuronal cells is blocked by ICP34.5, which binds to cellular protein phosphatase 1α leading to dephosphorylation of eIF-2α-P (He *et al.*, 1997). The HSV-1 mutant with deletion in $\gamma_1$34.5 gene have an approximately 3000-fold decreased ability to replicate in CNS, but it is still able to replicate in cell culture (Chou *et al.*, 1990). HSV-1 viruses with mutations in $\gamma_1$34.5 gene have been studied for their use as a vector for gene therapy and the development of engineered vaccine, as reviewed in Hukkanen *et al.* (2010) and Manservigi *et al.* (2010). Besides mutations in $\gamma_1$34.5 gene, variation in genes

encoding glycoprotein gD and gB have been shown to affect the neuroinvasiveness (Izumi & Stevens, 1990; Yuhasz & Stevens, 1993).

Latency

A unique biological property of HSV-1 is the ability to establish latency in non-dividing sensory neuronal cells. The viral genome is circularized after the release into the nucleus and the viral genome resides as an episomal DNA. During latency only a small proportion of HSV-1 DNA is expressed, coding for the latency-associate transcripts (LATs) (Stevens *et al.*, 1987; Wagner *et al.*, 1988). Although the LAT region has not been shown to encode any protein, several pathogenic functions of LAT have been demonstrated, such as neuronal survival and anti-apoptotic functions, virulence, establishment of latency, and reactivation from latency (Hill *et al.*, 1990; Leib *et al.*, 1989; Perng *et al.*, 2000; Thompson & Sawtell, 1997; Thompson & Sawtell, 2001). Recently, HSV-1 has been shown to hijack the histone deacetylase (HDAC) 1 or 2/ RE-1 silencing transcription factor (REST) / corepressor of REST (CoREST) 1 – complex to silence itself in neurons (Du *et al.*, 2010).

Reactivation of HSV-1 from latency can be induced by several factors, such as stress, UV light exposure, hormonal factors, tissue injuries and other infections. The molecular mechanism of reactivation has not yet been fully defined. Based on recent studies, HSV-1 transactivator VP16 is necessary for reactivation of HSV-1 in sensory neurons (Thompson *et al.*, 2009; Thompson & Sawtell, 2006). HSV-1 reactivates from only a few neurons at the same time. The infectious virus is carried anterogradely by axonal transport from the ganglia to epithelial cell, usually near the site of primary infection. The fate of the neuron is not clear after reactivation (Roizman *et al.*, 2007).

2.1.4 Immune response

HSV-1 infection induces a variety of innate and adaptive immune responses in the host, reviewed in Chew *et al*. (2009), Hukkanen *et al.* (2010) and Melcjorsen *et al.* (2009). In addition, at the level of infected cell, HSV-1 infection evokes cellular responses, such as apoptosis, autophagy, and changes of the cell cycle regulators or factors associated with gene silencing. The first line of defense in the body is the physicochemical barrier formed by the epithelium of the skin or the mucosa and by the body fluids covering the mucosal surfaces. Because healthy epithelium prevents HSV-1 from invading the most susceptible metabolically active basal cells, HSV-1 infects the host via mucosal surfaces or slightly damaged skin. Body fluids, such as saliva and tears, contain antiviral molecules, which can prevent viral infection or reduce the amount of HSV-1 inoculum reaching the epithelium. These antiviral soluble molecules include i.e. lactoferrin, defensins and cysteine protease inhibitor cystatin D (Andersen *et al.*, 2003; Gu *et al.*, 1995; Jenssen *et al.*, 2008; Välimaa *et al.*, 2009). Complement and natural antibodies inactivate HSV-1 in the bloodstream (Da Costa *et al.*, 1999). Cell types involved in the innate immune system include dendritic cells (DCs), macrophages, neutrophils, natural killer (NK) cells and γδ T cells, reviewed in Chew et al. (2009). The type I interferons (IFNs) and the Stat1 IFN signaling molecule are

important factors in the antiviral response against HSV-1 (Leib *et al.*, 1999; Melchjorsen *et al.*, 2009; Pasieka *et al.*, 2009).

Innate response – Toll-like receptors

The innate recognition of virus is mediated through interaction of pathogen-associated molecular patterns (PAMPs) with host cell pattern recognition receptors (PRRs). There are several classes of PRRs, such as Toll-like receptors (TLRs), retinoic acid inducing gene I (RIG-I)-like receptors (RLR) and Nod-like receptors (NLRs), reviewed in Kumagai & Akira (2010). The interaction between PAMP and PRR activates downstream pathways leading to expression of type I IFN genes and secretion of IFN. Secreted IFN binds to its receptor in the target cell. This triggers signaling through the Jak/Stat pathway and the IFN-stimulated genes (ISGs), such as dsRNA-dependent PKR, are activated, reviewed in Chew *et al.* (2009).

Toll-like receptors (TLRs) recognize different PAMPs, such as viral DNA and proteins, reviewed in Akira *et al.* (2006). Various immune cells, such as DCs, B cells and specific type of T cells, express TLRs. The rate of TLR expression is modulated in response to pathogens, various cytokines and environmental stresses. Extracellular TLRs, such as TLR1, 2, 4 and 6, are expressed on the cell surface, while intracellular TLRs, such as TLR3, 7, 8 and 9, are expressed in intracellular compartments, like endosomes. During pathogen infection, recognition of PAMP by TLR recruits adaptor protein, such as myeloid differentiation factor 88 (MyD88), to the cytoplasmic portion of the TLR. This triggers downstream signaling cascade and production of pro-inflammatory cytokines and chemokines. (Figure 4)

TLR2, 3 and 9 are suggested to be involved in HSV-1 recognition in different cells. TLR9 recognizes unmethylated CpG-DNA and senses HSV-1 in murine interferon-producing cells (IPC), also called plasmacytoid dendritic cells (pDCs) (Krug *et al.*, 2004b). TLR2 mediates the production of inflammatory cytokines in response to HSV-1 infection. This leads to the development of lethal viral encephalitis (Kurt-Jones *et al.*, 2004). In addition, HSV-1 has been shown to be dually recognized by both TLR2 and TLR9 in DCs resulting in IL-6 and IL-12 production (Sato *et al.*, 2006). Recently, TLR3 was shown to be involved in defense against severe HSV-1 infections of the central nervous system (CNS) indicating indirectly the involvement of the endosomal dsRNA sensor TLR3 in recognition of HSV-1 (Zhang *et al.*, 2007).

Recognition of HSV-1 by TLRs stimulates various intracellular pathways leading to activation of several transcriptional factors, such as nuclear factor–κB (NF-κB) and interferon (IFN) regulatory factors (IRFs) (Kaisho & Akira, 2006) (Figure 4). Cytoplasmic adaptor molecules, such as MyD88, associated with the intracytoplasmic region of the TLRs mediate the TLR-signaling cascade. MyD88 can associate with all TLRs, except from TLR3, and the MyD88-dependent pathway in TLR7/9 signaling induces both inflammatory cytokines and type I interferons (IFNI) (Hemmi *et al.*, 2003). The MyD88-independent pathway can be stimulated by TLR3 and TLR4, which associate with another adaptor protein, TIR domain-containing adaptor protein inducing IFN-β (TRIF). This interaction leads to IFN regulatory factor (IRF)-3 or NF-κB activation (Kaisho & Akira, 2006). To evade the innate response, particularly HSV-1

infection-induced IFN response of the host, HSV-1 has developed several molecular strategies (Melchjorsen *et al.*, 2009; Paladino & Mossman, 2009). For example, the immediate early proteins ICP0 and ICP27 evade the IFN response by inhibiting the IRF3 activation and IRF3-mediated ISG induction and the tegument protein *vhs* blocks the monocytic DC activation by TLR-independent recognition pathway (Cotter *et al.*, 2010).

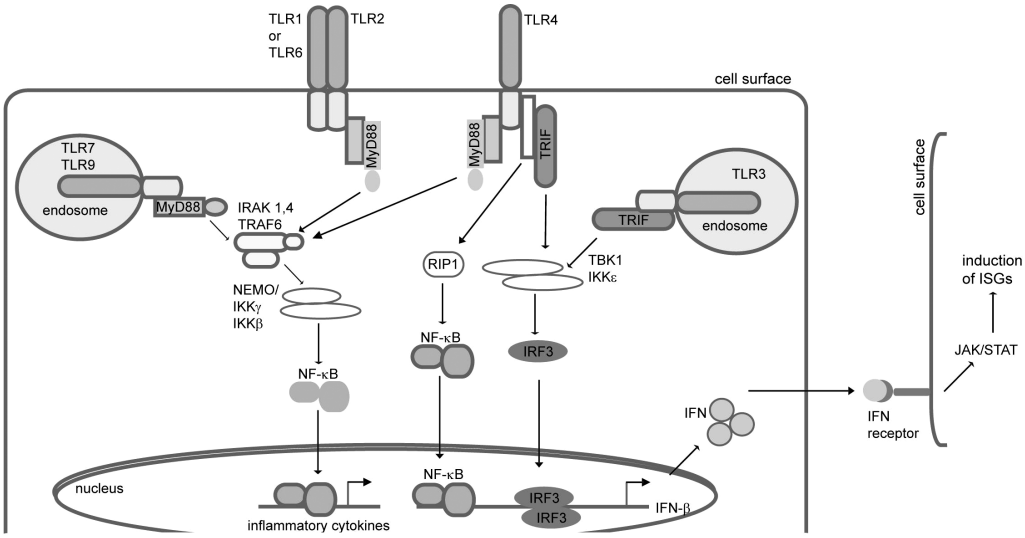

Figure 4. Schematic representation of Toll-like receptors in innate response.

2.2 Measles virus

2.2.1 Structure and genome

Measles virus (MV) causes a highly contagious childhood infection that spreads through respiratory route (Griffin, 2007). MV is a relatively large, enveloped, negative-stranded RNA virus belonging to the Morbillivirus genus in the virus family *Paramyxoviridae*. The assembly of nucleocapsid takes place in the cytoplasm and the infective virions are assembled at the plasma membrane. MV infection triggers an efficient immune response and the infection is associated with transient but strong immunosuppression that contributes to secondary infections and mortality (Griffin, 1995).

In the MV virion the genomic RNA is encapsidated by the nucleoprotein (N), phosphoprotein (P) and large protein (L) forming nucleocapsid (Figure 5). The nucleocapsid is surrounded by the envelope, which consist of the transmembrane hemagglutinin (H), and fusion (F) glycoproteins. The inner surface of the lipid envelope is covered with the matrix protein (M). The size of the virion range of 100-300 nm in diameter.

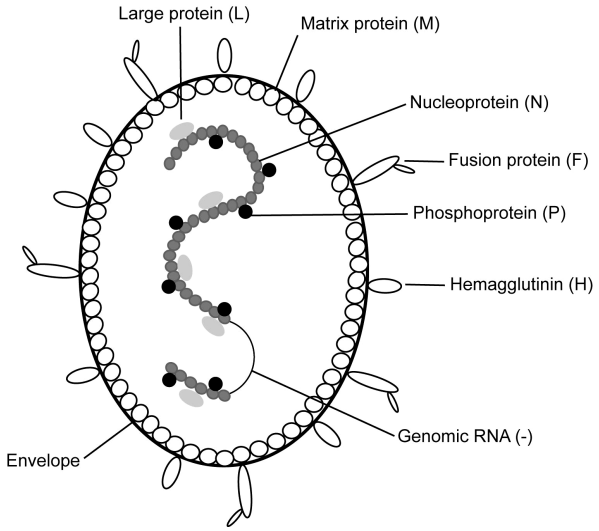

Figure 5. Schematic structure of measles virus virion.

The MV genome is composed of 15 894 nucleotide long, single-stranded RNA with negative polarity (Figure 6). The genome encodes for six structural proteins (N, P, M, F, H, and L) and for two non-structural proteins (C and V) (Griffin, 2007). Leader and trailer sequences at the 3´and 5´ends, respectively, have no coding capacity, but they are suggested to function as regulators of transcription and replication. In addition, the 3´ leader sequence is suggested to be recognized by the retinoic acid inducing gene I (RIG-I), and this interaction would trigger the early IFN response (Plumet *et al.*, 2007). Also, cellular proteins have binding sites in the untranslated RNA sequences (Leopardi *et al.*, 1993).

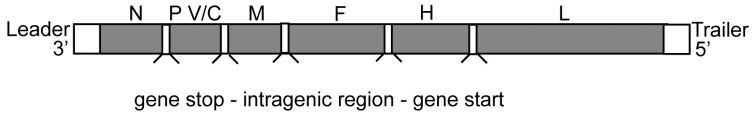

Figure 6. Schematic representation of measles virus RNA genome.

During the MV infection, the N mRNA is first transcribed and the N protein is the most abundant protein of MV proteins. N binds both to RNA and to P protein and it is required for transcription and replication. The P protein is a polymerase cofactor, which links L to N to form replicase complex. The P protein is abundant in the infected cells, but in the virion only small amount of P protein is present. In addition to P proteins, the P gene encodes C and V proteins, which are not necessary for replication, but have other essential functions. The C protein interferes with innate immune response via inhibition of IFN signaling, modulates viral polymerase activity and is involved in the prevention of cell death (Bankamp *et al.*, 2005; Reutter *et al.*, 2001; Shaffer *et al.*, 2003; Takeuchi *et al.*, 2005). The V protein is diffusely distributed in the cytoplasm of infected cells and it affects N-P interaction. Also, the V protein interferes with IFN signaling (Ohno *et al.*, 2004; Palosaari *et al.*, 2003; Takeuchi *et al.*, 2003). Deletions of C or V genes decrease the replication of MV in various cell types and reduces the

amount of released virus (Escoffier *et al.*, 1999; Patterson *et al.*, 2000; Takeuchi *et al.*, 2005; Tober *et al.*, 1998; Valsamakis *et al.*, 1998). The M protein is likely to interact with the intracytoplasmic region of one or both of transmembrane glycoproteins, modulates the targeting and fusiogenic capacity of the envelope glycoproteins, and directs release of virus (Blau & Compans, 1995; Naim *et al.*, 2000). The F protein mediates the fusion of the infected cells. In addition to F protein, fusion requires the expression of H and binding of H to a cell surface receptor (Zhang *et al.*, 2005). The fusion of infected cells leads to formation of multinuclear giant cells, the syncytia. The MV-induced formation of the syncytia has been shown to amplify type I IFN response indicating that multinuclear giant cells contribute to the antiviral immune response (Herschke *et al.*, 2007). The L protein is present in small quantities in the infected cell, and it interacts with P protein, and is part of the nucleocapsid.

2.2.2 Replication

The replication of MV takes place in the cytoplasm of the host cell. The viral H protein binds to cell surface receptor and after attachment, the envelope of the virion fuses with the plasma membrane. The nucleocapsid is released to the cytoplasm. The viral RNA polymerase participate both in primary transcription of mRNAs and synthesis of positive-stranded anti-genome. The genomic negative-stranded RNA is used as a template. The transcription of mRNAs initiates at the N gene start signal. The RNA polymerase ends transcription at the stop signal and continues transcription at the start signal of the next gene after the gene-separating junction (Dowling *et al.*, 1986; Rima *et al.*, 1986). Except for this, the P gene encodes both full-length copy of P mRNA, but also the C mRNA by alternative translational initiation at an overlapping reading frame (Bellini *et al.*, 1985), and V mRNA by a process of RNA editing (Cattaneo *et al.*, 1989). Since the polymerase occasionally detaches from the template at the junctions, the efficiency of transcription is weaker towards the 5´end resulting in less abundant L mRNA as compared with N mRNA (Cattaneo *et al.*, 1987a; Cattaneo *et al.*, 1987b; Ray & Fujinami, 1987). The transcribed mRNAs are translated to viral proteins early after infection and the proteins seem to be required for the replication of MV genome.

For the replication of MV genome, a full-length complementary copy, called anti-genome, is produced. This positive-stranded anti-genome serves as a template for the production of progeny RNA. The newly synthesized RNA is encapsidated by N protein forming the helical ribonucleoprotein structure, with which the P-L protein complexes associate (Mountcastle & Choppin, 1977; Stallcup *et al.*, 1979). The viral membrane proteins H and F are glycosylated in the endoplasmic reticulum, where several folding enzymes and molecular chaperons of the host cell assist the conformational maturation (Plemper *et al.*, 2001). The H and F proteins are transported to Golgi apparatus, where the proteins are further modified, and finally to plasma membrane. The interaction of M protein on the inner surface of the plasma membrane with the cytoplasmic domains of the glycoproteins and with the nucleocapsid initiates virus assembly and the budding process. MV is likely to utilize the cellular cytoskeleton structures for cytoplasmic transportation of viral components and the new virus particles (Berghäll *et al.*, 2004).

2.2.3 Pathogenesis

Measles is a childhood infection spread by the respiratory route. Symptoms, such as fever, cough, runny nose, conjunctivitis and small white spots inside the cheek (Koplik´s spots), begin to appear after an incubation period of 10 to 14 days. The maculopapular rash develops in a few days and it co-incides with the appearance of the immune response and the initiation of virus clearance. After recovery, life-long immunity to reinfection is achieved. Despite an efficient vaccine, MV is still an important global pathogen. MV causes lymphopenia and depresses cell-mediated immunity. MV infection leads to profound long-lasting immunosuppression, which increases susceptibility to secondary infections causing approximately 200 000 deaths every year mainly in developing countries (WHO, 2008). Serious complications include blindness, encephalitis, severe diarrhoea, otitis and pneumonia. The most severe, but rare complication, is fatal degenerative disease of the central nervous system called subacute sclerosing panencephalitis (SSPE). The incubation period after MV infection is approximately 7 to 10 years. SSPE leads to death 1 to 3 years after the onset of symptoms (Griffin, 2007).

In the initial infection, MV replicates in tracheal and bronchial epithelial cells and pulmonary macrophages of the respiratory tract (Sakaguchi *et al.*, 1986). From the respiratory tract MV is transported to local lymphatic tissues, which are prominent sites of replication. MV infection causes formation of giant cells in lymphatic tissue and the amplification of virus results in the appearance of a viremia and MV spreads through the blood to variety of organs, such as skin, conjuctiva, kidney and lung. Monocytes are the primary blood cells infected with MV, but also T and B cells can be infected (Esolen *et al.*, 1993). Resting lymphocytes and dendritic cells produce little virus unless stimulated (Fugier-Vivier *et al.*, 1997; Vainionpää *et al.*, 1991)

To date two host cell molecules, the transmembrane glycoprotein CD46 and the human signaling lymphocyte activating molecule (SLAM, CD150), have been identified as MV receptors. SLAM is the major entry receptor and it determines the tropism of MV for immune cells. In addition, SLAM is involved in the induction of immunomodulation and immunosuppression as well as the systemic spread of MV to organs (Ludlow *et al.*, 2009). Wild-type MV appears to use mainly SLAM, but it is also able to bind to CD46 at a lower affinity (Manchester *et al.*, 2000; Massé *et al.*, 2002). Laboratory-adapted and vaccine strains are able to use both CD46 and SLAM as receptors. Recently, extracellular matrix metalloproteinase inducer (CD147/EMMPRIN) has been shown to act as a functional entry receptor for MV on epithelial cells (Watanabe *et al.*, 2010). In addition, cyclophilin, the cellular ligand for CD147, was found in MV virions and the inhibition of cyclophilin incorporation attenuated SLAM-independent infection on epithelial cells. This indicates that MV infection occurs via CD147 and virion-associated cyclophilin independently of hemagglutinin (Watanabe *et al.*, 2010).

2.2.4 Immune response

MV infection induces innate and adaptive immune responses in the infected host, which results in several of the clinical manifestations of measles. Innate responses contribute to control of MV replication during the incubation period, but the onset of

symptoms coincides with the appearance of MV-specific adaptive immune responses. MV infection leads to a strong immunosuppression, reviewed in Avota *et al.* (2010). The immunosuppression can continue for many weeks after apparent recovery.

At the initiation of the MV infection the innate immune response is activated by recognition of the incoming MV. TLR3 has been shown to be up-regulated in MV-infected cells, and hence TLR3 might be involved in the recognition of MV (Berghäll *et al.*, 2006; Tanabe *et al.*, 2003). On the other hand, MV protein V prevents TLR7/9-mediated IFN induction (Pfaller & Conzelmann, 2008). Also, gene expression of the recognition receptor of dsRNA, namely melanoma differentiation-associated gene 5 (mda-5), and RIG-I are activated (Berghäll *et al.*, 2006). Several studies have shown that MV induces production of IFN-α and IFN-β, reviewed in Hahm (2009). In addition, MV activates NF-κB and IRF-3 signaling pathways (Helin *et al.*, 2001; tenOever *et al.*, 2002). Produced IFN decreases MV replication and increases expression of major histocompatibility complex (MHC) class I antigen and TLR3 on infected cells (Leopardi *et al.*, 1992; Tanabe *et al.*, 2003). Also, IFN-inducible myxovirus resistance protein A (MxA) decreases the MV replication (Schnorr *et al.*, 1993).

During adaptive immune response antibodies against viral proteins are produced to vast majority of viral proteins, but the most abundant antibody is against the N protein (Graves *et al.*, 1984). T lymphocyte-mediated immune response plays a critical role in recovery from measles. CD8+ and CD4+ T cells are activated during measles and the CD8+ T cell memory is established by infection of MV (Jaye *et al.*, 1998; Nanan *et al.*, 1995; van Binnendijk *et al.*, 1990). Both CD8+ and CD4+ T cells produce IFN-γ that activates macrophages and in addition, CD4+ T cells produce interleukin (IL)-2 that promotes T-cell proliferation (Griffin & Ward, 1993; Griffin *et al.*, 1990). IFN-γ can suppress MV replication *in vitro*, thus it may have a direct antiviral effect.

Despite the fact that measles has been known for over hundred years (von Pirquet, 1908), the mechanisms of immunosuppression are still not fully clarified. It is probably a multifactor process and likely to increase susceptibility to secondary infections and mortality. MV causes lymphopenia and the number of lymphocytes may reduce even to 10 % of normal values (Okada *et al.*, 2000). However, lymphopenia is not the only factor contributing to immunosuppression since lymphopenia is also found in other virus infections associated with no immunosuppression. Mechanism behind immunosuppression may include suppression of cytotoxic functions of lymphocytes (Casali *et al.*, 1984; Galama *et al.*, 1980), the reduced proliferation response to mitogens and antigens of peripheral blood mononuclear cells (PBMC) (Galama *et al.*, 1980; McChesney *et al.*, 1988) and altered cytokine production and cell cycle arrest in the G_0/G_1 phase (Engelking *et al.*, 1999; McChesney *et al.*, 1988; McChesney *et al.*, 1987; Naniche *et al.*, 1999). Also, MV has been found to induce apoptosis in Vero and pro-monocytic cells (Esolen *et al.*, 1995), in different type of cells of the central nervous system of SSPE-patients (McQuaid *et al.*, 1997), and in dendritic cells (Fugier-Vivier *et al.*, 1997). The interaction of MV H protein with CD46 has been suggested to impair the capacity of dendritic cells and monocytes to produce pro-inflammatory cytokine IL-12, which is important for initiation of the cellular immune response (Fugier-Vivier *et al.*, 1997; Karp, 1999; Marie *et al.*, 2001). Also, SLAM-mediated interaction of MV and

dendritic cells decrease IL-12 production through TLR4 activation (Hahm *et al.*, 2007). Among H protein, N protein appears to be involved in immunosuppression by inducing cell cycle arrest and apoptosis (Laine *et al.*, 2005; Laine *et al.*, 2003).

2.3 Programmed cell death

2.3.1 Types of programmed cell death

Programmed cell death plays an important role during development, homeostasis and immune regulation of multicellular organisms. As a part of immune response, programmed cell death is generally induced against pathogen infections. On the other hand, pathogens have developed strategies to modulate cell death of host. Thus far, four different types of programmed cell death have been described, including apoptosis, necrosis, autophagic cell death and pyroptosis, reviewed in Duprez *et al.* (2009). In Table I, the morphological features and molecular mechanism typical for each type of programmed cell death are listed.

Table 1. Different modes of cell death.

Cell death mode	Morphological features and molecular mechanisms
Apoptosis	Rounding up of the cell Reduction of cellular and nuclear volume Chromatin condensation DNA fragmentation Little or no modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by resident phagocytes Intrinsic or extrinsic pathways of apoptosis Recruitment of apoptotic caspases Regulation of apoptosis by Bcl-2 family members
Necrosis	Cytoplasmic swelling Rupture of the plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condensation Activation of RIP1 Formation of RIP1-RIP3 pro-necrotic complex Activation of necrotic mediators, such as ROS, calcium, calpains and cathepsins 'Necroptosis', term used for RIP1-dependent programmed necrosis
Autophagy	No chromatin condensation Massive vacuolization of the cytoplasm Accumulation of double-membraned autophagic vacuoles Little or no uptake by phagocytic cells Starvation-induced inhibition of mTOR
Pyroptosis	Osmotic pressure: water influx, cell swelling, lysis Loss of mitochondrial membrane potential Loss of plasma membrane integrity Chromatin condensation DNA fragmentation Formation of inflammasomes Recruitment of caspase-1 Pore formation in the plasma membrane Induction of inflammatory response

Modified from Duprez et al. (2009) and MacFarlane (2009).

Apoptosis

Apoptosis is a genetically controlled process of cell suicide involved in the regulation of homeostasis, tissue development, and the immune system to eliminate cells that are no longer useful. Apoptosis plays also a protective role eliminating aberrant cells created by DNA damage or those infected by viral pathogens. A variety of stimuli can provoke apoptosis, among them metabolic disturbances due to chemical insults, virus infections or developmental cues. The concept of apoptosis was introduced in 1972 by Kerr, Wyllie and Currie to describe a form of cell death distinct from necrosis (Kerr *et al.*, 1972). Programmed cell death by apoptosis is characterized by chromatin condensation, DNA fragmentation, phosphatidylserine exposure, plasma membrane blebbing, cell shrinkage, formation of apoptotic bodies and activation of caspase cascade (Fadok *et al.*, 1998; Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Wyllie *et al.*, 1972). Apoptotic caspases are involved in the initial signaling events (initiator caspases) and downstream proteolytic cleavages (executioner caspases), that characterize the apoptotic phenotype. Also, the proteins of Bcl-2 family participate in the process of apoptosis controlling mitochondrial integrity (Duprez *et al.*, 2009; MacFarlane, 2009).

Apoptosis can be induced either by intrinsic or extrinsic pathway (Duprez *et al.*, 2009; MacFarlane, 2009) (Figure 7). The intrinsic pathway can be stimulated by various stimuli, such as DNA damage or other intracellular stress signals at the mitochondrial level. The intrinsic pathway is regulated by the Bcl-2 protein family, reviewed in Youle & Strasser (2008). The Bcl-2 family is divided to three groups based on the Bcl-2 homology (BH) domain. The BH3-only proteins, such as Bid, Bim, and Bad, contain a single BH3 domain and are pro-apoptotic. Multiple BH domains containing Bcl-2 proteins are either pro-apoptotic, such as Bax, Bak and Bok, or anti-apoptotic, such as Bcl-2 and Bcl-XL. Apoptosis can be triggered by BH3-only proteins by inducing the oligomerisation of Bax and/or Bak or by binding to and inhibiting the anti-apoptotic Bcl-2 family proteins. Apoptosis triggered by pro-apoptotic members of the Bcl-2 family causes permeabilization of the outer mitochondrial membrane leading to release of cytochrome c (cyt c) from the intermembrane space. In the cytosol, cyt c interacts with both the adaptor molecule Apaf-1 and ATP forming a multimeric protein complex called the apoptosome (Cain *et al.*, 2002; Cain *et al.*, 1999). The apoptosome recruits and activates caspase-9 leading to activation of downstream executioner caspases, caspase-3, -6 and -7, and the manifestation of the apoptotic phenotype. In addition, other pro-apoptotic proteins released from the mitochondria, such as second mitochondria-derived activator of caspases/direct IAP binding protein with low PI (Smac/Diablo), are involved in the apoptotic cell death. Smac/Diablo induces apoptosis by blocking the effects of the inhibitor of apoptosis proteins (IAPs), such as XIAP (van Loo *et al.*, 2002). IAPs can block apoptosis signaling either by binding to caspases and thus inhibiting the caspase cascade or by contributing to the NF-κB-mediated expression of anti-apoptotic genes (LaCasse *et al.*, 2008).

In the extrinsic pathway of apoptosis, specific pro-apoptotic ligands, such as Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), bind to death receptors, such as Fas and TRAIL-R, on the surface of target cells. The death receptors belong to the TNF receptor superfamily of proteins, some of which have a

cytoplasmic death domain that can trigger the activation of apoptosis, reviewed in Nagata (1997). The binding of ligand to its pro-apoptotic receptor leads to recruitment of adaptor molecule, such as Fas-associated death domain (FADD), which in turn recruits caspase-8 to the activated Fas-receptor to form death-inducing signaling complex (DISC). This leads to activation of the initiator caspases -8 and -10 and triggering of apoptosis (Figure 7).

Extrinsic pathway

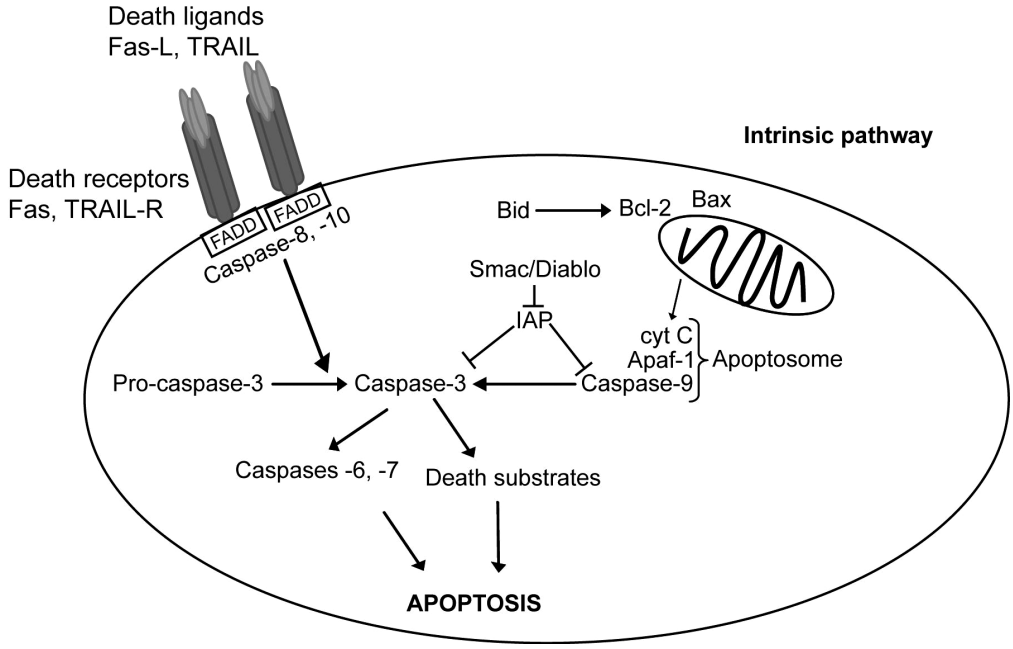

Figure 7. Intrinsic and extrinsic pathways of apoptosis.

Necrosis

Previously, necrosis has been considered as an accidental and uncontrolled type of cell death without signaling events. Currently, accumulating evidence suggest, that necrosis is programmed and well-regulated type of cell death. Necrotic cell death is characterized by cytoplasmic and organelle swelling, loss of membrane integrity and the release of cellular contents into extracellular space. Since caspases are not involved in necrosis, necrotic cell death can be thought as a back-up cell death pathway in the conditions when caspase activation has became complicated. Different stimuli can trigger necrosis by binding to death receptors on the surface of the cell. Stimulation of the receptor leads to the activation of receptor-interacting protein 1 (RIP1), which is a crucial initiator of death receptor-mediated necrosis (Festjens *et al.*, 2007; Zheng *et al.*, 2006). In fact, the term necroptosis describes the RIP1-dependent programmed necrosis (Degterev *et al.*, 2005). The kinase activity of RIP1 activates a wide range of necrotic mediators, such as reactive oxygen species (ROS), calcium, calpains and cathepsin. RIP1 interacts with RIP3, which is also crucial upstream

activating kinase that regulates RIP1-dependent necroptosis (Cho *et al.*, 2009; He *et al.*, 2009; Zhang *et al.*, 2009). Necroptosis can be inhibited by Necrostatin-1 (Nec-1) which targets RIP1 (Degterev *et al.*, 2008; Degterev *et al.*, 2005). In addition to death receptor-mediated necrosis, triggering of pathogen recognition receptors, such as TLRs, NLRs and RLRs, can lead to necrosis (Duprez *et al.*, 2009).

Autophagy

Autophagy is an evolutionary conserved catabolic pathway that delivers cytoplasmic constituents into lysosomes (Duprez *et al.*, 2009; MacFarlane, 2009). The cellular components, such as damaged organelles, insoluble proteins and lipid droplets, are degraded and recycled in autophagic processes, which regulate cellular homeostasis, cell death and survival and lipid metabolism. A typical morphological feature of autophagy is the extensive formation of double-membrane vesicles, called autophagosomes. These autophagosomes contain proteins and organelles and they have been found in dying cells. This implies that autophagy accompanies rather than causes cell death (Kroemer & Levine, 2008). Several types of autophagy have been described. In the classical pathway of autophagy, mammalian target of rapamycin (mTOR) is inhibited in response to starvation or rapamycin-treatment. This leads to autophagosome formation and eventually the autophagosome fuses with a lysosome. The content of autophagosomes is degraded by lysosomal hydrolases. Autophagy shares various molecular regulators with apoptosis. Depending on the cellular settings, autophagy can either serve a survival pathway suppressing apoptosis or lead to death in collaboration with apoptosis or as a back-up mechanism (Eisenberg-Lerner *et al.*, 2009).

Pyroptosis

Pyroptosis is a recently recognized caspase-1-dependent form of programmed cell death, reviewed in Labbé & Saleh (2008). Microbial pathogens induce pyroptosis in monocytes, macrophages and DCs, reviewed in Bergsbaken & Fink (2009). Pyroptotic cells have biochemical and morphological features of both apoptotic and necrotic cells. In pyroptosis, cells lose their mitochondrial membrane potential and plasma membrane integrity and cytoplasmic contents are released into the extracellular milieu. Also, DNA is fragmented and nuclei become condensed. During pyroptosis, recognition of bacterial or viral signals induces the formation of inflammasomes, which activates caspase-1 via adaptor molecule. Caspase-1 activation leads to pore formation in the plasma membrane, maturation of IL-1β and IL-18 resulting in the induction of an inflammatory response and cleavage of cellular substrates, such as cytoskeletal proteins and caspase-7 (Duprez *et al.*, 2009).

2.3.2 Herpes simplex virus type 1-induced programmed cell death

HSV-1-induced apoptosis

HSV-1 induces apoptosis in the host cell at early stage of the infection (Aubert & Blaho, 1999; Koyama & Adachi, 1997; Nguyen & Blaho, 2009; Sanfilippo & Blaho, 2006; Sanfilippo *et al.*, 2004). HSV-1 induces apoptosis prior to 1 hour post infection

and apoptosis blocking proteins are synthesized between 3 to 6 hours post infection (Aubert & Blaho, 1999). This period corresponds approximately to the transition from immediate early to early phase of viral gene expression. To delay the apoptotic dying of the host cell until the HSV-1 has replicated, HSV-1 have evolved several strategies to block the HSV-1-induced apoptosis at multiple steps of infection (Aubert & Blaho, 1999; Galvan & Roizman, 1998; Koyama & Miwa, 1997). Thus, in HSV-1-infected cell there is a complex balance between the pro-apoptotic and anti-apoptotic factors, reviewed in Nguyen and Blaho (2009).

The extent of HSV-1-induced apoptosis is cell type-dependent. This implies, that HSV-1-induced apoptosis is regulated by different cellular factors, such as caspases, Bcl-2 family members and NF-κB (Ahmed et al., 2002; Asano et al., 2000; Aubert & Blaho, 2003; Branco & Fraser, 2005; Galvan & Roizman, 1998; Nguyen et al., 2007). Caspases-3 and -9 have been shown to mediate HSV-1-induced apoptosis (Aubert et al., 2007; Kraft et al., 2006) whereas HSV-1-induced apoptosis is not caspase-8-mediated in epithelial cell line (Aubert et al., 2007). In addition, the cytochrome c is released from mitochondria in a caspase-independent manner during HSV-1-induced apoptosis (Aubert *et al.*, 2007). These findings suggest that HSV-1 induces apoptosis through the intrinsic pathway of apoptosis.

The apoptosis blocking proteins of HSV-1 include immediate early proteins ICP4, ICP22 and ICP27, ICP10, late protein kinase Us3, glycoproteins gD and gJ, and the latency associated transcripts (Aubert & Blaho, 1999; Aubert *et al.*, 1999; Jerome *et al.*, 1999; Leopardi & Roizman, 1996; Leopardi *et al.*, 1997; Perng *et al.*, 2000; Zhou *et al.*, 2000) (Figure 8). The late protein kinase Us3 of HSV-1 have been shown to block apoptosis induced by exogenous agents, by over-expression of pro-apoptotic proteins, such as Bad, and by infection with defective viruses, such as HSV-1 deleted for ICP4 gene (Benetti *et al.*, 2003; Benetti & Roizman, 2007; Jerome *et al.*, 1999; Leopardi *et al.*, 1997; Munger *et al.*, 2001; Munger & Roizman, 2001; Ogg *et al.*, 2004). The target of Us3 is so far unknown. However, it has been shown that Us3 activates protein kinase A (PKA) by phosphorylation. Activated PKA or both PKA and Us3 inhibit apoptosis (Benetti & Roizman, 2004). Us3 participates also in the phosphorylation of Bad in HSV-1-infected cells and thus blocks the pro-apoptotic activity of Bad (Cartier *et al.*, 2003). The Us3 transcriptional unit contain a smaller transcript designated Us3.5 (Poon *et al.*, 2006; Poon & Roizman, 2005). Both Us3 and Us3.5 proteins phosphorylate several viral and cellular proteins in HSV-1-infected cell and localize to mitochondria (Benetti & Roizman, 2004; Poon *et al.*, 2006). Much of the work above was done by Us3 transduction with baculoviruses. Some of the results have been confirmed in actual HSV-1 infections (Cartier *et al.*, 2003; Jerome *et al.*, 1999; Leopardi *et al.*, 1997).

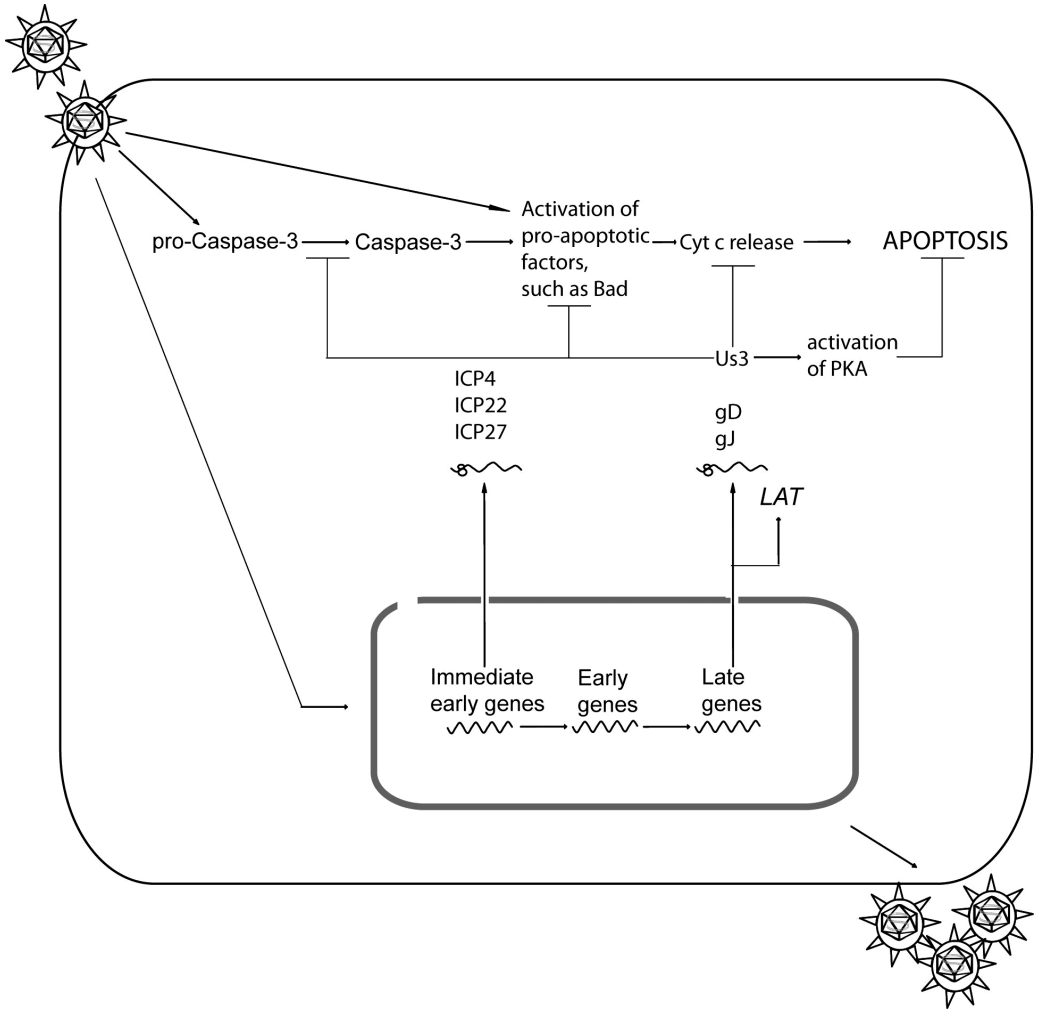

Figure 8. Schematic representation of HSV-1-induced apoptosis. HSV-1 infection induces signaling cascade which leads to apoptotic death of the host cell. However, several factors of HSV-1 can block apoptosis signaling. The protein kinase Us3 blocks apoptosis by inhibiting activation of caspase-3 or other pro-apoptotic factors, by inhibiting the release of cytochrome c (Cyt c) and activating the protein kinase A (PKA).

HSV-1-induced autophagy

In addition to apoptosis, HSV-1 infection induces autophagy in host cell. The HSV-1-induced autophagy is mediated via PKR pathway (see 2.1.3 Pathogenesis – Neurovirulence). The phosphorylation of the cellular factor eIF-2α is required for the PKR-mediated autophagy (Tallóczy *et al.*, 2002). HSV-1 is able to inhibit autophagy through the function of neurovirulence gene encoded late protein ICP34.5, which dephosphorylates the eIF-2α and binds to the essential autophagy protein Beclin 1 (Chou *et al.*, 1995; He *et al.*, 1996; Orvedahl *et al.*, 2007). In cells infected with HSV-1 viruses lacking neurovirulence gene $\gamma_1$34.5, the long-lived protein degradation and formation of autophagosomes is increased and the degradation of virions is enhanced (Orvedahl *et al.*, 2007; Tallóczy *et al.*, 2002; Tallóczy *et al.*, 2006). HSV-1 viruses

having a deletion of Beclin 1 binding domain (BBD) in the ICP34.5 are less capable of regulating the autophagosome formation and these viruses are neuroattenuated (Orvedahl *et al.*, 2007). Also, mice infected with HSV-1 with deletion in the ICP34.5 BBD are able to clear virus more efficiently and the stimulation of CD4+ T-cells is enhanced when compared with wild type virus-infected mice. This implies, that ICP34.5 plays an important role in the HSV-1 pathogenesis through its ability to inhibit autophagy and dampen the activation of CD4+ T-cells (Leib *et al.*, 2009).

2.3.3 Measles virus-induced programmed cell death

MV-induced apoptosis

Apoptosis has been suggested to participate, among other factors, in the MV-induced immunosuppression. MV infection leads to apoptosis in several types of cells, such as Vero and pro-monocytic cells (Esolen *et al.*, 1995), dendritic cells (Fugier-Vivier *et al.*, 1997), thymic epithelial cells (Laine *et al.*, 2003) and peripheral blood mononuclear cells (PBMCs) (Ito *et al.*, 1997; Okada *et al.*, 2000). It has been shown, that the induction of apoptosis in MV-infected PBMCs is triggered by interaction between the viral hemagglutinin protein and the cellular receptor SLAM and that the apoptosis is signalled via both intrinsic and extrinsic pathways of apoptosis recruiting caspase-3, -8 and -9 (Iwasa *et al.*, 2010). Since apoptosis induced by the laboratory-adapted strain of MV is not blocked by pan-caspase inhibitor while the apoptosis induced by wild type MV is blocked, it seems that the MV-induced apoptosis pathways may differ depending on the viral receptors CD46 and SLAM (Iwasa *et al.*, 2010). In addition to hemagglutinin protein, also viral nucleoprotein has been shown to be involved in induction of apoptosis (Laine *et al.*, 2005; Laine *et al.*, 2003). Recently, it has been shown that the protein kinase PKR restricts MV multiplication and inhibits MV-induced apoptosis through the action of viral protein C (Toth *et al.*, 2009a). This indicates that the C protein is able to antagonize the pro-apoptotic and antiviral activities of PKR. On the other hand, the RNA-specific adenosine deaminase 1 (ADAR1) of the host has been shown to suppress MV-induced apoptosis and activation of PKR, indicating that ADAR1 is pro-viral and anti-apoptotic host factor (Toth *et al.*, 2009b).

MV infection is associated with lymphopenia. The numbers of T cells and B cells in circulation are decreased during the rash, reviewed in Griffin (2010). MV-infected cells can induce apoptosis in uninfected bystander lymphocytes and this can, among others, result in MV-induced lymphopenia. MV-infected antigen-presenting dendritic cells have been shown to induce apoptosis in uninfected co-cultivated T cells (Fugier-Vivier *et al.*, 1997). In addition, productive MV infection in thymic epithelial and myelomonocytic cells leads to thymocyte apoptosis in MV-infected SCID-hu mice (Auwaerter *et al.*, 1996). Non-infected PBMCs of measles patients have been shown to express high levels of apoptosis-associated molecules, such as CD95 or Fas and tumor necrosis factor-related apoptosis-inducing ligand-receptor (TRAIL) and these cells dies due to apoptosis (Okada *et al.*, 2000). The apoptotic dying of non-infected blood cells results in lymphopenia and thus is suggested to be one reason for MV-induced immunosuppression (Okada *et al.*, 2000).

In addition to lymphocytes, MV infection causes apoptosis in antigen presenting DCs. The apoptosis of DCs in MV-infected DC and T cell co-cultures has been shown to be Fas-mediated (Servet-Delprat *et al.*, 2000). Activated T cells express CD40 ligand, which enhances the replication of MV in DCs and the MV is released due to apoptotic death of DCs. Apoptosis is induced by the Fas ligand, which is produced by the activated T cells (Servet-Delprat *et al.*, 2000). Moreover, the mRNA and protein expression of TRAIL in DCs is induced by MV infection and the MV-infected DCs develop TRAIL-mediated cytotoxicity resulting in Fas-mediated apoptosis of DCs (Vidalain *et al.*, 2001; Vidalain *et al.*, 2000).

MV-induced autophagy

Recently, MV has been shown to induce autophagy through CD46 receptor-mediated pathway (Joubert *et al.*, 2009). MV infection increases the amount of autophagy marker LC3-II and induces de novo formation of autophagosomes in cell culture (Joubert *et al.*, 2009).

2.4 Cystatins and cysteine cathepsins - regulators of apoptosis

2.4.1 Cystatins

Cystatins are classical inhibitors of C1 cysteine proteases, reviewed in Ochieng & Chaudhuri (2010). The subfamily of C1 cysteine proteases includes proteases such as papains and cathepsins. The MEROPS protease database gives an excellent classification of proteases and their inhibitor according to their mechanism and sequence similarity (http://merops.sanger.ac.uk/index.shtml). Cysteine proteases and their inhibitors have mainly been discovered in Finland (Järvinen, 1976; Järvinen & Hopsu-Havu, 1975; Rinne *et al.*, 1985) in the 1970´s and 1980´s. Cystatins are divided in three types according to their distinct structural details and their distribution in the body. Type 1 cystatins, cystatin A and B, are generally expressed intracellularly. They are unglycosylated inhibitors of approximately 11 kDa lacking signal sequence and disulfide bonds. Type 2 cystatins include cystatins C, D, S, SA and SN. These cystatins have molecular masses ranging from 13 to 14 kDa and they contain signal sequence and disulfide bonds. Some of type 2 cystatins are glycosylated. Type 3 cystatins are kininogens with molecular weights in the range 88-114 kDa and they are glycosylated. Recently, a new group of cystatins have been identified with cystatin like sequences but without cysteine protease inhibitory properties (Ochieng & Chaudhuri, 2010).

Cystatins are widely distributed in the body. For example, cystatin A is a major intracellular inhibitor in skin (Järvinen, 1978), cystatin C is the dominating inhibitor in extracellular fluids (Abrahamson, 1994) and cystatin D is excreted in saliva and tears (Abrahamson, 1994). Cystatins are involved in several diverse biological functions, such as cell proliferation and differentiation, antigen processing and presentation, interleukin and nitric oxide production, protein degradation and cell death and survival (Keppler, 2006; Ochieng & Chaudhuri, 2010). In addition, cystatins have been shown

to inhibit virus replication. Salivary cystatins, cystatin C and D, are able to inhibit coronavirus and herpes simplex virus replication (Björck, 1990; Collins & Grubb, 1998; Gu *et al.*, 1995). Among these biological functions, cystatins play key roles in the progression of a variety of tumors and neurodegenerative diseases, reviewed in Ochieng & Chaudhuri (2010).

2.4.2 Cysteine cathepsins

Cathepsins are intracellular proteases located in endolysosomal vesicles. They are divided to three subgroups, cysteine, aspartate and serine, according to their active-site amino acid. Most of the lysosomal cysteine cathepsins, such as cathepsin B, H, L and S, belong to the cysteine protease C1 family, which members are inhibited by cystatins. Cathepsins degrade proteins optimally at acidic pH, but they can function also in neutral pH outside the lysosome. For example, cathepsin B can function either as a carboxypeptidase or as an endopeptidase depending on the pH. In addition to protein degradation and turnover, cysteine cathepsins are involved in processes such as antigen presentation, lysosomal-mediated apoptosis, cancer progression and TLR-mediated signaling, reviewed in Colbert *et al.* (2009). The substrates of cysteine cathepsins include i.e. Bcl-2 family members, granzymes A and B, endocytic TLR9 and other cathepsins (Colbert *et al.*, 2009; Matsumoto *et al.*, 2008).

Cathepsins have been shown to promote several types of virus infections in the host cells, reviewed in Vasiljeva et al. (2007). In HSV-1 infection, cathepsin B has been shown to mediate the cleavage of the viral origin binding protein (OBP) to yield OBPC-1, which is needed for the DNA replication of HSV-1 (Link *et al.*, 2007). Based on recent studies, HSV-1 infection has been shown to increase cysteine cathepsin expression. The expression of cathepsin H mRNA was increased during latency and reactivation in neuronal cells of mice (Kent & Fraser, 2005). Also, cathepsin J mRNA expression is increased in neuronal cell culture during quiescent infection of HSV-1 (Danaher *et al.*, 2008).

2.4.3 Regulation of apoptosis by cystatins and cysteine cathepsins

Cystatins have been shown to be involved in regulation of apoptosis induced by different stimuli. Cystatin A suppresses ultraviolet B irradiation (UVB)-induced apoptosis of keratinocytes by inhibiting the activation of caspase-3 (Takahashi *et al.*, 2007). It also prevents the cathepsin B activation and apoptosis in bile salt-treated rat hepatoma cell line (Jones *et al.*, 1998). Moreover, cystatin A has been shown to block rhabdovirus-induced apoptosis in carp cell line (Björklund *et al.*, 1997). Cystatin B inhibits TRAIL-induced apoptosis in human melanoma cells (Yang *et al.*, 2010). Thymocytes of cystatin B-deficient mice are more sensitized to staurosporine-induced caspase-dependent apoptosis (Kopitar-Jerala *et al.*, 2005) and these mice have increased expression of both apoptosis and glial activation genes (Lieuallen *et al.*, 2001). Oxidative stress stimulates an increase in cystatin C expression in cultured neurons suggesting that cystatin C is able to regulate oxidative stress-induced apoptosis (Nishio *et al.*, 2000). Indeed, expression of cystatin C in neuronal cells prevents oxidative stress-induced cell death (Nishiyama *et al.*, 2005).

Cathepsins have been shown to act as mediators of apoptosis, reviewed in Colbert *et al.* (2009) and Yamashima & Oikawa (2009). The release of cathepsins from lysosomes into the cytosol results in apoptosis (Colbert *et al.*, 2009; Yamashima & Oikawa, 2009). There is a quantitative relationship between the amount of lysosomal rupture and mode of cell death. Apoptosis is induced due to low stress intensities triggered limited lysosomal membrane permeabilization (LMP), while high intensity stresses lead to generalized lysosomal rupture and necrosis (Brunk *et al.*, 1997). The LMP can be induced by different stimuli, such as cathepsins, caspases, DNA damage and viral proteins, reviewed in Boya & Kroemer (2008). The loss of lysosomal integrity may directly activate caspases leading to apoptosis. Also, LMP may trigger the mitochondrial outer membrane permeabilization (MOMP)-associated caspase pathway or MOMP- and caspase-independent pathways of apoptosis (Boya & Kroemer, 2008). The LMP-stimulated pathway of apoptosis is cell type-dependent. The mechanism(s) controlling LMP or the hierarchy within the apoptosis signaling cascade are not yet fully understood. However, accumulating data suggest that cathepsins are involved in the LMP-induced apoptosis.

Cathepsins involved in LMP-stimulated apoptosis are those cathepsins that remain active at neutral pH, such as cathepsin B. In the cathepsin-mediated apoptosis, the proteolysis of cellular components by cathepsins triggers apoptosis hallmarks like MOMP and caspase activation. MOMP can be induced by Bcl-2 family member Bid, which is first cleaved by cathepsins, such as cathepsin B, H, L or S (Cirman *et al.*, 2004). The cleavage of Bid results in cytochrome c release from mitochondria (Cirman *et al.*, 2004). Cathepsin B-dependent Bid cleavage has been observed in various cell lines, reviewed in Boya & Kroemer (2008). In addition, LMP can initiate caspase-independent pathway of apoptosis. Instead of cytochrome c release from mitochondria, the pro-apoptotic apoptosis-inducing factor (AIF) translocates from mitochondria to nucleus, where it induces DNA degradation, reviewed in Modjtahedi *et al.* (2006). The AIF-mediated apoptosis is cell type-dependent and occurs only when caspases are inhibited or not activated.

Cathepsin-mediated apoptosis can be blocked with various inhibitors. Cystatin A has been shown to prevent cathepsin B activation and apoptosis in bile-salt-treated rat hepatoma cell line (Jones *et al.*, 1998) and cathepsin B-mediated death receptor-triggered apoptosis in fibrosarcoma cells (Foghsgaard *et al.*, 2001). It has been shown, that mice with cystatin C deficiency have enhanced cysteine protease activity and their smooth muscle cells dies of apoptosis (Schulte *et al.*, 2010). Also, cystatin B deficient mice show loss of cerebellar granular cell neurons due to apoptosis (Lehesjoki, 2003; Pennacchio *et al.*, 1998; Shannon *et al.*, 2002). Taken together, tightly regulated balance between cystatins and cathepsin is essential for the cell survival.

3 AIMS OF THE STUDY

Cell death by apoptosis is one of the host responses against virus infection. On the other hand, viruses have developed different strategies either to prevent or actively use apoptosis in virus infections for their own purposes. Molecular details of programmed cell death in virus infections have been an extensively researched topic during the last decade. When this study started, herpes simplex virus type 1 (HSV-1) and measles virus (MV) were known to induce apoptosis but the mechanisms were unclear. Therefore, one aim of this study was to investigate the mechanisms of HSV-1- and MV-induced apoptosis. Recent data about the potential role of cystatins in virus-induced apoptosis led to study if cystatins inhibit virus-induced apoptosis in HSV-1- and MV-infected cells. In addition, the effects of cystatins on virus replication were investigated. Accumulating reports on Toll-like receptor signaling associated with virus infections set aim to investigate the innate immune response in virus-infected host cell. Knowledge in HSV-1-induced cell death, as well as in the modulation of immune response by HSV-1, is required for the development of viral vectors for gene therapy. Gaining knowledge in the mechanism of MV-induced cell death is important in order to understand the mechanism of MV-caused immunosuppression.

The specific aims of the study were:

1. To study the mechanisms of HSV-1 deletion virus- and MV-induced programmed cell death.

2. To study the effects of cystatins and the roles of cysteine cathepsins in HSV-1- and MV-induced cell death and viral replication.

3. To study, how HSV-1 deletion virus infections modulate innate immune response in infected host cell.

4 MATERIALS AND METHODS

4.1 Viruses (I-IV)

For herpes simplex type 1 (HSV-1) infections, wild-type and recombinant HSV-1 viruses were used (II-IV). The structures of HSV-1 viruses are shown in Figure 9. The virus R7041 has a deletion in Us3 and it is derived from the wild-type HSV-1 (F) (Purves *et al.*, 1987). The R7306 is a wild-type like rescue virus of the R7041 with an insertion of Us3 gene (Purves *et al.*, 1987). The d120 is derived from the wild-type HSV-1 (KOS) having deletions in Us3 and in the both copies of ICP4 (DeLuca *et al.*, 1985). The HSV-1 stocks were grown in Vero cells (American Type Culture Collection, ATCC, CCL-81). The d120 was grown in ICP4 transfected Vero E5 cells provided by Dr. DeLuca (University of Pittsburgh, USA). The virus titers from the culture supernatants were between $5x10^8$-$1x10^{10}$ plaque forming units (PFU)/ml. Cells were infected with HSV-1 at a multiplicity of infection (moi) of 0.05 (II), 1 (III) or 5 (II-IV).

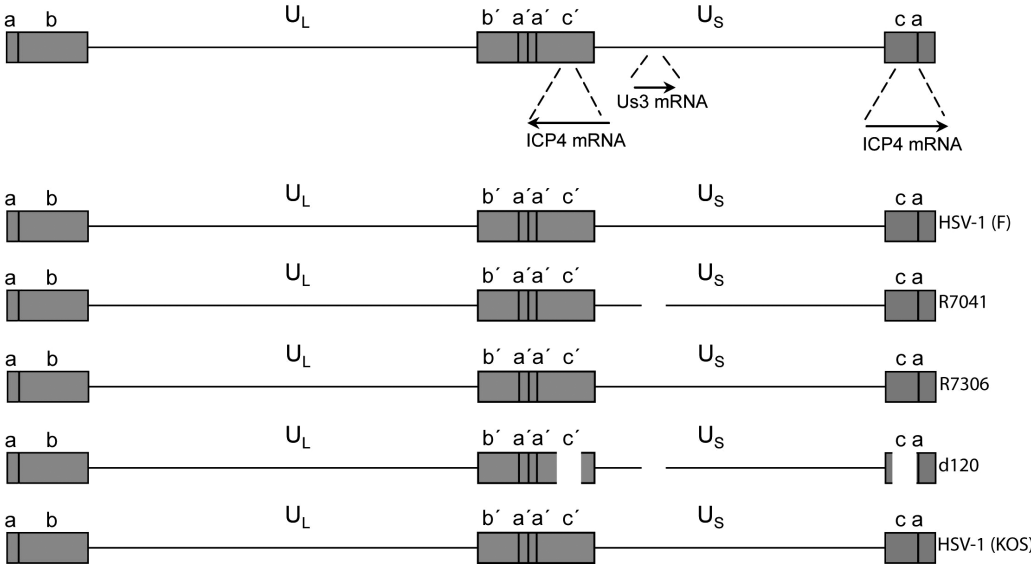

Figure 9. Genomic structures of HSV-1 viruses.

For measles virus (MV) infections, wild-type laboratory-adapted strain of MV (Vainionpää *et al.*, 1978) with a high infectivity titer (>$1x10^6$ PFU/ml) was used (I). The inoculum MV was propagated in Vero cells, and the production of MV was assayed by plaque titration. Cells were infected with MV at a moi of 3.

4.2 Cells and cell lines (I-IV)

Peripheral blood mononuclear cells (PBMC) were isolated from blood drawn from healthy adults using Ficoll-Isopaque (Pharmacia) density centrifugation. PBMC were cultured at the concentration of 1×10^6 ml^{-1} in RPMI 1640 medium (Gibco) containing 10% fetal calf serum (FCS, Bioclear) 1% glutamine, and gentamicin (I). Monocytes were enriched from PBMC by plastic adherence. The non-adherent cells were removed by several washings, and the purity of the monocyte population (70-80%) was determined by flow cytometry. Human epithelial cells (HEp-2) were cultured in DMEM (Gibco) supplemented with 7% FCS and gentamicin (II). Human monocytic (U937) cells were cultured at the concentration of 1×10^6 ml^{-1} in RPMI 1640 medium containing 10% FCS, 1% glutamine and gentamicin (III, IV). Cells were maintained at 37°C in 5% CO_2.

4.3 Reagents (I-IV)

Phytohemagglutinin (PHA) (Difco Laboratories) was used to stimulate PBMC cells at a concentration 25 $\mu g ml^{-1}$ (I). Cystatin A was purified from human post-mortem spleen according to the method of Järvinen & Hopsu-Havu (Järvinen & Hopsu-Havu, 1975) at the Department of Forensic Medicine, University of Turku, Finland (II). The purity of cystatin A was confirmed by gel electrophoresis and immunoblotting using an anti-cystatin A antibody. The preparation was free of other studied cystatins. Cystatin C and D were produced in *E. coli* expression systems (Abrahamson *et al.*, 1988; Freije *et al.*, 1993). Cystatin A was used at a concentration 125 nM (II). Cystatins C and D were used at concentrations 100 nM (II). Cathepsin B substrate (Z-Arg-Arg-AMC), cathepsin H substrate (H-Arg-AMC), cathepsin B+L substrate (Z-Phe-Arg-AMC), and cathepsin S substrate (Z-Val-Val-Arg-AMC) were purchased from Bachem and they were diluted in DMSO (II, IV). Cathepsin B inhibitor was used to block the cathepsin B activity in cathepsin L activity assays (IV). Inhibitors of caspase-3 (Z-DEVD-FMK), cathepsin B (CA-074Me), cathepsin H (H-Leu-CMK), cathepsin L (Z-Phe-Tyr(t-Bu)-DMK), cathepsin S (Z-Val-Val-Nle-DMK) and necroptosis (Necrostatin-1, Nec-1) were diluted in DMSO (IV). Cathepsin inhibitors were purchased from Bachem, caspase-3 inhibitor was purchased from R&D Systems and necroptosis inhibitor Nec-1 from Tocris Bioscience.

4.4 Cell viability, apoptosis and necroptosis (I-IV)

For cell viability studies, 60 000 of U937 or HEp-2 cells were grown on 96-well plate and the number of viable cells was studied with the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions.

For apoptosis studies, $1\text{-}5\text{x}10^5$ of U937 or PBMC cells were harvested at different time points post infection (p.i.). The DNA fragmentation was studied using propidium iodide (PI) staining (I). The cells were washed with phosphate buffered saline (PBS), incubated overnight at +4 °C in hypotonic solution containing 0.1% Triton-X 100 and

0.1% sodium citrate solution, and 30 μg ml^{-1} PI. The number of apoptotic cells was measured with the double staining of an early apoptosis marker Annexin-V-Fluos (Caltag) and PI to differentiate between apoptotic and necrotic cells. The staining was performed according to the manufacturer´s protocol (I, III-IV). The percentage of apoptotic and necrotic cells was analyzed with Cell Quest™ software (Becton Dickinson) from 10 000 cells collected with FACScan® flow cytometer (Becton Dickinson).

To study the proportion of necroptotic cells (IV), 60 000 of U937 cells were cultured on 96-well plate and treated with necroptosis inhibitor, Nec-1, at a concentration of 50 μM beginning 3h prior the infection. The cell viability was measured at early and late time points of infection as described above.

4.5 Rapid virus culture (II)

To detect replicative HSV-1 in the infected HEp-2 cell monolayers, rapid-culture method with immunostaining for HSV-1 gC was used as described earlier (Ziegler *et al.*, 1988). To measure the amount of released HSV-1, the cell culture media collected from the infected HEp-2 cell cultures were diluted tenfold and distributed on Vero cells. After 18h incubation, the Vero cells were immunostained for HSV-1 gC antigen.

4.6 RNA extraction, production of cDNA and quantitative real-time PCR (III)

In study III, the RNA of $2x10^6$ U937 cells was extracted using the TRIZOL reagent (Invitrogen) or TriPure reagent (Roche) according to the manufacturer´s protocol. The cDNA was synthesized using M-MLV reverse transcriptase (Promega) and random hexamer primers for 1h at +37 °C. For quantitative real-time PCR, QuantiTect™ SYBR® Green system (Qiagen), forward and reverse primers for each target of interest, and 2 μl of the cDNA or diluted PCR standard was run in Rotor-Gene™ 6000 instrument (Corbett Life Science). The PCR protocol consisted of an initial incubation for 15 min at +95 °C followed by PCR cycling using a three step cycle at +95 °C for 15 sec, at +60 °C (or +55 °C for MyD88) for 30 sec and at +72 °C for 45 sec for a total of 40 cycles. Cellular mRNA changes during the HSV-1 infections were measured with quantitative real-time PCR of cellular β-actin mRNA as described earlier (Mäkelä *et al.*, 2006). For external PCR standard construction, cDNA transcripts of RNA isolated from stimulated human PBMC or U937 cells were used. The copy numbers of standards were calculated as described earlier (Broberg *et al.*, 2003). For each PCR run, dilution series of standards of 10^1 to 10^8 copies per reaction were used.

4.7 Flow cytometric analysis of infected cells (I, III-IV)

For flow cytometric analysis, 10 000 cells were collected with FACScan® flow cytometer (Becton Dickinson) and analyzed with Cell Quest™ software (Becton Dickinson).

Infected cells (I)

In study I, MV-infected PBMC were collected at different time points after infection, washed with PBS, permeabilized with 0.3% saponin for 15 min, incubated with 1% normal rabbit serum for 5 min followed by incubations with polyclonal MV nucleocapsid antibodies (Department of Virology, University of Turku, Finland) and fluorescein-conjugated anti-rabbit antibodies (Caltag, USA).

Cell surface proteins (I)

To detect cell surface proteins by flow cytometry, cells were washed with PBS and incubated with rhodamine-conjugated anti-human CD3 antibody (Caltag) phycoerythrin-conjugated anti-human CD14 (Pharmingen), monoclonal anti-human Fas or FasL antibody (Pharmingen) and fluorescein-conjugated anti-mouse antibody (Caltag), or fluorescein-conjugated anti-human TNFR1 antibody (R&D Systems) for 30 min at +37 °C.

Intracellular proteins (III)

For intracellular staining of TLR3, $1x10^6$ of U937 cells were collected and fixed with 3% paraformaldehyde for 15 min at RT and permeabilized with 0.1% Triton-X in PBS 100 for 5 min. After washing the cells with 0.5% bovine serum albumin (BSA) in PBS the cells were incubated with monoclonal antibody to TLR3 (Axxora) and with Alexa Fluor 488 goat anti-mouse immunoglobulin (Invitrogen Molecular Probes). For MxA intracellular staining, $1x10^6$ of U937 cells were fixed and permeabilized as described earlier (Halminen *et al.*, 1997). The MxA protein was detected with rabbit anti-MxA serum (Ronni *et al.*, 1993) and fluorescein-conjugated goat anti-rabbit immunoglobulin (Caltag).

4.8 Fluorescence microscopy (II)

Adherent human epithelial HEp-2 cells were cultured on 12 mm glass coverslips. At different time points p.i., the cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature (RT), permeabilized with 0.1% Triton-X 100 in PBS for 15 min at RT and stained for DNA with Hoechst 33258 DNA stain (1 μg/ml in 25% ethanol/75% PBS) for 15 min. Cells were analyzed using a Leica DM L microscope and Leica IM50 Image Manager.

4.9 Western blotting (I, III)

Total protein from 2-3x10^6 cells was extracted with the RIPA-buffer (50 mM Tris-HCl buffer, pH 8.0, containing 1% NP-40, 0.4% Na-deoxycholate, 0.1% SDS, 150 mM of NaCl, 1 mM of phenylmethylsulphonyl fluoride, 10 μgml^{-1} of leupeptin and 10 μgml^{-1} aprotinin) (I) or with the ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas) (III). Protein concentrations were determined from the supernatants using the Bio-Rad Protein Assay Kit according to the Bradford method (I). In study I, aliquots containing equal amounts of proteins were separated by 12% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). Protein transfer was verified with Ponceau S staining. In study III, protein samples were separated with NuPAGE Electrophoresis System on a 10% polyacrylamide gel in Tris-Glycine native running buffer (25 mM Tris base, 192 mM Glycine, pH 8.3). Proteins were transferred to Hybond ECL Nitrocellulose membrane (Amersham Biosciences) using NuPAGE Transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1mM EDTA, pH 7.2). The equal protein loading was confirmed by blotting for GAPDH from the same samples using a denaturing gel. After blocking the blots overnight at +4 °C with 5% non-fat dry milk in PBS (I) or with Tris-buffered saline tween-20 (TBS-T) (III), the blots were incubated with monoclonal antibody to human Bcl-2 (Dako) (I) or polyclonal antibody to human IRF-3 (Santa Cruz Biotechnology) (III). Antibody binding was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Santa Cruz Biotechnology) (I) or with HRP-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc.). Proteins were visualized using the enhanced chemiluminescence system (ECL, Amersham Biosciences).

4.10 Lysosomal membrane stability assay (IV)

The permeabilization of lysosomes was studied with acridine orange staining as described earlier (Zhao *et al.*, 2001). Acridine orange exhibits red fluorescence when it is highly concentrated, such as in intact lysosomes. Thus percentage of lysosomal membrane permeabilization can be evaluated from the percentage of decreased red fluorescence. For acridine orange staining, U937 cells (0.5x10^6) were infected with HSV-1 and stained with acridine orange (Invitrogen) at a concentration of 5 μg/ml for 15 min at +37 °C in RPMI-1640 medium. After washed twice with RPMI-1640 medium, the red fluorescence of the cells was studied with FACScan® flow cytometer. For analysis, 10 000 cells were collected and analyzed with Cell Quest™ software. The reduced red fluorescence correlated with the reduced number of the intact lysosomes.

4.11 Cathepsin activity assay (II, IV)

For cathepsin assay, HEp-2 (II) and U937 (IV) cells were plated on black 96-well Isoplates (PerkinElmer, USA) and grown overnight. Cathepsin B, H, L and S activities were analyzed at different time points after infection as described earlier (Hulkower *et al.*, 2000). Cells were washed with PBS and incubated with pericellular assay buffer

(PAB, Hank's balanced salt solution lacking sodium bicarbonate and containing 0.6 mM $CaCl_2$, 0.6 mM $MgCl_2$, 2 mM L-cysteine and 25 mM Pipes, pH 7.0). After 30 minutes, the buffer was replaced with fresh PAB containing 100 µM of cathepsin B substrate, 50 µM cathepsin H, 100 µM cathepsin L or 25 µM cathepsin S substrate and 0.1 % Triton X-100 to liberate total cellular cathepsin activity. Victor 1420 Multilabel counter (Perkin Elmer) was used to monitor the fluorescent product formation (355 nm / 460 nm) at +37˚C for 20 min. Specificity of the assay was enhanced by using specific inhibitors.

4.12 Caspase activity assay (I, IV)

In study I, caspase activity of PBMC was performed as described earlier (Zhirnov *et al.*, 1999) using a colourimetric caspase substrate set containing substrates for caspase-1, -3 and -6 and for granzyme B (Calbiochem). At different time points after infection, $1.5x10^7$ cells were lysed in PBS containing 20 nM Tris-HCl (pH 7.6) and 4 mM dithiotreitol and the cell lysate was sonicated. The protein concentration was determined from the supernatants with Bio-Rad Protein Assay Kit. For colourimetric caspase assay, 60 µg of protein and 40 µg of caspase substrate were mixed and incubated for 4h at +37 °C. After centrifugation, the caspase activity was measured by Titertek Multiscan (Labsystems) at 405 nm as intensity of the colour of free para-nitroanilide (pNA).

In study IV, 60 000 of U937 cells were plated on black 96-well Isoplates (Perkin Elmer) and grown overnight. Caspase-3 activity was analyzed at different time points after infection. Cells were washed with PBS, incubated with lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA and 0.1% TritonX-100) on ice for 30 min. After cell lysis 50 µM of caspase-3 substrate (Z-DEVD)2-rhodamine 110 (Bachem) in a 50 µl of reaction buffer (20 mM Pipes pH 7.4, 4 mM EDTA, 0.2% CHAPS, and 10 mM DTT) was added onto cells and the cells were incubated at +37 °C for 30 min. The progress of fluorescent product formation was monitored with Victor 1420 Multilabel Counter (Perkin Elmer) at +37 °C for 60 min.

4.13 Statistical analyses (II-IV)

Statistical analyses were performed with SAS software. Dunnett generalized linear model procedure was used to compare pairwise values of cystatin-treated cells with values of untreated cells (II). Non-parametric one-way analysis of variance and Wilcoxon scores was used for real-time PCR analyses (III) and Student´s t-test for flow cytometric analyses (III, IV) and for the assays of cell viability and cathepsin and caspase activity (IV). The values of deletion viruses were compared pairwise to the values of their rescue or parental viruses or to the values of untreated cells. Values of $P<0.05$ were considered statistically significant.

5 RESULTS

5.1 Virus-induced cell death

5.1.1 Herpes simplex virus type 1 ICP4 and Us3 (d120) or Us3 (R7041) deletion virus induce apoptosis (II-IV)

Herpes simplex virus type 1 (HSV-1) induces apoptosis at early stage of infection. However, apoptosis is blocked by proteins synthesized shortly after infection (Nguyen & Blaho, 2009). For example, the late protein kinase Us3 and the immediate-early protein ICP4 participate in blocking of the HSV-1-induced apoptosis (Leopardi & Roizman, 1996; Leopardi *et al.*, 1997; Munger & Roizman, 2001). In this work, HSV-1 Us3 deletion mutant (R7041) and ICP4 and Us3 deletion mutant (d120) virus-induced apoptosis was studied in human epithelial and monocytic cell lines. To study, if R7041 and d120 induce apoptosis in human epithelial cell line (HEp-2), HEp-2 cells were stained with Hoechst and the number of fragmented nuclei was calculated. The numbers of apoptotic cells in uninfected, wild type HSV-1 (F)-infected, R7041- and d120-infected cells were similar at an early time point of infection (6h). However, the amount of apoptotic cells was clearly increased in R7041- and d120-infected HEp-2 cells at late time point of infection (16h-21h p.i.) when compared with uninfected or wild type HSV-1 (F)-infected cells (II, Figure 2). The percentage of apoptotic cells was approximately 15% in R7041-infected and 27% in d120-infected HEp-2 cells while in uninfected and HSV-1 (F)-infected cells the percentage of apoptotic cells varied from 4% to 7%. The effects of ICP4 deletion on HSV-1-induced apoptosis are discussed in more details later.

In addition, R7041 and d120 viruses induced apoptosis in monocytic cells (U937). The percentage of apoptotic cells was studied with Annexin-V and propidium iodide double staining. Thus, apoptotic and necrotic cells could be differentiated. The amount of apoptotic cells was significantly increased in R7041- and d120-infected U937 cells when compared with uninfected, rescue- or parental virus-infected cells (III, Figure 5). The percentage of apoptotic cells was approximately 60% in R7041-infected cells (5 moi) while in its rescue virus (R7306)-infected cells the percentage of apoptotic cells was approximately 27%. In d120-infected U937 cells, the percentage of apoptotic cells was approximately 70% (5 moi), whereas in its parental virus (KOS)-infected cells the percentage of apoptotic cells was only 23%. Thus R7041 and d120 induced significant increase in the number of apoptotic U937 cells. These findings were supported with similar results of the percentage of apoptotic cells (IV, Figure 1b) and the relative proportion of viable cells in R7041- and d120-infected U937 cells. The relative number of viable cells was significantly decreased in R7041- and d120-infected cells when compared with their rescue or parental virus-infected cells at late time point of infection ($P=0.004$ and $P<0.001$, respectively) (IV, Figure 1a).

5.1.2 Herpes simplex virus type 1 ICP4 and Us3 deletion virus (d120) induces necroptosis (IV)

In addition to apoptosis, the HSV-1-induced programmed necrosis or necroptosis was studied. Necroptosis is a recently described type of cell death, which shares some regulatory mechanisms with apoptosis (Declercq *et al.*, 2009; Degterev *et al.*, 2005; Tait & Green, 2008). To study the proportion of necroptosis in HSV-1-induced cell death, U937 cells were treated with necroptosis inhibitor (Necrostatin, Nec-1) beginning 3h before the infection. The viability of the U937 cells was measured at late time point of infection. The relative number of viable cells was significantly increased in d120-infected cells treated with Nec-1 when compared with untreated d120-infected cells ($P<0.001$) (IV, Figure 1c).

5.1.3 Measles virus induces apoptosis (I)

Measles virus induces apoptosis in different cell lines (Auwaerter *et al.*, 1996; Esolen *et al.*, 1995; Fugier-Vivier *et al.*, 1997; Ito *et al.*, 1996; McQuaid *et al.*, 1997). The proportion of apoptotic cells can be measured by propidium iodide staining and flow cytometry. The broad DNA peak of apoptotic cells can be distinguished from the narrow peak of viable cells (Nicoletti *et al.*, 1991). To investigate, if measles virus induces cell death in human peripheral blood mononuclear cells (PBMCs), the DNA peaks in MV-infected PBMCs were analyzed. MV induced DNA fragmentation (broad peak) in PBMCs already 1 day p.i. (I, Figure 1). The percentage of DNA fragmentation was clearly increased in MV-infected PBMCs when compared with uninfected PBMCs 1 day p.i. (10% and 2%, respectively) (I, Figure 1). The proportion of DNA fragmentation was higher in MV-infected PBMCs in comparison with uninfected cells also 3 and 6 day p.i. In order to study, if productive measles virus replication increases the number of DNA fragmentation, the PBMCs were stimulated with phytohaemagglutinin (PHA). It has been shown, that MV replicates productively in PHA-stimulated PBMCs only (Vainionpää *et al.*, 1991). The percentage of DNA fragmentation was clearly increased in PHA-stimulated MV-infected PBMCs when compared with PHA-stimulated uninfected cells 2 and 3 day p.i. (42% and 58%; 9% and 14%, respectively) (I, Figure 1).

Since MV infects mainly monocytes (Salonen *et al.*, 1988), the cell phenotype undergoing apoptosis was studied. For this, MV-infected PBMCs were stained with either monocyte marker CD14 or thymocyte and T lymphocyte marker CD3 and with an early apoptosis marker Annexin-V. The percentage of apoptotic monocytes varied from 11% to 31% in MV-infected and from 18% to 44% in uninfected PBMCs (I, Figure 2). Thus, MV infection did not induce apoptosis in monocytes. However, the number of apoptotic lymphocytes was notably increased in MV-infected PBMCs when compared with uninfected PBMCs (I, Figure 3a). The percentage of apoptotic lymphocytes ranged from 19% to 25% in MV-infected PBMCs during the days 1-6 p.i. while in uninfected cells the percentage of apoptotic lymphocytes ranged from 11% to 19%. Only 2% of the lymphocytes were infected with MV (I, Figure 3a) whereas the percentage of MV-infected monocytes was 35% (I, Figure 3b) on day 3 p.i. suggesting, that MV-induced lymphocyte apoptosis occur via indirect mechanism. This finding was

further supported by the experiment, in which monocytes were first isolated by plastic adherence and then infected with MV. When these MV-infected monocytes were co-cultivated with uninfected non-adherent lymphocytes, the amount of apoptotic lymphocytes was clearly increased (35%) when compared with lymphocytes co-cultivated with uninfected adherent cells on day 3 p.i. (I, Figure 4). In addition, the number of MV-infected lymphocytes remained low (1%) (I, Figure 4).

Since the replication of MV is highly productive in human monocytic cell line (U937) (Helin *et al.*, 1999), the MV-induced cell death was studied also in U937 cells. First, the number of viable cells in MV-infected and uninfected U937 cells were measured at different time points of infection. Figure 10 represents the relative proportion of viable cells in MV infection in comparison with uninfected cells. The number of viable cells was significantly decreased in MV-infected U937 cells 2 and 3 day p.i. (68% and 32%; P=0.035 and P=0.001, respectively) when compared with uninfected cells (100%).

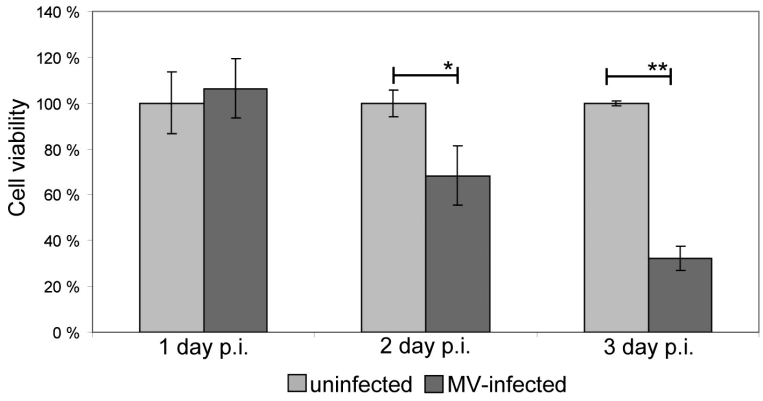

Figure 10. MV infection causes cell death in U937 cells. The number of cell viability was measured 1, 2 and 3 day p.i. with CellTiter-Glo luminescent cell viability assay kit. Data are means±SD of the relative proportion of the number of viable U937 cells in comparison with uninfected cells. Data was collected from three parallel reactions. (*:P<0.05, **:P<0.01)

Thus, MV induces significant cell death in MV-infected U937 cells 2 and 3 day p.i. To study, if MV induces cell death through apoptotic signaling, U937 cells were collected 2 and 3 day p.i. and stained with an early apoptosis marker Annexin-V. The number of apoptotic cells was increased in MV-infected cells when compared with uninfected cells on day 2 p.i. (31% and 12%, respectively) (P=0.083) (Figure 11). Moreover, on day 3 p.i., the number of apoptotic cells was significantly increased in MV-infected cells in comparison with uninfected cells (50% and 8%, respectively) (P=0.016) (Figure 11).

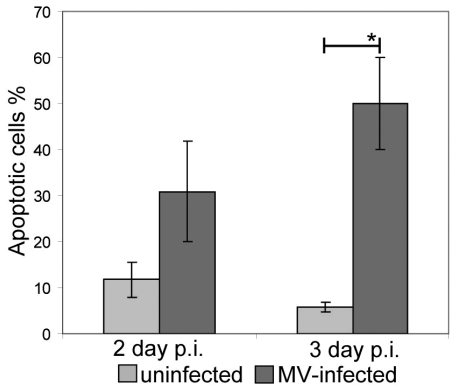

Figure 11. MV induces apoptosis in U937 cells. The percentage of apoptotic cells was studied 2 and 3 day p.i. with an early apoptosis marker Annexin-V and flow cytometer. The number of apoptotic cells was significantly increased in MV-infected cells when compared to uninfected cells 3 day p.i. (*:P<0.05)

In addition to apoptosis, MV-induced necroptosis was studied. The Nec-1 treatment did not significantly increase the number of viable cells on day 1, 2 or 3 p.i. (data not shown). This result indicates that MV-induced cell death in U937 cells is not caused by necroptosis.

5.2 Cathepsins in virus infection

5.2.1 Herpes simplex virus type 1 induces cathepsin B and H activation (II, IV)

Different stimuli, such as virus infections, induce lysosomal membrane permeabilization (LMP) and the release of lysosomal cathepsins into cytosol. To study, if HSV-1 infection induces LMP in infected monocytic cells, U937 cells were collected at different time points of infection and stained with acridine orange. Acridine orange accumulates in lysosomes and exhibits red fluorescence. During LMP acridine orange is released to cytosol, in where it fluoresces green light. To measure the LMP, the intensity of red fluorescence of the acridine orange stained U937 cells was analysed with flow cytometer.

LMP was significantly increased in d120-infected U937 cells at late time point of infection when compared with HSV-1 (KOS)-infected and uninfected U937 cells (P<0.001 and P<0.001, respectively) (IV, Figure 2). In d120-infected cells the percentage of LMP was approximately 70% while in its parental virus HSV-1 (KOS)-infected and uninfected cells it was approximately 15% (IV, Figure 2). In addition, in R7041-infected U937 cells LMP was significantly increased when compared with its rescue virus R7306-infected or uninfected U937 cells (P<0.001 and P<0.001, respectively) (IV, Figure 2).

To further study, if the increased lysosomal membrane permeabilization lead to increased cathepsin activities in R7041- and d120-infected U937 cells, cathepsin B, H, L and S activities were measured in HSV-1-infected U937 cells. Cathepsin B activity was significantly increased in d120-infected U937 cells at late time point of infection when compared with uninfected or parental virus HSV-1 (KOS)-infected cells (P=0.012 and P=0.019, respectively) (IV, Figure 3). Cathepsin H activity was also increased when compared with uninfected cells (P=0.048) (IV, Figure 3). Cathepsin B activation was also studied in HSV-1-infected HEp-2 cells (II). However, in HEp-2 cells the cathepsin B activity was not significantly increased in d120- or R7041-infected cells in comparison with uninfected cells (II, Figure 3).

5.2.2 Cathepsin B, L and S mediate herpes simplex virus type 1 ICP4 and Us3 (d120) deletion virus-induced cell death

Since d120 and R7041 infections resulted in increased cathepsin activities, the roles of cathepsins as mediators of HSV-1-induced apoptosis were studied. U937 cells treated with cathepsin H or L inhibitor did not show increased number of viable cells (IV, Figure 4). In contrast, the proportional number of viable cells was significantly increased in d120-infected cells treated with cathepsin B or S inhibitor (P=0.024 and P=0.023, respectively) (IV, Figure 4). In addition, the effects of cathepsin inhibitors on the number of apoptotic cells were studied in R7041- and d120-infected U937 cells. In d120-infected U937 cells treatment with cathepsin L inhibitor significantly decreased number of apoptotic cells when compared with untreated d120-infected cells (P=0.007) (IV, Figure 4). In contrast, no significant difference in the number of viable or apoptotic cells between cathepsin inhibitor-treated or untreated cells was seen In R7041-infected cells (data not shown). To ensure that cathepsin inhibitors really blocked cathepsin activities, cathepsin activities were studied in cathepsin inhibitor-treated U937 cells. Cathepsin B, H, L and S inhibitor treatments clearly blocked the cathepsin activities (data not shown).

5.2.3 Measles virus induces cathepsin B, H, L and S activation

There was no difference in LMP between MV-infected and uninfected U937 cells on day 1, 2 or 3 p.i. (data not shown) indicating that MV infection does not induce LMP. Although lysosomal membranes seemed to remain intact, cathepsin B, H, L and S activities were significantly increased in MV-infected U937 cells when compared with uninfected cells (Figure 12). Since MV significantly decreased the viability of U937 cells on day 2 and 3 p.i., the cathepsin activity values were proportionated to the number of viable cells. Figure 12 shows the relative cathepsin B, H, L and S activities in viable U937 cells. Cathepsin B and H activities were notably increased in MV-infected U937 cells in comparison with uninfected cells on day 2 and 3 p.i. (B: P=0.013 and P=0.009; H: P<0.001 and P<0.001, respectively). In addition, cathepsin L activity was significantly increased on day 3 p.i. (P=0.014) and cathepsin S activity was increased on day 1 and 3 p.i. (P=0.002 and P<0.001, respectively).

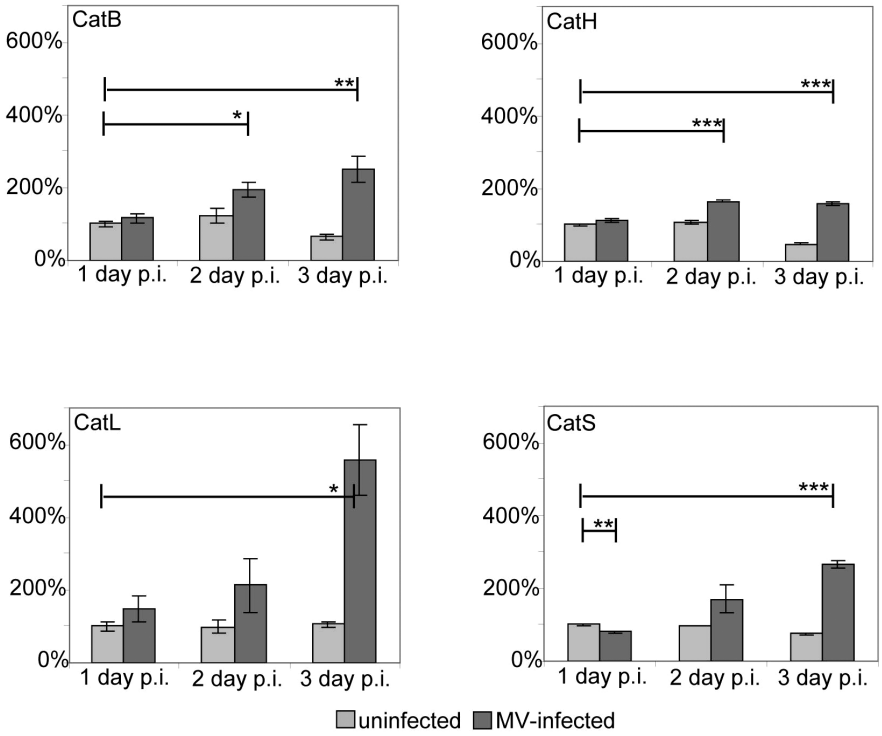

Figure 12. Measles virus increases cathepsin B, H, L and S activities in U937 cells. Cathepsin B, H, L and S activities were studied with cathepsin specific fluorescent substrates on day 1, 2 and 3 p.i. Bars represent means±SD of the relative amount of fluorescent product formation per minute in viable cells in comparison with uninfected cells from four parallel reactions. (*:P<0.05, **:P<0.01, ***:P<0.001)

5.3 Herpes simplex virus type 1 and measles virus induce caspase-3 and -8 activation (I, IV)

Since both HSV-1 and MV induced cell death and increased cathepsin activities in U937 cells, the possibility that increased cathepsin activity resulted in increased caspase-3 and -8 activities was studied in HSV-1- and MV-infected U937 cells. In HSV-1-infected U937 cells, the caspase-3 activity was remarkably increased in d120-infected cells at late time point of infection when compared with parental virus HSV-1 (KOS)-infected or uninfected cells (P<0.001 and P<0.001, respectively) (IV, Figure 5). Moreover, the caspase-8 activation was remarkably increased in d120-infected U937 cells when compared with HSV-1 (KOS)-infected or uninfected cells (P<0.001 and P<0.001, respectively) (Figure 13).

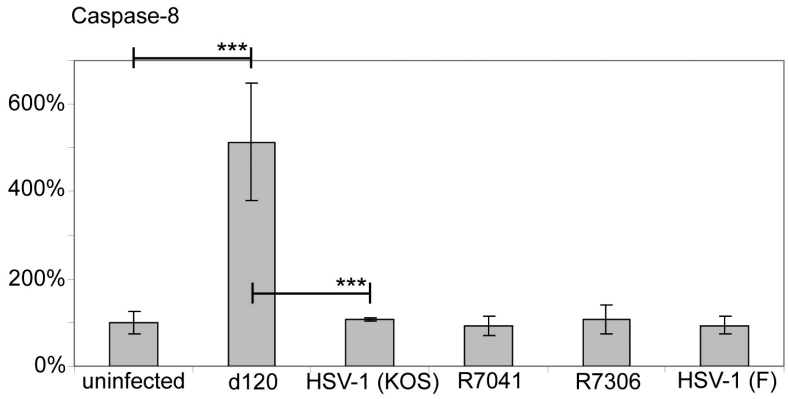

Figure 13. d120 induces caspase-8 activation in U937 cells. Caspase-8 specific fluorescent-conjugated substrate was used to measure caspase-8 activity in HSV-1-infected U937 cells at 21h p.i. Bars represent the means±SD of fluorescent product formation in viable cells in comparison with uninfected cells from four parallel reactions. (***:P<0.001)

To explore, if increased caspase-3 acitivity in d120-infected U937 cells was induced by the increased cathepsin activity, d120-infected cells were treated with cathepsin inhibitors and studied for caspase-3 activity. The caspase-3 activity was significantly decreased in cathepsin L inhibitor-treated U937 cells when compared with untreated cells (P=0.019) (IV, Figure 6). However, caspase-3 activity was not altered in cathepsin B, H or S inhibitor-treated U937 cells when compared with untreated d120-infected cells (IV, Figure 6). Treatment with caspase-3 inhibitor significantly blocked the activity of caspase-3 (Figure 14). However, there was no significant decrease in the amount of apoptotic cells in caspase-3 inhibitor-treated d120-infected cells (Figure 14).

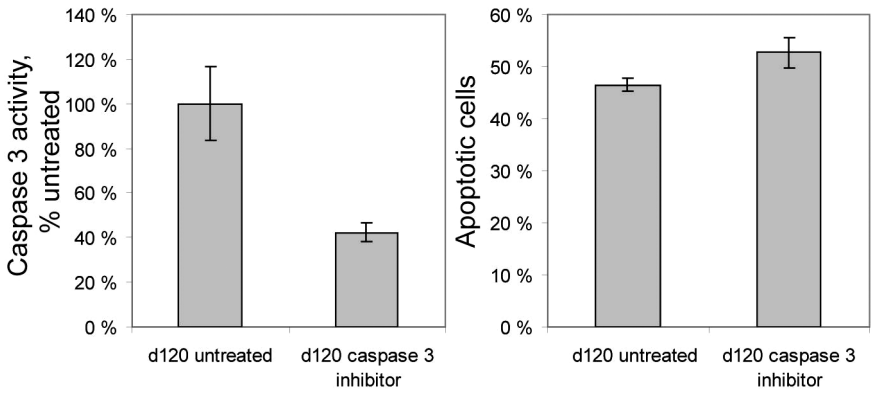

Figure 14. Caspase-3 acitivity and the amount of apoptotic cells in caspase-3 inhibitor-treated U937 cells.

In MV-infected U937 cells, caspase-3 activity was significantly increased when compared with uninfected cells on day 2 and 3 p.i. (P<0.001 and P=0.007,

respectively) (Figure 15). In addition, caspase-8 activity was remarkably increased in MV-infected cells in comparison with uninfected cells on day 1, 2 and 3 p.i. (P=0.027, P<0.001 and P<0.001, respectively) (Figure 15).

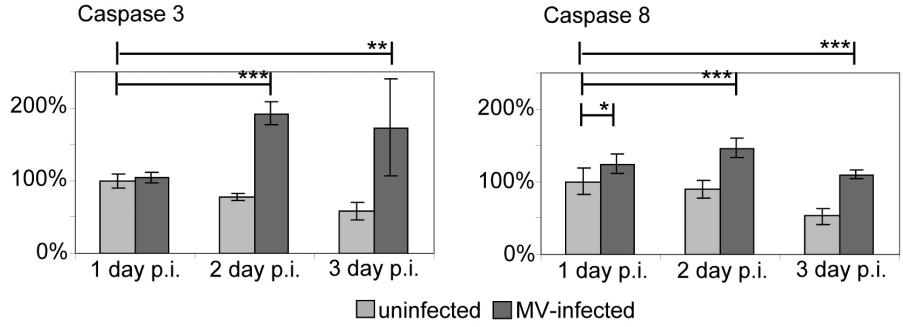

Figure 15. Measles virus induces caspase-3 and -8 activation in U937 cells. Caspase-3 and -8 specific fluorescent-conjugated substrates were used to measure caspase-3 and -8 activity in MV-infected U937 cells on day 1, 2 and 3 p.i. Bars represent the means±SD of fluorescent product formation in viable cells in comparison with uninfected cells from four parallel reactions. (*:P<0.05, **:P<0.01, ***:P<0.001)

Caspase activation was also studied in MV-infected PBMCs. MV increased both caspase-3 and caspase-6 activation in unstimulated and PHA-stimulated PBMCs on day 1, 2 and 4 p.i. (I, Figure 5). In addition, MV increased granzyme B activation (I, Figure 5) and the protein expression of Bcl-2 (I, Figure 6). Also, the expression of Fas receptor on lymphocytes was significantly increased in MV-infected PBMCs (98%) when compared with uninfected PBMCs (48%) (I, Figure 7).

5.4 Cystatins inhibit cathepsin activity, virus-induced apoptosis and virus replication (II)

Cysteine protease inhibitors, cystatins, inhibit papain-like cysteine proteases such as cathepsin B, H, L and S. Thus, the roles of cystatins in regulation of cathepsin B, H, L and S activities in infected U937 cells were studied. Also, the possible inhibition of virus-induced cell death was studied. In HSV-1-infected U937 cells, cystatin treatment did not significantly decrease cathepsin B, H, L or S activities in HSV-1-infected U937 cells (data not shown). However, in d120-infected HEp-2 cells, cystatin C and D treatment significantly decreased cathepsin B activity at late time point of infection when compared with untreated d120-infected cells (P=0.001 and P=0.002, respectively) (II, Figure 3). Also, cystatin D significantly decreased the amount of d120-induced apoptosis in HEp-2 cells (P=0.038) (II, Figure 2).

In addition, the effects of cystatins on virus replication and virus release in HEp-2 cells were studied. Since d120 is not able to replicate in HEp-2 cells due to the deletion of ICP4, viral replication was investigated in R7041- and HSV-1 (F)-infected HEp-2 cells. The R7041 production was notably decreased in HEp-2 cells treated with cystatin C and D (P=0.006 and P=0.001, respectively) (II, Figure 1). In addition, cystatin C and D

treatment significantly decreased the proportion of released virus in R7041-infected (P=0.026 and P=0.005, respectively) and HSV-1 (F)-infected HEp-2 cells (P=0.002 and P00.002, respectively) (II, Figure 2). Also, cystatin A treatment decreased the release of HSV-1 (F) in HEp-2 cells (P=0.010) (II, Figure 1).

In MV-infected U937 cells, cystatin C significantly decreased cathepsin H activity on day 2 and 3 p.i. when compared with MV-infected untreated U937 cells (P=0.011 and P=0.011, respectively) (Figure 16). Also, cystatin C significantly decreased cathepsin S activity on day 3 p.i. (P=0.005). In addition, cystatin D treatment significantly decreased cathepsin B activity on day 1 p.i. (P=0.006), cathepsin H activity on day 1 and 2 p.i. (P=0.003 and P<0.001, respectively) and cathepsin S activity on day 1 and 3 p.i. (P=0.022 and P=0.009, respectively) (Figure 16). However, the number of apoptotic U937 cells was not decreased in MV-infected U937 cells treated with cystatins (Figure 17).

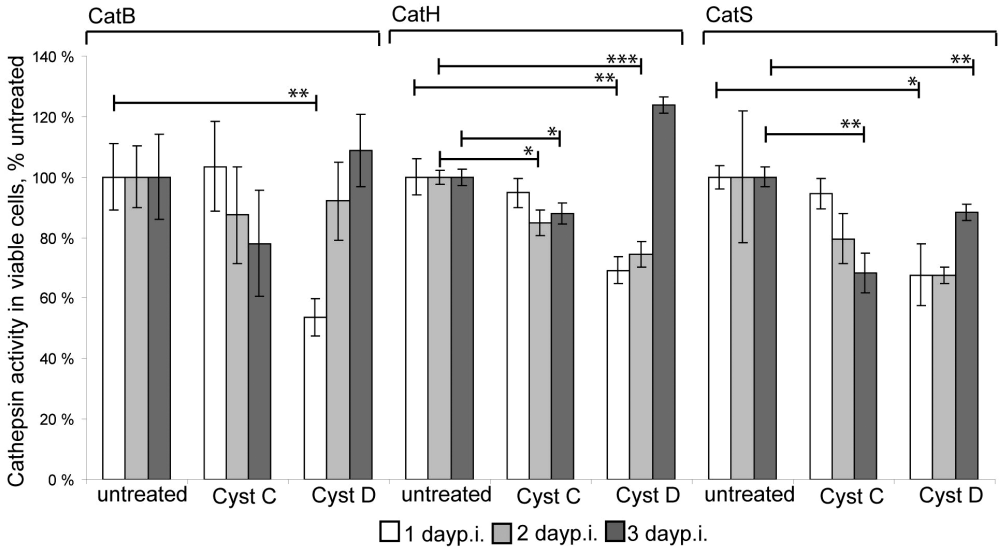

Figure 16. Cystatin C and D decrease cathepsin B, H and S activity in MV-infected U937 cells. Cathepsin B, H and S activities were studied with cathepsin specific fluorescent substrates in untreated or cystatin C- and D-treated cells on day 1, 2 and 3 p.i. Bars represent means±SD of the relative amount of fluorescent product formation per minute in viable cells in comparison with untreated cells from four parallel reactions. (*:P<0.05, **:P<0.01, ***:P<0.001)

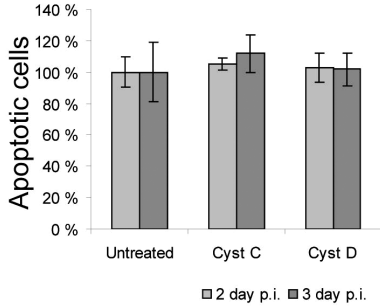

Figure 17. The amount of apoptotic cells in MV-infected U937 cells treated with cystatin C and D 1 day p.i.

5.5 Herpes simplex virus type 1 ICP4 and Us3 (d120) and Us3 (R7041) deletion viruses influence Toll-like receptor responses (III)

Toll-like receptors (TLRs) have been shown to initiate innate responses to HSV-1. In order to study the TLR gene expression in HSV-1-infected U937 cells, the mRNA levels of TLR2, TLR3, TLR4 and TLR9 were studied with quantitative real-time PCR. The expression of TLR3 mRNA was significantly increased in R7041-infected U937 cells when compared with its parental virus HSV-1 (F)-infected cells (1 moi, P=0.021) or with uninfected cells (5 moi, P=0.045) at late time point of infection (III, Figure 1a). In addition, the expression of TLR3 mRNA was significantly increased in d120-infected cells when compared with its parental virus HSV-1 (KOS)-infected cells at an early time point of infection (5 moi, P=0.033) (III, Figure 1a). Moreover, the TLR3 mRNA expression level was notably increased in d120-infected cells when compared with uninfected cells at late time point of infection (1 moi, P=0.009 and 5 moi, P=0.020, respectively) (III, Figure 1a). In contrast, the expression level of TLR4 mRNA was significantly decreased in d120-infected U937 cells when compared with its parental virus HSV-1 (KOS)-infected cells at late time point of infection (1 moi, P=0.024 and 5 moi, P=0.024, respectively) (III; Figure 1b). In addition, the TLR4 mRNA expression level was significantly decreased in d120-infected cells when compared with uninfected cells at late time point of infection (1 moi, P=0.025 and 5 moi, P=0.015) and in R7041-infected cells when compared with uninfected cells at early time point of infection (5 moi, P=0.045) (III, Figure 1b). There were no statistically significant difference in the expression levels of TLR2 (data not shown) and TLR9 (III, Figure 1c) mRNAs in HSV-1-infected U937 cells.

To study, if the increased TLR3 mRNA level resulted in increased TLR3 protein levels in R7041- and d120-infected U937 cells, the intracellular TLR3 was measured with flow cytometer. The percentage of TLR3 positive cells was significantly increased in R7041-infected U937 cells when compared with its rescue virus R7306-, its parental virus HSV-1 (F)-infected and uninfected cells (P<0.001, P<0.001 and P<0.001, respectively) (III, Figure 2a). Moreover, the number of TLR3 positive cell was significantly increased

in d120-infected cells when compared with its parental virus HSV-1 (KOS)-infected and uninfected cells (P=0.002 and P=0.001, respectively) (III, Figure 2a).

To further study the effect of increased intracellular expression of TLR3 on downstream factors of the signaling pathway, the activation of IRF-3 was examined in infected U937 cells. R7041 and d120 infection induced the dimerization of IRF-3 at early time point of infection (III; Figure 3). In addition, the rescue virus R7306 induced a weak dimerization of IRF-3. Since the activated IRF-3 induces, among others, the IFN-β gene, the expression levels of type I IFN mRNAs were studied. The expression of IFN-β mRNA was significantly increased in R7041-infected U937 cells when compared with the wild type HSV-1 (F) at late time point of infection (1 moi, P=0.016) (III, Figure 4a). In addition, the expression of IFN-β mRNA was significantly increased in d120-infected U937 cells when compared with its parental virus HSV-1 (KOS)-infected cells at early and late time points of infection (5 moi, P=0.017 and 5 moi, 0.024, respectively) (III, Figure 4a). Also, the expression levels of IFN-β mRNA were significantly increased in R7041- (1 moi, p<0.001 and 5 moi, P=0.007), d120- (1 moi, P=0.015 and 5 moi, P<0.001) and HSV-1(F)-infected (5 moi, P<0.001) cells at late time point of infection when compared with uninfected cells (III, Figure 4a). In addition, the IFN-α mRNA levels were significantly increased in R7041- (1 moi, P=0.031), d120- (1 moi, P=0.001 and 5 moi P=0.003) and HSV-1 (F)-infected (5 moi, P=0.025) cells when compared with uninfected cells (III, Figure 4b).

To study, if increased IFN-β mRNA level resulted in functional IFN-β, the amount of the IFN-inducible MxA protein in HSV-1-infected U937 cells was measured. The percentage of MxA positive cells was increased in R7041-infected U937 cells when compared with its rescue virus R7306-infected (5 moi, P<0.001) and its parental virus HSV-1(F)-infected (1 moi, P=0.003 and 5 moi, P=0.033) or uninfected cells (5 moi, P=0.040) (III, Figure 4c). In addition, the intracellular expression of MxA was significantly increased in d120-infected cells when compared with its parental virus HSV-1 (KOS)-infected cells (P=0.012) (III, Figure 4c).

6 DISCUSSION

In this work cell death induced by two different viruses, the double stranded DNA-virus HSV-1 and the negative-stranded RNA-virus MV, has been studied. Both of these viruses are able to establish long-term infections. HSV-1 causes latent infection in the nervous system and MV is able to establish persistent infections both *in vivo* and *in vitro* in many cell types. Based on the results of this study, HSV-1 and MV induced apoptosis in monocytic cells (U937). While wild-type HSV-1 was able to prevent apoptosis in U937 cells, MV induced, but was not able to prevent apoptosis in these cells. In addition, deletion mutant HSV-1-induced cell death was partially caused by necroptosis, but in MV-infected cells necroptosis could not be detected. In the following paragraphs the characteristics of HSV1- and MV-induced apoptosis are discussed in more details.

6.1 Herpes simplex virus type 1-induced cell death

6.1.1 Apoptosis

As a response to HSV-1 infection, apoptosis signaling is triggered in the host cell. However, HSV-1 has several anti-apoptotic factors which are able to prevent the dying of the host cell before the replication of HSV-1 is completed. Thus, there is a complex balance between pro- and anti-apoptotic factors in the HSV-1-infected cell (Nguyen & Blaho, 2009). HSV-1-induced apoptosis is blocked, besides other anti-apoptotic HSV-1 factors, by the late protein kinase Us3 and the immediate-early protein ICP4 (Leopardi & Roizman, 1996; Leopardi *et al.*, 1997; Munger & Roizman, 2001). For example, Us3 activates PKA and phosphorylates the same targets as PKA, such as Bad, Bid and pro-caspase-3 (Benetti *et al.*, 2003; Benetti & Roizman, 2004; Hagglund *et al.*, 2002; Munger & Roizman, 2001; Ogg *et al.*, 2004). Thus, HSV-1 viruses lacking ICP4 and Us3 genes are not able to prevent apoptosis in the host cell as efficiently as the wild-type HSV-1 viruses. In this study, we have investigated HSV-1 Us3 deletion mutant (R7041) and ICP4 and Us3 deletion mutant (d120) virus-induced apoptosis in human epithelial and monocytic cell lines.

The HSV-1-induced apoptosis in human epithelial cell line (HEp-2) was studied by Hoechst staining. The number of fragmented nuclei corresponded to the number of apoptotic cells. As expected, wild-type HSV-1 infection did not increase apoptosis in HEp-2 cells, but the amount of apoptosis was increased in R7041- and d120-infected cells at late time point of infection when compared with uninfected or wild type HSV-1-infected cells. The proportion of apoptotic cells was higher in HEp-2 cells infected with ICP4 and Us3 deletion virus than in HEp-2 cells infected with Us3 deletion virus, indicating that both ICP4 and Us3 participate together in blocking the HSV-1-induced apoptosis in human epithelial cells. The functional ICP4 is needed for the expression of β genes and therefore for all post α genes. Thus, in ICP4 deletion virus-infected cells just α genes are predominantly expressed (Roizman *et al.*, 2007). The HSV-1 apoptosis inhibitors encoded by the β and γ genes are therefore lacking.

In addition to HEp-2 cells, HSV-1 Us3 deletion viruses induced significant cell death in human monocytic cells (U937). The amount and type of cell death was studied with flow cytometer. Apoptotic and necrotic cells were differentiated with Annexin-V and propidium iodide double staining. HSV-1-induced cell death in U937 cells was mainly apoptotic, since the majority of dying cells were Annexin-positive but propidium iodide-negative. In R7041- and d120-infected U937 cells the proportion of apoptotic cells was significantly increased when compared with their rescue or parental virus-infected cells. These results were supported with the finding, that the relative proportion of viable cells in R7041- and d120-infected U937 cells was clearly decreased when compared with rescue or parental virus-infected cells. The amount of HSV-1-induced apoptosis was higher in U937 cells than in HEp-2 cells indicating that the HSV-1-induced apoptosis and the extent of apoptosis is cell type dependent. Also, it implies that cellular factors participate in the regulation of HSV-1-induced cell death.

6.1.2 Necroptosis

Necroptosis is recently described type of caspase-independent programmed cell death (Degterev *et al.*, 2005). So far, little is known about necroptosis in virus-infected cells. Recently, Upton *et al.* showed that murine cytomegalovirus infection induces RIP3-dependent necrosis and that the viral inhibitor of RIP activation (vIRA) interferes with necroptosis (Upton *et al.*, 2010). In addition, the RIP1-RIP3 complex has been shown to be induced during vaccinia virus infection (Cho *et al.*, 2009). Cho *et al.* suggested, that the RIP3 control programmed necrosis by initiating the pro-necrotic kinase cascade, which is necessary for the inflammatory response against virus infections (Cho *et al.*, 2009).

To study, if HSV-1-induced cell death is in part caused by necroptosis, we analyzed the proportion of viable cells in necroptosis inhibitor (Nec-1)-treated and untreated HSV-1-infected cells. The treatment with Nec-1, in other words the blocking of the necroptosis, clearly increased the amount of viable U937 cells in d120-infected cells when compared with untreated d120-infected cells. This indicates that the d120-induced cell death is partially caused by necroptosis. Thus, the d120-induced cell death is a combination of apoptosis and necroptosis. Necroptosis has been described as a back-up mechanism to kill virus-infected cells if apoptotic pathway is blocked and it may promote the release of intracellular factors, which may boost innate immune response (Declercq *et al.*, 2009). In the case of d120-infected U937, the apoptosis signaling was not blocked, since the majority of the dying cells were apoptotic. Moreover, necroptosis inhibitor treatment did not increase the viability of R7041-infected cells indicating that there was no necroptosis in R7041-infected cells. This may suggest that since HSV-1 virus with deletions both in ICP4 and Us3 induced necroptosis but HSV-1 virus with only Us3 deletion did not, ICP4 may contribute, in addition to blocking apoptosis, to blocking necroptosis in infected host cell. On the other hand, since the ICP4 and Us3 deletion virus induces a massive apoptosis in infected cells, necroptosis could be considered as a back-up system for cell death in these cells. Thus, virus with ICP4 and Us3 deletion first induces apoptosis, which in turn could induce necroptosis. This hypothesis can be further studied using caspase inhibitors, which block caspase-dependent apoptosis but not necroptosis in ICP4 and

Us3 deletion virus-infected cells. The amount of necroptosis can then be studied using necroptosis inhibitor and by comparing the proportion of cell viability between inhibitor-treated and untreated cells.

6.1.3 Mechanism of herpes simplex virus type 1-induced cell death

The HSV-1-induced apoptosis has been reported to be mediated via caspase-3, -7 and -9, but not via caspase-8 in human epithelial cells (Aubert et al., 2007; Kraft et al., 2006). In this study, the activity of caspase-3 and -8 was measured in HSV-1-infected U937 cells. Activity of caspase-3 was significantly increased in d120-infected U937 cells when compared with uninfected or parental virus-infected cells, whereas R7041 infection did not increase caspase-3 activation. Moreover, activity of caspase-8 was significantly increased in d120-infected cells. Since caspase-3 and -8 activities were not significantly increased in Us3 deletion virus-infected cells but they were increased in ICP4 and Us3 deletion virus-infected cells, it seems that functional ICP4 is needed for preventing the caspase-3 and -8 activities in HSV-1-infected U937 cells.

Although the activity of caspase-3 was significantly decreased with caspase-3 specific inhibitor treatment, the number of apoptotic cells was not decreased in d120-infected U937 cells treated with inhibitor. Based on this finding it could be suggested that d120-induced apoptosis in U937 cells is rather caspase-8- than caspase-3-mediated. Caspase-7 or -9 activities were not measured in this study and thus the roles of caspase-7 and -9 in d120-induced apoptosis can not be excluded.

6.2 Measles virus-induced cell death

6.2.1 Apoptosis

MV infection induces strong immunosuppression, which can lead to secondary infections and mortality. Lymphopenia during natural measles has been suggested to be one of the mechanisms contributing to immunosuppression. The number of lymphocytes is significantly decreased as a consequence of MV infection (Okada *et al.*, 2000). Since lymphopenia is also found in other virus infections with no immunosuppression, there must be other factors besides lymphopenia involved in MV-induced immunosuppression. Among others, apoptosis has been suggested to play role in MV-induced immunosuppression.

In this study, MV-induced cell death in human PBMCs was studied *in vitro*. First, the DNA fragmentation in MV-infected cells was studied with propidium iodide staining. MV induced DNA fragmentation in relative small population of unstimulated PBMCs when compared with uninfected cells. Since MV does not replicate productively in lymphocytes unless stimulated with mitogen (Vainionpää *et al.*, 1991), PBMCs were stimulated with PHA. The amount of DNA fragmentation was clearly increased in MV-infected PHA-stimulated PBMCs, when compared to uninfected PHA-stimulated PBMCs.

Lymphocytes have been reported to die due to apoptosis in MV-infected cell cultures (Auwaerter *et al.*, 1996; Fugier-Vivier *et al.*, 1997; Okada *et al.*, 2000). Thus, the cell population undergoing MV-induced apoptosis in PBMCs was studied. Although the number of MV-infected monocytes was relatively high, the amount of apoptotic monocytes was not increased in MV-infected PBMCs when compared with uninfected cells. On the contrary, the proportion of apoptotic lymphocytes was increased in MV-infected PBMCs when compared with uninfected cells at different time points after infection. However, the majority of the lymphocytes stayed uninfected. These findings implied that in PBMC cell culture MV infects monocytes, which then induce apoptosis in uninfected bystander lymphocytes. To confirm these results, monocytes were isolated from lymphocytes with plastic adherence and then infected with MV. MV-infected monocytes were co-cultivated with uninfected non-adherent lymphocytes and the proportion of apoptotic lymphocytes was studied. Co-cultivation with MV-infected monocytes increased the amount of apoptotic lymphocytes when compared with lymphocytes co-cultivated with monocytes without infection. Since the amount of MV nucleocapsid expressing lymphocytes was only 1%, lymphocytes co-cultivated with MV-infected monocytes did not get infected. Based on these results it can be concluded that MV-infected monocytes induce apoptosis in uninfected bystander lymphocytes.

Since MV replicates productively in U937 cells, MV-induced cell death was also studied in U937 cells. The proportion of viable U937 cells was significantly decreased in MV-infected U937 when compared with uninfected cells. The cell death was caused by apoptosis, since in MV-infected cells the amount of apoptotic cells was significantly increased. The necroptosis inhibitor treatment did not increase the viability of MV-infected cells, indicating that necroptosis is not associated with MV-induced cell death in U937 cells.

6.2.2 Mechanism of measles virus-induced cell death

To study the mechanisms of MV-induced apoptosis in uninfected bystander lymphocytes, the activation of caspase-1, -3, -6, and granzyme B in unstimulated and PHA-stmulated PBMCs were investigated. Granzyme B is a key effector molecule of cytotoxic lymphocytes and it promotes caspase-dependent or –independent cell death in response to virus infections, reviewed in Hoves *et al.* (2010). In this study, MV infection induced the activation of granzyme B, caspase-3 and -6 in PHA-stimulated PBMCs. Thus, these results suggest that in response to MV infection, granzyme B is activated, which triggers caspase-3 and -6-dependent pathway of apoptotic signaling. In unstimulated MV-infected PBMCs granzyme B activity was not clearly increased. However, the activity of caspase-3 was increased in these cells. This implies that apoptosis signaling in MV-infected PBMCs is triggered also by other mechanism.

The homeostasis of peripheral T cells is maintained by activation-induced cell death (AICD) (Xu & Shi, 2007). AICD can be induced by various antigens and it is needed in vigorous immune response. Death receptor Fas and its ligand FasL are the main mediators of AICD (Xu & Shi, 2007). Fas and TRAIL death receptors have been found on the surface of uninfected lymphocytes derived from measles patients (Okada *et al.*,

2000). In this study, almost all (98%) lymphocytes expressed Fas receptor on the cell surface at day 3 p.i. in MV-infected PBMCs. However, the number of apoptotic lymphocytes was limited (approximately 23% at day 3 p.i.). This suggests that the expression of FasL could be restricted and thus apoptosis would be induced only in a limited number of lymphocytes.

In addition to possible restriction of FasL expression, there could be intracellular factors, such as anti-apoptotic Bcl-2 family members, which block the MV-induced apoptosis in lymphocytes. In fact, the expression of anti-apoptotic Bcl-2 was increased in MV-infected PBMCs, suggesting that MV-induced apoptosis is at least in part inhibited with Bcl-2 protein. This finding differ from the results from Ito *et al.*, who showed that there is no change in Bcl-2 expression in MV-infected unstimulated PBMCs when compared with uninfected PBMCs (Ito *et al.*, 1997). The difference between the results can be explained by the different time course of the experiment. In this study, the Bcl-2 protein levels were up-regulated already 24 h p.i. and the levels started to decline after 48 h p.i. This indicates that in PBMCs there is a rapid anti-apoptotic response against MV infection.

To study the mechanism of MV-induced apoptosis in U937 cells, the activity of caspase-3 and -8 were measured. The activity of caspase-3 and -8 were significantly increased in MV-infected U937 when compared with uninfected cells. These findings suggest that both the intrinsic and extrinsic pathways of apoptosis are triggered in MV-infected U937 cells. To confirm this hypothesis, further experiments with caspase-specific inhibitors should be done. If caspase inhibitors block or decrease the amount of MV-induced apoptosis in U937 cells, the MV-induced apoptosis can be considered as caspase-dependent. Also, in the MV-infected U937 cells, there could be cellular anti-apoptotic factors, that block caspase-mediated signaling pathway. For example, the roles of Bcl-2 family members in MV-induced apoptosis should be examined in more details.

6.3 Cysteine cathepsins in herpes simplex virus type 1 and measles virus infection

Different stimuli, such as viruses, can trigger lysosomal membrane permeabilization (LMP) (Boya & Kroemer, 2008). In consequence, cathepsins are released from lysosomes into the cytosol and this may result in apoptosis (Colbert *et al.*, 2009; Yamashima & Oikawa, 2009). To study, if lysosomal cathepsins are involved in HSV-1- and MV-induced apoptosis in U937 cells, the cysteine cathepsin B, H, L and S activities were measured. First, the possible virus infection triggered LMP was studied in infected cells. The lysosomal membrane permeabilization was significantly increased in R7041- and d120-infected cells when compared with uninfected and rescue or parental virus-infected U937 cells, suggesting that lysosomal cathepsins were released into the cytosol. On the contrary, in MV infection, LMP was not increased when compared with uninfected cells. Currently, little is known about the role of LMP in virus-induced apoptosis. So far, LMP has been reported to be involved in

HIV-1- and parvovirus H1-induced apoptosis (Di Piazza *et al.*, 2007; Laforge *et al.*, 2007).

Although cathepsins degrade proteins optimally at acidic pH, they can function also in neutral pH outside the lysosome. The activities of released cathepsins in the cytosol were studied in HSV-1-infected U937 cells. In d120-infected cells the activity of cathepsin B was clearly increased when compared with uninfected or parental virus HSV-1 (KOS)-infected cells. Also, there was significant difference in cathepsin H activity between d120-infected and uninfected cells. Cathepsin B activity was also studied in epithelial cells, but no difference in cathepsin B activity was seen between d120- or R7041-infected and uninfected HEp-2 cells. This indicates that virus-induced activation of cathepsins is cell type-dependent.

Surprisingly, although MV infection did not increase LMP, it significantly increased cathepsin B, H, L and S activities in infected U937 cells. These divergent findings may be explained by the method used for cathepsin acitivity measurement. In this study, pericellular cathepsin activities were measured. To get more precise result from the cathepsin activities in the cytosol of apoptotic cell, an activity-based probe should be used as described by Pratt *et al.* (2009).

Since cathepsins are able to mediate apoptosis, the roles of cathepsins in virus-induced apoptosis were studied. Blocking cathepsin H or L activity with cathepsin specific inhibitor did not increase cell viability in d120-infected U937 cells. Instead, cathepsin B or S inhibitor treatment increased the amount of viable cells in d120-infected U937 cells. On the other hand, cathepsin B or S inhibitors did not decrease the amount of apoptosis. These findings may imply that the increased cell viability in cells treated with cathepsin B and S inhibitors is due to inhibition of d120-induced necroptosis, but not apoptosis.

6.4 Cystatins in herpes simplex virus type 1 and measles virus infection

Cystatins inhibit cysteine proteases, such as cathepsin B, H, L and S, and they have been reported to inhibit apoptosis induced by different stimuli. Thus, the roles of cystatin A, C or D in the inhibition of virus-induced cathepsin activity and virus-induced apoptosis were studied. Cystatin A, C or D treatment did not decrease cathepsin B, H, L or S activity or d120-induced apoptosis in U937 cells. However, in d120- and wild type HSV-1-infected HEp-2 cells cathepsin B activity was significantly decreased with cystatin C and D treatment. In addition, cystatin D treatment decreased cathepsin B activity in R7041-infected HEp-2 cells. Also, the amount of apoptotic cells was decreased in d120-infected HEp-2 cells treated with cystatin D. These results indicate that cystatins inhibit both HSV-1-induced cathepsin activity and virus-induced apoptosis, but that the inhibition is cell type-dependent. In this study, cells were treated with cystatin A, C or D. The diverse results between U937 and HEp-2 cells could be explained by the different endogenous expression of cystatins. The mRNA and protein expression of cystatin A, C and D should be studied in more details in U937 and HEp-2 cells. Also, the uptake of cystatins into the infected cells should be further studied.

In MV-infected U937 cells, cystatin C and D decreased the activity of cathepsin B, H and S. However, the treatment with cystatins did not decrease the amount of apoptotic cells in MV-infected U937 cells. This may imply, that MV-induced apoptosis is not mediated via cathepsins in spite of increased cathepsin activities in MV-infected U937 cells. On the other hand, because cystatins blocked the cathepsin activities only to some extent, the cystatin concentration used was probably not sufficient for apoptosis blocking. In any case, the mechanism of MV-induced apoptosis in U937 cells should be studied in more details. To study, if MV-induced apoptosis is cathepsin-mediated, the amount of apoptosis in cathepsin inhibitor-treated and untreated cells should be compaired.

Since cathepsins promote several types of virus infections in host cells (Vasiljeva *et al.*, 2007), inhibition of cathepsin activity by cystatins could lead to decrease in virus production. As described above, cathepsin B activity was decreased in HSV-1-infected cells treated with cystatin C and/or D. Thus, the inhibition of virus production by cystatin C and D treatment was studied in HEp-2 cells. Because of ICP4 mutation, d120 replicates productively only in Vero E5 cells expressing ICP4. Thus, the virus replication in R7041- and its parental virus HSV-1 (F)-infected HEp-2 cells was studied. The virus production and the amount of virus release were significantly decreased in cystatin C and D treated R7041- and HSV-1 (F)-infected HEp-2 cells. Also, cystatin A treatment decreased the relase of HSV-1 (F). Based on these results, cystatin C and D interfere with HSV-1 replication in HEp-2 cells. The inhibition of cathepsin B activity by cystatins could in part explain these results. Cathepsin B has been shown to mediate the cleavage of the viral origin binding protein (OBP) to yield OBPC-1, which is needed for the DNA replication of HSV-1 (Link *et al.*, 2007). In addition, HSV-1 infection has been shown to increase cysteine cathepsin expression (Szpara *et al.*, 2010), suggesting that cathepsins contribute to HSV-1 infection. Since cystatin treatment decreased both the amount of HSV-1-induced apoptosis and viral replication in HSV-1-infected cells, it is possible that the initiatiton of apoptosis is beneficial for the replication of HSV-1 in infected cells as discussed in Blaho (2004).

6.5 Modulation of innate immune response by herpes simplex virus type 1

Toll-like receptors (TLRs) have an important role in innate immune response against several virus infections. So far, TLR2, 3 and 9 have been shown to be involved in HSV-1 recognition in different cell types (Krug *et al.*, 2004a; Krug *et al.*, 2004b; Kurt-Jones *et al.*, 2004; Sato *et al.*, 2006; Zhang *et al.*, 2007). Recognition of HSV-1 by TLRs leads to activation of several transcriptional factors. To interfere with the innate response of the host, HSV-1 has developed various molecular strategies (Melchjorsen *et al.*, 2009; Paladino & Mossman, 2009; Tsitoura & Epstein, 2010). For example, the late protein kinase Us3 of HSV-1 contributes to the post-translational modification of IFN receptors (Liang & Roizman, 2008), the immediate early ICP0 protein inhibit the induction of IFN-stimulated genes (Eidson *et al.*, 2002; Lin *et al.*, 2004; Melroe *et al.*,

2004; Melroe *et al.*, 2007), and the the tegument protein *vhs* blocks the production of IFNs (Cotter *et al.*, 2010).

In this study, the innate response in U937 cells against HSV-1 infection was studied. ICP4 and Us3 deletion virus d120 and Us3 deletion virus R7041 increased both TLR3 mRNA and protein expression in infected U937 cells when compared with parental or rescue virus-infected or uninfected cells. Moreover, d120 and R7041 infections induced activation of IRF-3 and increased expression of type I IFNs in U937 cells. Also, the expression of type I inducible MxA protein was significantly increased in d120- and R7041-infected U937 indicating that the functional type I IFN was produced in these cells. Since HSV-1 viruses with deletion in ICP4 and Us3 or in Us3 induced innate immune response in U937 cells, it can be suggested that HSV-1 induces innate immune response in U937 cells, but the response is interfered with Us3 and/or ICP4 of the virus. Thus, Us3 and/or ICP4 might act as inhibitor of TLR3-mediated signaling. So far, TLR3 pathway inhibitor has not been associated with HSV-1. Since TLR3 has been shown to directly trigger caspase-dependent apoptosis in human cancer cells (Salaun *et al.*, 2006), the d120- and R7041-induced apoptosis in U937 cells could be explained, at least in part, by the triggering of TLR3- and caspase-mediated signaling pathway in d120- and R7041-infected cells.

7 CONCLUSIONS

In this study, the type and mechanisms of HSV-1- and MV-induced cell death were studied. HSV-1 ICP4 and Us3 (d120) and Us3 deletion (R7041) viruses induced apoptosis in human epithelial and monocytic cells. In addition, d120 induced necroptosis in monocytic cells. To our knowledge, necroptosis has not been reported before in HSV-1 infections. Caspase-3 and -8 activities were increased in d120-infected monocytic cells. These findings could imply that ICP4 and Us3 of HSV-1 are together involved in regulation of caspase-3 and -8 activities in HSV-1-infected cells. Also, apoptosis induced by d120 could be caspase-mediated which remains to be elucidated.

MV induced apoptosis in human monocytic cells and in human peripheral blood mononuclear cells. MV infected monocytes, which induced apoptosis in uninfected bystander T lymphocytes. This may imply that monocytes interact with uninfected T lymphocytes via cell surface molecules and induce apoptosis by indirect mechanism. Apoptotic dying of the uninfected lymphocytes may contribute to the pathogenesis of MV-induced immunosuppression. The activities of caspase-1, -3, -6 and -8 were increased in MV-infected human peripheral blood mononuclear cells and in monocytic cells implying that caspases may participate in MV-induced apoptosis. MV induced the expression of death receptor Fas on the cell surface of lymphocytes. This could mean that MV induces apoptosis via extrinsic pathway.

The cathepsin activities were increased in HSV-1 and MV-infected monocytic cells suggesting that cathepsins may be involved in HSV-1- and MV-induced apoptosis. The inhibition of cathepsin activities with specific inhibitors or with cystatins increased the cell viability or decreased the amount of apoptosis in HSV-1-infected monocytic and epithelial cells. This would imply that cathepsins are involved in HSV-1-induced apoptosis or necroptosis.

The innate immune response in HSV-1-infected host cell was also investigated. The mRNA expression of TLR3 and type I IFNs were increased in d120- and R7041-infected monocytic cells. Also IRF-3 was activated in d120- and R7041-infected cells, suggesting that the downstream factors of the TLR3 signaling pathway were stimulated. Also, type I IFN inducible MxA protein level was increased indicating that functional type IFN was produced. Based on these results, ICP4 and Us3 genes of HSV-1 are involved in the control of TLR3 response in monocytic cells.

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