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Innate Immune Recognition of RNA-Virus Infection in Human Macrophages

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(née Rintahaka)

ACADEMIC DISSERTATION

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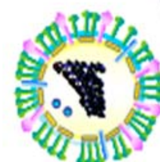

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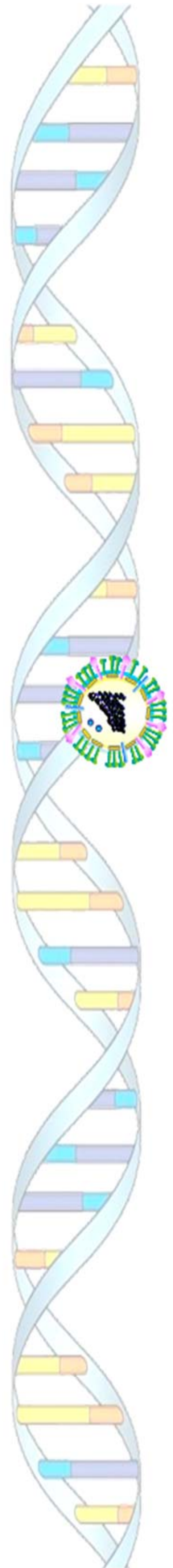

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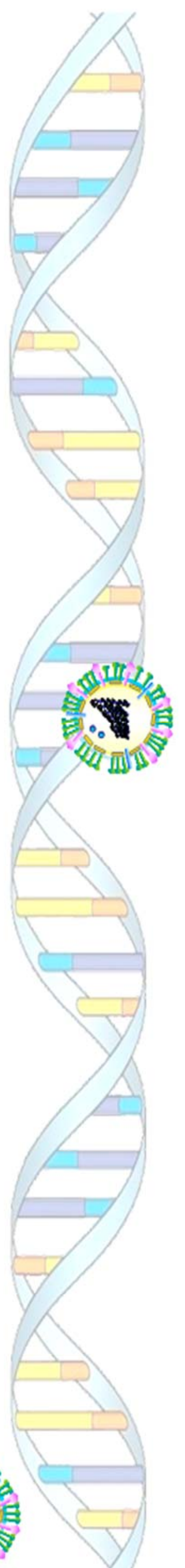
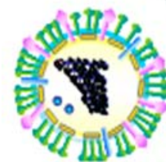


Every good and perfect gift is from above,
coming down from the Father of the heavenly lights,
who does not change like shifting shadows.

James 1:17

Jokainen hyvä anti ja jokainen täydellinen lahja
tulee ylhäältä, taivaan tähtien Isältä, jonka luona
ei mikään muutu, ei valo vaihdu varjoksi.

Jaak 1:17



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Abstract

Innate immunity and host defence are rapidly evoked by structurally invariant molecular motifs common to microbial world, called pathogen associated molecular patterns (PAMPs). In addition to PAMPs, endogenous molecules released in response to inflammation and tissue damage, danger associated molecular patterns (DAMPs), are required for eliciting the response. The most important PAMPs of viruses are viral nucleic acids, their genome or its replication intermediates, whereas the identity and characteristics of virus infection-induced DAMPs are poorly defined.

PAMPs and DAMPs engage a limited set of germ-line encoded pattern recognition receptors (PRRs) in immune and non-immune cells. Membrane-bound Toll-like receptors (TLRs), cytoplasmic retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain-like receptor (NLRs) are important PRRs involved in the recognition of the molecular signatures of viral infection, such as double-stranded ribonucleic acids (dsRNAs). Engagement of PRRs results in local and systemic innate immune responses which, when activated against viruses, evoke secretion of antiviral and pro-inflammatory cytokines, and programmed cell death *i.e.*, apoptosis of the virus-infected cell.

Macrophages are the central effector cells of innate immunity. They produce significant amounts of antiviral cytokines, called interferons (IFNs), and pro-inflammatory cytokines, such as interleukin (IL)-1β and IL-18. IL-1β and IL-18 are synthesized as inactive precursors, pro-IL-1β and pro-IL-18, that are processed by caspase-1 in a cytoplasmic multiprotein complex, called the inflammasome, before they are biologically active and secreted. The signals and secretory routes that activate inflammasomes and the secretion of IL-1β and IL-18 during virus infections are poorly characterized. The main goal of this thesis was to characterize influenza A virus-induced innate immune responses and host-virus interactions in human primary macrophages during an infection.

It was found that extracellular dsRNA and cytoplasmic dsRNA differed strikingly in their capacity to elicit IFNs, IL-1β, IL-18, and apoptosis in human primary macrophages. Recognition of extracellular dsRNA *via* TLR3 induced only modest production of IFNs, whereas pro-IL-1β was robustly synthesized. However, extracellular dsRNA was insufficient alone to trigger caspase-1 activation or IL-1β and IL-18 secretion. In contrast, cytoplasmic dsRNA elicited marked expression of IFNs, caspase-1 activation, and subsequent processing of constitutively produced pro-IL-18, and IL-18 secretion. Nonetheless, TLRs were needed to augment the weak cytoplasmic RNA-induced expression of pro-IL-1β in order to achieve significant secretion of IL-1β. Interestingly, cytoplasmic dsRNA stimulus and influenza A virus infection, but not TLRs, were able to activate caspase-3, the ultimate executioner of apoptosis. This apoptotic response was intimately associated with the activities of RIG-I/mitochondrial antiviral signalling protein (MAVS). The results indicate that the cytoplasmic RNA recognition pathway elicited a strong antiviral cytokine response, secretion of IL-18, and apoptosis, but IL-1β could only be released from cytoplasmic dsRNA-stimulated or influenza A virus-infected human primary macrophages if TLRs were simultaneously activated. Overall, the results highlighted the importance of cytoplasmic PRRs for antiviral innate immune responses.

To elucidate virus-infection-induced inflammasome activation further, secreted proteins, secretomes, from cytoplasmic dsRNA-stimulated human primary macrophages were analysed with high-throughput mass spectrometric (MS)-based methods. Cytoplasmic dsRNA induced subtle secretion of lysosomal proteases cathepsins which have been postulated to promote inflammasome activation and apoptosis. Further characterization revealed that inflammasome activation preceded apoptosis, and that cathepsin D was simultaneously secreted with inflammasome components before cathepsin B was secreted. Pharmacological inhibition of cathepsin B abolished IL-18 secretion and apoptosis in response to cytoplasmic dsRNA stimulus and encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV) infections. Moreover, gene silencing experiments targeted against cathepsin D suggested role for cathepsin D in cytoplasmic dsRNA-induced inflammasome activation. These results highlighted the importance of cathepsins in mediating the innate immune responses against RNA-viruses.

Two-dimensional electrophoresis (2DE) coupled with MS was performed to better elucidate influenza A virus-induced innate immune responses in human primary macrophages. Compared to controls, subcellular proteomes of influenza A virus infected cells displayed significant changes in mitochondria and cytoplasm. In particular, the cytoskeleton was significantly reorganized, including caspase-3-mediated fragmentation of the actin network, and translocation of intact and fragmented actins into mitochondria. In addition to proteins of influenza A virus, RLR signalling components, RIG-I, TRIM25, and TRADD translocated sequentially to mitochondria during infection. Disruption of actin network with actin depolymerizing agent, cytochalasin D, prevented *e.g.*, transcription of IFNs, but not caspase-3 activation or viral protein expression. The results pointed to a requirement of intact actin for IFN-signalling, and emphasized the importance of interactions between actin and mitochondria in the regulation of innate immune responses during influenza A virus infection.

In order to obtain a more global view on host-virus interactions in response to influenza A virus infection, quantitative high-throughput subcellular proteomic and secretome profiling were performed. Dramatic alterations were observed. Influenza A virus infection altered the abundance and location of more than 1000 proteins in nuclear, mitochondrial, and cytoplasmic proteomes at early time points of infection. Furthermore, several nuclear, mitochondrial, and lysosomal proteins were detected in cytoplasmic proteomes and secretomes, and among the secreted proteins, several potential DAMPs were detected. In an attempt to uncover host-virus-responses and possible regulatory proteins important for inflammasome activation, a protein-protein interaction network from intracellular and inflammation-associated proteins of influenza A virus-infected human primary macrophages was constructed *via* bioinformatics. Based on results from the network analysis and functional studies, cathepsin B, $P2X_7$-receptor, and Src tyrosine kinases appeared to be involved in the influenza A virus-induced pro-inflammatory cytokine response of IL-1β and IL-18.

In summary, this thesis provides interesting insights into innate immune responses against RNA-viruses and has characterized host-virus interactions during influenza A virus-infection in human primary macrophages.

Tiivistelmä

Immuunivaste on luokiteltu toiminnallisesti luontaiseen ja hankittuun immuniteettiin. Luontainen immuniteetti on nopea ensivaste taudinaiheuttajaa vastaan, ja se edeltää lymfosyytteihin ja vasta-aineisiin perustuvaa pitkäkestoista hankittua immuniteettia. Luontaisen immuniteetin toiminta perustuu liukoisiin ja solukalvoissa oleviin tunnistereseptoreihin. Tärkeitä tunnistereseptoriperheitä ovat solukalvojen Toll-kaltaiset reseptorit (TLRs), sekä liukoiset soluliman RIG-I:n kaltaiset reseptorit (RLRs) ja Nukleotidi-sitoutumis- ja oligomerisaatioalueen -kaltaiset reseptorit (NLRs). Tunnistereseptorien sitoutuminen patogeenien rakenteisiin tai infektion ja kudosvaurion yhteydessä soluista vapautuviin vaarasignaaleihin aktivoi luontaisen immuniteetin. Tärkein tunnistereseptoreita aktivoiva viraalinen rakenne on viruksen nukleiinihapot, perimäaines tai sen replikaatiotuotteet. Virusinfektion aikana solusta vapautuvat vaarasignaalit ovat huonosti tunnettuja. Tunnistereseptorien aktivoituminen johtaa usein systeemiseen ja paikalliseen antiviraalisten sytokiinien ja tulehdusvälittäjäaineiden erittymiseen ja infektoituneen solun ohjelmoituun solukuolemaan, apoptoosiin. Ilman luontaista immuniteettiä hankittu immuniteetti ei voi muodostua kohdattua taudinaiheuttajaa vastaan.

Makrofagit ovat keskeisiä luontaisen immuniteetin soluja, jotka tuottavat antiviraalisia sytokiinejä ja tulehdusvälittäjäaineita, kuten interferoneja (IFN) ja interleukiineja (IL). IL-1β ja IL-18 ovat elimistön tärkeimpiä tulehdusvälittäjäaineita. Ne tuotetaan biologisesti toimimattomina esiasteina, jotka kaspaasi-1 prosessoi soluliman inflammasomi-rakenteessa biologisesti aktiivisiksi. Signaalit, jotka johtavat inflammasomin aktivaatioon ja sitä seuraavaan IL-1β ja IL-18 erittymiseen virusinfektiossa, ovat huonosti karakterisoituja. Väitöskirjatyön päätavoitteena oli tutkia luontaisen immuniteetin aktivoitumismekanismeja ja isäntä-virus vuorovaikutusta influenssa A virus -infektoiduissa ihmisen primääri makrofageissa.

Virusinfektio tuottaa soluun ribonukleiinihappoja (RNAs). Solun ulkopuolinen RNA usein fagosytoidaan ja tunnistetaan TLR-välitteisesti, kun taas solulimassa oleva viraalinen RNA aktivoi soluliman RLR- ja NLR-tunnistereseptoreja. Makrofageissa solun ulkopuolinen ja soluliman viraalinen RNA aiheuttivat huomattavan erilaiset antiviraali sytokiini- ja tulehdusvälittäjäainevasteet. Solun ulkopuolisen viraalisen RNA:n tunnistaminen TLR-reitin kautta aikaansai merkittävän pro-IL-1β:n tuotannon, muttei huomattavaa IL-1β eritystä tai IFN:n ilmentämistä. Soluliman viraalisen RNA:n ja influenssa A-viruksen tunnistaminen soluliman tunnistereseptorien välityksellä puolestaan indusoivat voimaakkaan IFN:n ilmentämisen, kaspaasi-1 aktivaation ja IL-18 erityksen. Virusinfektion aikainen IL-1β:n tuotto ja eritys vaati kuitenkin TLR- ja soluliman tunnistereseptorien yhteistoiminnan. Soluliman viraalinen RNA ja influenssa A-virus, muttei solun ulkopuolinen RNA, aktivoivat myös kaspaasi-3:a ja sen välittämää ohjelmoitua solukuolemaa, apoptoosia. Kaspaasi-3:n aktivaatio ja apoptoosi kytkeytyi vahvasti soluliman RLR-tunnistereseptorien toimintaan. Tulokset osoittavat soluliman tunnistereseptorien olevan avainasemassa puolustautumisessa viruksia vastaan.

Soluliman viraalisen RNA:n tunnistaminen aiheutti makrofageissa huomattavaa proteiinieritystä, jota tutkittiin laaja-alaisesti proteomiikan menetelmin. Erittyneiden

proteiinien joukossa oli lysosomaalisia proteaaseja, katepsiineja, joiden tiedetään osallistuvan sekä inflammasomin aktivaatioon että apoptoosiin. Tarkempi karakterisointi osoitti soluliman viraalisen RNA:n aktivoivan inflammasomin ennen apoptoosia ja katepsiini-D:n erittyvän stimuloiduista soluista yhdessä inflammasomi-komponenttien kanssa ennen katepsiini-B:tä. Katepsiini-D:n ilmentämisen hiljentäminen vähensi soluliman viraalisen RNA:n aikaansaamaa IL-18 eritystä. Lisäksi katepsiini-B proteiinin toiminnan farmakologinen estäminen rajoitti huomattavasti inflammasomin aktivaatiota ja apoptoosia, myös Encephalomyocarditisvirus (EMCV)- ja Vesicular stomatitis-virus (VSV) -infektiossa. Tulokset korostavat katepsiinien tärkeyttä luontaisen immuniteetin vasteissa virusinfektion aikana.

Kaksiulotteista proteiinien elektroforeettista erottelua yhdistettynä proteiinien massaspektometri (MS)-pohjaiseen tunnistukseen hyödynnettiin solunsisäisten proteiinimuutoksien havainnollistamiseksi influenssa A-virus -infektoiduissa makrofageissa. Infektoituneiden solujen mitokondrioiden ja solulimojen proteomit erosivat huomattavasti kontrolli solujen vastaavista. Etenkin solutukirangassa havaittiin suuria muutoksia: aktiinitukiranka pilkkoutui kaspaasi-3-välitteisesti ja aktiinisäikeet siirtyivät solulimasta mitokondrioihin. Influenssa A-viruksen proteiinien lisäksi myös RLR-reitin signalointimolekyylit, RIG-I, TRIM25 ja TRADD, siirtyivät solulimasta kyseisiin organelleihin infektion edetessä. Aktiinitukiverkon polymerisaation estäminen sytokalasiini-D:llä vähensi antiviraalisten sytokiinien ilmentymistä. Yhdiste ei kuitenkaan vaikuttanut kaspaasi-3:n aktivaatioon tai virusproteiinien tuotantoon influenssa A-virus -infektoiduissa soluissa. Tulokset viittaavat yhtenäisen aktiinitukiverkoston olevan tärkeä IFN:ien ilmentymiseen, mutta ei apoptoosin tai virusreplikaation etenemiseen. Mitokondrioiden ja aktiinien välinen vuorovaikutus ilmentänee niiden osallistumista luontaisen immuniteetin vasteiden säätelyyn influenssa A-virus -infektoiduissa soluissa.

Viimeisessä osatyössä influenssa A-virus-makrofagi-vuorovaikutusta profiloitiin kokonaisvaltaisella, kvantitatiivisella MS-pohjaisella proteomiikalla aikapisteittäin. Soluista analysoitiin soluliman, tuman ja mitokondrioiden proteiinifraktiot sekä sekretomit. Kvantitatiivinen proteomiikka-analyysi paljasti yli tuhannen proteiinin määrän ja paikan muutoksen jo varhaisessa vaiheessa influenssa A-virus -infektiota. Useita tuman, lysosomien ja mitokondrioiden proteiineja havaittiin solulimasta, mikä viittaa vilkkaaseen soluorganellien väliseen proteiiniliikenteeseen ja näiden organellien mahdolliseen solukalvojen lisääntyneeseen läpäisevyyteen tai vaurioon. Myös huomattava määrä vaarasignaaleja erittyi infektoituneista soluista. Tunnistetuista ja tulehdukseen liittyvistä solunsisäisistä proteiineista luotiin bioinformatiikan keinoin proteiini-proteiini vuorovaikutusverkosto, jonka avulla selvitettiin isäntä -virus -vuorovaikutusta ja mahdollisia inflammasomin säätelyproteiineja. Vuorovaikutusverkoston ja funktionaalisten kokeiden tulokset osoittivat katepsiinien, $P2X_7$-reseptorin ja Src-tyrosiinikinaasien osallistuvan inflammasomin aktivaatioon influenssa A-virus-infektiossa ihmisen primääri makrofageissa.

Väitöskirjan tulokset antavat kattavan kuvan luontaisen immuniteetin aktivaatiomekanismeista RNA-virusinfektion aikana ja isäntä-virus -vuorovaikutuksesta influenssa A-virus -infektoiduissa ihmisen primääri makrofageissa.

Abbreviations and a mini-glossary

For convenience and in contrast to the customary form, in certain case
the abbreviated name will be presented first in the text.

2'-5'OAS, 2'-5'-oligoadenylate synthetase:
an enzyme that converts adenosine triphosphate (ATP) into
2'-5'-oligoadenylates in response to interferon (IFN) signal.

5'-ppp, 5'-triphosphate:
three phosphate molecules at the 5' end of ribonucleic acid (RNA) or
deoxyribonucleic acid (DNA) molecules.

AIM2, absent in melanoma-2:
a cytoplasmic pattern recognition receptor (PRR) belonging to
AIM-like receptors (ALRs).

ASC, apoptosis-associated speck-like protein containing CARD:
an essential component in all inflammasomes.

ATP, adenosine triphosphate:
the main energy-transfer molecule in the cell used by enzymes and
structural proteins in many cellular processes. Cellular respiration in mitochondria
is the largest source of ATP in eukaryotic cells.

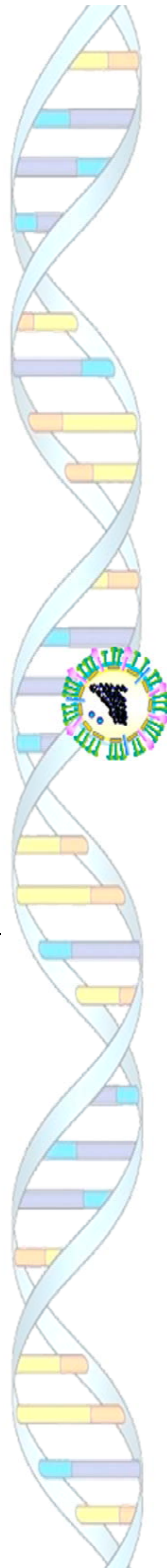
BAX, B-cell CLL/lymphoma 2 (Bcl-2)-associated X protein:
a pro-apoptotic protein involved in dissipation of mitochondrial membrane potential.

Bcl-2, B-cell CLL/ lymphoma-2:
an anti-apoptotic protein which binds and inhibits pro-apoptotic functions of Bax.

Bid, BH3 interacting domain death agonist:
Proteolysis of Bid into its **t**runcated variant (**t**-Bid) promotes apoptosis.

CARD, caspase recruitment domain:
an important protein domain found *e.g.,* in retinoic acid -inducible gene-I
(RIG-I), melanoma differentiation-associated gene-5 (MDA-5), mitochondrial
antiviral signalling protein (MAVS), and in caspases.

Caspase, **c**ysteine-dependent **asp**artate-directed prote**ases**:
a family of cysteine proteases that play essential roles in apoptosis,
inflammation, and inflammation-promoting cell death, pyroptosis.



Cyt c, cytochrome c:
a small heme-containing protein which is an essential component of electron transport chain. It loosely associates with the inner membrane of the mitochondrion, and its release into cytoplasm triggers apoptosis.

DAMP, danger associated molecular pattern:
endogenous molecules that signals for danger.

DNA, deoxyribonucleic acids:
the genetic code of *flora* and *fauna*, with the exception of RNA viruses. Information coded in DNA is passed onto the next generation during cell division or replication.

ds, double-strand:
complementary pairing of single-stranded (ss) nucleotides into ds-variants.

EMCV, encephalomyocarditis virus:
a positive sense ssRNA virus of *Picornavirus* family that causes encephalitis and myocarditis, mainly in its natural host, rodents.

FADD, Fas-Associated protein *via* death domain:
an adapter molecule which is part of death-inducing signaling complex (DISC). FADD also interacts with MAVS.

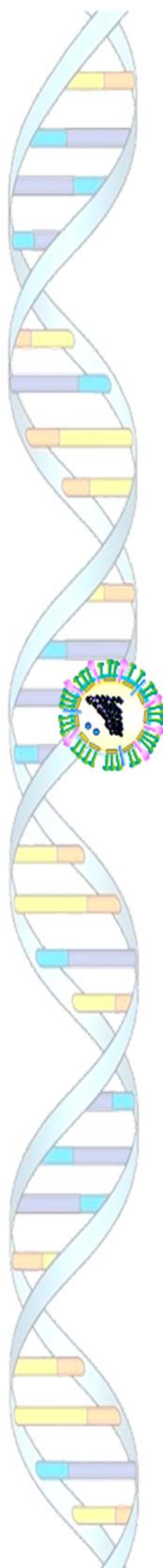
HA, hemagglutinin:
an important structural capsid protein of influenza viruses. HA mediates binding of virus to a permissive host cell.

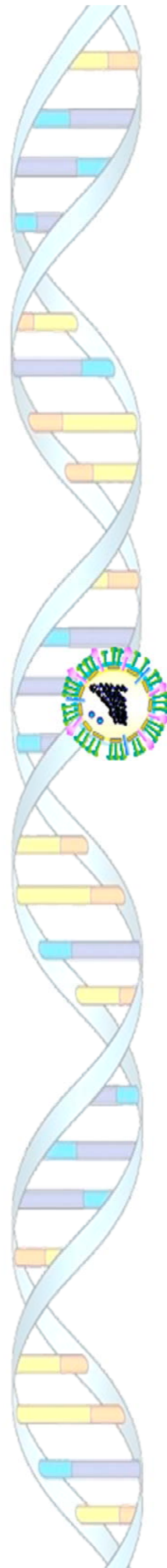
HSP, heat shock protein:
a protein that assists in correct folding, function, and stability of other proteins.

HCV, hepatitis C virus:
a positive-sense ssRNA virus of *Flaviviridae* family. Hepatitis C virus causes hepatitis C in humans.

HMGB1, high mobility group box-1 protein:
a chromatin-binding nuclear protein with versatile functions. Extracellularly HMGB1 is a DAMP.

IFNs, interferons:
antiviral cytokines which have been subcategorized into three classes; type I-III IFNs.





IL, interleukin:
a group of important cytokines. The term *interleukin* derives from (*inter*-), meaning communication, and (-*leukin*) indicating that these proteins are mainly produced and targeted by leukocytes.

IL-1Ra, IL-1 receptor (IL-1R) antagonist:
IL-1Ra blocks access of IL-1 to its receptor IL-1R.

IRF, IFN regulatory factor:
a family of transcription factors that, upon phosphorylation, translocate into nucleus and activate expression of genes containing IFN-stimulated response elements (ISREs).

LGP2, Laboratory of genetics and pathology-2:
a member of RIG-I-like receptors (RLRs). LGP2 is a RNA helicase which regulates functions of RIG-I and MDA-5.

LPS, lipopolysaccharide:
a cell wall component of gram-negative bacteria.

LRR, leucine rich-repeat:
Structural motif of proteins composed of 20-30 tandem copies of amino acid sequences rich of leucines. LRRs are the ligand-binding part of several PRRs.

M2, Matrix protein-2:
an essential ion channel protein of influenza A virus particle.

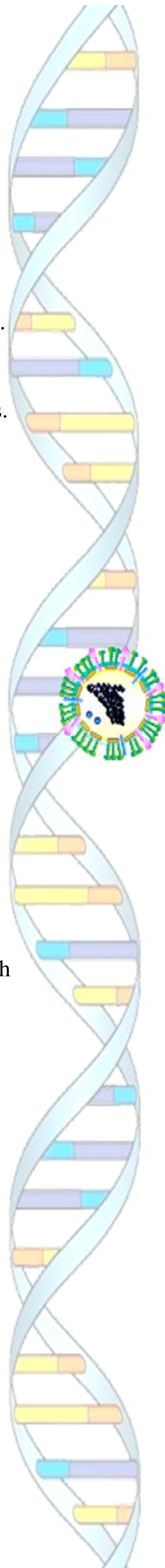
MAVS, mitochondrial antiviral signalling protein
(or alternatively IPS-I/VISA/CARDIF): a downstream signalling protein of RLRs.

MDA-5, melanoma differentiation-associated gene-5:
a member of RLR group of cytoplasmic PRRs.
MDA-5 detects dsRNA derived from viruses.

mRNA, messenger RNA:
carries coding information derived from DNA (or from negative sense ssRNAs of viruses) for synthesis of proteins.

MyD88, Myeloid differentiation primary response gene 88:
an adapter signaling protein for all Toll-like receptors (TLRs), except for TLR3.

MS, mass spectrometry:
an analytical technique that measures mass-to-charge ratio of charged particles for elucidation of their chemical structures.



NA, neuraminidase:
an enzyme found on the surface of influenza virus particles, and essential for virus propagation.

NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase:
a membrane-bound enzyme complex which generates superoxide. Superoxides may spontaneously form hydrogen peroxides, *i.e.*, reactive oxygen species (ROS).

NF-κB, nuclear factor kB:
transcription factor needed for expression of many inflammation-associated genes.

NLR, nucleotide-binding oligomerization domain (NOD)-like receptor:
a large group of proteins that function as cytoplasmic PRRs.

NLRP3, NOD-like receptor protein-3:
an important member of NLR group of cytoplasmic PPRs.

PAMP, pathogen associated molecular pattern:
invariant structural motifs common to pathogens but not to mammalian cells.

PKR, dsRNA-activated protein kinase:
detects foreign cytoplasmic dsRNA in cells.

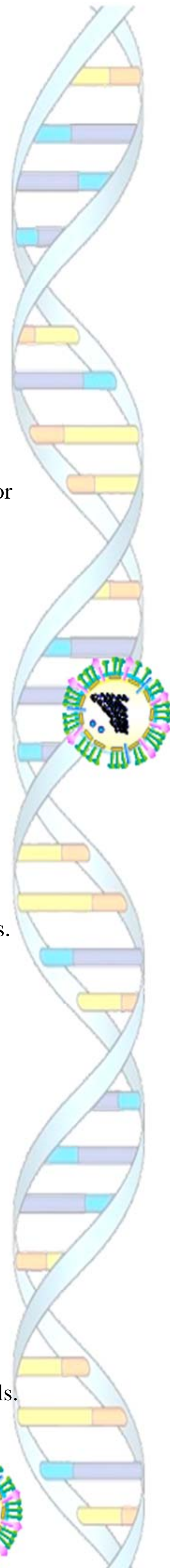
Poly(I:C), polyinosinic-polycytidylic acid:
a synthetic mimetic of dsRNA.

RNA, ribonucleic acid:
A chain of nucleotide units, comprising of a nitrogenous base (generally adenine, cytosine, guanine, or uracil), a ribose sugar, and a phosphate, covalently joined to each other by ester bonds. Different types of RNAs are required for synthesis of proteins.

RNAseL, ribonuclease-L:
2'-5'OAS and 2'-5'-oligoadenylate-dependent ribonuclease,
a component of IFN-regulated system which mediates the antiviral and antiproliferative roles of IFNs.

RIG-I, retinoic acid-inducible gene-I:
a member of RLRs of cytoplasmic PRRs. RIG-I detects cytoplasmic RNA mainly of viral origin.

RLR, RIG-I-like receptor:
a group of cytoplasmic PRRs (*i.e.*, RIG-I, MDA-5, and LGP2) specialized in the recognition of cytoplasmic RNAs.



ROS, reactive oxygen species:
chemically-reactive molecules containing oxygen. Oxygen ions and peroxides are produced in large amounts from different parts of cell during stress responses.

SLE, systemic lupus erythematosus:
severe systemic autoimmune disease affecting multiple parts of the body.

STAT, signal transducer and activator of transcription:
Family of transcription factors which are necessary for expression of IFNs.

***si*RNA**, small interfering RNA:
effector molecules of RNA interference (RNA*i*), a post-transcriptional mechanism for inhibition of gene expression of specific genes. *si*RNA binds to complementary nucleotides found in mRNA molecules and interferes or blocks their translation.

ss, single-stranded:
RNA or DNA molecules that contain only one strand of nucleotides.

TIR-domain, Toll/IL-1R-domain:
A protein domain found in *flora* and *fauna*.
In mammals, TIR-domains are found from *e.g.*, TLRs and IL-1R.

TLR, toll-like receptor:
a membrane-bound PRR group mediating recognition of several types of PAMPs.

TRADD, Tumor necrosis factor receptor type 1-associated *via* death domain:
an adapter protein essential for RIG-I-mediated signalling and induction of apoptosis.

TRAF3, tumor necrosis factor receptor-associated factor-3:
an important signal transduction molecule for TLRs and RLRs.

TRIF, TIR-domain-containing adapter-inducing IFN-β:
a signalling adapter protein downstream from TLR3 and -4.

TRIM25, tripartite motif protein-25:
an E3 ubiquitine ligase which ubiquitinates RIG-I and enables RIG-I's downstream signalling.

vRNP, viral ribonucleoprotein:
a complex containing viral genomic RNA and associated nucleoproteins.

VSV, vesicular stomatitis virus:
a negative sense ssRNA virus of *Rhabdovirus* family that infects insects and mammals.

Original publications

This thesis is based on the following publications, which are referred to by Roman numerals in the text.

I Johanna **Rintahaka**, Daniel Wiik, Panu E. Kovanen, Harri Alenius, and Sampsa Matikainen. (2008). Cytosolic Antiviral RNA Recognition Pathway Activates Caspases 1 and 3. *The Journal of Immunology, 180:1749-57*.

II Tiina Öhman, Johanna **Rintahaka**, Nisse Kalkkinen, Sampsa Matikainen, and Tuula A. Nyman. (2009). Actin and RIG-I/MAVS Signaling Components Translocate to Mitochondria upon Influenza A Virus Infection of Human Primary Macrophages. *The Journal of Immunology, 182:5682-92*.

III Johanna **Rintahaka**, Niina Lietzén, Tiina Öhman, Tuula A Nyman, and Sampsa Matikainen. (2011). Recognition of Cytoplasmic RNA Results in Cathepsin-Dependent Inflammasome Activation and Apoptosis in Human Macrophages. *The Journal of Immunology, 186:3085-3092*

IV Niina Lietzén, Tiina Öhman*, Johanna **Rintahaka***, Ilkka Julkunen, Tero Aittokallio, Sampsa Matikainen, and Tuula A Nyman. (2011). Quantitative Subcellular proteome and Secretome Profiling of Influenza A Virus-Infected Human Primary Macrophages. *PLoS Pathogens, 7:e1001340*

*Equal contribution

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Review of the literature

1. Innate immunity: the primary sensor of "self" and "non-self"

Immunity is conceptually categorized into the innate and acquired immune systems. Innate immunity is the first line of defence against pathogenic insults found in all classes of plant and animal life (Nurnberger et al., 2004). It functions continuously, supervises the well-being of organisms, and launches innate immune reactions within minutes to hours after the first pathogenic insult.

Innate immunity consists of physical, chemical, humoral, and cellular defence mechanisms. The first physical obstacle that an invading microbe has to confront is often the intact epithelial cell lining which covers external surfaces and lines the internal cavities of vertebrates, such as respiratory, genital, and gastrointestinal tracts. In the lungs, an organ targeted by respiratory viruses, physiological and chemical protection is provided by cilia and the mucous lining: cilia move inhaled foreign particles trapped in the mucus present in the trachea toward the oral cavities to be swallowed and digested in the acidic pH of stomach. Furthermore, lung epithelial cells produce substances that aid in the protection of respiratory tract from inhaled microbes, like mucus, antimicrobial peptides, and surfactants (Sadler and Williams, 2008). In the blood, an important part of humoral innate immunity is formed by certain plasma proteins produced mainly by hepatocytes, such as complement proteins. They cooperate with cellular part of the innate immunity in the identification, destruction, and clearance of pathogens, as well as in the removal of infected and damaged cells at the site of infection and tissue damage (Medzhitov, 2007; Meri, 2003).

While innate immunity operates immediately after first encounter with pathogens, long-term acquired immune responses, mediated by B- and T-lymphocytes in vertebrates, are effective only after several days. During these early days, innate immunity guides the development of proper type of acquired immune responses and provides an often life-long immunological memory. The immunological memory ensures that more efficient and faster acquired immune responses are evoked if the same pathogen is re-encountered in later life. Needless to say, defects in innate immunity often result in severe inflammatory conditions, autoinflammatory diseases, or high susceptibilities to pathogens, and at worst, the premature death of the infected individual (Castellino and Germain, 2006; Hoffman et al., 2001; Medzhitov, 2007; Picard et al., 2003).

1.1. Cells of the innate immunity

The most important part of the innate immunity is its cellular arm consisting of white blood cells *i.e.,* leukocytes, mainly of myeloid origin. These include monocytes and monocyte-derived macrophages and dendritic cells (DCs); mast cells, and granulocytes, such as neutrophils, eosinophils, and basophils. In addition, cytolytic NKs and subset of

γδT-cells of lymphoid origin can be functionally classified as part of the innate immunity (Medzhitov, 2007; Matzinger, 2002) (**Figure 1**).

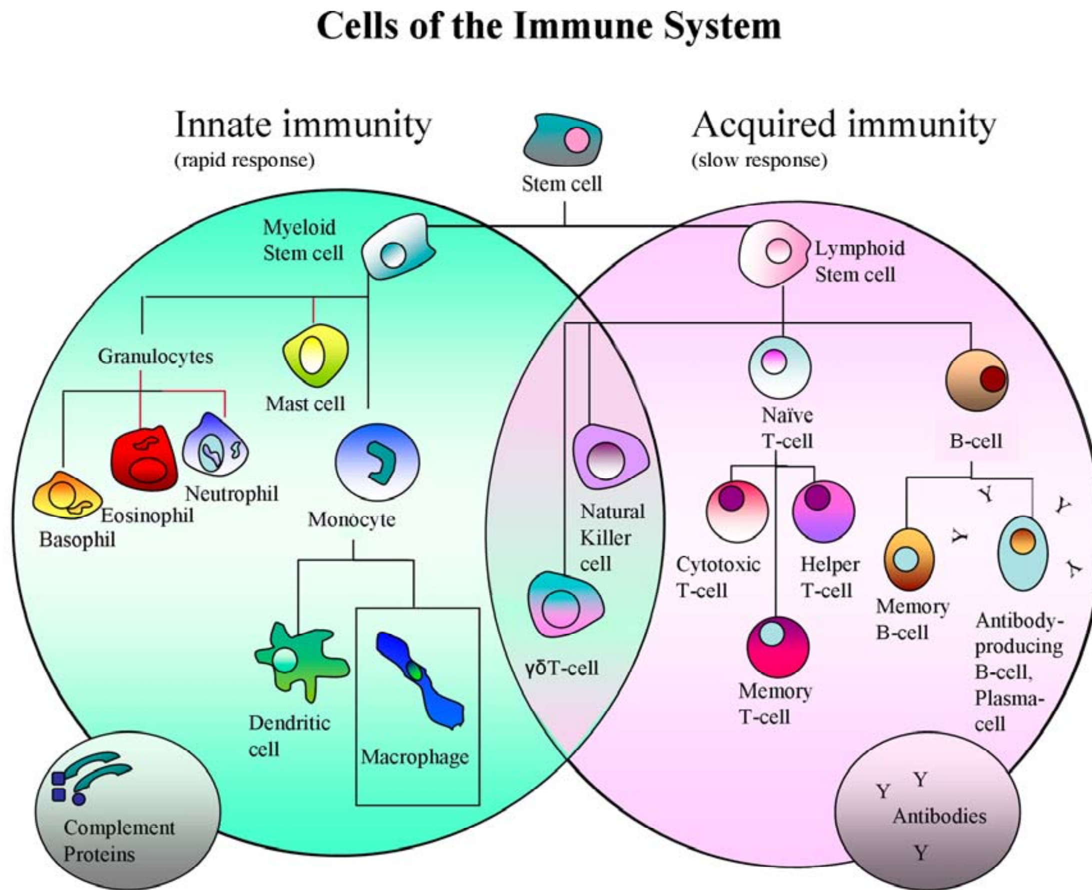


Figure 1 ***Cells of the immune System***. *Myeloid and B-cell progenitors develop in the bone marrow, whereas naïve T-cells differentiate mainly in the thymys. Monocytes represent 10% of leukocytes in human blood and serve as precursors for macrophages and DCs. A healthy human has approximately 450 monocytes/µl which represents a total of 2.25 billion monocytes in 5 litre of blood (Robbins and Swirski, 2010). For simplicity, only some of the distinct monocyte-, macrophage-, DC-, and T-cell types are depicted.*

The interaction of the humoral and cellular parts of the innate immunity with pathogens, pathogenic material, infected host cells, cell remnants, or other immunostimulatory molecules results in host cell activation. This includes *e.g.*, 1) secretion of soluble mediators, such as chemokines, antiviral- and pro- inflammatory cytokines, and endogenous danger signalling molecules, called danger-associated molecular patterns (DAMPs); 2) increased expression of different kinds of receptors and costimulatory molecules; and 3) elevated phagocytic or cytotoxic activities of certain host cell populations. Activated resident cells attract circulating leukocytes to the site of infection or tissue injury for the elimination of the infectious agents and for the initiation of tissue repair processes (Medzhitov, 2007; Mosser and Edwards, 2008) (**Fig. 2**).

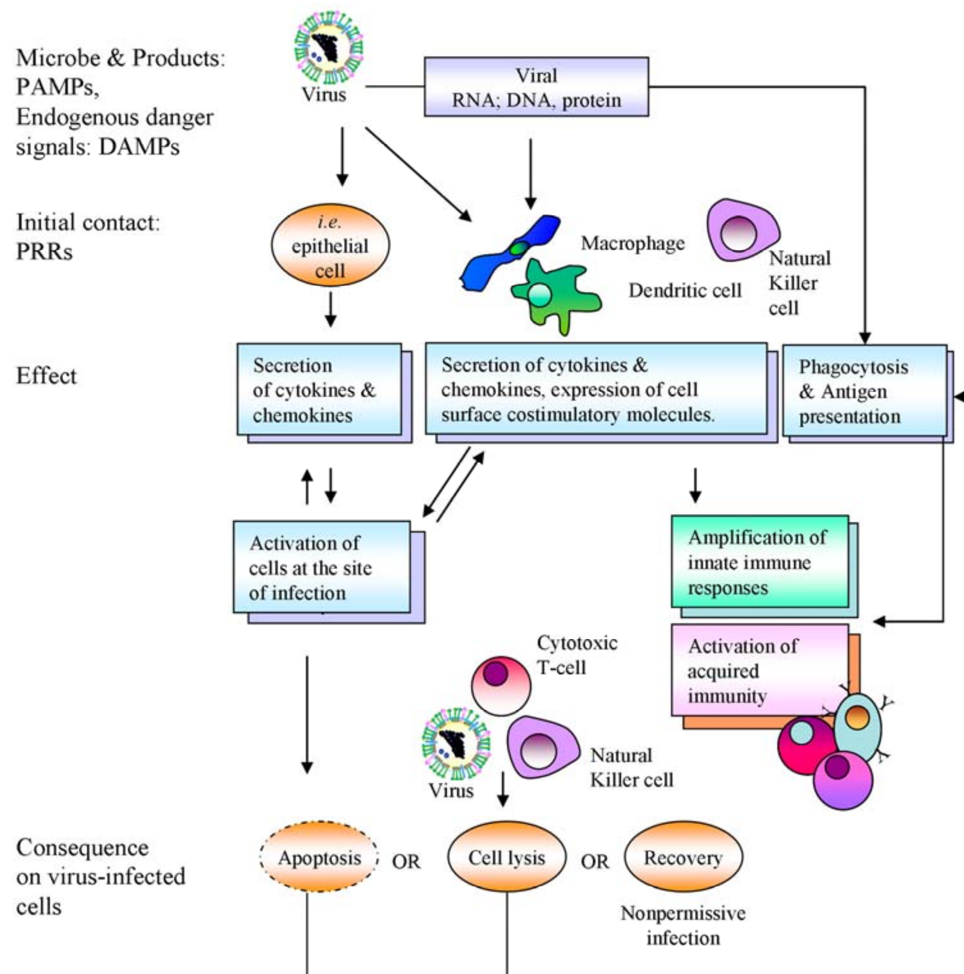


Figure 2 ***Orchestration of cellular innate immune responses against viruses***. *Initial contact of PRRs with PAMPs and/or DAMPs trigger humoral and cellular part of immune responses resulting in programmed cell death, i.e., apoptosis, lysis, or recovery of the infected host cell. Nonpermissive cells do not support viral replication, thus they are not prone to infection.*

1.1.1 Professional phagocytes and antigen presenting cells (APCs)

Monocytes, macrophages, DCs, and neutrophils are professional phagocytes (Savina and Amigorena, 2007). After recognition of pathogen, they phagocytose; *i.e.*, engulf and enclose foreign material into phagosomes; and trigger oxidative burst *i.e.*, the increased production of reactive oxygen species (ROS) to destroy the intruders. ROS can be either released into the local extracellular environment, or utilized intracellularly in phagosomes to break down the engulfed matter. A low pH and actions of lysosomal proteases are also important for phagocytic destruction inside phagosomes. The impressive phagocytic activity of macrophages can clear approximately 2×10^{11} erythrocytes each day. This accounts for recycling of almost 3 kg of iron and haemoglobin per year (Mosser and

Edwards, 2008)! Thus, in addition to host defence, phagocytes play an essentially part in the overall maintenance of homeostasis.

The primary effector cells of the innate immunity, DCs, and macrophages, are also professional antigen presenting cells (APCs). In other words, they present antigens to T-cells of the immune system in order to evoke acquired immunity. APCs establish proper T-cell responses during infection: they process and present antigens in complex with major histocompatibility complex (MHC) class I and II molecules to T-cells possessing cognate receptors specific for the antigen-MHC complex on the APC (Iwasaki and Medzhitov, 2010; Le Bon et al., 2003; Sporri and Reis e Sousa, 2005). This is in contrast to the majority of cells in the body that present virus infection-associated peptides in MHC class I molecules to $CD8^{+}$T-cells only after they have been infected themselves. However, *uninfected* APCs may capture antigens through *phagocytosis* and present these *exogenous* peptides to effector T-cells. Therefore, APCs' function is essential for the host defence also against viruses that do not infect APCs. The presentation of viral antigens, especially by DCs, involves maturation of naïve $CD8^{+}$T-cells into cytotoxic $CD8^{+}$T-cells and the differentiation of naïve $CD4^{+}$T-cells to helper T-(Th)-cells of which there are various kinds (*e.g.*, Th_1, Th_2, Th_{17}). These DC-T-cell-interactions in conjunction with cytokines, are key for full effector activities of T-cells, since other APCs trigger mainly previously activated effector T-cells of acquired immunity (Castellino and Germain, 2006; Rock and Shen, 2005). If DCs present "self-antigens", the result is normally self-tolerance, for example through deletion of autoreactive T-cells. Thus, in addition to the fact that functions of APCs are highly important for proper development of T-cell responses against infectious agents, they also prevent the development of autoimmune reactions against self-derived material, as well as maintain tolerance to harmless environmental stimuli and commensal micro-organisms (Flint et al., 2004h).

1.2. Activation of innate immunity by pathogenic microbes: pathogen associated molecular patterns (PAMPs)

PAMPs are conserved and invariant structural components or metabolic products of pathogenic protozoa, fungi, bacteria, and viruses that are not commonly expressed or found in the animal kingdom (Janeway, 1989; Medzhitov, 2007). Since PAMPs are essential for microbes, they are not prone to structural modifications, and conversely, are competently distinguished as "non-self" and danger. PAMPs are proteins, oligosaccharides, lipids, and nucleic acids in varying compositions. A classical PAMP is lipopolysaccharide (LPS), a component of outer membrane of gram-negative bacteria. It is composed of several oligosaccharides and a lipid molecule, designated lipid A, which is the actual bioactive component of LPS (Beutler and Rietschel, 2003).

1.2.1. Viral PAMPs

Viruses are obligatory parasites that cannot produce infectious progeny virus particles, *i.e.*, virions, independently of their host. In fact, the whole virion is created of components derived from metabolic processes of the host cell, and thus it poses a major challenge to the immune system. As a consequence, protein, lipid, or carbohydrate modifications restricted only to viruses are in scarce or do not exist. Instead, part of the innate immunity has specialized to detect viral nucleic acids, which are thought to serve as the primary PAMPs of viruses at present. Indeed, in order to replicate and produce *de novo* virion components, viruses must first undress their virion-coding sequences, deoxyribonucleic acids (DNA) and/or ribonucleic acids (RNAs), from their viral capsids and present them to the host's transcriptional and protein synthetic machineries (The general protein synthesis scheme is presented in **figure 3**). Furthermore, viral nucleic acids, especially if present in the cytoplasm, are a relatively early sign of active viral replication in a cell, and this is one further reason why viruses are primarily recognized by virtue of their nucleic acids.

The immunostimulatory nature of viral nucleic acids is due to their divergent structure, unusual location, or abundance in the host cell. In terms of viral and bacterial DNA, they contain often unmethylated cytidine-guanoside-rich, so called CpG, motifs that are rarely present in mammalian DNA (Hemmi et al., 2000; Krug et al., 2004). Moreover, the nucleic acid products polymerized by viral polymerases do not necessarily resemble those polymerized by the host polymerases. In additon, eukaryotic host DNA is highly coiled and tightly packed into chromosomes and is only occasionally located in the cytoplasm: it is normally sequestered in nuclei and mitochondria (except during the prometaphase stage of cell division when the nuclear membranes disappear and chromosomes are released into the cytoplasm). Furthermore, if endogenous DNA is found in the extracellular space, cytoplasm, or endolysosomal compartment, then the host's nucleases can degrade these unwanted self-nucleic acids which means that the host is mainly devoid of free intra- and extracellular DNA (Vilaysane and Muruve, 2009). This is in clear contrast to the situation with certain DNA-containing pathogens which replicate in the cytoplasm *e.g.*, to *Listeria monocytegenes*- and vaccinia virus-infections, in which foreign DNA molecules enter the cytoplasm, and this facilitates distinction between DNA of "self" and "non-self" (Rathinam et al., 2010).

Recognition of viral RNA seems to be more complex than that of microbial DNA: vertebrate cells contain many different species of RNA molecules, such as messenger RNA (mRNA), transfer RNA (tRNA), small nuclear RNAs (snRNAs), ribosomal RNAs (rRNAs), and several regulatory RNAs, such as microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) (Brosnan and Voinnet, 2009). In addition to specialized functions, these RNAs have unique structures since both their bases and sugars can be modified in numerous ways as they mature. Processes that affect the molecular make-up of RNAs include splicing (for certain mRNAs and tRNAs), capping (a special 5'-end methylguanosine structure, 5'-cap, in mRNAs and snRNAs), polyadenylation (3'-polyA-tail in mRNAs), phosphorylation (5'-monophosphate in tRNAs and rRNAs), and editing with unusual bases, such as pseudouridine (all RNAs except mRNAs) (Perry, 1981; Karikó et al., 2005; Koski et al., 2004). Furthermore, unlike DNA molecules, RNAs are

not confined to the nuclei or mitochondria, but shuttle between different cellular compartments like nuclei, endoplasmic reticulum (ER), and cytoplasm. Nonetheless, the innate immune system is able to discriminate between "self-" and "non-self-RNAs".

Several important molecular features of viral RNA are not commonly found in eukaryotic RNAs *e.g.*, 1) three phosphates in the 5'-end of RNA molecules (abbreviated as 5'-ppp) (albeit the abundantly expressed polymerase III transcript of the host, namely 7SL RNA, also contains 5'-ppp (Zieve et al., 1977)); 2) covalently linked proteins at the end of RNA molecules, such as the V-proteins of paramyxoviruses; 3) lack of 2'-O-methylation in 5'-ends of viral RNAs; 4) nucleoside modifications, such as N6-methyladenosine; and in some cases, 5) extensive secondary structures (*e.g.*, polioviruses) (Andrejeva et al., 2004; Flint et al., 2004a; Hornung et al., 2006; Karikó et al., 2005; Pichlmair et al., 2006; Weber et al., 2006; Zust et al., 2011). The localized increase in the amount of RNAs produced during viral infection is also critical for lowering the threshold for activation of innate immunity (Lecture of Taniguchi T. in the 4th International HMGB1 Symposium 2010 in Helsinki, June 20-23).

However, although the immunostimulatory activity of RNA was recognized already in the 1960's (Field et al., 1967a; Field et al., 1967b; Tytell et al., 1967), it was only recently that the mechanism for its detection and subsequent immunoactivation was identified (Alexopoulou et al., 2001; Andrejeva et al., 2004; Yoneyama et al., 2004) (see 1.4.).

1.2.1.1. Double-stranded RNA (dsRNA)

Double-stranded RNAs play crucial roles in cells. They are structures found from key metabolic and catalytic RNAs of protein synthesis pathways (*e.g.*, different tRNA and ribozymes, respectively). In addition, dsRNAs function in the regulation of gene expression (*e.g.*, splicing and in translational repression and gene silencing). For viruses, dsRNAs are also essential: they are obligatory replication intermediates for many RNA-viruses and have to be exponentially produced for productive infection. Furthermore, replication of several different DNA viruses produces dsRNAs as a result of converging bidirectional transcription. Moreover, parts of the synthesized ssRNAs can unintentionally anneal and form dsRNA motifs (Jacobs and Langland, 1996; Kumar and Carmichael, 1998). The amount of dsRNAs produced during an infection cycle depend on the virus strain (Marcus and Sekellick, 1977; Pichlmair et al., 2006; Weber et al., 2006). The classification of viruses and more information about production of viral dsRNAs and mRNAs during virus replication is presented in **figure. 4**.

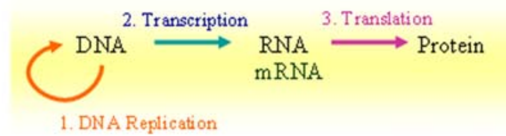


Figure 3 ***General flow of events from genes to proteins.*** *1) DNA is duplicated for the daughter cells in synthesis-phase of cell division. 2) During interphase, cell growth requires unwinding of dsDNA into its ss-form so that transcription factors can gain access to the specific gene, and de novo protein synthesis from the subsequently formed mRNA may begin. Messenger RNA is transcripted in the nucleus, 3) transported to the cytoplasm where ribosomes translate it into protein. Splicing and miRNA-mediated gene silencing in the nucleus and cytoplasm, respectively, are post-transcriptional mechanisms that affect quality and stability of mRNAs, respectively.*

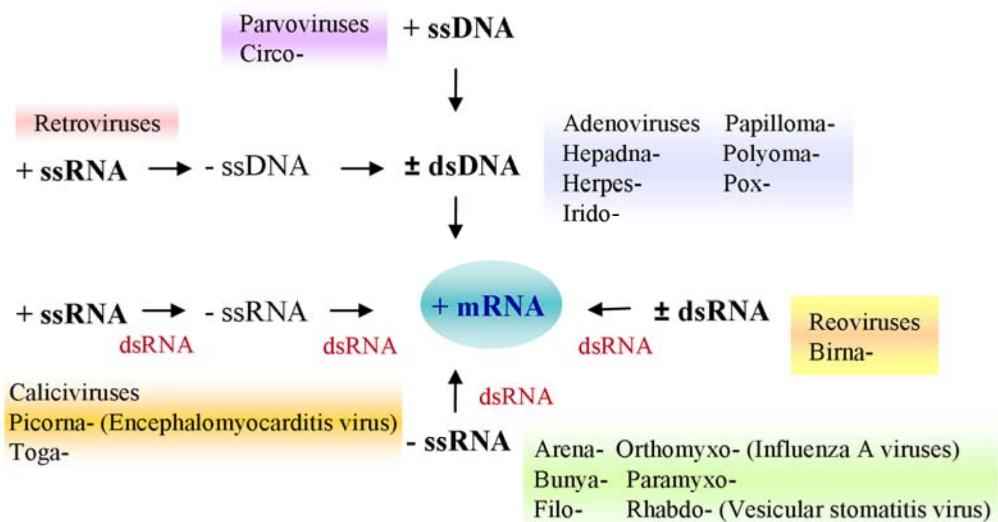


Figure 4 ***The Baltimore classification of viruses and production of dsRNAs and mRNAs during viral replication.*** *Viruses are classified according to the nature (ss- or ds-DNA/RNA) and polarity (+/-/±) of their genome. All viruses must produce mRNAs (+sense RNAs) and exponentially multiply their genetic code in order to synthesize viral proteins and attain productive infection. The dsRNAs are obligatory replication intermediates for most types of viruses. Double-stranded DNA-viruses produce dsRNAs by convergent transcription whereas dsRNA viruses copy both of their RNA strands through dsRNAs intermediates. The genome of positive sense (+) ssRNA viruses are directly used as mRNA, but exponential replication occurs via dsRNAs. The genomes of negative sense (-) ssRNA viruses are in a complementary orientation to that of mRNA, and for that reason, their replication also generates dsRNA. Moreover, parts of the ssRNA genomes can anneal and form dsRNA motifs. The viruses used in this study are shown in parenthesis next to the virus family in which they have been classified (Flint et al., 2004i)*

1.3. Activation of innate immunity by endogenous host molecules: danger associated molecular patterns (DAMPs)

Productive virus infection often significantly alters cellular metabolism and cause tissue damage *e.g.*, *via* cell lysis and cell death. Stressed cells produce, modify, and release endogenous molecules which are recognized as danger signals that activate host immune responses and induce inflammation (Shi et al., 2003). These kinds of molecules are called DAMPs. As with PAMPs, DAMPs are antigenic because of their deviant structure, location and/or abundance. According to the danger hypothesis proposed by Polly Matzinger in 1994, the immune system is more concerned with danger and damage than with foreignness, and therefore it is also activated by danger signals of endogenous origin (Matzinger, 1994; Matzinger, 2002). This concept reconciled the paradox of immune activation during infection and sterile injury. Thus, innate immune responses can be initiated not only by recognizing PAMPs but also by detecting DAMPs. Indeed, infected cells can stimulate NK cells or the complement system with subsequent antiviral innate immune responses without directly interacting with viral PAMPs. Furthermore, the complement- and NK-cell-mediated lysis of infected cells and the subsequent spillage of cell contents into cell surroundings produce more DAMPs, further amplifying the immune responses. However in general, it is thought that both PAMPs and DAMPs are required for mounting effective innate immune responses.

One of the best defined DAMPs are monosodium urate crystals. They are spontaneously formed from urid acid, a product of purine catabolism, which is abundantly released from cells at sites of tissue injury. Monosodium urate crystals accumulates in excess in joints during pathogenesis of gout (Martinon et al., 2006; Shi et al., 2003). At present, virus-infection-associated DAMPs are poorly characterized.

1.4. Pattern recognition receptors (PRRs)

Unlike the acquired immunity, which is based on somatic gene arrangements and somatic hypermutations of antigen receptors that generates a diverse set of receptor specificities for detecting immunostimulatory molecules, the innate immunity rely only on a small number of germ-line encoded receptors, called pattern recognition receptors (PRRs) (Janeway, 1989; Medzhitov, 2007). PRRs can be secreted, membrane-bound, or cytoplasmic. They are expressed in especially high numbers on monocytes, macrophages, and DCs, and they coordinate innate immune reactions. Binding of PAMPs to structural motives of signalling-adapted PRRs activate intracellular signalling cascades that lead to changes in gene expression and metabolism of the cell. This prompts the host to mount the appropriate defence against the pathogen.

The most extensively studied signalling-capable PRRs are membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and cytoplasmic retinoic acid inducible gene-I (RIG)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Creagh and O'Neill, 2006; Geijtenbeek and Gringhuis, 2009). Recently, a new cytoplasmic PRR group involved in recognition of foreign

cytoplasmic DNA was proposed to be included among the above four and was named absent in melanoma (AIM) -like receptors (ALRs) (Unterholzner et al., 2010). In addition to these, there exist numerous scavenger receptors which act as PRRs. They primarily assist in phagocytosis of PAMPs or costimulation of TLRs, such as dsRNA-binding protein SR-A (Scavenger receptor class-A) and CD36, respectively (Areschoug and Gordon, 2009). Importantly, the simultaneous activation of different PRR class members is often necessary for the initiation of effective innate immune responses.

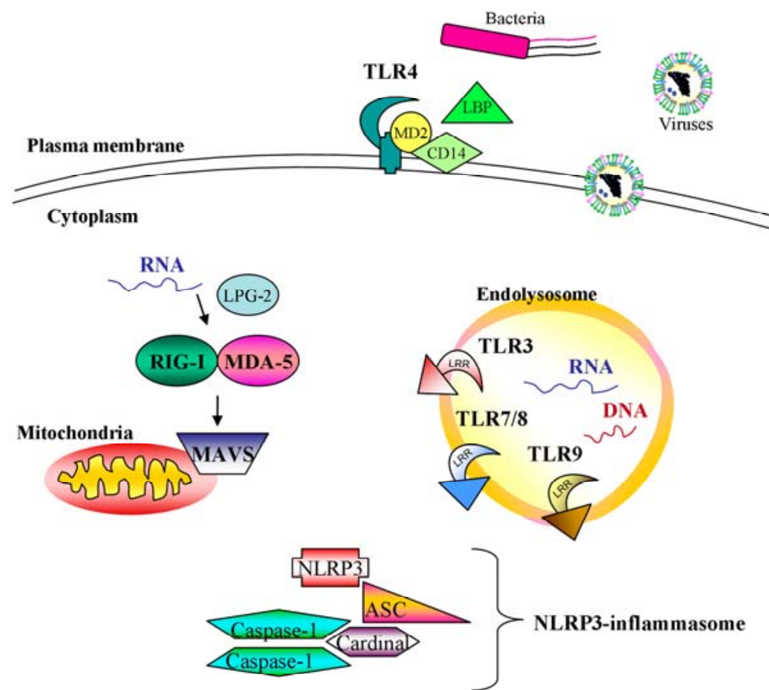


Figure 5 ***Important pattern recognition receptors during virus infection.*** *Upon recognition of PAMPs and/or DAMPs, membrane-bound TLRs, and cytoplasmic RLRs and NLRs trigger signaling cascades that culminate in the induction of innate immune responses. TLR8 and the inflammasome component called cardinal are expressed only in humans.*

1.4.1. Toll-like receptors (TLRs)

A fruit fly, *Drospohila melanogaster,* paved the way for the initial identification of receptors that elicit immune responses (Lemaitre, 2004; Valanne et al., 2011). In 1996, *Drosophila* carrying mutations in a receptor called '*Toll*', were found to be highly susceptible to fungal infections (Lemaitre et al., 1996). Furthermore, the intracellular domain of *Drosophila* '*Toll*' had structural similarities and functional parallels in the activation of immune responses with the mammalian type I interleukin (IL)-1 receptor (IL-1R). For these reasons, this protein domain, having essential roles in immune signalling, was named the **T**oll/**I**L-1**R** (TIR)-domain (Gay and Keith, 1991; Lemaitre, 2004). Importantly, it was also noted that the extracellular part of *Drosophila Toll* contained

repetitive leucine rich repeats (LRRs), a feature they share with several PRRs from animals to plant species, and thus, these have been suggested to be important for recognition of pathogens (Creagh and O'Neill, 2006; Medzhitov et al., 1997; Nurnberger et al., 2004).

The first human 'Toll' was characterized one year later in 1997 (Medzhitov et al., 1997), and soon nine other 'Tolls', named as Toll-like receptors (TLRs), had been identified in humans (Chaudhary et al., 1998; Chuang and Ulevitch, 2001; Chuang and Ulevitch, 2000; Du et al., 2000; Heil et al., 2004; Hemmi et al., 2000; Rock et al., 1998; Takeuchi et al., 1999). Importantly, it was found that mammalian TLRs and *Drosophila* '*Toll*' signalled through conserved signalling cascade culminating in the expression of inflammatory genes and antimicrobial peptides (the NF-κB-pathway; see 2.2.3.1 and **Fig. 8**). This suggested that the pathway operated in organisms from insects to humans in immune signalling (Lemaitre et al., 1996; Medzhitov et al., 1997; Rosetto et al., 1995). Indeed, a loss-of-function mutation in the mouse homologue of certain "human Toll" gene, namely *tlr4,* was found to result in an unresponsiveness to bacterial LPS (Poltorak et al., 1998). This finding led also to characterization of the receptor involved in the recognition of this endotoxin of gram negative bacteria (namely, TLR4), which had puzzled researchers for a long time (Beutler and Rietschel, 2003). The knocking-out of other TLRs in mice revealed that TLRs serve as PRRs for the detection of a diverse variety of PAMPs.

Membrane-bound TLRs survey in different sites of cell and recognize a vast number of microbial components *via* their LRR-protein domains that point toward the extracellular milieu of plasma membrane or to the topologically equivalent lumen of membrane-enclosed intracellular compartment, such as the lumen of endolysosomes (Kawai and Akira, 2010) (See **Fig. 5**). Recognition of PAMPs by TLRs results in immune signalling *via* receptor homo- or heterodimerization. In addition in some cases, the activation of accessory cell surface- or soluble proteins is required. For example, heterodimerization of TLR2 with TLR1 and TLR6 potentiate the recognition and discrimination between tri- and diacetylated lipopeptides, respectively, of gram-negative and -positive bacteria, protozoa, mycobacteria, yeast, mycoplasma, and certain viruses (like herpes simplex virus and vaccinia virus (Barbalat et al., 2009; Farhat et al., 2008; Kurt-Jones et al., 2004), whereas binding of TLR2 to cell surface receptors, such as CD36 and Dectin-1, further extends its ligand range to different PAMPs (Goodridge and Underhill, 2008; Hoebe et al., 2005). TLR4, in turn, uses accessory proteins, such as LPS-binding protein (LBP), and LRR-containing MD2 and CD14, for efficient responsiveness to LPS (Shimazu et al., 1999; Tobias et al., 1986; Wright et al., 1990). Importantly, TLR4 can recognize also structural features of some viruses like vesicular stomatitis virus (VSV) and DAMPs (Georgel et al., 2007; Miller et al., 2005). At present, it is the only TLR reported to become activated by inorganic molecules, nickel cations, which are the causative factor in nickel allergy affecting approximately 30% of the general population (Schmidt et al., 2010; Thyssen and Menne, 2010).

1.4.1.1. Nucleic acid-sensing TLRs in viral recognition

TLR3, -7/8, and -9 are the most important TLRs for the detection of viruses: they are dedicated to recognizing nucleic acids (and their chemical mimetics) originating from the extracellular environment: dsRNAs (polyinosinic-polycytidylic acid (poly(I:C)), uridine- or uridine/guanosine-rich ssRNAs (imidazoquinone R848), and unmethylated CpG-nucleotides of linear DNA strands (synthetic CpG-DNAs) (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2002; Hemmi et al., 2000; Lee et al., 2003; Lund et al., 2003; Lund et al., 2004). With some exceptions, nucleic acid-sensing TLRs reside primarily in endolysosomal compartments (endosomes, lysosomes, and in endolysosomes) where phagocytosed and endocytosed material is gathered. In addition, viral components sequestered from cytoplasm to endolysosomes *via* a process called autophagy, may trigger endosomal TLRs (Orvedahl and Levine, 2008). Thus, positioning TLRs into endolysosomes is related to their function: nucleic acid-sensing TLRs sample material entering cells mainly from outside the cells. In addition that the acidic environment of endolysosomes releases nucleic acids from the interior of viral capsids, this site concentrates extracellular nucleic acids in order to achieve more efficient recognition of them by TLRs. The compartmentalization may also help in the determination of the source of nucleic acids (Barton et al., 2006; Nishiya et al., 2005; Johnsen et al., 2006): under normal conditions, several nucleases degrade nucleic acids in the extra- and intracellular spaces which means that endosomes are mainly devoid of endogenous RNA or DNA molecules, as described in 1.2.1 (Vilaysane and Muruve, 2009).

Interestingly, the altered gene number of nucleic acid-sensing TLRs may predispose to the development of autoimmune diseases, as suggested by studies conducted in murine models of systemic lupus erythematosus, implying that the functions of TLRs have to be tightly controlled in order to maintain immune homeostasis (Deane et al., 2007; Subramanian et al., 2006).

1.4.2. Retinoic acid-inducible gene-I (RIG-I) -like receptors (RLRs)

In most cell types, detection of cytoplasmic RNA molecules is carried out by DExD/H-(Asp-Glu-X-His/Asp)-box RNA helicases called RLRs: RIG-I, melanoma differentiation-associated gene 5 (MDA-5) and LGP2 (Laboratory of Genetics and Physiology 2) (Andrejeva et al., 2004; Kato et al., 2005; Yoneyama et al., 2005; Yoneyama et al., 2004). In general, DExD/H-box helicases have various functions in transcription, mRNA splicing, translation, ribosome biosynthesis, and RNA decay (Schwer, 2001). Initially, RIG-I, MDA-5, and LGP2 were independently cloned by separate groups, but it was not before functional screenings and assays made by Yoneyama *et al.*, that these DExD/H-box RNA helicases were linked to antiviral defence and innate immunity (Yoneyama et al., 2005; Yoneyama et al., 2004).

RIG-I was cloned as *all-trans* retinoic acid-inducible gene in 1997 (Sun, 1997). Additional work revealed that the helicase domain of RIG-I displayed high homology to a

helicase in a nematode (roundworm) (Tabara et al., 2002). This helicase was part of a RNA-induced silencing complex functioning in dsRNA-mediated post-transcriptional gene silencing. Soon, it was found that LPS and interferon (IFN)-γ upregulated RIG-I pointing to its involvement in inflammation and innate immunity (Imaizumi et al., 2002; Imaizumi et al., 2004). MDA-5, in turn, was characterized as a new caspase recruitment domain (CARD)-containing protein with pro-apoptotic functions (Kovacsovics et al., 2002). The expression of MDA-5 was induced by IFNs and the helicase had dsRNA-dependent ATPase activities and melanoma growth suppressive properties (Kang et al., 2002). The gene encoding for LGP2 was found from the same gene locus which encoded important transcription factors, signal transducers and activators of transcription (STAT) -3 and -5 (Stat3/Stat5), for proliferation and differentiation of mammary glands (Cui et al., 2001). By the year 2005, all these DExD/H-box RNA helicases had been demonstrated to detect cytoplasmic dsRNA and share unique antiviral functions in innate immunity (Andrejeva et al., 2004; Yoneyama et al., 2005; Yoneyama et al., 2004).

RIG-I and MDA-5 induce primarily antiviral cytokines, IFNs, in response to the presence of viral RNAs in cytoplasm. They are the actual effector molecules of RLRs because LGP2 lacks two N-terminal CARDs necessary for downstream signalling. Instead, LGP2 presumably modulates the functions of RIG-I and MDA-5 (Murali et al., 2008; Rothenfusser et al., 2005; Satoh et al., 2010; Venkataraman et al., 2007; Yoneyama et al., 2005). Furthermore, the fully efficient IFN response to various RIG-I dependent viruses requires LGP2, with the notable exception of influenza A virus and an artificial mimetic of viral cytoplasmic dsRNA, poly(I:C) (Satoh et al., 2010). RIG-I and MDA-5 interact with a shared signalling molecule found mainly at the mitochondrial membrane *via* homotypic CARD-CARD-interactions. Four distinct research groups independently and simultaneously characterized and named this signalling partner as mitochondrial antiviral signalling protein (MAVS), CARD adapter-inducing IFN-β (Cardif), virus-induced signalling adapter (VISA), and IFN-β promoter stimulator-1 (IPS-1) (Loo et al., 2006; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). (Here, the name MAVS is utilized). Although, the original reports indicated that RIG-I and MDA-5 have structural similarities and respond equally well to viral dsRNA (Yoneyama et al., 2005; Yoneyama et al., 2004), later knock-out studies in mice revealed differences in their specificities against different viruses (Kato et al., 2006; Loo et al., 2008). Thus, RIG-I and MDA-5 are not completely functionally equivalent.

1.4.2.1 RNA recognition of RIG-I and MDA-5

RIG-I has the ability to detect several negative-strand ssRNA viruses, such as influenza A virus, VSV, and Sendai virus; and positive-strand ssRNA viruses like Japanese encephalitis virus. Subsequently, RIG-I triggers IFN-signalling (Kato et al., 2006). Remarkably, even less than 20 molecules of viral RNA can be sensed by RIG-I (Zeng et al., 2010). RIG-I detects primarily RNAs carrying 5'-ppp (Hornung et al., 2006; Pichlmair et al., 2006). This structure is absent in host RNAs (except the 7SL RNA, depicted earlier) because eukaryotic RNAs are capped, or the 5'-ppp is post-transcriptionally removed or

otherwise modified in the nucleus (*e.g.*, 5'-ppp is removed from mRNAs, rRNAs, and tRNAs or the last base in RNA containing the free 5'-ppp is modified) (Perry, 1981). In contrast, several viral polymerases and T7-bacteriophage, also used for *in vitro* transcription in molecular biology, can polymerize 5'-ppp. Interestingly however, 5'-ppps are not solely sufficient to activate the antiviral actions of RIG-I: chemically synthetized 5-ppp ssRNAs do not activate RIG-I, suggesting that the enzymatic synthesis of RNAs produces also some dsRNA by-products or structures that are required for activation of RIG-I (Schlee et al., 2009; Schmidt et al., 2009). Indeed, chemically synthesized dsRNA with or without 5'-ppp or 5'-monophosphate, and short *si*RNAs lacking two nucleotide 3'-overhangs engage RIG-I (Kato et al., 2008; Lu et al., 2010; Marques et al., 2006; Takahasi et al., 2008). Thus, full responsiveness of RIG-I towards the viral RNAs seems to depend on 5'-ppp in conjunction with short blunt-end dsRNA regions (Lu et al., 2010; Pichlmair et al., 2006). Recently Rehwinkel *et al.,* demonstrated that the *natural agonist* of RIG-I was RNAs containing short blunt-end, short dsRNA, and at least one 5'-ppp (Rehwinkel et al., 2010). These kinds of partially complementary panhandle regions at the 5'- and 3'-ends of RNAs are found in the genomes of negative sense ssRNA viruses, like influenza A virus (Hsu et al., 1987).

Interestingly, RIG-I is also capable of recognizing indirectly RNA virus infections by detecting short cleavage products of endogenous ssRNAs made by host ribonuclease-L (RNaseL) (Malathi et al., 2007) (See 1.5.2.). Moreover, RIG-I is unexpectedly involved in the cellular DNA-sensing machinery, which is poorly characterized at present: RIG-I is engaged when host polymerase III detects cytoplasmic adenosine and thymidine-rich DNAs and uses them as a template to synthesize dsRNA molecules with 5'-ppp (Ablasser et al., 2009a). This kind of RNA recognition appears to be relevant for the detection of small non-protein coding DNA molecules of a DNA-virus Epstein-Barr virus (termed as EBER1 and -2 RNA), as well as for the recognition of other DNA-viruses, such as adenovirus and herpes simplex virus (Ablasser et al., 2009a; Chiu et al., 2009).

MDA-5 is the primary sensor for coronaviruses, such as severe acute respiratory syndrome coronavirus and picornaviruses, like poliovirus and encephalomyocarditis virus (EMCV) (Gitlin et al., 2006; Kato et al., 2006; Zust et al., 2011). These viruses subvert RIG-I-mediated recognition by having either covalently bound protein attached to their 5'-RNA-end or by mimicking functions homologous to and associated with the formation of 5'-cap structure. However, even these measures are insufficient to evade recognition by MDA-5. Importantly, MDA-5 recognizes viral mRNAs that lack 2'-O-methylation in their 5'-end (Zust et al., 2011). In contrast to viral RNAs, this molecular structure is present in the 5'-cap-elements of mammalian mRNAs. MDA-5 is also the primary sensor of viruses with long dsRNA stretches (over 1 kb in length) and of long cytoplasmic dsRNA polymers, like poly(I:C), which has been used for decades as an artificial mimic of viral infection (Kato et al., 2008).

Importantly, RIG-I and MDA-5 may cooperate in the detection of viruses. For example, cytoplasmic poly(I:C) is length-dependently sensed also by RIG-I: while long (~ 4000-8000 bp) cytoplasmic poly(I:C) strands activate MDA-5, the short ones (median size 300

bp) engage RIG-I (Kato et al., 2008). The recognition of cytoplasmic poly(I:C) by RIG-I is due to activities of a dsRNA specific endonuclease, Dicer, which partially digests poly(I:C) and subsequently converts it into a substrate of RIG-I. This finding also suggests that long viral RNAs primarily detected by MDA-5 may also be sensed by RIG-I. In addition to RIG-I, RNAseL cleavage products also engage MDA-5 after duplex formation (Malathi et al., 2007). Furthermore, certain viruses contain RNA structures or sequences that activate both RIG-I and MDA-5 (Broquet et al., 2010). These kinds of viruses include segmented dsRNA virus reovirus and positive-strand RNA viruses like West Nile virus and dengue virus (Broquet et al., 2010; Fredericksen and Gale, 2006; Loo et al., 2008). Furthermore, RIG-I has crucial role in the initial detection of West Nile virus infection, whereas MDA-5 amplifies and maintains the response. Taken together, RLRs-mediated recognition and distinction of cytoplasmic RNAs between host and virus, and between different virus families, seem to be very complex, but very accurate processes.

1.4.3. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and inflammasomes

NLRs are cytoplasmic receptors involved in the detection of PAMPs and DAMPs and their activities are often licensed by other NLRs within the family or by other PRRs (Davis et al., 2010; Martinon et al., 2009). At present, a total of 22 NLR proteins have been characterized in mammals and classified into four distinct groups based on their structure: 1) NODs; 2) NOD, LRR, and pyrin domain-containing proteins (NLRPs); 3) NLRC4 (NLR family, CARD domain containing-4), formerly known as IPAF and NAIPs (NLR family, apoptosis inhibitory protein); and 4) CIITA (Class II Transactivator). Interestingly, NLR orthologues are present in plant disease resistance genes, and these gene products are intended to sense activities or cellular processes that are commonly associated with microbial infections, rather than in the direct detection of PAMPs (Nurnberger et al., 2004). (This indirect recognition of pathogens is also found in *Drosophila*: proteases activated by the microbial insult cleave the cytokine Spätzle, which then binds to *Drosophila Toll* triggering the antimicrobial response (Lemaitre, 2004; Lemaitre et al., 1996).) The importance of NLRs in antiviral responses is underlined *e.g.*, by the recent discovery of viral homologue of NLRP1, encoded by Kaposi's sarcoma-associated herpesvirus, namely Orf63 (open reading frame 63) (Gregory et al., 2011). This viral protein can antagonize the functions of NLRP1. Furthermore, NLR proteins, such as NLRX1 (NLR family, X1) and NLRC5 (NLR family, CARD containing-5), may interact with RLRs and negatively regulate their functions (Moore et al., 2008; Cui et al., 2010).

NLRs are expressed in autorepressed forms. After direct or indirect binding of their LRR-domains with ligands (with the exception of NLRP10 which lacks LRRs (Ting et al., 2008)), LRRs oligomerisize in an ATP-dependent manner, and large multiprotein molecular platforms, approximately 700 kDa in mass are formed (Agostini et al., 2004; Martinon et al., 2002; Martinon et al., 2009). The name "inflammasome" is used to describe the protein complexes that confer precursor processing of IL-1-family of pro-inflammatory cytokines into their biologically active forms, hence the name

"inflammasome". Although various inflammasomes exist (*e.g.*, NOD2/NLRP1-, IPAF/NLRC4-, RIG-I/ASC-, and AIM2-inflammasomes), they all comprise at least effector protein **c**ysteine-dependent **asp**artate-directed prote**ase** (caspase)-1 and adapter protein apoptosis-associated speck-like protein containing CARD (ASC). In humans, the structure is also completed by another adapter molecule called cardinal (Agostini et al., 2004). The importance of inflammasomes is related to the function of activated caspase-1. It processes precursors of IL-1 family of pro-inflammatory cytokines, namely pro-IL-1β and pro-IL-18, into their biologically active forms that are then secreted (Cerretti et al., 1992; Thornberry et al., 1992). The name for each inflammasome is given according to the "sensor"-molecule which likely recognizes PAMPs and/or DAMPs. In inflammasome research, attention has focused on NOD-like receptor protein-3 (NLRP3), formerly called NALP3, cryopyrin, or PYPAF1 (Ting et al., 2008). Its function is associated with the development of inflammation in many non-infectious and infectious diseases.

1.4.3.1. NLRP3-inflammasome

NLRP3 is predominantly expressed in immune and epithelial cells, and in bone degrading cells, osteoblasts, thus, in strategically important cell types for health and disease (Dowds et al., 2004; Feldmeyer et al., 2007; Guarda et al., 2011; Martinon et al., 2009; McCall et al., 2008; Watanabe et al., 2007). The functional importance of NLRP3 in innate immunity is emphasized in patients who carry a single point mutation in the NLRP3-encoding gene, *CIAS*. These individuals suffer from rare hereditary autoinflammatory illnesses in which an array of tissues and organs (lungs, gastrointestinal tract, skin, and central nervous system) are affected due to the overactivation of NLRP3-inflammasome (Agostini et al., 2004; Hoffman et al., 2001; Manji et al., 2001; Martinon et al., 2009).

Many potential PAMPs and DAMPs activate NLRP3 oligomerization. Whole pathogens which have been demonstrated to activate the NLRP3-inflammasome include *Neisseria gonorrhoeae* and *Staphylococcus aureus* (Duncan et al., 2009; Kankkunen et al., 2009; Mariathasan et al., 2006). In addition, hyphaes *i.e.*, the long and branching filamentous structures of *Candida albicans*; bacterial pore-forming toxins; and hemozoins *i.e.*, crystallized waste products of heme formed by parasite *Plasmodium falciparum*, activate NLRP3 (Dostert et al., 2009; Joly et al., 2009; Mariathasan et al., 2006). Importantly, acute infection with RNA and DNA viruses, such as influenza A virus and Sendai Virus, as well as modified vaccinia virus Ankara, results in NLRP3-inflammasome activation (Allen et al., 2009; Delaloye et al., 2009; Ichinohe et al., 2009; Kanneganti et al., 2006; Thomas et al., 2009). The exogenous particulates or chemicals that trigger NLRP3-inflammasome indicate that the action of NLRP3 is important in the development of certain work-related diseases (silica, asbestos), contact hypersensivity reactions (certain skin irritants), actions of vaccines (vaccine adjuvant alum), and certain pharmaceuticals (*e.g.,* antibiotics) (Allam et al., 2011; Cassel et al., 2008; Dostert et al., 2008; Eisenbarth et al., 2008; Hornung et al., 2008; Kool et al., 2008; Sharp et al., 2009; Watanabe et al., 2007). Interestingly, also electromagnetic radiation *e.g.,* ultraviolet B-light, activates NLRP3 and subsequently causes sunburn-associated inflammation (Feldmeyer et al.,

2007). Furthermore, also the host-derived molecules, DAMPs, activate NLRP3. These include crystals of cholesterol and monosodium urate, aggregates of fibrilliar peptide amyloid-β, and oligomers of islet amyloid polypeptide (Duewell et al., 2010; Halle et al., 2008; Martinon et al., 2006; Masters et al., 2010). These molecules have been linked with the development of atherosclerosis and arthritis, gout, Alzheimer's disease, and type II diabetes, in this same order. The vast structural divergence of these NLRP3 activating PAMPs and DAMPs and their close association to cellular stress responses suggest that they induce the common changes required for NLRP3-inflammasome assembly and subsequent caspase-1 activation. The molecular mechanisms that orchestrate inflammasome activation during virus infections are poorly understood.

1.4.3.2. Inflammasomes in virus recognition

Currently, at least three distinct inflammasomes have been characterized to function in antiviral defence: AIM2-, NLRP3-, and RIG-I/ASC-inflammasomes (Poeck et al., 2009; Rajan et al., 2011). In addition, there is preliminary evidence for existence of MDA-5/ASC-inflammasome, but this awaits further elucidation (Poeck et al., 2009; Rajan et al., 2011). DNA-viruses, such as vaccinia virus and mouse cytomegalovirus are detected by the AIM2-inflammasome (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Rathinam et al., 2010), whereas the recognition of RNA viruses seems to be more complex. For example, the pro-inflammatory cytokine response against EMCV, has been reported to be MDA-5 and NLRP3-dependent or governed by NLRP3 alone (Poeck et al., 2009; Rajan et al., 2011). Similarly, VSV has been shown to activate either RIG-I/ASC- or NLRP3-inflammasome (Poeck et al., 2009; Rajan et al., 2011). The reasons for discrepant results are unclear at present. Furthermore, in regards of influenza A virus infection, the host response to the virus is NLRP3-independent during first week post-infection, while in later stages it is NLRP3-dependent (Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009). At the moment, the requirement of RIG-I for inflammasome activation during these phases is not known. Moreover, also cytoplasmic poly(I:C)-induced inflammasome activation has been reported to be NLRP3-dependent with or without concomitant requirement of MDA-5 (Poeck et al., 2009; Rajan et al., 2010). Collectively, these results highlight the variabililty in the requirement of cytoplasmic PRRs to activate caspase-1 and to elicit inflammation in response to RNA-viruses. Furthermore, they point to the existence of multiple pathways of activating inflammasomes, partly depending on the virus family and the genus. It is not known, why different inflammasome structures are utilized to elicit pro-inflammatory responses against distinct virus families and within a virus family. Presumably, they serve to safeguard synthesis and the secretion of IL-1 family of pro-inflammatory cytokines under different circumstances.

1.5. Other dsRNA-activated effector proteins of the innate immunity

In addition to nucleic acid-sensing TLRs and cytoplasmic PRRs recognizing RNAs, also other effector molecules detect viral RNAs. However, their expression levels are low in uninfected cells, and need to be boosted before their full effectiveness can be attained. The initial recognition of viral RNA results in type I IFNs production by RLRs in most cell types and by TLR7/8 (but not *via* TLR3) in plasmacytoid DCs (pDCs) (Diebold et al., 2004; Kato et al., 2005; Kato et al., 2006; Lund et al., 2004). Type I IFNs trigger expression of hundreds of IFN-inducible genes which constitute an important antiviral part of innate immunity. Three of these IFN-inducible antiviral effectors have been extensively studied.

1.5.1. Double-stranded RNA-activated Protein kinase (PKR)

PKR was one of the first antiviral dsRNA-binding proteins characterized (Kerr et al., 1977). It is activated by most viruses, and consequently, viruses counteract its antiviral actions through a variety of mechanisms (Langland et al., 2006). The importance of PKR during viral infections is vital since PKR phosphorylates and subsequently inactivates elF2α, an initiator of translation (Meurs et al., 1992). Thus, activity of PKR suppresses the translation not only of viral, but also of host mRNAs. If translational block continues for a long time, the infected cell will ultimately die, but in so doing, it will slow down the spread of viral infection, and the organism itself may be spared. Phosphorylation of elF2α triggers also autophagy, one form of cellular intrinsic defence mechanism in which constituents of cytoplasm and mal-functioning components and organelles are digested by lysosomal enzymes in autophagolysosomes. Subsequently, the resulting building blocks are recycled in anabolic reactions within the cell (Talloczy et al., 2002). Programmed cell death can also be considered as another form of the cell's intrinsic defence mechanisms, and it is also partly mediated by PKR (Balachandran et al., 1998).

1.5.2. 2'-5'-oligoadenylate synthetase (2'-5'OAS) and ribonuclease-L (RNAseL) system

Activation of 2'-5'OAS by dsRNA results in conversion of ATP to free phosphate molecules and formation of 2'-5'-linked oligoadenylates of various lengths (Hartmann et al., 2003). These trigger activity in the RNA-degrading enzyme RNaseL. Cytoplasmic RNaseL catalyses degradation of self- and non-self ssRNAs resulting in a halt of protein synthesis and engagement of RIG-I and MDA-5 by the 3'-phosphorylated RNA degradation products (Malathi et al., 2007). Not only does the RNaseL system amplify the activation of RLR pathway, but RNAseL has also been implicated in the induction of apoptosis (Li et al., 2004).

1.5.3. Mx protein A (MxA)

In contrast to PKR, 2'-5'OAS, and RNAseL that function broadly against several viruses, small IFN-inducible GTPases, MxA and MxB proteins in humans, defend against negative-strand RNA viruses like influenza A virus and VSV (Pavlovic et al., 1990). Human MxA interacts with proteins of influenza A virus in the nucleus where MxA inhibits the early transcription of the viral genome (Turan et al., 2004).

2. Innate immune responses against viruses

Innate immune responses against viruses are initiated when virus attaches to the surface of a permissive cell and invades the cell. If the viral infection outpaces the cell's intrinsic innate defences, more global host responses will be experienced. Often inflammation appears when infected cells die or lyse, and innate and acquired immune responses develop. If the infection is spread from the initial inoculation site, second and third rounds of replication may occur in other parts of the body. If the infection is cleared, the host will return to its normal, pre-infection state **(Fig. 2)**. However, dramatic differences exist in patterns of infection, ranging from acute to lifelong persistent infections. The extent of activation of innate and acquired immune responses determines the host's immunity to subsequent infections by the same virus. The most important innate immune responses of cells against viruses include antiviral and pro-inflammatory cytokine responses, and apoptosis of the virus-infected cell. Only the acute phase of infection will be considered here.

2.1. Antiviral cytokine response: Interferons (IFNs)

Mammals, birds, reptiles, and fish synthesize IFNs, which have high antiviral, anti-proliferative, and immunomodulatory properties. IFNs were discovered first by Isaacs and Lindenmann in 1957, who observed that chicken cells exposed to influenza A virus contained a substance that "interfered" with infection of other chicken cells by a live influenza A virus, hence the name "interferon" (Isaacs and Lindenmann, 1957). Nowadays, more than 20 IFN genes are known, and they are classified into three groups. In humans, type I IFNs (often abbreviated as IFN-α/β) contain multiple IFN-α genes (at least 13) and single copies of IFN-β, IFN-ω, -κ, and -ε genes (Génin et al., 2009). Type II IFNs comprises solely of IFN-γ. It is secreted by activated cells of the innate and acquired immunity: NK cells and T-cells, respectively (Renauld, 2003). In animal models of virus infection, the outcome of viral pathogenicity can be more severe upon disruption of IFN-α/β response than when IFN-γ signalling is abrogated (Muller et al., 1994), implying that type I IFNs play the major role in defence against viruses, as well as against some other microbes (Freudenberg et al., 2002). The Type III IFNs represent for IL-28A (or IFN-λ2), IL-28B (or IFN-λ3), and IL-29 (or IFN-λ1) (Kotenko et al., 2003). These antiviral

cytokines have same biological effects than type I IFNs since, apart from their receptors (IFNAR1 and -2, and IL-28R and IL-10Rβ, respectively), they share the same signal transduction pathways (*i.e.*, IFN-stimulated gene factor-3 (ISGF3); see 2.1.2.) (Hengel et al., 2005; Renauld, 2003; Sheppard et al., 2003).

2.1.1. Systemic and context-specific effects of IFN-α/β

Plasmacytoid DCs account for only 0.5% of peripheral blood mononuclear cells in humans, but they are still the principal cell type responsible for producing systemic concentrations of IFN-α/β (Colonna et al., 2004; Kumagai et al., 2007; Siegal et al., 1999). In contrast, the RLR pathway, predominating in all other cells types, contributes to local IFN-α/β production (Kato et al., 2005; Kato et al., 2006). Indeed in mice, the primary IFN-α-producing cell type in response to pulmonary RNA viruses is the alveolar macrophage (Kumagai et al., 2007). The importance of type I IFNs is highlighted by the fact that almost all nucleated cells produce these agents and also are able to respond to them.

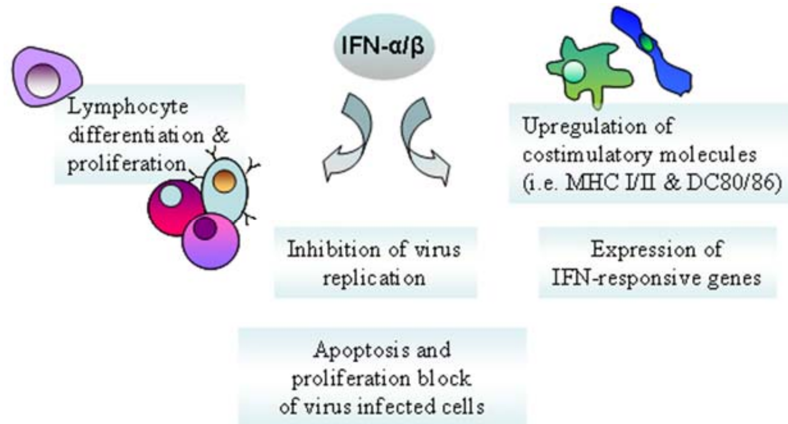


Figure 6 ***Immunomodulating effects of IFN-α/β****. In order to restrict microbial infection, both non-immune cells (such as resident cells at the site of infection) and cells of the innate- and acquired immunity respond by versatile manner to IFNs. Adapted from Solis et al., (2006).*

The systemic IFNs have context- and cell-specific effects. Cells that encounter type I IFNs without any other cues of the infection induce the expression of more than 300 IFN-inducible genes which are involved in the detection and elimination of incoming microbes (de Veer et al., 2001). However, when the IFN-signalling is associated with other signs of infection, cells may also become sensitive to apoptotic signals (Tanaka et al., 1998; Kaiser et al., 2004). IFNs induce macrophages and DCs to express surface receptors for costimulation and antigen-presentation, such as CD80/86 and MHCI/II, respectively (Luft et al., 1998). Noticeably, IFN-α/β permit DCs to present antigens directly without the help of $CD4^{+}$T-cells to cytotoxic- $CD8^{+}$T-cells; stimulate effector and memory functions of

cytotoxic- $CD8^+$T-cells; enhance the cytolytic activity of NK cells; and directly improve clonal expansion of $CD4^+$T-cells (Havenar-Daughton et al., 2006; Kolumam et al., 2005; Le Bon et al., 2003; Nguyen et al., 2002). Furthermore, IFN-α/β and DCs enable B cells to undergo antibody isotype switching and differentiation into antibody-producing plasma cells (Le Bon et al., 2001). In summary, IFNs-α/β represent a crucial link between myeloid and lymphocytic cell populations, as well as between innate and acquired immune reactions (**Fig. 6**). The ultimate goal of secreted IFNs' is to achieve the elimination of infected cells.

2.1.2. Induction of IFN-α/β: two phases

The primary antiviral IFN response is initiated by the activation of certain TLRs and RLRs resulting in the expression of IFN-α/β. This early antiviral response is relatively weak, and is followed by a more potent, secondary phase of IFN induction in a positive feedback manner. The secondary IFN-response begins when the *de novo* synthetized IFN-α/β bind to their cognate receptors *via* auto- or paracrine manner resulting in transcription of IFN-stimulated genes (Honda et al., 2005; Marie et al., 1998; Sato et al., 2000).

Both IFN-α/β phases are predominantly induced through sequential activation of transcriptional activators, called interferon regulatory factors (IRFs)-3 and -7 (Sato et al., 2000). The first wave induces primary production of IFN-β and IFN-α1 (IFN-α4 in mice) through the transcriptional activator IRF3 which is constitutively produced in many cell types (Marie et al., 1998; Solis et al., 2006). When a critical threshold is exceeded, *i.e.*, when enough *de novo* IRF7, IFN-βs, and IFN-α1s have been synthesized and freshly produced IFN-α/β have bound to their cognate receptors (IFNAR1 and IFNAR2) and signalled, the second state of IFN-α/β synthesis takes place. The receptor engagement activates Janus kinases (JAK, or "Just another kinase") (namely, Jak1 and Tyk2) which phosphorylate STAT1 and -2 transcription factors. Subsequently, STAT1 and -2 heterodimerize and interact with IRF9. Next, the formed cytoplasmic complex, called ISGF3, translocates into the nucleus, binds to genes containing IFN-stimulated response elements (ISREs), and induces their expression (Renauld, 2003). ISRE-containing genes include other IFNs, IRF7, RLRs, PKR, 2'-5'OAS, RNAseL, and MxA, to name but a few (**Fig. 7**).

The initial IRF3 activation and resulting IFN-β and IFN-α1s production are vital for antiviral responses because IRF3 and IRF7 cannot efficiently form homo- or heterodimers and activate robust production of antiviral cytokines and IFN-inducible genes unless the originally low expression level of IRF7 (due to its short-half life) is augmented (Sato et al., 2000).

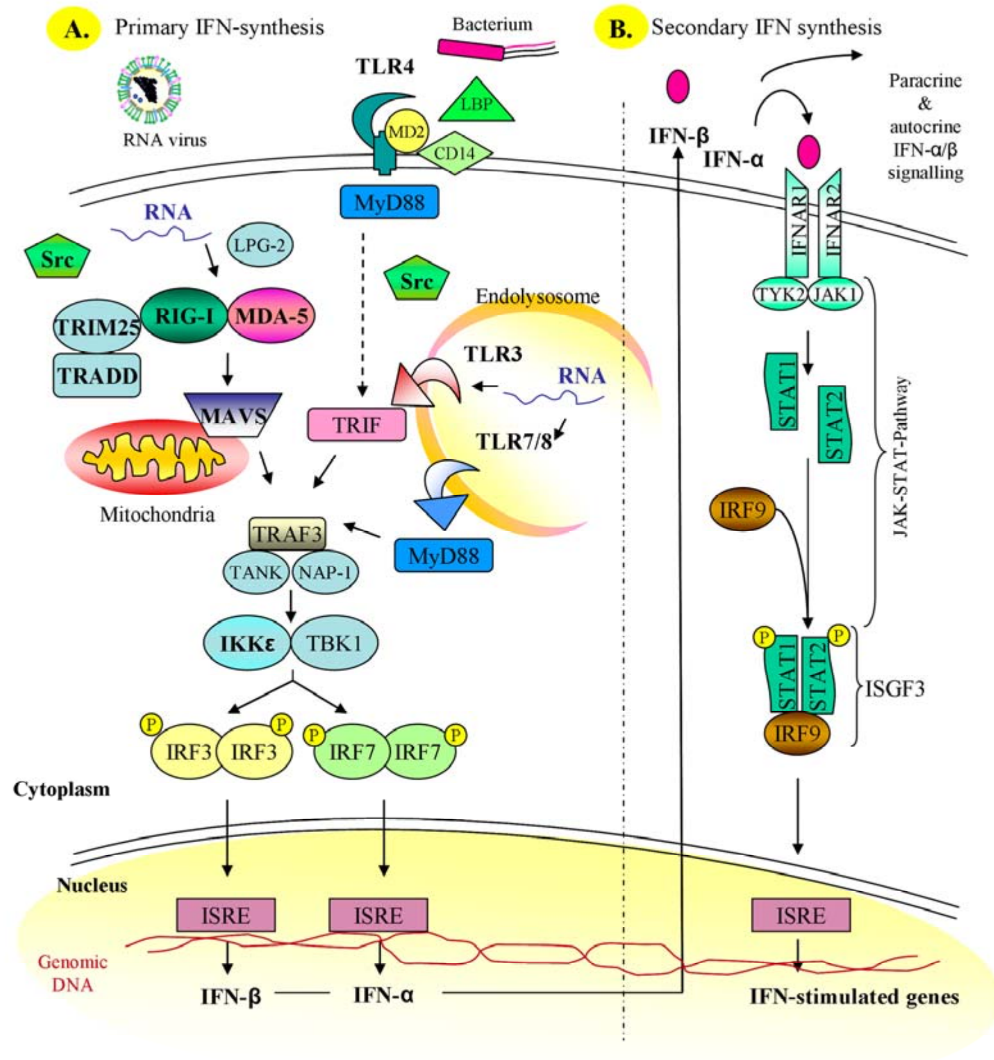


Figure 7 ***Simplified view of RNA-virus-induced (A) primary and (B) secondary signal transduction pathways for expression of IFN-α/β and IFN-stimulated genes, respectively.*** *(A) Engagement of TLR3, -4, -7/8, and of RIG-I and MDA-5 induce IFN-α/β expression through TRIF-, MyD88/TRIF-, MyD88-, or MAVS-dependent pathways, respectively, all culminating ultimately in the activation and translocation of IRF3/IRF7 into the nucleus. (B) The secreted and de novo produced IFN-α/β signal via IFNRA1/2s and Jak-Stat pathways to initiate expression of IFN-responsive genes and subsequently to pave the way for more powerful IFN-responses. Molecules marked in bold have been addressed in the original publications I-IV.*

2.1.3.1 IFN-α/β production mediated through TLRs

In general, the induction of IFN-signalling through TLRs does not require that the cell itself is infected: phagocytic cells take in material from the extracellular environment that may engage TLRs in the endolysosomal compartment, as described earlier in 1.1.1 and 1.4.1.1. Upon ligand binding to TLRs, the TIR (**T**oll/**I**L-1**R**)-domain of TLRs recruits other TIR-domain containing adapter proteins which are important for IFN production.

The most important of these are MyD88 (myeloid differentiation primary response gene-88) and TRIF (TIR-domain containing adapter inducing IFN) (Kawai et al., 1999; Kawai and Akira, 2010; Medzhitov et al., 1998; Yamamoto et al., 2003), both of which have their own specific accessory proteins which link them to IRF3 and/or IRF7 activation. Apart from TLR3, which signals solely through TRIF, the other IFN-induction capable-TLRs use adapter protein MyD88 for IFN induction. However, TLR4 utilizes sequentially both MyD88 and TRIF for IFN-signal transduction (Kagan et al., 2008) (**Fig. 7**).

It has been noted that only PRRs signalling from the interior of the cell are potent for IFN-α/β production for some unclear reason. This compartmentalization probably aids in the discrimination between self and non-self (Barton et al., 2006; Nishiya et al., 2005; Johnsen et al., 2006). Interestingly, TLR2 and -4 located on the plasma membrane, are also capable of IFN induction but only after they have been internalized into the endosomal compartment (Barbalat et al., 2009; Kagan et al., 2008). The mechanistic explanation lies probably in TRAF3 (tumor necrosis factor (TNF) receptor-associated factor-3), a cytoplasmic signal transduction molecule downstream of TRIF and MyD88 (Oganesyan et al., 2006). TRAF3 is unable to recruit itself in the close vicinity of PRRs on the cell surface, thus, the receptors upstream of TRAF3 must be internalized first (Kagan et al., 2008). Why cell surface TLRs initiate IFN-signalling in the first place is not understood, though it may harness IFN production for the enhancement of T- and NK-cell functions when there is some infection.

The IFN-signal transduction pathway downstream of MyD88 and TRAF3 continues through several IRAKs (IL-1R associated kinases) and ubiquitin protein ligases TRAFs (Kawai et al., 1999; Kawai and Akira, 2010; Medzhitov et al., 1998), whereas the components downstream of TRIF seem to involve TBK1 (TANK-binding kinase-1) and IKKε (inducible IκB kinase ε) (Fitzgerald et al., 2003; Kawai and Akira, 2010). The full activation of the IRF3 in response to TLR3 engagement requires yet additional kinases: PI3K (phosphatidylinositol-3-kinase), NAP1 (Nak-associated protein-1), and proto-oncogene tyrosine kinase c-Src (Johnsen et al., 2006; Sarkar et al., 2004; Sasai et al., 2005). Both MyD88 and TRIF-dependent pathways are subjected to strict regulation by highly conserved proteins called ubiquitines (Keating and Bowie, 2009). Ubiquitines are post-transcriptionally attached to specific signalling proteins by ubiquitin protein ligases in order to regulate the stability, function, and intracellular localization of signalling proteins. In addition to ubiquitines, sphingolipids have been shown to be able to modulate signalling *via* TLRs recently, highlighting the complexity of the regulation of the TLR signalling pathway (Napolitano and Karin, 2010) (**Fig. 7**).

2.1.3.2 IFN-α/β production mediated through RLRs

The cytoplasmic RLR pathway leading to IFN-β production is dependent on interactions of CARDs between RIG-I/MDA-5 and MAVS, as described in 1.4.2 (Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). The efficient IFN production in response to RIG-I and MDA-5 agonists is dependent primarily on mitochondrial localization of MAVS and on the formation of high-order signalling complex within the mitochondrial membrane. This

requirement was elucidated in cells infected with hepatitic C virus: NS3/4 protein of the virus was found to block IFN-α/β production by cleaving MAVS from the mitochondrial membrane, which subsequently prevented the association of several essential proteins in close proximity to MAVS (Loo et al., 2006). Important signalling molecules of RIG-I/MDA-5/MAVS-pathway include TBK1, IKKε, TRAF3, TRADD (TNF- receptor-1-associated death domain protein), and c-Src (Hemmi et al., 2004; Johnsen et al., 2009; Michallet et al., 2008b; Saha et al., 2006). Thus, the signal transduction pathways of TLR and RLR for IFN-α/β expression seem to converge at the level of TRAF3 and TBK1/IKKε (Hemmi et al., 2004; Oganesyan et al., 2006; Saha et al., 2006)(**Fig. 7**).

As with TLR-induced IFN-α/β production, RLR signalling is also full of interesting details to achieve efficient antiviral response. For example, cells that have low initial expression level of RIG-I, a DEAD box polypeptide-3 helicase DDX3X may serve as the initial sensor for RNA-mediated IFN production (Oshiumi et al., 2010). Furthermore, peroxisomal MAVS may establish an early and transient antiviral defence response to delay viral replication, before more robust and prolonged IFN-signalling from mitochondrial MAVS is evoked (Dixit et al., 2010). Moreover, regulators of autophagy Atg (**au**t**o**p**h**agy related)-5-Atg12 conjugate, complement receptor gC1qR (receptor for globular head domain of complement component C1q), NLRC5, and NLRX1 negatively regulate RIG-I and MDA-5 (Cui et al., 2010; Jounai et al., 2007; Xu et al., 2009, Moore et al., 2008), suggesting that distinct cell's intrinsic defence processes and effectors of innate immunity are being coregulated to mount the appropriate intensity of antiviral responses.

Ubiquitination plays an important part also in RIG-I-mediated IFN-α/β production. The tripartite motif protein-25 (TRIM25) and RNF135 (Ring finger protein-135) ubiquitine ligases attach polyubiquitins to RIG-I to enable RIG-I's downstream signalling, whereas ubiquitin ligase RNF125 negatively regulates the RLR-pathway (Arimoto et al., 2007; Gack et al., 2007; Oshiumi et al., 2009; Zeng et al., 2010). In addition to these ubiquitin ligases, the aforementioned proteins, and the RLR family member LGP2 (presented in 1.4.1), there are numerous other proteins which regulate and determine the strength and duration of RLR signalling, implying that IFN-signalling is sophisticatedly fine-tuned in infected cells (Zhong et al., 2008).

2.2. Virus-induced pro-inflammatory cytokine response

In addition to antiviral cytokines, recognition of viruses promotes the secretion of pro-inflammatory cytokines and elicits subsequent inflammatory responses. The latin word for inflammation, *inflammare*, means "set on fire". An acute inflammation is a vigorous response to injurious stimuli and an attempt to remove the cause. The four classical signs of inflammation; redness, heat, swelling, and pain, were already described in the 1st century *Anno Domini* by the Roman Doctor Cornelius Celsus and published in the book of De Medicina (Libby, 2007). The inflammatory symptoms can be attributed to increased blood flow, capillary permeability, influx of phagocytic cells, and tissue damage. The extent and nature of virus-induced inflammation depends on the primary site of infection and type of the infecting virus: 1) strong inflammatory responses are restricted in

privileged inflammatory sites, as in brain and testicles; 2) noncytopathic viruses do not necessarily induce strong inflammatory responses; and 3) some viruses encode proteins that bypass or reduce activation of inflammatory responses.

Chronic inflammation ensues if innate immunity is dysfunctional or inefficient in clearing the inflammatory agent. The continuous inflammation of tissues is often destructive and leads to serious inflammatory diseases. In addition, virus infection may predispose host with certain genetic background to autoimmune diseases. For example, certain polymorphisms in the *MAVS*-encoding gene are linked to susceptibility to systemic lupus erythematosus, and MDA-5 alleles present in the general populations are associated with type I diabetes, whereas rare loss of function mutations in *MDA-5* correlate with resistance to the autoimmune disease (Liu et al., 2010; Nejentsev et al., 2009; Shigemoto et al., 2009).

2.2.1. IL-1 family of pro-inflammatory cytokines

Interleukin-family of pro-inflammatory cytokines is central to inflammatory responses and innate immunity. The principal member of the family is IL-1β. Initially, IL-1β was characterized as the major fever-inducing agent, but today a plethora of other biological properties have been associated to this cytokine (Dinarello, 2010; Dinarello et al., 1977; Goshen and Yirmiya, 2009; Wolf et al., 2003). The linkage between innate immunity, IL-1β, and inflammation became obvious when the cytoplasmic domains of both IL-1R type I and TLRs (the TIR-domain), were found to be homologous, as described previously in 1.4.1. (Lemaitre, 2004; Martinon et al., 2009; Medzhitov et al., 1997, Valanne et al., 2011).

The IL-1 pro-inflammatory cytokine family consists of 11 members, both activators and inhibitors of inflammation and innate immunity. The original members of the IL-1 superfamily included IL-1β, IL-1α, and IL-1 receptor antagonist (IL-1Ra), the latter competes for receptor binding with IL-1α and IL-1β, and subsequently can block their immunobiological functions (Dinarello, 2009; Dinarello et al., 1981). In addition to these, IL-18, formerly known as IFN-γ-inducing factor, and IL-33 are part of the family and have been studied relatively well (Dinarello, 1999; Liew et al., 2010). The last six members of the superfamily; IL1F5, IL1F6, IL1F7, IL1F8, IL1F9, and IL1F10, share structural similarities to IL-1α and IL-1β, but their actions have not been so fully elucidated. Of these, IL-1F5 and IL-1F7 are believed to promote anti-inflammatory responses (Costelloe et al., 2008; Sharma et al., 2008).

The biology of IL-1 family cytokines is complex: the control over IL-1β- and IL-18-binding to their respective receptors and subsequent downstream signaling is exerted at both the extracellular and at the intracellular levels (see 2.2.3). Extracellularly, the functions of *de novo* secreted IL-1β and IL-18 can be coordinated by transmembrane and soluble receptors: binding of IL-1β to IL-1 receptor type II (IL-1RII) blocks IL-1β-signaling; a transmembrane protein SIGIRR (single immunoglobulin IL-1-related receptor) can suppress IL-1R- and TLR-mediated inflammatory responses in general, and naturally occurring soluble proteins, IL-1Ra and IL-18 binding protein (IL-18BP),

neutralize IL-1β and IL-18, respectively (Colotta et al., 1993; Eisenberg et al., 1990; Novick et al., 1999; Wald et al., 2003). The binding of the IL-1 family of cytokines to their respective receptors; IL-1R1 and IL-1R accessory protein (IL-1AcP) for IL-1β, and IL-18Rα and IL-18Rβ for IL-18, results in increased gene expression in the nucleus (Born et al., 1998; Greenfeder et al., 1995). This can be mediated *via* several signal transduction molecules, like mitogen activated protein kinases (MAPKs), and transcription factors, such as nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (**Fig. 8C and D**) (Dinarello, 1996; Serkkola and Hurme, 1993).

2.2.2. Systemic effects of IL-1β and IL-18

IL-1β and IL-18 are structurally and functionally most related and important cytokines within the IL-1 family. They impact on cells in central nervous system and periphery, and participate in the regulation of homeostasis and behavior (Besedovsky and Del Rey, 2010; Goshen and Yirmiya, 2009, Alboni et al. 2010). Blood monocytes, tissue macrophages, and DCs are the primary producers of IL-1β and IL-18 (Eder, 2009; Pirhonen et al., 1999).

Interleukin-1β indirectly affects (*via* prostaglandin E2 synthesis) the thermoregulatory center of hypothalamus in the brain and induces fever (Dinarello, 2005). This can be achieved at systemic concentrations of IL-1β as low as 1-10 ng *per* kg of body weight (Dinarello, 1996; Ogilvie et al., 1996). In the periphery, endothelial cells respond to IL-1β by producing IL-6 which contributes to vascular permeability and redness. IL-6, in turn, stimulates the synthesis of hepatic acute-phase proteins, and increases platelet production, and together with IL-1β augments number and mobilization of bone marrow cells, such as granulocyte progenitors and mature neutrophils. Increased vascular permeability results in a high protein content of plasma and subsequent swelling, as well as in infiltration of inflammatory cells to the site of infection *via* IL-1β-induced expression of adhesion molecules. IL-1β is also involved in increased pain perception, loss of weight, and fatigue (Dinarello, 1996; Dinarello, 2010; Wolf et al., 2003).

The biological functions of IL-18 are related to, but can also be opposite to that of IL-1β (Andoh et al., 2008; Dinarello, 1999). In addition, a commonly accepted and broad functional division is made by their ability to polarize naïve $CD4^+$T-cells: IL-1β drives predominantly inflammation-associated Th_1-type and autoimmune disease-affiliated Th_{17}-type acquired immune responses, while IL-18, formerly known as IFN-γ-inducing factor, promotes Th_1-type responses only in the presence of IL-12 and IFN-α (Chung et al., 2009; Okamura et al., 1995; Sareneva et al., 1998). However, in the absence of IL-12, IL-18 may also enhance allergy-coupled Th_2-type reactions (Dinarello, 2009; Xu et al., 2000). In general, the common "flu-like" symptoms associated with microbial infections are due to the actions of both of these pro-inflammatory cytokines. Indeed, mice defective in IL-1β or IL-18 signalling are more susceptible to severe influenza A virus infection than their wild type counterparts, showing that both of these pro-inflammatory cytokines are essential for antiviral defence (Kozak et al., 1995; Liu et al., 2004).

2.2.3. Production of IL-1β and IL-18: two phases

The production of IL-1 family of pro-inflammatory cytokines differs from other cytokines, ultimately because of their gene structure. 1) IL-1β and IL-18 are synthesized as immature pro-forms, pro-IL-1β and pro-IL-18, and 2) they lack N-terminal leader signal-peptides which would direct them to the classical ER-Golgi-secretory pathway. In contrast, the precursor-forms remain in the cytoplasm in inactive states after their synthesis. In order to become biologically active and secreted, two signals are most often needed, depending on the type of IL-1 cytokine family member and of cell in question. In macrophages, the first signal is required for transcription and translation of pro-IL-1β, but not that of pro-IL-18 which is continuously produced (Dinarello, 1999; Netea et al., 2009). The second stimulus, in turn, is neccessary for activation of caspase-1-mediated processing of these pro-forms to their biologically active equivalents and for their secretion. However, in contrast to macrophages, monocytes do not need the second signal for processing or secretion: they possess constitutively active caspase-1 (Netea et al., 2009). The reason most probably is that monocytes circulate in the pathogen-free environment of the blood rather than residing in areas where they would be exposed to exogenous stimuli, such as the alveolar space or mucosal lining. Thus, in macrophages, the vigorous inflammatory reactions are tightly controlled by the requirement for two distinct signals. Several proposed mechanisms exist for secretion of IL-1β and IL-18, but the process is still not completely understood (Eder, 2009).

The stability and translational efficiency of mRNAs, precursor processing, and binding of the mature IL-1β and IL-18 to their respective receptors determine the intensity of their biological responses. As with any gene to be transcripted and subsequently translated, the half-lives of its mRNAs and peptides will greatly influence on the final amount of protein produced. For example, the presence of bacterial endotoxins will elevate IL-1β mRNA within 15 minutes with a subsequent decline after 4 h of mRNA accumulation, whereas cells encountering IL-1β itself maintain half of their IL-1β mRNAs for over 24 h (Fenton et al., 1988; Schindler et al., 1990; Serkkola and Hurme, 1993). Furthermore, not all of the produced pro-forms are matured and secreted in healthy individuals: less than 10% of the pool of pro-IL-1β present in monocytes of blood is actually processed, and less than 5% of the total IL-1β produced is released from human monocytes (Dinarello, 2004; Hogquist et al., 1991). Furthermore, the secreted IL-1β can be captured up by type II decoy receptor (IL-1RII), SIGGRR, or soluble decoy receptors, such as IL-1Ra (Colotta et al., 1993; Dinarello, 2009; Eisenberg et al., 1990; Wald et al., 2003). Notwithstanding, the biologic responses to IL-1β can be evident when only 2% of IL-1Rs are occupied by IL-1β and IL-1AcP (Ye et al., 1992). Importantly, IL-1AcP binding to IL-1β and IL-1R is rarely rate limiting if IL-1AcP is expressed: it binds to the cytokine-receptor complex with high affinity (Dinarello, 1996; Greenfeder et al., 1995).

2.2.3.1. Transcription of pro-IL-1β and pro-IL-18

Similar to the situation during IFN-production, transcriptional activation of the *IL-1B*-gene by TLRs is mediated by TIR-TIR-domain contacts between the receptors and their adapter proteins. Depending on the TLR in question, MyD88 and/or TRIF can associate with different combinations of downstream signalling components like TRAM, IRAK1/2, -4, TRAF3 or -6 (Kawai and Akira, 2010). The subsequent activation of MAPK- and NF-κB-pathways is mediated by a complex containing TAK1 (tumor necrosis growth factor-β-activated kinase-), TAB (TAK-1-binding protein)-1, -2, and -3 (Ishitani et al., 2003). Most of these aforementioned proteins are also regulated by the levels of unconjugated free K63-linked polyubiquitin chains (Keating and Bowie, 2009). The TAK1/TAB-complex phosphorylates either MAPK kinase-6 or IκB kinase-β (IKKβ), the latter being part of IKK-complex containing IKKα, and IKKγ/NEMO (NF-κB essential modulator) (Wang et al., 2001). The activated MAPK-pathway triggers binding of the heterodimeric transcription factor AP-1 to promoter areas of pro-inflammatory cytokine genes in the nucleus, whereas the IKK-complex phosphorylates NF-κB inhibitory protein IκB and marks it for degradation (Ninomiya-Tsuji et al., 1999). Subsequently, NK-κB is translocated into the nucleus with a similar enhancing outcome on gene expression than AP-1 (**Fig. 8A**).

RLRs also activate the NF-κB pathway for expression of genes important for initiation and maintenance of the pro-inflammatory response. This occurs in a MAVS-dependent manner *via* TRADD, TRAF6, RIP1 (receptor interacting protein-1), and/or *via* adapters CARD9 and Bcl-10 (B-cell lymphoma/leukemia 10) resulting in the engagement of the IKK-complex (Kawai et al., 2005; Michallet et al., 2008a; Poeck et al., 2009; Xu et al., 2005). After recognition of dsRNA, an additional step for NF-κB-activation FADD (Fas-associated protein with the death domain)-mediated activation of caspase-8 and -10 (Takahashi et al., 2006). However, the precise molecular details are not completely understood.

2.2.3.2. Processing of pro-IL-1β and pro-IL-18

The early observations that cell lysis in a hypotonic buffer could promote conversion of pro-IL-1β to the mature cytokine, led to the identification of caspase-1 (Kostura et al., 1989; Thornberry et al., 1992; Martinon et al., 2002). However, it took several years to obtain the first biochemical evidence from the existence of the inflammasome complex, a platform for caspase-1-mediated pro-inflammatory events, in cytoplasm (Martinon et al., 2002).

As briefly mentioned, in macrophages, caspase-1, formerly known as IL-1β converting enzyme, proteolyses the precursors of IL-1β and IL-18, as well as IL-33, and possibly that of IL-1F7 (Cayrol and Girard, 2009; Dinarello, 2009; Kumar et al., 2002). However, only the cleavage of pro-IL-1β and pro-IL-18 into their mature forms, results in the induction of inflammatory responses: IL-33 is already biologically active as a precursor and it is inactivated by caspase-1, while IL-1F7 has anti-inflammatory effects. Caspase-1 itself is

synthesized as an inactive enzyme precursor, but if there are inflammatory stimuli, then it becomes recruited into inflammasomes which serve as scaffolds for the self-processing of pro-caspase-1 to its mature form, and for subsequent caspase-1-mediated processing of precursors of IL-1β and IL-18 (Agostini et al., 2004; Martinon et al., 2002).

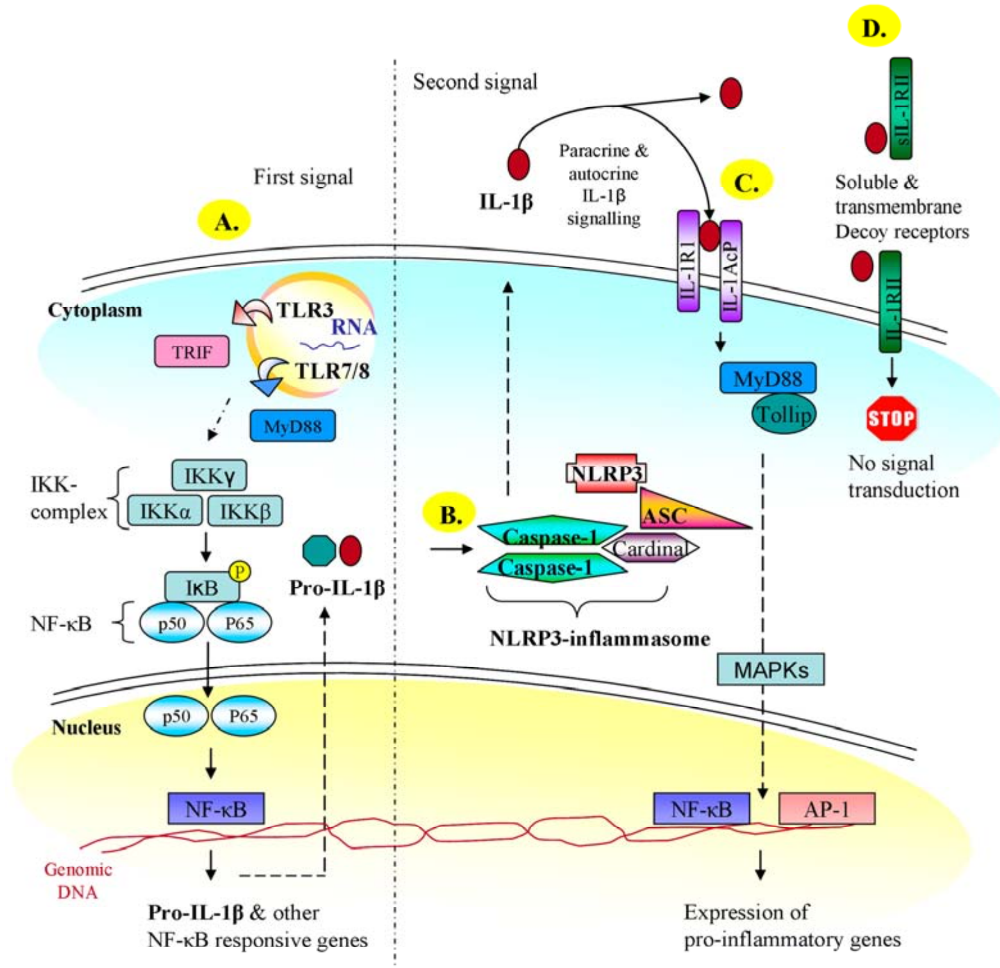


Figure 8 ***Simplification of IL-1β production, secretion, and signalling.*** *IL-1β synthesis is initiated by signal 1 that induces (A) transcriptional activation of pro-IL-1β gene which is followed by signal 2 resulting in (B) caspase-1-mediated processing of pro-IL-1β to biologically active equivalent in cytoplasmic multiprotein complex, called inflammasome. (C) Auto- or paracrine binding of secreted IL-1β to IL-1R-complex activates signalling cascades resulting in expression of important inflammation-associated genes. It is noteworthy that IL-1R1 and TLRs share TIR-domains and also partially the same signal transduction components. (D) Decoy receptors may block IL-1β signalling.*

In addition to intracellular processing of IL-1β and IL-18, also extracellular maturation of these pro-inflammatory cytokines may occur. Neutrophils and macrophages possess serine proteases proteinase-3, elastase, and cathepsin-G; mast cells have chymase; and cytotoxic T cells and NKs release granzymes that are able to process pro-IL-1β and pro-IL-18

(Coeshott et al., 1999; Dinarello, 1996; Sugawara et al., 2001). In addition, a role for matrix metalloprotease-9 has been proposed (Amantea et al., 2007). Furthermore, certain pathogens like *Candida albicans* release enzymes that promote the processing of pro-IL-1β into its active form (Beausejour et al., 1998).

2.2.3.3. Secretion of biologically active IL-1β and IL-18

As mentioned above, IL-1β and IL-18 do not contain leader-signal-peptides in their mRNA structures that would guide them to the classical secretory pathway consisting of ER- and Golgi-compartments (Eder, 2009; Rubartelli et al., 1990). In contrast, the secretion of these pro-inflammatory cytokines follows unconventional protein secretion pathways which are regulated by caspase-1 (Keller et al., 2008).

At present, five models for IL-1β and IL-18 release exist, all of which have arisen from studies with LPS-primed and ATP-stimulated monocytes, macrophages, and DCs. These models include 1) cytolysis, 2) fusion of multivesicular bodies with plasma membrane and subsequent release of IL-1β-containing exosomes, 3) release of microvesicles in a process called membrane blebbing, 4) export of these cytokines *via* large and specific membrane transporters, like pannexin-1, and 5) exocytosis through K^+-dependent mechanisms (Andrei et al., 2004; Brough and Rothwell, 2007; Hogquist et al., 1991; MacKenzie et al., 2001; Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007; Perregaux and Gabel, 1994; Petrilli et al., 2007; Qu et al., 2007; Qu et al., 2009).

According to Andrei *et al.*, pro-IL-1β is transported from cytoplasm in some still poorly known way to LAMP-1 (lysosomal-associated membrane protein-1) positive pre-terminal endocytic vesicles, where it co-localizes with caspase-1 and the aspartate-specific protease cathepsin D (Andrei et al., 2004). Thus, this observation indicates that IL-1β and caspase-1 are synchronously secreted after inflammasome activation. Furthermore, in addition to the fact that low intracellular K^+- and often high cytoplasmic Ca^{2+}-concentrations trigger inflammasomes (Andrei et al., 2004; Petrilli et al., 2007, Qu et al., 2007; Qu at al., 2009), these conditions also elicit the cation-dependent phospholipase cascade and exocytosis: phosphotidylcholine phospholipase C augments Ca^{2+}-levels resulting in increase in cytoplasmic Ca^{2+}, which in turn, activates Ca^{2+}-dependent phospholipase A_2, and exocytosis. In addition, a member of the Rab GTPase, a protein which has important roles in protein trafficking and secretion, namely Rab39a, is important for caspase-1-mediated IL-1β secretion (Becker et al., 2009). It is noteworthy that in addition to caspase-1, also other inflammasome components and unconventionally secreted proteins are simultaneously exocytosed upon inflammasome and caspase-1 activation (Feldmeyer et al., 2007; Keller et al., 2008; Martinon et al., 2002). Interestingly, also RIG-I is among these proteins (Kim and Yoo, 2008). While secretion of inflammasome components and RIG-I may possibly limit inflammasome activation (and/or cell death) in infected cells, the secretion of other unconventionally secreted proteins may further regulate the host response to an infective agent. Simultaneous transport of pro-IL-1β and caspase-1 and/or their abrupt secretion upon inflammasome activation may also be indicative of pyroptosis, a form of caspase-1-mediated

inflammatory cell death, which has the characteristics of both necrosis and apoptosis (Brough and Rothwell, 2007; Fernandes-Alnemri et al., 2007). Furthermore, intracellular bacteria can be cleared from infected cells *via* caspase-1-mediated manner (Miao et al., 2010).

2.3. Death of virus infected cell

Multiple potential cell death mechanisms may become activated during viral infection *i.e.*, necrosis, apoptosis, caspase-1-mediated pyroptosis, and regulated necrosis, called necroptosis. However, in many cases, it is hard and sometimes impossible to make a clear distinction between different cell death mechanisms in a given cell population during infection. Since apoptosis is an important regulator of viral replication and recovery of host from infection, it will be mainly considered here.

The principle of programmed cell death was described already in 1840s (Clarke and Clarke, 1996), but it was not until the early 1970s that the term 'apoptosis', derived from a Greek word describing falling leaves, was introduced to depict a special form of death of mammalian cells (Kerr et al., 1972). During homeostasis, apoptosis can be considered as an immunologically silent and non-inflammatory mode of cell death that preserves overall tissue and developmental homeostasis. It has been estimated that 50-70 billion cells die each day *via* apoptosis in the average human adult under normal conditions. Usually, apoptosis progresses quickly, and apoptotic cells are rapidly engulfed by professional phagocytes. Needless to say, deregulation of apoptosis and impaired clearance of apoptotic cells result in disease pathogenesis. In addition, the onset of apoptosis and removal of improperly functioning cells is also cost-effective during virus infection: elimination of infected cells often prevents the spread of virus since without the actions of the host cell, virus replication comes to an end. Furthermore, phagocytosis of virus-infected apoptotic cells elicits innate immune responses and may also well destroy the virus: TLRs are engaged by virus-derived materials and hydrolases degrade engulfed virus particles in the endolysosomal compartments of the professional phagocytes. Thus, apoptosis during viral infection can be regarded as a host's in-built, intrinsic, defence mechanism. This is also supported by the concept that many viruses encode anti-apoptotic proteins (Hay and Kannourakis, 2002).

However, viruses may also utilize apoptosis for their own benefit: the functions of anti-apoptotic proteins of viruses or activation of the host's anti-apoptotic proteins by viruses may provide more time for virus propagation. Furthermore, after the completion of virion assembly, the progression of apoptosis may disseminate the infection further. Indeed, phagocytosis of apoptotic cells or cell bodies containing intact viruses may propagate infection *via* cell-cell-contacts. This mode of action is particularly relevant during the late stages of infection, *e.g.*, being employed by human immunodeficiency virus. It was noticed that macrophages carrying intact human immunodeficiency viruses disseminated viruses to other $CD4^{+}$-cells throughout the body (Ghorpade et al., 1998). Another example is given by reconstructed pandemic influenza A virus of the year 1918. It was found to upregulate key components of the inflammasome and cell death-related

genes in macaques, and this has been speculated to have contributed to the severity and lethality of the 1918 virus (Cilloniz et al., 2009). Thus, the pathogenic nature of the virus may correlate with the extent of apoptosis. Furthermore, this can be partly explained by host responses causing tissue damage, such as the intense secretion of inflammatory cytokines and lymphocyte-mediated destruction of infected cells. Against all these backgrounds, the importance of appropriate regulation of apoptosis for host survival during the course of infection becomes obvious. Moreover, there must exist a fairly precise threshold for execution of apoptosis since there is no possibility of return once that threshold has been crossed.

2.3.1. Induction of cell death

Both internal (*e.g.*, changes in metabolism, growth, and cell cycle progression) and external cues (*e.g.*, presence or absence of certain hormones, growth factors, and cytokines, or agents released by cytotoxic lymphocytes) may initiate apoptosis. For long it has been known that dsRNA and IFNs sensitize cells, depending on their type, for programmed cell death (Tanaka et al. 1998; Kaiser et al., 2004). This is exemplified *e.g.*, by the action of IFN-inducible genes like tumour suppressor p53, PKR, and 2'5'-OAS-RNaseL system (Balachandran et al., 1998; Li et al., 2004; Takaoka et al., 2003). In terms of executors of apoptosis, the discovery of caspase-1 led onto the trail of other caspases that initiate or execute apoptosis. These are the so called initiator caspases: caspase-2, -8, -9, -10, and -12, as well as the effector caspases: caspases-3, -6, -7, and -14 (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Pop and Salvesen, 2009; Yuan et al., 1993). One should recall that the rest of the characterized caspases are inflammatory, *i.e.*, caspase-1, -4, -5 in humans, as well as caspase-11 in mice, a homologue of human caspase-4. It seems that inflammatory caspases are not directly involved in apoptosis, but rather may function in pyroptosis, in the inflammation-promoting mode of cell death. Since viral replication engages multiple parts of the host's metabolic processes, a variety of signals may trigger apoptosis, pyroptosis, as well as necroptosis. However, whereas the first two mentioned pathways converge in the activation of apoptotic and inflammatory caspases, respectively, necroptosis progresses independently of these proteases (Vandenabeele et al., 2010).

Initiation and progression of cell death is intimately linked to functions of mitochondria which seem to be sensors of well-being of cells (Ott et al., 2007; Zhou et al., 2010, Castanier et al., 2010). For example, not only are they important for the innate immunity, but they also are vital for key metabolic pathways in cells, such as the citric acid cycle, β-oxidation, and electron transport chain. These pathways convert fats, carbohydrates, and proteins to water and carbon dioxide and supply of metabolic energy in the form of ATP and NADPH (nicotinamide adenine dinucleotide phosphate). Furthermore, the biosynthesis of haem, some phospholipids, and iron-sulfur clusters for enzymes take place in mitochondria, and these organelles also participate in the regulation of calcium homeostasis and ROS production (Westermann, 2010; Ott et al., 2007). Naturally, agents that induce the mitochondrial membrane permeability, dissipating its

membrane potential, altering negatively its function, and causing its damage, undoubtedly evoke cell death.

Both extrinsic and intrinsic apoptotic pathways converge at the level of mitochondria (Garrido et al., 2006; Nakahira et al., 2011; Zhou et al., 2010). In addition, pyroptosis, and necroptosis may also involve mitochondria (Vandenabeele et al., 2010). The intrinsic, apoptotic cascade, also known as the mitochondrial pathway, includes proteolytic processing of the pro-apoptotic protein, Bid (BH3 interacting domain death agonist) into its **t**runcated variant (**t**-Bid) and its subsequent translocation with Bax (Bcl-2–associated X protein) from cytoplasm to the mitochondrial outer membrane. These proteins ultimately destabilize mitochondrial membranes, resulting in release of cytochrome c from the mitochondrial inner membrane space to the cytoplasm (Luo et al., 1998; Shimizu et al., 1999; Strasser, 2005). Then, in the presence of cytoplasmic ATP or dATP, an apoptosome, a high molecular weight complex containing Apaf1 (Apoptotic protease activating factor 1), cytochrome c, and pro-caspase-9 is oligomerized leading to subsequent caspase-9 activation (Li et al., 1997). Caspase-9 recruits pro-caspase-3 and mediates its processing into its active form. Finally, caspase-3, the terminal member of apoptotic caspase cascade, executes apoptosis (Bao and Shi, 2007; Nicholson et al., 1995).

The extrinsic apoptotic pathway, in turn, is often activated when specific cell surface receptors bind to pro-apoptotic ligands, such as TNF, FasLigand (TNF ligand superfamily member 6), or TRAIL (TNF-related apoptosis-inducing ligand). Subsequently, the death-inducing signalling complex (DISC), comprising *e.g.,* of FADD/TRADD, is formed (Hsu et al., 1996; Kischkel et al., 1995). In response to a cytoplasmic dsRNA trigger, DISC also recruits two initiator caspases, caspase-8 and -10 (Iordanov et al., 2005; Takahashi et al., 2006). Upon activation, caspase-8 may proteolyse Bid into t-Bid, resulting in the activation of caspase-3 and commitment to apoptosis, as described above (Luo et al., 1998; Strasser, 2005). Importantly, cytoplasmic dsRNA-induced apoptosis has also been reported to be independent of the extrinsic pathway, suggesting that there may well be an alternative pathway for DISC assembly and activation of the caspase cascade during viral infection (Iordanov et al., 2005). It is also worth mentioning that other caspases than caspase-8, as well as lysosomal proteases, cathepsins, may cleave Bid, providing further evidence that mitochondria are linked to the intrinsic and extrinsic apoptotic pathways through actions of Bid (Yin, 2006).

The mitochondrial connection for initiation of caspase-1-mediated pyroptosis is related to NLRP3, ATP, and/or ROS production (Elliott et al., 2009; Zhou et al., 2010). However, NLRP3, ATP, and ROS are also believed to be involved in necrosis and apoptosis (Chekeni et al., 2010; Ghiringhelli et al., 2009; Iyer et al., 2009; Willingham et al., 2009; Willingham et al., 2007). Furthermore, several viruses can induce cell death with necrotic features which are also shared by pyroptosis. Thus, as mentioned already before, the predominant cell death mechanism may be difficult to pinpoint during infection.

Necroptosis is an only recently characterized cell death mechanism, which may become active if caspase-mediated cell death mechanisms are blocked by the infecting virus (Degterev et al., 2005; Ray et al., 1992; Ray and Pickup, 1996). Necroptosis involves the active disintegration of mitochondrial, lysosomal, and plasma membranes, which presumably contains an autophagic and a mitochondrial component (Vandenabeele

et al., 2010). The intracellular signalling complex involved in necroptosis is similar to DISC presented above, but requires RIP1 and -3 for its full activity. The importance of necroptosis during virus-host responses is also highlighted by the fact that there are viruses which block RIP-signalling (Mack et al., 2008).

2.3.2. Nucleic acid-sensing TLRs, RLRs, and inflammasomes in cell death

The molecular pathways that cause activated PRRs to trigger caspase-3 during viral infection are not well known. At least all RNA-sensing TLRs have been reported to induce apoptosis *via* both intrinsic and extrinsic mechanisms (Salaun et al., 2006; Schon et al., 2004). It has been also observed that virus-induced TLR3 activation and the subsequent vigorous cytokine response have often detrimental outcomes to the host by disrupting the normal functions of tissues or by causing disproportionate tissue damage, suggesting that also other cell death mechanism than apoptosis may occur after TLR activation (Le Goffic et al., 2006; Wang et al., 2004).

The mitochondrial localization of MAVS and the involvement of mitochondria in apoptotic pathways suggest that RLRs may be directly linked to apoptosis. Several viruses cleave MAVS and/or MDA-5 and RIG-I to counteract the functions of RLR pathway (Barral et al., 2007; Barral et al., 2009; Lin et al., 2006). MAVS also contains specific cleavage sites for caspases and is proteolysed by them during apoptosis (Rebsamen et al., 2008; Scott and Norris, 2008; Yu et al., 2010). Furthermore, the cleavage of MAVS can be blocked by overexpression of the anti-apoptotic protein, Bcl-XL (B-cell lymphoma-extra large) (Scott and Norris, 2008). Moreover, the association of MAVS with FADD and RIP-1 may also imply that RLRs are tied to activation of the extrinsic apoptotic pathway and possibly also to necroptosis, respectively (Lad et al., 2008). In fact, the molecular pathways for virus-induced apoptosis are in many ways still poorly characterized.

Although, the large protein family of NLRs were initially identified through a homology search of the apoptosome regulator of human Apaf-1, and its equivalents in *Caenorhabditis elegans, Drosophila*, and plant cells (Manji et al., 2002), so far there is no direct molecular evidence for cross-talk between components of apoptosome and NLRP3-inflammasomes. However, activation of inflammasomes and apoptosomes display many similarities that might be interconnected. For example, increased extracellular ATP-concentrations trigger ATP-sensitive $P2X_7$ receptor and a pannexin-1 hemichannel resulting in reduced cytoplasmic ATP- and K^+-concentrations which are both features found in the inflammasome and apoptosome activation (Cain et al., 2001; Chekeni et al., 2010; Cruz et al., 2007; Pelegrin and Surprenant, 2006; Perregaux and Gabel, 1994; Petrilli et al., 2007). Furthermore, pannexin-1 is cleaved and subsequenly activated by caspase-3 and -7 during apoptosis, and the pancaspase inhibitor, z-VAD-FMK, suppresses ATP release through pannexin-1 from cells that otherwise might potentially amplify the apoptotic and inflammatory responses (Chekeni et al., 2010).

With respect to the other NLRs, it has been reported that anti-apoptotic proteins of the B-cell lymphoma 2 (Bcl-2) family, namely Bcl-2 and Bcl-XL, transiently bind to and inhibit NLRP1 oligomerization during bacterial infections (Bruey et al., 2007). Thus,

NLRP1-inflammasome activity is likely linked to the mitochondrial pathway. Furthermore, a NLR member, NLRX1, is constitutively localized within the mitochondria and has been suggested to induce formation of ROS in response to recognition of cytoplasmic dsRNA (Tattoli et al., 2008). ROS, in turn, may lower the apoptotic threshold by destabilizing mitochondrial membranes, resulting in cytochrome c leakage and triggering apoptosome assembly (Ott et al., 2007).

Albeit early works ascribed apoptotic functions to caspase-1, later NLRP3, ASC, and caspase-1 were linked to necrosis and pyroptosis in response to inflammatory signals (Fernandes-Alnemri et al., 2007; Li et al., 2009; Willingham et al., 2009; Willingham et al., 2007). In a human macrophage cell line, THP-1, LPS and a TLR7/8 agonist, R837, induced ASC dimerization, ASC-dimer oligomerization, and subsequent recruitment and activation of caspase-1 (Fernandes-Alnemri et al., 2007). This large (2 µmM in diameter) supramolecular assembly of ASC oligomers and caspase-1 was reported to be structurally distinct from inflammasomes and was called pyroptosome. Interestingly, only one pyroptosome per cell is formed within minutes in response to inflammatory signals resulting in an abrupt rupture of the plasma membrane and subsequent cell death (Fernandes-Alnemri et al., 2007). Similarly to the situation for inflammasome and apoptosome assembly, this involves depletion of intracellular concentration of K^+. Thus, also pyroptosome activation resembles those encountered in the activation of inflammasomes and apoptosome. The exact inflammatory signals regulating the assembling of pyroptosomes and inflammasomes, (as well as of apoptosomes) during microbial infections are gradually emerging (Martinon et al., 2009).

3. Influenza A virus infection and structure of the virus

Influenza viruses are enveloped RNA viruses belonging to the *Orthomyxoviruses.* They are further classified into influenza A, -B and -C viruses according to antigenic differences in their nucleocapsid protein (NP) and matrix proteins (M) (Murphy and Webster, 1996) (see **Fig. 10**). Influenza A virus infects birds and mammals, and it is the most pathogenic virus of the *Orthomyxoviruses* to humans (Hale et al., 2010). The name influenza was introduced by Italians during an outbreak in 1354 for which they blamed the influence "influentia" of the stars (http://www.naturalnews.com/017503.html).

Influenza A virus spread from person to person *via* aerosols, droplets, or by contact transmission (Weber and Stilianakis, 2008). The virus causes acute respiratory infections which can range from mild disease to fatal but rare viral pneumonia. Approximately 5-15% of the population living in the northern hemisphere will be infected with influenza viruses every year (Sanders et al., 2011). The productive replication of influenza A virus may result in severe acute respiratory illnesses, serious enough to require hospitalization or cause death. There were between 3-5 millions of these cases worldwide in 2010. The infection can be life-threatening, especially to people at the risk of developing secondary bacterial pneumonia, *e.g.*, immuno-compromised and pregnant women, or people suffering chronic illnesses, such as congestive heart failure or chronic obstructive pulmonary disease. However, usually most deaths occur in very young children or

individuals over 65 years, albeit elderly individuals in good health may also be protected from seasonal infections since they possess high-avidity antibodies generated against influenza viruses that they have experienced previously in their lifetime (Monsalvo et al., 2011).

The highly pathogenic nature of influenza A viruses is due to the extensive intra- and interspecies re-assortments and variation of their genomes (Holmes, 2008). Consequently, different influenza A virus strains are serologically categorized according to their two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA), against which the antibody responses are mainly targeted. The distinct antigenic subtypes of influenza A viruses are different configurations of H1 to H16 and N1 to N9. Seasonal epidemics are caused by influenza A virus H1N1, H2N2, and H3N2. The influenza A virus strain most extensively used in this study, A/Udorn/72, belongs to influenza A virus subtype H3N2.

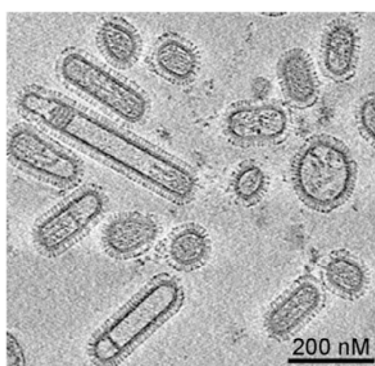


Figure 9 ***Influenza A viruses are pleiomorphic.*** *Tomogram sections of frozen-hydrated A/Udorn/72(H3N2) influenza A virions recorded in a single tomogram field. The picture is used with the permission of the authors (Calder et al., 2010)*

The primary cellular targets of influenza A viruses are epithelial cells of the upper respiratory tract, alveolar macrophages, and DCs. The virus binds to the terminal sialic acid moieties of oligosaccharide chains on the cell surface glycolipids or -proteins (in humans to sialic acid attached to galactose with an $\alpha(2,6)$-linkage) *via* its virion envelope protein, HA (Skehel and Wiley, 2000). Interestingly, the structure of the HA tip of H1N1 "swine flu" from the year 2009, achieved 95% similarity with the pandemic strain of the year 1918 influenza A virus, which killed more than 50 million people during the winter of 1919 (Wei et al., 2010). Fortunately and in contrast to the aforementioned pandemic strains, this HA subregion is glycosylated in seasonal viruses and elicits an effective antibody response. In addition to qualities of incoming viruses, genetic background, age, general health status, and immunity of the host will determine the outcome of the infection.

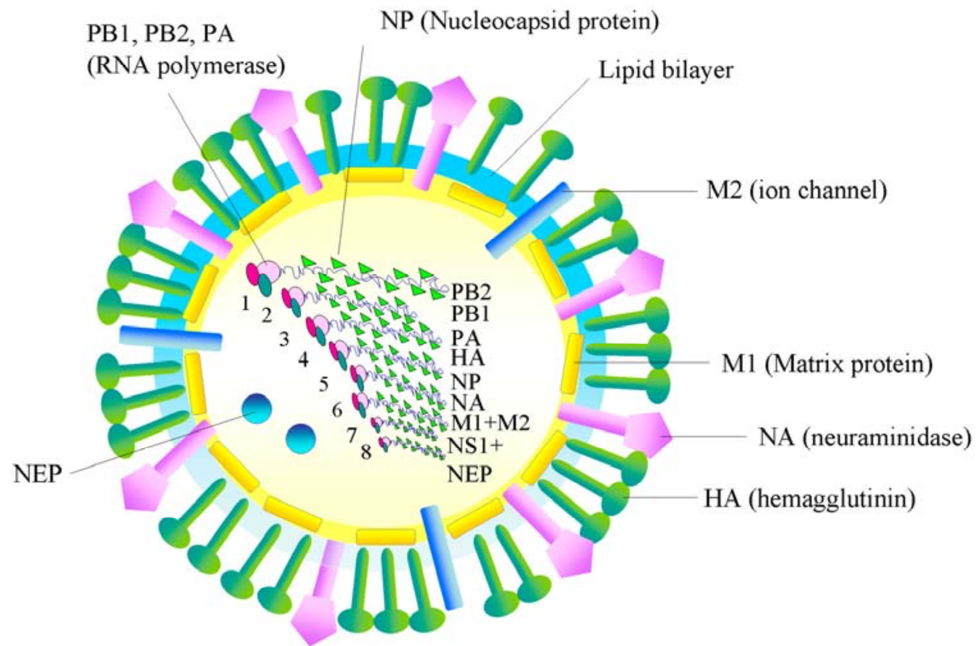


Figure 10 ***Schematic view of structure of influenza A virus.*** *The viral envelope has three types of membrane proteins: HA, NA, and M2. M1 protein forms the underlying nucleocapsid where vRNPs, consisting of eight negative sense ssRNAs, viral polymerase, and NPs are enclosed. The eight ssRNAs encode for the proteins necessary for viral replication. From each ssRNA segment, mRNAs are produced for the synthesis of the viral proteins indicated in the figure. The virion has approximately 500 trimers of HA and 100 tetrameres of NA on its surface, important for entry and egress of influenza A virus. Adapted from Kääriäinen and Ahola (2007).*

3.1. Replication of influenza A virus

Influenza viruses are unusual among the RNA viruses since their RNA synthesis takes place in the cell nucleus instead of the cytoplasm, meaning that its genome must be transported from the cell surface to the nuclear periphery and released from the virion's protein shell, called the nucleocapsid, and from RNA-bound proteins (Flint et al., 2004d; Nayak et al., 2004). The viral replication events begin when HA binds to terminal sialic acid moieties of the oligosaccharide chains on the host cell surface. This triggers internalization of the virus *via* receptor-mediated endocytosis resulting in the formation of endocytic vesicles. When these endocytic vesicles become acidified during their transport toward the nucleus, HA undergoes an acid-catalyzed membrane fusion (Flint et al., 2004b; Skehel and Wiley, 2000). In addition to the fact that the low pH induces conformational changes on HA, it triggers proton transport inside the virus particle through the virion's surface ion-channel M2. Acidification of the interior of the virion is the key for dissociation of the viral genome-protein complexes, called viral ribonucleoproteins (vRNPs), from the nucleocapsid wall, where they have been anchored *via* M1. A low pH is also required for loosening of the virion coat, and subsequent release of eight genomic RNAs which are covered with multiple NPs, into cytoplasm. The nuclear localization

signals present in NPs target vRNPs into nucleus (Flint et al., 2004d) (**Fig. 11**). In addition to NPs, vRNPs consist of trimeric viral RNA polymerase (which holds for polymerase basic protein (PB)-1 and -2 and polymerase acidic protein (PA)). PA, PB1, and PB2 are responsible for transcription and efficient initiation of translation of viral nucleic acids and mRNAs, respectively. Importantly, the viral polymerase is highly error-prone and partly responsible for the huge viral variation observed among influenza A viruses. It is worth mentioning that all the negative-sense ssRNA segments of influenza A virus contain 5'-ppp-ends, produced by viral RNA-dependent RNA-polymerase (Pichlmair et al., 2006).

Influenza A viruses have unusual replication strategies, such as cap-snatching, RNA splicing, use of overlapping reading frames or reinitiation of translation, and leaky scanning that all ensure that there are high amounts of distinct mRNAs produced for viral protein synthesis from a limited number of genomic sequences (Flint et al., 2004f). Furthermore, these strategies maximize the use of cellular protein synthetic machineries. Viral mRNA synthesis is initiated by viral polymerase that uses host-made 5'-capped RNA fragments, cleaved from host mRNAs, as primers (the 5'-cap snatching mechanism). The 5'-cap structure is necessary for the stability of mRNAs and the initiation of translation, and because influenza A virus does not encode any enzymes that synthesize 5'-cap-structures or otherwise manipulate the host's 5'-cap-synthesizing system, it cleaves the 5'-cap from host's mRNA molecules utilizing it for the production of its own mRNAs (Flint et al., 2004e). Subsequently, the translation of cellular mRNAs is hindered in the absence of 5'-cap-structures. This is an example of how the virus gains a competition advantage over the host in the use of the host's protein synthesis machinery. The *de novo* positive-sense RNAs (viral mRNAs) produced are transported, with or without splicing, from the nucleus to the cytoplasm, and used as "template" for viral protein synthesis.

Most of the influenza A virus proteins are translated by free ribosomes in the cytoplasm. However, mRNAs specifying HA, NA, and M2 are translated by ER-bound ribosomes and simultaneously inserted into the ER-Golgi pathway, and once there, they are post-translationally processed, glycosylated (HA and NA), and then transported through this classical secretion pathway to the plasma membrane. Part of the *de novo*-synthesized polymerase subunits (PB1, PB2, PA), and NPs, in turn, are imported back from cytoplasm into the nucleus, where they serve to support *exponential* replication of full length positive sense viral mRNAs and then of negative sense genomic ssRNAs of influenza A virus. During the late stages of infection, the vRNPs are assembled and transported to the cytoplasm in a M1- and nuclear export protein (NEP)-aided manner. The progeny vRNPs, HA, NA, and nucleocapsid proteins gather together for assembly and budding to the plasma membrane. The recently assembled virions obtain their envelope from the sites of host's plasma membrane which are rich of cholesterols and glycosphingolipids (Simpson-Holley et al., 2002). NA releases virions from the sialic acid residues of cell surface and prevents self-aggregation of the virus particles. The released virion seeks a new target.

The genome of influenza A virus also encodes an important multifunctional nonstructural protein 1 (NS1) which is synthesized in infected cells but is not incorporated into virions (Krug et al., 2003). Instead, it disturbs normal cellular functions to promote virus replication in infected cells. The primary function of NS1 is suppression of IFN-β

production. Firstly, NS1 binds ss- and dsRNAs, thus sequesters substrates *e.g.*, from PRRs and IFN-inducible antiviral effector proteins, such as PKR and 2'5'-OAS/RNase L, resulting in suppression of IFN-α/β synthesis and inhibition of other antiviral innate immune responses (Gack et al., 2009; Hatada et al., 1999; Mibayashi et al., 2007; Min and Krug, 2006; Wang and Krug, 1996). Secondly, NS1 also blocks IFN-α/β production in infected cells by interacting with RIG-I and E3 ubiquitin ligase TRIM25. NS1 prevents TRIM25-mediated K63-ubiquitination of a CARD of RIG-I and subsequent interaction of RIG-I with MAVS (Gack et al., 2009; Mibayashi et al., 2007). Thirdly, the viral protein inhibits transactivation of IFN-β promoter by IRF3, NF-κB, and AP-1 transcription factors, subsequently impairing the efficient expression of IFN-β (Ludwig et al., 2002; Talon et al., 2000; Wang et al., 2000). Interestingly, IFN-inducible genes display different sensitivities to NS1-mediated inhibition of their expression (Geiss et al., 2002). This may partly be due to NS1-mediated cleavage of the polyadenylation specificity factor, 30 kDa (CPSF30) (Nemeroff et al., 1998; Noah et al., 2003). CPSF30 processes the 3'-ends of cellular pre-mRNAs, which is necessary for efficient translation, splicing, and capping of host mRNAs. Subsequently, when these functions are compromised, translation of viral mRNAs is enhanced at the expense of translation of the host mRNAs. Moreover, more resources for 5'-cap snatching become available.

It is uncertain whether the binding of NS1 to RIG-I also affects the pro-inflammatory cytokine response through inhibition of RIG-I/ASC-inflammasome. However, certain NS1 variants can interact with caspase-1 and enhance secretion of IL-1β and IL-18, as well as promote apoptosis, suggesting that NS1 may play a role in activation of inflammasome-mediated events (Stasakova et al., 2005). To conclude, NS1 has a variety of effects on the progression of infection. This capacity varies between distinct influenza A virus strains, and subsequently poses a challenge to immunization programs and to the development of effective antiviral therapies against this virus.

Replication of influenza A virus is destructive to the host cell and the cell eventually dies because of the infection. It seems that the death of cells is attributable to the activities of caspases and the extensive inflammation response. Indeed, activation of caspase-3 is necessary for efficient propagation of influenza A viruses (Wurzer et al., 2003), and the presence of caspase cleavage motifs in influenza virus proteins has been shown to determine their immunopathogenicity (Zhirnov and Syrtzev, 2009). Furthermore, the extent of inflammation is an important determinator of life and death of host (Cilloniz et al., 2009; Le Goffic et al., 2006)

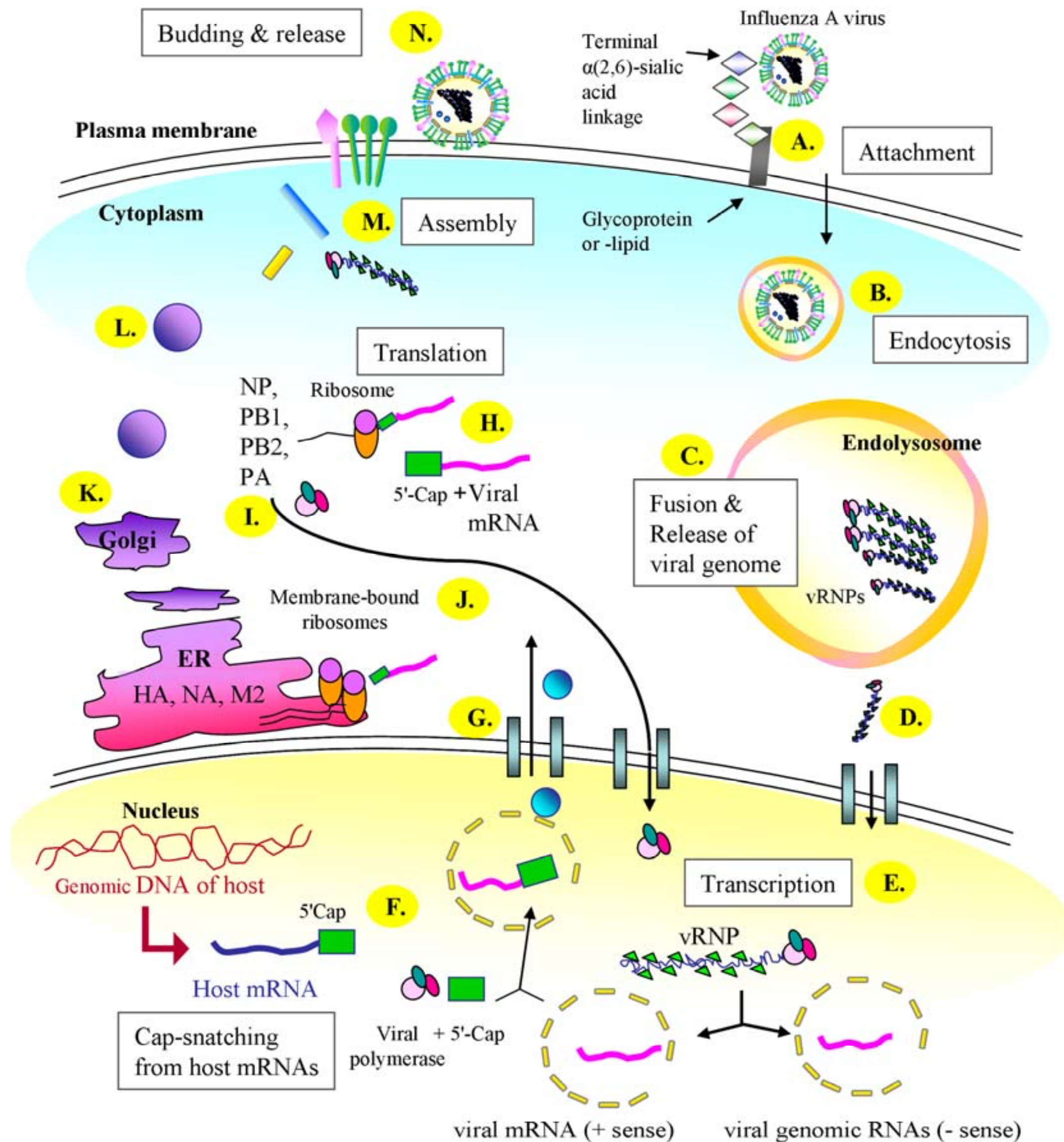


Figure 11 ***Key events during influenza A virus replication.*** *(A) Attachment. (B) Receptor-mediated endocytosis, (C) Fusion of the endocytic vesicle with acidic endosomes and subsequent breakdown of the virion capsid. (D) Release of vRNPs into the cytoplasm and their import into the nucleus. (E) Transcription of viral mRNAs and genome segments in the form of nucleocapsids. (F) The 5'-cap-snatching from recently transcripted host mRNAs to be attached to viral mRNAs. (G) NEP-assisted nucleocapsid export to cytoplasm. (H) Translation of NP, PA, PB1, and PB2 by the host's ribosomes in cytoplasm. (I) Nuclear import of viral polymerase for exponential viral RNA synthesis. (J) Viral mRNAs encoding HA, NA, and M2 are translated by ER-bound ribosomes, and (K) subsequently shunted into the ER-Golgi pathway for post-translational modifications, such as glycosylation. (L) HA, NA, and M2 are transported to the plasma membrane where (M) all the rest of the virion constituents have already gathered for virion assembly and budding. (N) NA detaches mature influenza A virus particles from the cell surface and prevents their self-aggregation.*

Aims of the Study

Macrophages play central roles in the innate immunity and microbial defence. They have a high expression level of PRRs and are markedly activated by PAMPs and DAMPs. The main goal of this study was to characterize the host response to influenza A virus infection in human primary macrophages. The molecular pathways involved in the activation of innate immune during virus infection were examined with the following aims;

- ❖ To characterize the cytoplasmic RNA-induced antiviral- and pro-inflammatory cytokine response, inflammasome activation, and apoptosis in human primary macrophages (I, III).

- ❖ To explore influenza A virus-induced subcellular changes by two-dimensional electrophoresis (2DE) -coupled with mass spectrometric (MS) analyses (II).

- ❖ To delineate quantitatively influenza A virus-induced subcellular changes and secretomes in a global manner by high-throughput proteomics, and to elucidate host-virus-interactions and inflammasome activation in influenza A virus-infected human primary macrophages (IV).

Summary of Materials and Methods

The materials and methods of this study are described in more detail in the original publications I-IV.

1. Cells

1.1. Human primary macrophages

Human primary macrophages were obtained from leukocyte-rich buffy-coats from healthy blood donors. Peripheral blood mononuclear cells (PBMCs) were extracted by density gradient centrifugation. Monocytes were differentiated into macrophages by maintenance in Macrophage serum-free media supplemented with Granulocyte-macrophage colony-stimulating factor (GM-CSF) at 10 ng/ml and antibiotics, as described more detail in I-IV and by Sareneva et al., (1998). After five days of culturing, the cells were used in the experiments. Each macrophage sample represents a pool of separately stimulated cells from three different blood donors. All results are representatives of three independently but similarly performed experiments.

1.2. Other cell types

Table 1. *Use of other cell types for indicated purposes during the study.*

Other Cell Types	Description	Reference
HaCaT (from American Type Culture, Collection, ATCC)	I*n vitro* spontaneously transformed keratinocytes from histologically normal skin used for transfection experiments	I
Primary T-cells	Purified and activated from non-adherent PBMCs, maintained in 10% Fetal Calf Serum and IL-2 to study IL-18-induced responses	I
293T (ATCC)	Embryonic kidney cell line used for propagation of genetically engineered retroviruses	I
Embryonated eggs	Utilized for influenza A virus growth	I, II, IV, (Pirhonen et al., 1999)
L929 (ATCC)	Mouse fibroblast cells for propagation of EMVC and VSV	III, (Ohman et al., 2010)

2. Viruses

2.1. Influenza A virus

Influenza A virus (A/Udorn/72(H3N2)) is an enveloped and morphologically filamentous-shaped virus (**Fig. 9**). In addition to Udorn, also another influenza A virus strain (A/Beijing/353/89(H3N2)) was utilized in this study (IV). Viruses were cultured in 11-day-old embryonated eggs, as described by Pirhonen et al., (1999). The cells were stimulated with 2,56 hemagglutination U/ml and as depicted in I, II, and IV. Influenza A viruses were kindly provided by Professor Ilkka Julkunen.

2.2. Other RNA viruses

Table 2. *Other RNA viruses used in this study. More details are found from mini-glossary and from the indicated references.*

Virus	Description	Reference
EMCV	Picornavirus, a positive sense ssRNA virus	III
VSV	Rhabdovirus, a negative sense ssRNA virus	III

Both virus stocks were a kind gift from Dr. Jesper Melchjorsen, and used at 1 multiplicity of infection (MOI), as described in III.

3. Synthetic RNA polymers

3.1. Double-stranded RNA mimetic poly(I:C)

Poly(I:C) is a long synthetic analog of dsRNA (**Fig. 12**). It is a polymer of polyinosinic: polycytidylic acids and has been used for decades to mimic RNA virus infections. It contains 5'-mono- or diphosphate ends (Grunberg-Manago et al., 1955). The commercially available product is heterogenous in nature and lot-to-lot variations commonly occur. The molecular weight range is 100,000-200,000 which corresponds roughly to 125-250 base pairs. Cells can be stimulated with either extracellular or intracellular poly(I:C) (Ablasser et al., 2009b; Alexopoulou et al., 2001; Yoneyama et al., 2004). Transfection of poly(I:C) with lipofectamine 2000 was used for intracellular delivery of poly(I:C) into the cell cytoplasm. Poly(I:C) was used at 10 µg/ml unless otherwise mentioned (I-III), and was

introduced into the cell cytoplasm according to instructions of the manufacturer (Sigma-Aldrich, St. Louis, Mo).

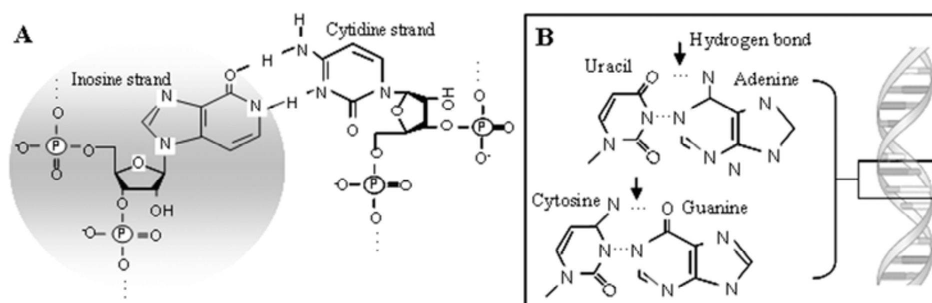
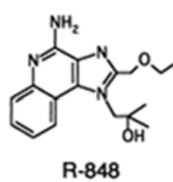


Figure 12 ***Chemial structure of (A) poly(I:C) and (B) base-pairing of RNA strands****. (B) Uracil and adenine and cytidine and guanine are most often base-paired between ssRNA molecules. The ribose-sugars of RNA are not shown in B.*

3.2. Single-stranded RNA mimetic R848



Resiquimod (R848) is a low molecular weight compound of the imidazoquinoline family. R848 has potent antiviral and antitumour-properties in animal models and it is being evaluated *e.g.,* for treatment of genital warts and superficial basal cell carcinoma in humans (Gupta et al., 2004). R848 signals through TLR7/8 (Hemmi et al., 2002; Jurk et al., 2002). The compound was purchased from Invovogen (San Diego, CA).

Figure 13 ***Chemical structure of R848.***

4. Exogenous cytokines

To study effects of exogenous IFN-α/β, IFN-β (Betaferon) was provided to cells at 100 international unit (IU)/ml as described in I. GM-CSF was given to PBMC-derived monocytes at 10 ng/ml to *in vitro* differentiate them into macrophages and to maintain the obtained pool of cells. IFN-β and GM-CSF were purchased from Schering Plough (Espoo, Finland) and ImmunoTools (Friesoythe, Germany), respectively.

5. Inhibitors

Several inhibitors were utilized to delineate the roles of their known targets during innate immune response to viruses.

Table 3. *Chemical inhibitors, their targets, and manufacturers. More detailed information of their use is presented in the references (I-IV).*

Inhibitor	Description	Company	Reference
Ca-074 Me	Cathepsin B inhibitor	Calbiochem	III, IV
Cytochalasin D	Promotes conditions for actin depolarization and may block endocytotic trafficking	Sigma-Aldric	II
Cathepsin inhibitor III	Selectively inhibits cathepsin B, L, S, and papain	Calbiochem	III
EST (E-64d)	Irreversible inhibitor of cysteine proteases	Calbiochem	III
z-VAD-FMK	Pancaspase inhibitor of caspase-1, -3, -4, and -7	Santa Cruz Biotechnology	II
z-FA-FMK	Inhibitor of cathepsin B and L	Calbiochem	IV
AZ11645375	$P2X_7$ receptor inhibitor	Sigma-Aldric	IV
PP2	Src tyrosine kinase inhibitor	Sigma-Aldric	IV

6. Antibodies (Abs)

Table 4. *Abs used in this study. More information is found in the indicated references*

Abs against	Manufacturer	Reference
Actin	Santa Cruz Biotechnology	II
Annexin A1	Santa Cruz Biotechnology	IV
ApoE	Santa Cruz Biotechnology	IV
ASC	Millipore	III
β-Amyloid (APP) (20.1)	Santa Cruz Biotechnology	IV
BID	Cell Signaling Technology	III
Caspase-1p10	Calbiochem	I-III
Caspase-1p10	Santa Cruz Biotechnology	IV
Caspase-	Sigma-Aldrich	I-III
Caspase-3p17/19	Cell Signaling Technology	I-IV
Cathepsin B	Calbiochem	III, IV
Cathepsin D (C-20)	Santa Cruz Biotechnology	III, IV
Cathepsin Z	Santa Cruz Biotechnology	IV
Cytochrome c	Santa Cruz Biotehcnology	IV
Galectin-3	Santa Cruz Biotechnology	IV
GAPDH	Santa Cruz Biotechnology	IV
Histone H1	Santa Cruz Biotechnology	IV
HMGB1	Santa Cruz Biotechnology	IV
HSP90	Cell signaling Technology	IV
H3N2	kindly provided by Prof. Ilkka Julkunen	II, IV
IFIT3	BB Transduction Laboratories	IV
IKKε (Q-15)	Santa Cruz Biotechnology	II
IL-1β	kindly provided by Prof. Ilkka Julkunen	I
IL-18	MBL	I
IL-18	(Pirhonen et al., 1999)	I-IV
LAMP-1	Santa Cruz Biotechnology	IV
MAVS	kindly provided by Prof. Ilkka Julkunen	II
MDA-5	kindly provided by Dr. Paul B. Fisher	II
$P2X_7$ (H-265)	Santa Cruz Biotechnology	IV
RIG-I	kindly provided by Prof. Ilkka Julkunen	I, II
TRADD (A5)	Santa Cruz Biotechnology	II
TRIM25	BD Transduction Laboratories	II
VDAC-1 (D-16)	Santa Cruz Biotechnology	IV
XIAP	Cell Signaling Technology	II

7. Methods

Only a brief description of central methods is provided. See also **Table 5** for summary of all methods utilized in the original publications.

7.1. RNA isolation and complementary DNA (cDNA) synthesis (I-III)

Total cellular RNA was isolated by RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A total of 0.5 µg of RNA was reverse transcribed into cDNA by a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) in a 25 µl reaction mixture containing optimized reverse transcription buffer, random primers, deoxyNTP mixture, and MultiScribe reverse transcriptase. The conditions for cDNA synthesis were: annealing at 25° C for 20 min. and synthesis at 37° C for 120 min. The reverse transcription reaction was performed in an Applied Biosystems' Thermal cycler version 2720.

7. 2. Quantitative real-time polymerase chain reaction (real-time PCR) and data analysis (I-III)

For quantitative real- time PCR, TaqMan analysis was done in a 96-well optical reaction plate in ABI PRISM 7500 Fast Sequence Detector (Applied Biosystems). The cDNA was amplified in 11 µl of 1 x TaqMan Fast universal PCR master mix with pre-developed TaqMan® assay primers and probes sets which were designed and optimized according to Applied Biosystems' guidelines. For each sample, PCR amplification of the endogenous 18S rRNA was determined to control for the amount of cDNA added according to the manufacturer's instructions (Applied Biosystems) and to allow for normalization between the samples. The thermocycling conditions consisted of an initial step of 95ºC for 20 s, 40 cycles of denaturation at 95ºC for 3 s, and annealing and extension steps at 60ºC for 30 s. Real time-PCR was performed at least in duplicate for each cDNA product. No template control (NTC), in which molecular grade water was used instead of the template, was included in each assay.

The real- time PCR data was developed by using Sequence detector system version 1.4 software (Applied Biosystems). The cycle threshold value (C_T) of a sample, that is the cycle number at which measured fluorescence was greater than the manually fixed threshold fluorescence in the amplification plot, was selected according to the manufacturer's guidelines. Relative units (RU) were calculated by a comparative C_T method. First, ΔC_T, the difference between the C_T value of the target amplicon and 18S rRNA, was determined for each sample. Second, a calibrator ΔC_T value of 40, obtained from NTC, was subtracted from average C_Ts of 18S rRNAs to obtain $\Delta\Delta C_T$. Finally, the amount of target normalized to an endogenous control which was relative to the NTC-calibrator, was calculated from equation $2^{-\Delta\Delta CT}$. Standard curve and statistics were generated in GraphPad Prism 4 Software (San Diego, CA).

7. 3. Protein extraction for Western blot analysis (I-IV)

Cells were suspended into a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, and 25% ethylene glycol supplemented with Complete mini protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) and homogenized by ultrasound sonication. Total of 10 µg of proteins were separated on 15% SDS-PAGE (Sodium dodecyl sulfate polyAcrylamide gel electrophoresis) at 200 V, and transferred on Immobilon-P transfer membranes (Millipore, Bedford, MA). Blots were stained with Ponceau red to confirm equal loading and transfer of the protein samples. Membranes were blocked in PBS containing 5% non-fat milk and stained with Abs of interest at +4 ºC for 18 h. Subsequently, membranes were washed with PBS-Tween (0.05%) and incubated at Room Temperature (RT) for 1 h with appropriate Horseradish peroxidase-conjugated secondary Abs (Dako A/S, Glostrup, Denmark.) Proteins were visualized by an enhanced chemiluminescence system (PerkinElmer™ Life Sciences, Zaventem, Belgium).

7. 4. Enzyme-linked immunosorbet assay (ELISA) (I-IV)

Cytokine concentrations of IL-1β and IL-18 were determined with ELISA according to the manufacturer's instructions. Human IL-1β Eli-pair and IL-18 ELISA were purchased from Diaclone (Besançor Cedex, France) and Medical & Biological Laboratories Co (Naka-ku Nagoya, Japan), respectively.

7. 5. Immunofluorescence staining and confocal microscopy (II)

For indirect immunofluorescence staining, human macrophages were grown on coverslips. After 12 h infection, cells were incubated with 1 µM MitoTracker Red580 (Molecular Probes, Eugene, OR) for 30 min at 37ºC cell incubator at 5% CO_2. The cells were fixed with 4% paraformaldehyde in PBS and permeabilized with ice-cold acetone for 5 min. The aldehyde groups were quenched with 50 mM NH_4Cl for 20 min. Cells were treated with specific Abs for 1 h at RT followed by treatment with secondary, species-specific Abs conjugated with Alexa488 (Molecular Probes) for 1 h. The samples were mounted in Mowiol and viewed under a Leica TCS SP5 confocal microscopy. A HCX APO 63x/1,30 (glycerol) objective was used and images were processed using the LAS AF program (Leica application Suite advanced Fluorescence) and Adobe Photoshop.

7. 6. Co-immunoprecipitation (II)

Macrophages were infected with influenza A virus for 18 h or left untreated. Cells were lysed with 500 µl lysis buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA and protease inhibitor cocktail (Roche Diagnostics). Lysates were precleared with Dynabeads Protein A (Invitrogen, Carslbad, CA) at +4°C for 1 h and

incubated with the RIG-I Ab overnight. Immune complexes were captured on protein A beads and the immunoprecipitates were washed three times with lysis buffer and once with PBS. The bound proteins were eluted with Laemmli sample buffer and subjected to SDS-PAGE. Separated proteins were blotted onto PVDF-membrane and analyzed with TRIM25-Ab.

7.7. Transient transfection of RIG-I and TLR3 constructs into HaCaT cells (I)

HaCaT cells, obtained from ATCC (Boras, Sweden), were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamate, and antibiotics (all from GIBCO, Carslbad, CA). TLR3 expression plasmid was purchased from InVivoGen (Toulouse, France). The RIG-I and ΔRIG-I plasmids have been presented previously (Imaizumi et al., 2002; Matikainen et al., 2006). The RIG-I expression vectors were transfected into HaCaT cells using FuGENE 6 transfection reagent, as specified by the manufacturer (Roche Diagnostics).

7.8. Retroviral infection of HaCaT cells by MAVS cDNA (I)

MAVS cDNA was amplified (5'-prime: CCG TTT GCT GAA GAC AAG ACC TAT AAG TAT and 3'-prime: CTA GTG CAG ACG CCG GTA CAG CAC CA) using Pfu polymerase (Stratagene, Cedar Creek, TX), and cloned into modified retroviral pMSCVpuro vector (Invitrogen) where the puromycin gene had been replaced by human CD8 cDNA. N-terminal HA-epitope tag sequence was inserted into the MAVS cDNA. The parental and MAVS-encoding vectors were simultaneously transfected with pCL retroviral packaging vector and pVSVG-envelope protein into human embryonic kidney 293T cells using standard calcium phosphate precipitation method to produce retroviruses (Naviaux et al., 1996). After 48 h of transfection, growing HaCaT cells were combined with culture supernatant containing retrovirus and polybrene (8 µg/ml), and centrifuged at 2500 rpm at RT for 45 min. A second infection was conducted 24 h later after which the cells were used for experiments. The infection level was verified by flow cytometry and with R-Phycoerythrin-conjugated anti-CD8-Ab (Becton Dickinson, Frankling Lakes, NJ). The average infectivity was 78 % for parental vector and 40 % for vector encoding MAVS.

7.9. Small interfering RNA (*si*RNA) experiments (III, IV)

After five days of cell culture in 12-well plates, macrophages were transfected with 100 nM (III) or 200 nM (IV) non-targeting control *si*RNA (AllStars Negative Control *si*RNA) and with 50 nM (III) or 100 nM (IV) of each of the two different *si*RNAs (Hs_CTSD_1 and Hs_CTSD_3 (III), or Hs_P2RX7_1 and Hs_P2RX7_2 (IV)) by using HiPerFect Transfection Reagent (all from Qiagen) according to the manufacturer's instructions. After

4 h of *si*RNA treatment, fresh macrophage medium was supplied to the cells. On the following day, macrophages were left unstimulated or stimulated with **t**ransfected poly(I:C) (poly(I:C)-**t**) (III) or infected with influenza A virus (IV) for 8 and 9 h, respectively. Cell culture supernatants were collected and total proteins extracted for ELISA and western blot analyses, respectively.

7.10. APOPercentage apoptosis assay (III, IV)

The percentage of apoptotic cells was assayed with APOPercentage Apoptosis Assay according to manufacturer´s instructions (Biocolor Life Science Assays, Carrickfergus, UK). Photographs were taken with an Olympus DP70 digital microscope camera connected to an Olympus IX71 light microscope using DP Controller (version 2.2.1.227) and DP Manager (version 2.2.1.195) software (Espoo, Finland). The stained (apoptotic) and unstained cells were manually counted, and the percentage of apoptotic cells was calculated.

7.11. Subcellular fractionation of infected cells and concentration of cell culture media for secretome analyses (II-IV)

Macrophages were infected with influenza A virus, strain A/Udorn/72 (H3N2) in complete Macrophage-SFM medium. The studied cells were lysed or fractionated into mitochondrial, cytoplasmic, and/or nuclear fractions. Mitochondrial and cytoplasmic fractions were isolated by Qproteome^{TM} Mitochondria Isolation Kit (Qiagen) and the cytoplasmic fractions were further purified with 2-D Clean-Up Kit (GE Healthcare, Helsinki, Finland) (II, IV). Nuclear fractions were isolated by Qproteome^{TM} Nuclear Protein Isolation Kit (Qiagen) and the insoluble and soluble nuclear protein fractions were combined before analysis (IV). About 1 x 10^7 cells were used for all isolations (II, IV). In the secretome analyses, the cells grown in complete Macrophage-SFM medium were washed three times with PBS, after which the cells were stimulated in RPMI growth media supplemented with 1mM HEPES, L-Glutamine, and antibiotics (GIBCO) (III-IV). The growth media were collected and concentrated in Amicon Ultra centrifugal filter devices (Millipore), and analysed further by Western blotting or MS-based proteomics (III-IV).

7.12. Two-dimensional (2DE) electrophoresis and protein identification (II)

The first dimension of 2DE was performed with 11 cm pH 4-7 immobilized pH gradient-strips (Bio-Rad, Helsinki, Finland) and the second dimension with Criterion Tris-HCl 8-16% gradient precast gels (Bio-Rad). Isolated mitochondrial and cytoplasmic fractions from influenza A virus infected and untreated macrophages were dissolved in 200 µl rehydration buffer (7M urea, 2M thiourea, 3.5 % CHAPS, 0.6 % dithiothreitol, 0.5 % IPG-

buffer 4-7) and the proteins were absorbed onto immobilized pH gradient-strips for 24 h at RT. Isoelectric focusing to a total of 55 kVh was done at 20°C, and the focused strips were equilibrated two times at RT for 15 min with SDS-equilibration buffer containing 6M urea, 50 mM Tris-HCl, pH 6.8, 2 % SDS, 20 % glycerol, and 10 mg/ml dithiothreitol for the first equilibration, and 25 mg/ml iodoacetamide for the second equilibration. SDS-PAGE was run at 200V using a Criterion-electrophoresis unit (Bio-Rad) at +4°C. Proteins were detected with MS-compatible silver staining(O'Connell and Stults, 1997). Only those protein spots that were reproducibly clearly up- or downregulated in at least three independent experiments were chosen for identification.

For identification, the proteins were in-gel digested with trypsin and the resulting peptides were analyzed by peptide-mass fingerprinting (PMF) as previously described (Nyman et al., 2001; Shevchenko et al., 1996). The mass spectra were acquired using an Ultraflex TOF/TOF instrument (Bruker Daltonik, Bremen, Germany) in the positive ion reflector mode. Database searches were done with publicly available Mascot search engine against NCBInr database (http://www.matrixscience.com). The search criteria were: human-specific taxonomy; trypsin digestion with one missed cleavage allowed; carbamidomethyl modification of cysteine as a fixed modification and oxidation of methionine as a variable modification; peptide tolerance maximum ±50 ppm. All of the protein identification scores were significant ($p<0.05$).

7.13. Secretome characterization by gel-based mass spectrometry (MS) (III)

Cell culture supernatants were collected and concentrated as described in 7.7., followed by protein purification using the 2D Clean-Up Kit (GE Healthcare). Next, proteins were separated using SDS-PAGE and visualized with silver-staining. For protein identification, whole gel lanes were cut into 30 pieces of equal size, proteins were in-gel digested with trypsin and the resulting peptides were analyzed by nanoLC-MS/MS (nano-liquid chromatography-tandem mass spectrometry) as described previously (Ohman et al., 2010). The LC-MS/MS data was searched with in-house Mascot version 2.2 through a ProteinPilot 3.0 interface against the SwissProt database (version 56.12). The search criteria for Mascot searches were as presented above in 7.12.

7.14. Isobaric tag for relative and absolute quantification (iTRAQ)-labeling and MS (IV)

Mitochondrial, cytoplasmic or nuclear fractions or secretomes of uninfected control cells and influenza A virus infected cells at given time-points were labeled with 4plex iTRAQ. The samples were first dissolved into 43 µl of iTRAQ dissolution buffer and 2 µl of each sample was run into an SDS-PAGE gel. When evaluating the intracellular fractions, equal protein amounts of each sample were taken for the iTRAQ analyses based on the silver stained gels. In the secretome analyses, the whole samples were labeled. Protein alkylation, trypsin digestion, and labeling of the resulting peptides were done according to the manufacturer's instructions (AB Sciex, Copenhagen, Denmark). After labeling, the

samples were pooled, dried and dissolved into 20 mM KH_2PO_4 (pH 3). Labeled peptides were fractionated by strong cation exchange chromatography (SCX) on an Ettan HPLC (high-performance liquid chromatography) system (Amersham Biosciences, Uppsala, Sweden). Each SCX fraction containing labeled peptides was analysed twice with nano-LC-ESI-MS/MS (nano-liquid chromatography-electrospray ionization-tandem mass spectrometry) analysis (using Ultimate 3000 nano-LC (Dionex, Amsterdam, Netherlands) and QSTAR Elite hybrid quadrupole time-of-flight mass spectrometer (AB Sciex) with nano-ESI (approximately 22 SCX fractions for intracellular samples and 13 fractions for secretome). MS data were acquired automatically using Analyst QS 2.0 software. Information-dependent acquisition method consisted of a 0.5 s TOF-(time-of-flight)-MS survey scan of m/z (mass-to-charge ratio) 400-1400. From every survey scan, the two most abundant ions with charge states +2 to +4 were selected for product ion scans. Once an ion was selected for MS/MS fragmentation, it was placed on an exclusion list for 60 s.

7.15. Data analysis (IV)

Protein identification and relative quantitation were performed using ProteinPilot 2.0.1 software (AB Sciex). Data files from both technical replicates of an iTRAQ sample set were processed together. The search database was a self-built combination of Uniprot human protein sequences and Uniprot ssRNA negative-strand viruses sequences (both from the release 55.0, 02/08). The search criteria were: cysteine alkylation with MMTS (methyl methanethiolsulfonate), trypsin digestion, biological modifications allowed, thorough search, and detected protein threshold of 95% confidence (Unused ProtScore > 1,3). Additionally, automatic bias correction was used for intracellular fractions to correct for uneven protein loading. ProteinPilot identification and quantitation results were also manually checked: for each identified protein with at least two unique peptides with good quality MS/MS data were required, and MS/MS spectra with all reporter ion peak heights below 10 counts were manually removed from quantitation results. False discovery rates were calculated using a concatenated normal and reversed sequence database and a previously reported method (Yanai et al., 2009).

Proteins identified from each subcellular fraction were classified based on their Gene Ontology annotations using GeneTrail (Sato et al., 2009). Additionally, k-means clustering analysis was performed for the differentially regulated proteins in each subcellular fraction using Chipster, an open source data analysis tool (http://chipster.sourceforge.net). Clustering was done based on the relative quantitation results from the iTRAQ experiments, and a suitable number of clusters for each subcellular fraction was determined by studying the cluster profiles. Protein-protein interaction networks for a selected group of proteins were created using String (Elias and Gygi, 2007).

Table 5. *Methods of this study. More details are found from the references (I-IV)*

Method	Reference
Cell culturing:	
Monocyte isolation and differentiation into macrophages	I-IV
T-cell purification and culture	I
HaCat cell culture	I
Transfections:	
Retroviral infection for gene transfection	I
Transient gene transfection (with FuGene 6; Roche Diagnostics)	I
Poly(I:C) transfection (with Lipofectamine; Invitrogen)	I-III
*si*RNA transfection experiments (with HighPerfect; Qiagen)	III, IV
Basic Techniques of molecular biology and of protein analysis:	
RNA extraction, cDNA synthesis and real-time PCR (TaqMan)	I-III
Protein extraction, SDS-PAGE, Western blotting, and Enhanced Chemiluminicence	I-IV
Co-immunoprecipitation	II
IL-1β ELISA	I, IV
IL-18 ELISA	I-IV
Techniques used in proteomic experiments:	
Subcellular fractionation	II, IV
Cell culture media concentration	III, IV
2DE and MS	II
Protein in gel digestion, silver staining and MS	II, III
Protein digestion, iTRAQ labeling and MS	IV
Data analyses of proteomes and secretomes	II-IV
Stainings and microscopy:	
Mitochondrial staining (MitoTracker)	II
Immunofluoresence staining for confocal microscopy	II
Apoptotic cell staining (APOPercentage) for light microscopy	III

Results and Discussion

1. Human primary macrophages as a model to study innate immune responses

Macrophages are an important cell population which are responsible for eliciting innate immune responses during microbial encounter. Once they gain access to tissues, circulating blood monocytes differentiate into specific, morphologically and somewhat functionally distinct, macrophage cell populations. PBMC-derived $CD14^+$-monocytic cells differentiated with GM-CSF adopt classical phenotype, such as high surface expression of macrophage marker $25F9^+$ (data not shown), and are potent producers of *e.g.*, IL-1 and IL-12 (Mosser and Edwards, 2008). Furthermore, GM-CSF-derived primary macrophages has been reported to resemble that of human alveolar macrophages (Akagawa et al., 2006), thus providing an excellent and relatively cost-effective cell model with which to study innate immune responses against respiratory viruses, like influenza A virus.

2. Recognition of extracellular and cytoplasmic RNA (I)

Nucleic acid-sensing TLRs reside primarily in endolysosomal compartment where endocytosed virus particles and phagocytosed extracellular nucleic acids *e.g.*, from damaged cells, end up by poorly known mechanisms. In order to study extracellular RNA-elicitated innate immune responses, synthetic dsRNA and ssRNA mimetics, poly(I:C) and imidatsoquinole R848, respectively, were extracellularly administered to human macrophages to engage TLR3 and -8, respectively (Alexopoulou et al., 2001; Jurk et al., 2002). During the virus replication cycle, virus-derived RNAs also become often exposed to recognition *via* cytoplasmic PRRs. Since dsRNA is an obligatory replication intermediate of many viruses, often generated at some point during the infection (Jacobs and Langland, 1996; Kumar and Carmichael, 1998; Weber et al., 2006), poly(I:C)-polymer was tranfected into cell cytoplasm, denoted as poly(I:C)-t, to trigger the RLR pathway *i.e.*, to mimic virus replication in the cell.

2. 1. Extracellular and cytoplasmic dsRNA activate differentially synthesis of antiviral cytokines

2.1.1. Cytoplasmic dsRNA is a strong inducer of antiviral cytokines

Extracellular and cytoplasmic dsRNAs are known to activate an antiviral cytokine response (Alexopoulou et al., 2001; Diebold et al., 2003; Yoneyama et al., 2004). In human primary macrophages, endocytosis of extracellular poly(I:C) resulted in relatively

moderate synthesis of type I and III IFNs: IFN-β and IL-29, respectively. This transcriptional elicitation occurred early, at the 3 h time point, but was not sustained for a long period. However, a cytoplasmic dsRNA stimulus triggered forceful IFN-β and IL-29 mRNA expression which remained elevated up to 24 h. The results suggested that recognition of viral RNA in cytoplasms *via* RLRs is primarily responsible for the intense expression of IFNs during virus infection. Hence, entry or replication of viruses in cytoplasm, but not their presense in endolysosomes, seems to be the sign of active virus infection in an infected cell, and this consequently establishes an effective IFN response to protect the host.

2.2. Extracellular and cytoplasmic dsRNA regulate differentially transcription of pro-inflammatory cytokines IL-1β and IL-18

2.2.1. Extracellular dsRNA induces expression of pro-inflammatory cytokine IL-1β

Both extracellular and cytoplasmic RNA recognition pathways have been connected to NF-κB activation and production of pro-inflammatory cytokines (Alexopoulou et al., 2001; Yoneyama et al., 2005; Yoneyama et al., 2004). In human macrophages, extracellular dsRNA activated robust expression of IL-1β both at the mRNA and protein level. The transcriptional activation of IL-1β in response to extracellular dsRNA was dose-dependent, and the kinetics resembled those of cytoplasmic dsRNA-induced IFN-β production: pro-IL-1β mRNA levels were most pronounced at 3 h, and remained elevated still at the 24 h time point. The highest concentration of extracellular dsRNA also amplified constitutive expression of pro-IL-18 in human primary macrophages. However, in contrast to extracellular dsRNA, cytoplasmic dsRNA elicited only modest expression of IL-1β mRNA and protein without any effect on continuously expressed IL-18, suggesting that recognition of cytoplasmic RNA was not sufficient to promote efficient transcriptional activation of *IL-1β*-engoding gene. The results implied that TLR signalling is superior to RLRs in the initiation of transcription of the IL-1β precursor, and that the recognition of extracellular pathogens *via* TLRs competently prime cells for a vigorous IL-1β pro-inflammatory cytokine response.

2.3. Extracellular and cytoplasmic dsRNA regulate differentially processing and secretion of IL-1β and IL-18

2.3.1. Cytoplasmic dsRNA evokes efficient processing and secretion of IL-18

Caspase-1 is responsible for processing of pro-IL-1β and pro-IL-18 into their biologically active equivalents before they can be secreted by unconventional protein secretion pathway(s) (Dinarello, 2009; Keller et al., 2008). Caspase-1 itself is expressed as an

inactive 45 kDa (p45) precursor which undergoes autoproteolysis in inflammasomes to become enzymatically active heterodimer composed of p10 and p20 subunits (Cerretti et al., 1992; Thornberry et al., 1992). Albeit extracellular dsRNA induced transcription of pro-inflammatory cytokine IL-1β in human primary macrophages, extracellular dsRNA did not clearly activate caspase-1, assessed as formation of caspase-1p10 and p20 subunits and *via* the secretion of IL-1β and IL-18. The result indicated that recognition of endolysosomal RNAs were not directly connected to assembly and activation of inflammasomes in human primary macrophages.

In contrast to extracellular dsRNA and also other well-known ligands of TLRs (R848 and LPS), cytoplasmic dsRNA and influenza A virus-infection promoted clear and efficient caspase-1 activation which resulted in robust processing and secretion of IL-18. The result suggested that only viral RNA in cytoplasm may directly or indirecly trigger inflammasome in infected cells. Since cytoplasmic dsRNA- and influenza A virus-induced expression of pro-IL-1β was weak, the measured secretion of IL-1β was low. The reasons why cytoplasmic dsRNA and influenza A virus infection-induced only modest production of pro-IL-1β were not elucidated, but may be related to functional specialization of RLRs and TLRs in promotion of antiviral and pro-inflammatory cytokine expressions, respectively.

In order to verify that recognition of cytoplasmic dsRNA and influenza A virus-infection, resulted in secretion of biologically active IL-18 from human primary macrophages, media from poly(I:C)-t -stimulated or influenza A virus-infected cells were transferred to primary T-cells, and expression of IFN-γ mRNA was analysed by real- time PCR. In accordance with the knowledge that T-cells produce IFN-γ in response to IL-18 and IFN-α (Sareneva et al., 1998), IFN-γ was markedly induced and its expression could be blocked by neutralizing Abs against IL-18. Not only did the result exclude the possibility of influenza A virus to promote processing of IL-18 to a form that could not engage IL-18 receptors, namely IL-18Rα and IL-18Rβ, but it also elegantly demonstrated the functional relevance of macrophages in the interface between innate and acquired immune responses.

2.3.2. Effect of TLRs on virus infection-induced pro-inflammatory cytokine response

In contrast to IL-18 secretion, IL-1β was significantly secreted from cytoplasmic dsRNA-stimulated and influenza A virus infected human primary macrophages, only if the cells had been stimulated first with TLR-agonists. Concurrent exposure of macrophages with TLR agonists and cytoplasmic dsRNA-stimulus or influenza A virus-infection also elevated IL-18 secretion. The extent of the synergistic action varied depending on the TLR in question. The results clearly showed that TLRs augmented the initially weak cytoplasmic RNA-induced expression of pro-IL-1β and were necessary for significant virus-induced IL-1β secretion. In other words, it seemed that TLRs ensured the initiation of transcription of IL-1β-encoding gene (signal one), whereas recognition of cytoplasmic RNA contributed to the inflammasome assembly, caspase-1 activation, and secretion of IL-1 family of pro-inflammatory cytokines (signal two).

Firstly, the results indicated that without simultaneous recognition of extracellular and intracellular PAMPs (or DAMPs), pro-IL-1β, is not expressed and processed into mature protein and externalized in cells stimulated with poly(I:C)-t or infected with influenza A virus. The requirement for two signals presumably prevents the unintentional initiation of an intense pro-inflammatory cytokine response unless the host is severely infected. An interesting example of the cooperation of TLRs with cytoplasmic PRRs during virus-infection is given by Ichionahe *et al.*,: commensal microbiota in the intestine provide constitutive expression of pro-IL-1β and NLRP3, that is necessary for inflammatory responses in the lung during influenza A virus infection. Conversely, certain antibiotics alter the composition of intestinal microbes, resulting in weakened immune responses against influenza A virus (Ichinohe et al., 2011).

In the context of an ongoing infection, the results obtained firstly suggested that the pro-inflammatory cytokine response of the virus-infected cell may be amplified by phagocytosis of extracellular RNAs from injured or apoptotic neighbouring cells containing viral RNAs. This would be rather likely especially during late phases of infection when macrophages phagocytose damaged or apoptotic cells in order to clear the site of infection. This is also supported *e.g.,* by the concept of Nishiya *et al.,*: TLR3 and TLR7/8 preferentially localize near phagosomes containing apoptotic cell particles (Nishiya et al., 2005).

Secondly, since stimulation of human primary macrophages with the prototypic TLR4-ligand LPS amplified tremendously influenza A virus-induced IL-1β and IL-18 secretion, it can be speculated that concomitant inhalation of LPS, bacterial coinfection, or formation of TLR4-stimulating DAMPs, may contribute to the "cytokine storm", hypercytokinemia, in the infected host. Indeed, secondary bacterial infections are known to associate with severe illnesses and mortalities of influenza A virus-infected individuals (Morens et al., 2008). Moreover, oxidized phospholipids produced during acute lung injury with H5N1 avian influenza virus trigger TLR4 and are intimately involved in the pathogenesis of virus-induced severe acute respiratory distress syndrome (Imai et al., 2008). Thus, one might hypothesize that simultaneous engagement of TLRs by these kinds of DAMPs during influenza A virus infection, may maintain a major pro-inflammatory cytokine response in human primary macrophages.

At the level of infected individuals, pathogens have been reported to use TLR-mediated inflammation as a route of dissemination *i.e.*, to establish an infection in other parts of the body. Intense production of cytokines *via* TLRs may damage tissues, resulting in the spread of the virus (Cilloniz et al., 2009; Kash et al., 2006; Kurt-Jones et al., 2004). Consistent with this concept, TLR3 has been shown to play a pathological, rather than a protective, role during influenza A virus infections: TLR3-knockout mice infected with influenza A virus display lower levels of pro-inflammatory cytokines and diminished numbers of $CD8^{+}$T-cells in the bronchoalveolar airspace of lung than their wild-type counterparts (Le Goffic et al., 2006). The peripheral inflammatory responses against encephalitogenic and positive sense ssRNA virus, such as West Nile virus, are also initiated by TLR3-induced cytokines resulting in increased permeability of blood-brain barrier and subsequent lethal viral encephalitis (Wang et al., 2004). However in humans,

there also is one reported case in which loss-of-function mutation in TLR3 caused severe influenza A virus-associated encephalopathy (Hidaka et al., 2006).

3. Inflammasome activation in response to cytoplasmic RNA (III)

Both cytoplasmic dsRNA-stimulus and influenza A virus-infection promoted robust IL-18 secretion, without any necessary assistance from TLRs in human primary macrophages. For this reason, IL-18 secretion was utilized as a direct marker of inflammasome activation. In an attempt to characterize further the mechanism for engagement of inflammasomes during virus infection, macrophages were stimulated with the mimetic of cytoplasmic signature of viral infection, poly(I:C)-t, as above.

3.1. Cytoplasmic dsRNA induces secretion of inflammasome components caspase-1 and ASC

Time-course analysis of cytoplasmic dsRNA-induced secretion of IL-18 was performed in human primary macrophages. IL-18 was secreted at 3 h and increasingly thereafter, detected by ELISA from cell media and by western bloting from intracellular protein lysates of cytoplasmic dsRNA-stimulated cells. Accordingly, also the subunit of activated caspase-1 was observed from western blots of intracellular protein lysates starting at the same time point and at later times. At 6 h, IL-18 was significantly secreted, and the biologically active equivalent of the cytokine was observable also in western blots of extracellular, secreted, proteins. Furthermore, two core components of inflammasomes, caspase-1 and ASC, were also found in the extracellular protein lysates of cytoplasmic dsRNA-stimulated cells, indicating that the inflammasome had indeed been activated. Unfortunately, due to unavailability of specific Abs against NLRP3 and MDA-5, externalization of NLRP3 and MDA-5 could not be analysed. The most likely explanation for the secretion of core components of inflammasomes is to limit inflammatory response and cell death during infection. In addition to poly(I:C)-t, also a positive sense ssRNA virus, EMCV, and negative sense ssRNA virus VSV, induced IL-18 secretion, meaning that this phenomenon may well occur among viruses from different RNA-virus families.

3.2. Secretome characterization of cytoplasmic dsRNA-stimulated human macrophages

In addition to the fact that prolonged activation of caspase-1 results in secretion of inflammasome components, caspase-1 activation is known to trigger unconventional protein secretion (Keller et al., 2008). In an attempt to obtain more information about protein secretion in the response to cytoplasmic dsRNA, global secretion pattern of proteins, secretomes, from control and cytoplasmic dsRNA-stimulated cells were determined by qualitative MS-based proteomics. Human primary macrophages were either

left unstimulated or were stimulated with cytoplasmic dsRNA for 6 h and 18 h, after which the proteins from cell culture media were collected, concentrated, precipitated, and subjected to SDS-PAGE. The SDS-PAGE gels were cut into lanes and then into smaller equal-size pieces to identifiy the SDS-PAGE-separated proteins, and the fragments were in-gel digested with trypsin, and the resulting peptides were analysed with nanoLC-MS/MS.

This method revealed almost 300 proteins from cell culture supernatants of cytoplasmic dsRNA-stimulated cells at 6 h and more than 400 proteins at the 18 h time point, whereas the corresponding numbers in the control cells remained at approximately 200. Thus, recognition of dsRNA induced strong and early protein secretion, which was augmented as time progressed. This phenomenon is probably related to both active and passive processes mediated by prolonged caspase-1 activation and cell death. The elevated protein secretion might serve as a mechanism to regulate inflammation and acquired immune responses in the host during virus infection, as already mentioned in the review of the literature in 2.2.3.3., and will be discussed in more detail in 5.4.

Identification of proteins from cytoplasmic dsRNA-treated cells revealed many unconventionally secreted proteins and proteins associated with exosomes, transport, and secretion, suggesting that both conventional and caspase-1-mediated unconventional protein secretion pathways were in operation. In particular, there was significant secretion of Ras-related proteins, rabs. Since these small GTPases are involved in the regulation of vesicular transport in and between distinct organelles as well as in the endocytic apparatus (Zerial and McBride, 2001), their secretion implied that marked alterations had occurred in secretory routes in response to recognition of cytoplasmic dsRNA.

Importantly, several lysosomal proteins, like cathepsins and endogenous inhibitors of cysteine-specific proteases, cystatins (Turk and Stoka, 2007; Turk and Bode, 1991), were detected, indicating that recognition of cytoplasmic dsRNA destabilized also lysosomal membranes and subsequently resulted in the leakage of lysosomal proteins into cytoplasm. This, in turn, might potentiate inflammasome activation in infected cells, and it was studied in further detail.

3.3. Cathepsins are vital for cytoplasmic RNA-triggered inflammasome activation

3.3.1. Cathepsin D secretion precedes secretion of cathepsin B in response to cytoplasmic dsRNA

Secretome analysis suggested substantial changes in secretion of cathepsins and their endogenous regulators in response to cytoplasmic dsRNA-stimulus. To delineate further cytoplasmic dsRNA-induced effects on cathepsin regulation, secretion of individual cysteine- and aspartate-specific proteases, cathepsin B and cathepsin D, respectively, were analysed by western blotting. Interestingly, secretion of mature cathepsin B followed release of activated caspase-1 and ASC, suggesting that the inflammasome had been activated before significant secretion of cathepsin B occurred. Moreover, cytoplasmic

dsRNA-induced secretion of cathepsin D preceded that of cathepsin B, implying that also cathepsin D may take part in inflammasome activation, or alternatively, was localized in the same secretory vesicles than caspase-1, ASC, and IL-18, as postulated by Andrei *et al.,* (Andrei et al., 2004). To delineate this further, cells were prestimulated with the most commonly used cathepsin B inhibitor, Ca-074 Me, before they were treated with cytoplasmic dsRNA. Ca-074 Me almost totally blocked secretion of IL-18 and ASC in response to cytoplasmic dsRNA-stimulus. Moreover, it suppressed also EMCV- and VSV-induced IL-18 secretion, suggesting that cathepsin B is essential for inflammasome activation in response to cytoplasmic dsRNA, EMCV, and VSV, and conceivably in general during RNA-virus infections. Two other pharmacologically distinct cathepsin inhibitors, Est and CatIII, also abolished cytoplasmic dsRNA-elicited IL-18 secretion, supporting the results obtained with Ca-074 Me.

To elucidate further the role of cathepsin D in cytoplasmic dsRNA-induced inflammasome activation, expression of cathepsin D was silenced with cathepsin D-specific *si*RNAs. After silencing, human primary macrophages were stimulated with cytoplasmic dsRNA mimetic for 8 h, and then samples for western blotting and ELISA were prepared. Cathepsin D-specific *si*RNAs effectively reduced cytoplasmic dsRNA-induced protein expression of cathepsin D and secretion of IL-18. These were not observed with cells treated with non-targetting control *si*RNAs. The results indicated that cathepsin D is likely involved in cytoplasmic dsRNA-induced inflammasome activation. Similarly to the obtained results, β-amyloid- and cholesterol crystal-induced NLRP3-inflammasome-activation have also been reported to require lysosomal proteases other than cathepsin B for their activation (Duewell et al., 2010; Halle et al., 2008; Hornung et al., 2008).

What mechanism then might be involved in cytoplasmic dsRNA-induced inflammasome activation which is associated to direct or indirect activation of cathepsins? It has been hypothesized that cathepsins may convert inflammasome component(s) into their active conformation(s), or process unknown cytoplasmic factor(s) that subsequently promote inflammasome assembly and activation (Martinon et al., 2009). It is noteworthy that NLRP3 contains cleavage sites for caspase-1, cathepsin B, and cathepsin L (E. Latz, unpublished observation). Further work will be definitely needed to delineate the roles of the individual cathepsins in inflammasome activation. However, this is a challenging task because numerous cathepsins (*i.e.*, cathepsin B, C, H, F, K, L, O, S, V, W, and X/Z), and species-specific differences between mice and men exist that complicate knock-out studies (Turk and Stoka, 2007).

The high-throughput secretome data of cytoplasmic dsRNA-stimulated human macrophages revealed also secretion of cystatins, endogenous inhibitors of cysteine-specific proteases, like cathepsin B and caspase-1 (Turk and Stoka, 2007; Turk and Bode, 1991). In particular, cystatins-A and -B were detected, implying that the actions of cysteine specific proteases are tightly controlled during cellular stress and this might bring additional level of regulation for inflammasome activation during virus infection. Moreover, the aspartate-specific protease cathepsin D is able to inactivate cystatins

(Lenarcic et al., 1988; Lenarcic et al., 1991). Thus, cathepsin D might antagonise the inhibitory functions of cystatins on cysteine specific proteases, and in this way may promote indirectly cathepsin B-dependent inflammasome activation, or alternatively activities of caspase-1. Furthermore, in contrast to plant cells which also defend themselves by LRR-containing disease resistance proteins, mammalian cells do not encode proteases that would negatively regulate the actions of aspartate-specific proteases, like cathepsin D (Lison et al., 2006). It is not known why the extra-lysosomal actions of cathepsin D are not inhibited in mammals. Nonetheless, complex proteolytic cascades evidently regulate the activities of inflammasomes during viral infection.

4. Apoptosis in response to virus infection (I, III)

4.1. Extracellular and cytoplasmic dsRNA regulate differentially apoptosis (I, III)

Recognition of cytoplasmic RNA, but not that of extracellular dsRNA, evoked the appearance of apoptotic morphology in human primary macrophages, including cell shrinkage and rounding, and subsequent detachment of cells from their growth site. In contrast to stimulation of TLR3 with extracellular dsRNA (or activation of other TLRs together or alone with their respective agonists *i.e.*, LPS and/or R848), only cytoplasmic dsRNA and influenza A virus-infection activated caspase-3. This was evident as the appearance of caspase-3 p17/19 subunit of the activated heterodimeric enzyme (the other subunit being caspase-3 p12) (Fernandes-Alnemri et al., 1994; Nicholson et al., 1995). Furthermore, the biologically inactive fragment of IL-18, formed by caspase-3 from pro-IL-18 and of IL-18 (Akita et al., 1997; Pirhonen et al., 1999), was detected by western blotting from cytoplasmic dsRNA-treated cells, suggesting that caspase-3 was functionally active.

In addition to cytoplasmic dsRNA-stimulus and influenza A virus-infection, also EMCV and VSV induced caspase-3 activation in human primary macrophages, showing that infection with these RNA-viruses included activation of programmed cell death.

4.2. Cytoplasmic dsRNA elicits inflammasome activation before apoptosis (III)

Apoptosis was also assessed with a unidirectional dye-uptake bioassay which is based on membrane alterations during apoptosis (APOPercentage; III, IV). After overnight exposure with cytoplasmic dsRNA, approximately 50% of the live cells, still attached to the bottom of cell growth plates, had progressed to apoptosis. (Influenza A virus had slower kinetics: no apoptotic cells were detected after 6 h post-infection, and 19% of the cells infected with the virus had progressed to apoptosis at 12 h and 73% were apoptotic at 18 h (IV)). Importantly, a clear activation of caspase-3 occurred at 6 h in cytoplasmic dsRNA-stimulated human primary macrophages, three hours later than first markers of

inflammasome activation were detected, suggesting that pro-inflammatory cytokine response was initiated before apoptosis in virus infected cells. The inflammatory response prior to apoptosis is an effective mechanism to signal the presence of danger and to activate the cellular arm of the innate and acquired immunity, as well as resident cells at the site of infection.

4.3. Mechanism for cytoplasmic dsRNA-induced apoptosis (I, III)

In an attempt to clarify the molecular mechanism of cytoplasmic dsRNA-induced apoptosis, an actively dividing cell line, human keratinocytes, HaCaTs, were examined. RIG-I and MAVS-encoding genes were transiently overexpressed or stably transfected through retroviral gene transfer into the nuclei of HaCaTs, respectively. With both of these constructs and methods, the end-result was the same: caspase-3 was significantly activated and the cells died, clear evidence that RIG-I and MAVS are capable of triggering apoptosis (I).

It has been known for a long time that exogenous IFN-α/β can potentiate apoptosis (Tanaka et al., 1998; Kaiser et al., 2004), possibly *via* increased expression of IFN-inducible genes that are involved in apoptotic responses (Balachandran et al., 1998; Li et al., 2004). However, in HaCaTs, exogenous administration of IFN-β itself did not enhance apoptosis, suggesting that the production of IFN-α/β was not primary reason for the RIG-I/MAVS-induced apoptotic effects in these cells.

Similarly, Chattopadhyay *et al.*, subsequently reported that RIG-I elicited apoptosis through MAVS-TRAF3-TBK1 and *via* adapter molecules TRAF2 and TRAF6 without concomitant transcriptional activation of ISRE-containing genes by IRF3 (Chattopadhyay et al., 2010). In contrast, this pathway culminated in direct interaction of IRF3 with pro-apoptotic protein Bax and subsequent permeabilization of mitochondrial membranes. However, MAVS has also been tightly connected to signal transduction cascades that lead to IRF3-mediated trancriptional activation of pro-apoptotic genes, like Noxa and Puma (Besch et al., 2009; Lallemand et al., 2007). Furthermore, others have reported that the cytoplasmic dsRNA pathway involves death-ligand and death-receptor-independent DISC assembly and subsequent activation of the caspase cascade *via* interaction of MAVS with FADD, TRADD, and the apoptotic initiator caspases, caspase-8 and -10 (Iordanov et al., 2005; Takahashi et al., 2006). In addition, reovirus-induced activation of MAVS and IRF3 is connected to caspase-3 activation (Holm et al., 2007). It is also worth mentioning that previous studies described apoptotic functions to MDA-5 (Kang et al., 2004; Kang et al., 2002; Kovacsovics et al., 2002). Further evidence of involvement of RLRs in apoptotic responses is illustrated by the fact that there are viruses which cleave and subsequently inactivate RIG-I, MDA-5, and MAVS during infection (Barral et al., 2007; Barral et al., 2009; Lei et al., 2009; Rebsamen et al., 2008; Yu et al., 2010). This processing, mediated either by viral proteins or host caspases activated by the infecting virus, suggests that viruses do attempt to attenuate innate immune responses, including the initiation of apoptosis, in infected cells. Furthermore, the actions of anti-apoptotic proteins of host may regulate the functions of RLRs, implicating that RLR members have apoptotic functions.

For example, cleavage of MAVS during infection can be blocked by overexpression of Bcl-XL, and certain primary cells can be rescued from RIG-I/MDA-5-mediated apoptosis by Bcl-XL (Besch et al., 2009; Scott and Norris, 2008). Overal, these reports from other laboratories strongly support the results obtained here: RLR pathway integrates apoptotic signals during virus infection. The knowledge of the capacity of RIG-I/MAVS signalling pathway to promote apoptosis may have therapeutic significance in medicine.

Importantly, in addition to the fact that cathepsin B inhibitor Ca-074 Me significantly suppressed inflammasome activation in response to recognition of cytoplasmic dsRNA in human primary macrophages, it also abolished cytoplasmic dsRNA-, EMCV-, and VSV-triggered caspase-3 activation (III). Furthermore, the agent inhibited cytoplasmic dsRNA-induced proteolysis of Bid into t-Bid and the number of apoptotic cells. Thus, the obtained results with human primary macrophages implied that in addition to MAVS and RIG-I, also cathepsins are involved in the induction of apoptosis in response to the recognition of cytoplasmic RNA. Importantly, Blomgran *et al.*, and Kagedal *et al.,* have reported that cathepsins are important activators of oxidative stress-induced apoptosis in neutrophils and fibroblasts (Blomgran et al., 2007; Kagedal et al., 2001). Thus, it can be speculated that recognition of cytoplasmic RNA in human primary macrophages might trigger ROS production resulting in destabilization of lysosomal membranes, leakage of cathepsins into cytoplasm, proteolysis of Bid, and subsequent activation of pro-apoptotic proteins like Bax, breakdown of mitochondrial membrane integrity, cytochrome c release, and apoptosis (Garrido et al., 2006; Li et al., 1998; Luo et al., 1998; Strasser, 2005).

Overall, one might hypothesize that recognition of cytoplasmic RNA induces formation of multiprotein complexes, including RIG-I/MDA-5, MAVS, ASC/NLRP3, and/or t-Bid/Bax in the vicinity of mitochondria, from where the signals for antiviral and pro-inflammatory cytokine responses, and apoptosis are successively initiated or regulated. This concept is supported at least by three recent reports showing that MAVS regulates mitochondrial dynamics to facilitate the mitochondria-ER association required for antiviral signal transduction; NLRP3 and ASC localize to ER-membranes near to mitochondria after a stimulus; and NLRP3 is essential sensor of membrane permeability of mitochondria and subsequent release of cytochrome c in response to the appearance of danger signals (Castanier et al., 2010; Nakahira et al., 2011; Zhou et al., 2010). A hypothetical model for cytoplasmic RNA recognition pathway-induced inflammasome activation and apoptosis in human primary macrophages is presented in **figure 14**.

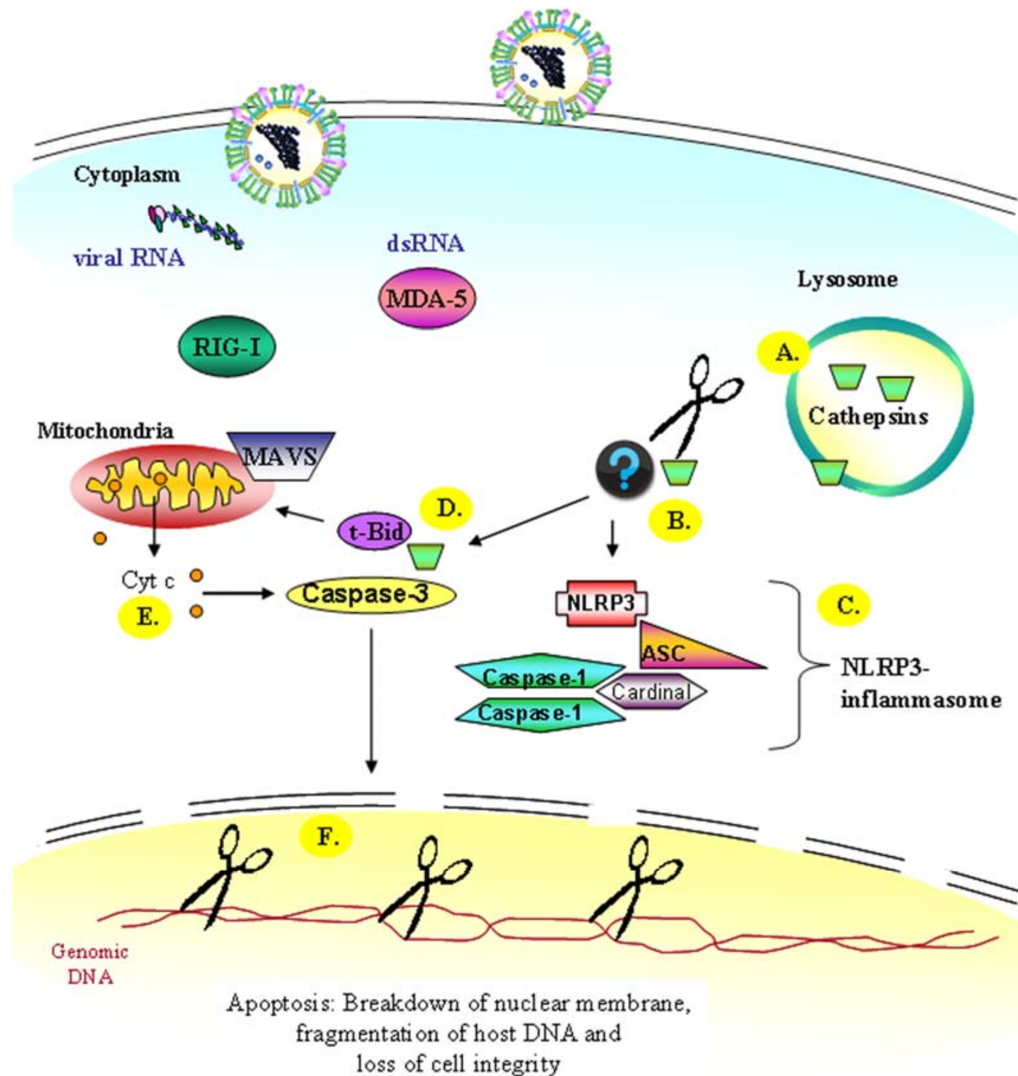


Figure 14 ***Hypothetical pathways for inflammasome activation and apoptosis in virus-infected cells.*** *Inflammasome activation preceded apoptosis in cytoplasmic dsRNA-stimulated cells. Host response to virus may destabilize membrane integrity of mitochondria and lysosomes. Lysosomal enzymes, such as cathepsins, leak into cytoplasm. There, (B) they may proteolyse an unknown host-factor(s) (or e.g., NLRP3), resulting in (C) inflammasome activation as well as apoptosis. (D) Indirect or direct cleavage of Bid by cathepsins promotes (E) mitochondrial membrane destabilization and subsequent release of cytochrome c into cytoplasm. (F) Caspase-3 is activated and apoptosis ensues. In addition to NLRP3-inflammasome, also other inflammasomes may be involved in cytoplasmic dsRNA-induced inflammasome activation.*

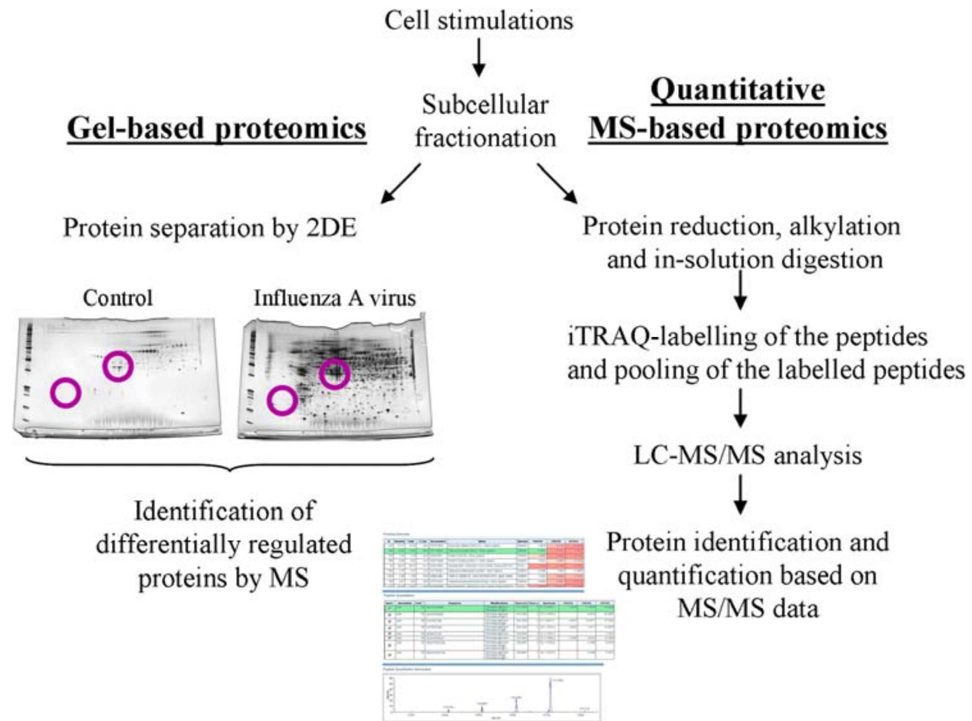


Figure 15 ***Methodological differences between 2DE-based- and quantitative high-throughput-iTRAQ-based proteomics.*** *In 2DE-based proteomics, differentially expressed proteins are cut out of the gels, in-gel digested into peptides, and identified by MS and database searches. Through this method, high-abundance proteins with clear differences in their expression compared to controls are best characterized. In quantitative high-throughput MS-based proteomics, proteins in solution are digested, labelled, pooled, and fractionated with liquid chromatography (LC). The resulting peptide fractions are analysed twice with MS to improve quality of protein identifications and quantifications. The MS data is further handled with bioinformatics. The quantitative MS-based proteomics is able to detect minimal changes on protein expression and post-translational modifications from a very limited amount of starting material.*

5. Subcellular proteome and secretome profiling of influenza A virus-infected human primary macrophages (II, IV)

Proteomics addresses the level of gene products in cells at certain time points, and may provide information about changes of protein abundances, locations, and modifications. In an attempt to characterize influenza A virus-induced effects at the proteome level, the infected cells were fractionated into different subcellular compartments and the fractions were individually analysed. First, cytoplasmic and mitochondrial proteomes were analysed by 2DE-based proteomics (II) (**Fig. 15**). Next, fast methodological development of proteomics enabled quantitative high-throughput subcellular and secretome profiling of influenza A virus-infected human primary macrophages, and consequently provided a more global view of the host-virus-interactions and innate immune responses during virus

infection. The quantitative proteomic data from nuclear, cytoplasmic, and mitochondrial proteomes of influenza A virus-infected macrophages at 6 h, 12 h, and 18 h post-infection were generated by MS-based proteomics, namely the iTRAQ-technique (**Fig. 15**). To complete characterization of influenza A virus-mediated events in human primary macrophages, also the secretomes at 6 h, 9 h, and 12 h post-infection were analysed with the same method.

5.1. Marked alterations in cytoplasmic and mitochondrial proteomes of influenza A virus-infected cells (II)

2DE coupled to MS revealed marked changes in cytoplasmic and mitochondrial protein fractions between infected and non-infected cells at 18 h after the onset of the influenza A virus infection. Several proteins were upregulated in both fractions in response to the infection. These included IFN-inducible MxA, and several stress- and apoptosis-associated proteins, such as heat-shock proteins (HSPs) and protein fragments produced by caspase-3, respectively. The result indicated that antiviral-, apoptosis- and stress-related processes were predominant in the cells during early phases of influenza A virus infection. Indeed, in terms of the antiviral cytokine response, the time-course analysis of influenza A virus infected cells revealed that mRNA and protein expressions of RIG-I were rapidly induced at 3 h and 6 h post-infection, respectively, before significant mRNA expression of IFN-β and IL-29 occurred, suggesting that high RIG-I expression preceded a vigorous IFN-response, and that cells adopted rapidly an antiviral cytokine status after influenza A virus infection.

The virus elicited also translocation and fragmentation of various cytoskeletal components, such as actins, α- and β-tubulins, vimentins, and gelsolins, pointing to the interaction of influenza A virus with its host resulted in marked cytoskeletal rearrangement. In particular, intact and fragmented actin molecules accumulated significantly in the mitochondrial protein fraction at 18 h post-infection. The extensive redistribution of the actin network in response to influenza A virus infection was confirmed also by examination with confocal microscopy.

Caspase-3 proteolyses cytoskeletal proteins like actin, gelsolin, and vimentin (Fischer et al., 2003; Gourlay and Ayscough, 2005). In line with this knowledge, caspase-3 activation was followed by the appearance of actin fragments and their translocation into mitochondria at 16 h post-infection. In accordance, inhibition of caspase-3 with the pancaspase inhibitor, z-VAD-FMK, but not with the caspase-1 and -4 inhibitor, z-YVAD (data not shown), blocked caspase-3 activation and clearly decreased actin fragmentation and its mitochondrial translocation. The result suggested that influenza A virus-activated caspase-3 is involved in the breakdown of the actin cytoskeleton, and the actin fragments produced were subsequently translocated to mitochondria during the infection.

The proteomic analysis of Liu *et al.*, also detected significant changes in actin network with avian influenza virus (H9N2) infection in a human cell line (Liu et al., 2008). Furthermore, others have reported that influenza A virus takes advantage of the

remodelling of the actin network for entering and budding in polarized epithelial cells (Simpson-Holley et al., 2002; Sun and Whittaker, 2007). Moreover, egress of filamentous influenza A viruses, *e.g.*, influenza A virus strain Udorn, can be reduced by actin depolymerizing agents cytochalasin D or jasplakinoline in this cell type (Roberts and Compans, 1998; Simpson-Holley et al., 2002), suggesting that the actin cytoskeleton organizes viral proteins at the site of viral exit. However, in the present experiments, cytochalasin D did not have any effect on the production of influenza A virus proteins, indicating that disruption of actin filaments did not disturb replication of the virus in human primary macrophages.

The significance in fragmentation and relocalization of actins may also be related to the utilization of actin for transport and sorting of viral RNAs and proteins (Simpson-Holley et al., 2002; Sundell and Singer, 1991). At least NP and M1 of influenza A virus are known to interact with cytoskeletal elements and apparently facilitate vRNP transport to the budding sites (Avalos et al., 1997). Interestingly, retroviruses and several negative strand RNA viruses also incorporate cellular actins into their virion particles, and other viruses use actin to propel themselves out of cells (Roper et al., 1998; Sagara et al., 1995). However, influenza A virus is not known to utilize actins in such properties.

Alternatively, the observed breakdown of the cytoskeleton by caspase-3 could also reflect cell's intrinsic defence mechanisms: efficient transcription, protein synthesis, movement of organelles, transport of proteins, vesicular cargoes, and individual proteins are dependent on an intact actin network (Miralles and Visa, 2006; Wickramarachchi et al., 2010), and without a cytoskeleton, the cell interior will collapse, shrink, and apoptosis will ensue. Furthermore, without proper cell-functions, cell replication is halted.

5.2. Translocation of RIG-I/MAVS signalling components from cytoplasms to mitochondria (II)

Cytoplasmic RNA recognition gathers several signalling components of the RLR pathway to close proximity of mitochondria (Gack et al., 2007; Lin et al., 2006; Michallet et al., 2008a). In accordance, it was found that influenza A virus infection, recognized by RIG-I, promoted translocation of TRIM25, IKKε, RIG-I, and TRADD into the mitochondrial protein fraction, whereas cytoplasmic dsRNA, triggering mainly MDA-5, induced hardly any or even no mitochondrial translocation at all of these above signalling components. This data is also in line with other reports which show that influenza A virus and the long cytoplasmic dsRNA polymer are mainly recognized by RIG-I and MDA-5, respectively (Kato et al., 2006; Pichlmair et al., 2006).

Interestingly, mitochondrial translocation of TRIM25 and TRADD followed translocation of intact actins from cytoplasm to mitochondria in response to influenza A virus infection. These events, in turn, preceded simultaneous translocation of RIG-I and fragmented actins into the mitochondrial fraction, suggesting that the actin cytoskeleton is intimately involved in the regulation of virus recognition and subsequent signalling, and could serve as a platform for immune-associated molecules. However, inhibition of caspase-3, and subsequent decreased fragmentation of actins and their translocation to

mitochondria did not hinder translocation of RLR-signaling molecules, RIG-I, TRIM25, or TRADD into the mitochondrial protein fraction. Thus, this suggested that RIG-I is translocated to mitochondria without the requirement for caspase-3-mediated actin fragmentation. To delineate this further, the cells were stimulated with cytochalasin D before the virus was inoculated, and the expression of antiviral cytokines was analysed with real- time PCR at 6 h post-infection. The pharmacological agent diminished mRNA expression levels of type I and III IFNs, as well as that of TNF-α suggesting that intact actins are involved in efficient expression of antiviral and pro-inflammatory cytokines. However, cytochalasin D had no effect on influenza A virus protein production or apoptosis, as determined with the Abs recognizing subunits of H3N2 influenza A virus and activated caspase-3, respectively. Although the importance of intact actins for transport of viral proteins was not elucidated, it was found that they accumulated in excess in mitochondria during the progression of infection. This observation highlighted the importance of mitochondrial location for viral proteins during infection in human primary macrophages. Indeed, PB2 of influenza A virus contains a mitochondrial localization signal, and PB2 has been recently found to accumulate in mitochondria and interact there with MAVS and to suppress IFN-signalling (Carr et al., 2006; Graef et al., 2010).

Collectively, the result suggested that an intact actin network supports efficient signalling for transcriptional activation of certain cytokines, such as IFNs. Furthermore, the close interaction between mitochondria and actin filaments might be involved in regulating the progression of influenza A virus infection.

Interestingly, Mukherjee *et al.*, have reported that RIG-I binds *via* its CARD to actin and facilitates actin polymerization, and overexpression of RIG-I induces cellular migration and wound healing of nonpolarized epithelial cell (Mukherjee et al., 2009). Kong *et al.,* also demonstrated that RIG-I binds to and co-localizes with actin, and IL-1β, LPS, and poly(I:C) enhance further this interaction (Kong et al., 2009). Furthermore, loss of RIG-I was found to impair phagocytosis of bacteria. In another study, genetic deletion of RIG-I led to a colitis-like phenotype, and similarly to the study of Kong *et al.*, increased the susceptibility to bacterial infections in mice (Wang et al., 2007). These examples suggest that the functions of RIG-I are linked to the cytoskeleton, and cytoskeletal changes which negatively impact on RIG-I may have profound effects on the clearance of infective agents from the surroundings, preventing the recovery from microbial infection, as well as interfering with normal mucosal signaling of the intestine. Thus, it seems that RIG-I has other roles beyond the elicitation of cytokines and apoptosis during complex host-virus-interactions, and these might include restoration of homeostasis after infection. To summarize, the early induction of RIG-I observed during influenza A virus infection both in human primary macrophages, as well as in HaCaTs (I), may be more than simply a prelude to the antiviral cytokine response and triggering of apoptosis.

5.3 Quantitative analyses of subcellular proteomes of influenza A virus-infected cells (IV)

The quantitative and more global proteomic analysis of influenza A virus-infected macrophages identified 3477 distinct proteins, of which 2466 were reliably quantified, and from these, 1321 were differentially regulated in the intracellular fractions of influenza A virus-infected human primary macrophages. Proteins with known cytoplasmic, mitochondrial, or nuclear location were detected mainly in their respective fractions. However, in addition to mitochondrial proteins, most lysosome- and ER-associated proteins identified were also found in the mitochondrial proteomes, impying that these subcellular organelles were enriched in mitochondrial protein fractions. Interestingly, large numbers of mainly nuclear proteins were found in the cytoplasmic fractions, pointing to heavy traffic of proteins between these cellular compartments during influenza A virus infection.

Indeed, the largest number of differentially regulated proteins was detected in the nuclear fractions, the nucleus being the main replication site of the virus. Over 300 proteins were up- and downregulated while approximately 200 proteins retained their nuclear abundance at 12 h and 18 h post-infection. Based on gene ontology annotations, the upregulated proteins originated from mitochondria, ER, Golgi, and cytoplams, and were functionally classified into proteins involved in gene expression, metabolism, Ca^{2+}-ion binding, transport, and signalling. The downregulated proteins, in turn, were mainly of nuclear origin, and functioned in gene expression, particularly in nucleotide metabolism and mRNA processing. Furthermore, approximately 5% of them were related to mRNA splicing, the process utilized by influenza A virus during its replication cycle (*e.g.,* NEP and M2 are generated *via* splicing from NS1- and M1-encoding mRNAs, respectively (Flint et al., 2004c), suggesting that the host response to influenza A virus might include downregulation of activities of *e.g.*, spliceosomes. Since several nuclear histones and other nuclear proteins were upregulated in the cytoplasmic protein fraction at 12 h and 18 h post-infection, influenza A virus infection likely promoted marked changes in the architecture of nuclei.

In addition to several nuclear proteins, lysosomal proteins including cathepsins, and mitochondrial inner membrane proteins, like components of electron transport chain, oxidative phosphorylation, and cytochrome c, were upregulated in the cytoplasmic proteomes of influenza A virus-infected cells. The results suggested that substantial subcellular alterations/damage had occured in these organelles likely impairing their functions during infection. For example, lysosomes are indispensable for destruction of phagocytosed pathogens and autophagy-mediated recycling of cellular components and malfunctioning organelles, while mitochondria are vital for innate immunity, apoptosis, energy production, and many other metabolic processes described earlier in the review of the literature (2.3.1.). If damaged, mitochondria in particular produce ROS and this is linked to the development and amplification of pro-inflammatory cytokine response and apoptosis of cell (Ott et al., 2007; Skulachev, 2000; Zhou et al., 2009). Indeed, classical signs of apoptosis were detected from influenza A virus-infected human primary macrophages demonstrating the progression of apoptosis: the amount of cytochrome c was

increased and that of Bax decreased in the cytoplasmic protein fraction during the course of the infection, and *vice versa* in the mitochondrial protein fraction. Western blot analysis of caspase-3 activation, as well as apoptotic staining of influenza A virus-infected cells (the APOPercentage assay), also confirmed induction of apoptosis in human primary macrophages.

In addition, the antiviral cytokine response prevailed. Increased levels of IFN-inducible proteins, such as ubiquitin-like-protein-ISG15 (ISG15) and IFN-induced protein with tetratricopeptide repeats (IFIT)-1 and -3 were detected in cytoplasmic protein fractions at 6 h post-infection and increasingly thereafter, suggesting that IFN-response was started immediately after recognition of the virus. (This concept was also in accordance with the time kinetic studies performed with real-time PCR presented in original publication II, and here in 5.1.). Furthermore, their increased expression is essential for host response to viruses: ISG15 stabilizes and activates various host proteins important for antiviral responses, such as RIG-I, and inhibits nuclear entrance of NS1 preventing replication of influenza A virus (Zhao et al., 2005; Zhao et al., 2010). The antagonistic actions of IFITs on influenza A virus are less clear at present: IFITs suppress the translation of viral RNAs lacking 2'-O-methylation in their 5'-cap that the influenza A virus is able to subtly subvert by acquiring 2'-O-methylated 5'-caps from host mRNAs (Daffis et al., 2011; Zust et al., 2011).

Although multiple antiviral effector proteins are involved in the initiation of translational block during infection, several influenza A virus proteins, such as HA, NA, and NP were significantly upregulated in the cytoplasmic and mitochondrial protein fractions at 12 h and 18 h post-infection, respectively, suggesting that the virus strongly counteracted the host responses, possibly *via* actions of NS1. The increased expression of viral proteins in those subcellular compartments was also observed by the western blotting technique in the previously described 2DE work (II).

5.4 Quantitative secretome analyses of influenza A virus-infected cells uncover secretion of important mediators of innate immunity (IV)

With regards to secretomes, influenza A virus induced substantial protein secretion already at 6 h post-infection which increased with the progression of time. Proteins with and without signal-peptides were secreted, indicating that both conventional protein secretion pathways, consisting of ER-*trans*-Golgi-network, as well as poorly characterized unconventionally secretion pathway(s) were functioning (Keller et al., 2008). Furthermore, influenza A virus infection elicited secretion of several proteins that were also identified from cytoplasmic dsRNA-stimulated cells (III), suggesting that virus infections may induce a common pattern of protein secretion. These proteins induced lysosomal cathepsins, their endogenous inhibitors, cystatins, as well as subunits of vacuolar-(v)-type proton ATPases and Ras-related proteins.

Vacuolar-type proton ATPases and Ras-related proteins, Rabs, regulate endosomal acidification or trafficking and recycling, respectively (Savina and Amigorena, 2007; Wada et al., 2008; Zerial and McBride, 2001). Interestingly, genome-wide RNA*i* screens

in *Drosophila* and in human lung epitlelial cells have also identified v-type proton ATPases as important host factors for influenza A virus infection (Hao et al., 2008; Karlas et al., 2010; Konig et al., 2010). Consistently with the knowledge that v-type proton ATPases are required for endosomal acidification, Hao *et al*., and Guinea *et al*., reported that the activity of v-type proton ATPases was essential for influenza A virus entry and replication (Hao et al., 2008; Guinea and Carrasco, 1995). With regards to Ras-related proteins, Bruce *et al.*, found regulator of actin dynamics, Rab11, to be essential for morphogenesis of filamentous influenza A virus particles, and Sieczkarski *et al.*, demonstrated that Rab5 and -7 were required for early and late endosomal trafficking of the virus and the subsequent establishment of influenza A virus infection (Bruce et al., 2010; Sieczkarski and Whittaker, 2003). Thus, secretion of v-type ATPases and Rabs may serve as an early attempt of the host to prevent replication of viruses: without functional v-type proton ATPases endosomal acidification, being required for membrane fusion between viral and host membranes and uncoating of the virus genome from its capsid is impaired, while without Rabs, no correct assembly or endosomal trafficking of virus occurred in infected cells. Since Rabs are involved in exosome biogenesis and traffic, their secretion may also be an indication of release of different kinds of membrane vesicles, including also apoptotic vesicles, from infected cells in order to mount pro-inflammatory cytokine response and material for antigen presentation.

In addition, soluble mediators of innate immune responses, including chemokines, such as CCL8 and CCL24 (chemokine (C-C motif) ligand 8 and -24, respectively), and proteases and proteinases, such as matrix metalloproteinase-9, metalloelastase, and plasminogen activator inhibitor, were secreted. Their annotated functions demonstrate the importance of macrophages in mediating innate immune responses *in vivo*, such as the attraction of leukocytes, stimulation of resident cells, and the regulation of extracellular matrix turnover at the site of infection or tissue damage, respectively (Coelho et al., 2005; Westermann et al., 2010). Furthermore, secretomes of influenza A virus (and of cytoplasmic dsRNA) contained also proteins that have previously been detected from secretory vesicles of apoptotic DCs (Thery et al., 2001; Thery et al., 2009). The protein composition of DCs' secretory vesicles was unique with both immunostimulatory and/or cell-protecting properties. This implies that also influenza A virus-infected human primary macrophages may regulate immune responses through increased protein secretion.

Human primary macrophages infected with influenza A virus secreted also several nuclear components such as histones, which might serve as antigens for self-reacting lymphocytes. For example, autoantibodies targetted against histones are found in systemic lupus erythematosus patients (Banchereau and Pascual, 2006). Although the mechanisms involved in the pathogenesis of autoimmune diseases in response to viruses are poorly known, the extracellular presence of these kinds of proteins might contribute to the development and/or progression of autoimmune diseases especially in individuals with autoimmune disease-prone genetic background.

One of the most intriguing findings of the secretome data was the identification of potential DAMPs, such as S100 proteins, HSPs, HMGBs, and galectin-3, from cell culture media of influenza A virus-infected macrophages. Secretion of certain DAMPs was also

verified by western blotting. According to the literature, these DAMPs were mainly unconventionally secreted, suggesting that their release from cells could be associated with caspase-1 and inflammasome activation (Keller et al., 2008; Mambula et al., 2007). Furthermore, since most of the aforementioned proteins have dual functions both intracellularly and extracellularly (Arnoys and Wang, 2007), to be discussed below, their secretion might critically regulate host-virus interactions, inflammation, and immune responses. Indeed, it has been shown that ASC/caspase-1 activation determines the development of acquired immune responses in influenza A virus-infected mice (Ichinohe et al., 2009; Ichinohe at al., 2011).

With respect to the identified DAMPs from secretomes of influenza A virus-infected macrophages S100A8 and S100A9 proteins were well represented. S100A8 and S100A9 are Ca^{2+}-binding proteins that undergo various post-translational modifications and form homo- and hetero-oligomers with specific functions (Lim et al., 2009). Intracellularly, they may be involved in the regulation of cytoskeletal rearrangements and redox balance, whereas extracellularly they have multiple anti-inflammatory and pro-inflammatory roles, and are released in abundance also during rheumatoid arthritis, inflammatory bowel disease, and cancer (Halayko and Ghavami, 2009). Furthermore, since S100A8/A9 induce cell proliferation, apoptosis, inflammation, collagen synthesis, and/or cell migration in resident and infiltrating airway cells, S100A8/A9 could promote airway remodelling and perhaps chronic airway inflammation during virus infection. Indeed, respiratory virus infections have been linked to the development of asthma and idiopathic pulmonary fibrosis (Hakonarson et al., 1999; Pulkkinen et al., 2009), to diseases in which all the aforementioned symptoms are common.

Another interesting protein group found in the influenza A virus secretome was HSP chaperones. HSPs assist in proper folding, function, and stability of their protein-binding partners, and their expression is elevated in response to cellular stress (Richter et al., 2010). HSPs were also observed during influenza A virus infection in the 2DE work (II). Many HSPs are intracellularly anti-apoptotic, and HSP90-α and HSP90 have been implicated in positive regulation of RIG-I's stability and activation of NLRP3, respectively (Matsumiya et al., 2009; Mayor et al., 2007), suggesting that their externalization may negatively impact on antiviral-, inflammatory, and/or apoptotic responses. Moreover, HSP70 is a guardian of lysosomal and mitochondrial membrane integrity (Kirkegaard et al., 2010; Stankiewicz et al., 2005). Interestingly, Hsc70 is known to interact with M1 of influenza A virus and without Hsc70, M1 and vRNPs are not exported from the nucleus resulting in incomplete replication of the virus (Watanabe et al., 2006). In addition, HSP90 binds to influenza A virus proteins (Momose et al., 2002). Its binding to PB2 stimulates viral RNA synthesis possibly by hindering self-aggregation of newly-produced PB2s or by otherwise stimulating the functions of PB2.

As described, HMGB1 and -2 are universal sentinels for nucleic acid-mediated innate immune responses and they operate upstream of RLRs and TLRs (Yanai et al., 2009). Cytoplasmic HMGB1 is also a crucial regulator of autophagy, being released from cells in response to apoptosis (Tang et al., 2010). Interestingly, extracellular HMGB1s have been associated with cancer and autoimmune diseases, like systemic lupus erythematosus and rheumatoid arthritis (Klune et al., 2008).

Galectin-3 is a β-galactoside-binding lectin which also has dual functions inside and outside cells, and is highly expressed and secreted, especially by macrophages (Liu et al., 1995). Intracellular galectin-3 seems to be vital for efficient phagocytosis, whereas extracellular galectin-3 attracts monocytes and macrophages, and induces superoxide production (Sano et al., 2003; Sano et al., 2000). Furthermore, since galectin-3 has a unique ability to associate with macrophages, it may facilitate the recognition of carbohydrate-containing PAMPs and promote the removal of pathogens *via* phagocytosis. The role of galectin-3 in direct binding and neutralization of influenza A virus glycoproteins (HA, and NA) is worthy of further characterization. In summary, secretion of DAMPs may have a major impact on the host response and subsequent prograssion and outcome of influenza A virus infection.

Importantly, IL-1β, IL-18, and all the aforesaid proteins are secreted unconventionally. This might be particularly important if the infecting agent disturbs mechanisms or the ultrastructure of organelles involved in conventional protein secretion. Many viruses encode proteins that block classical transport routes or induce dramatic reorganization of ER-*trans*-Golgi-network (Flint et al., 2004g). However, unconventional secretion of the aforementioned DAMPs (as well as IL-1β and IL-18) ensures that they are released without any constraints from cells upon caspase-1 activation, more or less independently of the structure and function of the conventional secretion pathway. Hence, unconventional protein secretion may also be considered as a cell's intrinsic defence mechanism. It is noteworthy that intracellular bacteria are expelled from infected cells as a result of prolonged caspase-1 activity (Miao et al., 2010).

5.5. Cathepsins, P2X$_7$ receptors, and Src kinases are involved in influenza A virus-induced inflammasome activation (IV)

Due to the cytoplasmic increase of cathepsins, as well as their secretion in influenza A virus infected human primary macrophages, it was decided to characterize their involvement in inflammasome activation. Similarly to cytoplasmic dsRNA-stimulated cells, it was found that significant secretion of cathepsin D preceded that of cathepsin B in influenza A virus-infected human primary macrophages. Moreover, treatment with the cathepsin B inhibitor Ca-074 Me, almost completely abolished influenza A viruses Udorn- and Beijing-elicited IL-18 secretion, as well as caspase-3 activation. The result suggested that cathepsins were important also for influenza A virus-induced inflammasome activation and apoptosis, equally well as they were for cytoplasmic dsRNA-, EMCV-, and VSV-triggered inflammasome activation and apoptosis. Interestingly, Ngamurulert *et al.*, reported the interaction of NS1 of influenza A virus (H5N1) with cathepsin B in a yeast two-hybrid system (Ngamurulert et al., 2009), suggesting that influenza A viruses may manipulate activities of cathepsin B for their own benefit, *e.g.*, by hindering cathepsin B-mediated host responses or by utilizing its proteolytic abilities to mature viral proteins.

In an attempt to delineate host-virus interactions further and potential regulatory proteins for inflammasome activation, a protein-protein interaction network, consisting of

inflammation-related proteins, was generated from proteins identified in the intracellular protein fractions of influenza A virus-infected human primary macrophages. NLRP3 was added to the protein interaction network since it has been reported to be important for activation of inflammatory response during influenza A virus infection (Allen et al., 2009; Thomas et al., 2009). The network showed that there was a direct link between ASC (illustrated as PYCARD in original publication IV; Fig. 5A), NLRP3, and $P2X_7$ receptor. Functional studies were conducted, and it was found that inhibition of ATP-sensing protein $P2X_7$ receptor with AZ11645373 suppressed influenza A viruses Udorn- and Beijing-induced IL-18 secretion. Treatment with the inhibitor reduced also IL-1β secretion in LPS-primed and influenza A virus-infected human primary macrophages. Furthermore, silencing the expression of $P2X_7$ receptor with *si*RNAs reduced inflammasome activation in response to virus infection. Importantly, also Ichinoce *et al.*, reported the partial involvement of $P2X_7$ receptor in inflammasome activation during influenza A virus infection in mice (Ichinohe et al., 2010).

It is known that binding of extracellular ATP to purinergic $P2X_7$ receptor at concentrations known to be present at sites of injury and inflammation, triggers a rapid and massive ATP- and K^+-effluxes from cells (Mariathasan et al., 2006; Mackenzie et al., 2001; Perregaux and Gabel, 1994; Perregaux et al., 2000; Petrilli et al., 2007) and promotes gradual formation of pannexin-1 membrane-hemichannel and inflammasome activation in response to different kinds of stimuli (Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007). K^+-efflux (and increased intracellular concentrations of Ca^{2+}) trigger secretion of the IL-1-family of pro-inflammatory cytokines, but may also result in cathepsin B and cathepsin D secretion (Andrei et al., 2004; Qu et al. 2007; Qu et al. 2009). Furthermore, the activation of $P2X_7$ receptor promotes a rapid rearrangement of actin resulting in membrane blebbing and exosome release and the creation of secretory routes available not only for IL-1 and IL-18, but also for other unconventionally secreted proteins (MacKenzie et al., 2001; Qu et al., 2007; Qu et al., 2009). Thus, in human primary macrophages, ATP released in response to influenza A virus-infection, and subsequently sensed by $P2X_7$ receptor might be a likely mechanism for inflammasome activation, secretion of IL-1β and IL-18, and of all the unconventionally secreted proteins. Furthermore, ATP-dependent opening of pannexin-1 allows the passage of small-sized ions and DAMPs across the plasma membrane into both directions (Chekeni et al., 2010; Marina-Garcia et al., 2008; Pelegrin and Surprenant, 2007), which might further contribute and intensify inflammasome activation in an infected cell, as well as in its neighbour. Moreover, the apoptotic response during influenza A virus infection may also be initiated by extracellular ATP acting *via* the $P2X_7$ receptor and pannexin-1 opening: reduced cytoplasmic concentrations of ATP and K^+ are required for apoptosome formation (Cain et al., 2001). In addition, elevated extracellular levels of ATP sensed by $P2X_7$ receptor on DCs are known to be able to activate the inflammasome in DCs, which is a necessary prerequisite for activation of cytotoxic $CD8^+$T cells (Ghiringhelli et al., 2009). Thus, sensing of extracellular ATP through the $P2X_7$ receptor may drive inflammasome activation and/or apoptosis in resident cells, as well as in APCs recruited to the site of infection, and could also represent a potential bridge between the innate and acquired immune responses.

Macrophages are professional phagocytes which kill microbes through increased ROS production (see review of the literature 1.1.1.). The enzymes responsible for ROS generation in the extracellular or luminal space are NADPH oxidases. NADPH oxidases are transmembrane proteins composed of several subunits that transport electrons across biological membranes in order to reduce oxygen to superoxide which is then converted to ROS (Bedard and Krause, 2007). The protein-protein interaction network, constructed from intracellular proteins of influenza A virus-infected macrophages, linked NLRP3 and ASC closely with NADPH oxidase subunits p22phox and gp91phox (illustrated as CYBA and CYBB, respectively, in the original publication IV; Fig. 5A). These proteins have been demonstrated to be involved in inflammasome activation through ROS production in response to various stimuli (Cruz et al., 2007; Dostert et al., 2009; Gross et al., 2009). The network analysis linked p22phox and gp91phox also to tyrosine kinase Src, a prototypic member of the nonreceptor membrane-associated tyrosine kinases. Src is involved in the regulation of oxidative signalling and it responds to ROS (Giannoni et al., 2010). Interestingly, Src has been shown to upregulate the expression of subunits of NADPH oxidases and to interact with these components (Giannoni et al., 2010; Touyz et al., 2003). It was found here that in influenza A virus-infected human primary macrophages, the Src inhibitor, PP2, almost completely abrogated the secretion of IL-18 and LPS-primed secretion of IL-1β. In addition, the influenza A virus strain Beijing was sensitive to PP2-mediated blockage of inflammasome activation, showing that this phenomenon may by a general feature of influenza A viruses. The results emphasized the significant role for Src (and possibly to that of ROS) in the regulation of the pro-inflammatory cytokine response in influenza A virus-infected human primary macrophages. In line with this result, Raung *et al.*, reported that IL-1β secretion and viral propagation in response to neurotropic Japanese encephalitis virus, recognized also by RIG-I, was sensitive to Src inhibition (Raung et al., 2007). Moreover Dostert *et al.*, and Shio *et al.*, demonstrated that malarial hemozoin-induced inflammasome activation and secretion of IL-1β were partly dependent on ROS, as well as required the contribution of Lyn, another member of the Src tyrosine kinase family (Dostert et al., 2009; Shio et al., 2009). Importantly, in addition to inflammasome signalling, efficient RIG-I signalling involves gp91phox and Src, and under certain circumstances, increased ROS (Johnsen et al., 2009; Soucy-Faulkner et al., 2010; Tal et al., 2009). One should not forget that Src is also vital for TLR3 signalling (Johnsen et al., 2006). Moreover, other members of Src kinases have been suspected of playing physiologically important roles during hepatitis C virus replication or pathogenesis (Macdonald et al., 2004). The precise role of Src in host response to influenza A virus infection is worthy of more detailed characterization.

Interestingly, patients suffering from chronic granulomatous disease lack the core component of all NADPH oxidases, namely p22phox, but are still capable of secreting normal amounts of IL-1β in response to the inflammasome trigger (van Bruggen et al., 2010). However, primary cells extracted from these patients, treated with ROS scavengers or general flavoprotein inhibitors, and then stimulated with inflammasome agonists, secrete greatly reduced amounts of IL-1β. These results of van Bruggen *et al.*, suggest that

ROS are involved in inflammasome activation, but their source is unclear. Indeed, ROS are known to be involved in different forms of inflammasome activation *e.g.*, in response to ATP, particulate matter, and many microbes (Cruz et al., 2007; Dostert et al., 2008; Eisenbarth et al., 2008; Gross et al., 2009; Kankkunen et al., 2010; Nakahira et al., 2011; Shio et al., 2009; Zhou et al., 2009; Zhou et al., 2010).

Mitochondria are a possible origin of intracellular ROS in the response of the organelle to stress (Ott et al., 2007; Zhou et al., 2010). The high-throughput proteomic data of influenza A virus-infected macrophages revealed marked mitochondrial (and lysosomal) alterations and/or damage, as described in 5.3. Thus, mitochondrial ROS might contribute to the inflammasome activation seen in influenza A virus-infected macrophages. In line with this hypothesis, Allen *et al.,* reported that influenza A virus-induced inflammasome activation was sensitive to inhibitors blocking ROS production (Allen et al., 2009). The mechanism for influenza A virus-induced ROS production has also been recently associated to the function of proton-selective ion channel M2 of the influenza A virus (Ichinohe et al., 2010; Lazrak et al., 2009). Particularly, Ichinohe *et al.*, linked the expression of M2 to increased ROS production, K^+-efflux, and inflammasome activation (Ichinohe at al., 2010). Although the mechanisms for M2-induced inflammasome triggering and elevated intracellular concentrations of ROS and reduced K^+ levels were not elucidated, the localization of M2 in acidified Golgi-compartment was essential for the process to occur.

Not only do ROS detrimentally affect on mitochondria, they may also destabilize the lysosomal membranes and this would subsequently result in cathepsin leakage into cytoplasm and cathepsin-dependent inflammasome activation and apoptosis. Furthermore, increased ROS levels may trigger the inflammasome during virus infection by directly modifying crusial cellular components that then are recognized as DAMPs. Indeed, minimally oxidized LDLs and oxidized phospholipids produced during the progression of infection or cellular stress, might be sensed as danger signals (Duewell et al., 2010; Imai et al., 2008; Masters et al., 2010; Miller et al., 2005). These kinds of DAMPs could also be responsible for the influenza A virus-induced inflammasome activation and/amplification of pro-inflammatory responses observed in human primary macrophages. **Figure 16** presents the current model for inflammasome activation in response to microbial infection and physical damage.

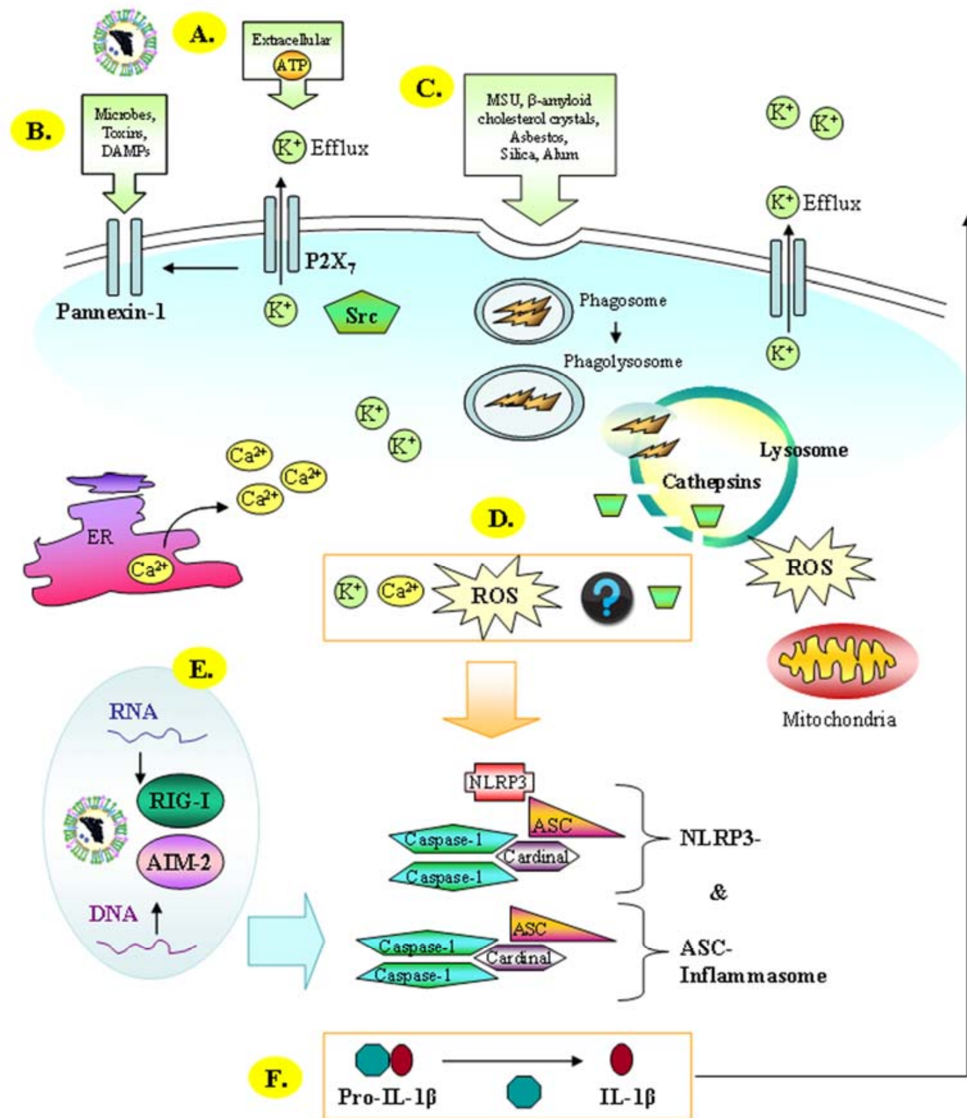


Figure 16 ***Prevailing model for inflammasome activation in response to numerous stimuli.*** *(A) Extracellular ATP released in response to danger or cell lysis binds to $P2X_7$ receptor. This results in K^+-efflux and activation of pore-forming pannexin-1 hemichannel. (B) The opening of pannexin-1 promotes entry of extracellular activators of the inflammasome inside the cell, as well as ATP- and K^+-efflux. (C) The phagocytosis of large particles, or other physical forces in cells during infection, or in the presence of damage, induces ROS production and lysosomal rupture followed by cathepsin leakage into cytoplasm. ER-stress releases also Ca^{2+}-cations into cytoplasm which might contribute to the creation of the conditions for inflammasome assembly. (D) Alterations in the equilibrium of general denominators for inflammasome activation, shown in the box, likely promote inflammasome activation. (E) Recognition of nucleic acids in the cytoplasm may trigger direcly NLRP3-independent or -dependent inflammasomes into action. (F) Caspase-1 launches a vigorous pro-inflammatory cytokine response and unconventional protein secretion. Potential DAMPs are released, which may provide conditions for inflammasome activation in neighbouring cells. The inflammation response becomes extended. Cell death may ensue and/or repair responses may be initiated in the resolution phase of the infection.*

Concluding Remarks

The presence of the molecular signature of viral infection, cytoplasmic RNA, triggers multiple cellular signalling pathways within the host. The immune response of the host, which viruses try to subvert and counteract in many ways, ultimately determines the outcome of infection. The main goal of this thesis was to elucidate the innate immune responses and complex host-virus interactions occurring during influenza A virus infection in an crucial effector cell type of innate immunity, human primary macrophages.

The involvement of membrane-bound TLRs and cytoplasmic PRRs in the elicitation of antiviral- and pro-inflammatory cytokines and the activation of apoptosis was characterized first. Recognition of extracellular dsRNA by TLR3 induced only a modest antiviral cytokine response, but resulted in intense expression of pro-IL-1β. The executioner of apoptosis, caspase-3, was not activated. This implied that phagocytosis of extracellular RNAs, originating from virus-infected cells, prepared uninfected cells for possible incoming danger without eliciting vigorous antiviral or pro-inflammatory cytokine responses or programmed cell death. In contrast, the hallmark of ongoing viral replication, the presence of viral RNA in the cytoplasm, efficiently induced type I and III IFNs, caspase-1 activation, and subsequent secretion of processed IL-18. Furthermore, caspase-3 and apoptosis were rapidly induced in response to recognition of cytoplasmic RNA, and these effects were linked to functions of RIG-I/MAVS at the molecular level. Thus, recognition of cytoplasmic RNA served as a marker of actual infection and it seemed to be a prerequisite for induction of efficient antiviral cytokine response, inflammasome activation, and apoptosis. Interestingly, IL-1β was not significantly secreted in response to cytoplasmic dsRNA stimulation or influenza A virus infection. Its abundant secretion occurred only if the TLR stimulus complemented the initially weak cytoplasmic RNA-induced pro-IL-1β expression first. The results suggested that an intense inflammatory response, which is potentially damaging to tissues, was engaged only after definite perception of severe infection, activating both endosomal as well as cytoplasmic PRRs in human primary macrophages. Overall, the results higlighted key roles for cytoplasmic PRRs during innate immune responses against viruses.

Inflammasome activation results often in secretion of its core components and of unconventionally secreted proteins that likely fine tune the inflammatory responses. In an attempt to characterize how the inflammasome responds to cytoplasmic dsRNA, a complete set of secreted proteins, secretomes, were analysed with high-throughput MS-based methods. Cytoplasmic dsRNA triggered robust protein secretion. Several lysosomal proteins, such as cathepsins, were identified, suggesting that lysosomal integrity had been impaired. Further characterization revealed that inflammasome activation preceded apoptosis, and that pharmacological inhibition of cathepsin B could abolish the secretion of IL-18 and halt the progression of apoptosis. Interestingly, cathepsin D was secreted simultanously with inflammasome components, and silencing of cathepsin D expression suppressed cytoplasmic dsRNA-induced inflammasome activation. These results implied that inflammasome activation in response to virus infection involved lysosomal

components, and that both cathepsin B and cathepsin D participated in this response. Importantly, EMCV- and VSV-induced inflammasome activation and apoptosis were also abolished by cathepsin B inhibition. As a whole, the results highlighted the importance of lysosomal proteases in orchestration of innate immune responses against RNA-viruses.

2DE-based proteomic analysis was performed to characterize alterations in subcellular proteomes of human primary macrophages during influenza A virus-infection. The virus induced marked changes in cytoplasmic and mitochondrial proteomes: IFN-inducible proteins and stress- and apoptosis-related ones were upregulated, and striking cytoskeletal rearrangements were observed. The constituents of cytoskeleton were caspase-3-dependently fragmented, and translocated from cytoplasm to mitochondria. Moreover, sequential translocation of intact and fragmented actin molecules from cytoplasm coincided with the ordered translocation of RLR signalling pathway members from cytoplasm into mitochondria. In addition, viral proteins accumulated to mitochondria during progression of infection, an evidence of their importance for their presence in mitochondria during infection. Interestingly, pharmacological breakdown of the actin network with cytochalasin D resulted in decreased IFNs expression, without any effect on the progression of caspase-3 activation or viral protein synthesis, suggesting that the antiviral cytokine response was dependent on intact actin cytoskeleton. The results implied close interaction of actin network with mitochondria in the regulation of antiviral responses during influenza A virus infection.

The host-virus-interactions were examined in more detail during influenza A virus-infection by conducting quantitative subcellular high-throughput proteome and secretome profiling. More than one thousand host proteins were differentially expressed and/or localized during infection in human primary macrophages. Several nuclear, lysosomal, and mitochondrial proteins were leaked or translocated into cytoplasm, and potential DAMPs were significantly secreted. In order to obtain a more comprehensive view on this robust host-virus interaction and on potential regulatory proteins for inflammasome activation, a protein-protein interaction network was constructed *via* bioinformatics from the identified intracellular and inflammation-related proteins of influenza A virus-infected cells. Functional studies were carried out, and the results suggested that cathepsins, $P2X_7$ receptor, and Src kinases participated in influenza A virus-induced inflammasome activation. Overall, the results demonstrated that multiple cellular pathways ensure activation of inflammasomes during influenza A virus infection.

The outcome of virus infection depends primarily on host-virus interactions. The functions of several PRRs and of individual influenza A virus proteins are rather well characterized. Furthermore, cellular responses to influenza A virus and other RNA viruses have been elucidated by genome-wide screening techniques, as well as 2DE- and more sophisticated MS-based proteomic methods in cell lines. However, few studies have globally adressed host-virus responses in human primary cells with modern high-throughput proteomic techniques, albeit these are an excellent way to understand the progression of viral infections and the related inflammatory responses in human hosts. This thesis strives to

meet these needs, and provides an interesting insight into inflammatory and antiviral innate immune responses during influenza A virus infection in human primary macrophages. In addition, the work presented here highlights the importance of macrophages in the development of immune responses of the host: infected cells secrete many proteins that undoubtedly contribute to antigen presentation, local inflammatory responses, as well as signalling the presence of danger to other parts of the body. Moreover, antigen presentation and cytokine secretion enhance the development of acquired immune responses, resulting in the clearance of pathogen and recovery from the infection. Future studies conducted in human primary cells will be necessary to delineate whether a common or rather specific protein secretion pattern is launched in response to distinct pathogens, virus families, and viruses within a virus species. Furthermore, an understanding of differences in proteomes and secretomes between infected individuals and possible identification of reliable biomarkers which can be associated with severe infection may be of significant clinical value. Perhaps in the future, secretome- and proteome profiling will become cost-effective techniques in diagnostics to be used to predict the outcome of infections and the choice of the appropriate antiviral treatments.

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