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ACTIVATION OF INNATE IMMUNE RESPONSE IN HUMAN MACROPHAGES BY HERPES SIMPLEX VIRUS-1 AND CRYSTALLIZED MONOSODIUM URATE

Juho Miettinen

ACADEMIC DISSERTATION

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“Consider a man riding a bicycle.
Whoever he is, we can say three things about him.
We know he got on the bicycle and started to move.
We know that at some point he will stop and get off.
Most important of all, we know that if at any point between
the beginning and the end of his journey he stops moving
and does not get off the bicycle he will fall off it.
That is a metaphor for the journey through life of any living thing,
and I think of any society of living things.”

William Golding

TABLE OF CONTENTS

ABBREVIATIONS

2-DE	two-dimensional gel electrophoresis
CCL	C-C motif chemokine
DAMP	danger-associated molecular pattern
ER	endoplasmic reticulum
EV	extracellular vesicle
HSP	heat shock protein
HSV	herpes simplex virus
ICP27	infected cell protein 27
IFIT	interferon-induced protein with tetratricopeptide repeats
IFN	interferon
ISG	interferon-stimulated gene
iTRAQ	isobaric tag for relative and absolute quantitation
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LPS	lipopolysaccharide
MDA5	melanoma differentiation-associated gene 5
MS	mass spectrometry
MSU	monosodium urate
MxA	myxovirus resistance protein A
NES	nuclear export sequence
NLR	NOD-like receptor
NLS	nuclear localization sequence
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene 1
RLR	RIG-I-like receptor
TLR	toll-like receptor
Viperin	virus inhibitory protein, endoplasmic reticulum-associated, interferon inducible

LIST OF ORIGINAL PUBLICATIONS

I **Miettinen J.J.**, Matikainen S., Nyman T.A.
Global secretome characterization of herpes simplex virus 1-infected human primary macrophages. *Journal of Virology*, 86, 23, 12770-12778 (2012)

- JM performed the experimental work, except for the Luminex assays, and drafted the manuscript.

II Välimäki E., **Miettinen J.J.**, Lietzén N., Matikainen S., Nyman T.A.
Monosodium urate activates Src/Pyk2/PI3 kinase and cathepsin dependent unconventional protein secretion from human primary macrophages. *Molecular & Cellular Proteomics*, 12, 3, 749-763 (2013)

- JM performed LC-MS/MS-based protein identifications from two-dimensional gels and reported these results in the manuscript. JM was also involved in the enrichment of extracellular vesicles (EV) from cell supernatants and in identifying EV-secreted proteins from these enriched fractions using Western blotting.

III **Miettinen J.J.,** Öhman T., Nygårdas M., Hukkanen V., Paludan S.R., Nyman T.A., Matikainen S.
Regulation of interferon response and apoptosis by HSV-1 protein ICP27 in human macrophages.
(Manuscript)

- JM performed most of the experimental work, except for the qRT-PCR of the HSV-1 genes. The iTRAQ-experiments JM did together with Öhman T. The manuscript was drafted by JM.

ABSTRACT

Innate immunity provides the first line of defence against invading pathogens, and can also be activated by endogenous danger signals released upon tissue damage or injury. Macrophages play an important role in innate immunity. They perform the immune surveillance of their immediate surroundings seeking out tissue damage and/or invading pathogens via pattern recognition receptors. Macrophages are activated upon detection of an invading pathogen or tissue damage, leading to the expression, and secretion of the proteins required for an efficient innate immune response.

Herpes simplex virus-1 (HSV-1) is a common human pathogen. It is a master of evading the host immune response leading to its ability to cause a life-long infection in its host. In most cases, HSV-1 does not cause any symptoms in the host, or causes nothing more than an occasional painful blister in the orofacial region. However, in certain cases when the host immune system is compromised, it can cause severe, even lethal, infections. Uric acid can act as an endogenous danger signal that cells can produce and release into the extracellular space after encountering a stress, including that caused by certain viral infections. A high amount of uric acid in cells can also be attributed to certain dietary habits. In the extracellular space if present at high enough concentrations and in the presence of sodium, uric acid can become crystallized into monosodium urate (MSU). MSU is the causative of gouty inflammation in the joints and is known to be phagocytosed by macrophages.

In this work, human monocyte-derived macrophages infected with HSV-1, or stimulated with MSU were studied using several proteomic methods combined with functional assays. The main goals were: to characterize the protein secretion pathways and the proteins being secreted from macrophages upon (1) HSV-1 infection, and (2) MSU stimulation; and (3) to characterize the functional mechanisms of HSV-1 infected cell protein 27 (ICP27) in the inhibition of the interferon (IFN) response and also inhibition of apoptosis during HSV-1 infection. The results revealed that robust extracellular vesicle-mediated unconventional protein secretion is induced by IFN-β priming in HSV-1-infected, and by MSU stimulation in Toll-like receptor 4 ligand, bacterial cell wall component lipopolysaccharide-primed human macrophages. The secreted proteins included endogenous danger signal proteins, and interferon-stimulated gene-encoded proteins. In addition, HSV-1 ICP27 protein requires both the nuclear localization sequence (NLS) and nuclear export sequence (NES) in order to inhibit the IFN response, whereas the NES, but not NLS, is required for the inhibition of apoptosis. The results also demonstrate that pattern recognition receptors RIG-I and/or MDA5 are involved in the HSV-1-induced activation of apoptosis.

To conclude, this thesis provides new information on the innate immune response induced by HSV-1 infection, and MSU stimulation, and also on the evasion mechanisms deployed by HSV-1 to avoid the innate immune response in human macrophages. These new results presented in this thesis may be exploited in several ways; in the development of new therapies against viral infections, and in the treatment of autoinflammatory diseases.

I REVIEW OF THE LITERATURE

1. INNATE IMMUNITY

The immune system of vertebrates can be divided into two separate, although interconnected, arms: the innate and adaptive immunity. Innate immunity provides the first line of defence against invading pathogens, and responds to endogenous danger signals released from injured cells.

The innate immune system consists of four different defence barriers: cellular, physical, humoral, and chemical. The cellular part of the innate immune system is mediated by the white blood cells, leucocytes, and it represents the most important defence mechanism in the innate immune system. The activation of leucocytes leads to the secretion of a variety of different cytokines, chemokines, and other proteins that are crucial for the regulation of the innate immune response. Even before the cellular part of the innate immune system is triggered, the intact epithelial cell lining provides a physical barrier that prevents invading pathogens from entering freely into the host organism. In vertebrates, it covers the external surfaces and lines the internal cavities, such as gastrointestinal, respiratory, and genital tracts. In the oral cavity, the humoral protection is achieved partly through the salivary glands which produce mucus that can trap a pathogen and can then be swallowed to the stomach. In the stomach, the acidic pH is a kind of chemical protection, and also intestinal enzymes may well destroy the pathogen. In addition to their barrier function, the epithelial cells of the oral cavity take part in the humoral innate immune defence by secreting antimicrobial peptides and lipids into the surrounding mucus (Dawson et al. 2013, Diamond et al. 2008). An important part of the humoral innate immunity is the complement system. The complement molecules in cooperation with the cellular part of the innate immune system participate in the identification, destruction, and clearance of pathogens and in the removal of infected and damaged cells at the site of infection (Medzhitov 2007).

In vertebrates, the adaptive immune system is mediated by B- and T-lymphocytes and is fairly slow, taking days to be effective after its first encounter with the pathogen. In contrast the innate immune response can be activated already within minutes from this first encounter which allows the innate immune response to keep the infection under control while the adaptive immune response is developing. The innate immune response is also necessary for the appropriate type of adaptive immune response to be mounted, and it provides an often life-long immunological memory. In the case of re-exposure, the existence of an immunological memory means thus a rapid and efficient immune response is available against the pathogen. Defects in the innate immune system can result in autoinflammatory diseases, severe inflammatory conditions, and increased susceptibility to infections, highlighting its importance for the host defence (Hoffman et al. 2001, Netea et al. 2012, Picard et al. 2003).

1.1 Cells of the innate immune system

Leucocytes, mainly of myeloid origin, form the most important part of innate immunity. They include monocytes, monocyte-derived macrophages and dendritic cells (DC), tissue macrophages, mast cells, and granulocytes (neutrophils, eosinophils, basophils). Innate lymphocytes, natural-killer (NK) cells and a subset of γδT-cells of lymphoid origin can also be considered functionally as belonging to the innate immune system (Gasteiger, Rudensky 2014, Medzhitov 2007, Matzinger 2002).

Phagocytes are cells that engulf and enclose foreign material, microbes, or dying cells into phagosomes. Thus, they function in host defence and have an important role in the maintenance of homeostasis. These cells are mainly monocytes, macrophages, DCs, and neutrophils. In addition to being phagocytes, macrophages and DCs also act as antigen presenting cells (APC). APCs present antigens to T-cells through the major histocompatibility complex (MHC) class I and II molecules,

ultimately leading to the activation of the adaptive immune system (Iwasaki, Medzhitov 2010, Le Bon et al. 2003).

1.1.1 Macrophages

Macrophages, the first innate immune cells to be identified, were discovered by Eliah Metchnikoff in 1866 (Chang 2009). Together with monocytes, they have been evolutionarily traced to the ancient invertebrate mononuclear phagocyte system (MPS) (Ottaviani, Franceschi 1997). The MPS has been defined as a family of cells of the innate immunity system derived from haematopoietic progenitor cells under the influence of specific growth factors (Hume 2012).

Macrophages are highly heterogeneous cells that can rapidly adjust their function in response to signals in their local microenvironment (Murray, Wynn 2011). They achieve this response by relying on various surface and secreted molecules, which monitor and respond to changes in their environment (Murray, Wynn 2011). Macrophages can be divided into different subpopulations based on their tissue localization (Davies et al. 2013, Sabin et al. 1925). If needed, circulating monocytes residing in the blood can migrate to sites of infection or tissue damage, leading to their differentiation into monocyte-derived macrophages. A wide range of tissue-resident macrophages are found in various tissues in which they perform their specific functions (Davies et al. 2013) e.g. the microglia in the central nervous system, the Langerhans cells in the skin, the Kupffer cells in the spleen, and the osteoclasts in bone.

Macrophages have long been considered to originate solely from the lineage-committed bone marrow precursors, which develop into monocytes and further into macrophages (Gordon, Taylor 2005). Recently this model has been supplemented with a new one which includes at least three lineages of macrophages (Hoeffel et al. 2012, Schulz et al. 2012, Wynn et al. 2013). In the mouse, during the first days of development, the embryonic yolk sack is the sole producer of macrophage progenitor cells which then give rise to many of the tissue-specific macrophages. Then, during embryonic development, the fetal liver takes over as the producer of macrophage progenitor cells and these give rise to some of the Langerhans cells. In the adult, the haematopoietic cells of the bone marrow are the source for the circulating blood monocytes and macrophages (Schulz et al. 2012, Wynn et al. 2013).

Macrophages have multiple roles in different biological processes such as development, homeostasis, tissue repair, and immunity (Murray, Wynn 2011, Wynn et al. 2013). They perform immune surveillance of their immediate surroundings for tissue damage and/or invading pathogens (Murray, Wynn 2011). Macrophages are activated upon detection of tissue damage or an invading pathogen with the activation occurring after the detection of triggering signals by recognition receptors or phagocytosis. The activation of macrophages leads to the secretion of a wide array of proteins including chemokines and cytokines, and other defence factors such as radical oxygen species (ROS) (Mosser, Edwards 2008). Macrophages can exist in a wide spectrum of activated phenotypes depending on the type of activating stimulus (Mosser, Edwards 2008).

1.2 Activation signals and receptors of the innate immune system

Activation of the innate immune response requires the detection of extrinsic or endogenous signals by the specific receptors of the innate immune system. The pathogen-derived extrinsic signals are referred to as pathogen-associated molecular patterns (PAMP) (Janeway 1989), and the endogenous signals as damage-associated molecular patterns or danger-associated molecular patterns (DAMP) (Matzinger 1994), and their receptors as pattern recognition receptors (PRR). Ligand binding to PRRs results in the activation of signalling cascades and leads to the production of cytokines, chemokines, antimicrobial proteins, or proteins involved in PRR modulation (Takeuchi, Akira 2010).

1.2.1 Pathogen- and damage-associated molecular patterns (PAMPs and DAMPs)

PAMPs are conserved and invariant structural components or metabolic products of viruses, bacteria, fungi, or protozoa. In the so-called self *vs* non-self (SNS) hypothesis, Janeway proposed that cellular receptors are able to detect PAMPs that are not expressed or found under normal conditions in the animal kingdom, which leads to the activation of the innate immune response (Janeway 1989, Medzhitov 2007). PAMPs represent essential molecules for microbes, and therefore are structurally conserved. They can be microbial nucleic acids, lipoproteins, glycoproteins, or other microbial membrane components such as lipopolysaccharides (LPS). When considering viral infections, the most typical viral PAMPs are their nucleic acids, DNA and RNA.

The danger hypothesis described by Polly Matzinger in 1994 states that in addition to the recognition of extrinsic PAMPs, the innate immune cells can recognize signs of tissue distress. This is mediated by endogenous molecules that are normally found inside cells but which can be released into the extracellular space following tissue damage or injury. The term "DAMP" was first coined by Seong and Matzinger in 2004 and it refers to both extrinsic PAMPs and the endogenous danger signal molecules (Seong, Matzinger 2004). Later it has been widely used in referring to endogenous danger signal molecules separately from the PAMPs. Thus, endogenous DAMPs are now considered as those molecules that are either actively secreted by activated inflammatory cells (Matzinger 1994, Matzinger 2002) or passively released from damaged tissue (Kono, Rock 2008, Lotze et al. 2007) to the extracellular space from cells in response to stress or injury (Table 1). They can be proteins (e.g. annexins, galectins, heat shock proteins (HSP), S100 proteins, high-mobility group box 1 protein (HMGB1)), nucleotides (e.g. adenosine), nucleotide metabolites (e.g. uric acid), or lipids (e.g. cholesterol crystals).

Table 1. Secreted endogenous danger-associated molecular pattern (DAMP) molecules

	Reference
Endogenous Proteins	
Annexins	(Gerke et al. 2005)
Galectins	(Sato et al. 2009)
Heat shock proteins	(Henderson, Henderson 2009)
HMGB1	(Lotze, Tracey 2005)
S100 proteins	(Foell et al. 2007)
Thioredoxin superfamily members	(Henderson, Henderson 2009)
Other Endogenous Molecules	
Nucleotides (e.g. adenosine)	(Bours et al. 2006)
Nucleic acids	(Barrat et al. 2005, Jounai et al. 2013)
Heparan sulphate	(Johnson et al. 2002)
Uric acid	(Shi et al. 2003)
Cholesterol crystals	(Rajamäki et al. 2010)

HMGB1, high-mobility group box 1 protein

Endogenous DAMPs signal to nearby cells about the potential danger leading to the emergence of appropriate defensive and protective measures. These may have proinflammatory functions (e.g. HMGB1) (Scaffidi et al. 2002) or in some cases anti-inflammatory effects (e.g. adenosine) (Bours et al. 2006). Some endogenous DAMPs can also act as chemokines (e.g. galectin-3) (Sano et al. 2000) by attracting immune cells to sites of stress or injury. The DAMPs that have a distinct intracellular and a separate chemotactic extracellular function form a subgroup called alarmins that include a variety of proteins (Bianchi 2007, Yang, Oppenheim 2004, Yang et al. 2013). These molecules promote not only the migration and recruitment of diverse types of cells via multiple mechanisms but also they can induce cell migration directly by interacting with their cognate receptors (Gi-coupled protein receptor, receptor for advanced glycation end products), or indirectly by stimulating production of chemokines, growth factors, and adhesion molecules by

activating the corresponding PRRs (Yang et al. 2013). At present, endogenous DAMPs associated with viral infections have been poorly characterized. There are indications that DAMPs, in addition to PAMPs, may be involved in the activation of antiviral responses in infected cells (Chew et al. 2009).

The secretion of endogenous danger signals can also occur in metabolic disorders leading to autoinflammatory diseases such as gout. Gout is considered as inflammatory arthritis triggered by crystallized uric acid, monosodium urate (MSU) (Rock et al. 2013). Under normal physiological conditions, extracellular uric acid levels stay well below a critical concentration and in solution i.e. the acid will not crystallize into MSU. A variety of dietary and hereditary factors can increase the levels of uric acid in some individuals and lead to the development of gout (Rock et al. 2013). Uric acid can also function as a DAMP since it is released from injured cells (Shi et al. 2003). Elevated levels of uric acid in the host cells have also been reported to occur in several viral infections (Boyes et al. 1989, Duhalde-Vega, Retegui 2011, Endo et al. 2008).

1.2.2 Pattern recognition receptors (PRRs)

PRRs are a diverse group of receptor proteins involved in the recognition of PAMPs and DAMPs (Adib-Conquy, Cavaillon 2007). PRRs can be divided into two major groups based on their cellular localization: membrane-bound receptors and cytosolic receptors. The best described group of PRRs are the membrane bound Toll-like receptors (TLR) (Kawai, Akira 2011). These receptors are localized either to the plasma membrane where they recognize extracellular ligands, or to the membranes of intracellular endosomal compartments. In addition to TLRs, there are also scavenger receptors which are membrane bound PRRs (Canton et al. 2013). The cytosolic receptors can be divided into further subclasses based on their structural similarities: AIM2-like receptors (ALR), NOD-like receptors (NLR), and RIG-I-like receptors (RLR). PRRs can also be divided into two groups based on their function: PRRs involved in pathogen binding and phagocytosis (e.g. macrophage receptor with collagenous structure, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin) known as scavenger receptors (Canton et al. 2013), and signalling PRRs (Hansen et al. 2011). Upon recognition of their ligands, signalling PRRs (e.g. ALRs, NLRs, RLRs, TLRs) (Figure 1) stimulate the signal transduction pathways that lead to gene expression activated by several key transcription factors. These transcription factors include nuclear factor (NF)-κB, activator protein 1, interferon regulatory factors (IRFs), and nuclear factor of activated T-cells (NFAT) (Lee, Kim 2007). Many genes are switched on by these transcription factors e.g. cytokines and chemokines, antimicrobial effectors, as well as genes involved in the induction of adaptive immunity. In particular, TLRs and RLRs are crucial in the production of type I IFNs and proinflammatory cytokines in a cell type-specific manner (Rasmussen et al. 2009, Takeuchi, Akira 2008).

1.2.3 Inflammasomes

Inflammasomes are protein complexes that are involved in the precursor processing of the IL-1-family of proinflammatory cytokines into their biologically active forms, and in inflammatory cell death (Gram et al. 2012). The canonical inflammasomes include three core components, the sensor receptor, adaptor apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1. The non-canonical inflammasomes differ in the type of associated caspases. In humans, only one non-canonical caspase-8 inflammasome has been described (Gringhuis et al. 2012), whereas in mice also non-canonical caspase-11 inflammasomes have been reported (Broz, Monack 2013). After binding of its ligand, such as PAMPs and DAMPs, the receptor interacts with the ASC protein. In the canonical inflammasome, the ASC protein also binds pro-caspase-1 and functions as a bridge between the receptor and the pro-caspase-1. The inflammasome complex formation brings multiple pro-caspase-1 proteins into close proximity and leads to their autocatalytic cleavage resulting in the

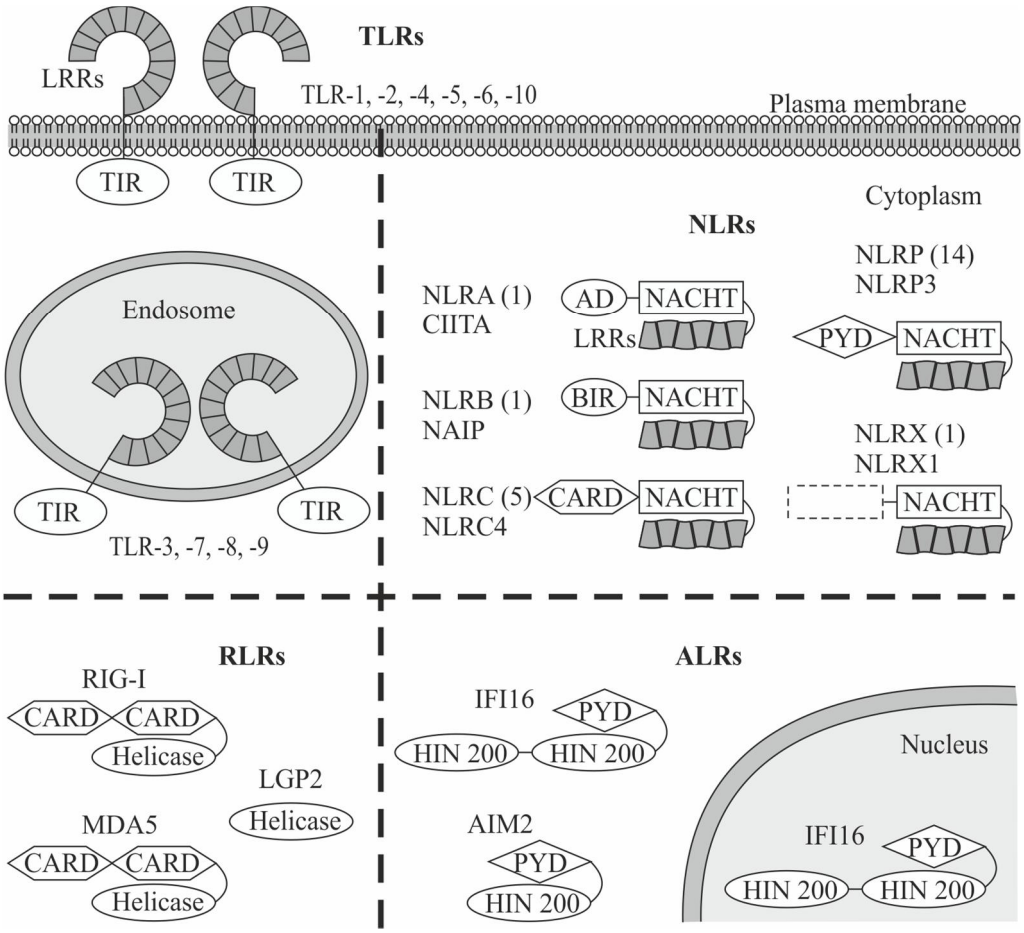

Figure 1. Signalling pattern recognition receptors and their domain structures (Modified from Gram et al. 2012). There are four subclasses of signaling PRRs: Toll-like receptors (TLR), NOD-like receptors (NLR), AIM2-like receptors (ALR), and RIG-I-like receptors (RLR) which sense microbial products and aberrant self, and activate transcriptional mechanisms that lead to phagocytosis, cellular activation and the release of cytokines, chemokines, and growth factors. The membrane bound TLRs are either localized on the plasma membrane or within the membrane structures of intracellular endosomal compartments. They recognize extracellular PAMPs such as LPS by TLR4, or endosomal PAMPs such as viral RNA or DNA by TLR3 and TLR9. Cytosolic NLRs can recognize PAMPs and DAMPs such as MSU by NLRP3. RLRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) recognize cytosolic exogenous RNAs, and LGP2 functions as a regulator of RIG-I and MDA5. ALRs AIM2 and IFI16 recognize cytosolic DNA, and can also recognize exogenous DNA in the nucleus. LRR, leucine-rich repeat; TIR, Toll/interleukin 1 receptor homology domain; AD, acidic activation domain; PYD, pyrin domain; BIR, baculovirus IAP repeat domain; CARD, caspase activation and recruitment domain.

activation of caspase-1. Active caspase-1 further cleaves pro-forms of interleukins 1β (IL-1β) and IL-18 into their mature proinflammatory forms that can then be secreted from the cell (Martinon et al. 2002). Active caspase-1 can also induce a form of inflammatory cell death known as pyroptosis (Latz et al. 2013).

There are several different types of inflammasomes characterized by the different receptors involved in the protein complex (Table 2 on page 6) (Latz et al. 2013). Each receptor has its specific ligands that can activate the inflammasome. The NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome is the best characterized of all currently known inflammasomes. Several DNA and RNA viruses are known to activate the NLRP3 and other inflammasomes. The DNA viruses that activate inflammasomes include the alphaherpesviruses varicella zoster virus (VZV) (Nour et al. 2011) and HSV-1 (Johnson et al. 2013, Muruve et al. 2008), adenoviruses (Barlan et al. 2011, Muruve et al. 2008), and myxoma virus (Rahman, McFadden 2011). The inflammasome activating RNA viruses include influenza A virus (Kanneganti et al. 2006), encephalomyocarditis virus, and vesicular stomatitis virus (Rajan et al. 2011). The molecular

mechanisms that are involved in inflammasome activation in response to viral infection are currently poorly understood.

Table 2. Different types of inflammasomes.

Receptor	Ligand	Reference
NLRP1	Anthrax lethal toxin (LT),	(Liao, Mogridge 2009)
	cytosolic muramyl dipeptide	(Martinon et al. 2002)
NLRP2	ATP	(Minkiewicz et al. 2013)
NLRP3	multiple:	
	PAMPs (e.g. viruses)	(Kanneganti et al. 2006, Nour et al. 2011, Rajan et al. 2011)
	DAMPs (e.g. MSU, ATP)	(Mariathasan et al. 2006, Martinon et al. 2006)
NLRP6	Unknown	(Grenier et al. 2002)
NLRP7	Bacterial lipopeptides	(Khare et al. 2012)
NLRP12	Unknown	(Vladimer et al. 2012, Wang et al. 2002)
NLRC4	Flagellin	(Franchi et al. 2006, Miao et al. 2006)
	Components of the T3SS	(Miao et al. 2010)
AIM-2	dsDNA	(Bürckstümmer et al. 2009, Fernandes-Alnemri et al. 2009, Hornung et al. 2009, Rathinam et al. 2010)
IFI-16	viral dsDNA	(Johnson et al. 2013, Kerur et al. 2011)
RIG-I	dsRNA	(Poeck et al. 2010)

ATP, adenosine triphosphate; PAMP, pathogen-associated molecular pattern; DAMP, damage-associated molecular pattern; MSU, monosodium urate; ds, double-stranded

1.3 Protein secretion by immune cells

Upon activation, immune cells secrete a variety of proteins such as different cytokines and chemokines, which are required for mounting an efficient immune response. There are several different ways in which eukaryotic cells in general can secrete proteins. Upon secretion, most proteins go through the endoplasmic reticulum (ER)-Golgi complex (Figure 2), which is referred to as the conventional or classical protein secretion pathway. Proteins secreted through this pathway contain an amino (N)-terminal or internal signal peptide. The signal peptide is recognized by the signal recognition particle that guides the protein into the ER lumen. From the ER, the protein is transferred to the Golgi apparatus via COPII-coated vesicles, and from the Golgi it is incorporated into a vesicle that fuses with the plasma membrane releasing its cargo into the extracellular space (Dancourt, Barlowe 2010). Conventional protein secretion can be either constitutive or regulated. In constitutive secretion, the protein is secreted by direct vesicle fusion with the plasma membrane at a constant rate in the normal cell state. Depending on the protein, the rate of expression may stay the same regardless of the cellular environment, or it may change in response to the appearance of some kind of cell stimulus (Stow, Murray 2013). In regulated conventional protein secretion the proteins are stored in vesicles within the cytoplasm and released from the cell after the fusion of the vesicles with the plasma membrane upon cell stimulation (Burgoyne, Morgan 2003).

Unconventional protein secretion does not involve the classical ER-Golgi complex pathway. This process is mostly used for the secretion of proteins lacking the signal peptide. The different unconventional protein secretion pathways can be divided into two groups based on the presence or absence of vesicular intermediates. It has also been postulated that the unconventional protein secretion pathways could be classified further into four different types based on their more detailed secretion mechanisms (Rabouille et al. 2012) (Figure 2). The non-vesicular pathways include types I and II unconventional protein secretion. Type I unconventional protein secretion is characterized by self-sustained protein translocation by lipid-induced oligomerization and membrane insertion. The secretion of fibroblast growth factor 2 is a prime example of this type of

unconventional protein secretion (Schäfer et al. 2004). Type II is characterized by ABC transporter-based protein secretion and is exemplified by the secretion of yeast mating type α protein (McGrath, Varshavsky 1989).

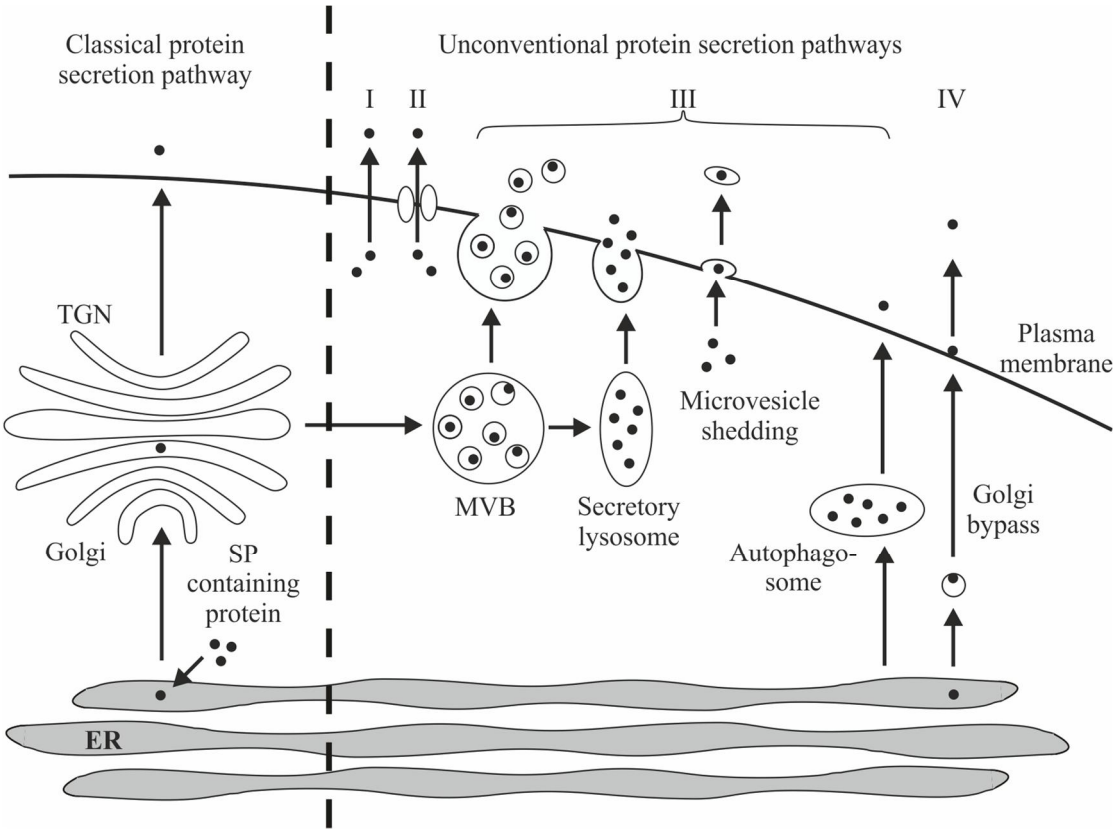

Figure 2. Protein secretion pathways in eukaryotic cells (Modified from Ding et al. 2012). Eukaryotic cells can secrete proteins either through classical (conventional) secretion pathways, or through unconventional secretion pathways. The classical secretion pathway requires that the secreted protein has a signal peptide (SP) that targets the protein to the endoplasmic reticulum (ER) and from there to the trans-golgi network (TGN) leading to its secretion. Proteins that lack the SP can be secreted through the unconventional secretion pathways, which can be divided into 4 different types. Types I and II of unconventional protein secretion are independent of intermediate vesicles. In type I secretion, the secreted protein translocates through the plasma membrane *via* lipid-induced oligomerization and membrane insertion. In type II, the protein is secreted by membrane-bound transporter proteins. In contrast to types I and II, types III and IV are intermediate vesicle-dependent release forms. Type III secretion can occur through at least four different routes. Smaller secretory vesicles can be located within larger so-called multivesicular bodies (MVBs) that can fuse with the plasma membrane and release their content into the extracellular space. Secretory lysosomes can fuse with the plasma membrane and release their cargo proteins for secretion. In microvesicle shedding, secreted proteins are packaged into vesicles at the plasma membrane and released out of the cell within the microvesicle. In autophagosome-mediated secretion, proteins within the autophagosome are transported to the plasma membrane in phagosome-like vesicles that fuse with the plasma membrane and release their content into the extracellular space. In type IV unconventional protein secretion, referred to as the Golgi bypass, proteins in the ER can be transported in ER-derived vesicles directly to the plasma membrane and released into the extracellular space.

Several proteins leave the cell via unconventional protein secretion through the vesicular pathway e.g. the type III unconventional protein secretion, which is characterized by intracellular vesicle mediated secretion, and type IV describing the secretion of plasma-membrane-resident proteins through Golgi bypass (Rabouille et al. 2012). The type III pathway is especially suited for unconventional secretion of soluble cytoplasmic proteins and there are several different ways in which a soluble cytoplasmic protein can be secreted from the cell via this pathway. Proteins can be transferred to a secretory lysosome as has been described for IL-1β, and released into the extracellular space by fusion of the lysosome with the plasma membrane (Andrei et al. 1999, Andrei

et al. 2004). Autophagosomes may also be able to function in vesicle-mediated protein secretion as has been demonstrated with the unconventional secretion of IL-1β (Dupont et al. 2011). Recently, extracellular vesicles (EV), exosomes and shedding vesicles have attracted increasing attention as mediators of unconventional protein secretion (Cocucci et al. 2009).

1.3.1 Extracellular vesicles (EVs)

There are two distinct types of EVs, exosomes and shedding vesicles that can be distinguished from each other based on their biogenesis (El Andaloussi et al. 2013). Exosomes are derived from the endosomal membrane compartment and are secreted by the fusion of multivesicular bodies (MVB) with the plasma membrane (Figure 2). Shedding vesicles are derived and directly shed from the plasma membrane. EVs were originally thought of only as a mechanism to remove cellular debris, but are now considered as multifunctional structures inherently involved in important cellular processes such as mediating intercellular communication, transferring molecular components, as well as in extracellular signalling (Buzas et al. 2014, Robbins, Morelli 2014). Through these functions, EVs released from both immune and non-immune cells have an important role in the regulation of the immune response.

There are many advantages should intercellular communication occur through EVs. Firstly, they permit the display of membrane proteins in a high localized concentration on the EV surface. Secondly, the vesicular structure makes it possible to transfer hydrophobic molecules within the EV membrane from one cell to another. Thirdly, EVs can be targeted to the recipient cells by specific ligands on the EV membrane, and fourthly, the EV cargo is protected from degradation by extracellular factors (Choi et al. 2014). EVs utilise different mechanisms to deliver complex signals to their target cells. They can induce receptor-mediated intracellular signal transduction by surface-expressed or surface-bound ligands, transfer surface receptors to target cells, and deliver functional proteins, lipids, and RNAs into the target cell by fusion with the plasma membrane or via internalization into the endocytic compartment (Ratajczak et al. 2006). Several different cell types have been shown to take up EVs, including macrophages, DCs, and epithelial cells (Lässer et al. 2011, Morelli et al. 2004, Obregon et al. 2009).

EVs can be considered as “signalosomes”, multifunctional signalling complexes for controlling various cellular functions (El Andaloussi et al. 2013). The signals mediated by EVs may play a role in the maintenance of normal physiology including, tissue repair (Gatti et al. 2011), immune surveillance (Raposo et al. 1996), and regulation of immune responses (Robbins, Morelli 2014, Thery et al. 2009). In the regulation of immune responses, EVs have been shown to be doubled-edged swords being involved in both activation and suppression. Their activating functions include antigen presentation to T-cells (Giri, Schorey 2008, Walker et al. 2009), activation of macrophages, monocytes, B cells, and NK cells (Baj-Krzyworzeka et al. 2007, Bhatnagar et al. 2007, Simhadri et al. 2008, Sprague et al. 2008). The immune response suppressive roles of EVs include the inhibition of T-cell activation and increasing regulatory T-cell numbers (Admyre et al. 2007), suppression of NK cell activity (Clayton et al. 2008), and prevention of monocyte differentiation into DCs as well as DC maturation (Eken et al. 2008, Yu et al. 2007).

2. INNATE IMMUNE RESPONSE TO VIRAL INFECTION

The innate immune response limits the viral replication in the tissues and via antigen presentation, it can activate the adaptive immune response (Egan et al. 2013). The mounting of an effective innate immune response against viral infection requires the detection of the invading viruses by PRRs, and intrinsic restriction factors. The intrinsic restriction factors such as tetherin, and interferon inducible transmembrane proteins (IFITMs) have a direct effect on the viral components to which they bind

(Yan, Chen 2012), whereas the PRRs function indirectly. Many of the intrinsic restriction factors are continuously expressed in the cell, ready to defend the cell against invading pathogens. The recognition of infecting viruses by host PRRs can induce two distinct outcomes: (1) activation of cytokines, especially type I IFNs, and other proteins that are secreted from the infected cell, and (2) formation of the inflammasome complexes involved in the processing of cytokines IL-1β and IL-18 into their mature forms and their secretion from the cell (Iwasaki 2012).

2.1 Type I interferons (IFNs)

IFNs are cytokines secreted by cells in response to different stimuli (Borden et al. 2007). They are classified into three different groups according to their amino acid sequence. Type I IFNs include several IFNs -α/-β, type II consists of only one IFN, the IFN-γ, and type III is made up of IFN-λs IL-29 and IL-28. In particular, type I IFNs, but also type-III IFNs, are recognized as potent antiviral cytokines (Randall, Goodbourn 2008). Type I IFNs are expressed by many different cell types and play a crucial role in antiviral responses (Borden et al. 2007, Isaacs, Lindemann 1957, Nagano, Kojima 1958). They are the master regulators of antiviral immunity and possess immunomodulatory properties (Goubau et al. 2013). The most significant producers of type I IFNs are professional innate immune cells, especially DCs that produce IFN-α (Hertzog, Williams 2013).

Type I IFNs are expressed in large amounts only after a specific stimulus, most often by viral infection (Hertzog, Williams 2013). This leads to the activation of the IFN circuit consisting of three distinct steps (Figure 3 on page 10). First, the recognition of PAMPs by PRRs evokes the activation of transcription factors, and production of type I IFNs (Figure 3A). In the second step, the secreted IFNs bind to their plasma membrane cognate receptors, IFN-α/β receptor (IFNAR), in an autocrine or paracrine manner (Figure 3B). In the final step, activation of the IFN receptor stimulates the transcription of hundreds of interferon-stimulated genes (ISGs) (Figure 3C). Binding of type I IFNs to the IFNAR activates signal transducer and activator of transcription (STAT) family members (Matikainen et al. 1999) (Figure 3B). IRF9 is recruited to the activated STAT1 and STAT2, and together they form the protein complex known as the IFN-stimulated gene factor 3 (ISGF3) (Platanias 2005) that induces the transcription of ISGs (Gonzalez-Navajas et al. 2012, Platanias 2005) (Figure 3C). In addition to the ISGF3 complex, there are also other STAT protein complexes that do not recruit IRF9. For example, the STAT1 homodimer can bind to IFN-γ activated site (GAS) enhancer elements in the promoters of ISGs to initiate gene transcription of proinflammatory and apoptotic ISGs (Gonzalez-Navajas et al. 2012, Platanias 2005).

2.2 Interferon-stimulated genes (ISGs)

The strict definition of an interferon-regulated gene (IRG) is a gene whose transcriptional output is either increased in the case of ISGs, or decreased in response to IFN, largely due to the presence of IFN-stimulated response elements and/or GAS sequences in the promoter and enhancer regions of the gene (Schneider et al. 2014). The proteins encoded by ISGs represent a diverse defence system against invading pathogens (MacMicking 2012). They are involved in eliminating viral components, inducing apoptosis in the infected cell, and conferring resistance to viral infection in uninfected cells. Nearly 2000 human and mouse IRGs have been identified so far (Samarajiwa et al. 2009), and most of them, nearly 1600, are ISGs (Hertzog et al. 2011). Type I IFNs clearly regulate most of the IRGs as the vast majority, nearly 1700, are regulated by them (Hertzog et al. 2011). Many of the ISG-encoded proteins have only a modest antiviral effect on their own (Schoggins et al. 2011) but their efficacy is increased when various ISG-encoded proteins are expressed in combination, suggesting that most of these proteins function as a unit in combination with other ISG-encoded proteins (Schoggins et al. 2011).

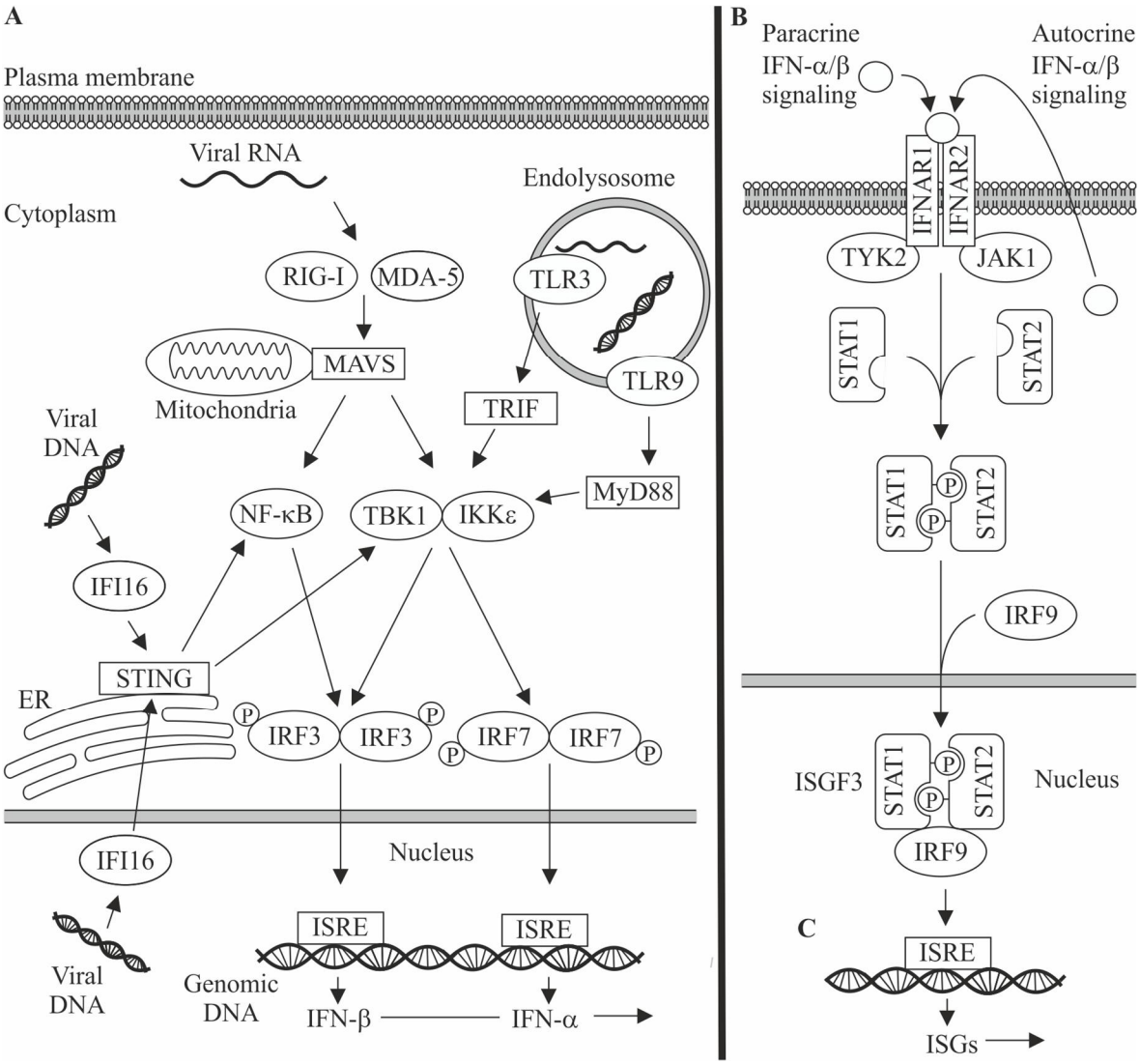

Figure 3. The interferon circuit. A) Recognition of PAMPs by different PRRs, such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), toll-like receptors (TLR), and γ-interferon-inducible protein 16 (IFI16) mediate their signals through adapter molecules (e.g. mitochondrial antiviral-signalling protein (MAVS), TIR-domain-containing adapter-inducing interferon-β (TRIF), myeloid differentiation primary response protein 88 (MyD88), stimulator of interferon genes protein (STING). This ultimately leads to the activation of different transcription factors resulting in the production of type I IFNs. B) Type I interferons (IFN) signal through the IFN-α/β receptor (IFNAR) which consists of two subunits, IFNAR1 and IFNAR2 that are constitutively associated with Janus kinase 1 (JAK1) and non-receptor tyrosine kinase (TYK2). Binding of type I IFNs to their receptor activates JAK1 and TYK2, which leads to the tyrosine phosphorylation and activation of signal transducer and activator of transcription (STAT) family members. Interferon regulatory factor 9 (IRF9) is recruited to STAT1 and STAT2, and together they form the IFN-stimulated gene factor 3 (ISGF3). C) The ISGF3 migrates to the nucleus where it binds to IFN-stimulated response elements (ISRE) in the promoters of interferon-stimulated genes (ISG) to initiate ISG transcription. NF-κB, nuclear factor NF-κ-B; TBK1, TANK-binding kinase 1; IKKε, inhibitor of NF-κB kinase subunit ε.

Proteins encoded by ISGs can operate in multiple cell types and at all successive stages of the viral life cycle, from viral entry to the release of viral particles from the host cell (MacMicking 2012, Schneider et al. 2014). ISG-encoded proteins involved in blocking viral entry and uncoating include MxA, which targets cytoplasmic viruses by binding to their viral capsid i.e. sequestering them and delivering them for proteosomal degradation (Haller, Kochs 2011). Inhibition of viral replication is mediated by protein kinase R (PKR), which can inhibit host and virus protein synthesis by phosphorylating eukaryotic initiation factor 2α (eIF2α) (Pindel, Sadler 2011). Phosphorylation inactivates eIF2α and thus blocks totally protein synthesis. ISG15 protein also

inhibits viral replication, but by a different mechanism i. e. it ISGylates newly synthesised viral proteins, which interferes with the ubiquitin in the viral proteins leading to their degradation (Morales, Lenschow 2013). ISG-encoded proteins involved in preventing viral assembly, budding and release include viperin (virus inhibitory protein, endoplasmic reticulum-associated, interferon inducible), also known as radical S-adenosyl domain containing 2 (RSAD2) which interferes with the assembly and egress of influenza A virus and hepatitis C virus particles through a mechanism that has not been fully characterized (Helbig, Beard 2014) but may occur through the disruption of ER-derived lipid rafts that transport viral envelope proteins to the plasma membrane.

3. HERPES SIMPLEX VIRUS-1 (HSV-1)

All herpesviruses are classified into a single order, the *Herpesvirales*, which is subdivided into three families, the *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae* (Davison et al. 2009, Davison 2010). A common feature of all herpesviruses is their ability to produce a life-long latent infection in their host from which they can be periodically reactivated to produce a lytic infection. HSV-1, also known as Human herpesvirus 1 (HHV1), belongs to the *Herpesviridae* family which can be divided further into three subfamilies, the *Alpha-*, *Beta-*, and *Gammaherpesvirinae*, according to the host cells in which they cause the latent infection (Szpara et al. 2013). In total there are nine pathogenic human herpesviruses. Three of these viruses, HSV-1 with HSV-2 and VZV, belong to the *Alphaherpesvirinae* subfamily. Originally they were distinguished from beta- and gamma-herpesviruses by their ability to latently infect sensory neurons, and more recently based on their genetic similarities (Mori, Nishiyama 2005). The HSVs differ from VZV, the causative agent of measles and shingles (herpes zoster), in their ability to continuously reactivate from latency and the route of primary infection.

3.1 Structure, entry, and replication

The intact HSV-1 virion is composed of four morphologically distinct structures (Grunewald, Cyrklaff 2006) (Figure 4 on page 12). The HSV-1 genome consists of approximately 80 genes that can be divided into three different groups based on their transcription kinetics. Immediate-early (IE) genes, also known as alpha(α)-genes, are transcribed between 2 and 4 hours post infection (hpi), early genes (E; beta(β)-genes) between 4 and 6 hpi, and late genes (L; gamma(γ)-genes) after 6 hpi (Honess, Roizman 1974, Honess, Roizman 1975). The IE gene products are involved in regulation of both host and HSV-1 gene transcription and also in combatting host responses against HSV infection. There are five IE gene products: infected cell protein 0 (ICP0), ICP4, ICP22, ICP27, and ICP47. E genes are involved in DNA metabolism and replication, and L genes code for structural and tegument proteins (Weir 2001).

HSV-1 primary infection occurs most often in the orofacial region, where it infects epithelial cells of the oral mucosa. The virus is transmitted during reoccurring lytic infection through close contact via saliva from one infected person to another. HSV-1 can enter into the host cell by two different means, either by direct membrane fusion, or by membrane-bound receptor-mediated interactions. In both cases, the entry of the virus leads to the release of the viral capsid and the tegument proteins into the host cell cytoplasm (Rahn et al. 2011). The capsid shields the viral genomic double-stranded (ds) DNA from the host cytosolic defence mechanisms and transports the viral genome along the microtubules to the host cell nucleus. Once there, the HSV genome is transferred from the capsid through nuclear pores into the nucleus, where it can be transcribed and replicated.

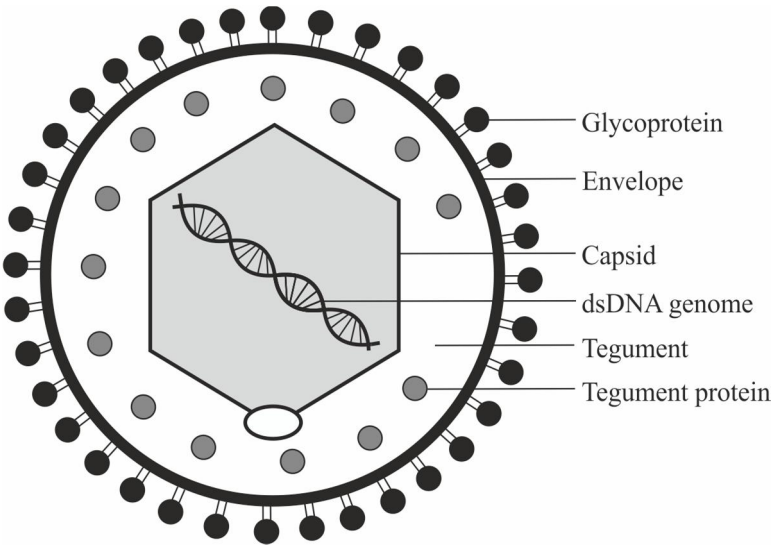

Figure 4. A simplified representation of the HSV-1 virion structure. The outermost structure of HSV-1 is the viral envelope consisting of a lipid double bilayer obtained from the host cell containing viral glycoproteins. Beneath the envelope lies the protein-rich tegument that is situated between the viral envelope and capsid. The HSV-1 double-stranded (ds) DNA genome is located within the capsid.

During the primary lytic infection, HSV-1 replicates in the epithelial cells from where it spreads to infect nearby sensory nerves. Once it has infected the sensory nerves, it spreads to the trigeminal ganglion, which is the primary site of the life-long HSV-1 latent infection, a dormant state of infection (Arduino, Porter 2008). However, HSV-1 can emerge periodically from its latent form and its reactivation triggers a lytic infection during which it most often travels back via the sensory neurons to the vicinity of the site of primary infection, where it infects nearby epithelial cells. The emergence from latency can be induced by several different stress factors such as fever, exposure to ultraviolet light, and trauma (Arduino, Porter 2008).

3.2 Immune response to HSV-1 infection

There are multiple PRRs that are involved in the detection of HSV-1 leading to the activation of the immune response (Table 3). The most prominent PAMP detected by these PRRs is the HSV-1 genomic DNA, although viral RNA and glycoproteins can also serve as PAMPs. The primary recruited immune cell mediators in HSV-1 infection include monocytes, neutrophils, DCs, and NK cells (Egan et al. 2013). Monocytes recruited to the site of HSV-1 infection differentiate into tissue macrophages and phagocytose released virions and apoptotic epithelial cells. Macrophages also function as “professional” APCs that present viral peptides to the cells of the adaptive immune system (Egan et al. 2013). At the sites of primary HSV-1 infection (Cheng et al. 2000, Mott et al. 2007) and in the trigeminal ganglia (Kodukula et al. 1999) macrophages attempt to limit the viral replication until the adaptive immune response can be activated. Infected cells such as epithelial cells secrete type I IFNs and other compounds that are chemotactic for activated macrophages. After their activation, the macrophages themselves secrete proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6, C-C motif chemokine 5 (CCL5; RANTES), type I IFNs, and nitric oxide (NO) (Egan et al. 2013). NO production occurs by the enzyme inducible nitric oxide synthase, which is activated by IFN-γ secreted by NK cells early in infection, and by CD8+ T, CD4+ T, and γδT-cells later in infection (Egan et al. 2013).

Table 3. PRRs involved in HSV-1 recognition

PRR	Ligand	Reference
Membrane-bound		
TLR		
TLR2	HSV gB & gH/L	(Leoni et al. 2012, Kurt-Jones et al. 2004)
TLR3	dsRNA	(Zhang et al. 2007)
TLR9	HSV genomic DNA	(Krug et al. 2004, Rasmussen et al. 2007)
Scavenger receptors		
MARCO	HSV gC	(MacLeod et al. 2013)
Cytosolic		
RLR		
MDA5	RNA	(Melchjorsen et al. 2010)
RIG-I	RNA	(Cheng et al. 2007)
ALR		
AIM2	dsDNA	(Bürckstümmer et al. 2009)
IFI16	HSV genomic DNA	(Conrady et al. 2012)
NLR		
NLRP3	n.d.	(Johnson et al. 2013)
Other		
DAI	dsDNA	(Takaoka et al. 2007)
RNA Pol III	HSV genomic DNA	(Chiu et al. 2009)
cGAS	dsDNA	(Sun et al. 2013)
DDX41	dsDNA	(Zhang et al. 2011c)
DDX60	dsDNA & dsRNA	(Miyashita et al. 2011)
DHX9	HSV genomic DNA	(Kim et al. 2010)
DHX36	HSV genomic DNA	(Kim et al. 2010)
Ku70/DNA-PK	dsDNA	(Zhang et al. 2011b)

gB, Glycoprotein B; ds, double-stranded; n.d., not determined

3.3 Modulation of the host immune response by HSV-1

HSV-1 has developed several strategies to allow the virus to evade host antiviral responses. The IFN response is an important host defence mechanism against which virtually all viruses have evolved evasion strategies. The four major IFN-antagonist strategies are (1) the general inhibition of gene expression/protein synthesis, (2) sequestration of molecules in the IFN circuit, (3) proteolytic cleavage of innate immune components, and (4) proteosomal degradation of these components (Versteeg, Garcia-Sastre 2010). Viruses, including HSV-1, have developed mechanisms to counteract the induction, signalling, and antiviral actions of the type I IFN-induced proteins (Versteeg, Garcia-Sastre 2010). The importance of type I IFNs in HSV infections is emphasised by the vast numbers of HSV proteins involved in the inhibition of IFN response (Melchjorsen et al. 2009, Paladino, Mossman 2009) (Table 4 on page 14) and by the susceptibility to severe HSV infections by IFN-receptor deficient mice (Leib et al. 1999).

In addition to the evasion of the IFN-response, HSV-1 has evolved several other immune evasion mechanisms to counteract other host antiviral responses e.g. the inhibition of apoptosis, inhibition of host gene expression, inhibition of autophagy and antimicrobial proteins, inhibition of the complement system, antigen presentation and APC function, and inhibition of PRR function. Table 5 (page 15) describes the multiple HSV proteins involved in these inhibition processes.

Table 4. HSV-1 proteins involved in the evasion of the host IFN response

HSV-1 protein	Effect	Reference
Inhibition of IFN production		
ICP0	Inhibits IRF3 nuclear accumulation and subsequent induction of type I IFNs	(Eidson et al. 2002, Lin et al. 2004, Melroe et al. 2004, Melroe et al. 2007)
ICP27	Inhibits IRF3 and NF-kB activation	(Melchjorsen et al. 2006)
ICP34.5	Prevents IRF3 activation and subsequent expression of Type I IFNs	(Verpooten et al. 2009)
Us3	Modulates TLR3 transcript levels	(Peri et al. 2008)
VP16	Abrogates IFN-β production by inhibiting NF-κB activation	(Xing et al. 2013)
Inhibition of IFN signaling		
ICP0	Antagonizes STAT1 activation	(Halford et al. 2006)
ICP0	Inhibits IRF7-mediated ISG induction	(Lin et al. 2004)
ICP27	Inhibits STAT-1α phosphorylation and subsequent nuclear translocation and ISG expression	(Johnson et al. 2008, Johnson, Knipe 2010)
ICP34.5	Induces eIF-2α dephosphorylation	(He et al. 1997)
UL13	Increases expression of SOCS-3	(Yokota et al. 2004)
Us3	Post-translationally modifies type II IFN receptor and subsequently inhibits ISG induction	(Liang, Roizman 2008)
Vhs	Inhibits STAT-1α phosphorylation	(Eisemann et al. 2007)
Vhs	Disrupts formation of STAT-1/-2/p48 complex	(Chee, Roizman 2004)
Inhibition of ISG production and function		
ICP0	Degrades ISG-encoded proteins (e.g. PML)	(Chee et al. 2003)
ICP34.5	Interferes with RNase L-independent rRNA degradation	(Sobol, Mossman 2006)
Us11	Inhibits PKR from phosphorylating eIF-2α	(Cassady, Gross 2002, Poppers et al. 2000)
Us11	Prevents the production of 2',5'-oligoadenylate and the subsequent activation of latent Rnase L	(Sanchez, Mohr 2007)
Vhs	Correlates with prevention of eIF-2α phosphorylation	(Pasieka et al. 2008)
Vhs	Interferes with the production of ISG transcripts during later stages of infection	(Liang, Roizman 2008)

3.4 Extracellular vesicles and HSV-1

During viral infections, EVs can confer an advantage for either the infecting virus (Meckes, Raab-Traub 2011, Silverman, Reiner 2011, Wurdinger et al. 2012), or for the host cell itself (Kesimer et al. 2009, Khatua et al. 2009). EVs allow some viruses to evade the host immune system. For example, HSV-1 glycoprotein B (gB) binds to human leukocyte antigen (HLA)-DR protein and routes the gB-HLA-DR complex into EVs for secretion out of the host cell rather than correctly targeting HLA-DR to the plasma membrane (Neumann et al. 2003). This allows HSV-1 to hijack the cellular antigen-presenting machinery, thus preventing further antigen presentation and increasing EV production. HSV-1 induces also the secretion of so-called non-infectious L particles which can be considered as EVs. These particles are HSV-1 virions containing the viral envelope with its glycoproteins and also the tegument proteins, but lacking the viral capsid and the dsDNA genome. L particles are thus incapable of forming a productive lytic infection in the host cell (McLauchlan, Rixon 1992, Rixon et al. 1992). L particles fuse with the host cell and release their contents, including the tegument proteins, into the host cell cytoplasm. In this way, they enhance HSV-1 replication by rendering the surrounding cells more susceptible to HSV-1 infection (Dargan, Subak-Sharpe 1997, McLauchlan et al. 1992). L particles have been proposed to reduce immune rejection by the host cells (Temme et al. 2010).

Table 5. HSV-1 proteins involved in the evasion of other host immune response mechanisms

HSV-1 protein	Effect	Reference
Inhibition of apoptosis		
gD	n.d.	(Zhou, Roizman 2001)
gJ	Inhibits caspase activation	(Jerome et al. 1999)
ICP4	n.d.	(Leopardi, Roizman 1996)
ICP22	n.d.	(Aubert et al. 1999)
ICP27	n.d.	(Aubert, Blaho 1999)
ICP34.5	Inhibits PKR activity	(He et al. 1997)
ICP34.5	Inhibits CTL-induced apoptosis by down-regulating cell surface Fas ligand	(Sieg et al. 1996, Sieg et al. 1997)
LAT	n.d.	(Perng et al. 2000)
Us3	n.d.	(Jerome et al. 1999, Leopardi et al. 1997, Munger, Roizman 2001)
Inhibition of host gene expression		
ICP0	Inhibits TLR-induced JNK and NF-kB activation	(Daubeuf et al. 2009)
	Causes cell cycle arrest and disturbed cellular gene expression	(Hobbs, DeLuca 1999)
ICP27	Reduces mRNA stability	(Mogensen et al. 2004)
	Inhibits splicing	(Bryant et al. 2001, Hardy, Sandri-Goldin 1994, Sciabica et al. 2003)
Vhs & VP16	Degrades RNA	(Strand, Leib 2004)
Unknown	Inhibits NFAT activation	(Scott et al. 2001)
Inhibition of autophagy and anti-microbial proteins		
ICP0	Down-regulades SLP1	(Fakioglu et al. 2008)
ICP0 & Vhs	Represses the expression of viral restriction factor ATRAX	(Jurak et al. 2012)
ICP34.5	Prevents autophagy by targeting Beclin-1	(Orvedahl et al. 2007, Talloczy et al. 2002)
Inhibition of complement, antigen presentation and APC function		
gB	Inhibits MHC II-mediated antigen presentation by inhibiting the expression of invariant chain and interacting with HLA-DR and HLA-DM	(Neumann et al. 2003)
gC	Binds to complement factor C3	(Friedman et al. 1984, Lubinski et al. 1999)
gE/I complex	Binds to the Fc domain of IgG	(Lubinski et al. 1998)
ICP47	Inhibits antigen presentation by MHC I	(Ahn et al. 1996)
Vhs	Inhibits DC maturation and reduced cytokine production	(Salio et al. 1999, Samady et al. 2003)
Vhs	Interferes with MHC I transport and reduces levels of MHC II	(Hill et al. 1994, Tigges et al. 1996, Trgovcich et al. 2002)
Inhibition of PRR function		
ICP0	Degrades IFI16	(Orzalli et al. 2012)
ICP0	Inhibits inflammasome activation	(Johnson et al. 2013)
Us11	Down-modulatesf RLR signaling pathways	(Xing et al. 2012)

n.d., not determined

3.5 Infected cell protein 27 (ICP27)

ICP27 is a multifunctional immediate-early phosphoprotein encoded by the HSV UL54 gene (Sandri-Goldin 2011). It has homologs in every mammalian herpesvirus that has been sequenced so far (Sandri-Goldin 2011). ICP27 has multiple different functions including the regulation of viral early and late gene transcription, inhibition of host gene transcription, and the inhibition of host antiviral responses. The different functions of ICP27 are made possible through its many different

interaction partner proteins as shown in Figure 5. The functions of ICP27 can be divided into two groups depending on the cellular localization of the protein in the host cell, i.e. nuclear and cytoplasmic functions. In the infected cell, ICP27 is localized during early infection mainly to the nucleus but later during the infection, it is also found in the cytosol (Lengyel et al. 2002, Soliman, Silverstein 2000). It can readily shuttle between the cytosol and nucleus (Mears, Rice 1998, Soliman et al. 1997), this process is made possible by its N-terminal nuclear export sequence (NES) (Sandri-Goldin 1998) and nuclear localization sequence (NLS) (Hibbard, Sandri-Goldin 1995, Mears et al. 1995) (Figure 5).

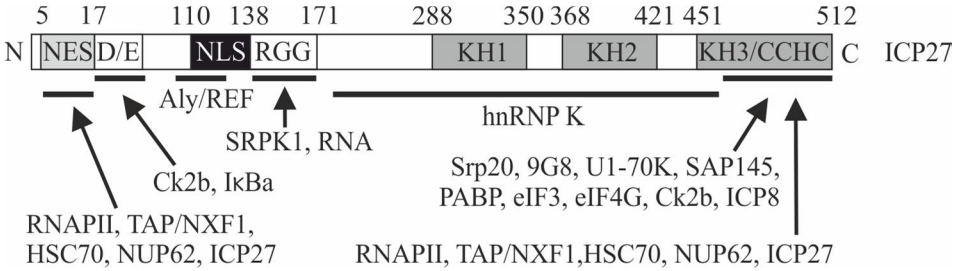

Figure 5. Domain structure of HSV-1 infected cell protein 27, and the binding sites for its interacting partners. ICP27 consists of an amino (N)-terminal nuclear export sequence (NES), an acidic domain (D/E), major nuclear localization sequence (NLS), RNA binding arginine- and glycine rich motif (RGG), three KH domains, and a zinc-finger-like domain (CCHC) at its carboxy-terminal end. The first 160 amino acid residues of the N-termini form a region that is highly flexible and unstructured allowing it to be intrinsically disordered or unfolded on its own. This region holds within it the NES, NLS, the RGG box, and binding sites for several ICP27 interaction partners such as RNA polymerase II (RNAPII), nuclear pore glycoprotein p62 (NUP62), nuclear RNA export factor 1 (TAP/NXF1), heat shock cognate 71 kDa protein (HSC70), Aly/REF export factor (Aly/REF), serine/arginine-rich protein-specific kinase 1 (SRPK1). ICP27 interaction partners are marked under the domain structure at their interaction sites.

The nuclear functions of ICP27 include the inhibition of host cellular pre-mRNA splicing, which contributes to the general inhibition of host cell protein synthesis (Bryant et al. 2001, Lindberg, Kreivi 2002, Hardy, Sandri-Goldin 1994, Sciabica et al. 2003). ICP27 interacts with and recruits RNA polymerase II (RNAPII) to viral replication sites in the nucleus in order to stimulate viral gene transcription (Dai-Ju et al. 2006, Zhou, Knipe 2002). Furthermore, it can enhance HSV infection by recruiting the cellular chaperone heat shock cognate 71 kDa protein (HSC70) into the nuclear virus-induced chaperone-enriched domains that play a role in nuclear protein quality control and thus possibly resolve interrupted transcription complexes in viral replication compartments (Li et al. 2008, Livingston et al. 2009). An important nuclear role of ICP27 is the viral mRNA export from the host nucleus to the cytosol for translation. Most HSV mRNAs lack introns making them less efficiently bound by the host nuclear mRNA export factors than spliced mRNAs. In order for the viral mRNAs to be exported from the nucleus, ICP27 functions as an mRNA export adaptor protein by binding to viral mRNAs and interacting with multiple host proteins involved in the host nuclear mRNA export. These host proteins include the mRNA export receptor nuclear RNA export factor 1 (TAP/NXF1) (Chen et al. 2005, Hernandez, Sandri-Goldin 2010), Aly/REF export factor, pre-mRNA-splicing factor SRp20, and splicing factor 9G8 (Escudero-Paunetto et al. 2010, Sciabica et al. 2003, Tian et al. 2013).

The main cytoplasmic function of ICP27 is the stimulation of viral mRNA translation by interacting with and recruiting cellular translational initiation factors to viral mRNAs. The translation initiation factors with which ICP27 can interact include poly A-binding protein, eukaryotic translation initiation factor 3 (eIF3), and eIF4G (Fontaine-Rodriguez et al. 2004, Larralde et al. 2006). There are also multiple ICP27 functions that have been described but for which the mechanism is either poorly characterized or completely unknown. These include the inhibition of the host IFN-response and the inhibition of apoptosis. ICP27 has been shown to inhibit the activation of nuclear factor NF-κ-B (NF-κB) and IRF3 leading to reduced IFN production (Melchjorsen et al.

2006) (Table 4). It has also been shown to prevent STAT1 phosphorylation in the cytosol, and STAT1 nuclear translocation (Johnson et al. 2008) (Table 4). The exact mechanisms of inhibition are unknown, although a soluble ICP27 dependent IFNAR antagonist has been reported to be involved in the STAT1 inhibition (Johnson, Knipe 2010). The mechanisms by which ICP27 inhibits apoptosis remain unknown (Nguyen, Blaho 2007).

4. MASS SPECTOMETRY-BASED PROTEOMICS IN THE STUDY OF INNATE IMMUNITY

The proteome is defined as all the proteins expressed in a cell or tissue at a certain time point (Wilkins et al. 1996) and the corresponding field of research is called proteomics. The main goal of proteomics is to identify all the proteins in living organisms and to characterize their functions. Mass spectrometry (MS)-based proteomics has undergone enormous development. The number of proteins that can be studied in a single MS-based proteomics experiment is now in the order of thousands (Geiger et al. 2012). This technique can be applied in many different areas of proteomics such as in the study of protein expression, post-translational modifications (PTM), or protein-protein interactions (Meissner, Mann 2014). The ability to measure protein expression, PTMs, and protein-protein interactions by using MS-based proteomics allows the study of complex biological systems on the protein level. The innate immune system is a good example of a complex system where the use of different MS-based proteomic methods has already generated valuable new information (Rebsamen et al. 2013).

Quantitative MS-based proteomics has been used in the study of host innate immune response in different experimental settings, including in the activation of the innate immune response upon different viral infections. Human macrophages infected with influenza A virus (Lietzén et al. 2011) and with human immunodeficiency virus-1 (Haverland et al. 2014) have been studied using quantitative MS-based proteomics. In addition, human foreskin fibroblasts infected with human cytomegalovirus (Weekes et al. 2014), and A549 cells infected with influenza A virus (Dove et al. 2012) and respiratory syncytial virus (Dave et al. 2014) have been investigated using different quantitative MS-based proteomics methods. So far, proteomics studies of HSV-1-infected cells have utilized mainly two-dimensional gel electrophoresis (2-DE)-based methods, and have been conducted during early HSV-1 infection. These studies have focused on the intracellular changes occurring in the whole cell extract (Antrobus et al. 2009), in the cytosolic and microsomal fractions (Santamaria et al. 2009), in the nuclear fraction (Sanchez-Quiles et al. 2011), and in ribosomal fraction or acidic proteins (Greco et al. 2000). In contrast, quantitative MS-based proteomic studies on HSV-1-infected macrophages have not been conducted.

MS-based proteomic characterization of protein secretion involved in innate immunity has also been an area of interest. The effect of bacterial LPS stimulation on protein secretion from macrophages has been investigated by several groups recently in which the whole secretome has been analyzed in a quantitative manner (Eichelbaum, Krijgsveld 2014, Meissner et al. 2013, Öhman et al. 2014). The quantitative analysis of secretomes from human macrophages stimulated with the fungal cell wall component, β-glucan (Öhman et al. 2014), and infected with influenza A virus (Lietzén et al. 2011) have also been performed. Protein secretion from cells stimulated with MSU (Martinon et al. 2006, Martinon 2010), or infected with HSV-1 (Melchjorsen et al. 2002, Melchjorsen, Paludan 2003, Melchjorsen et al. 2006, Melchjorsen et al. 2010, Mikloska et al. 1998) have been examined, but these investigations have focused on only a few selected proteins at the same time, mainly cytokines and chemokines which have been analyzed using enzyme-linked immunosorbent assays (ELISA), and semi-quantitative Western blotting. Protein secretion induced by HSV-1 infection or MSU stimulation has not been studied previously in a manner where the whole secretome would have been investigated.

II AIMS OF THE STUDY

Macrophages play an important role in the innate immune response against microbial infections, and in several autoinflammatory disorders. This study was conducted to obtain new insights on the mechanisms of innate immune responses activated in human monocyte-derived macrophages by the DNA virus HSV-1, and by MSU, the crystallized extracellular form of the DNA metabolite uric acid using MS-based proteomics combined with functional studies.

The specific aims of the study were:

1. to characterize the secretome of HSV-1 infected human primary macrophages and to discover novel secreted host factors involved in antiviral defence

2. to characterize the secretome of LPS- and MSU-stimulated human primary macrophages to obtain a global view of macrophage response to combined DAMP and PAMP stimulation

3. to characterize the functional mechanisms of HSV-1 ICP27 protein in the inhibition of IFN response, and apoptosis during HSV-1 infection

III MATERIALS AND METHODS

The materials and methods used in this study are summarized below. For each method, the relevant original publications contain detailed descriptions, and these are indicated by their Roman numerals.

1. Cell culture and stimulations

Human primary macrophages: Peripheral blood mononuclear cells (PBMC) were isolated from leucocyte-rich buffy coats obtained from healthy blood donors, as previously described (Pirhonen et al. 1999). In brief, human PBMCs were isolated using density gradient centrifugation and the mononuclear cell layer containing the monocytes was collected. Monocytes were left to differentiate into macrophages in macrophage serum-free medium supplemented with 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and antibiotics. After five to six days of cell culturing, depending on the experimental setup, the macrophages were used in the experiments. Each sample represents a pool of separately stimulated macrophages from three different blood donors (I, II, III).

Exogenous cytokines: When studying the effects of exogenous IFN-β during HSV-1 infection, IFN-β (Betasferon) was given to macrophages at a concentration of 200 international units (IU)/ml for 4 h prior to HSV-1 infection (I). GM-CSF was delivered to PBMC-derived monocytes at 10 ng/ml to *in vitro* differentiate them into macrophages and to maintain the obtained pool of cells (I, II, III).

Bacterial lipopolysaccharide (LPS): Macrophages were activated by priming with 100 ng/ml LPS from *Escherichia coli* for 18 or 21 hours, depending on the experiment, before MSU stimulation (II).

Cathepsin nhibitor Ca-074 methyl ester (Ca-074 Me): Ca-074 Me was used at a concentration of 25 μM for 1 h prior to MSU stimulation (II), or dLeu infection (III).

(S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser(S)Lys4-OH trihydrochloride (Pam_3Cys): Macrophages were primed with 1 μg/ml Pam_3Cys for 4 hours before MSU stimulation or HSV-1 infection (I).

Monosodium urate (MSU): MSU was used in the stimulation of macrophages at a concentration of 100 μg/ml for 3 or 6 hours, depending on the experiment, after Pam_3Cys (I), or LPS priming (II).

Herpes simplex virus-1 infections: Wild type (wt) KOS strain of HSV-1 or HSV-1 ICP27 mutant virus dLeu and d3-4 were used at a multiplicity of infection (MOI) of 1.

Table 6. List of viruses used in this work

Virus	Description	Reference	Used in
HSV-1 (KOS)	wild type	(Smith 1964)	I, III
d3-4 (KOS)	ICP27 mutant lacking the NLS	(Mears et al. 1995)	III
dLeu (KOS)	ICP27 mutant lacking the NES	(Lengyel et al. 2002)	III

NLS, nuclear localization sequence; NES, nuclear export sequence

2. Extracellular and cytosolic protein enrichment

Secretome protein enrichment: In the experiments where secretome proteins were enriched, macrophages were grown prior to stimulations in complete Macrophage–SFM medium. Before starting the stimulations, macrophages were washed three times with Dulbecco's phosphate-buffered saline to completely remove serum from the cultivation vessels. The macrophages were stimulated in RPMI growth media supplemented with 10 mM Hepes, L-glutamine and antibiotics. For the whole secretome, the growth media were collected and concentrated with Amicon Ultra centrifugal filter devices (Millipore) with 10 kDa nominal molecular weight cut-off (NMWO). The concentrated media were either used directly for Western blot analysis or purified with 2-D Clean-Up Kit (GE Healthcare) for MS-based proteomic analysis (I, II).

Extracellular vesicle (EV) enrichment: In the EV enrichment from the cell supernatant, the collected growth media was first subjected to concentration in Amicon Ultra centrifugal filter devices with a 100 kDa NMWO, and the flow-through was further concentrated using filter devices with 10 kDa NMWO (II).

Cytosolic fractionation: For cytosolic fractionation, approximately 10 million cells were used. The macrophage cytosolic fractions were isolated using QProteome Nuclear Protein Isolation Kit (Qiagen) (III).

3. Proteomics

3.1 Quantitative analysis using isobaric tags for relative and absolute quantitation (iTRAQ) labelling

4plex iTRAQ (AB Sciex) labelling was used for relative quantitation of proteins in HSV-1-infected macrophages (I, III). In the secretome analysis, an equal volume of cell supernatant was taken for the analysis, and in the cytosolic fraction analysis, equal amounts of protein were taken from each sample for the analysis based on silver stained gels. Cysteine reduction, alkylation and protein in-solution digestion were performed for each sample followed by iTRAQ-labelling of the resulting peptides. Digestion and labelling were done according to the manufacturer's instructions. After labeling, the peptide mixtures were prefractionated with strong cation exchange chromatography (SCX). The SCX separations were done using Ettan HPLC system (Amersham Biosciences) using a PolySULPHOETHYL A column (200 x 2.1 mm, PolyLC). The LC was operated at 0.2 ml/min and 20 mM KH_2PO_4 buffer (pH 3) was used with a gradient of 0-0.4 M KCl in 35 min. The eluting sample was collected in 1 ml fractions and the fractions containing peptides were analyzed using nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC-ESI-MS/MS).

3.2 Gel-based methods used in proteomic experiments

Two-dimensional gel electrophoresis (2-DE): 2-DE was used for the separation of secretome proteins in LPS- and MSU-stimulated macrophages (II). 11 cm pI 3-10 immobilised pH gradient-strips (BioRad) were used as the first dimension and Criterion Tris-HCl 10-20% precast gels (BioRad) as the second dimension. The gels were silver stained in a mass spectrometry compatible manner. Spot detection, matching and intensity-based quantitation were conducted using ImageMaster 2D Platinum version 6.0 (GE Healthcare). Spots with at least a 2-fold difference in expression between LPS-primed, and LPS-primed MSU-stimulated samples were considered differentially expressed and were selected. Proteins in the selected spots were in-gel digested with

trypsin, the resulting peptide samples were desalted using mC18 Zip Tips (Millipore) and the proteins were identified using nano-LC-ESI-MS/MS.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): in addition to 2-DE, SDS-PAGE was also used for the separation of secretome proteins in LPS- and MSU-stimulated macrophages (II).

3.3 Mass spectrometry

Nano-LC-ESI-MS/MS analyses of tryptic peptides were performed using Ultimate 3000 nano-LC system (Dionex) combined with QStar Elite hybrid quadropole time-of-flight mass spectrometer (TOF-MS) (AB Sciex) (I, II, III). In the analyses, the sample was first injected into ProteCol C18 trapping column (0,15x10mm; 3 um; 120Å) (SGE) and the column was washed with 0.1% trifluoroacetic acid (TFA) for 10 min. Subsequently, the peptides were separated in a PepMap100 C18 analytical column (0,075x150mm; 5um; 100Å) (LC Packings/Dionex) using a linear gradient of 0-40% acetonitrile in 0.1% formic acid. The time duration of the gradient varied between 20 min and 120 min depending on the sample. Mass spectral analyses were performed in the positive ion mode. MS data were acquired using Analyst 2.0 software (AB Sciex) and an information-dependent acquisition method. The method consisted of a 0.5 s TOF-MS survey scan of m/z 400-1400 followed by MS/MS scans of the two most abundant ions with charge states +2 to +4. Once an ion was selected for fragmentation, it was placed on an exclusion list for 60 s.

3.4 Database searches

Two different database search engines, Mascot (Matrix Science) (in-house Mascot version 2.2) (II) and Paragon from ProteinPilot version 2.0 (I) and 4.0.1 (III) (AB Sciex), were used for protein identifications based on MS data. Searches were conducted against NCBI or UniProt/SwissProt databases. Similar search parameters were used in all the searches. False discovery rates for the identifications were calculated using a target-decoy strategy based on searches against concatenated normal and reversed protein sequence databases (Elias, Gygi 2007).
For iTRAQ-based quantitative analysis, only Paragon was used. Raw MS data from both technical replicates of an iTRAQ sample set were processed together to improve the quality of quantitation data. Finally, those proteins with a more than 1.5-fold or less than 0.67-fold difference (cytosolic fractions) (III) or a more than 2-fold difference (secretomes) (I) between control and infected sample were considered as being differentially expressed.

4. Bioinformatic methods

After retrieving a list of protein identifications (and quantitation data) from a database search engine, supplementary steps in the data analysis were performed to assist in interpreting the results. Protein classification based on their biological functions, cellular localization, and pathway associations were done using GeneTrail (Backes et al. 2007) (I, II, III). In addition, more detailed analyses were performed using different specific databases. For the secretome datasets, ExoCarta (Mathivanan, Simpson 2009, Mathivanan et al. 2012), SignalP (Nielsen et al. 1997, Petersen et al. 2011), and Interferome database (Samarajiwa et al. 2009) were used (I, II). The updated Interferome database v2.0 (Rusinova et al. 2013), and ApoptoProteomics database (Arntzen, Thiede 2012) were used for the HSV-1-infected macrophage cytosolic proteome datasets (III).

5. Other methods

Table 7. Other methodological techniques used in this work

Method	Used in
Enzyme-linked immunosorbent assays (ELISA)	I, II
Luminex assay	I, II
Quantitative reverse transcription PCR (qRT-PCR)	II, III
siRNA transfection of cells	II, III
Western blotting	I, II, III

6. Antibodies

Table 8. Antibodies used in this work. More information can be found in the indicated references

Antigen	Antibody	Source or Reference	Used in
Annexin A1	Mouse monoclonal	Calbiochem	II
ASC	Rabbit polyclonal	Millipore	II
cleaved Caspase-3	Rabbit polyclonal	Cell Signaling	III
Cathepsin B	Rabbit polyclonal	Calbiochem	II
Cathepsin D	Goat polyclonal	Santa Cruz Biotechnology	II, III
Galectin-3	Mouse monoclonal	Santa Cruz Biotechnology	I, II
GAPDH	Mouse monoclonal	Santa Cruz Biotechnology	I, II, III
gB (HSV-1)	Mouse monoclonal	Virusys Corporation	III
gD (HSV-1)	Mouse monoclonal	Santa Cruz Biotechnology	I, III
gH (HSV-1)	Rabbit polyclonal	a kind gift from prof. Gabriella Campadelli-Fiume	III
HISTH1	Goat polyclonal	Santa Cruz Biotechnology	III
HISTH2B	Rabbit polyclonal	Abcam	III
HSP27	Goat polyclonal	Santa Cruz Biotechnology	I
HSP90	Rabbit polyclonal	Cell Signaling	I, II
IFIT2	Rabbit polyclonal	Sigma Aldrich	I, III
IFIT3	Mouse monoclonal	BD Biosciences	I, II, III
IL-1β	Rabbit polyclonal	(Pirhonen et al. 1999)	I, II
IL-18	Guinea pig polyclonal	(Pirhonen et al. 1999)	II
ISG15	Rabbit monoclonal	Cell Signaling	III
LAMP1	Mouse monoclonal	Santa Cruz Biotechnology	II
MDA5	Rabbit polyclonal	(Lin et al. 2006)	III
MxA	Rabbit polyclonal	(Ronni et al. 1993)	I, II, III
RIG-I	Guinea pig polyclonal	(Imaizumi et al. 2002)	III
S100-A9	Mouse monoclonal	Santa Cruz Biotechnology	I
STAT1 p84/p91	Rabbit polyclonal	Santa Cruz Biotechnology	I
Viperin	Rabbit polyclonal	Enzo Lifesciences	III

IV RESULTS AND DISCUSSION

Macrophages play an important role in the initiation of host defence during microbial infection and in the response to other dangerous molecules either endogenous or exogenous in their origin. Differentiation of PBMC-derived monocytes with GM-CSF leads to the appearance of a classical macrophage phenotype which is capable of producing many pro-inflammatory cytokines such as IL-1 and IL-18 (Mosser, Edwards 2008). GM-CSF-derived human macrophages represent a suitable primary human cell model with which to study the innate immune responses against viral infections, and its activation by endogenous danger signal molecules (Akagawa et al. 2006). In this work, human macrophages were used to elucidate the innate immune responses elicited by HSV-1 infection (I & III) and MSU stimulation (II). Proteomic methods including iTRAQ (I, III), 2-DE (II), and Gel-LC-MS/MS (II) with MS were used in combination with bioinformatic tools (Figure 6 on page 24). In addition, functional studies were performed using chemical stimulators (I), pharmacological inhibitors (II, III), and siRNA silencing (III).

1. Secretome characterization of HSV-1-infected, and MSU-stimulated human primary macrophages (I, II)

The activation of the innate immune response is mediated by the early detection of exogenous PAMPs and/or endogenous DAMPs by the cellular PRRs (Kono, Rock 2008) leading to the activation of immune cells. After activation, immune cells can secrete a variety of proteins which can act in a paracrine and/or exocrine manner. These proteins are involved in the further activation and regulation of the immune response, and in the recruitment of other immune cells to the site of infection and tissue damage. Macrophages, in addition to being phagocytes, after activation can exert a role in the secretion of a wide range of different molecules such as cytokines, chemokines, anti-microbial proteins, and endogenous danger signal molecules. The secretomes of macrophages activated by DNA virus HSV-1, and by MSU, the crystallized extracellular form of the DNA metabolite uric acid have not been characterized previously using quantitative MS-based proteomic methods.

1.1 IFN-β priming is required for robust protein secretion from HSV-1-infected macrophages (I)

Type I IFNs have long been known to play a crucial role in the antiviral response against viral infections by inducing the expression of many ISGs (Borden et al. 2007). Cells infected with HSV-1, including epithelial cells, secrete type I IFNs which function in both paracrine and autocrine manners. The paracrine secretion of type I IFNs can activate an antiviral state in the surrounding uninfected cells and thus protect them against a potential infection. The analysis of (i) IFN-β-primed, (ii) HSV-1-infected, and (iii) IFN-β-primed HSV-1-infected human macrophage secretomes identified a total of 1094 human proteins in the secretomes, from which 516 had good-quality quantification information. The total protein amounts in the secretomes of (i) IFN-β-primed and of (ii) HSV-1-infected macrophages did not differ significantly from the control secretome (I, Fig. 1c). In contrast, in the (iii) IFN-β-primed HSV-1-infected macrophage secretome, there was a significantly higher amount of proteins exhibiting increased secretion (I, Fig. 1b & c). This clearly demonstrates that IFN-β priming is required for efficient protein secretion from HSV-1-infected human primary macrophages. The requirement for IFN-β priming could be considered as a mechanism by which the host cell can regulate the intensity of the pro-inflammatory response

depending on the severity of viral infection. The absence of extracellular IFN-β could indicate that the infection is not severe and thus does not require an intense antiviral secretory response.

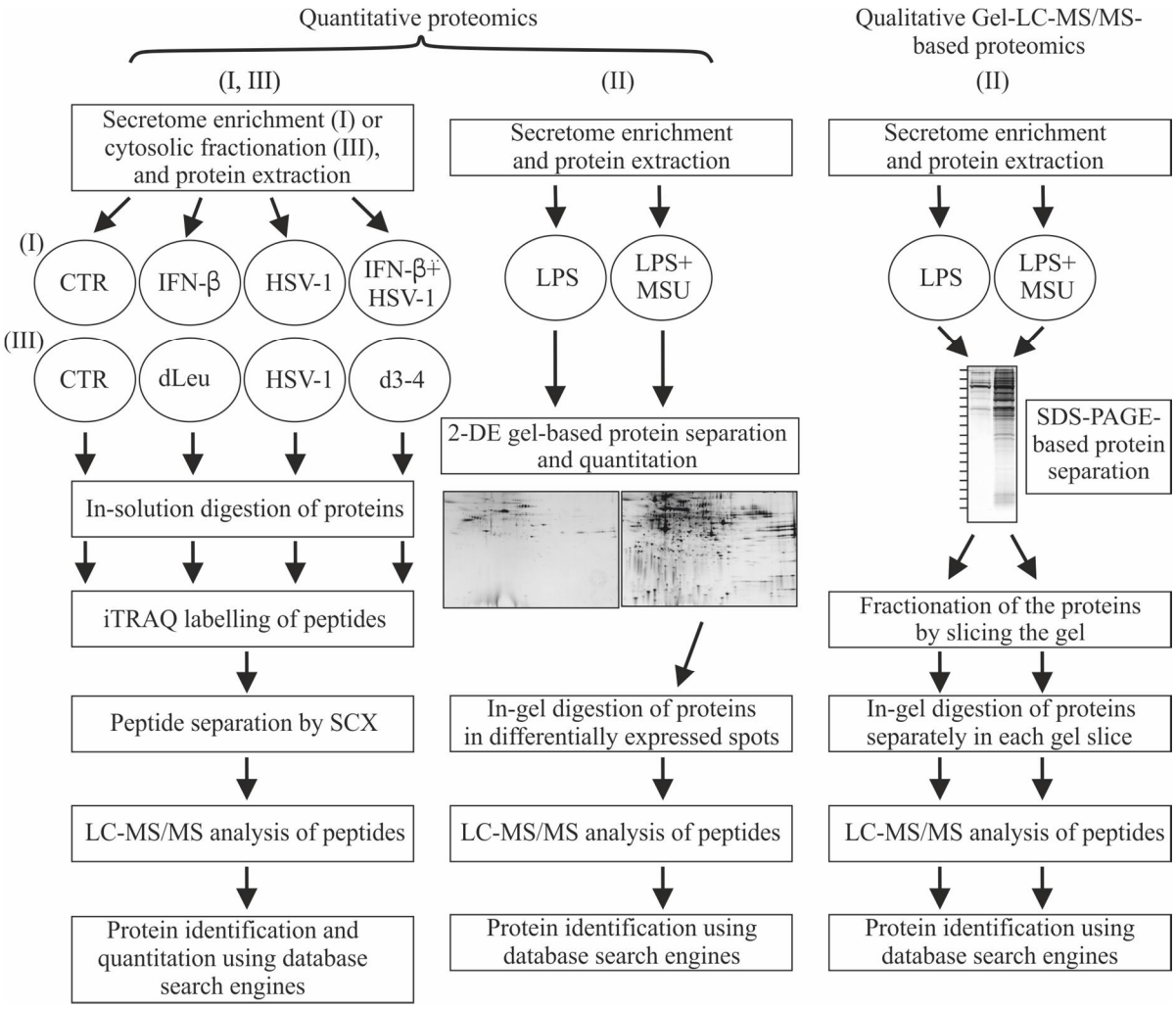

Figure 6. Proteomic methods used in this work. For quantitative proteomics both isobaric tags for relative and absolute quantitation (iTRAQ) labelling (I, III), and two-dimensional gel electrophoresis (2-DE) (II) were used. In addition, qualitative Gel-LC-MS/MS-based proteomics using SDS-PAGE was used (II). In the iTRAQ-labeling-based quantitative proteomics approach, the protein samples are first digested in-solution into peptides followed by iTRAQ-labeling of the peptides and fractionation of the peptides by strong cation exchange chromatography (SCX). The peptides in each fraction are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and proteins are identified and quantified using database search engines. In the 2-DE-based quantitative proteomics approach, the proteins in the samples are first separated by 2-DE and quantified based on the protein staining differences in the 2-DE gels from the different samples. The differentially expressed protein spots are selected and in-gel digested into peptides. The peptides in each spot are analyzed by LC-MS/MS and proteins are identified using database search engines. In the qualitative Gel-LC-MS/MS-based proteomics approach, the proteins in the samples are first separated by one-dimensional SDS-PAGE. They are fractionated by slicing the gel into small pieces, and proteins in each gel piece are in-gel digested into peptides. The peptides are analyzed by LC-MS/MS and proteins are identified using database search engines.

1.2 MSU stimulation induces significant protein secretion from macrophages (II)

Bacterial cell wall component LPS is a TLR4 ligand and is a potent activator of macrophages (Dobrovolskaia, Vogel 2002, Nau et al. 2002). Secretomes of macrophages stimulated with LPS

alone, and with LPS priming prior to MSU stimulation were analyzed using both quantitative 2-DE-based proteomics, and qualitative Gel-LC-MS/MS-based proteomics (Figure 6). There were a total of 238 differentially expressed protein spots in the LPS-primed MSU-stimulated macrophage 2-DE gel when compared to the LPS-primed macrophage gel (II, Fig. 1). In the Gel-LC-MS/MS-based identifications, there were a total of 428, and 937 identified human proteins in the LPS-primed, and LPS-primed MSU-stimulated macrophage secretomes, respectively (II, Fig. 1). These results indicate that MSU stimulation of LPS-primed macrophages triggers significant protein secretion from macrophages, whereas LPS priming alone induces only modest protein secretion.

1.3 HSV-1 infection and MSU stimulation induce extracellular vesicle-mediated unconventional protein secretion from macrophages (I, II)

Protein secretion by eukaryotic cells can occur either through classical or unconventional protein secretion pathways (Dancourt, Barlowe 2010, Rabouille et al. 2012). GO annotation analysis according to the cellular compartment of the secreted proteins after HSV-1 infection of IFN-β-primed (I, Fig. 3b), and MSU stimulation of LPS-primed macrophages (II, Fig. 2a) revealed that most of the secreted proteins were annotated as being intracellular. Further analysis using SingnalP demonstrated that only a minority of the secreted proteins in both secretomes were predicted to have a signal sequence (I, Fig. 3c; II, Fig. 2c). This indicated that unconventional protein secretion could be a potential alternative pathway for the secretion of these proteins. Proteins identified in both secretomes were compared to the extracellular vesicle (EV) protein database ExoCarta. In the (iii) IFN-β-primed HSV-1-infected macrophage secretome, nearly 80% of the proteins with increased secretion were found in ExoCarta database (I, Fig. 3c). In addition, the majority (70%) of secreted proteins in LPS-primed MSU-stimulated macrophages were found in ExoCarta (II, Fig. 2c). These results clearly reveal that the robust protein secretion induced in both (iii) IFN-β-primed HSV-1-infected, and LPS-primed MSU-stimulated macrophages occurred through EV-mediated unconventional protein secretion pathways. Similarly, influenza A virus infection (Lietzén et al. 2011), and β-glucan stimulation (Öhman et al. 2014) of human primary macrophages have been reported to increase protein secretion through EV-mediated unconventional secretion pathways.

Experiments were designed to evaluate whether the proteins were being secreted through EV-mediated unconventional protein secretion mechanisms. Thus, EVs were enriched by ultrafiltration from cell supernatants collected from unstimulated, LPS-primed, MSU-stimulated, and LPS-primed MSU-stimulated macrophages. The EV-enriched fractions were analyzed using Western blotting for known EV-mediated unconventionally secreted proteins (annexin A1, galectin-3, HSP90). The levels of these three proteins were increased in the fractions enriched for EVs in the MSU-stimulated, and LPS-primed MSU-stimulated macrophage secretomes (II, Fig. 3a) confirming that MSU stimulation had activated unconventional protein secretion mediated by EVs from human primary macrophages. Taken together, these results suggest that EV-mediated cell-to-cell communication is utilized by activated macrophages as a general signalling mechanism.

Some viruses, such as poliovirus, can block classical protein secretion by interfering with the ER-trans-Golgi-network (Beske et al. 2007). The unconventional protein secretion routes that are evident in HSV-1-infected (I) and also in influenza A virus-infected (Lietzén et al. 2011) macrophages, could provide an alternative form of protein secretion in situations where the classical protein secretion mechanisms have been disrupted. Thus, in these virus infections, the unconventional protein secretion pathways could be considered as an additional host cell intrinsic defence mechanism.

1.4 Cathepsins and unconventional protein secretion in MSU-stimulated, and in HSV-1-infected macrophages (I, II)

Several cathepsins (B, D, S, and Z) were identified in the LPS-primed, LPS-primed MSU-stimulated, and also in the IFN-β-primed and/or HSV-1-infected macrophage secretomes. There were increased amounts of cathepsins D, S, and Z in the LPS-primed MSU-stimulated secretome when compared to the LPS-primed secretome (II, Table S1). Interestingly, the secretion of cathepsins B, D, S, and Z was reduced in (ii) HSV-1-infected secretome, while that of cathepsin D secretion was increased in (iii) the IFN-β-primed HSV-1-infected macrophage secretome (I, Table S1). These results illustrate significant differences in the secretion of cathepsins between (iii) IFN-β-primed HSV-1-infected (I) and LPS-primed MSU-stimulated (II) macrophages.

The intracellular expression of the pro-enzyme and enzymatically active mature form of cathepsins B and D measured by Western blotting revealed equal amounts of the mature forms of both cathepsins in control, LPS-primed and/or MSU-stimulated cells (II, Fig 4a). The mature form of cathepsin B was detected only in the LPS-primed MSU-stimulated macrophage secretome, whereas mature cathepsin D was detectable in both LPS-primed and/or MSU-stimulated macrophage secretomes (II, Fig 4b). Cathepsin inhibition has been shown to inhibit the activation of the NLRP3 inflammasome (Halle et al. 2008, Hornung et al. 2008, Kankkunen et al. 2010, Rajamäki et al. 2010). Inflammasomes and active caspase-1 have been postulated to mediate activation of unconventional protein secretion (Feldmeyer et al. 2007, Keller et al. 2008). ASC, caspase-1, and IL-18 were identified (II, Table 1, Fig. 4b & d), and the secretion of IL-1β and IL-18 was increased (II, Fig. 4b) in the LPS-primed MSU-stimulated macrophages demonstrating that the NLRP3 inflammasome was activated by MSU stimulation. The role of cathepsins in the unconventional protein secretion pathway was explored by inhibition of cathepsin activity, which reduced the overall protein secretion from LPS-primed MSU-stimulated macrophages (II, Fig. 4c). In addition, the secretion of known unconventionally secreted proteins IL-1β and IL-18 was completely inhibited (II, Fig. 4d), and the secretion of ASC was reduced in LPS-primed MSU-stimulated cells due to cathepsin inhibition (II, Fig. 4d). In addition, due to cathepsin inhibition, the secretion levels of interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), galectin-3, and lysosome-associated membrane glycoprotein 1 were reduced (II, Fig. 4d). The secretion of conventionally secreted proteins CCL2-4 was not affected by inhibition of cathepsin activity (II, Fig 4e). These results indicate an important role for cathepsins in the activation of unconventional protein secretion mediated by MSU stimulation.

1.5 The activity of Src, Pyk2, and PI3 kinases are essential for MSU-induced protein secretion in macrophages (II)

The activity of Src kinases is essential for inflammasome activation during influenza A virus infection (Lietzén et al. 2011). In LPS-primed MSU-stimulated macrophages after Src kinase inhibition, secretion of IL-β was not detected (II, Fig. 5a), and the overall protein secretion from LPS-primed MSU-stimulated cells was significantly reduced (II, Fig 5b). The inhibition of Src kinase in LPS-primed MSU-stimulated macrophages also resulted in the absence of any mature forms of cathepsin B and D in the cell culture supernatants (II, Fig 5c). Src, Pyk, and PI3 kinases are known to be involved together in certain signaling cascades in macrophages and neutrophils (Fuortes et al. 1999, Okigaki et al. 2003). As Src kinase was shown to have an essential role in protein secretion from macrophages during MSU stimulation, the roles of Pyk and PI3 kinase in this process were also investigated. They also were involved in the protein secretion from macrophages during MSU stimulation (II, Fig. 5d-i). Together these results show that the activity of Src, Pyk2, and PI3 kinases are essential for MSU-induced unconventional protein secretion. Src, Pyk2, and PI3 kinase act upstream of the cathepsins before the involvement of lysosomes. Their function has been linked

to phagocytosis (Araki et al. 1996, Berton et al. 2005, Owen et al. 2007) which may indicate that they are activated by phagocytosis of MSU.

1.6 Inhibition of inflammasome activation by HSV-1 infection (I)

The maturation of IL-1β into its biologically active form by HSV-1 infection has been reported in mice, indicating that HSV-1 can activate the inflammasome (Muruve et al. 2008). Inflammasome activation in HSV-1-infected human macrophages was investigated. Previous studies have revealed that IL-1β production in influenza A virus-infected macrophages is TLR-dependent (Rintahaka et al. 2008). Thus, TLR2 ligand Pam_3Cys was used to pre-treat macrophages before HSV-1 infection. MSU stimulation after Pam_3Cys pre-treatment was used since MSU is known to activate the NLRP3 inflammasome (Martinon et al. 2006) and this triggers the secretion of the biologically active form of IL-1β. MSU stimulation after Pam_3Cys pre-treatment caused a robust secretion of IL-1β from human macrophages, whereas HSV-1 infection after Pam_3Cys pre-treatment caused only modest secretion of IL-1β (I, Fig. 6a). To determine the biological activity of the secreted IL-1β, Western blotting was used to examine the concentrated cell supernatants, and total cell lysates from macrophages pre-treated with Pam_3Cys and/or infected with HSV-1, and pre-treated with Pam_3Cys and stimulated with MSU. HSV-1 infection alone, or Pam_3Cys pre-treatment together with HSV-1 infection did not result in the secretion of the biologically active form of IL-1β from human macrophages at 18 hpi (I, Fig. 6b). These results clearly demonstrate that inflammasomes are not activated upon HSV-1 infection in human macrophages during the later stages of infection. This indicates that HSV-1 can inhibit the activation of inflammasomes.

Recently it has been shown that PRRs γ-interferon-inducible protein 16 (IFI16) and NLRP3 are involved in inflammasome activation during early HSV-1 infection, and that later this activation can be inhibited by HSV-1 (Johnson et al. 2013). HSV-1 ICP0 protein was shown to be involved in the degradation of IFI16, and in addition, caspase-1 was shown to be trapped in actin clusters through an unknown mechanism, both contributing to the inhibition of inflammasome activation (Johnson et al. 2013). The results by Johnson *et al.* confirm results obtained in this work, that HSV-1 is capable of inhibiting inflammasome activation later in HSV-1 infection. These results also show that HSV-1 infection of human macrophages can activate unconventional protein secretion in the absence of inflammasome activation.

1.7 Secretion of endogenous danger signal proteins by macrophages in response to HSV-1 infection, and MSU stimulation (I, II)

Many cellular proteins have dual functions both inside the cell and in the extracellular space (Arnoys, Wang 2007). Endogenous danger signal proteins constitute one group of such proteins, which initiate their danger signal functions after being either actively secreted or passively released from cells to the extracellular space following injury or stress (Kono, Rock 2008, Lotze et al. 2007, Matzinger 1994, Matzinger 2002). Many of the endogenous danger signal proteins are known to be secreted from cells through the unconventional secretion pathways (Bianchi 2007). In the secretomes of both (iii) IFN-β-primed HSV-1-infected (I, Table 2), and LPS-primed MSU-stimulated (II, Table 2) macrophages, there were many endogenous danger signal proteins including annexins, galectins, HSPs, S100-proteins, and thioredoxin superfamily members. The secretion of 29 known or putative endogenous danger signal proteins was increased in the (iii) IFN-β-primed HSV-1-infected macrophages (I, Table 2) and 19 of such proteins in LPS-primed MSU-stimulated cells (II, Table I). Many of them had increased secretion also in the (ii) HSV-1-infected macrophage secretome (I, Table S1). Furthermore, many of the same endogenous danger signal proteins have been identified in influenza A virus-infected (Lietzén et al. 2011), and in the β-glucan-stimulated (Öhman et al. 2014)

human macrophage secretomes (Table 9). This indicates that endogenous danger signal proteins have an extensive role in many different inflammatory reactions.

1.7.1 Annexins

Intracellular annexins are known as Ca^{2+} sensors that translocate to the cellular membranes should there be an elevation in cytosolic Ca^{2+} levels (Draeger et al. 2011). They also function as regulators of plasma membrane architecture (Monastyrskaya et al. 2009), and inflammation (Lizarbe et al. 2013). In addition, annexins can be found in the extracellular space either attached to the cell membrane or in soluble forms. In both the (iii) IFN-β-primed HSV-1-infected (I), and LPS-primed MSU-stimulated (II) macrophage secretomes, there were six annexins (A1, A2, A4, A5, A6, and A11) (I, Table 2; II, Table 1 & Fig. 3a). The same annexins have also been identified with increased secretion in the influenza A virus-infected (Lietzén et al. 2011), and β-glucan-stimulated (Öhman et al. 2014) macrophage secretomes (Table 9**)**. Annexin A1 released by neutrophils has an extracellular anti-inflammatory function by inhibiting leucocyte migration (Solito et al. 2003). Cell membrane attached annexin A2, and A5, function as viral receptors in inducing the infection through their binding with viral surface components (Hertogs et al. 1993, Huang et al. 1996, Yang et al. 2011). In view of the capability of some of the annexins to bind viral surface components, it is possible that they may exert some extracellular antiviral function after their binding to the viral surface structures. For example, this might hinder the ability of the virus to enter the host cell by preventing viral interactions with host viral receptors, or induce the phagocytosis of these "marked" viruses. It is essential to ensure that the host immune response is kept in balance during its activation by viral infections, or by other stimuli such as MSU. The anti-inflammatory role of annexin A1 suggests that other extracellular annexins may well possess similar anti-inflammatory functions and perhaps also regulate the inflammatory response.

1.7.2 Galectins

Galectins are β-galactoside-binding lectins with dual functions found both inside and outside cells (Sato et al. 2009). The secretion of several galectins, including galectin-1 and -3, were increased in (iii) IFN-β-primed HSV-1-infected (I, Table 2 & Fig. 5b), and galectin-1, -7, and -9B from LPS-primed MSU-stimulated (II, Table 1 and Fig. 3a) macrophages. Galectin-1 and -3 have also been shown to be secreted from influenza A virus-infected (Lietzén et al. 2011), and β-glucan-stimulated (Öhman et al. 2014) macrophages, and galectin-9B from β-glucan-stimulated macrophages (Table 9). Both galectin-1 and -3 are secreted by activated macrophages (Liu et al. 1995, Sato, Hughes 1994, Zuniga et al. 2001), and HSV-1 infection induces their secretion (Gonzalez et al. 2005, King et al. 2009). Extracellular galectin-1 has anti-inflammatory functions and reduces macrophage activation and leucocyte recruitment (Rabinovich et al. 2002), whereas galectin-3 contains chemotactic functions attracting monocytes and macrophages (Sano et al. 2000). Induced galectin-1 secretion after HSV-1 infection has been postulated to be beneficial for the virus due to its anti-inflammatory functions (Gonzalez et al. 2005). In contrast, galectin-3 has opposing functions since it recruits inflammatory cells to the site of infection. It is possible that the balance between the secreted extracellular galectin-1 and -3 may regulate the direction of the inflammatory response. It is tempting to speculate that there may also be other possible extracellular functions of galectins. Galectin-1 has been shown to inhibit the viral envelope fusion to the host cell membrane in Nipah virus infection (Levroney et al. 2005). It has been speculated that this inhibition is mediated by galectin-1 interaction with Nipah virus N-glycans attached to its membrane glycoprotein (Lee 2007). Galectin β-galactoside-binding capabilities could facilitate the recognition of carbohydrate-containing viral PAMPs and promote the removal of these viral pathogens via phagocytosis, or prevent viruses from entering the host cell. The functions of extracellular galectin-7 and -9B are not known, but their

Table 9. Endogenous danger signal proteins with increased secretion in macrophages activated with different stimuli

	IFN-β+ HSV-1	LPS+ MSU	influenza A virus[a]	β-glucan[b]
Annexins				
Annexin A1	x	-	x	x
Annexin A2	x	-	x	x
Annexin A4	x	x	x	x
Annexin A5	x	x	x	x
Annexin A6	x	x	x	x
Annexin A11	x	x	x	x
Galectins				
Galectin-1	x	x	x	x
Galectin-3	x	-	x	x
Galectin-7	-	x	-	-
Galectin-9B	-	x	-	x
Heat shock proteins				
60-kDa heat shock protein, mitochondrial (HSP60)	x	-	-	x
78 kDa glucose-regulated protein (GRP78)	x	-	x	x
Endoplasmin (Gp96)	x	x	x	x
Heat shock 70 kDa protein 1A (HSP71)	x	-	x	x
Heat shock 70 kDa protein 4	x	-	x	x
Heat shock cognate 71 kDa protein (HSC70)	x	-	x	x
Heat shock protein 105 kDa	x	x	x	x
Heat shock protein beta-1 (HSP27)	x	x	x	x
Heat shock protein HSP 90-alpha	x	-	x	x
Heat shock protein HSP 90-beta	x	-	x	x
HSPE1 protein (HSP10)	x	-	x	x
Putative heat shock protein HSP 90-alpha A4	-	x	-	-
S100-A proteins				
Protein S100-A4	x	x	x	x
Protein S100-A6	x	-	x	x
Protein S100-A8	x	-	x	x
Protein S100-A9	x	-	x	x
Protein S100-A10	-	x	x	x
Protein S100-A11	x	x	x	x
Thioredoxin superfamily members				
Peroxiredoxin-1	x	-	x	x
Thioredoxin	x	x	x	x
Thioredoxin reductase 1, cytoplasmic	x	-	x	x
Other proteins				
Hepatoma-derived growth factor	-	x	x	x
Nucleophosmin	x	x	x	x
Parathymosin	-	x	x	x
Peptidyl-prolyl cis-trans isomerase A	x	-	x	x
Putative annexin A2-like protein	-	x	-	-
Putative heat shock protein HSP 90-alpha A4	-	x	-	-
Thymosin beta-4-like protein 3	-	x	x	-

(Lietzén et al. 2011[a], Öhman et al. 2014[b])
x, increase in secretion; -, no increase in secretion

secretion upon MSU stimulation from LPS-primed macrophages suggests they might also be involved in the regulation of inflammatory responses.

1.7.3 Heat shock proteins

HSPs are generally recognized for their intracellular chaperone functions i.e. they are assistants of proper protein folding, and as guardians of protein quality standards. They are also known as stress proteins (Richter et al. 2010) and as such, many HSPs have extracellular functions (Henderson, Pockley 2010). Because of their extracellular functions, they have been considered as endogenous danger signals (Bianchi 2007). Several HSPs had increased secretion in both the (iii) IFN-β-primed HSV-1-infected (I, Table 2 & Fig. 5c), and LPS-primed MSU-stimulated (II, Table 1, Fig. 3a) macrophages. There were a total of eleven HSPs with increased secretion in the (iii) IFN-β-primed HSV-1-infected cells. The secretion of most of these HSPs has been shown to be increased also in human macrophages infected with influenza A virus (Lietzén et al. 2011), and stimulated with β-glucan (Öhman et al. 2014) (Table 9). Only four HSPs were identified for which there was increased secretion in LPS-primed MSU-stimulated cells (Table 9). Extracellular HSPs have both pro- and anti-inflammatory functions. They can mediate macrophage activation (e.g. HSC70), and induce the secretion of different cytokines (e.g. HSP60) (Henderson, Henderson 2009, Osterloh, Breloer 2008). HSPs can also inhibit monocyte or lymphocyte activation (e.g. Gp96, HSPE1, HSP27, HSP60) (Cohen-Sfady et al. 2005, Henderson, Pockley 2010, Zanin-Zhorov et al. 2003). Extracellular HSPs clearly have a broad range of functions depending on the cellular environment in which they are located. Their roles in HSV-1 infection and MSU stimulation have been poorly characterized and require further investigation.

1.7.4 S100 proteins

S100 proteins are a diverse group of proteins with many different functions inside and outside the cell (Donato et al. 2013). The secretion of S100 proteins was increased in both the (iii) IFN-β-primed HSV-1-infected (I, Table 2 & Fig. 5c), and LPS-primed MSU-stimulated (II, Table 1) macrophages. S100-A4 and -A11 were secreted from both (iii) IFN-β-primed HSV-1-infected, and LPS-primed MSU-stimulated macrophages. Extracellular S100-A4 exerts various effects on leucocyte migration, and can stimulate cytokine production (Donato et al. 2013). The role of extracellular S100-A11 has not been characterized. There were several S100-A proteins whose secretion was increased in (iii) IFN-β-primed HSV-1-infected, but not in LPS-primed MSU-stimulated cells, including S100-A8 and -A9. They have been shown to be secreted also during influenza A virus infection (Lietzén et al. 2011), and β-glucan stimulation (Öhman et al. 2014) from human macrophages (Table 9). S100-A8 and -A9 have multiple anti- and pro-inflammatory functions in the extracellular space (Donato et al. 2013). Their role in viral infections is not known, but based on their known extracellular pro- and anti-inflammatory roles, they might be involved in controlling the immune response. S100-A10 secretion was increased in LPS-primed MSU-stimulated cells, but not in (iii) IFN-β-primed HSV-1-infected cells. S100-A10 has been shown to be involved in macrophage recruitment in response to inflammatory stimuli (O'Connell et al. 2010).

1.7.5 Thioredoxin superfamily members and other endogenous danger signal proteins

Thioredoxin superfamily members function intracellularly as oxidoreductases with the ability to catalyse oxidative protein folding via protein-protein interactions and covalent catalysis, acting as chaperones and isomerases (Henderson, Henderson 2009). The secretion of thioredoxin was increased in both (iii) IFN-β-primed HSV-1-infected (I, Table 2), and LPS-primed MSU-stimulated macrophages (II, Table S1). Extracellular thioredoxin is a unique chemoattractant for human leucocytes and it is known to be released by mononuclear phagocytes (Bertini et al. 1999, Nakamura

et al. 2001). The secretions of both peroxiredoxin-1 and thioredoxin reductase-1 were increased only in (iii) IFN-β-primed HSV-1-infected macrophages (Table 9). Extracellular peroxiredoxins have been implicated as ways of providing protection against ROS (Robinson et al. 2010). ROS are produced by macrophages during HSV-1 infection (Fujioka et al. 2000) and the secretion of peroxiredoxin-1 might be a cellular protective measure against ROS during HSV-1 infection. The secretion of the thioredoxin superfamily members was also increased in influenza A virus-infected (Lietzén et al. 2011), and β-glucan-stimulated (Öhman et al. 2014) human macrophages (Table 9), indicating that the extracellular thioredoxin superfamily members have a role in viral infections and in inflammatory responses in general**.** There were also other endogenous danger signal proteins with increased secretion (e.g. Hepatoma-derived growth factor, nucleophosmin) in the (iii) IFN-β-primed HSV-1-infected (I, Table 2), and LPS-primed MSU-stimulated macrophages (II, Table 1, Table S1) (Table 9). Several of these proteins have also been shown to be secreted from influenza A virus-infected (Lietzén et al. 2011), and β-glucan-stimulated (Öhman et al. 2014) human macrophages.

Together these results suggest that many of the endogenous danger signal proteins are secreted by activated macrophages through unconventional protein secretion pathways in response to multiple different stress factors. Additionally, they may have a major impact on host response and subsequent progression and outcome of viral infections, and inflammatory disorders.

1.8 Secretion of ISG-encoded proteins by macrophages in response to HSV-1 infection, and MSU stimulation (I, II)

When a macrophage recognizes an infecting virus via PRRs it often leads to the activation of the IFN-response. Type I and III IFNs form an important group of antiviral cytokines. Their secretion from infected cells in both paracrine and exocrine manners induces the transcription of ISGs (Borden et al. 2007) (Figure 2). In the absence of IFN stimulation, some ISGs can also be induced by other stimuli such as viral infections (Schneider et al. 2014). Bioinformatic analysis of proteins with increased secretion in (iii) IFN-β-primed HSV-1-infected macrophages using GO annotations identified many proteins with a biological function in immune and inflammatory response (I, Fig. 4b). Similarly, in LPS-primed MSU-stimulated macrophages, KEGG-pathway analysis revealed enrichment in those pathways involved in defence responses (II, Fig. 2f). Many ISG-encoded proteins were identified among the proteins with increased secretions in (iii) IFN-β-primed HSV-1-infected macrophage secretome (e.g. IFIT2, IFIT3, MxA, and STAT1) (I, Fig 5b, Table 1, Table S3). Their secretion was not increased as considerably in (i) IFN-β-primed, or (ii) HSV-1-infected cells (I, Table S3). Interestingly, several ISG-encoded proteins were also secreted from LPS-primed MSU-stimulated macrophages (II, Table 1 & Fig. 3d). Since certain viral infections can induce uric acid formation in the host cell (Boyes et al. 1989, Duhalde-Vega, Retegui 2011, Endo et al. 2008), it is noteworthy that MSU can stimulate the secretion of ISG-encoded proteins. The secretion of ISG-encoded proteins induced by MSU stimulation could be an indication that there might be a link between the virus-induced uric acid formation and host cell antiviral response. Uric acid could function as an additional antiviral signal that allows activation of antiviral responses in situations where other antiviral signalling mechanisms have been inhibited by the infecting virus. Secretion of ISG-encoded proteins has also been shown in influenza A virus-infected (Lietzén et al. 2011), and β-glucan-stimulated (Öhman et al. 2014) human macrophages (Table 10 on page 32) suggesting that macrophages secrete ISG-encoded proteins in response to several different stimuli.

1.8.1 Extracellular functions of ISG-encoded proteins

IFNs are known to induce the expression of ISGs which include several PRRs involved in HSV-1 recognition. Exposure of macrophages to type I IFNs has been shown to induce the intracellular expression of TLRs (Miettinen et al. 2001, Sirén et al. 2005, Tissari et al. 2005) and RLRs

(Rasmussen et al. 2009). The increased expression of these PRRs in macrophages sensitises the cells to detect HSV-1 infection and enhances their antiviral response after HSV-1 recognition. This may also explain the enhancing effect of IFN-β priming on ISG-encoded protein secretion from HSV-1-infected macrophages. The presence and functions of ISG-encoded proteins in the extracellular space have been poorly characterized. One of the few groups of ISG-encoded proteins that have been suggested to have an extracellular function is the 2´5´-oligoadenylate synthases (OAS) (Kristiansen et al. 2010). Interestingly OASs were not detected among the many ISG-encoded proteins that were secreted from (iii) IFN-β-primed HSV-1-infected (I, Table 1 & Table S3), or LPS-primed MSU-stimulated (II, Table 1) human macrophages. Certain other ISG-encoded proteins have also been shown to be secreted from cells in response to different stimuli. ISG15 is secreted from human macrophages in response to influenza A virus infection (Lietzén et al. 2011) (Table 10), and from monocytes and lymphocytes after IFN-β-stimulation (D'Cunha et al. 1996b, Knight, Cordova 1991). ISG15 has been shown to induce IFN-γ secretion from exposed cells leading to the activation of NK cells (D'Cunha et al. 1996a). The ISG15 secreted from (iii) IFN-β-primed HSV-1 infected (I, Table 1), and LPS-primed MSU-stimulated (II, Table 1) macrophages might thus have a role in the activation of NK cells also during these stimulations, as NK cells are involved in both antiviral defence and also in gouty inflammation (Gerosa et al. 2002, Vogel et al. 2014).

Table 10. ISG-encoded proteins with increased secretion in human macrophages activated with different stimuli

	IFN-β+ HSV-1	**LPS+ MSU**	**Influenza A virus[a]**	**β-glucan[b]**
Caspase-1	-	x	-	x
Complement factor B	x	x	-	x
Cytosol aminopeptidase	x	x	x	x
Galectin-3-binding protein	x	-	-	-
Galectin-9	-	x	-	x
IFIT1	x	x	-	-
IFIT2	x	x	-	-
IFIT3	x	x	-	x
Indoleamine 2,3-dioxygenase 1	-	x	-	-
Interferon-induced 35 kDa protein (IFI35)	-	x	-	x
Interferon-induced guanylate-binding protein 1 (GBP1)	-	x	x	x
Interferon-induced guanylate-binding protein 2 (GBP2)	-	x	x	x
ISG15	x	x	x	-
Microtubule-associated protein RP/EB family member 1	x	x	x	x
MxA	x	x	-	x
MxB	-	x	-	-
Proteasome subunit alpha type-3	x	x	x	x
Proteasome subunit beta type-9	x	-	-	x
STAT1	x	x	-	x
Talin-1	x	-	x	x
Ubiquitin/ISG15-conjugating enzyme E2 L6	-	x	-	-
Zyxin	x	x	x	x

(Lietzén et al. 2011 [a], Öhman et al. 2014[b])
x, increase in secretion; -, no increase in secreion

The ovine homolog of human MxA, oMx1, has been shown to be secreted by ovine uterine epithelial cells (Toyokawa et al. 2007a). Its extracellular function is unknown, but intracellular oMx1 has been postulated to function as a mediator of unconventional protein secretion and to terminate in the extracellular space through this secretory function (Toyokawa et al. 2007b). The secretion of MxA was increased from both (iii) IFN-β-primed HSV-1-infected (I, Table 1 & Fig. 5b), and LPS-primed MSU-stimulated (II, Table 1 & Fig. 3d) macrophages, and it has been shown to

be secreted from β-glucan-stimulated human macrophages (Öhman et al. 2014) (Table 10). Considering the robust unconventional protein secretion activated by these stimulations, one could speculate that the extracellular presence of MxA could mirror its role in the regulation of unconventional protein secretion also in human macrophages. One cannot of course exclude the possibility that extracellular MxA might also have an antiviral role, as it is known to have antiviral functions inside the cell (Schneider et al. 2014).

There are four known human IFIT proteins, IFIT1, -2, -3, and -5. They have been claimed to possess multiple intracellular antiviral and inflammatory functions (Diamond, Farzan 2013, Vladimer et al. 2014), but their extracellular role has not been characterized. Increased amounts of IFIT1, -2, and -3 were detected in the secretomes of (iii) IFN-β-primed HSV-1-infected (I, Table 2 & Fig. 5b), and LPS-primed MSU-stimulated (II, Table 1 & Fig. 3d) human macrophages. Recently it has also been shown that IFIT3 is secreted from β-glucan-stimulated human macrophages (Öhman et al. 2014) (Table 10). Intracellular IFIT1 can bind viral 5´PPP-RNA and prevent its translation; furthermore IFIT2 and IFIT3 have been reported to bind IFIT1 forming together a protein complex (Pichlmair et al. 2011). Intracellular IFIT1 can also bind 2´O-unmethylated RNA and block its translation (Habjan et al. 2013). It could be that secreted IFITs function as extracellular PRRs that can recognize viral nucleic acids. The increased amounts of IFIT1, -2, and -3 that were also present in the LPS-primed MSU-stimulated (II), and IFIT3 in β-glucan-stimulated macrophage secretome suggest that they might alternatively have a more general stress-related function, perhaps functioning as endogenous danger signal proteins.

Taken together, these results on the secretion of ISG-encoded proteins from macrophages indicate that rather than merely having only intracellular functions these proteins may also be active in the extracellular space. In addition to having antiviral roles in the extracellular space, the extracellular ISG-encoded proteins may also be involved in inflammation and/or in the regulation of unconventional protein secretion. Further work will be required to clarify their extracellular roles in viral infections and in inflammatory responses in general.

1.9 Chemokine secretion from macrophages upon HSV-1 infection, and MSU stimulation (I, II)

Although the majority of the proteins in HSV-1-infected, and in MSU-stimulated macrophage secretomes were secreted through the unconventional pathways, there were also classically secreted proteins present in the extracellular space. Chemokines are known to be secreted through the classical pathway. They attract inflammatory cells to the sites of infection, and inflammation. Chemokines were secreted from (iii) IFN-β-primed HSV-1-infected (I, Fig. 5a), and LPS-primed MSU-stimulated (II, Fig. 3b, Table 1) macrophages. In the HSV-1-infected macrophage secretomes, there was only one chemokine C-X-C motif chemokine 10 (CXCL10) that could be quantified by the MS-based proteomic analysis. However, the Luminex assay for chemokines CCL2-5 confirmed also their presence in the secretomes, and that the amounts of all four chemokines were highest in the (iii) IFN-β-primed HSV-1-infected macrophage secretome (I, Fig. 5a), as was the case with CXCL10. The secretion of the same five chemokines was also increased in LPS-primed MSU-stimulated cells (II, Table 1, Fig. 3b) and their amounts were much greater than in (iii) IFN-β-primed HSV-1-infected cells. There were also other chemokines with increased secretion in LPS-primed MSU-stimulated macrophages (II, Table 1). These results confirm the already observed phenomenon that a second signal is required for the appearance of robust protein secretion also with conventionally secreted proteins in both LPS-primed, and HSV-1-infected macrophages.

The key secretome results from this work are summarized in Figure 7 (page 34). Robust EV-mediated unconventional protein secretion was induced by IFN-β priming in HSV-1-infected (I), and by MSU stimulation in LPS-primed (II) human macrophages. The secretion of endogenous danger

signal proteins, and ISG-encoded proteins was elevated in both (iii) IFN-β-primed HSV-1-infected (I), and LPS-primed MSU-stimulated (II) macrophages. Another important finding was that HSV-1 was able to inhibit activation of the inflammasome (I), and that the activity of Src/Pyk2/PI3 kinases and cathepsins were essential for the unconventional protein secretion from MSU-stimulated macrophages (II).

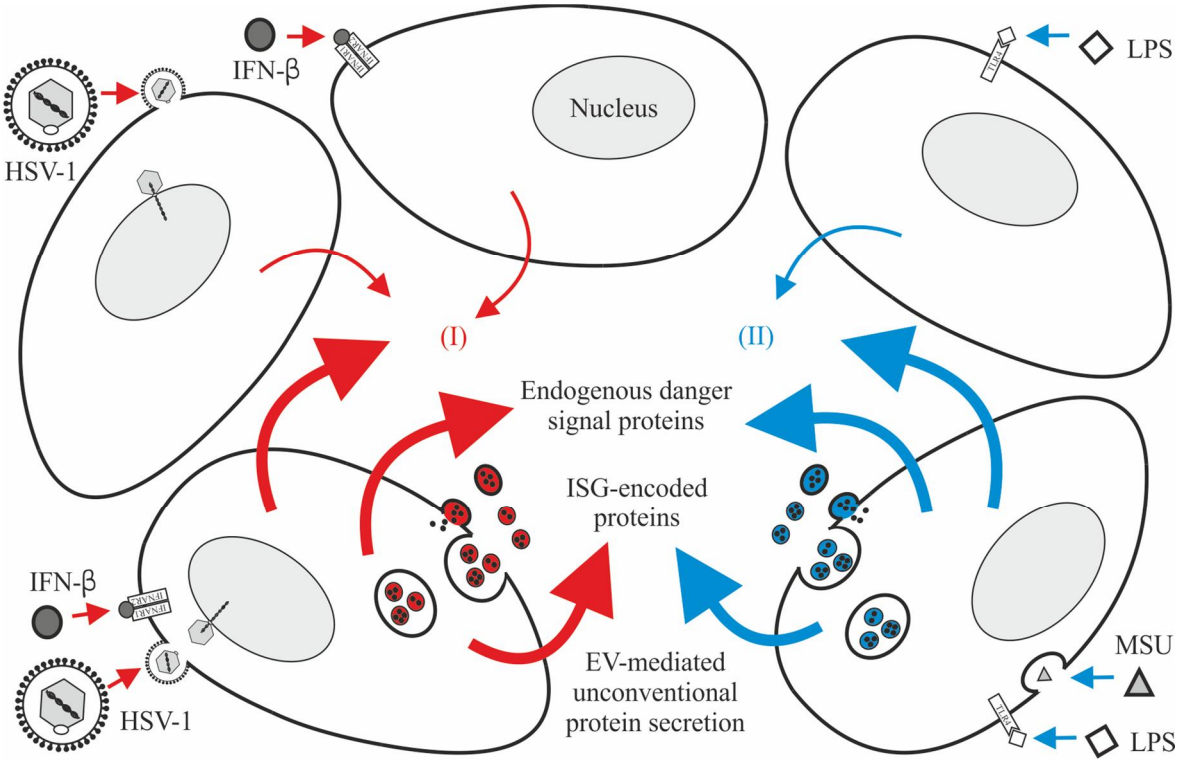

Figure 7. Summary of the key secretome results from macrophages stimulated with IFN-β and/or infected with HSV-1, and stimulated with LPS or first primed with LPS and then stimulated with MSU. Robust protein secretion is induced only after interferon (IFN)-β-priming in Herpes simplex virus 1 (HSV-1)-infected (I), and monosodium urate (MSU) stimulation in lipopolysaccharide (LPS)-primed (II) human macrophages indicated by thick arrows. There is also some protein secretion from IFN-β-stimulated (I), HSV-1-infected (I), and LPS-stimulated (II) macrophages indicated by thin arrows. Protein secretion from macrophages after IFN-β priming and/or HSV-1-infection is indicated with red arrows (I), and after LPS priming and MSU stimulation with blue arrows (II). Most of the proteins are secreted through the extracellular vesicle (EV)-mediated unconventional protein secretion pathway (I, II). The secretion of endogenous danger signal proteins and interferon-stimulated gene-encoded proteins is increased in both INF-β-primed HSV-1-infected (I), and LPS-primed MSU-stimulated (II) macrophages.

2. Role of ICP27 protein in the inhibition of the IFN response and apoptosis in HSV-1-infected human primary macrophages (III)

In order for a virus to replicate successfully in the host cell and to infect more of the surrounding cells, it often needs to tamper with the host innate immune response mechanisms. HSV-1 can adopt multiple different evasion mechanism including the inhibition of the IFN-response and inhibition of apoptosis during a lytic infection (Melchjorsen et al. 2009, Nguyen, Blaho 2007) (Tables 4 & 5). ICP27 is a multifunctional HSV-1 protein playing these kinds of roles in both of these inhibition processes (Sandri-Goldin 2011). It has both a nuclear and cytoplasmic presence in the host cell and it can translocate between the two cell compartments via its NES- and NLS-sequences. The roles of ICP27 NES- and NLS-sequence in the inhibition of the IFN-response have not been previously characterized, and their roles in the inhibition of apoptosis have been characterized only partially.

2.1 Cytosolic proteome characterization of wt HSV-1-, dLeu-, and d3-4-infected macrophages

The cytosolic proteomes of human macrophages infected with wt HSV-1, or ICP27 N-terminal mutant virus dLeu, and d3-4 (Table 6) (III, Fig. 1a) were characterized using iTRAQ labeling combined with LC-MS/MS (Figure 6). A total of 825 human (III, Fig. 2c & Table S2) and 17 HSV-1 (III, Table 1) proteins were identified. Quantification data was obtained from 752 human and 14 HSV-1 proteins, respectively (III, Fig. 1c). There were more down-regulated human proteins in the wt HSV-1-infected macrophage cytosolic proteome than in dLeu-, and d3-4-infected cells (III, Table S2) this difference being especially apparent in the down-regulation of ribosomal proteins (III, Table 2). HSV-1 can inhibit host protein expression through several viral proteins (Melchjorsen et al. 2009). In the dLeu- and d3-4-infected macrophages, there was reduced HSV-1 protein expression when compared to the wt HSV-1-infected cells (III, Table 1). These results are in line with data published by Lengyel *et al.*, and Mears *et al.*, demonstrating that dLeu, and d3-4 viruses show reduced viral replication efficiency (Lengyel et al. 2002, Mears et al. 1995). This could also be reflected on the differences of host protein expression levels between wt HSV-1- and the mutant virus-infected cells.

The wt HSV-1-infected macrophage cytosolic proteome included two human proteins with significant down-regulation in their expression levels: apolipoprotein E (ApoE) and transferrin receptor protein 1 (TfR1) (III, Table 2**)**. ApoE expression was down-regulated in all three, wt HSV-1, dLeu, and d3-4 virus infections. Especially in wt HSV-1-infected macrophages, its expression was down-regulated dramatically. This was also reflected in the HSV-1-infected macrophage secretome where there was reduced secretion of ApoE (I, Table S1). Classical activation of macrophages by proinflammatory cytokines such as IFN-γ, TNF-α, and IL-1β can down-regulate ApoE production in macrophages (Colton et al. 2005, Zhang et al. 2011a, Zhang et al. 2010). It has been proposed that extracellular ApoE competes with HSV-1 for binding to heparan sulfate proteoglycans (Itzhaki, Wozniak 2006) (III, Table 2). This could explain why ApoE is down-regulated in the wt HSV-1 infected cell cytosol and secretome. TfR1 was down-regulated in wt HSV-1 infected macrophages but its expression did not change after dLeu or d3-4 infection. It is involved in cellular iron metabolism (Drakesmith, Prentice 2008), and its expression is known to be decreased in macrophages in response to inflammatory ligands (Kim, Ponka 2000). The roles of ApoE and TfR1 in HSV-1 infection, as well as the role played by ICP27 in the inhibition of their expression are not clear.

The levels of annexin A1, A2, A5, and A11 were up-regulated in the wt HSV-1-infected macrophage cytosolic proteome (III, Table 2). Annexin A1 and A5 were also up-regulated in dLeu- and d3-4-infected cells although not as strongly as after wt HSV-1 infection. Annexin A2 and A11 expression levels did not change after mutant virus dLeu- and d3-4 infections. Annexin A2 has been implicated in viral infections having a role in viral entry and intracellular trafficking (Dziduszko, Ozbun 2013, Yang et al. 2011), and in the creation of infectious viral particles (Backes et al. 2010, Ma et al. 2004). The roles of annexins in HSV-1 infection have not been characterized, although annexin A2 has been identified in the intact HSV-1 virion tegument (Padula et al. 2009), and this protein has been shown to be enriched in HSV-1 infected cell nuclei (Sanchez-Quiles et al. 2011). The present results suggest that annexins play a role during HSV-1 infection in human macrophages.

2.2 Inhibition of the IFN response by HSV-1 ICP27 protein in macrophages (III)

ICP27 is involved in inhibition of the IFN response during HSV-1 infection where it inhibits the activation of transcription factors IRF3 and NF-kB, leading to the inhibition of type I and type III IFN production (Melchjorsen et al. 2006) and the subsequent loss of their antiviral functions (Melchjorsen et al. 2009). ICP27 can also prevent IFN signaling by inhibiting STAT-1α

phosphorylation and its subsequent nuclear translocation which is required for IFN-mediated ISG expression in infected cells (Johnson et al. 2008). This has been shown to be mediated by an unknown soluble IFNAR1 antagonist protein that is secreted from HSV-1-infected cells in an ICP27-dependent manner (Johnson, Knipe 2010).

2.2.1 ICP27 NES and NLS are required for the inhibition of IFN response by HSV-1

The comparison of reliably quantified cytosolic proteins from HSV-1-infected macrophage cytosol with the Interferome database revealed 12 differentially expressed interferon-regulated proteins (III, Table 2) including antiviral ISG-encoded proteins IFIT2 and MxA. Both proteins were up-regulated in dLeu- and d3-4-infected human macrophages, but not in wt HSV-1 virus infection. In addition, several other ISG-encoded proteins were also up-regulated in response to dLeu and d3-4 infection, including IFIT3, ISG15, and viperin (III, Fig. 2a & S1). These results show that the inhibition of the IFN response is impaired in dLeu- and d3-4-infected macrophages, indicating that NES- and NLS-sequences are required for efficient inhibition of the IFN response.

The mRNA expression kinetics of type I IFNs (IFN-α/-β) and type III IFN (IL-29) from uninfected, wt HSV-1-, dLeu-, and d3-4-infected macrophages were compared at different time points. During wt HSV-1 infection, the mRNA levels for the three IFNs reached their peaks at 6 hpi after which they started to decline (III, Fig. 2b). In dLeu-, and d3-4-infected cells, the mRNA levels of all 3 IFNs were clearly higher at all measured time points in comparison with the wt HSV-1-infected cells. These results confirm that type I and type III IFN expression is up-regulated in dLeu- and d3-4-infected macrophages indicating that the inhibition of NF-κB, and IRF3 (Melchjorsen et al. 2006) are impaired, leading to the increased IFN production.

Analysis of mRNA expression kinetics of ISGs IFIT3 and viperin showed that there was a clear difference in their expression in dLeu- and d3-4-infected macrophages when compared to wt HSV-1 infection (III, Fig. 2b). In the wt HSV-1-infected macrophages, IFIT3 and viperin mRNA expression stayed at very low levels at all measured time points, whereas in dLeu- and d3-4-infected cells, their expression was clearly up-regulated (III, Fig. 2b). The protein expression kinetics of IFIT2, IFIT3, ISG15, MxA, and viperin were also compared in uninfected, wt HSV-1-, and dLeu-infected macrophages at different time points (III, Fig. 2c). After wt HSV-1 infection, the expression of IFIT2, IFIT3, MxA, and viperin remained at a low constant level even at 24 hpi (III, Fig. 2c), whereas ISG15 briefly peaked at 6 hpi and then returned to its basal level (III, Fig. 2c). After dLeu infection, for all of the five measured ISG-encoded proteins, the expression levels increased already after 6 hpi and were still increasing after 24 hpi, and were much higher than in the wt HSV-1-infected cells (III, Fig. 2c). These results confirm that also the IFN-mediated signalling, which is inhibited by ICP27 in wt HSV-1-infected cells (Johnson et al. 2008, Johnson, Knipe 2010), is active and functioning in dLeu- and d3-4-infected cells. These results clearly demonstrate that the ICP27 N-terminal NES- and NLS-sequences are both required for the inhibition of the IFN response at the level of IFN production, and also at the level of ISG induction. They also suggest that the inhibitory function of ICP27 requires its translocation to the nucleus and back to the cytosol. Considering the known functions of nuclear ICP27, that require both the NES- and NLS-sequences, one could speculate that the observed effect of dLeu and d3-4 infection is related to ICP27's nuclear role in promoting the export of viral intronless mRNAs into the cytosol (Sandri-Goldin 2011).

2.2.2 Viperin expression in HSV-1 infection

Viperin has been proposed to mediate its functions through the disruption of ER-derived lipid rafts that transport viral envelope proteins to the plasma membrane (Tan et al. 2012, Wang et al. 2007). It is also involved in preventing viral replication, as well as assembly, budding and release from the host cell. Viperin is a versatile protein with many functions which interfere with the life cycles of multiple viruses (Helbig, Beard 2014). Interestingly, the expression of viperin was induced in dLeu-

and d3-4-infected macrophages but not in wt HSV-1-infected cells (III, Fig. 2). In order to investigate its role in HSV-1 replication, viperin was silenced via delivery of siRNA into human macrophages prior to dLeu, and d3-4 infection. The expression levels of mRNAs for HSV-1 genes encoding ICP27 (IE), ICP8 (E), gB (L), and VP16 (L), were quantified. No significant changes were detected in the expression levels of these HSV-1 genes after 6 hpi of dLeu or d3-4 infection in viperin-silenced human macrophages (III, Fig. 3a**)**. Protein expression levels were also measured using Western blotting for HSV-1 glycoproteins gB, gD, and gH to determine the role of viperin in HSV-1 replication at 18 hpi (III, Fig. 3b). No changes were detected in HSV-1 glycoprotein expression after dLeu or d3-4 infection in viperin-silenced cells. These results reveal that viperin does not have any effect on HSV-1 replication, but do not exclude the possibility that it could have some other anti-HSV functions, such as inhibiting HSV-1 assembly, or budding and release from the host cell. The results also suggest that ICP27 could be involved in the inhibition of viperin expression. Recently it has been shown that HSV-1 is able to inhibit viperin expression by virion host shut-off protein (VHS)-mediated degradation of viperin mRNA (Shen et al. 2014). Viperin was also shown not to have any effect on HSV-1 replication in viperin over-expression experiments (Shen et al. 2014). Interestingly, ICP27 has been shown to interact with VHS (Taddeo et al. 2010) although the ICP27 interaction site is not currently known. It could be speculated that there is a link between the VHS and ICP27 interaction and in the degradation of viperin mRNA. This link may be disrupted in dLeu- and d3-4-infected cells leading to reduced degradation of viperin mRNA and in the increased expression of viperin protein.

2.3 ICP27 NES is required for the inhibition of apoptosis in HSV-1 infection (III)

Early in HSV-1 infection apoptosis is activated, but very soon the virus begins to inhibit this phenomenon (Aubert et al. 1999, Nguyen, Blaho 2007, Sanfilippo, Blaho 2006). There are multiple HSV-1 proteins that have been shown or postulated to participate in the inhibition of apoptosis, including ICP27 (Aubert, Blaho 1999, Nguyen, Blaho 2007) although its mechanism is not understood. It has been shown that the ICP27 C-terminus is required to allow HSV-1 to inhibit apoptosis in infected cells, and the N-terminal NES-sequence has been suggested to have a role in this process (Aubert et al. 2001, Lengyel et al. 2002). Activation of apoptosis in the HSV-1 ICP27 NES deletion mutant dLeu-infected cells has not been previously studied. To investigate the role of ICP27 NES- and NLS-sequences in inhibition of apoptosis in human macrophages, the cytosolic proteomes from wt HSV-1-, dLeu-, and d3-4-infected macrophages was compared to the ApoptoProteomics database (Arntzen, Thiede 2012). There were a total of eleven apoptosis-related proteins in which there was statistically significant up- or down-regulation (III, Table 2). There were also other apoptosis-related proteins with clear changes in their expression in the human macrophage cytosol**.** Multiple histones were up-regulated only in dLeu-infected cells (III, Fig. 4a & b). Histones are known to be released from the nucleus into the cytosol during apoptosis (Wu et al. 2002). The triggering of apoptosis in dLeu-infected cells was confirmed by analyzing the activation of caspase-3 in HSV-1-infected human macrophages at 18 hpi (III, Fig. 4b). Increased levels of active caspase-3 were detected only in the dLeu-infected human macrophages indicating that there had been activation of apoptosis. These results show that dLeu infection induces the activation of apoptosis in human primary macrophages and thus confirms the requirement of the ICP27 N-terminal NES-sequence in the inhibition of apoptosis by HSV-1. The requirement that NES should be present to allow ICP27 to inhibit apoptosis suggests that ICP27 protein needs to be in the host cell's cytosol if it is to inhibit apoptosis. However the mechanism by which cytosolic ICP27 can inhibit apoptosis remains unknown.

Considering that ICP27 has both nuclear and cytosolic functions, it is also possible that the nuclear properties of ICP27 are involved in the inhibition of apoptosis. ICP27 has been speculated to elicit its anti-apoptotic functions indirectly through its ability to up-regulate early and

late anti-apoptotic viral gene products (Nguyen, Blaho 2007). Cells infected with dLeu display defects in both viral gene expression and viral replication (Johnson, Sandri-Goldin 2009, Lengyel et al. 2002, Strain, Rice 2011). dLeu ICP27 has also been shown to be unable to interact with several of wt ICP27s interaction partners involved in the regulation of viral gene products. These include RNAPII (Dai-Ju et al. 2006), HSC70 (Li et al. 2008), TAP/NXF1 (Johnson, Sandri-Goldin 2009), and nuclear pore glycoprotein p62 (Malik et al. 2012). The failure of dLeu ICP27 to interact with these proteins could lead to diminished levels of HSV-1 early and late anti-apoptotic viral gene products, leading to significant defects in the inhibition of apoptosis caused by dLeu infection.

Cathepsins are known to act in concert with caspases in apoptotic cell death (Johansson et al. 2010). They have been implicated in the triggering of apoptosis by several different RNA viruses (Lietzén et al. 2011, Rintahaka et al. 2011). Cathepsins were also identified in the macrophage cytosolic proteome after HSV-1 infection (III, Table 2 & Fig. 4c). The cytosolic expressions of cathepsins D and Z were down-regulated by wt HSV-1 infection whereas no change was observed in dLeu- or d3-4-infected macrophages (III, Fig. 4c). Pretreatment of macrophages with the cathepsin inhibitor, Ca-074Me, prior to infection with dLeu showed reduced caspase-3 activation in human macrophages (III, Fig. 4d) indicating a possible link between cathepsin activation and ICP27. This suggests that ICP27 could inhibit cathepsin activation during HSV-1 infection, and also that it plays a role in the inhibition of apoptosis.

2.4 RLRs RIG-I and MDA5 in HSV-1-mediated activation of apoptosis (III)

RLRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are known to recognize viral pathogens including HSV-1 in the cytosol by binding viral RNA (Melchjorsen et al. 2010, Rasmussen et al. 2009). RIG-I has also been shown to have a role in the activation of apoptosis by sensing Sendai virus and paramyxovirus infections, and transfected dsRNA (Chattopadhyay et al. 2010, Rintahaka et al. 2008). MDA5 has also been indicated as participating in the induction of apoptosis (Kang et al. 2004). The roles of RLRs MDA5 and RIG-I in activation of apoptosis by HSV-1 infection were characterized in human macrophages. Simultaneous silencing of MDA5 and RIG-I gene expression prior to dLeu infection reduced caspase-3 activation when compared to macrophages silenced using control siRNA and infected with dLeu (III, Fig. 5). In contrast, silencing of IFI16 exerted no effect on caspase-3 activation during dLeu infection. These results suggest that MDA5 and/or RIG-I are involved in the activation of apoptosis in response to HSV-1 infection.

The HSV-1 ICP0 mRNA transcript has been shown to be the activator of apoptosis during early HSV-1 infection (Sanfilippo, Blaho 2006). Currently the host receptor(s) that recognizes the ICP0 transcript and leads to activation of apoptosis is unknown. It is tempting to speculate based on the capability of RIG-I and MDA5 to bind viral RNA and to activate apoptosis that one or both of the receptors could recognize HSV-1 ICP0 transcripts in the cytosol and be responsible for the HSV-1-mediated activation of apoptosis. HSV-1 Us11 late protein has recently been shown to bind RIG-I and MDA-5 and to prevent their downstream signaling, leading to inhibition of IFN-β production by some unknown mechanism (Xing et al. 2012). Interestingly, dLeu-infected cells have been reported to have reduced expression of Us11 (Strain, Rice 2011). As the wt HSV-1 virus can inhibit apoptosis after a brief activation period, it could be that Us11 plays a role in this inhibition process through its interactions with RIG-I and MDA-5, perhaps by preventing them in some way from binding to the ICP0 transcript. It could be speculated that cells infected with dleu, having less Us11 protein, cannot properly inhibit RIG-I and MDA5 from binding to the ICP0 transcript and this causes increased activation of apoptosis.

The key results from the quantitative proteomic analysis of the cytosolic proteome of human macrophages infected with wt HSV-1, and mutants d3-4 and dLeu are shown in Figure 8. These

results reveal that dLeu and d3-4 are unable to inhibit the host IFN response in macrophages, indicating that both the ICP27 NES- and NLS-sequences are required for the inhibition of the IFN response. In addition, dLeu infection, but not d3-4 infection, activates apoptosis, confirming that the ICP27 NES-sequence is required for the inhibition of apoptosis. In addition, also the results indicating that RLRs RIG-I and/or MDA5 seem to participate in the activation of apoptosis induced by HSV-1 infection are of importance.

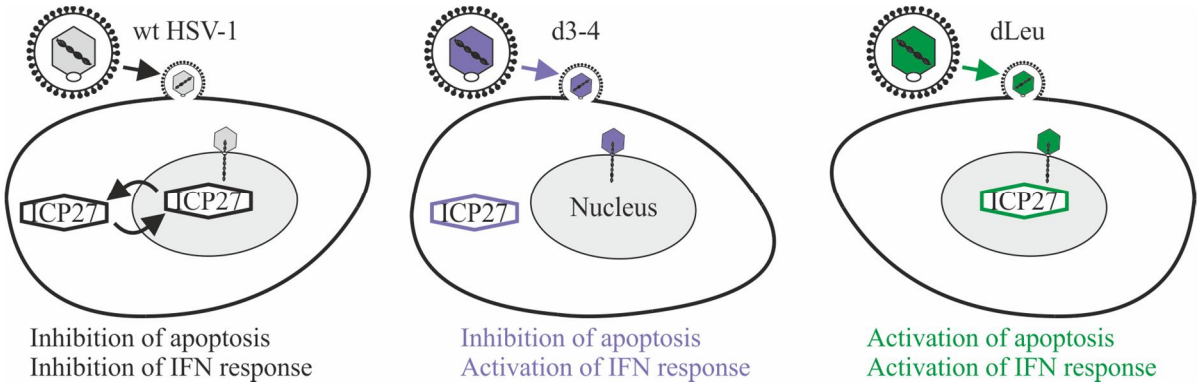

Figure 8. Role of ICP27 protein in the inhibition of the IFN response and apoptosis in HSV-1-infected human primary macrophages. The wt Herpes simplex virus 1 (HSV-1) infected cell protein 27 (ICP27) (black outline) possesses both the nuclear export sequence (NES) and nuclear localization sequence (NLS) required for its nuclear import and export. The ICP27 which contains both NES- and NLS-sequences is capable of inhibiting both apoptosis, and the interferon (IFN) response. The ICP27 mutant virus d3-4 has an amino (N)-terminal deletion of the NLS-sequence causing an enrichment of ICP27 (violet outline) in the cytosol. d3-4 virus is able to inhibit apoptosis, but is not able to inhibit the IFN response (III). The ICP27 mutant virus dLeu has an N-terminal deletion of the NES-sequence causing an enrichment of ICP27 (green outline) within the nucleus. dLeu virus is not able to inhibit apoptosis, or the IFN response (III). These results clearly show that the ICP27 NES- and NLS-sequences are both required for the inhibition of the IFN response, and that NES-, but not the NLS-sequence, is required for the inhibition of apoptosis.

V CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The innate immune system can be activated by exogenous and endogenous factors. HSV-1 is a common virus causing a life-long infection. In many individuals, it does not display its pathological side instead it remains in its latent form after the primary infection. However, in other infected individuals it can cause re-occurring painful although not life-threatening infections. Finally, in individuals with a compromised immune system, such as patients with immune deficiencies due to a disease or specific therapies, HSV-1 can cause severe infections. Furthermore, individuals with certain genetic deficiencies are more prone to frequent and more severe HSV-1 infections. At present, there is no cure available for HSV-1 infection, only therapies that can alleviate the symptoms and shorten the time of an acute infection.

Endogenous molecules that are involved in the activation of the innate immune system can cause autoinflammatory diseases and participate in exogenous pathogen infection-induced signaling. One endogenous molecule, uric acid, is involved in both the autoinflammatory disease gout and in certain viral infections. The crystallized extracellular form of uric acid, MSU, is a potent activator of the innate immune response, and is the triggering factor for gouty inflammation. One important part of the innate immune response in general is the secretion of a variety of proteins and other molecules by activated immune cells. Currently, the proteins secreted by these activated immune cells and the mechanisms by which they are secreted have been characterized only partially.

Proteomics provides excellent tools for the large-scale characterization of proteins from different types of samples including intracellular and extracellular cell fractions (e.g. cytosolic, extracellular proteins). The continued developments in these technologies can be predicted to continue, providing ever more useful research methods for the study of proteins. These methodologies already allow the quantitative measurement of differences in the expression of thousands of proteins between different samples in one experiment, the study of protein PTMs and protein-protein interactions. The integration of the data gathered using these different proteomics methods with the data obtained with other fields of “omics” (e.g. genomics, transcriptomics) will lead to a deeper understanding of different biological processes.

In this thesis, new light has been shed on protein secretion by human macrophages after activation of innate immune response by the DNA virus HSV-1 infection, as well as by stimulation with MSU, the crystallized extracellular form of the DNA metabolite uric acid. In addition, the mechanisms by which HSV-1 inhibits the innate immune response were investigated. This work provides the first quantitative MS-based proteomic characterization of secretomes from human macrophages either infected with HSV-1, or stimulated with MSU. These results show that the robust protein secretion from macrophages occurring after the activation of inflammatory response requires either IFN-β priming prior to HSV-1 infection or MSU stimulation after LPS priming. The protein secretion from macrophages occurred mainly via EV-mediated unconventional secretion pathways, indicating that these pathways are being utilized by macrophages in mediating the many different inflammatory responses induced by a variation of stimuli. The secretion of endogenous danger signal proteins and ISG-encoded proteins was especially increased in IFN-β-primed HSV-1-infected, and LPS-primed MSU-stimulated macrophages, indicating that there are similarities in the profiles of the proteins being secreted. The inflammasome was not activated by HSV-1 infection in human macrophages, suggesting that HSV-1 can inhibit the activation of inflammasomes, and revealing that the inflammasome is not involved in the unconventional protein secretion during HSV-1 infection. HSV-1 ICP27 N-terminal NES- and NLS-sequences were required for inhibition of the IFN response, indicating that the translocation of this protein back and forth between the host cell nucleus and cytosol is needed during this process. The NES-sequence was required for the inhibition of apoptosis suggesting that one of the functions of ICP27 in the cytosol is to mediate this inhibition. The RLRs RIG-I and/or MDA5 were shown to have a role in the

activation of apoptosis during HSV-1 infection, possibly being the first identified PRRs that play a role in activation of apoptosis after HSV-1 infection.

In the future, it will be important to clarify the role of EV-mediated unconventional protein secretion during innate immune responses activated by different viruses and other inflammatory stimuli. This will be needed in order to elucidate which proteins are secreted specifically by distinct stimuli, and which are secreted in a more general fashion through these pathways. The extracellular biological functions of the secreted proteins should also be addressed in more detail, e.g. clarifying the extracellular functions of many ISG-encoded proteins that currently are not known. Further studies will be needed to determine how HSV-1 ICP27 achieves the inhibition of IFN response and apoptosis. Although the requirement of NES- and NLS-sequences in the inhibition of IFN response, and NES-sequence in inhibition of apoptosis is evident, the molecular basis for this phenomenon needs to be further elucidated. In-depth knowledge on the innate immune response mechanism of actions during viral infections and autoinflammatory diseases, and on the viral countermeasures against the host response will lay a solid foundation for the development of novel, more efficient, therapies.

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