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# Activation of Innate Immune Responses by Toll-Like Receptors and Influenza Viruses

DEPARTMENT OF INFECTIOUS DISEASES
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DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
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# Activation of Innate Immune Responses by Toll-Like Receptors and Influenza Viruses

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## ACADEMIC DISSERTATION

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*To Samuel and Joosua*

## Abstract

Influenza viruses are human respiratory pathogens that cause seasonal epidemics and pandemics. The host restricts the virus infection by inducing immune responses aiming at virus clearance. The immune response has two arms. The innate immunity is the first line defense mechanism that is activated immediately after the recognition of the pathogen. The adaptive immunity, which consists of humoral and cell-mediated immunity, takes more time to develop. The epithelial cells of the respiratory tract and innate immune cells, such as macrophages and dendritic cells, are equipped with a plethora of receptors and signaling molecules that are designed for pathogen recognition. These receptors include Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). The pathogen recognition by these receptors leads to the activation of complex cellular signaling cascades that culminate in the production of cytokines, small proteins that mediate the communication between cells. In influenza infection, one important class of cytokines is interferons (IFNs) which induce the production of antiviral proteins that are able to inhibit virus infection. On the other hand, influenza viruses are capable of evading innate immune surveillance and there are differences between influenza virus types or strains in their immune evasion mechanisms.

In this thesis work influenza virus-induced IFN responses were studied in human macrophages and dendritic cells *in vitro*. Firstly, we showed that in macrophages influenza B virus infection induced a very early IFN-β and IFN-λ1 gene expression that coincided with the nuclear entry of the virus and the activation and nuclear import of IFN regulatory factor 3 (IRF3). This early activation did not take place in influenza A virus-infected cells. Furthermore, our study indicated that RIG-I receptor was essential for the early IFN gene expression. Secondly, we compared the cytokine responses induced by pandemic H1N1 influenza A virus to the ones induced by seasonal influenza A viruses in human macrophages and dendritic cells. We showed that the pandemic influenza A virus induced weak IFN responses but was highly sensitive to the antiviral actions of IFNs.

During the infection, different types of microbial structures are present and can be recognized by different cellular receptors. Another aim of this thesis was to elucidate the mechanism of receptor cooperation in inducing synergistic cytokine production. We confirmed the previous findings that TLR3 or TLR4 together with TLR7/8 induces synergistic interleukin (IL)-12 and IFN gene expression in human dendritic cells. We studied, which regulatory factors bound to IL-12 and IFN-λ1 gene promoters during a synergistic stimulation and which cell signaling pathways took part in the cytokine production. We conclude that at the transcriptional level, several different IRF proteins and cell signaling pathways cooperate in the

synergistic IL-12 and IFN-λ gene expression. In addition, we propose that IFNs produced after stimulation of the TLR3 pathway induce the expression of TLR7 receptor and other cell signaling components that create a positive feedback loop that further augments the cytokine and IFN production during synergistic stimulation.

This thesis discusses the host-pathogen interactions in the human system and clarifies the cell signaling pathways leading to synergistic cytokine gene expression. Moreover, the early events in influenza B virus infection and IFN responses induced by pandemic H1N1 influenza A virus are described. More detailed knowledge of the human innate immune responses induced by host-pathogen interactions is needed for the development of effective vaccines and antiviral treatments against influenza virus.

Keywords: innate immunity, influenza, interferon, cytokine, Toll-like receptor, macrophage, dendritic cell

## Tiivistelmä

Influenssavirukset aiheuttavat vuosittaisia epidemioita ja ajoittain ilmaantuvia pandemioita. Influenssavirusinfektio saa aikaan hengitystietulehduksen, joka voi johtaa vakavaan tautiin perussairailla, immuunipuutteisilla tai iäkkäillä henkilöillä. Ihmisen immuunipuolustuksen tehokas aktivoituminen virusinfektiossa johtaa yleensä taudin paranemiseen. Elimistön immuunipuolustus voidaan jakaa synnynnäiseen eli luontaiseen sekä hankittuun eli adaptiiviseen immuniteettiin. Luontainen immuniteetti käynnistyy nopeasti heti taudinaiheuttajan havaitsemisen jälkeen, kun taas adaptiivisen vasta-aine- ja soluvälitteisen imuniteetin käynnistyminen tapahtuu hitaammin. Hengitysteiden epiteelisolut ja luontaisen immuniteetin solut, kuten makrofagit ja dendriittisolut, tunnistavat mikrobirakenteita reseptoreillaan. Tunnistus johtaa solunsisäisten viestintäketjujen käynnistymiseen, luontaisen immuniteetin geenien luennan aktivoitumiseen ja solujen viestiaineiden, sytokiinien tuottoon. Sytokiinit ohjaavat immuunipuolustuksen toimintaa monin tavoin; influenssainfektiossa tärkeitä sytokiinejä ovat interferonit, jotka saavat aikaan useiden virusta rajoittavien proteiinien tuotannon. Toisaalta influenssavirus kykenee monin tavoin estämään elimistön immuunipuolustuksen käynnistymistä ja toimintaa.

Tässä työssä tutkittiin influenssavirusinfektion aikaansaamia interferonivasteita ihmisen immuunisolumallissa. Näytimme, että influenssa B-virus laukaisee hyvin nopean interferonivasteen, mikä tapahtuu selvästi influenssa A-viruksen aktivoimaa vastetta aikaisemmin. Ihmisen makrofageissa tämä aktivaatio tapahtuu jo ennen kuin influenssa B-virus alkaa monistua. Osoitimme solunsisäisen, viruksen RNA molekyylejä tunnistavan RIG-I-reseptorin olevan tärkeä aikaisen interferonivasteen käynnistymiselle. Toisessa työssä vertasimme interferonivasteita pandeemisen H1N1-alatyypin influenssa A-viruksen ja kausi-influenssavirusten kesken. Tutkimus osoitti, että pandeeminen virus laukaisi heikon sytokiinivasteen ihmisen immuunisoluissa, mutta solujen esikäsittely interferoneilla esti pandeemisen viruksen monistumista tehokkaasti.

Soluilla on useita erilaisia reseptoreita, jotka voivat tunnistaa eri mikrobirakenteita infektion aikana. Yksi tämän työn tavoitteista oli tutkia Tollin kaltaisten reseptoreiden (TLR) yhteistoimintaa ihmisen valkosolumalleilla. Tuloksemme vahvistivat muiden tutkijoiden aiemmat havainnot siitä, että solujen stimulointi TLR3 tai TLR4 reseptorin välityksellä samanaikaisesti TLR7/8 reseptorin kanssa saa aikaan voimakkaan interleukiini 12 ja interferoni λ1 tuotannon. Synergian mekanismia selvitettäessä havaittiin, että useiden eri transkriptiotekijöiden ja solunsisäisten viestintäketjujen samanaikainen aktivaatio selitti voimakasta sytokiinituotantoa soluissa. Havaitsimme myös, että TLR3:n välityksellä tapahtunut

interferonituotanto lisäsi TLR7:n ja TLR7-välitteisen viestintäketjun proteiinien tuotantoa, mikä voi osaltaan edesauttaa viestintäketjujen aktivaatiota ja voimistaa sytokiinituotantoa mikrobi-infektion tai TLR-stimulaation aikana.

Tämä väitöskirjatyö tarkastelee isäntäsolun ja taudinaiheuttajan välisiä vuorovaikutussuhteita ja valottaa synergistisen sytokiinituotannon mekanismeja ihmisen immuunisoluissa. Lisäksi tutkimus kuvaa influenssa B-virusinfektion aikaisia vaiheita ja pandeemisen H1N1 influenssa A-viruksen laukaisemia sytokiinivasteita. Tämän työn tarkoituksena on ollut osaltaan lisätä ihmisen luontaisen immuniteetin aktivoitumisen yksityiskohtaista tuntemusta, mikä on edellytys turvallisten ja tehokkaiden rokotteiden ja viruslääkkeiden kehittämiselle.

Avainsanat: luontainen immuniteetti, influenssavirus, interferoni, sytokiini, Tollin kaltainen reseptori, makrofagi, dendriittisolu

## Contents

## List of original papers

**I** **Mäkelä SM**, Östelund P, Westenius V, Latvala S, Diamond MS, Gale M Jr, Julkunen I. RIG-I signaling is essential for influenza B virus-induced rapid interferon gene expression. J. Virol. 2015; 89(23):12014-25.

**II** Österlund P, Pirhonen J, Ikonen N, Rönkkö E, Strengell M, **Mäkelä SM**, Broman M, Hamming OJ, Hartmann R, Ziegler T, Julkunen I. Pandemic H1N1 2009 influenza A virus induces weak cytokine responses in human macrophages and dendritic cells and is highly sensitive to the antiviral actions of interferons. J. Virol. 2010; 84(3):1414-22.

**III** **Mäkelä SM**, Strengell M, Pietilä TE, Österlund P, Julkunen I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. J. Leukoc. Biol. 2009; 85(4):664-72.

**IV** **Mäkelä SM**, Österlund P, Julkunen I. TLR ligands induce synergistic interferon-β and interferon-λ1 gene expression in human monocyte-derived dendritic cells. Mol. Immunol. 2011; 48(4):505-15.

## Candidate's independent contribution to this work

I SM, PÖ and IJ designed the study. SM performed most of the experiments and analyzed the results with some help from VW and SL. MD and MG provided knock-out mouse cells and helped with the writing of the manuscript. SM, PÖ and IJ wrote the manuscript.

II PÖ, JP, MS, TZ and IJ designed the study. PÖ, JP, MS, ER and SM performed the experiments and analyzed the results. NI, MB and ER analyzed virus sequences. AH and RH provided reagents. PÖ, JP, TZ and IJ wrote the paper.

III SM, MS, PÖ, TP and IJ designed the study. SM performed experiments and analyzed the results. SM and IJ wrote the manuscript.

IV SM, PÖ and IJ designed the study. SM performed most of the experiments and analyzed the results with help from PÖ. SM, PÖ and IJ wrote the manuscript.

## Abbreviations

| | |
|---|---|
| AIM | absent in melanoma |
| ALR | AIM2-like receptor |
| APC | allophycocyanin |
| AP-1 | activator protein 1 |
| ATF | activating transcription factor |
| ATP | adenosine triphosphate |
| BDCA | blood dendritic cell antigen |
| CARD | caspase recruitment domain |
| cDC | classical/conventional dendritic cell |
| cDNA | complementary DNA |
| cGAS | cyclic GMP-AMP synthase |
| CHX | cycloheximide |
| CLR | C-type lectin receptors |
| CPSF | cleavage and polyadenylation specific factor |
| CRM1 | cellular chromosome region maintenance protein 1 |
| cRNA | complementary ribonucleic acid |
| CSF | colony-stimulating-factor |
| Ct | comparative threshold |
| CTD | C-terminal domain |
| CTL | cytotoxic T lymphocyte |
| DAMP | danger-associated molecular pattern |
| DC | dendritic cell |
| DC-SIGN | dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin |
| Dectin-1 | DC-associated C-type lectin 1 |
| ds | double-stranded |
| EEA1 | early endosome antigen 1 |
| EGFR | epidermal growth factor receptor |
| ELISA | enzyme-linked immunosorbent assay |
| FC | flow cytometry |
| FCS | fetal calf serum |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| GP | glycoprotein |
| HA | hemagglutinin |
| HPAI | highly pathogenic avian influenza |
| IF | immunofluorescence |
| IFIT | IFN-induced protein with tetratricopeptide repeats |
| IFITM3 | IFN-inducible transmembrane protein 3 |
| IFN | interferon |
| IκB | inhibitor of nuclear factor κ-B |
| IKK | inhibitor of nuclear factor κ-B kinase |
| IL | interleukin |
| IRAK | IL-1 receptor-associated kinase |
| IRF | IFN regulatory factor |

| | |
|---|---|
| ISG | IFN-stimulated gene |
| ISGF3 | IFN-stimulated gene factor 3 |
| ISG15 | ubiquitin-like protein ISG15 |
| IU | international unit |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase |
| LAMP | lysosome-associated membrane glycoprotein |
| LGP2 | laboratory of genetics and physiology 2 protein |
| lncRNA | long noncoding RNA |
| LPAI | low pathogenic avian influenza |
| LPS | lipopolysaccharide |
| LRR | leucine-rich repeat |
| MAL | MyD88 adaptor-like protein |
| MAM | mitochondrial-associated membrane |
| MAPK | mitogen-activated protein kinase |
| MAVS | mitochondrial antiviral-signaling protein |
| MDA5 | melanoma differentiation-associated protein 5 |
| mDC | myeloid dendritic cell |
| MDP | monocyte/macrophage and dendritic cell progenitor cells |
| MEF | mouse embryonic fibroblast |
| MHC | major histocompatibility complex |
| moDC | monocyte-derived dendritic cell |
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| MxA | IFN-regulated resistance GTP-binding protein MxA (human) |
| Mx1 | myxovirus resistance protein 1 (mouse) |
| MyD88 | myeloid differentiation primary-response protein 88 |
| M1 | matrix protein |
| M2 | M2 proton channel protein |
| NA | neuraminidase |
| NEP | nuclear export protein |
| NF-κB | nuclear factor of κ light polypeptide gene enhancer in B-cells |
| NLK | NEMO-like protein kinase |
| NLR | NOD-like receptor |
| NLRC5 | NOD-like receptor family CARD domain containing 5 |
| NLRP3 | LRR- and pyrin domain-containing protein 3 |
| NLS | nuclear localization signal |
| NOD | nucleotide-binding oligomerization domain |
| NP | nucleoprotein |
| NS1 | non-structural protein 1 |
| OAS | 2′-5′-oligoadenylate synthase |
| PA | polymerase acidic protein |
| PAMP | pathogen-associated molecular pattern |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PB1 | polymerase basic protein 1 |

| | |
|---|---|
| PB2 | polymerase basic protein 2 |
| pDC | plasmacytoid dendritic cell |
| PDTC | pyrrolidine dithiocarbamate |
| pfu | plaque-forming unit |
| PI3K | phosphatidylinositol-3-kinase |
| PKR | protein kinase R |
| poly(I:C) | polyinosinic:polycytidylic acid |
| PRR | pattern recognition receptor |
| RIG-I | retinoic acid-inducible gene I protein (probable ATP-dependent RNA helicase DDX58) |
| RLR | RIG-I-like receptor |
| RNAseL | ribonuclease L |
| SARM | sterile alpha and HEAT/Armadillo motif protein |
| SDS | sodium dodecyl sulphate |
| siRNA | small interfering RNA |
| ssRNA | single-stranded RNA |
| STAT | signal transducer and activator of transcription |
| STING | stimulator of IFN genes |
| TAB | TAK1-binding protein |
| TAK | transforming growth factor-β-activated kinase 1 |
| TBK1 | TANK-binding kinase 1 |
| Th | T helper cell |
| TICAM1 | TIR-domain-containing molecule 1 |
| TIR | Toll/IL-1 receptor |
| TIRAP | TIR domain containing adaptor protein |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |
| TRAF | TNF receptor-associated factor |
| TRAM | TRIF-related adaptor molecule |
| TRIF | TIR domain-containing adaptor protein inducing IFN-β |
| TRIM | tripartite motif-containing protein |
| TYK2 | tyrosine kinase 2 |
| V-ATPase | vacuolar ATPase |
| VLP | virus like particle |
| vRNA | viral RNA |
| vRNP | viral ribonucleoprotein |
| vtRNA | vault RNA |
| WB | Western blotting |
| WT | wild type |

# 1 Introduction

Viruses were discovered more than 100 years ago. Since then it has become evident that viruses outnumber all cellular forms of life both in quantity and genetic diversity (Koonin and Dolja, 2013). Viruses are ancient obligate parasites that infect cells from all domains of life. They have greatly contributed to the evolution of humankind starting from the emergence of the first primordial cell (Forterre, 2006). Viruses are comprised of genetic material, RNA or DNA, a protein coat called a capsid and in some cases a host cell-derived lipid membrane, an envelope. Many viruses are human pathogens and thus studying the pathogenesis of viral infections and virus-host cell interactions is of great scientific and practical importance. On the other hand, the analysis of the biology of viral infections has taught us many aspects of cell biology. Presently, viruses are widely used as vaccines and potential gene therapy vectors as well as tools in biotechnology.

Viruses depend on the cellular machinery to complete their life cycle and hence the host-pathogen interplay builds up complex networks of interactions between the cellular and viral proteins. Viruses have evolved sophisticated means to manage with a minimal number of genes and yet turn the cell into a virus copy machine to produce thousands of infectious particles per cell. As this is often harmful to the host, host cells have also developed numerous means to fight against viral infection. These defense mechanisms are called the immune system which includes two arms, innate and adaptive immunity. The innate immune response is rapidly activated and rather unspecific reaction against an invading pathogen. The adaptive immunity takes a longer time to develop, but it is highly specific because of the antigen-specific humoral and cell-mediated immune responses. The activation of adaptive immunity is also able to generate immunological memory.

During the years that viruses were first described, the discovery of antibodies (reviewed in Llewelyn et al., 1992) initiated the studies on adaptive immunity. The topic of this thesis, innate immunity, was introduced later and the concept of pattern recognition receptors being able to distinguish self from non-self was presented by Charles Janeway in 1989 (Janeway, 1989). The first pattern recognition receptors were found in the drosophila fly (Lemaitre et al., 1996) and in human (Medzhitov et al., 1997) and since then a plethora of different receptors have been described that survey the extracellular and intracellular space of the cells for the potential microbial and other danger signals. The sensing of danger by the cell triggers the production and secretion of small messenger proteins, cytokines, which are essential in the communication between cells and in directing the cell movement and activation. Highly complex cellular signaling pathways carry the information of pathogen

recognition into the cell nucleus to initiate gene expression aiming at the production of proteins needed for the defense responses against microbial pathogens. The research conducted during the past 20 years has immensely increased our knowledge on innate immune signaling pathways activated by pathogen recognition receptors.

This thesis discusses the influenza virus-induced cytokine gene expression in human innate immune cells i.e. dendritic cells (DCs) and macrophages. The early events in influenza virus infection were analysed in the context of activation of cell signaling pathways leading to interferon (IFN) gene expression. Moreover, the activation of IFN induction by different influenza A virus strains was characterized. In addition, the cooperation of different pattern recognition receptors, namely Toll-like receptors (TLRs), in inducing cytokine expression was studied by analysing the cell signaling pathways required for the synergistic activation of cytokine gene expression.

# 2 Review of the literature

## 2.1 Influenza viruses

Influenza viruses are respiratory pathogens that cause seasonal epidemics and occasionally pandemics in the human population. World Health Organization estimates that influenza virus infection leads to the death of 250 000 - 500 000 humans every year, infecting 5 % - 10 % of the population (Keitel et al., 1997; Neuzil et al., 2002; Thompson et al., 2003; Thompson et al., 2009). During pandemics, a new type of influenza virus against which the human population has limited immunity, is able to spread from human to human, often causing significant morbidity. Previous influenza pandemics include the 1918 Spanish influenza, the 1957 Asian influenza, the 1968 Hong Kong influenza, and the 2009 (A) H1N1 swine influenza. Humankind constantly faces the fear of a new deadly influenza pandemic arising, as novel reassortant avian influenza viruses such as H5N1 and H7N9 are infecting people in the south-east Asia.

Presently, it is possible to fight pandemics with vaccines, which in the most recent 2009 H1N1 pandemics turned out to be successful. Vaccines against seasonal influenza are available and if the next year's circulating viruses match the vaccine strains well, the vaccines give effective protection against the infection. The problem with influenza A viruses is that they evolve rapidly; the high error rate of the viral RNA polymerase causes mutations in the virus leading to antigenic drift. In addition, the different influenza subtypes change gene segments with each other in

coinfection, giving rise to new reassortant viruses in a process called antigenic shift. As a result, the vaccine does not confer long-lasting immunity but needs to be taken yearly. Other possible treatments include antiviral drugs such as amantadine, rimantadine and neuraminidase inhibitors. The drawback with the drugs is that influenza viruses have developed resistance to these and thus novel ways to control influenza virus infection are needed.

Influenza viruses belong to the family *Orthomyxoviridae* and are classified into influenza A, B and C viruses. Wild aquatic birds are the natural host of influenza A viruses, but the viruses are able to infect many different mammals, including humans. Influenza A viruses are subtyped according to the antigenic properties of their surface glycoproteins; 18 different hemagglutinin (HA) and 11 neuraminidase (NA) types are presently known (Neumann and Kawaoka, 2015). Of these subtypes, H1-H16 and N1-N9 are found circulating in bird populations but only H1N1, H2N2 and H3N2 viruses have caused annual epidemics in humans so far. The influenza H17N10 (Tong et al., 2012) and H18N1 (Tong et al., 2013) viruses were recently discovered from bats. Influenza B viruses only infect humans and some marine mammals. Two distinct evolutionary lineages circulate in humans, called Victoria and Yamagata lineages. Influenza C viruses infect humans and pigs but are rare and thus less is known about them. Recently, a novel influenza virus was found in swine and cattle, resulting in the proposal to form a new influenza D virus genus (Collin et al., 2015; Sreenivasan et al., 2015).

### 2.1.1 Influenza virus structure

Influenza A and B viruses are pleomorphic enveloped viruses with a negative orientation single-stranded RNA (ssRNA) genome divided into eight segments. From these segments up to 16, or in the case of influenza B, 11 different proteins are encoded. The viral RNA segment forms a viral ribonucleoprotein (vRNP) complex (Fig.1) where nucleoprotein (NP) monomers are associated with the viral RNA and together form an antiparallel double helix structure (Eisfeld et al., 2015). The 5´and 3´ends of the RNA segment are partially complementary and form a panhandle structure which is bound by the viral polymerase complex consisting of single PB1, PB2 and PA proteins. The vRNP complexes are packaged into a virus particle that consists of matrix protein (M1) shell that is surrounded by the host-derived lipid membrane. The viral glycoproteins HA and NA are embedded in the membrane, as well as the ion channel protein M2 (Lamb and Lai, 1981). Influenza B virus has an additional ion channel NB. Influenza viruses also code a minor structural protein, nuclear export protein (NEP) (Yasuda et al., 1993) and a nonstructural protein NS1 that is not packaged in the viruses but is present in infected cells. NS1 is the best known multifunctional virulence factor of influenza virus and it is able to restrict IFN responses, for example (Krug, 2015). In addition, some influenza A virus

strains encode accessory proteins such as PB1-F2 (Chen et al., 2001; Conenello et al., 2007), PB1-N40 (Wise et al., 2009), PA-X (Jagger et al., 2012), PA-155, PA-182 (Muramoto et al., 2013) and PB2-S1 (Yamayoshi et al., 2015) that have been shown to regulate virus-host interactions or interfere with the defense systems of the host.

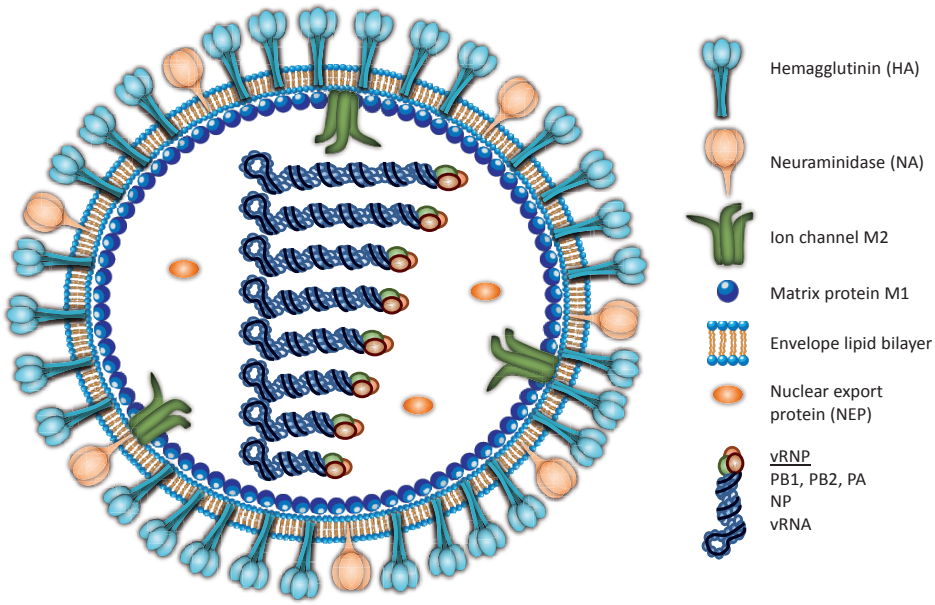


**Figure 1. Influenza A-virus structure.** (Adapted from Eisfeld et al., 2015; Wang and Palese, 2009). Influenza virus genome segments are organized into vRNP structures where the viral RNA (vRNA) is associated with multiple NP proteins and a polymerase complex comprising PB1, PB2 and PA proteins. These vRNPs are packaged in a virion that is build of M1 matrix protein, includes M2 ion channel proteins and nuclear export proteins (NEP) and is surrounded by a host cell-derived lipid envelope. Viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are embedded in the lipid bilayer.

### 2.1.2 Influenza virus entry and replication cycle

Influenza viruses spread via respiratory droplets, aerosols or surfaces contaminated with nasal secretions. Influenza viruses infect humans via the respiratory tract where the virus HA binds to sialic acid residues in the cell surface receptors of the airway epithelial cells. In addition to sialic acids, influenza viruses may utilize other receptors for binding or entry such as DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Londrigan et al., 2011; Wang et al., 2008), mannose binding lectins (Reading et al., 2000; Upham et al., 2010) and epidermal growth factor receptor (EGFR) (Eierhoff et al., 2010). Human influenza A viruses prefer glycoproteins with α2,6 -linked sialic acids which are found in the human upper respiratory tract (Shinya et al., 2006). Avian influenza A viruses instead bind to α2,3 sialic acid residues which are less abundant in the human respiratory tract

and found on the surface of type II pneumocytes residing at alveolar and bronchial level in the human lungs (Shinya et al., 2006). It is thought that this partially determines the species specificity of the influenza A viruses. Influenza viruses use the host cell machinery for all the steps in the entry process of the virus and several human proteins have been shown to be essential for the infection to take place (Konig et al., 2010).

After the adsorption of the virus particle to the cell surface, the viral particle is endocytosed via receptor-mediated endocytosis (Fig. 2) (Matlin et al., 1981). The best characterized route into the cell for influenza A virus is clathrin-mediated endocytosis (de Vries et al., 2011; Lakadamyali et al., 2004) but also macropinocytosis (de Vries et al., 2011) and clathrin- and caveolin-independent endocytosis (Sieczkarski and Whittaker, 2002) have been proposed. It has been shown that filamentous influenza viruses prefer macropinocytosis (Rossman et al., 2012). After the internalization of the virus, it travels via early and late endosomes towards the perinuclear space. The journey has been shown to involve three stages (Lakadamyali et al., 2003). First, an actin-dependent transport takes the endosome containing the virion to the cell periphery, then a dynein-directed movement is observed and finally a microtubule-dependent movement into the perinuclear space takes place. As the endosome matures, the pH decreases from 6.8-5.9 in early endosomes to 6.0-4.8 in late endosomes (Huotari and Helenius, 2011). The acidic environment induces conformational change in the HA (White and Wilson, 1987) and enables the fusion between the viral and endosomal membranes leading to the release of the nucleocapsid. The optimal pH for the fusion varies between different HA subtypes (Galloway et al., 2013) and the human isolates fuse in lower pH than the avian isolates even within the same subtype. The pH drop also opens up the viral M2 ion channels (Wharton et al., 1994) and the subsequent acidification of the virion results in the dispersion of the M1 matrix proteins (Fontana and Steven, 2013; Zhirnov, 1990) and vRNPs are released into the cytosol in the process called uncoating. Recently, the uncoating of the virion was shown to be dependent on the proteasome machinery of the cell (Banerjee et al., 2014). After uncoating, vRNPs are imported into the nucleus for viral replication and transcription to start. The nuclear import of the vRNPs relies on the nuclear pore complex and importin proteins which associate with the nuclear localization signal (NLS) of the NP proteins (Melen et al., 2003; O'Neill et al., 1995).

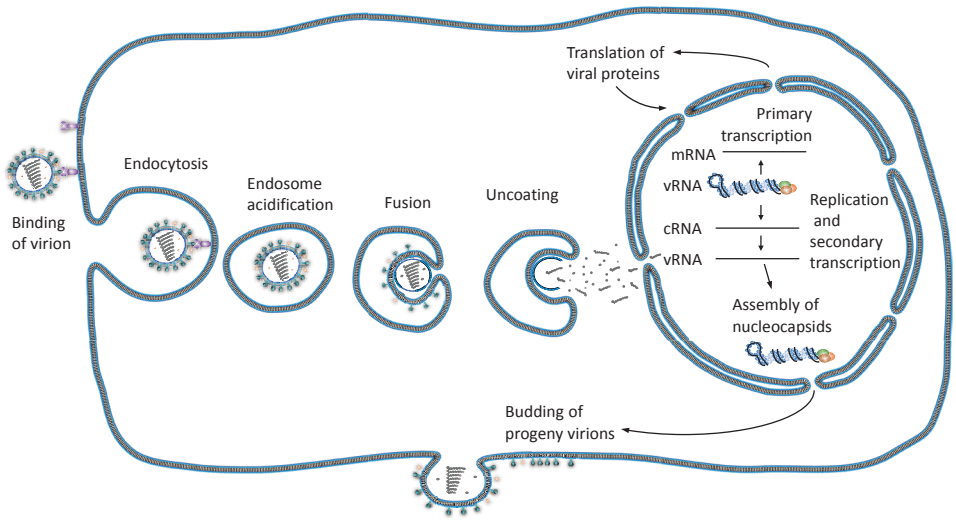


**Figure 2. Influenza virus entry and replication cycle.** (Adapted from Julkunen et al., 2001; Shi et al., 2014). Influenza virus binds sialic acid containing receptors of the cell and is endocytosed via receptor-mediated endocytosis. The acidification of the endosome prepares the virion for the fusion event, where the hemagglutinin facilitates the fusion between viral and endosomal membranes, releasing the nucleocapsid. The acidification also enables the dispersion of M1 protein and uncoating of the vRNPs. After nuclear entry of the vRNPs, the primary transcription takes place to transcribe viral mRNAs which are translated into viral proteins in the cytoplasm. Following nuclear import of newly synthesized viral proteins, viral replication and secondary transcription takes place and the assembled nucleocapsids are exported to the cell cytoplasm. The viral proteins and vRNPs gather to the plasma membrane for the budding of the progeny virions.

After the entry into the nucleus, viral polymerase proteins bound to the viral gene segments start the primary transcription of the viral mRNA (Jorba et al., 2009). The process does not require *de novo* protein synthesis (Mark et al., 1979), but is dependent on cellular mRNA synthesis; the virus steals the caps from the cellular pre-mRNAs to be used as primers by the viral polymerase complex in a process called cap-snatching (Plotch et al., 1979; Plotch et al., 1981). The primary transcription produces viral mRNAs which are then translated by the cellular ribosomes in the cytoplasm. Once newly synthesized polymerase proteins and NP have been transported into the nucleus, viral replication may take place (Vreede et al., 2004). First a full-length complementary RNA (cRNA) needs to be made to amplify the viral genomic vRNA, which is then assembled into progeny vRNPs. In the late phase of infection, the newly formed vRNPs are exported into the cytoplasm by cellular chromosome region maintenance protein 1 (CRM1)-dependent nuclear export process (Elton et al., 2001). Influenza viruses can also induce the enlargement of the nuclear pores that promotes the export of vRNPs (Muhlbauer et al., 2015). vRNPs are then transported to the plasma membrane via RAB11 recycling endosomes and microtubule networks (Amorim et al., 2011; Eisfeld et al., 2011; Momose et al., 2007; Momose et al., 2011). In addition to the vRNPs, the

viral proteins are also recruited to the plasma membrane and the budding of the progeny virions takes place (Noda and Kawaoka, 2010). The viral particle is released from the cell membrane once the NA cleaves the sialic acid residues of the host cell.

## 2.2 Innate immunity

The host defense against pathogens has been categorized into innate and adaptive immunity. Innate immunity is the omnipresent arm of the immunity which is unspecific and activated rapidly after recognition of danger. Innate immunity includes the physical barriers of skin and mucosa, the chemical barriers of different antimicrobial substances secreted on epithelial surfaces, the complement system and different innate immunity effector cells that are capable of recognizing and eliminating invading pathogens. The activation of innate immunity is a prerequisite for the activation of adaptive immunity which is activated rather slowly and comprises the immunological memory and specific antibodies against a certain pathogen. The antigen presenting cells of innate immunity, namely macrophages and DCs, are a crucial bridge between innate and adaptive immunity.

### 2.2.1 Macrophages and dendritic cells

Macrophages and DCs are mononuclear phagocytes that act as sentinels throughout the body. It was historically believed that both of these cell types can originate from a common precursor in the bone marrow and that monocytes are the link between the progenitor cells and tissue-resident macrophages and DCs (Mildner et al., 2013). It has now become increasingly evident that macrophages and DCs are a very versatile and heterogeneous group of cells with different origins. Just now, attempts are made to revise the classification of mononuclear phagocytes and unify the nomenclature (Guilliams et al., 2014; Guilliams and van de Laar, 2015; Tussiwand and Gautier, 2015; Wynn et al., 2013).

Resident macrophages in different tissues such as Langerhans cells in the skin, microglia in the brain, Kupffer cells in the liver, osteoclasts in the bone, alveolar macrophages etc., have great functional diversity and important roles in tissue repair, homeostasis and immunity. Many of these resident macrophages are self-renewable and originate from the yolk sac and/or fetal liver during embryogenesis (Gomez Perdiguero et al., 2015; Wynn et al., 2013). A third source of macrophages is the bone marrow-derived monocyte/macrophage and DC progenitor (MDP) cells that give rise to monocytes via common monocyte precursor (cMoP) (Hettinger et al., 2013). Macrophages have great phagocytosing potential and are able to engulf and degrade pathogens as well as cell debris. Thus especially the bone marrow-originated macrophages are able to differentiate into inflammatory macrophages,

also called M1 macrophages to distinguish them from deactivated M2 or healing macrophages (Italiani and Boraschi, 2014). Macrophages express a wide array of receptors aimed at recognition of pathogens discussed in the next section. Both alveolar and monocyte-derived macrophages express sialic acid receptors that bind influenza viruses (Yu et al., 2011). Whether these cell types support a productive influenza infection varies between different influenza strains and cell types (Short et al., 2012). Monocyte-derived macrophages but not alveolar macrophages are productively infected with seasonal influenza viruses (van Riel et al., 2011; Yu et al., 2011).

DCs originate from MDPs in the bone marrow via common DC precursor (CDP) (Naik et al., 2007; Onai et al., 2007) and pre-DC cells (Ginhoux et al., 2009; Liu et al., 2009). Pre-DCs give rise to classical DCs (cDCs), also called conventional or myeloid DCs (mDCs), and plasmacytoid DCs (pDCs) (Siegal et al., 1999). cDCs are the most important antigen presenting cells as they are highly phagocytic as immature cells and have a strong migratory and cytokine-producing capacity after maturation. cDCs that have encountered a pathogen, upregulate major histocompatibility complex (MHC) molecules and costimulatory receptors to become matured, and move to tissue-draining lymph nodes to present antigens to T cells. cDCs are categorized by their surface markers into cDC1 and cDC2 subtypes (Guilliams et al., 2014). Human cDC1 cells are blood DC antigen (BDCA) 3 positive and mouse cDC1 cells express CD103 or CD8, whereas human cDC2 cells are BDCA1 (CD1c) positive and mouse cDC2 cells express CD11b or CD4 (Guilliams et al., 2014; Mildner and Jung, 2014). These subsets are functionally distinct; cDC1 cells are superior in inducing Th1 type T cell responses whereas cDC2 cells more likely induce Th2 or Th17 T cell responses. However, based on surface markers, tens of different DC subtypes have been described, thus the great heterogeneity in the expression levels of different markers and different cell maturation stages complicates the definition of DC subsets. pDCs are specialized cells that are capable of producing high amounts of type I IFNs in response to viral infection (Nakano et al., 2001; Siegal et al., 1999). They are, however, less efficient as antigen presenting cells compared to other DC types. pDCs are found in the bone marrow and peripheral organs (Reizis et al., 2011). DCs are recruited to the lungs during influenza infection (Aldridge et al., 2009; McGill et al., 2008) and are essential for activation of innate immunity and subsequent T cell responses (Ballesteros-Tato et al., 2010; GeurtsvanKessel et al., 2008; Kim and Braciale, 2009). Monocyte-derived DCs (moDCs) express sialic acid receptors (Thitithanyanont et al., 2007) and are infected with seasonal and pandemic influenza viruses (Bender et al., 1998; Osterlund et al., 2005; Perrone et al., 2008), but only infections with H5N1 viruses are productive (Perrone et al., 2008; Thitithanyanont et al., 2007).

Monocytes circulate in the blood, express chemokine and adhesion receptors and are recruited in high numbers into inflamed tissues upon infection (Mildner et al., 2013). According to present views, monocytes are not just progenitor cells circulating in the blood, but are effector cells of their own, capable of reacting to pathogenic stimuli (Mildner et al., 2013). Monocytes can differentiate into macrophage- and DC-like cells under many inflammatory stimuli, and these populations are different from tissue-resident DC or macrophage populations (Geissmann et al., 2010; Italiani and Boraschi, 2014; Segura et al., 2013; Wynn et al., 2013). Because a limited number of human cDCs or macrophages are found in blood and tissues, monocytes have been widely used for research as they can be differentiated into monocyte-derived macrophages and moDCs using the growth factors colony-stimulating-factor (CSF)-1 (or granulocyte-macrophage (GM)-CSF) and GM-CSF + interleukin (IL)-4, respectively (Sallusto and Lanzavecchia, 1994). In mouse, similar cells are acquired from bone marrow with GM-CSF and these cells have been called bone marrow-derived DCs (BMDCs) (Inaba et al., 1992; Lutz et al., 1999). However, a recent study showed that this method produces a highly heterogeneous mix of macrophages and DCs, in terms of both surface markers and functionality (Helft et al., 2015). These results have questioned the use of BMDCs as a DC model and it may be a similar case for moDCs and macrophages in humans. However, as monocytes recruited into the site of inflammation can differentiate into macrophage- and DC-like cells (Cheong et al., 2010; Mildner et al., 2013; Serbina et al., 2003; Zigmond et al., 2012) *in vivo*, *in vitro*-differentiated cells can still be a relevant model at least for the studies concerning host-pathogen interactions. Nevertheless it is clear that the mononuclear phagocyte system is very complex; the cell populations are extremely diverse and dynamic and responsive to the cytokine milieu in the tissue.

### 2.2.2 Pathogen recognition by innate immunity

An important duty of innate immunity is to distinguish self from non-self in order to respond to danger signals appropriately. How this is accomplished has been an area of intense research in the field of innate immunity. Human cells have a multitude of receptors used for surveillance of extracellular and intracellular milieu for the presence of foreign microbial structures. These receptors have been named pattern recognition receptors (PRRs), and the structures they sense are termed pathogen associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs). Several classes of PRRs have been described: TLRs, C-type lectin receptors (CLRs), retinoic acid inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and absent in melanoma (AIM) 2-like receptors (ALRs) (Brubaker et al., 2015).

#### *2.2.2.1 Toll-like receptors (TLRs)*

TLRs are germline encoded transmembrane proteins that recognize various microbial structures in the extracellular space and in the endosomal pathway (Kawai

and Akira, 2010). The ligand binding domain consists of leucine-rich repeats (LRRs) and the cytoplasmic Toll IL-1 receptor (TIR) domain is responsible for signaling (Liu et al., 2008). To date 10 functional TLRs have been described in man. Mice also encode for three additional TLRs numbered from TRL11 to TLR13. TLR10 is not functional in mouse and TLR11, TLR12 and TLR13 are lost from the human genome. The representative ligands and localization of each TLR are presented in the Table 1. The localization of PRRs is an important aspect in controlling the fine balance between recognizing self from foreign pathogens without inducing unnecessary inflammation or even autoimmunity (Perkins and Vogel, 2015). In addition, receptors are localized so that they recognize relevant microbial patterns; receptors recognizing nucleic acid structures all reside in the intracellular or endosomal compartments of the cell thus recognizing viruses and intracellular pathogens, whereas many bacterial cell wall components are recognized by cell surface receptors.

TLR signaling is initiated when ligand binding induces TLR dimerization and the dimerized TIR-domains can recruit adaptor proteins (Fig. 3) (Narayanan and Park, 2015). Five adaptor proteins associated with TLRs are known, namely myeloid differentiation primary-response protein 88 (MyD88) (Medzhitov et al., 1998), TIR-domain-containing adaptor protein inducing IFN-β (TRIF) (Yamamoto et al., 2002) also known as TIR-domain-containing molecule 1 (TICAM1) (Oshiumi et al., 2003), MyD88 adaptor-like protein (MAL) (Fitzgerald et al., 2001) also known as TIR-domain-containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM) (Fitzgerald et al., 2003b) and inhibitory sterile α and HEAT/Armadillo motif containing protein (SARM) (O'Neill et al., 2003). The differential adaptor protein usage by different TLRs (see Table 1. and Fig. 1) leads to differential downstream activation events. TLRs that use TRIF adaptor, namely TLR3 and TLR4, induce both type I IFN and inflammatory cytokine responses whereas the cell surface TLRs employing MyD88 induce mainly inflammatory cytokines.

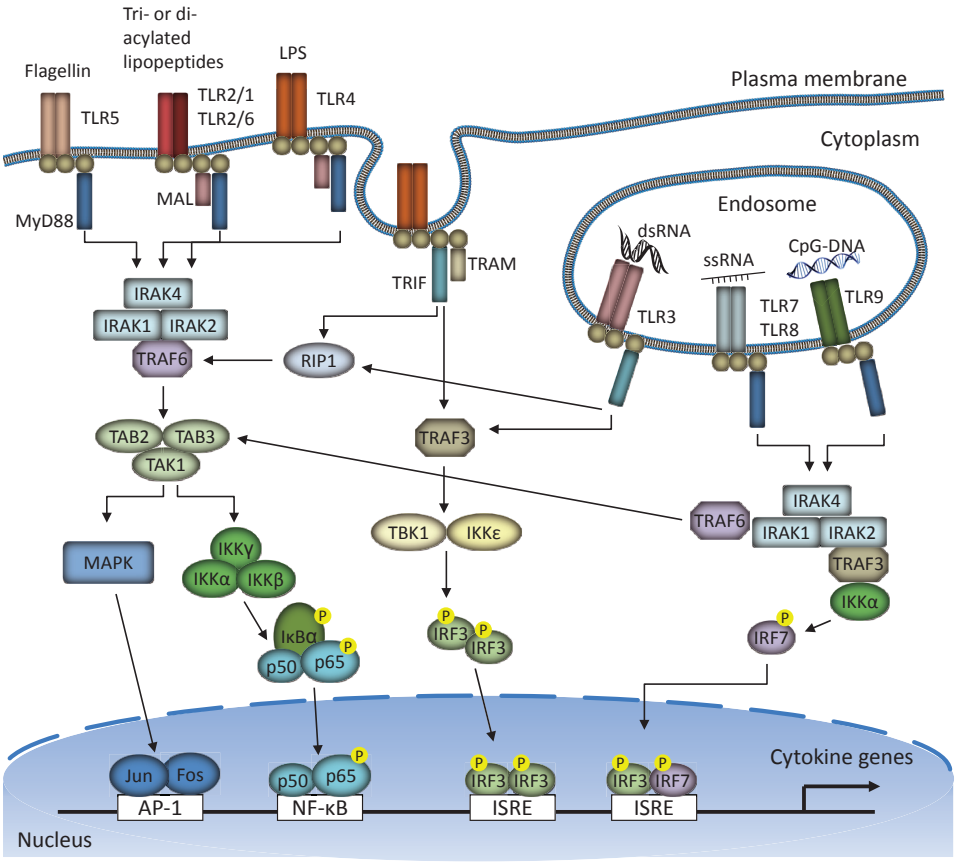


**Figure 3. TLR signaling pathways.** (Adapted from O'Neill et al., 2013). TLRs are transmembrane proteins expressed on the plasma membrane or in the endosomal membranes. TLRs recognize various microbe-derived ligands of which some prototypic ligands are indicated in the figure. The cytosolic domains of the receptors are associated with different adaptor proteins (MyD88, MAL, TRIF, TRAM) that are activated upon ligand binding. The activation triggers complex cellular signaling pathways leading to the activation of downstream kinases (IRAKs, TRAFs, MAPKs, IKKs) via different phosphorylation and ubiquitinylation events. These signaling pathways result in the activation of transcription factors such as IRFs, NF-κB (p50/p65) and Jun/Fos that are imported to the nucleus to bind their respective promoter elements in the cytokine genes thus inducing cytokine gene expression.

**Table 1. Toll-like receptors.**

| TLR | Localization (cell type) | Ligand | Origin of ligand | Adaptor | Reference |
|---|---|---|---|---|---|
| TLR1/2 | cell surface (monocytes, macrophages DCs, B cells) | Triacyl lipopeptides | Bacteria | MyD88, MAL | (Ozinsky et al., 2000; Takeuchi et al., 2002) |
| TLR2/6 | cell surface (monocytes, macrophages, mast cells, DCs, B cells) | Diacylated lipoprotein<br>Peptidoglycan<br>Lipoteichoic acid<br>Zymosan | Various pathogens<br>Gram+ bacteria<br><br>Fungi | MyD88, MAL | (Kang et al., 2009; Ozinsky et al., 2000) |
| TLR3 | Endosome (B-cells, T cells, natural killer cells, DCs)<br>Cell surface (fibroblasts) | dsRNA | Viruses | TRIF | (Alexopoulou et al., 2001) |
| TLR4 | Cell surface (monocytes, macrophages, DCs, mast cells, intestinal epithelium) | LPS<br><br>Fusion protein<br><br>Envelope protein | Gram- bacteria<br><br>Respiratory syncytial virus<br><br>Mouse mammary-tumor virus | MyD88, MAL, TRIF, TRAM | (Kurt-Jones et al., 2000; Medzhitov et al., 1997; Poltorak et al., 1998; Rassa et al., 2002) |
| TLR5 | Cell surface (monocytes, macrophages, DCs, intestinal epithelium) | Flagellin | Bacteria | MyD88 | (Hayashi et al., 2001; Smith et al., 2003) |
| TLR7 | Endosome (monocytes, macrophages, DCs, B cells) | Imidazoquinoline<br>Loxoribine<br>ssRNA | Synthetic<br>Synthetic<br>Viruses, bacteria | MyD88 | (Heil et al., 2003; Heil et al., 2004; Hemmi et al., 2002; Mancuso et al., 2009) |
| TLR8 | Endosome (monocytes, macrophages, DCs, mast cells) | Imidazoquinoline<br>ssRNA | Synthetic<br>Viruses, bacteria | MyD88 | (Eberle et al., 2009; Heil et al., 2004; Jurk et al., 2002) |

| | | | | | |
|---|---|---|---|---|---|
| TLR9 | Endosome (monocytes, macrophages, DCs, B cells, T cells) | Unmethylated CpG DNA | Bacteria, viruses | MyD88 | (Hemmi et al., 2000) |
| TLR10 | Endosome? (monocytes, macrophages DCs, B cells, T cells) | unknown | *Listeria monocytogenes* Influenza virus | MyD88 | (Chuang and Ulevitch, 2001; Lee et al., 2014; Regan et al., 2013) |
| TLR11* | Endosome (macrophages, DCs, kidney and bladder epithelial cells) | Profilin-like proteins Uropathogenic bacteria | *Toxoplasma gondii* | MyD88 | (Yarovinsky et al., 2005; Zhang et al., 2004) |
| TLR12* | Endosome (macrophages, DCs, B cells, T cells) | Profilin | *Toxoplasma gondii* | MyD88 | (Koblansky et al., 2013) |
| TLR13* | Endosome (macrophage, DCs) | 23S rRNA | Bacteria | MyD88 | (Li and Chen, 2012; Oldenburg et al., 2012) |

(Adapted from Akira and Takeda, 2004; Narayanan and Park, 2015).

* TLR11, TLR12 and TLR13 have only been found in the mouse and they are absent in the human system.

#### *2.2.2.2 RIG-I-like receptors (RLRs)*

RLRs are cytoplasmic sensors of RNA viruses expressed by nearly all cell types (Chan and Gack, 2015; Kell and Gale, 2015; Yoneyama et al., 2004; Yoneyama et al., 2015). RLR family comprises three RNA helicases: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RLRs bind RNA structures with the DExD/H-box domain and C-terminal domain (CTD) whereas signaling takes place via N-terminal caspase activation and recruitment domains (CARDs). As LGP2 lacks these signaling domains, it is thought to act as a regulator of RIG-I and MDA5 signaling (Komuro and Horvath, 2006; Rothenfusser et al., 2005; Saito et al., 2007; Satoh et al., 2010). The optimal ligand for RIG-I is believed to be short (<300 bp) dsRNA or panhandle RNA with 5´ triphosphate moiety (Baum et al., 2010; Hornung et al., 2006; Pichlmair et al., 2006; Schlee et al., 2009), although RIG-I has been shown to interact with RNA of various

lengths and end modifications (Goubau et al., 2014). MDA5, instead recognizes longer (>2000 bp) dsRNA molecules (Feng et al., 2012; Kato et al., 2008) and no known specific RNA modifications are needed for the recognition. RLRs have been shown to recognize many different types of RNA viruses (Errett et al., 2013; Gitlin et al., 2010; Kato et al., 2006; Loo et al., 2008) and are thus essential in inducing an antiviral state in response to virus infection.

#### *2.2.2.3 Other pattern recognition receptors (PRRs)*

NLRs are a large family of cytosolic receptors inducing inflammation, autophagy and cell death (Barbe et al., 2014). They comprise three functional domains: an N-terminal domain for signal transduction, a central domain for oligomerization and a C-terminal LRR domain for ligand binding. Upon activation NLRs form large protein complexes and especially the LRR- and pyrin domain-containing protein (NLRP) subgroup receptors scaffold into multiprotein complexes termed inflammasomes (Martinon et al., 2002). The inflammasome activates caspases, proteases that are important mediators of apoptosis and necrosis. The activation of caspase-1 leads to the proteolytic cleavage of proIL-1β and proIL-18, secretion of mature IL-1β and IL-18, and subsequently to a special type of cell death, pyroptosis (Bergsbaken et al., 2009). NLRs recognize a vast array of bacterial ligands and host cell-derived danger-associated molecules (Schroder and Tschopp, 2010). NLR called nucleotide oligomerization and binding domain (NOD2) receptor also has a role in viral infections (Lupfer et al., 2014; Sabbah et al., 2009).

ALRs are the most recently established class of PRRs and they act as cytosolic DNA sensors (Paludan and Bowie, 2013). The founding member AIM2 (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009) recognizes dsDNA, activates the inflammasome and induces IL-1β and IL-18 production. In addition to ALRs, several putative cytosolic DNA sensors have been described (Unterholzner, 2013). The primary DNA sensing receptor in the cytosol is cyclic GMP-AMP synthase (cGAS) (Li et al., 2013; Sun et al., 2013) and its activation leads to IFN expression via adaptor protein stimulator of IFN genes (STING) (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Sun et al., 2009; Zhong et al., 2008). STING is also a receptor for cyclic dinucleotides that are bacterial second messenger molecules (Burdette et al., 2011). Normally, DNA is contained in the nucleus but can be found in the cytosol as a result of microbial infection, aberrant host DNA metabolism or uptake of apoptotic cellular debris.

CLRs are best known for their role in anti-fungal immunity and they recognize carbohydrates, such as β-glucan, which is present in the fungal cell wall (Dambuza and Brown, 2015). The most studied CLR is DC-associated C-type lectin (Dectin)-1 (Brown and Gordon, 2001) which has a role in phagocytosis, autophagy, respiratory burst and production of inflammatory lipids, cytokines, chemokines and IFNs

(Dambuza and Brown, 2015). In addition to anti-fungal immunity, CLRs take part in recognition of dead cells, tumors and bacteria, especially mycobacteria. It is suggested, that they also regulate homeostasis, autoimmunity and allergy.

### 2.2.3 Cytokines

Cytokines are pleiotropic signaling molecules that are vital in cell communication. They facilitate many different immunity-related functions, such as cell differentiation, cell trafficking, antigen presentation and immune cell activation. Cytokines act in an autocrine or paracrine fashion via receptors recognizing cytokines in the same or in a neighboring cell, respectively. In some instances, cytokines may have systemic effects. Many different cell types secrete cytokines and express cytokine receptors on the cell surface. Cytokines include interferons (IFNs), interleukins (ILs), chemokines, growth factors, tumor necrosis factor (TNF) family members and adipokines. Two features define cytokines: pleiotropy, meaning that one cytokine elicits diverse functionality and redundancy, meaning that different cytokines exert overlapping activities (Ozaki and Leonard, 2002). Cytokine responses can be synergistic if two simultaneous signals induce a stronger cytokine response than two individual stimulators. One signal can also act antagonistically by reducing the cytokine response induced by another signal.

IL-12 is a heterodimeric proinflammatory cytokine that comprises p35 and p40 subunits and signals via IL-12 receptor which is composed of IL-12Rβ1 and IL-12Rβ2 subunits (Presky et al., 1996). IL-12 signaling leads to the activation of Janus-activated kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) which phosphorylate transcription factors signal transducer and activator of transcription 1 (STAT1), STAT3, STAT4 and STAT5 (Trinchieri, 2003). IL-12 produced by DCs and macrophages promotes the differentiation of naïve CD4 T cells into T helper 1 (Th1) cells (Macatonia et al., 1995; Manetti et al., 1993), linking the innate and adaptive immunity. Many microbial products induce the expression of IL-12p35 and IL-12p40 subunits which both are needed to produce bioactive IL-12p70 (Goriely et al., 2008). IL-12p35 expression is tightly controlled and it is a limiting factor in IL-12p70 production whereas IL-12p40 is secreted in excess over IL-12p35 and also as a homodimer (Goriely et al., 2008). Several studies have reported a synergistic IL-12 production in response to TLR stimulation via different TLRs (Bohnenkamp et al., 2007; Gautier et al., 2005; Napolitani et al., 2005). Such synergism offers a way to control proinflammatory cytokine production; only when multiple TLRs or other PRRs are triggered, high amounts of cytokines are produced.

### 2.2.4 Interferons

IFN was the first cytokine described in 1957 as it was found to interfere with the influenza virus infection (Isaacs and Lindenmann, 1957). Since then a great deal of

research into the importance of IFNs has been carried out and three classes of IFNs have been described. Type I IFNs include 13 different functional subtypes of IFN-α and IFN-β, IFN-ω, IFN-ε and IFN-κ; IFN-γ is the only member of type II IFNs and type III IFNs consists of IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4 (McNab et al., 2015; Prokunina-Olsson et al., 2013). IFNs induce the expression of hundreds of IFN-stimulated genes (ISGs) that are essential in inducing an antiviral state in IFN-stimulated cells. In addition to IFNs' direct antiviral effects, IFNs regulate many important aspects in innate immunity against viral infection such as the expression of PRRs, apoptosis, cytokine production, cell differentiation, polarization and proliferation.

The receptor that binds type I IFNs is composed of IFNAR1 and IFNAR2 chains (Domanski and Colamonici, 1996; Novick et al., 1994) that are ubiquitously expressed in tissues. Similarly, IFN-γ has a heterodimeric receptor IFNGR (Pestka et al., 1997), which is broadly expressed. The type III IFN receptor is expressed mainly in epithelial cells and consists of IFNLR1 and IL10R2 (Kotenko et al., 2003; Sheppard et al., 2003). The effects of IFNs are mediated via JAK1 and TYK2, whose interaction leads to the phosphorylation of STAT1 and STAT2 proteins. STAT1 and STAT2 form a dimer that recruits IFN regulatory factor 9 (IRF9) to make up the IFN-stimulated gene factor 3 (ISGF3) complex that can bind the promoter regions of ISG (Darnell et al., 1994; Qureshi et al., 1995).

Type I and III IFNs are expressed once the cells sense the presence of a pathogen with their PRRs. Both bacterial and viral ligands are able to induce type I and III IFN responses and the two types of IFNs share the transcription factors needed for their gene expression (Decker et al., 2005; Osterlund et al., 2005; Osterlund et al., 2007; Pietila et al., 2010). Type I IFNs induce DC maturation, enhance antigen presentation and increase the ability of DCs to prime T cells (Ito et al., 2001; Le Bon et al., 2003; Montoya et al., 2002). Type I IFNs have many effects on T cell proliferation, survival and effector cell differentiation (Havenar-Daughton et al., 2006; Kolumam et al., 2005; Le Bon et al., 2006; Marshall et al., 2010). The outcome, however, seems to depend on the timing of IFNAR and T cell receptor activation which determines which STAT proteins are activated (Crouse et al., 2015). IFN-λs share many biological functions with type I IFNs; although, as the receptor of IFN-λ is expressed mainly in epithelial cells, the antiviral actions of IFN-λs may vary depending on the site of infection (Iversen and Paludan, 2010).

### 2.2.5 Signaling pathways controlling cytokine gene expression

The activation of innate immunity is controlled tightly, because an immune response improperly adjusted may have a detrimental impact on the host. Cytokine production is mainly regulated on the transcriptional level by several transcription factor

families that bind to their respective promoter elements in the cytokine genes. The well-known signaling cascades leading to cytokine gene expression are the IRF, nuclear factor of κ light polypeptide gene enhancer in B-cells (NF-κB) and mitogen-activated protein kinase (MAPK)-pathways.

The MyD88 adaptor-dependent pathway is activated by all TLRs except TLR3. Upon TLR activation, MyD88 interacts with IL-1 receptor-associated kinase 1 (IRAK1), IRAK2 and IRAK4 forming a macromolecular complex where IRAK4 can phosphorylate IRAK1 and IRAK2 (Fig. 3) (Kawagoe et al., 2008; Lin et al., 2010; Motshwene et al., 2009). TNF receptor-associated factor 6 (TRAF6) is then activated, K63-polyubiquitinated (Deng et al., 2000) and binds TAK1-binding protein 1 (TAB1), TAB2 and TAB3 which leads to transforming growth factor-β-activated kinase 1 (TAK1) recruitment and activation. TAK1 can phosphorylate inhibitor of NF-κB kinase β (IKKβ), which then phosphorylates inhibitor of NF-κB α (IκBα) (Wang et al., 2001). The proteasomal degradation following K48-polyubiquitination of IκBα leads to the release of NF-κB components (p50/p65), which can then translocate to the nucleus to activate genes bearing NF-κB binding sites. TAK1 can also activate MAPK kinase 4/7 (MKK4/7), MKK3/6 (Wang et al., 2001) and MKK1/2, which can activate Jun N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK) 1 and ERK2, respectively. The MAPK signaling cascades eventually activate activating protein (AP)-1 transcription factor composed of dimers formed by Jun, Fos or activating transcription factor (ATF) subunits. The activation of NF-κB and AP-1 leads to the production of cytokines such as IL-12, IL-6 and TNF-α.

TLR3 and TLR4 use the TRIF adaptor protein to activate a MyD88-independent pathway (Fitzgerald et al., 2003b). TRIF is able to activate NF-κB and MAPK pathways via TRAF6 or TRAF6/RIP1 leading to the activation of downstream kinases as in the MyD88-dependent pathway (Fig. 3). The TRIF pathway also leads to the activation of an important transcription factor IRF3, which induces IFN gene expression. This happens through the activation of TRAF3, which K63-ubiquitinates TANK-binding kinase 1 (TBK1) and IκB kinase ε (IKKε), which subsequently phosphorylate IRF3 (Fitzgerald et al., 2003a; Hacker et al., 2006; Hemmi et al., 2004; McWhirter et al., 2004). The phosphorylated IRF3 forms dimers that translocate into the nucleus to bind IFN-stimulated response element (ISRE) sites in IFN gene promoters (Lin et al., 1998).

The cytoplasmic RLRs signal via CARD domains that interact with the CARD of the mitochondrial antiviral-signaling protein (MAVS) adaptor protein (Fig. 4), also known as IPS-1, VISA or Cardif (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). In steady state, the CARD domains of RIG-I are folded over one another to block signaling (Kowalinski et al., 2011). Upon biding of its RNA

ligands RIG-I undergoes an ATP-dependent conformational change rendering the CARD domains available for signaling. CARDs are then dephosphorylated (Wies et al., 2013) and CTD is ubiquitinylated by Riplet (Oshiumi et al., 2013). Tripartite motif-containing protein 25 (TRIM25) ubiquitin ligase adds ubiquitin to the CARDs in RIG-I which leads to RIG-I oligomerization and MAVS binding (Gack et al., 2007; Jiang et al., 2012) in the mitochondrial associated membrane (MAM). As a result, TBK1 and IKKε are activated, leading to the activation of IRF3 and NF-κB (Seth et al., 2005; Xu et al., 2005) which are translocated into the nucleus to drive the gene expression of IFNs and pro-inflammatory cytokine genes. RIG-I regulation has been studied extensively and many levels of regulation have been described (Chan and Gack, 2015; Errett and Gale, 2015; Kell and Gale, 2015), such as post-transcriptional mechanisms including alternative splicing of RIG-I (Gack et al., 2008) or MAVS mRNA (Brubaker et al., 2014; Lad et al., 2008) and microRNA-mediated negative feedback regulation of signaling pathway components (Hou et al., 2009). MDA5 signals similarly via MAVS and TBK1/IKKε but the details are still unclear.

#### *2.2.5.1 IRFs*

Currently, nine proteins are known in the mammalian IRF family of transcription factors (Ikushima et al., 2013). The most important transcription factors in RLR signaling and TRIF-dependent signaling regulating IFN gene expression are IRF3 and IRF7. IRF3 is constitutively expressed (Au et al., 1995). TBK1-mediated phosphorylation of the serine residues in the auto-inhibitory region of IRF3 allows it to dimerize and translocate into the nucleus (Fitzgerald et al., 2003a; Lin et al., 1998). IRF7 expression level, on the other hand, is low in most cells but strongly inducible with type I IFNs. IRF7 thus contributes to the positive feedback loop of IFN signaling (Sato et al., 2000a). IRF7 also undergoes a phosphorylation event in order to be imported into the nucleus (Marie et al., 2000). Moreover, IRF3 and IRF7 can form heterodimers that bind ISRE sites in the gene promoters (Au et al., 2001). In pDCs, which express IRF7 constitutively, MyD88-dependent signaling also leads to IRF7 activation via TLR7 and TLR9 (Honda et al., 2005a; Honda et al., 2005b; Kerkmann et al., 2003).

The first IRF discovered was IRF1 which is involved in IL-12 gene expression in cooperation with IRF8 (Liu et al., 2003; Liu et al., 2004; Maruyama et al., 2003; Masumi et al., 2002). The role of IRF8 in the development of mononuclear cells has been extensively studied (Tamura et al., 2015). In addition, IRF8 regulates IFN response as IRF8 is needed for the late phase IFN gene expression in mouse DCs (Tailor et al., 2007). Recently, IRF8 was shown to cooperate with STAT1 in inducing the activation of many IFN-β-activated genes (Mancino et al., 2015). Several studies have indicated that IRF5 is important in IFN induction (Barnes et al., 2001; Barnes et al., 2003; Lazear et al., 2013; Schoenemeyer et al., 2005; Yanai et al., 2007). IRF5 also takes part in the induction of proinflammatory cytokine genes

via MyD88-dependent signaling pathway (Takaoka et al., 2005). IRF4 can compete with IRF5 in binding MyD88 (Negishi et al., 2005) and is thus considered a negative regulator of TLR signaling. IRF9 is best known as a subunit of ISGF3, which it forms together with STAT1 and STAT2.

#### *2.2.5.2 NF-κB*

The NF-κB transcription factor family consists of subunits p65 (RelA), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2) which form hetero- or homodimers (Brady and Bowie, 2014). The inhibitory IκB proteins bind the dimers formed by different NF-κB subunits and the activation of NF-κB takes place when IκB is degraded by the proteasome. PRR ligands or proinflammatory cytokines activate the classical (canonical) NF-κB pathway where the IKK complex phosphorylates p65 and IκBα (Zandi et al., 1997) leading to degradation of IκBα and release of p65 or c-Rel -containing dimers. The alternative (non-canonical) NF-κB pathway is activated by TNF family members other than TNF-α, leading to IKKα-mediated phosphorylation of p100, the precursor for p52 (Senftleben et al., 2001). Proteolytic maturation of p52 allows it to form a dimer with RelB and to translocate into the nucleus. The maturation of p105 into p50 takes place constitutively (Fan and Maniatis, 1991).

#### *2.2.5.3 MAP kinases*

The MAPK signaling cascade comprises many sequentially activated kinases; a MAPK kinase kinase activates MAPK kinase (MKK), which then phosphorylates MAPK (Arthur and Ley, 2013). In mammalian cells 14 different MAPKs are known: six ERK-, four p38- and three JNK-family proteins and one NEMO-like protein kinase (NLK). At present, kinases downstream of MAPKs have also been described. The MAPK pathway is very complex and its different branches have both pro-inflammatory and anti-inflammatory (negative regulation) functions in immunity. Often MAPK-regulated pathways are cell type specific.

## 2.3 Influenza virus and the host

Influenza viruses enter the host via the respiratory tract and are first encountered by the mucus covering the epithelium. The primary target cells of influenza viruses are epithelial cells in the respiratory tract. In addition, influenza viruses infect immune cells, such as macrophages and DCs, which reside underneath the epithelial layer. IFNs (Cella et al., 1999; Ronni et al., 1995), proinflammatory cytokines (Chan et al., 2005) and chemokines (Matikainen et al., 2000; Veckman et al., 2006) are produced by these cells in response to the infection, and both local and systemic inflammatory responses are activated. Chemokines produced by epithelial cells and immune cells recruit neutrophils, monocytes and natural killer (NK) cells to the infection site to phagocytose and destroy virus-infected cells (Herold et al., 2006; Perrone et al., 2008). Antigen presenting cells take up viral antigens and migrate to the lymph

nodes to present them to T cells, thus inducing the activation of adaptive immunity against influenza.

The outcome of the influenza virus infection in the host depends on many factors. On the host side underlying diseases, age, pregnancy, host genetic background and previous influenza infections affect the severity of the infection. On the other hand, some influenza strains are more virulent than others and the case fatality rate of influenza viruses differs from less than 0.1 % up to 60 %. The reasons for this are largely unknown, but for example virulence factors, properties of the viral polymerase or glycoproteins and host specificity of the virus may play a role.

### 2.3.1 Innate immune recognition of influenza virus

RLRs play a crucial role in RNA virus recognition as mice deficient in this pathway are highly susceptible to infections with various RNA viruses (Kato et al., 2006). RIG-I, which is expressed by nearly all cell types, is the most important receptor inducing IFN gene expression during influenza virus infection (Fig. 4). Viruses have developed many means to counteract RIG-I which imply that it must be a central player in the innate immunity against viruses. It has been proposed that in vitro transcribed (Hornung et al., 2006) and influenza virus replication-derived (Pichlmair et al., 2006) 5´-triphosphate ssRNAs are ligands for RIG-I. However, it is now thought that a short stretch of dsRNA is essential for RIG-I recognition (Schlee et al., 2009) and such a dsRNA structure, termed a panhandle is present in the influenza virus vRNPs due to complementarity of 5´ and 3´ termini of the influenza virus genome segments. Recently, it has been shown that RIG-I readily recognizes the panhandle structure present in the incoming virus (Weber et al., 2015). In addition, 5´-triphosphate bearing defective interfering particles generated during influenza infection are efficient ligands for RIG-I (Baum et al., 2010; Tapia et al., 2013). Influenza virus activates caspase-1 and caspase-3 which leads to the secretion of IL-18 (Pirhonen et al., 1999; Pirhonen et al., 2001; Rintahaka et al., 2008) via RIG-I, and in primary lung epithelial cells RIG-I is the key factor inducing IL-1β (Pothlichet et al., 2013) thus giving rise to a term RIG-I-inflammasome (Chen and Ichinohe, 2015).

Studies using mouse embryonic fibroblasts (MEFs) deficient in MDA5 protein have shown that MDA5 receptor is dispensable for influenza-induced IFN responses (Kato et al., 2006; Loo et al., 2008). In contrast, chicken cells which lack RIG-I (Barber et al., 2010; Zou et al., 2009), can sense avian influenza viruses with MDA5 receptor (Liniger et al., 2012). A recent report using an *in vivo* RNAi screening revealed a contributing role for MDA5 in the *in vivo* response to influenza A virus lacking NS1 (Benitez et al., 2015); MDA5 was important in amplifying the host antiviral response even though it did not contribute to type I IFN induction. In

addition, a biased acute inflammatory response in the lungs of MDA5 deficient mice has been observed (Kim et al., 2014). The influenza virus-specific ligands that are recognized by MDA5 are not known as it is thought that dsRNA is not generated during negative sense ssRNA virus replication (Weber et al., 2006).

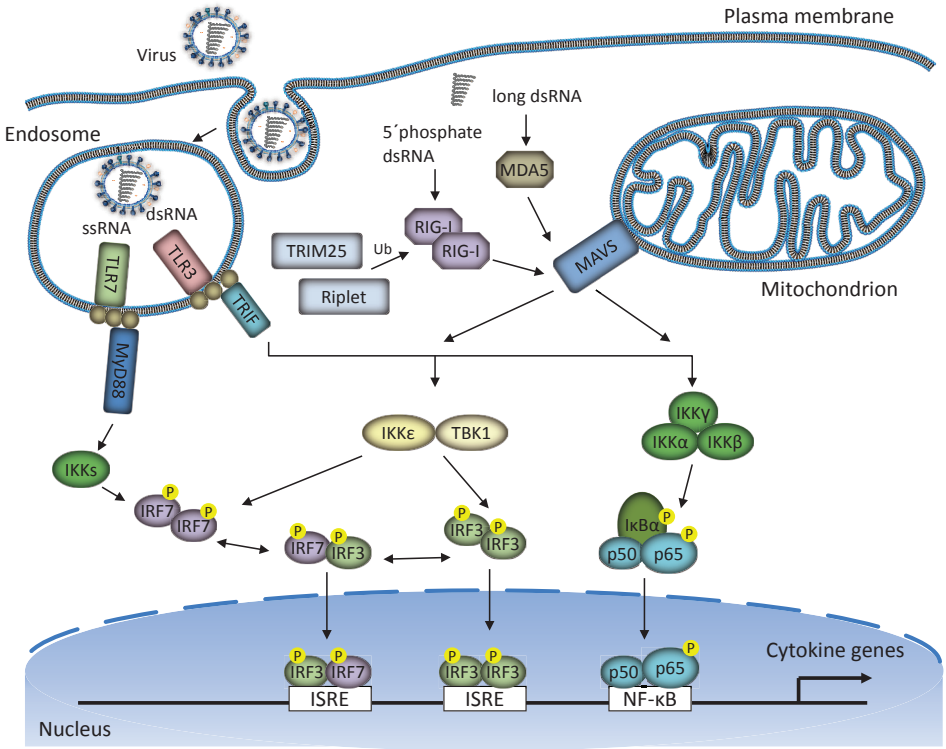


**Figure 4. Virus-induced signaling pathways.** (Adapted from Jiang et al., 2015). RNA viruses are recognized by endosomal PRRs TLR3 and TLR7/8 which bind dsRNA and ssRNA, respectively. For more detailed TLR signaling see Fig. 3. The cytosolic RNA receptors include RIG-I which is triggered by 5´phosphate containing dsRNA and MDA5 which binds longer dsRNA molecules. The receptors activate the downstream kinases (IKKs, TBK1) via adaptor proteins (MyD88, TRIF, MAVS) leading to the phosphorylation and activation of IRF- and NF-κB-family transcription factors. The transcription factors are subsequently imported into the nucleus and bind the promoter elements in the cytokine genes to induce gene expression.

Endosomal TLR7 and TLR8 recognize ssRNA structures and are potent PRRs in the recognition of influenza virus. However, during a successful influenza virus entry, virions are likely remaining intact in the endosomal compartments and thus inaccessible for TLR ligand binding. If some viruses are not able to escape the endosomal pathway before lysosomal degradation, TLR recognition may happen. It is possible that NP and polymerase proteins always associated with the ssRNA genome of influenza, interfere with TLR ligand binding. During phagocytosis of influenza-infected cells, pDCs have been shown to recognize the ssRNA genome of

the virus via TLR7 (Diebold et al., 2004; Lund et al., 2004). In addition, TLR7 and pDCs are essential for whole inactivated influenza virus vaccine-induced cytotoxic T lymphocyte (CTL) responses (Budimir et al., 2013). Much of the knowledge on TLR recognition has been acquired from knock-out mouse studies. It was earlier believed that TLR8 is not functional in mice and thus the function of TLR8 in influenza virus recognition is still unclear. TLR3 recognizes dsRNA, which is not generated during influenza virus infection, because of the helicase UAP56 (Wisskirchen et al., 2011). TLR3 deficient mice sustain higher viral loads and reduced leukocyte infiltrations in the lungs which indicate that TLR3 does, however play a role in influenza recognition (Le Goffic et al., 2006). Intriguingly, although its ligand is still unknown, TLR10 was involved in influenza virus-induced innate immune responses in human macrophages (Lee et al., 2014).

Several studies have demonstrated the importance of NLR protein NLRP3 in influenza infection (Allen et al., 2009; Ichinohe et al., 2009; Thomas et al., 2009). NLRP3-inflammasome activation led to caspase-1 activation and IL-1β production in influenza virus-infected cells. Viral RNA (Thomas et al., 2009), the nonstructural protein PB1-F2 (McAuley et al., 2013) and M2 ion channel protein (Ichinohe et al., 2010) are involved in NLRP3-inflammasome activation. A recent paper suggested that another NLR protein NLRC5 played a role in influenza infection by cooperating with RIG-I receptor (Ranjan et al., 2015) to induce IFN-β. It is not known whether NLRC5 is able to bind any influenza virus-specific ligands.

### 2.3.2 Influenza virus-induced cell signaling

Influenza virus triggers multiple signaling pathways during the course of infection. Some of these pathways are antiviral, some virus-supportive or even both depending on the step of infection and the time of activation. Phosphatidylinositol-3-kinase (PI3K) signaling has been shown to regulate influenza virus entry (Ehrhardt et al., 2006). PI3K is a kinase that phosphorylates membrane phospholipids and it regulates cell metabolism, proliferation and survival (Neri et al., 2002). The activation of PI3K leads to the activation of a downstream effector protein Akt (Vanhaesebroeck et al., 2012). Influenza A NS1 is able to activate PI3K to prevent apoptosis (Ehrhardt et al., 2007b; Hale et al., 2006) and thus enable efficient virus replication. It has also been shown that PI3K takes part in the RIG-I- and IRF3-mediated signaling pathway that induces type I IFN responses in influenza virus-infected cells (Hrincius et al., 2011).

Autophagy is a mode of “self-eating” targeted to harmful proteins or pathogenic protein aggregates in the cytosol. Only lately it has been recognized that viruses can take advantage of the autophagy machinery (Dreux and Chisari, 2010). Influenza A virus induces autophagosome formation (Zhou et al., 2009) and NS1, HA (Zhirnov

and Klenk, 2013) and especially M2 proteins (Gannage et al., 2009) are involved in the regulation of autophagy. In the study by Gannage and coworkers M2 protein was shown to prevent the autophagosome fusion with lysosome resulting in autophagosome accumulation in the cells. It has been postulated that by regulating autophagy, influenza virus can evade the host immune response as the autophagic cell death results in lower immunogenicity of the virus (Zhang et al., 2014).

Autophagy is connected with another cellular event induced by influenza, a programmed cell death, apoptosis (Zhang et al., 2014). It seems that autophagy can inhibit apoptosis, as in the autophagy-deficient cells, influenza A virus-induced apoptotic cell death was increased (Gannage et al., 2009). Influenza virus is known to downregulate apoptosis in an NS1-dependent way (Jackson et al., 2010; Zhirnov et al., 2002), but whether this depends on the PI3K-Akt pathway (Ehrhardt et al., 2007a; Hale et al., 2006) or not (Jackson et al., 2010) is contradictory. PI3K plays a role both in autophagy and apoptosis (Zhang et al., 2014) hence more research into this topic is needed to create a complete picture.

The roles of PRRs RIG-I, MDA5, TLR3 and TLR7/8 in influenza virus recognition are discussed in Section 2.3.1 and the cytokine signaling pathways downstream of these receptors in Section 2.2.5 (see also Fig. 4). In addition to inducing cytokine production, some of these pathways have been indicated to take part in other aspects of influenza virus-host crosstalk. MAPK pathway Raf/MKK/ERK is needed for the nuclear export of influenza vRNP complexes (Marjuki et al., 2006) and blocking this pathway reduces influenza propagation (Pleschka et al., 2001). Another signaling pathway affecting influenza virus growth is the NF-κB-pathway (Nimmerjahn et al., 2004; Wurzer et al., 2004) for which multiple molecular mechanisms have been described (Ludwig, 2009).

### 2.3.3 Antiviral responses against influenza virus

The major soluble mediators of antiviral responses are IFNs, whose action is largely conveyed by the antiviral proteins encoded by the ISGs. These factors are often ready-made in the cell and restrict virus entry, replication or assembly by directly interacting with the virus. The fist ISG found to constrain influenza virus infection was the myxovirus resistance gene *Mx* (Staeheli et al., 1986). The mouse proteins are called Mx1 and Mx2 of which only Mx1 blocks influenza infection (Haller et al., 2009). The human counterparts, MxA and MxB are IFN-inducible and MxA inhibits influenza A infection (Turan et al., 2004; Xiao et al., 2013). MxA has been shown to interact with NP (Turan et al., 2004) and inhibit viral replication (Pavlovic et al., 1992). MxA can also block influenza A infection at the level of incoming virus by preventing the nuclear localization of viral vRNPs (Xiao et al., 2013).

Another important protein restricting influenza virus infection is an IFN-inducible transmembrane protein 3 (IFITM3) (Brass et al., 2009). IFITM3 blocks influenza virus entry at late endosomal stage by interfering with the formation of a fusion pore between the viral and endosomal membrane (Desai et al., 2014). It has been suggested that patients with severe influenza infection are more likely to have a genetic defect in IFITM3 gene (Everitt et al., 2012; Zhang et al., 2013) but opposing data exist (Mills et al., 2014). Nevertheless, mice lacking IFITM3 are highly susceptible to influenza viruses (Bailey et al., 2012).

Additional antiviral proteins against influenza virus are the 2′-5′-oligoadenylate synthase (OAS) and ribonuclease L (RNAseL) that cooperate to cleave viral ssRNA (Silverman, 2007). Other known antiviral proteins with numerous functions are protein kinase R (PKR), IFN-induced protein with tetratricopeptide repeats (IFIT)-proteins, viperin, thetherin, TRIM22 and ISG15 (Iwasaki and Pillai, 2014; Pulendran and Maddur, 2015). A novel influenza virus restriction mechanism is virus inhibition by host cell-derived microRNAs (miRNA). At least miR136, miR323, miR491, miR654 and let-7c have been shown to interfere with influenza A virus replication (Ma et al., 2012; Song et al., 2010; Zhao et al., 2015).

### 2.3.4 Viral countermeasures against host

As viruses and the host cells have evolved hand in hand, viruses have developed means to counteract the antiviral defense of the host. In the case of influenza, NS1 protein has been a horn of plenty in regard to its functions for inhibiting the cellular defense against influenza virus. NS1 is a small 26 kDa protein with an RNA binding domain and an effector domain (Krug, 2015). It is not present in the virion and the virus is able to replicate in the absence of it. NS1 protein sequences between influenza A viruses are very conserved, however there are major functional differences between different virus strains (Kochs et al., 2007; Kuo et al., 2010). NS1 interferes with IFN gene expression by inhibiting IRF3 (Talon et al., 2000), NF-κB (Wang et al., 2000) and AP-1 (Ludwig et al., 2002) activation but the mechanisms are yet obscure. NS1 has been shown to bind many factors in the RIG-I pathway: RIG-I itself (Guo et al., 2007; Mibayashi et al., 2007; Opitz et al., 2007), TRIM25 (Gack et al., 2009) and Riplet (Rajsbaum et al., 2012). A long known feature of the influenza NS1 is its capability to inhibit the cellular mRNA production. This happens via NS1 binding of the cleavage and polyadenylation specificity factor (CPSF30) (Nemeroff et al., 1998) which interferes with the 3′end formation of pre-mRNAs. Influenza NS1 also binds and inhibits antiviral proteins produced by the cell, PKR (Li et al., 2006; Min et al., 2007) and OAS (Min and Krug, 2006). As discussed earlier, NS1 has been shown to regulate host cell apoptosis by interacting with PI3K.

An additional influenza A virus-encoded virulence factor is PB1-F2 (Chen et al., 2001), which induces apoptosis and inhibits type I IFN induction (Varga et al., 2011). It contributes to the virulence of several pandemic viruses such as the 1918 H1N1, the 1957 H2N2 and the 1968 H3N2, and the highly pathogenic avian influenza (HPAI) H5N1 viruses (Conenello et al., 2007; McAuley et al., 2010). However, the 2009 pandemic H1N1 virus does not encode PB1-F2 (Ozawa et al., 2011). From PB1 mRNA a second polypeptide is synthesized named PB1-40 (Wise et al., 2009) but the significance of the variant protein in the context of virus fitness is not known. Another polymerase protein-derived virulence factor is PA-X (Jagger et al., 2012) which has a role in shut-off of cellular gene expression (Hayashi et al., 2015). Recently two other proteins translated from influenza A virus PA mRNA were described (Muramoto et al., 2013) and termed PA-N155 and PA-N182. They may have functions in the replication cycle of the virus as viruses lacking PA-N155 and PA-N182 replicated more slowly. A very recent study found a splicing variant also for the polymerase subunit PB2, PB2-S1, which was able to inhibit RIG-I-dependent IFN pathway (Yamayoshi et al., 2015). It is probable that even more viral proteins encoded by spliced mRNAs will be found in the near future thus explaining in part the diversity of influenza virus-induced responses in the cell and strain-specific features of influenza viruses.

In recent years it has become evident that innate immune responses are also regulated by the expression of non-protein-coding RNAs such as miRNAs. Interestingly, it was shown in 2014 that long noncoding RNA (lncRNA) is important in successful influenza A virus infection (Winterling et al., 2014). IFNs have been shown to induce the expression of specific lncRNAs which were also induced by NS1 deficient influenza A virus, but not with wt influenza A virus (Carnero et al., 2014). Moreover, NS1 has been shown to induce the expression of vault RNA (vtRNA) (Li et al., 2015), which is another type of noncoding RNA. The expression of vtRNA interferes with PKR activity leading to enhanced virus replication (Li et al., 2015).

# 3 Aims of the study

Innate immune cells such as macrophages and DCs express various pattern recognition receptors that are employed for the recognition of microbial pathogens, including influenza viruses. The sensing of pathogen structures triggers the activation of intracellular signaling cascades leading to the production of cytokines and antiviral IFNs. During infection, several different pathogen-derived molecules are present that can activate different pattern recognition receptors in a cooperative manner to induce additive or synergistic production of cytokines. In this study the mechanisms of synergistic IL and IFN expression induced by different TLR ligands were studied. Another research aim was to analyze the induction of IFNs by different influenza viruses in human macrophages and DCs.

The specific aims of this study were:

- To study the early events of influenza B virus infection and influenza B virus-induced IFN responses in human macrophages (I).
- To characterize IFN induction by the novel 2009 pandemic H1N1 influenza virus in human macrophages and DCs (II).
- To elucidate the synergistic mechanisms of IL-12 cytokine gene expression induced by different TLRs (III).
- To analyze the synergy between RNA-recognizing TLRs in inducing IFN-λ1 gene expression in human DCs (IV).

# 4 Materials and methods

## 4.1 Ethics statement

Adult human blood was obtained from anonymous healthy blood donors from the Finnish Red Cross Blood Transfusion Service (permission renewed yearly). Animal immunizations related to this study were approved by the Ethical Committee of National Institute for Health and Welfare (permission KTL 2008-02).

## 4.2 Cell culture

### 4.2.1 Differentiation of macrophages and DCs from peripheral blood-derived monocytes (I, II, III, IV)

Human peripheral blood mononuclear cells (PBMC) were purified from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki). The cells were isolated by density gradient centrifugation over a Ficoll-Paque gradient (Amersham Biosciences) (Veckman et al., 2004). To obtain monocytes for macrophage differentiation, mononuclear cells were allowed to adhere to plates for 1 h at +37°C in RPMI-1640 (Sigma-Aldrich) supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM HEPES. Non-adherent cells were removed by washing with cold PBS and remaining monocytes were cultured in macrophage/serum-free medium (Life Technologies) supplemented with antibiotics and human rGM-CSF, 10 ng/ml (Nordic Biosite). The cells were differentiated into macrophages for 7 days changing fresh culture media every 2 days.

To obtain moDCs, Ficoll-Paque gradient centrifugation was followed by Percoll gradient (Amersham Biosciences) centrifugation (Veckman et al., 2004). The top layer containing monocytes was collected, and the remaining T and B cells were depleted using anti-CD3 and anti-CD19 magnetic beads (Dynal). Monocytes were allowed to adhere to plates for 1 hour at +37°C in RPMI-1640 supplemented as above. Adhered monocytes were washed with PBS and immature DCs were generated by cultivating cells in RPMI-1640 medium supplemented with 10 % FCS (Integro), 10 ng/ml human rGM-CSF, 20 ng/ml human rIL-4 (R&D Systems) and antibiotics. The cells were cultivated for 6 days and fresh media was added every 2 days.

### 4.2.2 Mouse embryonic fibroblast cells (I)

Primary wild type (wt) and *Irf3*$^{-/-}$*Irf7*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and *Rela*$^{-/-}$*Rel*$^{-/-}$*Nfκb1*$^{-/-}$ (NF-κB knock-out) MEF cell line were kindly provided by Dr. A. Hoffmann, Signaling Systems Lab, Los Angeles. Primary wt (*Rig-I*$^{+/+}$), *Rig-I*$^{-/-}$, *Mda5*$^{-/-}$ and *Rig-I*$^{-/-}$*Mda5*$^{-/-}$ MEFs were obtained from mouse embryos and they were immortalized by rigorous passaging protocol to obtain wt and knock-out cell lines. The MEF cell lines were cultured in DMEM-medium supplemented with 0.6 µg/ml penicillin, 60 µg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES and 10 % FCS.

## 4.3 Viruses and infection experiments (I, II)

Viruses used in the study are listed in the Table 2. All viruses were grown from a $10^{-5}$ dilution of stock virus in allantoic cavities of 11-day-old embryonated chicken eggs at +34°C for 3 days. Influenza stock virus titers were determined by a plaque assay on MDCK cells (Osterlund et al., 2012) or the infectivity of the virus stocks in human DCs and macrophages was determined by analyzing the percentages of virus-infected cells with flow cytometry (II) or immunofluorescence (I). The propagation of the A/Finland/553/2009 virus stock and the infection experiments with this virus were carried out under BSL-3 conditions at that time. The infection experiments are described in more detail in the materials and methods sections of the original publications (I, II).

**Table 2. Viruses used in the study**

| Virus | Type | HA titer | PFU/ml | Used in |
|---|---|---|---|---|
| A/Finland/553/2009 | H1N1 | 16 | | II |
| A/Brisbane/59/07 | H1N1 | 128 | | II |
| A/NewCaledonia/20/99 | H1N1 | 256 | | II |
| A/WSN/33 | H1N1 | 128 | | II |
| A/Udorn/72 | H3N2 | 256 | | II |
| A/Beijing/353/89 | H3N2 | 128 | $6x10^7$ (MDCK) | I |
| B/Shangdong/7/97 | | 64 | $18x10^7$ (MDCK) | I |
| Sendai virus | Cantell strain | | $6x10^9$ (moDC) | I |

## 4.4 Reagents

### 4.4.1 Antibodies and cytokines

The antibodies used in the study for different applications or for the neutralization of IFN during experiments are defined in the Table 3. IFNs used for priming the cells or for studying the IFN sensitivity of influenza viruses and cytokines used for moDC and macrophage differentiation are listed in the Table 4.

**Table 3. Antibodies**

| Antibody | Application | Source/reference | Used in |
|---|---|---|---|
| Guinea pig anti-IRF1 | WB | (Matikainen et al., 1996) | III, IV |
| Rabbit anti-p-IRF3 (ser396) | WB | #4947 Cell Signaling Technology | I, IV |
| Rabbit anti-IRF3 | WB | #4302 Cell Signaling Technology | I |
| Rabbit anti-IRF3 | WB, IF | (Osterlund et al., 2005) | I, III, IV |
| Rabbit anti-IRF4 | WB | sc-28696 Santa Cruz | III |
| Rabbit anti-IRF5 | WB | (Pietila et al., 2007) | III |
| Guinea pig anti-IRF7 | WB | (Osterlund et al., 2005) | III, IV |
| Goat anti-IRF8 | WB | sc-6058X Santa Cruz | III, IV |
| Rabbit anti-IRF9 | WB | sc-496 Santa Cruz | III |
| Rabbit anti-STAT1 | WB | sc-346 Santa Cruz | III |
| Rabbit anti-STAT2 | WB | sc-839X Santa Cruz | III |
| Rabbit anti-p50 | WB | sc-7178 Santa Cruz | III |
| Rabbit anti-p65 | WB | sc-372 Santa Cruz | III |
| Rabbit anti-IκBα | WB | #9242 Cell Signaling Technology | I |
| Rabbit anti-p-p38 | WB | #9211 Cell Signaling Technology | III |
| Rabbit anti-p38 | WB | #9212 Cell Signaling Technology | III |
| Mouse anti-p-p44/p42 | WB | #9106 Cell Signaling Technology | III |
| Rabbit anti-p44/p42 | WB | #9102 Cell Signaling Technology | III |
| Rabbit anti-p-Akt | WB | #9271 Cell Signaling Technology | III |
| Rabbit anti-Akt | WB | #9272 Cell Signaling Technology | III |

| | | | |
|---|---|---|---|
| Rabbit anti-p-c-Jun | WB | #9261 Cell Signaling Technology | III |
| Rabbit anti-IFITM3 | WB | AP1153a Abgent | I |
| Rabbit anti-MxA | FC, WB | (Ronni et al., 1993) | I, II |
| Rabbit anti-actin | WB | sc-10731 Santa Cruz | I |
| Mouse anti-nucleolin | WB | sc-8031 Santa Cruz | III |
| Rabbit anti-GAPDH | WB | #2118 Cell Signaling Technology | I |
| Mouse anti-EEA1 | IF | 610456, BD Biosciences | I |
| Mouse anti-LAMP1 | IF | sc-20011, Santa Cruz | I |
| Guinea pig anti-NP IBV | IF | | I |
| Rabbit anti-NP IBV | WB | (Osterlund et al., 2012) | I |
| Rabbit anti-NS1 IBV | WB | (Osterlund et al., 2012) | I |
| Guinea pig anti-NP IAV | IF | | I |
| Rabbit anti-NP IAV | WB | (Osterlund et al., 2012) | I |
| Rabbit anti-NS1 IAV | WB | (Osterlund et al., 2012) | I |
| Rabbit anti-GP H1N1 | FC | (Julkunen et al., 1983) | II |
| Sheep anti-IFN-α and -β | IFN-neutralization | (Mogensen et al., 1975) | IV |

WB,Western blotting; IF, immunofluorescence; FC, flow cytometry; GP, glycoprotein.

**Table 4. Purified cytokines**

| Cytokine | Concentration used | Source/reference | Used in |
|---|---|---|---|
| Recombinant IFN-α2 | 1, 10, 100 IU/ml | Schering-Plough | II |
| Recombinant IFN-β | 1, 10, 100 IU/ml | Schering-Plough | II, IV |
| Recombinant IFN-λ1 | 10 ng/ml | ZymoGenetics | IV |
| Recombinant IFN-λ1 | 1, 10, 100 ng/ml | (Dellgren et al., 2009) | II |
| Recombinant IFN-λ3 | 1, 10, 100 ng/ml | (Dellgren et al., 2009) | II |
| Human recombinant GM-CSF | 10 ng/ml | Nordic Biosite | I- IV |
| Human recombinant IL-4 | 20 ng/ml | R&D Systems | I-IV |

### 4.4.2 TLR ligands

TLR ligands used in the study are summarized in the Table 5.

**Table 5. TLR ligands used in the study**

| TLR | Ligand | Description | Concentration used | Source/reference | Used in |
|---|---|---|---|---|---|
| TLR1/2 | Pam3CSK4 | a synthetic tripalmitoylated lipopeptide | 100 ng/ml | InvivoGen | III |
| TLR3 | PolyI:C | Polyinosic-polycytidylic acid, mimics dsRNA | 30 µg/ml | Sigma-Aldrich | III, IV |
| TLR4 | LPS | Lipopolysaccharide from *Escherichia coli* serotype O111:B4 | 100 ng/ml | Sigma-Aldrich | I, III, IV |
| TLR5 | Flagellin | Flagellin from *Salmonella enterica* sv. Typhimurium | 500 ng/ml | InvivoGen | III |
| TLR7/8 | R848 | imidazoquinoline compound | 5-10 µM | Alexis Biochemicals | III, IV |

### 4.4.3 Pharmacological inhibitors

Inhibitors used in the study and their cellular targets are summarized in the Table 6.

**Table 6. Pharmacological inhibitors**

| Inhibitor | Concentration used | Cellular target | Source/reference | Used in |
|---|---|---|---|---|
| Bafilomycin A1 (BafA1) | 10 nM | vacuolar H+ ATPase | Sigma-Aldrich | I |
| Cycloheximide (CHX) | 10 µg/ml | *de novo* protein synthesis | Sigma-Aldrich | I, IV |
| Dynasore | 80 µM | dynamin | Sigma-Aldrich | I |
| Ly294002 | 50 µM | PI3K | Calbiochem | III, IV |

| | | | | |
|---|---|---|---|---|
| PDTC | 100 µM | NF-κB | Alexis Biochemicals | III, IV |
| PD98059 | 10 µM | MEK1 | Calbiochem | III, IV |
| SB202190 | 5-10 µM | p38 | Alexis Biochemicals | III, IV |
| SP600125 | 10 µM | JNK | Alexis Biochemicals | III, IV |

## 4.5 Quantitative real time PCR (qRT-PCR)

Total cellular RNA was isolated from MEFs, moDCs or macrophages derived from three to four pooled donors using TRIZOL and the Qiagen Rneasy Mini kit. One µg of total cellular RNA was reverse transcribed into cDNA in TaqMan RT buffer with 5.5 mM $MgCl_2$, 500 µM dNTPs, 2.5 µM oligo d(T)16 or random hexamers, 0.4 U/µL RNase inhibitor and 1.25 U/µL MultiScribe reverse transcriptase (Applied Biosystems). cDNA samples were then amplified in TaqMan universal PCR master mix buffer (Applied Biosystems) with Gene Expression system assay mix oligonucleotides (Applied Biosystems) shown in the Table 7 to analyze mRNA levels. Each cDNA sample was amplified in duplicate or triplicate with Stratagene Mx3500P (Agilent Technologies). The mRNA levels were normalized against human 18S rRNA (I, II), mouse GAPD (I) or β-actin (III, IV) mRNA and the amounts of the studied mRNA relative to unstimulated cells were calculated with ΔΔCt –method.

**Table 7. qRT-PCR gene expression assays**

| Gene Assay | ID | Source/reference | Used in |
|---|---|---|---|
| IFN-α1 | Hs00256882_s1 | Applied Biosystems | II, IV |
| IFN-β1 | Hs00277188_s1 | Applied Biosystems | I, II, IV |
| IFN-β1 (mouse) | Mm00439552_s1 | Applied Biosystems | I |
| IFN-λ1 | Hs00601677_g1 | Applied Biosystems | I, II, IV |
| IFN-λ2/3 | Hs00820125_g1 | Applied Biosystems | II, |
| TNF-α | Hs00174128_m1 | Applied Biosystems | I, II |
| TNF-α (mouse) | Mm00443260_g1 | Applied Biosystems | I |
| IL-6 | Hs00174131_m1 | Applied Biosystems | III, IV |

| | | | |
|---|---|---|---|
| IL-10 | Hs00174086_m1 | Applied Biosystems | III |
| IL-12p35 | Hs00168405_m1 | Applied Biosystems | III |
| IL-12p40 | Hs00233688_m1 | Applied Biosystems | III, IV |
| IL-23p19 | Hs00372324_m1 | Applied Biosystems | III |
| IL-27p28 | Hs 00377366_m1 | Applied Biosystems | III |
| IL-27 EBI3 | Hs00194957_m1 | Applied Biosystems | III |
| CXCL10 | Hs00171042_m1 | Applied Biosystems | I, II, IV |
| CXCL10 (mouse) | Mm00445235_m1 | Applied Biosystems | I |
| Mx1 | Hs00182073_m1 | Applied Biosystems | II, IV |
| IFITM3 | Hs01922752_s1 | Applied Biosystems | I |
| IRF7 | Hs00242190_g | Applied Biosystems | IV |
| TLR3 | Hs00152933_m1 | Applied Biosystems | IV |
| TLR4 | Hs01061963_m1 | Applied Biosystems | IV |
| TLR7 | Hs00152971_m1 | Applied Biosystems | IV |
| TLR8 | Hs00607866_mh | Applied Biosystems | IV |
| RIG-I | Hs00225561_m1 | Applied Biosystems | I |
| β-actin | Hs99999903_m1 | Applied Biosystems | III, IV |
| 18S | 4308329 | Applied Biosystems | I, II |
| GAPDH (mouse) | 4352339E | Applied Biosystems | I |
| IAV M1 | | (Ward et al., 2004) | II |
| IAV NP | | (Osterlund et al., 2012) | I |
| IBV NP | | (Osterlund et al., 2012) | I |
| IAV NS1 | | (Osterlund et al., 2012) | I |
| IBV NS1 | | (Osterlund et al., 2012) | I |

## 4.6 ELISA (II, III, IV)

Cytokine levels for IL-1β, IL-6, IL-10, IL-12p70 and TNF-α from cell culture supernatants were analysed by fluorescent bead immunoassay using FlowCytomix Human Th1/Th2 10-plex kit (Bender Medsystems). IL-12p70 levels were determined by Elipair kit (Biosite) and by IL-23 ELISA (Bender Medsystems).

CCL19, CCL20 and IFN-λ1 were measured with a Duoset kit ELISA (R&D Systems), IFN-α and IFN-β with kits from PBL Biomedical Laboratories and TNF-α and CXCL10 by using Ab pairs and standards from BD Pharmingen (San Diego, CA).

## 4.7 Oligonucleotide precipitation assay (DNA affinity binding) (III, IV)

TLR ligand-induced activation of transcription factors was studied by oligonucleotide DNA affinity binding method (Osterlund et al., 2005) using ISRE, mutated ISRE and NF-κB binding site-specific oligonucleotide sequences from the IL-12p35 promoter, and ISRE binding site-specific oligonucleotide sequences from the IFN-λ1 promoter (Osterlund et al., 2007). Equal amounts of cells were harvested and nuclear extracts were prepared. The forward oligonucleotide was 5′–biotinylated and a BamHI site was added as a spacer. The oligonucleotides used were IL-12p35 ISRE wt (5′-GGATCCAGTAACTGCGAACATTTCGCTTTCATTTTGGGCCGAGCTGGAGG CGGCGGGG), IL-12p35 ISRE mutant (5′-GGATCCAGTAACTGCGA**C**CA**C**T**G**CGC**G**T**GCAGG**T**C**GGGCCGAGCTGGA GGCGGCGGGG; mutations in bold), IL-12p35 NF-κB (5′-GGATCCAAGAGACCAGAGTCCCGGGAAAGTCCTGCCGCGCCT) and IFN-λ1 ISRE (5′-GATTTCAGTTTCTCTTTCCTTCTTGA) sites from DNA Technology A/S. Oligonucleotides were incubated at +4°C for 2 hours with streptavidin-agarose beads (Pierce). The unbound oligonucleotide was washed after which the protein lysates were incubated with the agarose-bound oligonucleotides at +4°C for 2 hours followed by washing of the unbound proteins. Oligonucleotide-bound proteins were released in SDS sample buffer followed by Western blotting using specific antibodies.

## 4.8 Western blot analysis (I, III, IV)

Oligonucleotide binding assay samples or aliquots of whole cell lysates were separated on 10 % or 12 % SDS-PAGE using the Laemmli buffer system. Whole cell lysates were prepared by lysing cells in TN buffer containing 25 % ethylene glycol, passive lysis buffer from Promega or RIPA buffer all with 0.5 mM DTT, 1 mM $Na_3VO_4$, and Complete protease inhibitor mixture. Proteins were electrotransferred onto Immobilon-P membranes (Millipore) or Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) which were blocked with 5 % milk in PBS (or in TBS for Cell Signaling antibodies) and stained with specific antibodies listed in the Table 3. HRP-conjugated secondary antibodies

(DakoCytomation) were used and proteins were visualized on HyperMax films using an ECL plus system (GE Healthcare).

## 4.9 Immunofluorescence microscopy (I)

Macrophages were differentiated on glass coverslips and infected with influenza viruses at a multiplicity of infection (MOI) of 30, incubated on ice for 1 hour, after which the virus inoculum was removed and replaced with warm media to allow virus entry to the cells at 37°C 5 % $CO_2$. After indicated times the cells were fixed with 3 % paraformaldehyde at room temperature for 20 min, washed with PBS, permeabilized with 0.1% Triton X-100 for 5 min, washed and blocked with 0.5% BSA in PBS for 30 min. The cells were stained for 45 min in PBS containing 0.5% BSA at 37°C with primary antibodies listed in the Table 3. Secondary antibodies were FITC-labelled goat anti-guinea pig, Rhodamine Red-X-labelled goat anti-rabbit and Rhodamine-labelled goat anti-mouse (Jackson ImmunoResearch Laboratories). The coverslips were washed with PBS containing 0.05% Tween 20, then with distilled water, and mounted in 25 % Mowiol (Polysciences) in 25 mM Tris-HCl, pH 7.5, 50 % glycerol, and 2.5 % 1,4-diazabicyclo[2.2.2.]octane. The cells were visualized under a Leica TCS SPE confocal microscope with 63x NA 1.40 oil objective with 1 Airy unit pinhole maintaining the same image acquisition settings for all acquired images. The cells in the infectivity experiments were analyzed with a Zeiss Stallion fluorescence microscope with a Hamamatsu ORCA-Flash 4.0 LT sCMOS camera and a 20x 0.4 NA objective by using Slidebook 6 software.

## 4.10 Flow cytometric analysis (II)

Flow cytometry was used to analyze the purity of monocyte-derived macrophages and DCs, and the infectivity of influenza viruses in moDCs and macrophages. The cell samples for the flow cytometry analysis were prepared as described previously (Veckman et al., 2004). The cells obtained from different blood donors were separately infected and pooled after virus infections followed by fixation with 4 % paraformaldehyde for 15 min at RT. In addition, the cells used for intracellular MxA analysis were permeabilized with 0.1 % Triton X-100 for 5 min at RT. The expression of viral glycoproteins on the cell surface and MxA was measured using cross-reactive anti-influenza A virus H1N1 glycoprotein-specific antibodies or anti-human MxA antibodies listed in the Table 3 and secondary FITC-labelled anti-rabbit antibodies (Caltag Laboratories). The cells were analyzed with FACSCanto II flow cytometer using FACSDiva software (Becton Dickinson). The purity of DC and macrophage populations were confirmed by staining noninfected cells with phycoerythrin (PE)-anti-CD2, PE-Cy7-anti-CD14 (Becton Dickinson), FITC-anti-

CD80, PE-anti-CD83, allophycocyanin (APC)-anti-CD86 (Caltag Laboratories) and APC-H7-anti-HLA-DR antibodies (Becton Dickinson).

# 5 Results and discussion

## 5.1 Human monocyte-derived macrophages and dendritic cells as a cell model to study innate immune responses

Throughout this study, our cell model has been human blood-derived $CD14^+$ monocytes differentiated into cells resembling DCs and macrophages with the help of growth factors. This is a well-established method (Sallusto and Lanzavecchia, 1994) of acquiring a large number of relatively homogenous cells cost-effectively, as only small amounts of different human DC subtypes and macrophages are found in the blood. During the differentiation process, many genes are upregulated or downregulated differentially between monocyte-derived macrophages and DCs (Lehtonen et al., 2007). However, genome-wide gene expression profiling has shown that human *in vitro*-differentiated moDCs and monocyte derived macrophages cluster closer together than moDCs and blood $BDCA3^+$ DCs, $BDCA1^+$ DCs or pDCs (Robbins et al., 2008). Demonstrating that the monocyte-derived cells are a relevant cell model in the human system, an inflammatory DC population was described that resembled *in vitro* moDCs and was likely derived from monocyte population (Segura et al., 2013). In addition, in steady state, a monocyte-derived macrophage population was recently described in the human skin (McGovern et al., 2014). *In vivo*, monocyte-derived effector cells infiltrate into infection sites, recognize pathogens and produce cytokines in response to infection in concert with epithelial cells, tissue resident immune cells and different dendritic cell subtypes (reviewed in Schlitzer et al., 2015). Instead of using monocultures for infection studies, cocultures of epithelial cells and immune cells could be used as it is known that interactions between the different cell types affect the outcome of the immune response (Duell et al., 2011; Qu et al., 2003).

## 5.2 Activation of innate immune responses by influenza viruses

### 5.2.1 Influenza virus recognition by the host cell (I)

The evolution of the virus and the host is a continuous game of hide and seek. The recognition of the pathogen is the first event that needs to take place in order to induce a proper immune response. The virus tries to avoid the recognition by the host cell or manipulate the subsequent signaling pathways for its own benefit. Our group had shown before that there is a clear kinetic difference in the IFN responses induced by influenza A and B viruses in human moDCs (Osterlund et al., 2012). Influenza B virus induced IRF3-dependent IFN-β and IFN-λ1 gene expression very

early upon entry, before replication of the virus, as evidenced by experiments with UV-treated virus. However, UV-treated and thus replication incompetent influenza A virus did not induce IFN. This indicated that already the incoming influenza B virus particles were sensed by moDCs whereas influenza A virus was able to evade the early recognition or interfered with the pathways leading to IRF3 activation and IFN gene expression. Most likely viral NS1 did not play a role in the difference between the viruses as it is not expressed during the first hours of infection. Österlund and coworkers also showed that both influenza A and B NS1 proteins were able to inhibit IFN-λ1 gene expression to the same extent in promoter luciferase assay (Osterlund et al., 2012).

We wanted to study the difference between influenza A and B viruses further and concentrated on influenza B virus, which is far less studied than influenza A. We wanted to know at which point of the entry influenza B virus activates IRF3 and which PRR is responsible for detecting influenza B virus. One possible approach was to use immunofluorescence microscopy. However, moDCs were not a good cell model for immunofluorescence since the cells are semiadherent. Macrophages instead are highly adherent cells and also a biologically relevant cell model as they reside under the epithelial cell layer in the influenza virus infection sites and are infected by influenza viruses. At first we confirmed, that also in human macrophages influenza A and B viruses induce kinetically different IFN gene expression patterns (Fig.1 in I). Likewise in moDCs, also in macrophages influenza B virus induced the activation, i.e. the phosphorylation of IRF3 already at 1 to 2 hours, whereas in influenza A virus-infected cells, the IRF3 activation took place at later time points of 4 to 8 hours (Fig. 2 in I).

To visualize the virus at the time when IRF3 was activated we differentiated macrophages on coverslips for immunofluorescence assay, infected the cells on ice for a synchronized infection (Martin and Helenius, 1991) and fixed cells at different time points up to three hours post infection in 37 °C. Confocal microscope images showed that at the zero time point influenza B virus NP was seen at the plasma membrane (Fig. 3A and Fig. 5 upper left panels in I), at 15 minutes inside the cell in granules and at 45-60 min time points NP started to accumulate into the nucleus. The kinetics of nuclear translocation of IRF3 indicated that its activation coincided with the influenza B virus nuclear entry. In influenza A virus-infected macrophages no IRF3 nuclear translocation was seen before 3 hours of infection (Fig. 3B in I).

The kinetics of influenza A virus entry has been studied with high content imaging (Banerjee et al., 2013) where the nuclear import of influenza A virus occurred after one hour post infection and peaked at 3.5 hours in A549 cells. Other studies have shown that influenza A virus enters the nucleus at 1 hour post infection (HeLa cells) (Sieczkarski and Whittaker, 2003), 35 min post infection (CHO-cells) (Martin and

Helenius, 1991) or even at 20 minutes post infection (MDCK-cells) (Chou et al., 2013). The discrepancy in the kinetics is partly dependent on the host cell type used (Lakadamyali et al., 2004) and likely also the amount of virus stocks and the used antibody reagents. The entry kinetics of influenza B virus in macrophages has not been studied before. In macrophages influenza B virus seemed to enter the nucleus somewhat faster than influenza A (Fig. 3B in I) but the kinetics did not explain the apparent difference in IRF3 activation.

To further elucidate at which step of the entry process influenza B virus activates IRF3 we took advantage of known inhibitors of cellular processes that are involved in influenza virus entry. BafA1 is an inhibitor of vacuolar $H^+$ type ATPase and it prevents the acidification of the endosome which is a prerequisite for the fusion to happen between viral and endosomal membranes. CHX blocks protein synthesis by inhibiting translational elongation. Influenza virus requires newly synthetized viral proteins for the replication of its genome and thus CHX arrests viral replication. IRF3 activation and nuclear accumulation of viral NP did not take place in BafA1-treated and influenza B virus-infected macrophages (Fig.5 lower panels, in I). However, CHX-treatment did not affect influenza B-virus induced IRF3 activation. This means that viral replication is not needed for the IRF3 activation but most likely the virus needs to escape the endosomal compartment. In the presence of CHX, the primary transcription can still take place by the viral RNA polymerases in the incoming vRNPs. Thus our results do not confirm, whether viral mRNA transcription is needed for IRF3 activation. However, previous results from infections with UV-treated influenza B virus that induces robust IFN responses (Osterlund et al., 2012) suggest that it is the incoming vRNP structures that trigger IRF3 activation.

A recent study using inhibitors, such as CHX, actinomycin D and α-amanitin to block viral primary transcription and 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole to block viral RNA export from the nucleus, showed that the primary transcription and nuclear export are essential for the influenza A virus-induced IFN responses (Killip et al., 2014). As readout they used IFR3 phosphorylation in A549 cells at eight hour time point. In our experimental setup, at eight hour time point the influenza B-virus induced IRF3 phosphorylation had already declined whereas influenza A virus induced maximal IRF3 activation at that time point (Fig. 2 in I). In addition in the study describing the IRF3 activation by the incoming nucleocapsids of influenza A (Weber et al., 2015), IRF3 was activated at one hour time point. It may be that in the case of influenza A viruses, RIG-I can recognize the incoming viral nucleocapsids but the subsequent signaling pathways are not activated because the viral polymerase itself can prevent the activation of RIG-I signaling pathway (Liedmann et al., 2014). Liedmann and coworkers found a motif in PB1 and PA polymerase proteins that was linked to virus-induced early activation of IRF3. The

authors showed that the IFN antagonism by the polymerase proteins depended on the interactions between the viral polymerase complex and RIG-I. Why this mechanism seems to inhibit the IFN activation by the incoming virus but not the RIG-I recognition and subsequent IFN response after virus replication is not yet known. This may be due to the idea discussed by Killip and coworkers that some presently unidentified viral transcription products are responsible for RIG-I activation after viral entry has taken place (Killip et al., 2014). Human H3N2 influenza strain A/Beijing/353/89 used in this thesis does not carry the complete motif in PB1 and PA needed for IFN antagonism but other such adaptations may be found in influenza virus polymerase proteins.

Macrophages are a good model to study innate immune recognition of pathogens as they readily express a wide array of different PRRs. The relevant receptors that bind influenza virus-derived ligands leading to IFN signaling are the cytosolic receptor RIG-I which senses 5´triphosphate ssRNA with a short dsRNA panhandle structure and endosomal TLR7 and TLR8 that recognize ssRNA. MDA5 and TLR3 clearly have some role in influenza virus infection (see Section 2.3.1.) although influenza virus is not expected to express dsRNA molecules during its replication (Weber et al., 2006; Wisskirchen et al., 2011). Since these PRRs are dispensable in influenza virus infection, they are not discussed here. Human macrophages express TLR8 whereas the expression of TLR3 and TLR7 is low. However, TLR3 and TLR7 are induced in influenza infection via type I IFN (Miettinen et al., 2001). The incoming influenza B virus could be recognized via TLR7/8 in the endosomal compartments after endocytosis. Yet, viral ssRNA is likely protected by the viral envelope and M1 protein and thus perhaps not accessible for TLR recognition. The viral particle should be first degraded in the lysosomal compartment by the host cell enzymes/proteases. As BafA1 also prevents the acidification of lysosomes, it would prevent the TLR7/8-induced IFN activation in influenza virus-infected cells. Moreover, the endosomal trafficking and acidification-dependent cleavage is needed for the functionality of endosomal TLRs (Lee and Barton, 2014). Regardless, TLR7/8 employs the adaptor protein MyD88 and its signaling does not lead to direct activation of IRF3 (Fig. 3). This excludes TLR7/8 as a putative receptor inducing early IFN gene expression in influenza B virus-infected macrophages.

Proteins in pimary macrophages are hard to knock down and we were not successful in inhibiting RIG-I protein expression with small interfering RNAs (siRNAs). Therefore we used MEFs lacking RIG-I, MDA5 or both receptors to study whether RIG-I is the receptor responsible for the early IFN induction by influenza B virus. MEFs have been reported to express TLRs from 1 to 9 (Kurt-Jones et al., 2004). We generated immortalized cell lines from the primary fibroblasts by rigorous passaging method. The kinetic difference between influenza A and B viruses in inducing IRF3 activation was clearly present in wt mouse fibroblasts (Fig. 4B in I) where influenza

B virus induced IRF3 phosphorylation at 3 hour time point and influenza A at 8 hour time point. As expected, RIG-I was the dominant receptor inducing IFN responses as the phosphorylation of IRF3 (Fig. 8B in I) or IFN-β gene expression (Fig. 8A in I) did not take place in the influenza B virus-infected RIG-I$^{-/-}$ cells. In MDA5 deficient cells, influenza B induced IRF3 phosphorylation as well as in the wt cells, however, in influenza A virus-infected MDA5 knock-out cells IRF3 phosphorylation was reduced (Fig. 8B in I). Our results indicate that RIG-I is the essential receptor for the early influenza B virus-induced IFN activation. Thus influenza B virus infection resembles that of the highly pathogenic avian influenza virus (H5N1) infection, which induces robust and early RIG-I-dependent IFN responses in human macrophages (Hui et al., 2011).

It is still not entirely clear, which influenza-derived ligands trigger RIG-I signaling in the context of infection. The question is not easy to answer as influenza viruses inhibit RIG-I recognition and RIG-I-induced signaling in many ways. In addition, influenza strains adapted to one host species may not show similar ability to restrict the activation of innate immune responses in the new host. The ds panhandle structure of influenza vRNP acts as a promoter region for viral replication and contains wobble pairs and mismatches and as such is not an optimal RIG-I ligand (Liu et al., 2015a). When stabilizing mutations were introduced to the panhandle, RIG-I activation and IFN induction were increased but viral mRNA synthesis was decreased (Liu et al., 2015a). As RIG-I is essential for inducing antiviral innate immune response against influenza, and since influenza virus is effectively blocking its functions, using optimized RIG-I agonists as prophylaxis against influenza has been studied (Chiang et al., 2015). Promising results have been obtained in mice by using these agonists as adjuvants in influenza vaccines (Beljanski et al., 2015). Virus like particle (VLP) vaccine, containing H5N1 HA and NA and the optimized RIG-I agonists as an adjuvant, greatly improved humoral and cellular immune responses to the vaccine and protected mice against lethal challenge with H5N1 virus (Beljanski et al., 2015).

### 5.2.2 Influenza virus-induced IFN responses in human macrophages and DCs (II)

In 2009 a novel swine origin H1N1 influenza virus caused a first influenza pandemic of the 21$^{st}$ century. As little was known about the characteristics of the pandemic H1N1 (pH1N1) virus in the human system, we decided to analyze the activation of IFN responses in human moDCs infected with the new virus. The Finnish virus isolate A/Finland/553/2009 used in the study was genetically almost identical to the initial isolate A/California/04/2009. The pH1N1virus readily infected moDCs (Fig. 1A in II) and replicated in moDCs with similar efficacy compared to the seasonal human H1N1 strains (Fig. 1B in II). When IFN gene expression was

studied, pH1N1 induced similar IFN mRNA levels as the seasonal human H1N1 strains (Fig. 2A in II), however the levels were much lower than those induced by laboratory adapted strains A/WSN/33 (H1N1) and A/Udorn/72 (H3N2). Nevertheless, pH1N1-induced IFN-α, IFN-β and IFN-λ1 protein production was barely detectable in moDCs, whereas seasonal H1N1 viruses or the H3N2 virus did produce IFNs (Fig. 2B in II). We also analyzed the infectivity and IFN gene expression in pH1N1-infected human macrophages in comparison to moDCs (Fig. 3 in II) and found no significant differences between the cell types.

Several *in vitro* studies have shown weaker (Chan et al., 2010; Mukherjee et al., 2011; Patel et al., 2011; Zeng et al., 2011) or similar (Lee et al., 2010) immune responses in cells infected with the 2009 pandemic virus compared to the seasonal H1N1 virus-infected cells. Consistent with our study, pandemic H1N1 induced weaker IFN-β gene expression than seasonal H3N2 virus or prototypic laboratory-adapted PR8 H1N1virus in human lung tissue (Wu et al., 2012). The authors showed that pandemic H1N1 virus suppressed RIG-I gene and protein expression but there was no difference in the proinflammatory response such as IL-6 gene expression. A comprehensive study analyzed the transcriptional response in human moDCs infected with pandemic H1N1 viruses (A/Brevig Mission/1/1918, A/California/4/2009) and seasonal H1N1 viruses (A/New Caledonia/20/1999, A/Texas/36/1991) (Hartmann et al., 2015). The authors showed that the 2009 pandemic H1N1 virus induced lower IFN-β gene expression than the seasonal 1999 H1N1 virus; however, the other seasonal H1N1 and the 1918 pandemic H1N1 induced even lower IFN-β gene expression levels. ISGs were induced clearly slower during the 2009 pandemic H1N1 virus infection. They also found a cell response signature that distinguished the seasonal viruses from the pandemic viruses, as the seasonal viruses caused a dramatic mRNA loss in the host cell starting 4 hours post infection (Hartmann et al., 2015). Another transcriptional analysis comparing the early innate immune responses induced by the pandemic H1N1 virus (A/Mexico/4108/2009) to ones induced by a seasonal H1N1 virus (A/Brisbane/59/2007) stated that the pandemic H1N1 virus induced a stronger inflammatory response than the seasonal H1N1 in human bronchial epithelial cells (NHBECs) (Paquette et al., 2014). Similarly, in human macrophages the pandemic H1N1 virus showed higher proinflammatory cytokine expression compared to PR8 H1N1 virus, but lower IFN-α and again, RIG-I expression was attenuated (Ramirez-Martinez et al., 2013).

Clearly there are significant virus strain- and host cell-specific differences in IFN and immune responses elicited by influenza A viruses. It may be that circumventing the early innate immune response and thus inducing less IFN in the host cells is crucial for successfull crossing of the species barrier. A recent paper indicated that the 2009 pandemic H1N1 virus is not able to subvert the innate immune responses in

porcine airway epithelial cells as efficiently as a true swine influenza virus (Krishna et al., 2015). The 2009 pandemic H1N1 strain replaced the seasonal H1N1 strains and still circulates among human population in addition to seasonal H3N2 and influenza B viruses. There is evidence that the pandemic H1N1 virus is acquiring human-adapting mutations which contribute to the severity of the virus infections in patients (Elderfield et al., 2014).

### 5.2.3 Differences in the activation of innate immune responses by different influenza viruses (I, II)

Apparently, influenza viruses activate innate immune responses differently and this variation has been studied from several perspectives: 1) differences between influenza A HA-NA subtypes, 2) pandemic vs. seasonal viruses, 3) avian influenza vs. human influenza, 4) low pathogenic avian influenza (LPAI) vs. HPAI viruses, 5) influenza A vs. influenza B viruses. It seems that in most cell types, influenza virus infection is sensed via RIG-I/MAVS/IRF3 pathway but the subsequent IFN responses elicited by the pathway differ in their kinetics and magnitude. Several mechanisms add to the complexity of the host-pathogen interactions.

One reason for the differences between the virus-induced responses is that not every cell in the population responds similarly to the virus infection. There is stochasticity in every level of the cell signaling pathway commencing from the viral ligand recognition all the way to the transcription of IFN genes (Zhao et al., 2012). Only now the methods in biotechnology have begun to enable gene expression analysis at a single cell level, and advanced live-cell-imaging techniques have revealed cell population dynamics in virus infections. The reasons for the variability in single cell responses are largely unknown. Only a fraction of virus-infected cells produces IFNs and the paracrine signaling from the first infected cell affects the bystander cells (Patil et al., 2015). In addition, the host cell population context affects many aspects of virus infection; whether the cell is growing in the middle of the population or on the edge, what is the cellular state or cell density (Snijder et al., 2009). It is well known that IFNs increase the expression of PRRs and cell signaling components in PRR signaling pathways (Miettinen et al., 2001; Osterlund et al., 2005; Siren et al., 2005; Siren et al., 2006; Tissari et al., 2005). The upregulation of several receptors and cell signaling components can sensitize the bystander cells and lower the threshold for virus-induced IFN expression. Even if IFN and maybe other cytokine responses induced by virus infection are stochastic in the cell population, IFNs induce ISGs in every cell via the JAK/STAT-pathway (Zhao et al., 2012). In the study by Zhao and coworkers, higher amounts of Sendai virus transcription and replication products and defective interfering particles were present in IFN producing cells. Another study using Sendai virus found out that virus MOI affects the IFN induction profile in cells (Zaritsky et al., 2015). This was dependent on the

differential activation of the JAK/STAT signaling via IFNAR by the different virus amounts. Thus when comparing different influenza strains, great care has to be taken in analyzing viral stocks, as differences in the infectivity of the stock virus may affect the results. Furthermore, if one influenza strain replicates more efficiently than the other, it may induce stronger and/or earlier IFN responses.

The replication efficiency does not explain the differences in IFN expression between different influenza A viruses in this study (Fig. 2 in II) as all viruses replicated equally well in human moDCs (Fig. 1B in II). It is more likely that the viruses differ in their ability to inhibit IFN gene expression via their virulence factors or they carry genomic features aimed at evading the functions of host viral restriction factors. Several such features have been described for the 2009 pandemic H1N1 virus. The NS1 of the 2009 pandemic H1N1 efficiently inhibits IFN gene expression; however it is not able to block host gene expression (Hale et al., 2010). Yet, A/California/04/2009 strain expresses PA-X protein that mediates host cell shut-off and inhibits IFN-β expression in the lungs of infected mice (Hayashi et al., 2015). The 2009 pandemic virus also carries mutations in NP protein that provide MxA resistance (Manz et al., 2013). The pH1N1 was highly sensitive to the antiviral actions of type I IFNs as shown by the IFN-dose dependent inhibition of viral M1 gene expression (Fig. 4B in II) and cell surface glycoprotein expression (Fig. 4A in II) in moDCs. The inhibitory effect of IFN pretreatment was similar in pH1N1 virus infected cells and seasonal H1N1 virus-infected cells. Hence, when the antiviral state has been established in the cells, the host cell efficiently restricts the replication of the pandemic virus. This implicates that the pandemic H1N1 virus is not better than the seasonal H1N1 virus in evading the IFN-mediated host antiviral mechanisms.

Although there is significant variance between different influenza virus infections *in vitro*, the clinical picture of influenza virus infections does not always vary accordingly. For example the HPAI H5N1 virus induces high IFN gene expression in the host; nevertheless the so-called cytokine storm is not protective but contributes to higher pathogenicity (de Jong et al., 2006). On the other hand, an avian influenza virus H7N9 that has caused severe disease in humans induces very low IFN responses in host cells (Arilahti et al., 2014). Intriguingly, the H5N1 and the pandemic H1N1 viruses have totally different phenotypes in TLR3 knock-out mice; in TLR3 deficient mice H5N1 is less pathogenic than in wt mice, whereas such a difference is not seen with the pandemic H1N1 virus (Leung et al., 2014). In the host organism many factors influence the outcome of the infection such as the transmission efficiency of the virus, the virus adaptation to the host including receptor binding, polymerase/replication efficiency, virulence factors, the interplay of different innate immune cells, the activation of adaptive immunity, possible cross

reacting antibodies against the virus, host genetic background and other yet unknown factors.

## 5.3 TLR ligand-induced cytokine responses in human immune cells (III, IV)

### 5.3.1 Cooperation of TLR signaling pathways

During the infection, many different PAMPs are likely available for recognition by plethora of PRRs located in different cellular compartments. This redundancy allows for rigorous control over activation of innate immunity which is of utmost importance in clearing the infection but avoiding an inappropriate inflammatory response. Many studies have demonstrated that stimulating cells with two PRR ligands simultaneously leads to a stronger cytokine response than triggering only one receptor. However, only a limited number of genes is regulated by this synergy (Napolitani et al., 2005), one of the most studied being IL-12. We also wanted to address the question of synergy and what mechanisms regulate synergistic cytokine response in human moDCs and macrophages.

First we analyzed the cytokine response in human moDCs (Fig. 1A in III) and macrophages (Fig. 1B in III) by stimulating them with a range of TLR ligands (Table 5) alone or pairwise. In moDCs TLR7/8 ligand R848 together with TLR3 ligand polyI:C or TLR4 ligand LPS induced a synergistic IL-6, IL-10, IL-12p70 and TNF-α cytokine secretion. In macrophages such synergy was not observed as R848 alone induced a maximal cytokine response. When the cytokine gene expression was analyzed by qRT-PCR, also macrophages induced synergistic IL-6, IL-10 and IL-12p35 gene expression at the late time point of 24 hours with the aforementioned TLR ligand combinations. In moDCs the synergy was evident earlier at 9 hour time point. In most studies synergy has been detected when a TLR that employs MyD88 adaptor protein (all TLRs except TLR3) is combined with TLR that is coupled to TRIF adaptor (TLR3 or TLR4) (Bagchi et al., 2007; Bohnenkamp et al., 2007; Gautier et al., 2005; Hume et al., 2001; Napolitani et al., 2005; Sato et al., 2000b; Suet Ting Tan et al., 2013; Yi et al., 2001). Thus it has been postulated that both MyD88-dependent and TRIF-dependent pathways are needed for synergistic signaling. To support this hypothesis, it has been proposed that triggering TLR4, which activates both MyD88 and TRIF pathways, is a synergistic event on its own (Krummen et al., 2010).

Most of the studies analyzing the synergy between different TLR receptors have been examining the proinflammatory cytokine responses. However, the endosomal TLRs recognizing nucleic acid structures are important in detecting viral infections. Therefore we wanted to study whether also IFNs are produced synergistically in

response to dual TLR stimuli. Indeed, we detected synergistic IFN-α, IFN-β, IFN-λ1 and IFN-λ2/3 gene expression in response to polyI:C+LPS, polyI:C+R848 or LPS+R848 in moDCs (Fig. 1A in IV). However, the synergy was not evident in all three time points studied and in 3 hours polyI:C and LPS induced efficient IFN-β and IFN-λ1 on their own. We also analyzed IFN-β and IFN-λ1 protein expression from the cell culture supernatants (Fig. 1C in IV). R848, which did not induce IFN gene or protein expression alone, augmented polyI:C-induced cytokine production. In addition, when IL-12p70 and IFN-λ1 protein expression in moDCs was analyzed after stimulation with polyI:C+R848 and LPS+R848 with several concentrations, IFN-λ1 protein expression was induced efficiently with polyI:C alone but 10 μM R848 increased IFN-λ1 protein expression only weakly (Fig. 5, polyI:C lower panel on the left, LPS lower panel on the right, unpublished). However, polyI:C or LPS did not induce IL-12p70 alone but dose-dependently increased R848-induced IL-12p70 production (Fig. 5, upper panels, unpublished). Clearly, IL-12p70 and IFNs are regulated differently by the synergistic TLR pathways.

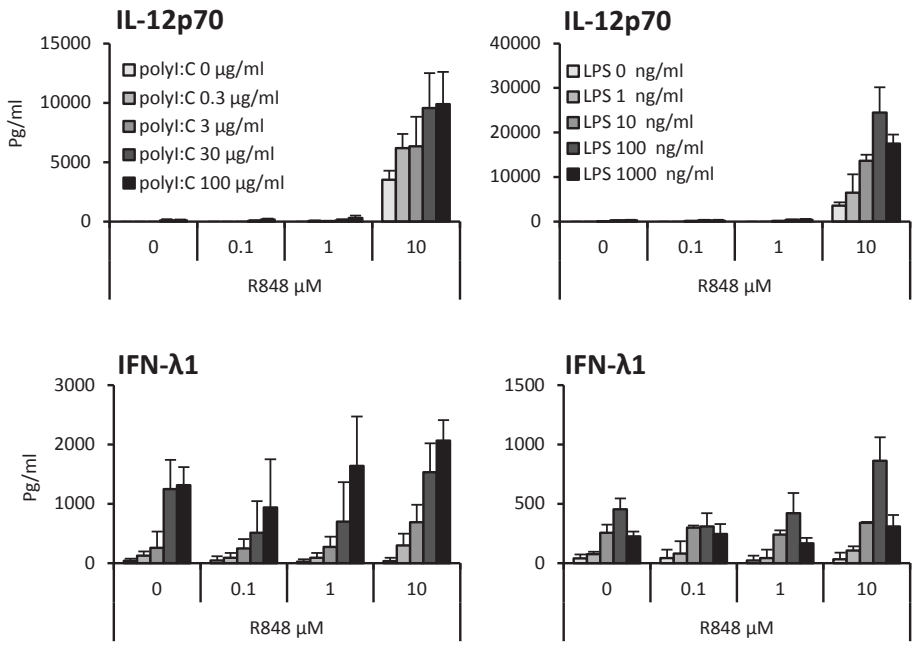


**Figure 5. Dose response of dual TLR ligand stimulation in moDCs.** Cell culture supernatants were harvested from moDCs of three blood donors after stimulation with different TLR ligand combinations for 24 hours with concentrations indicated in the figure. IL-12p70 and IFN-λ1 protein levels (pg/ml) were analyzed with ELISA kits and the data is presented as the means +SD. PolyI:C+R848 on the left panels, LPS+R848 on the right panels.

A recent paper showed that in murine antigen presenting cells, preconditioning cells with polyI:C augmented IFN-α and IFN-β production induced by R848 or TLR9 ligand CpG DNA (Hotz et al., 2015). The authors also stated that although pretreatment with polyI:C increased the cytokine responses induced by another TLR ligand, it inhibited subsequent RLR signaling. In addition, the effect was seen with virus infection which also sensitized the cells for TLR signaling but inhibited signaling via RLR pathway. The authors discussed that after the initial virus infection, blocking of RLR signaling and the sensitizing of TLR7 pathway would serve to induce efficient IL-12 production and subsequent Th1 cell differentiation. Our results support this theory, as in our human cell model polyI:C increased TLR7 and TLR8 expression (Fig. 3A in IV) and IL-12p70 protein was produced efficiently with R848 whereas polyI:C alone induced very little IL-12p70 (Fig. 5, upper left panel). Whether polyI:C treatment can also block the RLR signaling pathway in the human system requires further studies.

### 5.3.2 Transcriptional regulation of IL-12 and IFN-λ1 genes

We went on studying the mechanism of IL-12 synergy in human immune cells. Previously it had been proposed that sustained MAPK c-Jun (Napolitani et al., 2005) or MAPK p38 (Bohnenkamp et al., 2007) signaling is taking place in moDCs stimulated with polyI:C+R848. Furthermore, IFN autocrine-paracrine loop was shown to be involved in the synergistic effect (Gautier et al., 2005). As the promoter region of IL-12p35 was already analyzed (Goriely et al., 2006; Liu et al., 2003; Liu et al., 2004) we carried out oligonucleotide precipitation experiments to analyze the binding of transcription factors to the IL-12p35 ISRE and NF-κB promoter elements in human moDCs and macrophages. In moDCs, polyI:C or R848 both induced the binding of several IRF and STAT proteins to the ISRE site, but polyI:C-induced binding was stronger and took place at earlier time points than R848-induced binding for all IRFs studied except IRF8 (Fig. 4A in III). In contrast, in macrophages polyI:C did not induce the binding of IRF1, IRF3, IRF9 or STAT1 onto IL-12p35 ISRE site (Fig. 5A in III). R848, however induced the binding of IRF1 and IRF3, and the binding of IRF7 and IRF8 was stronger in R848-stimulated cells compared to polyI:C stimulated cells (Fig. 5A in III). Altogether, we did not observe a synergistic binding of transcription factors to the ISRE site in polyI:C+R848 stimulated moDCs but in macrophages the binding of IRF1, IRF7, IRF9 and STAT1 were induced better with the dual stimulus compared to a single stimulus. In moDCs the transcription factor activation took place earlier than in macrophages and was induced via both polyI:C and R848 reflecting the earlier and much stronger IL-12p35 gene expression seen in Fig. 2 in III. IRF1 (Liu et al., 2003), IRF3 (Goriely et al., 2006) and IRF8 (Liu et al., 2004) have been shown to be important for IL-12p35 gene expression and our results confirm these findings.

We also studied IRF1, IRF7 and IRF8 binding to the IFN-λ1 promoter ISRE site in moDCs and the polyI:C+R848 stimulus induced a stronger binding of all these IRFs compared to stimulation with a single ligand (Fig. 6A in IV). Consequently, at the transcriptional level the activation of multiple IRFs via TRIF- and MyD88-dependent pathways may contribute to the synergy. A very recent paper analyzed polyI:C+R848-induced synergistic IL-12p40 and IL-6 gene expression in murine macrophages and showed with oligonucleotide precipitation and siRNA experiments that IRF1, JunB and C/EBPβ transcription factors were responsible for the synergistic effect (Liu et al., 2015b). Probably, in the case of many cytokines, the synergy is regulated at the transcriptional level. Yet an additional enhancing mechanism requires attention and that is chromatin remodeling. Qiao and coworkers showed that IFN-γ is able to prime chromatin by inducing a sustained binding of STAT1 and IRF1 to the TNF, IL-6 and IL-12p40 gene promoter and enhancer regions (Qiao et al., 2013). The priming alone did not induce transcription but enhanced the transcription induced by TLR4 stimulation synergistically. We noticed that polyI:C+R848-stimulation induced ~400 fold induction of IFN-γ mRNA and up to 100 pg/ml IFN-γ protein production in moDCs from some donors (data not shown). Thus IFN-γ-dependent chromatin remodeling may well be a contributing factor in the TLR synergy and it remains to be investigated, whether other IFNs have similar effects.

Many of the MAPK signaling components have been linked to the synergistic cytokine expression. We analyzed the involvement of different MAPK signaling pathways in moDCs with immunoblot analysis of phosphorylated signaling components and treating cells with pharmacological inhibitors. Both polyI:C and R848 induced the phosphorylation of p38, c-Jun (JNK-pathway) and Akt (PI3K-pathway) (Fig. 6A in III). p44/p42 (ERK-pathway) was constitutively phosphorylated until a 9 hour time point, where the phosphorylation declined in the polyI:C- and R848-stimulated cells. Inhibitors of NF-κB, p38 and PI3K completely abolished the TLR ligand-induced IL-12p35 gene expression (Fig. 6B in III) and IL-12p70 protein expression (Fig. 6C in III) but also JNK and ERK inhibitors reduced IL-12 expression to some extent. A study using mouse bone marrow-derived DCs showed an identical inhibition pattern for IL-12p70 induced with LPS+R848 (Mitchell et al., 2010). We did not observe sustained c-Jun phosphorylation (Napolitani et al., 2005), p38 phosphorylation (Bohnenkamp et al., 2007) or ERK phosphorylation (Suet Ting Tan et al., 2013) with polyI:C+R848 stimulus, however we confirm that all these pathways play a role either directly or indirectly in TLR ligand-induced cytokine gene expression.

Very little is known about MAPK pathways in the context of IFN gene expression and it has been thought that IFN gene expression is regulated mainly via IRF, STAT and NF-κB pathways. We treated moDCs with the aforementioned inhibitor panel

and measured polyI:C and R848-induced IFN-β and IFN-λ1 gene expression. Inhibition of PI3K and p38 pathways abolished the TLR-induced IFN gene expression (Fig. 7 in IV). Interestingly, H5N1 influenza virus-induced IFN-β and IFN-λ1 gene expression was also inhibited by p38 inhibitor in human macrophages (Hui et al., 2009) and similarly p38 inhibition reduced H5N1 virus induced IFN-β gene expression in endothelial cells (Borgeling et al., 2014). In addition, a recent extensive study indicated that PI3K and especially p38 pathways have roles in IFN-λ1 gene expression induced by various types of RNA molecules (Jiang et al., 2015). Clearly, more research into the vast network of different MAPK pathways is needed to clarify their role in IFN gene expression.

### 5.3.3 IFN feedback loop in the cooperation of TLR signaling pathways

There are different views of the importance of IFN feedback loop in the cooperation of TLRs. Two groups claimed that IFNs are not responsible for the mechanism of synergy, as stimulating the cells with exogenous IFN-β along with the TLR ligands did not substantially increase IL-12p70 (Napolitani et al., 2005) or TNF (Bagchi et al., 2007) production. Gautier and colleagues, however, showed that in STAT1 or IFNAR knock-out mouse macrophages, synergistic IL-12p70 production was greatly reduced thus pointing to the dependence on IFN feedback loop (Gautier et al., 2005). We studied the synergistic IL-12p35 and IL-12p40 gene expression in moDCs in the presence of IFN-β or IFN-λ1. It has been shown that the synergy is most potent if the TLR3 or TLR4 ligands are added before the TLR7/8 ligand (Napolitani et al., 2005; Suet Ting Tan et al., 2013). Moreover, polyI:C and LPS but not R848 induce a rapid IFN-β and IFN-λ1 gene expression (Fig. 2A in IV). For these reasons we took a different approach and instead of stimulating the cells with IFNs simultaneously with the TLR ligands we primed moDC with IFN-β or IFN-λ1 for 4 hours prior to TLR stimulation. The priming increased TLR-induced IL-12p35 and IL-12p40 gene expression (Fig. 6, unpublished), IFN-β more potently than IFN-λ1. In addition, the priming augmented IFN-β and IFN-λ1 gene expression as well (Fig. 5B in IV). Treating moDCs with antisera against IFN-α/β also decreased the TLR-induced IFN-β and IFN-λ1 gene expression to some extent (Fig.4 in IV).

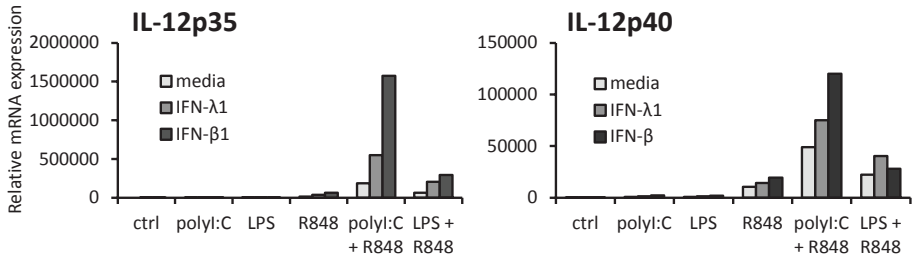


**Figure 6. Priming with IFN increases synergistic IL-12 gene expression in moDCs.** Human moDCs from four blood donors were primed with IFN-λ1 (10 ng/ml) or IFN-β (100 IU/ml) for 4 hours after which the cells were stimulated with polyI:C (30 μg/ml), R848 (10 μM) or their combinations for an additional 8 hours. The cells were pooled and total cellular RNA was isolated for cDNA synthesis and qRT-PCR analysis. The values were normalized against β-actin mRNA and relative mRNA levels were calculated with ΔΔCt-method using untreated cells as a calibrator.

As we know that the expression of TLR3 and TLR7 in human moDC is rather low but induced by IFNs (Osterlund et al., 2005), we studied the TLR gene expression in response to TLR ligand stimulation. PolyI:C induced the expression of TLR3, TLR4, and TLR8 but especially that of TLR7 (Fig. 3A in IV). The polyI:C-induced IFN contributed to TLR gene expression as priming with IFN-β or IFN-λ1 amplified TLR3, TLR7 and TLR8 gene expression (Fig. 5A in IV) and anti-IFN-α/β sera reduced TLR7 gene expression (Fig. 3B in IV). We propose that triggering the TLR3/TRIF pathway induces a rapid IFN gene expression which upregulates TLR and IRF7 (Fig. 5A in IV) expression and sensitizes the cells to TLR7/8 ligation and also amplify the TLR3 pathway signaling, leading to higher IFN-β, IFN-λ1 and IL-12 expression (Fig. 7). Supporting our hypothesis a very recent study using sorted human $BDCA^+$ DCs and pDCs showed that IFN-dependent upregulation of TLR7 (BDCA+ DCs) or TLR3 (pDCs) was involved in synergistic IL-6 production induced by R848 and polyI:C (Kreutz et al., 2015).

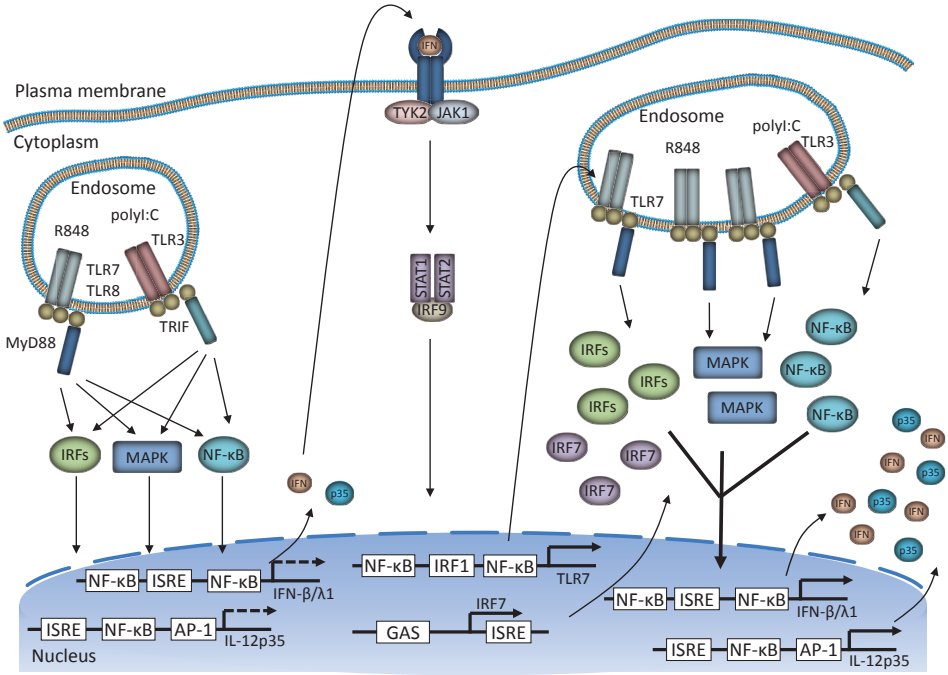


**Figure 7. A proposed model for TLR synergy.** First, triggering the TLR3 and TLR7/8 pathways leads to the activation of IRF-, MAPK- and NF-κB-pathways. The TLR7/8/MyD88-pathway induces the IL-12p35 expression and the TLR3/TRIF pathway induces IFN gene expression. The autocrine IFN signaling activates ISGF3 (STATs+IRF9) transcription factor that subsequently activates TLR7 and IRF7 gene expression and protein production. These will further amplify the TLR signaling pathways leading to stronger signaling and higher production of IFNs and IL-12p35 in later time points.

In summary, the synergistic activation of TLRs takes place when both MyD88- and TRIF-dependent pathways are triggered. These pathways lead to the activation of partially overlapping signaling cascades (Fig. 3) leading to an optimal activation of IRF, NF-κB and MAPK-pathways. IFNs induced mainly via TRIF-dependent pathway further enhance the synergistic cytokine gene expression by upregulating TLRs and downstream components of the signaling pathways such as transcription factors. The production of proinflammatory cytokines and IFNs can however lead to immunopathology. That is why the host must carefully control the expression of these mediators and induce the production only if the pathogen is sensed via different receptors in several cellular compartments. Furthermore, both positive and negative feedback loops in the cell signaling offer means of rigorous regulation.

# 6 Concluding remarks

In this thesis influenza virus- and TLR ligand-induced IFN and cytokine gene expression was studied in human primary macrophages and moDCs. We studied the very early virus-host interaction and induction of IFNs by influenza B virus in human macrophages. We found clear differences in the early innate immune activation by influenza B virus compared to influenza A virus. Influenza B virus induced early IFN gene expression and IRF3 activation upon entry into macrophages. In contrast these early responses were absent in influenza A virus-infected macrophages. Furthermore, we characterized IFN responses induced by different H1N1 influenza viruses compared to the novel 2009 pandemic H1N1 influenza and showed that the pandemic virus was highly sensitive to the actions of IFNs. Finally, we found differences between cell types (macrophages vs. moDCs) in the regulation of TLR ligand-induced cytokine gene and protein expression. MoDCs were more potent inducers of the synergistic IL-12 cytokine expression compared to macrophages and the difference was reflected to the transcription factor activation induced by different TLR ligands. Our studies illuminated and further confirmed cell signaling mechanisms involved in the synergistic IL-12 and IFN gene expression in the human system.

Despite the great advances in elucidating mechanisms of innate immune system activation during the past decade, many unanswered questions remain. There is still a great many receptors without known ligands and most likely additional PPRs are still to be found. Even though many innate immune signaling pathways are well defined, controversy exists, not least because of cell-type or organism-specific regulation. We have just started to realize the complexity of host-pathogen interactions of seemingly simple pathogens, such as viruses. The multitude of different receptors, signaling pathways and cytokines forming complex networks sets up a need for systems biology approach. A great deal of basic research into the innate immunity is necessary to establish the framework for further translational research.

The advantages of defining PRR-triggered cell signaling pathways leading to the activation of innate immunity are numerous. Already various types of TLR ligands are used as vaccine adjuvants (Maisonneuve et al., 2014) and in the future, cocktails of different PRR ligands could be used to induce optimal humoral and cellular immunity. Secondly, efficient DC-activating adjuvants are greatly needed for the use of DC-based therapy against cancer (Bloy et al., 2014). Thirdly, TRL7/8 ligand Imiquimod is already in use for the treatment of warts and skin cancer, thus detailed knowledge of PRR signaling pathways may give rise to additional antiviral or

anticancer treatment modalities. Fourthly, thorough understanding of influenza virus-induced cytokine gene expression may shed light on the mechanisms of hypercytokinemia induced by highly pathogenic influenza viruses and direct the development of novel cures for infectious diseases in general. Finally, it is becoming increasingly clear that the innate immune responses are closely coupled to almost every human disease including autoimmune diseases, infectious diseases and cancer. Elucidating the mechanisms of host-pathogen interactions and innate immunity is crucial for learning to manipulate these mechanisms for the benefit of human health.

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Sanna Mäkelä
Helsinki, February 2016

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