Avian influenza virus infection in human innate immune cells

Veera Westenius
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A doctoral dissertation completed for the degree of Doctor of Science (Technology) to be defended, with the permission of the Aalto University School of Chemical Engineering, at a public examination held at the lecture hall M1 of the school on 22 March 2019 at 12.

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Influenza A viruses are one of the most significant pathogens to humans. These viruses cause recurrent epidemics and occasional devastating pandemics. Avian influenza viruses (AIVs) circulate constantly among birds and some of these viruses are able to infect humans. Most human cases have been caused by H7N9, H5N1, H7N7 and H9N2 subtypes of influenza A viruses. H9N2 virus infections have been generally mild in humans. H5N1 and H7N9 viruses have caused a severe acute respiratory distress syndrome (ARDS) in several patients, and among humans, mortality rates are around 50% and 40%, respectively. AIVs pose a major pandemic threat, because practically all humans lack pre-existing immunity against these viruses. Innate immune responses are the first barrier against AIV infections prior to adaptive immunity being activated. In the present study, innate immune responses have been characterized in human primary innate immune cells induced by avian influenza viruses. H5N2, H7N3, H7N9 and H9N2 low pathogenic AIV strains were used, which had been isolated either from birds or humans and highly pathogenic AIV (H5N1) strains of human origin.

Macrophages and dendritic cells are the central cells of innate immunity. The present study shows that avian isolates of H5N2, H7N3 and H9N2 viruses are able to infect and replicate in human dendritic cells. Also, human isolates of H5N1, H7N9 and H9N2 viruses infected and replicated in dendritic cells, but in their ability to spread in human immune cells there was remarkable difference between different virus subtypes. It was evident, that the H5N1 viruses were able to spread extremely efficiently and eventually infected the whole cell culture starting from very low virus dose. H5N1 virus infection was productive in human monocyte-derived macrophages and dendritic cells, indicating that the infection produced new infective virus particles. Seasonal influenza H3N2 virus and H7N9 AIV infections seemed to be productive only in macrophages but not in dendritic cells. It was also noted that H5N1 virus particles were more often propagation competent than H3N2 or H7N9 viruses. In addition, innate immune responses induced by the different influenza virus strains were investigated. The data shows that low pathogenic H5N2, H7N3 and H9N2 AIVs induced interferon and pro-inflammatory cytokine responses and antiviral protein expression in a similar fashion as the seasonal influenza H3N2 virus. Surprisingly, interferon and pro-inflammatory cytokine responses were impaired in the H7N9 virus infection. In contrast to the H7N9 virus-induced deficient responses, H5N1 virus triggered very strong cytokine responses, which is known as a "cytokine storm".

In conclusion, the results of these studies show that currently circulating avian influenza viruses are able to infect human cells. The results also indicate that H5N1 and H7N9 viruses interfere with human innate immune signaling but by totally different mechanisms.

**Keywords** Influenza A virus, avian influenza, macrophages, dendritic cells, innate immunity


**ISSN (printed)** 1799-4934  **ISSN (pdf)** 1799-4942

**Location of publisher** Helsinki  **Location of printing** Helsinki  **Year** 2019

**Pages** 137  **urn** http://urn.fi/URN:ISBN:978-952-60-8424-4
The work reported in this thesis was carried out at the Expert Microbiology Unit, Department of Health Security, National Institute for Health and Welfare (THL) in Helsinki during the years 2012-2018. Some experiments were also made at the Finnish Food Safety Authority (Evira) in Helsinki. Juhani Eskola and Markku Tervahauta, the former and present heads of the institute, and the head of the unit Carita Savolainen-Kopra are thanked for providing excellent working facilities. I am grateful to the Sigrid Jusélius Foundation, the Academy of Finland, the Jenny and Antti Wihuri Foundation, the Finnish Cultural Foundation, the Foundation for Research on Viral Diseases, and the Oskar Öflunds Stiftelse sr for their financial support.

My greatest gratitude goes to my thesis advisors, docent Pamela Österlund and professor Ilkka Julkunen. Pamela, you have been an amazing supervisor and scientist and I could not even dream of a better supervisor than what you have been. Thank you for having been there for me when I have needed guidance. Ilkka thank that you gave an opportunity to a technical student and took me in your group and always supported and encouraged me even through difficult times. Pamela and Ilkka, it is a pleasure to work with you! I would also like to thank my supervising professor Katrina Nordström who had lead me to the world of microbiology when I started my studies in TKK, and has always been so friendly and helpful to me.

The preliminary examiners of my thesis, professor Søren Paludan and associate professor Juha Huiskonen, are thanked for reviewing this thesis and fluent review process. I am grateful to docent Thedi Ziegler for the idea to start to study avian influenza viruses; it has been an extremely interesting area!

I thank deeply the former and present members of our lab. The members of “juniorihuone” made life inside and outside the lab much funnier. Janne, Esa, Sinikka and Laura thanks for all the ”kuukausiskumppa” and ”leffamaraton” events and also for the support and help that you have given to me in the lab. Sanna and Krister, thank you that you have guided and helped me in the microscopy. I want to thank Hanna for all the trouble and time that you have used for me in BSL3, it really means a lot for me that there is someone who I trust so much as you. I am grateful also for the all present and former technical people in our lab who have contributed to my work, especially Nina, Jossu, Riitu and Teija. I would also like to thank people from Evira (present Finnish Food Authority), especially Anita Huovilainen, for rewarding collaboration.
I want to thank the groups of my dearest friends, “maailman pienimmät
teekarit” and “Puumalan tytöt”. Sarita, Kaisa, Maija and Tiia, thanks that you
have shared the student times and “swing in the sine waves” with me and also
life after student days. Pia and Anu, I am very happy that you have been my
friends through our life. Thanks that all you six girls have shared the sorrows
and happiness of life with me in spite of distance, you are important to me. I
am very grateful to Emil for the support that you have given me during these
years and that you have so positive an attitude towards my thesis project. Eri-
tyisen kiitollinen olen äitille ja iskälle, Miinalle sekä Maija-mimmolle, että
olette kaikki nämä vuodet tukeet ja kannustaneet minua opinnoissani. Äiti
ja iskä, ilman teidän tukea en olisi nyt tässä enkä voi ikinä kiittää teitä kaikesta
kylläksi. And last but not least, I want to thank with all of my heart my beloved
partner Antti for his love, patience and encouragement during the last year. I
am fortunate to have you in my life and that I can share it with you. Together,
with hand in hand we can walk through both sunny and cloudy days.

Helsinki, 31 January 2019
Veera Westenius
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<th>Full Form</th>
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<tbody>
<tr>
<td>AIV</td>
<td>Avian influenza viruses</td>
</tr>
<tr>
<td>aMφs</td>
<td>Alveolar macrophages</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein-1</td>
</tr>
<tr>
<td>BSL</td>
<td>Biosafety level</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase activation and recruitment domains</td>
</tr>
<tr>
<td>CCL3</td>
<td>Chemokine (C-C motif) ligand 3</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional DC</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>ECDC</td>
<td>European Centre for Disease Prevention and Control</td>
</tr>
<tr>
<td>E-MEM</td>
<td>Eagle's Minimum Essential Medium</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>Evira</td>
<td>Finnish Food Safety Authority (since 1/1/2019 the Finnish Food Authority)</td>
</tr>
<tr>
<td>FC</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FFU/ml</td>
<td>Focus forming units per milliliter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid</td>
</tr>
<tr>
<td>HPAI</td>
<td>Highly pathogenic avian influenza</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A viruses</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence assay</td>
</tr>
<tr>
<td>IFIT</td>
<td>IFN induced protein with tetratricopeptide repeats</td>
</tr>
<tr>
<td>IFITM</td>
<td>IFN-induced transmembrane protein</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferons</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>IRAT</td>
<td>Influenza risk assessment tool</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>IFN-stimulated gene</td>
</tr>
<tr>
<td>ISGF3</td>
<td>Interferon-stimulated gene factor 3</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-stimulated response element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenic avian influenza</td>
</tr>
<tr>
<td>M1/2</td>
<td>Matrix 1/2 protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signalling protein (is also known interferon-beta promoter stimulator 1 (IPS-1)/ CARD adaptor inducing IFN-β (CARDIF) / virus-induced signalling adaptor (VISA))</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MGL</td>
<td>MΦ galactose-like lectin</td>
</tr>
<tr>
<td>MMR</td>
<td>MΦ mannose receptor</td>
</tr>
<tr>
<td>moDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>moMΦ</td>
<td>Monocyte-derived macrophage</td>
</tr>
<tr>
<td>MΦs</td>
<td>Macrophages</td>
</tr>
<tr>
<td>MxA</td>
<td>Human myxovirus resistance protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-kappa-B essential modulator</td>
</tr>
<tr>
<td>NEP/NS2</td>
<td>Nuclear export protein/non-structural protein 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like receptor family pyrin domain containing 3</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide binding and oligomerization domain</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS1</td>
<td>Non-structural protein 1</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligoadenylate synthetase</td>
</tr>
<tr>
<td>P</td>
<td>Phospho</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PB1/2</td>
<td>Polymerase basic 1 or 2 protein</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid DC</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptors</td>
</tr>
<tr>
<td>PVDF</td>
<td>Hybond-P polyvinylidene difluoride</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene-I</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor-interacting protein 1</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SA</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF family member–associated NF-κB activator</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
</tbody>
</table>
TCID50  Tissue culture infective dose 50
Th    T helper
THL   National Institute for Health and Welfare
TIR   Toll/interleukin-1 receptor
TLR   Toll-like receptor
TNF   Tumor necrosis factor
TRAF  TNF receptor-associated factor
TRIF  Toll-IL-1 receptor domain (TIR)-containing adapter inducing IFN-β
TRIM25 TRIM-containing protein 25
TYK2  Tyrosine kinase 2
UD    Unpublished data
vRNA  Viral RNA
vRNP  Viral ribonucleoprotein
WB    Western blotting
WHO   World Health Organization
wt    Wild type
List of publications

This doctoral dissertation consists of a summary and of the following publications which are referred to in the text by their Roman numerals. Additionally, some unpublished results are presented.

Veera Westenius (née Arilahti): Westenus, V., Arilahti, V.


Author’s contribution

**Publication 1:** Efficient replication and strong induction of innate immune responses by H9N2 avian influenza virus in human dendritic cells

The author designed the study together with PÖ and IJ. The author performed the experiments and analysed the results with technical support from SM and PÖ. The author wrote the paper and the editing of the paper was done with PÖ and IJ. SM and TZ commented on the paper.

**Publication 2:** Novel avian influenza A (H7N9) virus induces impaired interferon responses in human dendritic cells

The study was designed by the author, PÖ and IJ. The author performed the experiments and analysed the results with technical support from PÖ, SM and JT. The author and PÖ wrote the paper and the editing of the paper was done with author, PÖ and IJ. SM and JT commented on the paper.

**Publication 3:** Highly pathogenic H5N1 influenza A virus spreads efficiently in human primary monocyte-derived macrophages and dendritic cells

The author designed the study together with PÖ and IJ. The author performed the experiments and analysed the results with minor technical support from SM and PÖ. The author wrote the paper and the editing of the paper was done with PÖ and IJ. SM commented on the paper.
1. Introduction and objectives of the thesis

The main objective of this thesis was to assess the pandemic potential of avian influenza viruses by studying viral replication and spreading of, and innate immune responses induced by avian influenza viruses in human immune cells. As the experimental part of the thesis was performed with dangerous avian influenza viruses in a bio safety level 3 laboratory, the additional aim was to develop biosafety practices and methods to increase the preparedness for emerging viral threats in Finland.

The specific objectives of this study were:

1. To compare innate immune responses in human immune cells induced by seasonal influenza and low pathogenic avian influenza viruses.
2. To characterize innate immune responses in human primary innate immune cells induced by avian influenza strains that have shown high mortality in humans.
3. To characterize highly pathogenic avian influenza virus replication and spreading in human primary macrophages and dendritic cells.
2. Background

Influenza viruses are one of the most significant human pathogens, causing approximately 1 billion human cases and 300 000-500 000 deaths each year. The first influenza virus was isolated from a human patient in 1933, but it is likely that influenza viruses have been amongst us much longer. Hippocrates described an epidemic in 412 BC which is usually cited as the first influenza epidemic of human history. Since then, influenza viruses have caused multiple epidemics and worldwide pandemics. Influenza pandemics have occurred at irregular intervals and the most devastating influenza pandemic occurred in 1918 when an estimated 50 million people died. Although influenza viruses can infect many different host species, including humans, cats, dogs, pigs, horses and marine mammals, aquatic birds are the natural reservoir of influenza A viruses (IAV). Avian influenza viruses (AIVs), such as H5N1 or H7N9 subtypes, have infected humans with a mortality rate of as high as 50%, posing a major zoonotic threat to humans. Furthermore, all IAV pandemics in the 20th century have been of avian origin.

In humans, the epithelial cells of the respiratory tract are the primary target of influenza infection. However, in close proximity to the lung epithelium reside alveolar dendritic cells (DCs) and macrophages (Mφs) and influenza infection can spread to these immune cells as well. DCs and Mφs are known to be the key cell types to orchestrate effective host innate immune responses against a virus. Host innate immunity provides the first barrier against a virus and initiates pro-inflammatory responses. Interferons were discovered by Isaacs and Lindenmann in 1957, which was one of the most significant discovery in biology in the past century. The expression of interferons (IFNs) inhibits viral replication and promotes further activation of innate immunity. During the later stages of infection adaptive immunity takes care of the clearance of the virus. DCs have an essential role in linking the innate and adaptive immunity by acting as antigen presenting cells inducing the proliferation and activation of T cells.

2.1 Influenza A virus

Influenza A virus, together with influenza B, C and D viruses, belong to the Orthomyxoviridae family. IAV is a single-stranded and segmented negative-sense ribonucleic acid (RNA) virus. The genome of IAV contains eight segments which each encode at least one protein (ten essential proteins and
several strain-dependent accessory proteins) which are presented in Table 1. Each of the eight viral RNA (vRNA) segments is coated with numerous copies of viral nucleoprotein (NP) and complexed with a single polymerase complex, which consists of polymerase basic (PB)1, PB2, and polymerase acidic (PA) subunits. vRNA segments and polymerase complex form viral ribonucleoprotein (vRNP) –complexes. Eight vRNPs are located inside the virus particle. In addition to the RNP core, the influenza virus particle consists of a lipid envelope and a matrix protein layer. The matrix layer is composed of matrix (M) 1 protein. The envelope includes a host-derived lipid bilayer membrane. Viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) and in a lesser amount the ion channel M2 protein are embedded into the viral envelope. The envelope also contains host membrane proteins including both cytoplasmic and membrane-bound proteins. Also, it has been shown, that the nuclear export protein/non-structural protein 2 (NEP/NS2) is found in purified virions.

Table 1. Gene segments and proteins of influenza A virus.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Gene</th>
<th>Protein</th>
<th>Essential protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB2</td>
<td>PB2</td>
<td>Yes</td>
<td>Polymerase subunit, binding of the cap on host pre-mRNAs¹⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB2-S1</td>
<td>No</td>
<td>Modulates host innate immune responses, unknown function</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB2Δ¹⁹</td>
<td>No</td>
<td>Modulates immune responses</td>
</tr>
<tr>
<td>2</td>
<td>PB1</td>
<td>PB1</td>
<td>Yes</td>
<td>Polymerase subunit, addition of nucleotides during elongation¹⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PB1-N40</td>
<td>No</td>
<td>Probably affects viral replication</td>
</tr>
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<td></td>
<td></td>
<td>PB1-F₂</td>
<td>No</td>
<td>Apoptosis regulator, modulates host immune responses, regulates polymerase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PA</td>
<td>PA</td>
<td>Yes</td>
<td>Polymerase subunit, endonuclease activity¹⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PA-N182</td>
<td>No</td>
<td>Role in efficient virus replication</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PA-N155</td>
<td>No</td>
<td>Role in efficient virus replication and in virus pathogenicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PA-X²⁸-³⁰</td>
<td>No</td>
<td>Host shut-off activity, modulates host immune responses</td>
</tr>
<tr>
<td>4</td>
<td>HA</td>
<td>HA</td>
<td>Yes</td>
<td>Surface glycoprotein, receptor binding, fusion activities, budding, particle assembly¹⁴</td>
</tr>
<tr>
<td>5</td>
<td>NP</td>
<td>NP</td>
<td>Yes</td>
<td>RNA binding in RNP complex, viral RNA synthesis, RNP nuclear import¹⁴</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>Surface glycoprotein, enzymatic activity in virus release and prevention of aggregation¹⁴</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>M1</td>
<td>Yes</td>
<td>Matrix protein¹⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2</td>
<td>Yes</td>
<td>Membrane protein, ion channel¹⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M42</td>
<td>No</td>
<td>Can functionally replace M2</td>
</tr>
<tr>
<td>8</td>
<td>NS</td>
<td>NS1</td>
<td>Yes</td>
<td>Multifunctional protein¹⁴</td>
</tr>
</tbody>
</table>
IAVs are classified on the basis of the antigenic properties of HA and NA proteins into subtypes. To date, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) have been isolated from birds. In addition, subtypes H17 and H18 and N10 and N11 have been isolated from bats, but it seems that these H17N10 and H18N11 subtypes do not use sialic acids (SA) as their receptors (cf. 2.1.1 Influenza A virus life cycle). Despite numerous possible combinations of HA and NA proteins, currently only H1N1 and H3N2 subtypes are endemic in humans, and H2N2 virus circulated amongst humans from 1957 to 1968. Although all seasonal and pandemic influenza strains in the 20th and 21st centuries have been H1N1, H2N2 or H3N2 subtypes, many other subtypes have also been isolated from humans. These are presented in section 2.2.2 Avian influenza virus infections in humans.

Constant evolution of the HA and NA of IAVs is caused by antigenic drift and antigenic shift. Antigenic drift involves the accumulation of mutations in HA and NA genes. The amino acid changes enable the reinfection of the same host because antibodies against the original strain are unable to neutralize the mutant virus. Periodic epidemics are caused by antigenically drifted viruses, whereas antigenic shift is responsible for the emergence of IAV pandemic viruses. In antigenic shift, a new subtype of IAV emerges in human population by the exchange of vRNAs between two different strains during a coinfection in the same cell.

### 2.1.1 Influenza A virus life cycle

IAVs can be transmitted through airborne aerosols, large droplets or direct contact. IAVs replicate mainly in the epithelial cells of the respiratory tract in humans and other mammals or in the intestinal tract in birds. The HA protein of IAV binds to the terminal sialic acid residues of the glycoproteins or glycolipids, which are expressed on the surface of the cells. Recently, a novel motile mechanism has been found in IAVs. Using cooperation of HA and NA, IAV moves across the cell surface and migrates to the proper sialylated SA. It is generally accepted that the HA of avian influenza viruses have higher specificity for the \(\alpha\)-2,3-linked SAs whereas the HA of human IAVs prefer \(\alpha\)-2,6-linked SAs. In agreement with this is the observation that \(\alpha\)-2,6-linked SAs are predominantly expressed in the human upper respiratory tract, while \(\alpha\)-2,3-linked SAs are expressed in the human lower respiratory tract as well as in the upper and lower respiratory tracts and in the intestinal tract of most avian species, such as in ducks. However, some human isolates of IAVs like H9N2 from 1999, low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) H7N9 and 2009 pandemic H1N1 strains with D225G mutation in their HA are capable of binding to both \(\alpha\)-2,3- and \(\alpha\)-2,6-linked SAs. In addition, human and avian cells can contain both \(\alpha\)-2,3- and \(\alpha\)-2,6-linked SAs. In addition to SA, it was found that IAVs can also utilize C-type lectin and epidermal growth factor receptors. After binding to the receptor, IAV en-
ters into the cell via endocytosis. Endocytosis can occur by clathrin-mediated endocytosis\textsuperscript{51, 52} or by micropinocytosis (clathrin-independent pathway)\textsuperscript{53, 54}. After endocytosis, viral particles are released from late endosome by a pH-dependent physiological event. The low pH of endosome opens the M2 ion channels causing conformational changes in HA, acidifying the viral particle, uncoating and releasing vRNPs to the cytoplasm.\textsuperscript{14} In order for the fusion between viral and endosomal membrane to occur, the precursor form of the HA has to be cleaved into HA1 and HA2 subunits by host proteases\textsuperscript{55}. The optimal pH for the fusion has been shown to vary between HA subtypes, and human isolates require lower pH for the fusion than the avian isolates\textsuperscript{56, 57}. The viral RNA is imported into the nucleus by interaction between the nuclear localization signal of the NP proteins and the cellular importin-α/β\textsuperscript{58-60}. In the nucleus, the vRNAs are used as a template in the transcription of viral mRNAs followed by the productions of viral proteins. The viral polymerase complexes (PA, PB1, and PB2) start the primary transcription of viral mRNA\textsuperscript{61}. By cap snatching mechanism the polymerase complex obtains the primers, which are generated from host cell mRNAs, and this requires cooperation with cellular RNA polymerase II\textsuperscript{62, 63}. Viral mRNAs are transported into the cytoplasm for translation of viral proteins. After mRNA synthesis and translation, the newly synthesized PB1, PB2, PA and NP proteins are transported into the nucleus, and virus replication starts in the nucleus. The replication involves the transcription of positive-sense complementary RNA (cRNA) and the transcription of vRNA using cRNA as a template. In the nucleus viral proteins and vRNAs are assembled into vRNPs which are exported to the cytoplasm. The vRNPs are transported to the cell membrane for the viral assembly and new virus particles bud out from the cell. Although the segmented genome allows genetic reassortment and thus evolutionary advantage to IAVs, still all eight vRNAs must be packed into progeny virions for the virions to be replication-competent and thus fully infectious. It has been proposed that vRNAs of IAV are packed in a specific manner by a selective packaging mechanism\textsuperscript{64} but the mechanism is not fully characterized. Several studies have suggested that vRNPs are in a “1+7” configuration where one vRNP is surrounded by the seven other vRNPs\textsuperscript{65, 66}. In addition, it has been proposed that the association of NP with vRNA contribute to the virion packaging\textsuperscript{67, 68}, but, on the other hand, it has been postulated that specific packaging would rather be mediated by selective RNA-RNA interactions between vRNPs\textsuperscript{59, 70}. However, a recent study shows that within IAVs, 80% of the virions contain all eight vRNPs and that the exact proportion is strain specific\textsuperscript{71}.

2.2 Avian influenza viruses

2.2.1 Highly and low pathogenic avian influenza viruses

Avian influenza viruses are classified as highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) viruses based on the pathogenicity in chickens or by the molecular characteristics of the HA protein. The
World Organisation for Animal Health has defined the HPAI viruses as follows:

“Any influenza A virus that is lethal for six, seven or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid”

or

“any influenza A virus that has an intravenous pathogenicity index (IVPI) greater than 1.2.”

In addition:

“For all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequence of the connecting peptide of the haemagglutinin must be determined. If the sequence is similar to that observed for other HPAI isolates, the isolate being tested will be considered to be HPAI.”

To date all known HPAI viruses belong to H5 and H7 subtypes but only part of the H5 and H7 subtypes are HPAI. The classification of HPAI or LPAI virus do not take a stand on pathogenicity in humans, but usually human infections with HPAI viruses are more severe than infections with LPAI viruses. However, an exception is the H7N9 virus that emerged in humans in 2013. That was the first time when an LPAI virus was reported to cause severe clinical symptoms in humans like HPAI viruses. It was noticed that patients with LPAI H7N9 infection developed a severe pneumonia and an acute respiratory distress syndrome like in HPAI H5N1 infection.

2.2.2 Avian influenza virus infections in humans

Thus far H1N1, H2N2 and H3N2 IAV subtypes have caused epidemics and pandemics among humans. However, several different subtypes of AIV have caused human infections, and these subtypes are presented in Table 2. These viruses are typically transmitted directly from avian species to humans by close human contact to infected poultry, and transmission from human to human has been very limited. At least H5, H6, H7, H9 and H10 hemagglutinin subtype viruses have caused human infections, but the H5, H7 and H9 subtypes are predominant. The outcome of a human infection with H9N2 or other LPAI viruses, except those caused by the H7N9 virus, is typically a mild influenza-like illness with symptoms such as fever, cough, mild dehydration, coryza, vomiting, poor appetite and inflamed oropharynx. Only one fatal case has been reported among human infections with H9N2 subtype. However, infections with H5N1 or H7N9 subtypes have been associated with severe respiratory illness and infection in the lungs which might be observed as a severe pneumonia and an acute respiratory distress syndrome (ARDS). Human infections with H5N1 virus were reported for the first time in 1997, and to date H5N1 virus has caused 878 human cases including 460 fatal cases giving a mortality rate of approximately 50%. H7N9 virus was first detected in humans in 2013 and to date it has caused 1567 human infections including 615 fatal cases with a mortality rate of 40%. The emergence of H7N9 virus in humans was the first time when it was noticed that LPAI virus can cause a severe disease like HPAI H5N1 virus. In the beginning of 2017 H7N9 virus pos-
sessing multi-basic HA cleavage site was isolated from human patients. Multi-basic HA cleavage site contains several basic amino acids in the cleavage site and can be cleaved by ubiquitously expressed furin-like proteases. To date 32 isolates of HPAI H7N9 viruses have been isolated from humans including 16 fatal cases. An overview of human infections caused by avian influenza viruses are presented in the Table 2.

Table 2. Avian origin influenza viruses, excluding H1N1, H2N2 and H3N2 subtypes, isolated from humans and confirmed in the laboratory between 1996 to November 2018.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Year</th>
<th>Location</th>
<th>Pathogenicity</th>
<th>Number of human cases</th>
<th>Number of deaths</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5N1</td>
<td>1997</td>
<td>16 different countries</td>
<td>HPAI</td>
<td>878</td>
<td>460</td>
<td>6</td>
</tr>
<tr>
<td>H5N6</td>
<td>2014</td>
<td>China</td>
<td>HPAI</td>
<td>22</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>H6N1</td>
<td>2013</td>
<td>Taiwan</td>
<td>LPAI</td>
<td>1</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>H7N2</td>
<td>2002</td>
<td>USA, UK</td>
<td>LPAI</td>
<td>7</td>
<td>0</td>
<td>88, 89</td>
</tr>
<tr>
<td>H7N3</td>
<td>2004</td>
<td>Canada, UK, Mexico</td>
<td>HPAI, LPAI</td>
<td>5</td>
<td>0</td>
<td>90-92</td>
</tr>
<tr>
<td>H7N4</td>
<td>2017</td>
<td>China</td>
<td>LPAI</td>
<td>1</td>
<td>0</td>
<td>6, 93, 94</td>
</tr>
<tr>
<td>H7N7</td>
<td>1996</td>
<td>UK, Netherlands, Italy</td>
<td>HPAI, LPAI</td>
<td>93</td>
<td>1</td>
<td>95-97</td>
</tr>
<tr>
<td>H7N9</td>
<td>2013</td>
<td>China</td>
<td>LPAI, HPAI</td>
<td>1567</td>
<td>615</td>
<td>6</td>
</tr>
<tr>
<td>H9N2</td>
<td>1999</td>
<td>China, Bangladesh, Egypt</td>
<td>LPAI</td>
<td>45</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>H10N7</td>
<td>2004</td>
<td>Egypt, Australia</td>
<td>LPAI</td>
<td>9</td>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td>H10N8</td>
<td>2013</td>
<td>China</td>
<td>LPAI</td>
<td>3</td>
<td>2</td>
<td>99</td>
</tr>
</tbody>
</table>

* Year of the first occurrence in humans

### 2.2.3 Avian influenza viruses in Finland

Thus far, in Finland, there have been no reported human cases with avian influenza viruses. However, according to a screening in 2006-2010 and in 2014 by the Finnish Food Safety Authority (Evira), 12 different subtypes of avian influenza viruses were detected in wild waterfowl in Finland, including H5N2, H7N3, H9N2 and H3N8 subtypes, and all strains were LPAI viruses. In 2016, for the first time, Evira reported an HPAI virus in Finland as HPAI H5N8 virus was isolated from tufted duck on the island of Åland. Since then, a total of 17 HPAI H5N8 viruses have been isolated from several different bird species also from the mainland of the west coast of Finland. In 2018, HPAI H5N6 subtype was detected from Finnish birds. According to the European Centre for Disease Prevention and Control (ECDC), HPAI H5N8 and H5N6 viruses have been circulating in Europe, but fortunately no transmission to humans has observed in Europe. These H5N6 and H5N8 viruses detected in Europe in birds and poultry do cluster in clade 2.3.4.4.b based on the HA sequences, which is different from the clade 2.3.4.4.c of the HPAI H5N6 virus...
associated with human infections in China. There has been no mammalian adaptation detected among the European H5N6 viruses. Also, a recent study with the HPAI H5N8 clade 2.3.4.4.b virus shows that the H5N8 virus is not transmissible between ferrets and it replicates poorly in human lung cell cultures indicating only a low zoonotic potential.

2.2.4 Pandemic potential of avian influenza viruses

In the last century H1, H2 and H3 IAV subtypes have possessed the ability for efficient transmission among humans. Section 2.2.2 presents subtypes of AIVs which have infected humans. The IAV strain of zoonotic origin has the ability to cause a possible epidemic or pandemic, because humans lack pre-existing immunity against these strains, which are antigenically different from previously circulating strains. Indeed, all IAV pandemics in twentieth century have been of avian origin.

U.S. Centers for Disease Control and Prevention (CDC) has carried out an Influenza Risk Assessment Tool (IRAT) to assess the potential pandemic risk posed by influenza A viruses. Evaluation in 2011-2014 showed the highest potential pandemic risk for the LPAI A/Hong Kong/125/2017 (H7N9) virus with “moderate to high” risk. A/Vietnam/1203/2004 (H5N1) and A/Yunnan/14564/2015 (H5N6) – like viruses were evaluated as “moderate” risk. Thus, here are summarized some of the factors which affect to the pandemic potential of an avian influenza virus focusing on the LPAI H7N9 and HPAI H5N1 and H5N6 viruses as well as the HPAI H7N9 virus which emerged after the IRAT evaluation.

At least H5, H6, H7, H9 and H10 subtype viruses have shown sporadic and repeated inter-species transmission to humans (cf section 2.2.2) and thus are considered to represent pandemic threats. Wild waterfowl are regarded as a natural reservoir for AIVs and AIVs are able to spread across the world by long-distance migration of wild birds. AIVs are able to transmit from wild birds to ducks and further to domestic poultry. Infections with LPAI viruses are mostly mild and asymptomatic in both wild and domestic birds. In poultry LPAI virus can transform to an HPAI-like virus or poultry can be infected with an HPAI strain, causing high mortality among poultry. In the area where AIVs are endemic in poultry or domestic mammals, the backyard farming and live animal markets promote the emergence of reassortant AIVs and further transmission to humans.

Viral ability to keep evolving and reassorting contributes to their pandemic potential. H5 subtype has continued to evolve by reassorting with multiple different NA genes to form new subtypes such as H5N5, H5N6, H5N8. H9N2 subtype is prevalent in birds and it may reassorts often with other subtypes, an example of which is the 2013 LPAI H7N9 virus which is a reassortant with viral HA and NA genes from wild birds and internal genes from domestic poultry H9N2 viruses. LPAI H7N9 has evolved further to an HPAI form by gaining insertion of multiple basic amino acids in its HA cleavage site which is associated with enhanced virulence in chickens.
In order for an IAV to be able to infect the cell, viral HA protein needs to bind to its receptor on the cell surface. The receptor-binding domain of HA determines the receptor specificity, which defines the viral host range and tissue tropism. Some influenza strains, such as the human isolates of H9N2 from 1999 and LPAI and HPAI H7N9 are capable of binding to both α2,3- and α2,6-linked SAs 45-47. The study by Belser and colleagues indicate that some human isolates of HPAI H7N3 from 2004 and LPAI H7N2 from 2002-2003 viruses have increased affinity toward α2,6-linked SAs whereas HPAI H7N7 from 2003 maintained binding preference toward α2,3-linked SAs115. Some human isolates of the HPAI H5N1 viruses can also bind to both α2,3- and α2,6-linked SA receptors, in contrast to the avian isolates which recognize mainly the α2,3-linked SA receptors 116. The dual receptor binding ability increases the pandemic potential of the virus, because the virus is then able to infect cells in both upper and lower respiratory tract of humans.

Viral ability to transmit via aerosols or respiratory droplets (referred to ‘airborne transmission’) in ferrets or in other mammals indicates human to human transmission, and thus high pandemic potential of the virus. Thus far the ability of sustained human to human transmission has not been detected among H5, H7, H9 or H10 subtypes although small clusters of human to human transmission of HPAI H5N1 and LPAI H7N9 have been documented 117-121. HPAI H5N1 and H5N6 are not capable of airborne transmission in ferrets. On the contrary, LPAI H7N9 has shown limited transmissibility and HPAI H7N9 even stronger ability to transmit via respiratory droplets among ferrets. Furthermore, some avian isolates of H9N2 viruses have been shown to transmit by direct contact among ferrets. 57, 122-126 An airborne-transmissible virus might be generated by natural reassortment, if two viruses infect the same cell. It is generally considered that pigs may serve as a “mixing vessels” for the generation of reassortant viruses as the respiratory tract of pigs contains receptors for both avian and human type viruses 127, 128. Similarly, reassortant virus may also be generated in human cells which express both α2,3- and α2,6-linked SAs, like dendritic cells and macrophages. However, multiple studies have shown that reassortment of the H5N1 virus and human seasonal influenza virus do not easily result in a virus which is airborne-transmissible between mammals. There are also publications which indicate that, at least with some mutations, reassortment between avian H5N1 virus and human seasonal influenza virus can result in an airborne-transmissible virus between mammals. 129-133 The studies have shown that the ability of HPAI H5N1 virus to bind to mammalian receptors is alone not sufficient to transform into an airborne transmissible virus 133-135. Recent studies have identified some molecular markers in addition to receptor binding preference, which may have an effect on the airborne transmission in ferrets, and these include mutations in HA, NA, PB1, PB2 and NP proteins 133, 135-137.

Multiple molecular markers have been shown to be associated with the viral adaptation to humans. Glutamic acid to lysine substitution at position 627 (E627K) of the PB2 increases the polymerase activity and thus allows the virus to replicate in mammalian cells at relatively low temperatures. In addition, the
H99Y mutation in PB1 enhances the virus replication in mammalian cells 135-138-140. HA substitutions G186V, Q222L, G224S, and T156A affect the receptor binding specificity 57, 141. The fusion of the viral and endosomal membranes at virus entry is triggered by a decrease in endosomal pH. Human influenza virus HA is sensitive to a lower pH than the HA of an avian origin 56. H103Y mutation in HA has been shown to be associated with the induction of a membrane fusion at lowered pH 57. T318I substitution in HA also increases the sensitivity for lower pH as well as the thermostability of the virus 133. This is in line with the observation that viral membrane fusion at low pH is associated with a higher thermostability than what is seen with those viruses which fuse at higher pH 142. Human isolates of the HPAI H7N9 virus possess some of these molecular markers, such as the E627K substitution in PB2, and indeed, it shows an enhanced virulence in ferret and mouse models as compared to the LPAI H7N9 virus123, 143. However, the HPAI H5N6 also carries the mutation E627K in PB2, but it lacks the mutations required for stabilized HA in a mammalian host 122.

In addition to the above-mentioned risk factors, other factors may also affect the pandemic potential of the virus. Those include host restriction factors, such as host-encoded proteins with antiviral activity, which can target different steps in the virus lifecycle and limit the infection.

2.3 Innate immunity to influenza A virus infection

2.3.1 Innate immune cells

*Respiratory epithelial cells*

In humans, the target cells for IAV infection are the epithelial cells of the respiratory tract, whereas in birds the virus replication takes place mainly in the epithelium of the intestinal tract. However, IAV infection can spread to alveolar macrophages and DCs. The respiratory system is divided into the upper tract, which includes nasal sinuses, pharynx and part of larynx, and the lower tract, which includes the lower part of the larynx, trachea, bronchi, bronchioles and alveoli. The mucus layer protects the respiratory epithelial cells, which are the primary targets of IAV infection. The epithelial cells in nasal mucosa predominantly express the α-2,6-linked SAs, but also α-2,3-linked SAs are detected, whereas epithelial cells in paranasal sinuses, pharynx, trachea and bronchi mainly express the α-2,6-linked SAs 42. In the lower region of the respiratory tract the α-2,3-linked SAs are prevalent 42. IAV replication is normally restricted to the upper respiratory tract, but in fatal cases also the lower tract is infected 144.

In respiratory epithelial cells IAV infection is productive, and thus the infection leads to the production of a great number of new infectious particles. In response to IAV infection respiratory epithelial cells initiate antiviral responses by producing type I and III interferons (IFNs) in addition to various other cytokines and chemokines 145-149. The IAV infection in the epithelial cells induces the recruitment of innate immune cells, which can produce higher
Background

amounts of cytokines and chemokines and initiate adaptive immune responses.

Dendritic cells

Dendritic cells (DCs) have a crucial role in inducing innate immune responses and acting as the main link between innate and adaptive immune responses. DCs are widely distributed throughout the body, also in the IAV infection entry sites, thus the innate immune responses in DCs restrict viral infection. In addition, DCs are the major cell type which activates naïve T lymphocytes by presenting antigens and thus initiates the adaptive immune responses. DCs can phagocytose IAV-infected epithelial cells or they can directly be infected by IAV to acquire the presented antigen. From the site of infection DCs migrate to the secondary lymphoid tissues where DCs present viral antigens on MHC class I and II by interacting with T cell receptor on the CD4 and CD8 T cells. Viral recognition by pathogen recognition receptors (PRR) induces the expression of costimulatory molecules CD80 and CD86 on DCs, and the expression of cytokines that stimulate naïve T cells, all of which are required for T cell activation and differentiation.

DCs in humans can be divided into two main subsets: the plasmacytoid DC (pDC) and conventional DC (cDC) (also called myeloid DC). cDCs can be further divided into cDC1 (CD1c+) and cDC2 (CD141+) subset, which correspond to CD11b+ and CD103+ subsets in mice. In addition to pDCs and cDCs, there is also a class of DCs derived from monocytes (moDC) which are differentiated as a consequence of inflammation or infection. In mice, moDCs have been described during pulmonary influenza virus infection.

Human lung DCs are difficult to obtain, thus, for research purposes, it is typical to use cDCs or pDCs isolated directly from the blood. Alternatively, monocytes can be isolated from peripheral blood mononuclear cells (PBMC) and they can be differentiated in vitro to monocyte-derived DCs (moDC) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) -4. moDCs express both α-2,3- and α-2,6-linked SAs and thus they can be infected by both avian and human type influenza viruses. In addition, moDCs express also C-type lectin receptors, such as MΦ mannose receptor (MMR), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and MΦ galactose-like lectin (MGL), which can serve as targets for influenza virus entry.

Macrophages

Macrophages (MΦs) are found in most organs throughout the body and can be divided into subpopulations based on their anatomical location and functional phenotypes, such as osteoclasts in the bone, alveolar macrophages (aMΦs) in the lungs, and Kupffer cells in the liver. MΦs help the host to defend against pathogens and maintain immunological homeostasis. MΦs are heterogeneous and plastic cells which can change their function according to their environment. Based on surface receptors, gene signatures and secretion of inflammatory mediators, MΦs can be divided into classically activated MΦs (M1 MΦs) and alternatively activated MΦs (M2 MΦs). M1 MΦs acting as inflammatory
MΦs mediate host defence responses against microbes while M2 MΦs regulate tissue repair. In the lungs, alveolar MΦs phagocytose IAV-infected cells and produce cytokines and chemokines. In addition, monocyte-derived MΦs have been found in human skin. Like DCs, also MΦs are professional antigen-presenting cells.

Human aMΦs are relevant for studying IAV infections, particularly innate immune responses, but it is very challenging to obtain them in sufficient amounts for research purposes. From PBMC, isolated CD14+ monocytes can be differentiated in vitro to monocyte-derived MΦs (moMΦs) in the presence of GM-CSF, and thus moMΦs are more readily available than aMΦs. Human aMΦs as well as human moMΦs express both α-2,3- and α-2,6-linked SA receptors. In addition, aMΦs and moMΦs express C-type lectin receptors, such as MMR and DC-SIGN receptor. moMΦs express also another C-type lectin, MGL.

Other innate immune system cells
In addition to DCs and MΦs, also other innate immune cells, including monocytes, neutrophils and Natural killer (NK) cells, control influenza infection. Monocytes are circulating leukocytes which are present in the blood, bone marrow and the spleen and they are recruited to the site of infection. In IAV-infected tissues, monocytes can differentiate into moDCs and moMΦs induced by IAV infection. However, these virus-induced moDCs are distinguished from cDC as these fail to upregulate DC maturation markers but they still produce type I IFNs and IFN-stimulated gene (ISG) protein. Human monocytes can be roughly divided into two subsets based on the expression of CD14 and CD16. Inflammatory monocytes express CD14 in the absence of CD16. Patrolling monocytes express low levels of CD14 but high levels of CD16 and after recognition of the virus by Toll-like receptor (TLR)7 and TLR8 these cells produce tumor necrosis factor (TNF)-α, IL-1β and chemokine (C-C motif) ligand 3 (CCL3). Like MΦs, neutrophils arrive at early stages to the site of infection and they are capable of phagocytosing infected cells and produce cytokines. In mouse models, the importance of neutrophils has been displayed in controlling the replication and spread of IAV. Also, NK cells produce cytokines and they are able to lyse infected cells by releasing perforin and granzymes. The role and significance of NK T cells, innate lymphoid cells, mast cells, eosinophils and basophils in influenza infection in humans is still unclear, although mouse models indicate that at least NK T and innate lymphoid cells might have a role in influenza virus infection.

2.3.2 Innate immune responses against influenza A virus

Recognition of influenza A virus

Innate immune system detects IAV infection via host pathogen recognition receptors (PRRs) which recognize pathogen associated molecular patterns (PAMPs) of the virus. IAV is recognized by three classes of PRR: retinoic acid-inducible gene-I protein (RIG-I) like receptors, TLRs and nucleotide binding and oligomerization domain (NOD)-like receptors. RIG-I is the main receptor...
for the IAV detection and induction of type I IFN in conventional DCs, aMΦs and epithelial cells, while pDCs express high levels of TLR7\(^{170, 171}\). Low levels of TLR3 are expressed in MΦs, moDCs, cDCs and human primary respiratory epithelial cells\(^{173}\). After viral replication, viral single-stranded RNA (ssRNA) bearing 5′-triphosphate is generated which acts as a ligand for RIG-I \(^{172, 173}\). TLR3 and TLR7 are the foremost TLRs in IAV infection and are expressed inside the cell on the surface of endosome. TLR3 recognizes double-stranded RNA (dsRNA) in the endosome \(^{174}\) probably by recognizing RNA structures that are present in IAV infected and phagocytosed cells \(^{175}\). TLR7 recognizes ssRNA of influenza virions that are inside the endosome, and this recognition does not require viral replication\(^{176}\). The relevance of TLR8 in IAV infection is not defined, but it is known that TLR8 is expressed in human monocytes and macrophages and it is stimulated by its ligand ssRNA leading to the production of IL-12 but not IFN-α\(^{177}\). In addition to RIG-I and TLRs, also some NOD-like receptors, e.g. NOD-like receptor family pyrin domain containing 3 (NLRP3), have been shown to be activated during IAV infection \(^{178}\).

**Signalling pathways after IAV recognition**

After 5′-triphosphate-bearing RNA is recognized by RIG-I, RIG-I is activated and caspase activation and recruitment domains (CARDs) are exposed. E3 ligases, such as TRIM-containing protein 25 (TRIM25), modulate the CARD by dephosphorylation or ubiquitination \(^{179}\). These conformational changes enable CARDs to bind to the adaptor molecule, mitochondrial antiviral signalling protein (MAVS), via CARD-CARD interactions \(^{180, 181}\). MAVS is also known as interferon-beta promoter stimulator 1 (IPS-1) or CARD adaptor inducing IFN-β (CARDIF) or virus-induced signalling adaptor (VISA). MAVS aggregates in the outer mitochondrial membrane and after dimerization MAVS recruits adaptor molecules which leads to the activation of transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interferon regulatory factor (IRF)3 and IRF7. Activation of NF-κB occurs via receptor-interacting protein 1 (RIP1), TNF receptor-associated factor (TRAF)2 and TRAF6 mediators followed by the activation of inhibitor of nuclear factor κB (IκB) kinase (IKK)α-IKKβ- NF-κB essential modulator (NEMO)-complex. IKK-complex phosphorylates the inhibitor of NF-κB (IκBα) and IκBα is degraded. Active NF-κB dimers translocate into the nucleus and induce pro-inflammatory responses. Activation of IRF3 and IRF7 occurs via TRAF3-MAVS interaction and recruitment of the TRAF family member–associated NF-κB activator (TANK)/NEMO/IKKɛ/TANK-binding kinase 1 (TBK1) complex. \(^{182}\) Phosphorylation, dimerization and nuclear translocation of IRF3 and IRF7 leads to the expression of type I and III IFNs \(^{182-184}\).

Upon TLR3 activation, TLR3 interacts with the Toll-IL-1 receptor domain (TIR)-containing adapter inducing IFN-β (TRIF), which leads to the activation of IRF3 and NF-κB via TANK/NEMO/IKKɛ/TBK1 complex or IKKα-IKKβ-NEMO-complex, respectively, such as in the RIG-I pathway \(^{185}\). IAV recognition by TLR3 primarily regulates a pro-inflammatory response \(^{186}\).

In IAV recognition by TLR7, the myeloid differentiation primary response 88 (MyD88) protein associates with the Toll/interleukin-1 receptor (TIR) domain
of TLR7 via TIR-TIR interactions. MyD88 associates with the interleukin-1 receptor-associated kinase (IRAK)4 and IRAK1. IRAK4 phosphorylates IRAK1 and promotes their association with TRAF6 followed by activation of TAK1 which activates IKKα-IKKβ-NEMO-complex and mitogen-activated protein kinases (MAPK) by phosphorylation. IKKα-IKKβ-NEMO-complex activation leads to the activation of NF-κB as described above. MAPKs activation induces activation of activating protein-1 (AP-1) transcription factor, which consist of Jun and Fos subunits. TLR7 activation can also lead to the activation of IRF7. IRAK1 directly phosphorylates IRF7 and phosphorylated IRF7 translocates into the nucleus and induces the expression of IFNs and IFN-inducible genes. Schematic diagram for innate immune signalling in early phase of IAV infection is presented in Figure 1.

IAV induced NLRP3-mediated cytokine production requires three signals. Detection of IAV by TLR7 enhances the transcription of the genes encoding pro-IL-1β, pro-IL-18 and pro-caspase-1 acting as a signal one. Signal two is when proton flux through the viral M2 ion channel triggers NLRP3 inflammasome activation and the cleavage of pro-IL-1β and pro-IL-18. Accumulation of PB1-F2 virus proteins in the lysosomes of macrophages is the third signal and leads to the activation of NLRP3 inflammasome and production of IL-1β and IL-18.

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**Figure 1.** Schematic presentation of innate immune signalling in early phases of IAV infection. Hemagglutinin protein of the influenza A virus attaches to the sialic acid α-2,3 or α-2,6 galactose (SA α-2,3 or α-2,6 Gal) and virus enters the cell via endocytosis. Pathogen recognition receptors (PRR), RIG-I, TLR3 and TLR7, recognize the pathogen associated molecular patterns. Activated PRRs deliver signals to the adaptor molecules (TRIF, MAVS, MyD88). The activation of downstream kinases (IKK complex, TBK1 complex, RIP-1, IRAK and MAPK) occur via mediators (TRAF2, -3 and -6) leading to the activation of transcription factors (NF-κB, AP-1, IRF3 and IRF7). Transcription factors translocate into the nucleus and bind to the promoter elements leading to the expression of IFN-β, IFN-λ1 and chemokines.
Interferons

IFNs are classified into type I, II and III interferons. In humans type I IFNs include 13 different subtypes of IFN-α and a single subtype of IFN-β, -ε, -κ, and –ω. Type I interferons are widely produced by almost all cell types. IFN-γ is the only member of type II IFN. Type III IFNs are IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4. Type I and III interferons are the major IFNs secreted during influenza virus infection in vitro and in vivo and these IFNs have an essential role in antiviral responses against IAV. The receptor of type I IFN is composed of IFNAR1 and IFNAR2 chains while type III IFN receptor is composed of IFNLR1 and IL-10R2 chains. IFNAR1, IFNAR2 as well as IL-10R2 are widely expressed whereas the expression of IFNLR1 subunit is more restricted and mainly expressed in the epithelial cells of the respiratory tract, intestinal tract, and the liver. The production of type I and III interferons leads to the expression of IFN-stimulated genes by activation of the Janus kinase (JAK) - signal transducers and activators of transcription (STAT)-signalling pathway. Despite similarities between type I and III IFNs, they also show different characteristics against IAV. Galani and colleagues show that in mice IFN-λs are produced earlier than type I IFNs. In addition, type III IFNs induce the expression of antiviral genes but not those that activate inflammation, while type I IFNs induce antiviral responses and the expression of pro-inflammatory cytokines and chemokines mediating inflammation.

Pro-inflammatory cytokines and chemokines

In addition to interferon responses, activation of PRRs and transcription factors lead to the expression of pro-inflammatory cytokines such as IL-6, IL-1β, IL-18, TNF-α, and chemokines such as CCL2, CCL3, CCL4 and CXCL10. Pro-inflammatory cytokines are required for the establishment of inflammation, they cause fever and activate adaptive immune responses against the virus. IL-12 and IL-23 are essential pro-inflammatory cytokines which induce the development of the T helper (Th)1 and Th17 cells. Chemokines are chemo-attractant cytokines which recruit innate immune cells and thus promote the viral clearance. However, a massive pro-inflammatory cytokine and chemokine production, such as in HPAI H5N1 virus infection, might increase the viral pathogenicity and contribute to severe, even fatal, outcome of the infection.

JAK-STAT signalling pathway in interferon signalling

IFNs are produced in infected cells and they interact with IFN receptors in the plasma membrane in an autocrine or paracrine manner. This process activates Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathway which is also used by many other cytokines and growth factors to transduce their signals. In canonical JAK-STAT signalling pathway, type I IFNs are recognized by IFNAR2 which triggers the heterodimerization between IFNAR2 and IFNAR1. This leads to the autophosphorylation of IFNAR1-bound tyrosine kinase 2 (TYK2). Activated TYK2 phosphorylates IFNAR2-bound Janus kinase 1 (JAK1), which in turn phosphorylates STAT2 allowing...
STAT1 phosphorylation. Phosphorylated STAT1 and STAT2 form dimers and they dissociate from the receptors. STAT1-STAT2 heterodimers translocate into the nucleus and together with IRF9 form the interferon-stimulated gene factor 3 (ISGF3) -complex. ISGF3-complex binds to the promoter region of interferon-stimulated response element (ISRE) to initiate the transcription of ISGs. 213 The nuclear localization signal of STAT1 interacts with importin-α5, which is a member of the importin α family of nuclear localization signal receptors, and this interaction mediates the nuclear translocation of STAT1-complex 59, 214. As with type I IFNs, the interactions between type III interferons and their receptors activate the canonical JAK-STAT signalling pathway, leading to the phosphorylation of STAT1 and STAT2 and resulting in the formation of ISGF3 complex and induction of ISGs 198, 215. JAK-STAT signalling pathway is schematically presented in Figure 2.

**Figure 2.** Schematic diagram for innate immune signalling in later phases of IAV infection. Type I and III interferon receptors recognize IFN-β and IFN–λ1, which are secreted in the early phases of IAV infection (Figure 1). Activation of IFN receptors leads to the activation of ISGF3-complex and the expression of interferon stimulated genes (ISGs). IFN expression also induces a positive feedback loop via IRF7 expression which results in a stronger type I and III IFN expression.

**Interferon stimulated genes in IAV infection**

IFNs secreted from virus-infected cells induce an antiviral state in the neighbouring cells by the expression of ISGs. The activation of JAK-STAT signalling pathway leads to the expression of ISGs, such as human myxovirus resistance protein 1 (MxA), protein kinase R (PKR), IFN-stimulated gene 15 (ISG15) and IFN-induced transmembrane protein (IFITM)3, which restrict the viral entry, replication and assembly of viruses thus limiting IAV infection and spread. Mx protein was the first identified antiviral ISG which inhibits influenza virus infection 216. The human MxA protein interacts with viral NP having antiviral
activity and MxA is also necessary for preventing the transport of the viral genome into the nucleus. 2'−5'−oligoadenylate synthase (OAS) and RNaseL also have antiviral effects. The activation of OAS leads to the activation of RNase L, which can cleave viral and cellular ssRNAs. PKR phosphorylates the eukaryotic translation initiation factor 2α resulting in translational block and reduction of viral replication. In addition, PKR activates NF-κB pathway. The IFN-induced protein with tetratricopeptide repeats (IFIT) and IFITM genes are also stimulated by IFNs. IFITM proteins restrict viral entry and particularly IFITM3 protein has been shown to be essential for host defence against IAV by restricting viral entry and by that decreasing the replication of IAVs. ISG15-deficient mice have been shown to be more susceptible to IAV infection and ISG15 inhibits IAV infection in human cells.

2.4 Biosafety and biosecurity

Biosafety refers to the principles, technologies, and practices that are implemented to prevent the unintentional exposure to biological agents and toxins, and to prevent their accidental release. Biosecurity, however, refers to the protection, control and the accountability of laboratories for biological agents and toxins, in order to prevent their loss, theft, misuse, diversion of, unauthorized access or intentional unauthorized release.

National biosafety and biosecurity regulations and legislations vary from one country to another. Biosafety and biosecurity management measures are defined by national laws, international instructions, standards and internal regulations and instructions of different research institutes. European Union (EU) member states are obliged to implement EU Directives 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work and in Finland this directive is included to the decree on the protection of workers from risks related to exposure to biological agents at work (933/2017). According to decree 933/2017, in Finland it is obligatory to inform Regional State Administrative Agencies when starting work with biological agent from risk groups III and IV (the risk group are defined by each country by itself). Based on the Act on communicable diseases (1227/2016) it is obligatory to inform THL when importing biological agents to Finland from risk group III-IV. Evira grants import permissions for biological agents, which can cause diseases in animals (for example for avian influenza viruses). In Finland, there is no separate authority which supervises and grants permissions for laboratories at different biosafety levels (BSL1-4). Rather, clinical microbiology laboratories must be licensed by the Regional State Administrative Agency, based on the Act on Communicable Diseases (1227/2016 18§).
3. Materials and methods

3.1 Biorisk management and ethics

3.1.1 Biosafety and biosecurity

All infection experiments using infective human isolates of avian influenza viruses have been done in biosafety level 3 (BSL3) facilities of the National Institute for Health and Welfare (THL). The avian isolates of avian influenza viruses were handled in the BSL3 facilities of the Finnish Food Safety Authority (Evira). Inactivated samples of avian influenza viruses were handled under the BSL2 or -1 facilities of THL. Infection experiments with seasonal influenza viruses and all cell cultures were performed in a BSL2 laboratory.

The BSL-3 facility in THL was audited by Det Norske Veritas (Dr. Paul J. Huntly) in 2013. CWA 15793 Laboratory Biorisk Management Standard has been implemented in THL. The functions and rights of THL are regulated by the Act on the National Institute for Health and Welfare (668/2008) and the Act on communicable diseases (1227/2016). THL is obliged to follow Finnish decree 933/2017 and EU Directives 2000/54/EC. THL possesses import permission for HPAI and LPAI viruses for research, diagnostic and surveillance purposes which has been granted by Evira (permission no 8634/0527/2012).

To ensure biosafety and biosecurity aspects, the BSL3 facility of THL includes multiple protection barriers: negative air-pressure, HEPA-filtered exhaust air, airtight dampers on all ductwork, no drains inside the laboratory, limited and controlled access, exit through a change of clothing, and all waste is autoclaved. Working inside the BSL3 laboratory was always done in pairs with a third supervisor outside the laboratory. The staff used washable underclothes, single-use Tyvek coveralls, double gloves, FFP-3 masks, goggles, rubber shoes and protective sleeves for personal protective equipment when working with avian influenza viruses. Infective viruses were handled only inside the class II biosafety cabinets. The maximum working time was four hours at a time. The staff has to pass a physical examination regularly. The staff is trained to work in the BSL3 laboratory and regular working experience inside the BSL3 is obligatory. The voluntary staff has been vaccinated by annual seasonal influenza vaccine and H5N1 virus vaccine. To prevent creation of recombinant viruses, the different virus strains were always handled in separate biosafety cabinets.
All inactivation methods for different sample materials, including methanol and paraformaldehyde (PFA) fixed cells, cell lysates for extraction of ribonucleic acid (RNA) and protein samples, were tested with HPAI H5N1 virus and validated internally in THL to ensure that all samples were non-infectious when they were brought out from BSL3 facilities.

3.1.2 Ethics statement

Adult human blood was obtained from anonymous healthy blood donors through the Finnish Red Cross Blood Transfusion Service (permission is renewed annually). Animal immunizations related to this study were approved by the Ethical Committee of the National Institute for Health and Welfare (formerly The National Public Health Institute of Finland) (permission no. KTL 2008-02).

3.2 Cell culture and virus infection

3.2.1 Cell cultures

Different cell cultures used in study are presented in Table 3.

<table>
<thead>
<tr>
<th>Cell</th>
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<th>Source</th>
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<td>ATCC CCL34</td>
<td>Plaque assay</td>
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<td>Infection experiment</td>
<td>I-III</td>
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<td>b</td>
<td>Infection experiment</td>
<td>I</td>
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<td>Infection experiment</td>
<td>I</td>
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<tr>
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<td>c</td>
<td>HA</td>
<td>I-III</td>
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<tr>
<td>Red blood cell</td>
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<td>c</td>
<td>HA</td>
<td>III</td>
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<tr>
<td>Macrophage</td>
<td>primary</td>
<td>Human peripheral blood monocytes</td>
<td>a</td>
<td>Infection experiment</td>
<td>III</td>
</tr>
</tbody>
</table>

a Finnish Red Cross Blood Transfusion Service, Helsinki, Finland.
b Dr. A. Hoffmann, Signaling Systems Lab, San Diego.
c The Laboratory Animal Centre of the University of Helsinki
MDCK Madin-Darby canine kidney
MEF mouse embryonic fibroblasts
wt Wild type
Monocyte-derived dendritic cells and macrophages

Theuffy coats were obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Human peripheral blood mononuclear cells (PBMC) were isolated fromuffy coats by density gradient centrifugation over a Ficoll-Paque gradient (Amersham Biosciences) [228]. To obtain monocytes for dendritic cell differentiation Percoll gradient (Amersham Biosciences) centrifugation was also performed. The layer containing monocytes were collected and remaining T and B cells were depleted by using anti-CD3 and anti-CD19 magnetic beads (Dynal). Monocytes were allowed to adhere to plates (Sarstedt) for 1 h at +37°C in Roswell Park Memorial Institute (RPMI) medium 1640 (Sigma-Aldrich) supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES). Non-adhered cells were washed away with phosphate-buffered saline (PBS) and adherent monocytes were cultivated in RPMI 1640 medium supplemented as described above and with 10% fetal calf serum (FCS) (Integro), 10 ng/ml human recombinant GM-CSF and 20 ng/ml human recombinant IL-4 (R&D Systems) to generate immature moDCs. The cells were cultivated for 6 days, and fresh medium was added every 2 days.

To obtain monocyte-derived macrophages (moMϕ), PBMCs were allowed to adhere onto plates or glass coverslips for 1 h at +37°C in RPMI 1640 medium (Sigma-Aldrich) supplemented with penicillin, streptomycin, L-glutamine and HEPES as described above. The cells were washed with PBS to remove non-adherent cells and the remaining adherent monocytes were cultured in macrophage serum-free medium (Life Technologies) supplemented with penicillin, streptomycin and human recombinant GM-CSF (10 ng/ml; Nordic Biosite). The cells were differentiated into macrophages for 7 days and the fresh culture medium was changed every 2 days.

Cell lines

The wild type (wt) and the Irf3 -/- Irf7 -/- primary mouse embryonic fibroblasts (MEF) and the wt and the RelA -/- c-Rel -/- Nfκb1 -/- MEF cell line were cultured in Dulbecco’s Modified Eagle’s medium (D-MEM). A549 and Madin-Darby canine kidney (MDCK) cells were cultivated in Eagle’s Minimum Essential Medium (E-MEM). Both D-MEM and E-MEM medium contained 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES and 10% FCS.

3.2.2 Viruses

THL has an import permission from Evira for HPAI and LPAI viruses for research, diagnostic and surveillance purposes. All virus strains used in the study are listed in Table 4.
Materials and methods

Table 4. Viruses used in the study.

<table>
<thead>
<tr>
<th>Virus</th>
<th>abbr.</th>
<th>Isolate</th>
<th>Pathogenicity</th>
<th>BSL</th>
<th>Source</th>
<th>Passage</th>
<th>Culture</th>
<th>Study</th>
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<td>BSL3</td>
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<td>egg</td>
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<td>BSL3</td>
<td>a</td>
<td>egg</td>
<td>UD</td>
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<td>egg</td>
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</tbody>
</table>

UD Unpublished data
a Finnish Food Safety Authority (Evira)
b WHO Collaborating Centre for Reference and Research on Influenza, UK
c Molecular Virology, Erasmus MC - Department of Viroscience, Netherlands

The virus stocks were grown in the allantoic cavity of 10 to 11-day-old embryonated chicken eggs at +36 °C for 2 to 3 days or in MDCK cells as indicated in Table 8.

3.2.3 Virus infection (I-III)

To infect moDCs virus dilutions were added onto the cells without changing the growth medium. For infecting moMφs, A549, MDCK or MEF cells the growth medium was removed and virus dilution was added on cells. Cells were infected with different multiplicity of infection (MOI) for different time periods as indicated in the individual publications.

3.2.4 Cytokine priming of virus-infected cells (I-II)

To analyse antiviral actions of virus-induced cytokines against the studied viruses, the moDCs were treated with 1, 10 or 100 IU/ml of IFN-α or IFN-β or with 1, 10 or 100 ng/ml of IL-1β or with 0.5, 5 or 50 ng/ml of TNF-α for 24 h before infections. Used cytokines are listed in Table 6.
3.3 Virus titrations

3.3.1 Hemagglutination assay
Hemagglutination assay was done with a standard protocol using 0.5% turkey or guinea pig red blood cells.

3.3.2 Plaque assay (I, III)
To obtain the concentration of infective viruses as plaque forming units (PFU)/ml, a plaque assay was performed in studies I and III. The viruses were serially diluted and inoculated onto confluent MDCK cells on 6-well plates for 1 h at 37°C. After incubation cells were washed with PBS and covered with 1% agarose (peqLAB) (study I) or 1.2% Avicel microcrystalline cellulose (#RC-591 NF, FMC BioPolymers) (study III). Agarose and Avicel were diluted to Eagle-MEM and final suspension containing 0.3% Bovine Serum Albumin (BSA), 2 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin, 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine and 20 mM HEPES. After 1-6 days of incubation the plaques were counted. With Avicel the cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) and stained with diluted crystal violet after incubation and before plaque counting.

In study III the infective virus titers were determined from supernatant samples by Avicel plaque assay as described above with the exception that MDCK cells were on 24-well plates.

3.3.3 End-point dilution assay (II)
Virus titers were determined by the end-point dilution assay in study II. The viruses were serially diluted to E-MEM containing the same supplements as in the plaque assay. Viruses were inoculated to confluent MDCK cells on 96-well plates for 1-4 days at 37°C and virus titers were determined by observing the cytopathic effect caused by the virus. Tissue culture infective dose 50 (TCID50) titers were calculated by the Spearman and Kärber algorithm.

3.4 Gene expression studies

3.4.1 qRT-PCR (I-III)
RNA was extracted using the RNEasy Mini kit (Qiagen). Cell lysates for the total cellular RNA extraction from different donors were pooled and RNA isolation was done including DNase digestion (RNase-free DNase kit, Qiagen) (I-III). RNA from supernatant samples were isolated separately from each donor without DNase digestion (III).

0.5 or 1 μg of total cellular RNA was transcribed to cDNA using TaqMan Reverse Transcriptase kit (Applied Biosystems) with random hexamers as primers. The cDNA was amplified by PCR using TaqMan Universal PCR Mastermix and Gene Expression Assays (Applied Biosystems). The data was nor-
malized to endogenous gene 18S and GAPH for human cells and mouse cells, respectively. The gene expression assays that were used in the study are listed in Table 5. The relative gene expression was calculated with the ΔΔCT method according to instructions provided by Applied Biosystems. If the cell lysates were pooled for the RNA isolation, the variation between individual blood donors cannot be assessed.

In study III, RNA from supernatant samples were transcribed to cDNA using RevertAid H Minus Reverse Transcriptase (Thermo Scientific) kit according to the manufacturer’s instructions with RiboLock RNase inhibitor (Thermo Scientific) and random hexamers (Roche) as primers. qRT-PCR was performed using QIAGEN® QuantiTect™ Multiplex PCR No Rox Kit (Qiagen). The influenza specific probe that was used is listed in Table 5. The relative viral gene expression was calculated with the ΔCT method according to the instructions provided by Applied Biosystems. The statistical significance of differences between the groupings was determined by Student’s t-test.

Table 5. Gene specific qRT-PCR assays used in the study.

<table>
<thead>
<tr>
<th>Gene assay</th>
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<td>Eukaryotic</td>
<td>4308329</td>
<td>Applied Biosystems</td>
<td>I-III</td>
</tr>
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</table>
3.5 Protein expression analyses

3.5.1 Western blotting (I-III)

For analysing protein expression of different proteins in infected cells, the cells were harvested and moDCs or moMφs from different donors in each experiment were pooled. moDCs, moMφs and A549 cells were lysed with a passive lysis buffer (Promega) containing 1 mM Na3VO4. MEF cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2) containing 0.5 mM DTT, 1 mM Na3VO4 and a protease inhibitor mixture (Complete, Roche). Total cellular proteins were boiled in a Laemmli sample buffer, proteins were separated in SDS-PAGE gels and transferred into Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were stained as indicated in individual publications with antibodies that are listed in Table 7. Protein bands were visualized on HyperMax films using an ECL plus system (GE Healthcare). In study I, the protein band intensities were quantitated with ImageJ software (http://imagej.nih.gov/ij/) to turn pixel intensity into optical density. Protein expression data was normalized to loading controls and then presented as fold induction of the relative protein expression in relation to unstimulated samples.

3.5.2 ELISA (I-II)

VeriKine™ human interferon alpha Multi-subtype ELISA kit (PBL Assay Science) or VeriKine™ human IFN beta ELISA kit (PBL Assay Science) were used according to the manufacturer’s instructions to analyse the levels of secreted IFN-α and IFN-β. IFN levels in cell culture supernatants from different blood donors were analysed separately.

3.5.3 Flow cytometry (I, III)

For flow cytometric analysis moDCs were differentiated on 6-well plates and the cells were infected as indicated in individual publications. After infection, the cells from four different donors were harvested and handled separately. Cells were fixed with 90% methanol for 20 min (I) or with 4% paraformaldehyde (PFA) for 30 min and permeabilized with 0.1% Triton X-100 for 5 min (III). The cells were stained as indicated in individual publications and the antibodies used are listed in Table 7. The LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit (LifeTechnologies) was used to determine the viability of infected cells in study I. The cells were analysed with a FACSCanto II (Becton Dickinson) flow cytometer using FACSDiva software (Becton Dickinson). Cells from different blood donors were analysed separately.

3.5.4 Immunofluorescence microscopy (III)

For immunofluorescence microscopy macrophages were differentiated on glass coverslips. The cells were infected, fixed with 4% PFA for 30 min and
permeabilized with 0.1% Triton X-100 for 5 min and stained as indicated in the publication III. The antibodies used in immunofluorescence microscopy are listed in Table 7.

3.5.5 Propagation competent virus particle test (III)
moMφs in the class cover slips were infected with a MOI of 0.01. After 1 h incubation virus inoculation was replaced with 1.2% Avicel suspension, which was prepared as described in plaque assay. After 16 h incubation in +37°C, cells were fixed with 4% PFA, permeabilized with 0.1% Triton and stained with virus-specific antibodies (presented in Table 7). The infection events were calculated by immunofluorescence microscopy. The infection event was considered abortive when one or two adjacent cells were infected and productive when three or more adjacent cells were infected.

3.6 Protein-protein interactions
3.6.1 Importin binding assay (unpublished study)
Human glutathione S-transferase (GST)-importin α1, α3, α4, α5, α6 and α7 expression constructs were prepared as previously described by Melen and Fagerlund and their colleagues. GST-importin-α1, α3, α5 and α6 and GST control were expressed in E. coli BL21 cells, GST-importin- α4 and α7 were expressed by baculovirus in Sf9 cells and were purified as described previously. GST control and GST-importins were allowed to bind to the glutathione Sepharose (GE Healthcare #17-0756-01) and equal binding for each GST-importins were verified by SDS-PAGE with Coomassie G-250 dye staining (PageBlue™ Protein Staining Solution # 24620, ThermoFisher Scientific). The A/Vietnam/1203/2004 (H5N1) NS1 and A/Udorn/1972 (H3N2) NS1 genes were cloned into pcDNA3.1 expression vector and translated in vitro with TnT Sp6 Quick Master Mix (Promega #L209A), [35S] methionine label (Amersham Biosciences) and T7 RNA polymerase (ThermoFisher Scientific #EPO111) according to the manufacturer’s instructions. In vitro translated NS1 proteins were allowed to bind to Sepharose-immobilized GST or GST-importin fusion proteins on ice at 1 h and were washed. GST-importin- bound 35S-labeled proteins were separated by SDS-PAGE. The gels were fixed with Isopropyl alcohol/water/acetic acid (25:65:10)-mixture and treated with Amersham amplify fluorographic reagent (GE Healthcare #NAMP100) according to the manufacturer’s instructions and autoradiographed.

3.7 Statistics
3.7.1 Statistical analysis (I-III)
Statistical significance of differences of mean values from multiple data points between experimental groups was determined by Student’s t-test. Values of p < 0.05 were considered statistically significant and marked with * symbol.
3.8 Experimental settings

A typical experimental setting used in the study is described in Figure 3. First, monocytes were isolated from PBMC, the cells were plated in suitable plates and moMφs and moDCs were differentiated for 6-7 days before the infections. The cell lines were cultured 12-24 h before the infections. The cells were infected with IAVs for 0.5 h-48 h and the samples were collected. Samples were analysed by qRT-PCR, Western blotting (WB), flow cytometry (FC) or immunofluorescence microscopy (IFA).

Figure 3 Typical experimental setting used in the study.

3.9 Reagents

3.9.1 Cytokines

Cytokines used in the study are presented in Table 6.

Table 6. Cytokines used in the study.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Application</th>
<th>Source/reference</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant human GM-CSF</td>
<td>Cell culture</td>
<td>Gibco®</td>
<td>I-III</td>
</tr>
<tr>
<td>Recombinant human IL-4</td>
<td>Cell culture</td>
<td>Gibco®</td>
<td>I-III</td>
</tr>
<tr>
<td>Recombinant human IFN-α-2b</td>
<td>Priming experiments</td>
<td>Schering-Plough</td>
<td>I-II</td>
</tr>
<tr>
<td>Recombinant human IFN-β-1b</td>
<td>Priming experiments</td>
<td>Schering-Plough</td>
<td>I-II</td>
</tr>
<tr>
<td>Recombinant human TNF-α</td>
<td>Priming experiments</td>
<td>Biosource</td>
<td>I-II</td>
</tr>
<tr>
<td>Recombinant human IL-1β-2b</td>
<td>Priming experiments</td>
<td>Biosource</td>
<td>I-II</td>
</tr>
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</table>
### 3.9.2 Antibodies

Antibodies used in the study are presented in Table 7.

**Table 7. Antibodies used in the study.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Method</th>
<th>Produced</th>
<th>Source/reference</th>
<th>ID</th>
<th>Study</th>
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<td>WB</td>
<td>Rabbit</td>
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<td>234</td>
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<td>IAV M1</td>
<td>WB</td>
<td>Rabbit</td>
<td></td>
<td>234</td>
<td>I-III</td>
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<td>IRF3</td>
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<td>Rabbit</td>
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<td>202</td>
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<td>P-IRF3</td>
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<td>Guinea pig</td>
<td></td>
<td>235</td>
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<td>WB</td>
<td>Guinea pig</td>
<td></td>
<td>202</td>
<td>I-II</td>
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<td>P-STAT1</td>
<td>WB</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-7988</td>
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<td>P-P38 MAPK</td>
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<td>#9211L</td>
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<td>Rabbit</td>
<td></td>
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<tr>
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<td>InVitrogen (Caltag Laboratories)</td>
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<td>Guinea pig</td>
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<td>III</td>
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<td>IFA</td>
<td>Guinea pig</td>
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<td>239</td>
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<td>goat</td>
<td>Jackson</td>
<td>106-096-006</td>
<td>III</td>
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<tr>
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<td>goat</td>
<td>InVitrogen</td>
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<td>Anti mouse IgG/HRP</td>
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<tr>
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<td>WB</td>
<td>goat</td>
<td>InVitrogen</td>
<td>P0141</td>
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</tr>
</tbody>
</table>

Phospho (P); Western Blot (WB); Glycoprotein (GP); flow cytometry (FC); immunofluorescence microscopy (IFA)
3.9.3 Inhibitors

In study III, to determine the infectivity of studied viruses in primary infection in moMφs and moDCs, the infected cells were incubated with 20 nM oseltamivir carboxylate (#RO0640802-002, Roche) which is a neuraminidase inhibitor. Oseltamivir carboxylate is an active metabolite of oseltamivir phosphate.
4. Results and Discussion

100 years after the devastating 1918 Spanish flu pandemic, influenza A virus epidemics and pandemics are still threats to humans. In addition to frequent seasonal influenza epidemics, one remarkable threat is posed by avian influenza viruses which can cross the species barrier and infect humans. In spite of active research on IAVs there are still open questions about AIV infections and the pandemic potential of AIVs. Host innate immune responses against the virus are the major factors in restricting virus replication and spread, and they facilitate the clearance of the virus. Although epithelial cells of the respiratory organs are the major target of IAV infection, the circulating immune cells, such as DCs and Mϕs, have an important role in defending the host against the virus infection. This study describes avian influenza virus infection and innate immune responses induced by AIVs of human or avian origin in human innate immune cells.

4.1 Human cell models used in the study

Monocyte-derived macrophages and dendritic cells are widely used in infection studies in ex vivo experiments instead of alveolar macrophages or conventional or plasmacytoid dendritic cells. To mimic the abundant levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the lungs, in our studies moMϕ has been cultured in the presence of GM-CSF to provide more realistic model of an in vivo situation. However, studies have shown that moMϕs and aMϕs respond differently to IAV infection and the infectivity of these cells is different \(^\text{147, 240}\). It seems that with H5N1 virus the infectivity and cytokine responses are higher in moMϕs than aMϕs but cytokine levels induced by seasonal influenza seems to be similar in moMϕs and aMϕs \(^\text{147, 240}\). It has been proposed that moMϕs represent Mϕ recruited into the lungs during the infection and aMϕs correspond to a residential Mϕ population, thus moMϕs and aMϕs are both relevant cell models to study influenza infection \(^\text{240}\). moDCs are differentiated in the presence of GM-CSF and IL-4 and they represent immature DCs. These cells are similar to DCs which are found in peripheral tissues. Immature DCs can be differentiated into mature DCs for example by pro-inflammatory cytokines or LPS treatment\(^\text{241}\) but seasonal influenza infection induces DC maturation inefficiently \(^\text{202}\). Unfortunately, it is not clear
how well moDC model corresponds to lung resident DCs, however, moDCs provide a sufficient model to investigate IAV infection.

All experiments with moDCs or moMφs have been done with cells from three or generally from four voluntary blood donors and all experiments were repeated several times as indicated in each study. The blood donors were different in each experiment. In the present study, there is no additional background information on the donors, including vaccination status. This is unfortunate, however, on the other hand, our cell model is a good representation of the average healthy population in Finland with different vaccination status, age, sex, body mass index etc. Within each experiment for qRT-PCR or Western blot analyses, RNA and protein samples from different donors were pooled for increasing the total number of donors analysed. In most experiments it was impossible to analyse all donors separately due to the high number of samples. The advantage for pooling the donors are that we can analyse higher amount of samples and with lower expenses, but there is also some disadvantage of pooling the donors. There is definitely considerable variation in responsiveness between the donors and if we pooled the donors the strong responders might dominate in the average result. However, in the study by Pietilä and colleagues the comparison of pooled RNA samples to samples from individual donors was analysed. It was shown that the biological donor-to-donor variation is substantial but still the trend between the donors was very similar from one donor to another. On the other hand, in this study we have not investigated the absolute activation levels but, instead, we have been interested in differences in the activation levels induced by different IAV strains. Thus, the pooled samples with repeated experiments can be considered to represent a valid data source.

The alveolar epithelium consists of type I and II pneumocytes, although alveolar macrophages are also present. Type I pneumocytes cover over 95% of the alveolar surface and act as a barrier between air space and blood and are responsible for gas exchange and fluid homeostasis. Type II pneumocytes cover only 5% of the alveolar surface, they produce surfactant and may serve as reserve cells which can differentiate into type I pneumocytes. Although, both type I and II pneumocytes contribute to the defence against IAV, type II pneumocytes are more widely studied. Type II pneumocytes produce cytokines which are involved in the activation and differentiation of immune cells. These cells have been shown to express both class I and class II major histocompatibility complex molecules and thus to be able to present antigens to specific T cells. It has been reported, that in the lungs IAV predominantly infects type II pneumocytes, although there are differences between the strains in their ability to replicate and induce cytokine responses. A549 cell line is derived from a human pulmonary adenocarcinoma and it is widely used as a models cell system for type II pneumocytes. However, A549 cells do not reliably represent the primary type II pneumocytes because in A549 cells added trypsin is needed for efficient replication of seasonal influenza virus and A549 cells do not produce surfactant as type II pneumocytes do.
4.2 Virus quantification

To compare different influenza strains, an equal virus concentration is essential. The multiplicity of infection (MOI) was calculated by infectious dose in MDCK cells (in study with avian isolates and studies I-II) or in human immune cells (study III). Virus quantifications with different methods are presented in Table 8.

Table 8. Virus titers in different cells types.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Culture</th>
<th>HA (turkey RBC)</th>
<th>HA (guinea pig RBC)</th>
<th>TCID₅₀ (MDCK)</th>
<th>PFU/ml (MDCK)</th>
<th>FFU/ml (moMₘ)</th>
<th>FFU/ml (moDC)</th>
<th>Study</th>
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<tr>
<td>H5N2</td>
<td>E 64</td>
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<td>UD</td>
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<td></td>
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<tr>
<td>H7N3</td>
<td>E 128</td>
<td>4x10⁶</td>
<td>UD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9/529</td>
<td>E 128</td>
<td>2x10⁷</td>
<td>UD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9/384</td>
<td>E 256</td>
<td>2x10⁵</td>
<td>UD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3N2</td>
<td>E 128</td>
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<td>2x10⁷</td>
<td>3x10⁷</td>
<td>5x10⁶</td>
<td>I-III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9N2</td>
<td>E 512-1024</td>
<td>2x10⁸</td>
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<td></td>
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<tr>
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<td>E 128</td>
<td>10⁵-10⁶</td>
<td>7.8x10⁶</td>
<td>7.8x10⁷</td>
<td>III</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>M 64-128</td>
<td>10⁵-10⁶</td>
<td>II</td>
<td></td>
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<td></td>
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<tr>
<td>H7N9</td>
<td>E 1024</td>
<td>2x10⁶</td>
<td>4.7x10⁶</td>
<td>7.8x10⁷</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>10⁵-10⁶</td>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5N1/97</td>
<td>E 256</td>
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<td>2.8x10⁹</td>
<td>1.2x10⁹</td>
<td>III</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RBC Red blood cells
UD Unpublished data
E Egg stock
M MDCK stock

The hemagglutination assay was also performed to analyse virus titers. With the viruses of human origin the titers were determined both with turkey (avian) and guinea pig (mammalian) red blood cells to investigate the host adaptation by receptor specificity between human seasonal virus and AIVs (Table 8, Table 1 in III). We noticed that AIVs show similar titers both in turkey and guinea pig cells. Because the hemagglutination assay measures only the amount of virus particles (amount of HA protein) and does not provide information on the number of infectious particles, we also determined the infectious dose by plaque forming units per milliliter (pfu/ml) by the plaque assay. The result from plaque assay depends on viral ability to form plaques and it represents the infectivity in MDCK cells. Further, for more exact analyses we determined the infectivity of the viruses in both moMₘs and moDC, by immunofluorescence microscopy and flow cytometry, respectively (Table 8, Table 1 in III). The number of infective units is somewhat higher in moMₘs than in moDCs (Table 8, Table 1 in III) and it seems that moMₘs are more susceptible to IAV infection than moDCs (Table 8, Table 1 in III). Another explanation is technical, because with moMₘs the infection is performed in a smaller volume.
than infection with moDC, and thus the viral particles might reach the moMφs more easily.

4.3 Replication of avian influenza viruses in human primary dendritic cells and macrophages

To investigate whether the currently circulating avian isolates of influenza viruses are able to infect human cells, we infected moDCs with avian-isolated H5N2, H7N3, H9/529, H9/384 and human H3N2 virus (seasonal influenza virus for control) with a MOI of 0.2 for 24 h. Cells from four donors were collected at 1 h, 3 h, 7 h and 24 h after infection and RNA samples were analysed by qRT-PCR and protein samples by Western blotting. The viral M1 RNA increased during infection time with all studied viruses, which indicates that these viruses are able to infect and replicate in human moDCs although the expression level of M1 gene was lower in cells with avian isolate virus infection than in the H3N2 virus-infected cells (Figure 4, unpublished data). We confirmed the RNA result by analysing of the expression of viral proteins. We noticed that viral M1 and NP protein expression followed the replication kinetics with H5N2, H7N3, H9/529, H9/384 and H3N2 viruses (Figure 4, unpublished data). The result indicates that AIV that circulate in birds in Finland, can infect human cells, but the replication levels were lower than with human-adapted seasonal IAV. We also compared the replication of the H3N2 seasonal influenza virus with that of the human isolate of an avian-origin H9N2, H7N9 or H5N1/04 viruses. We infected moDCs with the viruses at a MOI of 1 for 24 h and collected RNA samples for qRT-PCR and protein samples for Western blot analyses. Our results show that H5N1, H7N9 and H9N2 viruses replicated efficiently and viral protein expression increased noticeably during the infection (Fig 1 in I and Fig 1 in II).
Results and Discussion

Figure 4. Replication of avian isolates of avian influenza H5N2, H7N3 and H9N2 subtypes. moDCs were infected with a human isolate of seasonal influenza virus A/Beijing/353/1989 (H3N2) or with avian isolates of avian influenza virus A/mallard/Finland/13748/07 (H5N2), A/teal/Finland/9201/10 (H7N3), A/mallard/Finland/13384/10 (H9N2) or A/teal/Finland/10529/10 (H9N2) with a MOI of 0.02 at 24 h. The cells were collected at different time points and cells from four donors were pooled. A) For viral RNA analysis, total cellular RNA was isolated and viral M1 gene expression was analysed by qRT-PCR. The values are normalized to 18S rRNA and presented as relative gene expression in relation to the 1 h sample, representing increases in the viral RNA levels during the time course of the infection. Statistical differences cannot be assessed, because samples from different donors were pooled. B) Viral protein M1 and NP expression was analysed from whole cell lysates by Western blotting. The GAPDH protein expression was analysed to control equal loading of the samples.

To further analyse HPAI H5N1 virus infection, we infected moDCs and moMφs with H3N2, H5N1/04 and H7N9 viruses at different MOI values for 24 h. During primary infection (under 6 h) we did not observe any clear difference in viral M1 gene expression between H3N2, H5N1/04 and H7N9 viruses (Fig 2 in III). Also, at 6 h post-infection, with all viruses, the percent of infected cells corresponded well to the determined focus forming units per milliliter (FFU/ml) and MOI values (Fig 1 A and C in III). Surprisingly, in multi-cyclic infection (at 24 h) we noticed dramatic differences between H3N2, H5N1/04 and H7N9 viruses. The percent of infected cells was clearly dependent on the viral dose in the beginning of the infection in H3N2 and H7N9 infected cells but in H5N1/04 virus infection the virus was able to spread and the percentage of infected cells reached the maximum level even with an extremely low MOI value (Fig 1 B and D in III). This was also seen with H5N1/97 virus, another strain of HPAI H5N1 virus, which indicates that this is a universal feature of HPAI H5N1 viruses (Fig 6 A and B in III). In addition, viral RNA levels and viral M and NP protein expression at 24 h post-infection were almost independent of the viral dose in the beginning of the infection in H5N1/04 virus-infected cells, whereas viral RNA and protein expression was dose-dependent in H3N2 and H7N9 virus infected-cells (Fig 2 in III). Our results are comparable with the observation that H5N1 virus infection in humans can lead to a
systemic infection and viral antigens are found from multiple organs beyond the respiratory system\textsuperscript{246}. H5N1 virus infection is also associated with a high viral load in humans, however, productive viral replication was detected only in the lungs\textsuperscript{81, 246}.

Our results, which show that both human and avian isolates of AIV are able to infect human immune cells, can be explained with the SA receptors as moDCs and moM\(\Phi\) express both \(\alpha\)-2,3- and \(\alpha\)-2,6-linked SAs \textsuperscript{155, 162}. On the other hand, based merely on this result, it is impossible to estimate whether these viruses are able to develop into a pandemic strain. Some human\textsuperscript{45} and avian isolates\textsuperscript{247} of H9N2 have been shown to have specificity also for \(\alpha\)-2,6-linked SA and thus these viruses might be able to infect the upper respiratory tract of humans. The receptor binding specificity to \(\alpha\)-2,6-linked SA in several subtypes and the better replication of H9N2 virus in human epithelial airway cells have been associated with Q226L mutation in the HA molecule (according to H3 numbering\textsuperscript{248})\textsuperscript{249-251}. Q226L mutation is present also in the virus used in our studies (A/Hong Kong/1073/99 (H9N2))\textsuperscript{251} which correlates well with our observation of an efficient replication of that strain in human cells. Based on the HA gene sequence from Gisaid (https://www.gisaid.org/), both avian strains H9/529/10 and H9/384/10 have Q in position 234 (position 234 including signal peptide and position 216 without signal peptide), which corresponds to 226 in the H3 numbering. However, it has recently been demonstrated that Q226L mutation in fact correlates poorly with the ability of the virus to bind \(\alpha\)-2,6-linked SAs\textsuperscript{252} so the receptor binding properties cannot be estimated merely based on Q226L mutation. Currently, we do not have information on receptor binding preferences of currently circulating IAVs in Finland.

4.4 The spread of avian influenza viruses in primary human macrophages and dendritic cells

For more closely analyse the spread of HPAI H5N1 virus infection in human cells, dilution series were made of the H5N1/04 and H5N1/97 virus stocks and moDCs were infected with MOI values from 1 to \(10^{-12}\) for 48 h. We analysed the percent of the infected cells by flow cytometry with virus-specific antibodies. Both H5N1/04 and H5N1/97 viruses spread extremely efficiently since still with MOI \(10^{-5}\) most of the cells were infected and the percent of the infected cells did not decrease to the same level with non-infected samples until with MOI \(10^{-8}\) (Fig 5 and 6 C in III). Based on the cell count in the well, FFU/ml value of virus stock and MOI of infection, theoretically there should be only one infective virus particle per well with the dilution of MOI \(10^{-6}\) but still the proportion of the infected cells were around 10\% (Fig 5 in III). This indicates that HPAI H5N1 virus is able to start a productive infection and eventually infect the whole cell culture starting from one infective virus particle.

In order for IAV to be infective, the precursor of HA, the HA0, has to be cleaved to HA1 and HA2 subunits. Proteases, which cleave the HA protein, are not encoded in the genome of IAV, so the cleavage of HA is made by host pro-
teases. The sequence of the cleavage site determines by which proteases HA0 can be cleaved. IAV infection induces upregulation of cellular trypsins by the production of pro-inflammatory cytokines\textsuperscript{253}. The cleavage can take place either in the trans-Golgi network within the cell or extracellularly on the cell surface. The cleavage of seasonal IAV occurs in mammals mainly extracellularly by airway proteases, such as trypsin Clara, mini-plasmin, pancreatic trypsin, cellular trypsins, porcine lung trypstat, TC30, transmembrane serine protease 2 (TMPRSS2) and human airway trypsin-like protease (HAT)\textsuperscript{55, 253-258}. However, these different proteases activate HA of different IAVs with a variable efficiency. Contrary to seasonal IAV and LPAI which bear monobasic cleavage site with a single arginine or lysine residue, HPAI viruses have polybasic cleavage site with several arginine or lysine residues. Instead of restricted cleavage in the airways, the HA cleavage of HPAI viruses occurs by ubiquitously expressed furin and proprotein convertases and this takes place in the trans-Golgi network \textsuperscript{259-261}.

We decided to analyse whether the HA is cleaved in H3N2, H5N1/04, H5N1/97 or H7N9 infected human innate immune cells by analysing the HA0, HA1 and Ha2 protein expression from total cellular proteins. H5N1/04 and H5N1/97 infected cells expressed notable amounts of cleaved HA, while in H3N2 and H7N9 virus-infected cells there was very little of cleaved HA detectable (Fig 3 and 6 in III). However, if we had analysed also the extracellular proteins, it might be that the amount of cleaved HA might be higher also in H3N2 or H7N9 infections, because the cleavage of monobasic cleavage site of HA occurs mainly extracellularly, except TMPRSS2 cleaves HA within the cell \textsuperscript{262}. We concluded that in H3N2 and H7N9 infected cells, the HA0 is not cleaved as efficiently as in H5N1 virus-infected cells, and thus we added TPCK-treated trypsin to the cell culture to activate the cleavage of HA0 of H3N2 and H7N9 viruses. Although the percent of the infected cells was slightly higher in samples treated with TPCK-trypsin, H3N2 and H7N9 viruses were not able to spread as efficiently as the H5N1/04 virus (Fig 4 in III). This indicates that an efficient cleavage of H5N1 HA is not the only reason for the efficient spread of that virus. This is consistent with other studies, which indicate that multibasic cleavage site is an important virulence factor, but not the only factor which caused the systemic spread of some IAV strains. In mice and ferrets infection with HPAI H5N1 virus, where the multibasic cleavage site was removed, led to a restricted respiratory tract infection instead of a systemic one \textsuperscript{263, 264} but, on the other hand, insertion of the multibasic cleavage site in human H3N2 virus did not increase its pathogenicity in ferrets\textsuperscript{265}. Li and colleagues have shown that infection with recombinant virus which have HA and NA gene from PR8 and internal genes from HPAI H5N1, did not lead to high viral loads in mouse models or enhanced replication in epithelial cells, but, instead, lead to increased replication in myeloid cells \textsuperscript{266} which indicates that IAV replication is differently regulated in different cell types by host factors and internal gene segments of seasonal IAV and HPAI virus contribute to virus replication.
4.4.1 Packing of virus particle and productivity of infection

Each of the eight IAV RNA segments are essential for productive infection where new infectious particles are released outside the cell. There are conflicting results on how high is the proportion of virions that possess all eight segments. Several studies indicate that most of virions possess eight RNA segments. But on the other hand, there are also contradictory results which in turn demonstrate that the majority of IAV virions express an incomplete set of viral genes. In addition, semi-infected particles, which deliver an incomplete set of viral genes to the cell, enhance the rate of reassortment and frequent co-infections in the same cell with incomplete virions resulting in a productive infection. We hypothesised that virus particles of HPAI H5N1 virus might be more often completely packed with all eight RNA segments and therefore H5N1 virus is able to spread so efficiently. Accordingly, we tested how many of the primary infective events were productive, in other words how many of the viral particles are propagation competent. By propagation competent virus particle test (study III and 269) we noticed that almost 30% of total infection events were productive with H5N1/04 virus while, significantly less, only about 10% of events, were productive with H3N2 and H7N9 viruses (Fig 7 in III). In addition, we noticed that clusters of H5N1 virus-infected cells were larger and consisted even 10 cells while infection events with H3N2 or H7N9 viruses consisted clearly fewer cells (Fig 7 B in III). Our results were consistent with previous studies which indicate that most of IAV particles are incompletely packed and they are not able to initiate a productive infection. Namely, even with the H5N1 virus 30% of total infection events were productive, which means that 70% of infection events were abortive. In our propagation competent virus test the infection was performed with very low MOI value of 0.01, and therefore it might not permit the co-infection with two or more virus particles which in turn could increase the likelihood of a productive infection.

The propagation competent virus particle test shows the percentage of propagation competent virus particles in the virus stock, but it does not indicate whether the infection is productive in human moMφs and moDCs. In epithelial cells IAV infection is generally considered to be productive leading to a release of new infectious viruses. However, in immune cells the results are contradictory and partially poorly investigated. To investigate the productivity of IAV infection in moMφs and moDCs, we collected the supernatant samples from H3N2, H5N1/04 and H7N9 virus-infected moMφs and moDCs at 1 h and 24 h after infection and analysed the infective virus particles by plaque assay in MDCK cells. The experimental setting was simple for moMφs, because as adherent cells the inoculated virus could be easily washed away after 1 hour. Thus we did not detect any infective virus particles in the 1 h sample and all infective virus particles at the 24 h time point were progeny viruses which were secreted out of the cells. With H5N1 virus-infected cells we detected 1 000 to 100 000-fold higher virus titers at 24 h post-infection, and in H3N2 or H7N9 virus-infected cells the virus titers were increased only 10 to 100-fold during the same follow-up time (Fig 8 C in III). To confirm this, we measured viral
RNA levels in the supernatant samples. In H3N2, H5N1/04 and H7N9 virus-infected moMφs viral RNA levels were clearly increased in supernatants during the infection (Fig 8 C in III). In moDCs, however, the experimental setting was more problematic because of the semi-adherent feature of moDCs as the inoculum could not be washed away. Therefore, the background in 1 h sample was much higher in moDC than in moMφs. H5N1 virus produced approximately 10,000 times higher virus titers after 24 h in contrast to H3N2 and H7N9 infected moDCs where virus titers even seemed to decrease during the follow-up time (Fig 8 B in III). Viral RNA in supernatants from moDCs increased significantly more in H5N1 infection compared to H3N2 or H7N9 infections (Fig 8 D in III). This data indicates that HPAI H5N1 virus infection is productive in human moMφs and moDCs, while H3N2 and LPAI H7N9 virus infections seem to be productive only in moMφs but not in moDCs.

Several studies are consistent with our results and indicate that H3N2 and H5N1 virus infections are productive in human moMφs 9, 240, 272 although the study by Friesenhagen and colleagues 273 indicates that H5N1 and H1N1 virus infections would be abortive in moMφ. There are also contradictory reports on the productivity of IAV infection in alveolar Mφ, as van Riel and colleagues 240 did not notice significant virus production with H5N1, H1N1 or H3N2 viruses, while Yu and colleagues reported productive infection only with H5N1 virus but not with H1N1 virus 147. Perrone and colleagues and Thitithanyanont and colleagues 274 reported that H5N1 virus infection in moDC is productive, which is in line with our result, while according to Bender and colleagues H1N1 virus infection in human DCs is abortive. Cline and colleagues have tested the ability of H1 to H16 viruses to productively replicate in immortalized murine macrophages RAW264.7 cells and noticed that only HPAI H5N1 viruses were able to create a productive infection 275. The productivity of H7N9 virus infection in human Mφs and DC has been extremely poorly investigated, and thus our results introduce valuable new knowledge of IAV infection in human immune cells.

4.5 Human innate immune responses induced by avian influenza viruses

In the respiratory tract the epithelial cells, DCs and Mφs recognize IAV by pattern recognition receptors, which initiate signalling cascades and activation of transcription factors leading to the production of antiviral factors, which restrict and suppress the infection. Although cytokine gene expression is essential in regulating the clearance of IAV infection, the excessive production of cytokines results in inflammation and even immunopathogenesis. The phenomenon “cytokine storm” is associated with a wide variety of infectious and non-infectious diseases. Infection with HPAI H5N1 virus or with 1918 H1N1 pandemic virus has been associated with overwhelming production of inflammatory cytokines and chemokines, leading to prolonged fever, lymphopenia, severe pneumonia and extensive lung damage 81, 276, 277. Thus, the magnitude and duration of cytokine responses have to be tightly regulated.
Although the immune system has multiple means to control virus infection, IAVs have also several strategies to evade the antiviral actions of the immune system. Especially the non-structural proteins of IAV modulate the immune responses of the host. The major inhibitor of innate immune responses is the multifunctional protein NS1, which may suppress antiviral responses, including IFN gene expression, as well as it regulates host gene expression. Also, PB1-F2 protein with N66S mutation has been shown to inhibit the induction of IFNs.

4.5.1 Activation of transcription factors by avian influenza viruses

Activation of transcription factors, such as NF-κB, IRF3 and IRF7, is a prerequisite for the expression of cytokines. To characterize the transcriptional regulation of IFN responses in an avian influenza virus infection, we analysed the expression of different transcription factors in virus-infected human immune cells. IRF3 is the main transcription factor regulating the early antiviral IFN genes. In an inactive form, IRF3 is constitutively expressed and it resides in the cell cytoplasm, but upon IAV recognition IRF3 gets phosphorylated, forms dimers and translocates into the nucleus. Unexpectedly, we noticed that the expression levels of phosphorylated (P)-IRF3 were lower in moDCs or moMφs infected with the human isolates of H9N2, H7N9 or H5N1 AIV than in H3N2 virus-infected moDCs or moMφs (Fig 3 in I, Fig 3 A in II and Fig 11 in III). Especially, in H7N9-infected human moDCs or moMφ the P-IRF3 was completely missing (Fig 3 A in II and Fig 11 in III), although H7N9 virus replicated efficiently in both cell types (Fig I in II and Fig 2 in III). Studies have shown that the NS1 protein of IAV, including NS1 of A/Anhui/1/2013 (H7N9) virus, can interfere with the activation of IRF3 and the subsequent IFN gene expression which may explain our result on the lack of P-IRF3 in H7N9 virus-infected cells. However, another mechanistic explanation for the inhibition of IRF3 activation could be an IAV-induced over-expression of MAPK phosphatase 5 which has been shown as a negative regulator of IRF3-type I interferon activation.

We noticed that in avian-isolated H5N2, H7N3, H9/529, H9/384 virus-infected moDCs virus replication levels were lower than in H3N2 virus-infected moDCs (Figure 4). Therefore, we proceeded to investigate whether the innate immune signalling is activated in cells infected with avian-isolated viruses by analysing P-IRF3 protein expression. As with human-isolated AIVs, P-IRF3 expression was lower in avian-isolated H5N2, H9/529, H9/384 virus-infected moDCs than in H3N2 virus-infected moDCs (Figure 5). In H7N3 virus-infected moDCs P-IRF3 protein expression peaked strongly at 7 h after the infection but otherwise P-IRF3 levels were low (Figure 5). P-IRF3 (Figure 5) levels in H5N2, H9/529, H9/384 virus-infected moDCs correlated well with our results on replication of these viruses (Figure 4), since vRNAs act as ligands to TLR3 and RIG-I (cf Figure 1) and thus lower expression of viral mRNA in H5N2, H7N3, H9/529, H9/384 virus-infected cells than in H3N2 virus-infected cells leads also to reduced activation of IRF3.
To further analyse the activation of transcription factors, we investigated the expression of IRF1 and IRF7. Similarly to IRF3, IRF7 resides in the cytosol in a latent form and during activation it undergoes phosphorylation, dimerization and translocation into the nucleus. In contrast to IRF3, IRF7 is expressed only in low amounts in cells. IRF7 is induced by IFNs through the activation of the ISGF3 transcription factor and IRF7 activation leads to the activation of IFN-α, –β and –λ genes whereas IRF3 activation induces mainly the expression of IFN-β and IFN-λ1 genes. The expression of IRF1 is also induced by IFNs, preferentially by IFN-γ. Unlike in P-IRF3 expression, we did not notice differences between H3N2 and H9N2-infected cells in the expression of IRF1 and IRF9 (Fig 3 in I). IRF7 expression was clearly lower in H7N9 and H5N1/04-infected cells than in H3N2-infected cells (Fig 3 A in II) which correlates with the level of IRF3 activation in these cells (Fig 3 A in II and Fig 11 in III) as IRF7 activation is known to be regulated by IRF3-mediated IFN production. In H5N1/04 and H7N9 infected cells IRF1 expression was almost completely lacking at 24 h despite of strong expression of IRF1 in the 8 h time point (Fig 3 in II). Several studies have associated NS1 protein of HPAI H5N1 virus with the suppression of host immune system. However, a recent study by Thube and colleagues indicates that decreased IFN signaling occurred independently of the viral NS1 and, instead, other viral proteins affected to the decrease in host antiviral state.

In addition to IRFs, NF-κB is also an essential transcription factor in IAV infection, and activation of NF-κB leads to the induction of antiviral and inflammatory responses. Because IκBα is an inhibitor of NF-κB and the degradation of IκBα indicates the activation of NF-κB, we analysed the activation of NF-κB by analysing the protein expression of IκBα. No differences were evident in the levels of IκBα in the cells infected with seasonal influenza H3N2 or with LPAI avian influenza viruses (Fig 3 in I and Fig 3 B in II). On the other hand, we noticed that in H5N1-infected cells IκBα was degraded faster than in H3N2-infected cells (Fig 3 B in II). To investigate further the importance of IRF3/7 and NF-κB transcription factors to the cytokine responses in H9N2 and H3N2 virus infection, we infected MEF cells lacking IRF3 and IRF7 or NF-κB genes with H3N2 or H9N2 viruses for 24 h. Viral and cytokine gene expression was analysed by qRT-PCR and it was noted that in H3N2 and H9N2 virus infections IFN-β and CXCL10 gene expression was reduced in IRF3/IRF7 or NF-κB KO cells, whereas in H3N2 infection TNF-α gene expression seemed to be independent of IRF3/IRF7 transcription factors (Fig 5 in I). The result indicates that in H9N2 and H3N2 virus infection both IRF3/IRF7 and NF-κB are essential factors for an intact cytokine responses. Although we did not notice any clear difference in IAV replication between wt or NF-κB KO cells (Fig 5 B in I), other studies indicate that NF-κB signalling promotes influenza virus infection. Consistent with these studies, our results also indicate that priming the cells prior to infection with pro-inflammatory cytokines TNF-α or IL-1β, which are regulated by NF-κB, promoted the replication of H3N2, H9N2 and H7N9 viruses (Fig 7 in I and Fig 6 in II).
For the activation of interferon-stimulated genes (ISGs) by the JAK-STAT-signalling pathway, STAT1 and STAT2 need to be phosphorylated. P-STAT2 expression was slightly stronger in an early time point (3 h) in H3N2-infected moDC as compared to cells with H5N2, H7N3, H9N2/529 or H9N2/384 infection (Figure 5). With all viruses P-STAT2 expression peaked at 7 h and the expression level decreased within 24 h (Figure 5). In H9N2 virus-infected cells, we noticed that P-STAT1 was expressed at slightly higher levels than in cells with H3N2 virus infection (Fig 3 in I). Moreover, it was evident that H3N2, H5N1/04 and H7N9 virus infection led to the phosphorylation of STAT2 (Fig 11 in III) although NS1 of H5N1 virus has been shown to reduce the activation of STAT2. It seems that seasonal influenza virus H3N2 induces P-STAT2 expression somewhat earlier than AIVs, but with all investigated viruses P-STAT2 expression faded away between 7 and 24 h (Figure 5 and Fig 11 in III). The results indicate that the expression of transcription factors varied between transcription factors and virus strains, but there were no clear disturbance in the activation of transcription factors in AIV infection. The exception was H7N9 virus which did not induce the activation of IRF3 and thus the expression of interferon stimulated transcription factors were also weak.

4.5.2 Cytokine responses induced by avian influenza viruses

Interferon induction is the major mediator of antiviral responses. Consequently we investigated interferon gene expression in AIV-infected cells by qRT-PCR. moDCs were infected with H3N2, H5N2, H7N3, H9N2/529 and H9N2/384 viruses with MOI values of 0.02 for 24 h cells were collected at different time points and total cellular RNA was isolated. First, IFN-β and IFN-λ1 gene expression was analysed which showed that avian isolates of AIVs induced IFN responses in a similar fashion to seasonal H3N2 virus albeit the H7N3 induced stronger IFN responses at a 7 h time point than other viruses (Figure 6). We also investigated IFN responses in moDCs induced by a human isolate of LPAI H9N2 virus. H9N2 virus induced IFN-β, -α1 and -λ1 expression...
as strongly as H3N2 virus (Fig 2 in I). However, it was evident that similar to the H7N3 avian isolate, also H9N2 virus-induced IFN gene expression peaked at 6-8 h after infection, whereas all other investigated avian isolates and seasonal IAV H3N2 peaked at the 24 h time point (Fig 2 in I and Figure 6). Thus, the avian isolate of H7N3 and the human isolate H5N2 induced IFN responses with different kinetics compared to the seasonal IAV H3N2 and avian isolates of H5N2 or H9N2 viruses. These results were consistent with our results on the activation of transcription factors (Fig 3 in 1, Figure 5). This likely indicates that the antiviral signalling is intact and the activation of IRF3, IRF7 and NF-κB leads to the expression of IFN genes. In H7N9-infected moDCs P-IRF3 expression was completely absent and IFN-β, -α1 and -λ1 expression was strongly impaired (Fig 2 in II). This in turn reflected to the expression of P-STAT2 (Fig 11 in III) and IRF7 (Fig 3 in II) consistently with the study by Knepper and colleagues286. Similar results were also obtained from our infection experiment in human alveolar epithelial cells A549 (Fig 5 in II). Recent studies indicate that IFN responses in H7N9/13 virus infection are inhibited by NS1280, 281. However, N66S mutation in PB1-F2 has been shown to increase the virulence of H1N1/1918 and HPAI H5N1 viruses294, and in addition, inhibit IFN responses279. Human isolates of H7N9 viruses encode the full length PB1-F2 protein, but A/Anhui/1/13 (H7N9) virus does not have the N66S mutation in PB2-F2 protein295. However, there may still be other, still unknown factors, which contribute to inhibition of IFN gene expression. Opposite to H7N9 virus, the H5N1/04 virus induced significantly stronger IFN gene expression than H3N2 or H7N9 viruses (Fig 2 in II). This correlates with the clinical findings of severe cytokinemia from patients with H5N1 virus infection81. Matthaei and colleagues reported that in human cells avian isolates of HPAI H5N1 induced stronger IFN responses than human isolates of HPAI H5N1 virus296. On the other hand, our studies with LPAI H9N2 viruses indicate that the origin of the virus from human or avian had no effect to the IFN gene expression levels, at least with the strains that were used our studies.

Figure 6. IFN-β and IFN-λ1 gene expression in moDCs infected with avian isolates of H5N2, H7N3 and H9N2 viruses. moDCs were infected with a human isolate of seasonal influenza virus A/Beijing/353/1989 (H3N2) or with avian isolates of avian influenza virus A/mallard/Finland/13748/07 (H5N2), A/teal/Finland/9201/10 (H7N3), A/mallard/Finland/13384/10 (H9N2) or A/teal/Finland/10529/10 (H9N2) with MOI values of 0.02 for 24 h. The cells were collected at different time points after infection and cells from four donors were pooled and total cellular RNA was isolated. IFN-β and IFN-λ1 gene expression was analysed by qRT-PCR. The values were normalized to 18S rRNA and presented as relative gene expression in relation to the RNA sample obtained from uninfected control moDCs. Statistical differences cannot be assessed, because samples from different donors were pooled.
To prove that IFN mRNAs are really translated to proteins and IFN signaling is intact, we measured secreted IFN protein levels from the supernatant of moDCs with AIV infection by ELISA. We noticed that IFN-β and IFN-α1 levels that were produced (Fig 2 B in I and Fig 2 B in II) correlated well with IFN gene (mRNA) expression levels. In H3N2, H5N1 or H9N2 virus-infected moDCs IFN gene expression was strong and also IFNs were produced in high levels (Fig 2 in I, Fig 2 in II and Fig 10 in III), whereas, in H7N9 virus-infected moDCs IFN gene expression was impaired (Fig 2 in II and 10 in III). Moreover, also the protein expression of IFNs were lower in H7N9 virus-infected moDCs than in H3N2 or H5N1 virus-infected moDCs (Fig 2 B in II).

The severe clinical outcome of HPAI H5N1 virus infection is associated with the overexpression of pro-inflammatory cytokines, resulting in so-called “cytokine storm” 81. Therefore, we also set out to investigate whether the cytokine storm is also seen with other AIVs in human cells. It has been shown that avian-origin H9N2 virus can infect and induce pro-inflammatory cytokine and chemokine production in human A549 cells 297, thus we decided to investigate pro-inflammatory responses in human moDCs. H7N3 virus-infected moDCs expressed higher levels of CXCL10, TNF-α and IL-1β genes than H3N2-infected cells, but H5N2, H9N2/384 or H9N2/529 viruses induced the expression of these genes at similar levels as those induced by the H3N2 virus (Figure 7). However, the kinetics of CXCL10, TNF-α and IL-1β gene expression was slightly faster in H5N2, H7N3, H9N2/384 or H9N2/529 virus-infected cells than in H3N2-infected cells. Pro-inflammatory cytokine gene expression peaked with almost all investigated avian-isolated viruses at 7 h (Figure 7) after infection whereas IFN gene expression typically peaked at 24 h after infection (Figure 6). Similar TNF-α and IL-1β mRNA expression peak at 6-7 h after infection was also seen with H9N2, H7N9 and H5N1/04-infected moDCs (Figure 4 in I and Fig 4 in II). Although there were similarities in pro-inflammatory cytokine gene expression kinetics between different AIV strains, the expression levels were completely different. The maximum levels of gene expression of CXCL10, CCL5, TNF-α and IL-1β in moDCs were similar in H9N2 and H3N2 virus-infected cells (Fig 4 in I), but with H7N9 virus the expression was significantly lower than in H3N2 or H5N1 virus-infected moDCs (Fig 4 in II) although, in the serum of H7N9-infected patients increased levels of chemokines and cytokines have been reported 46. The H5N1 virus induces very strong cytokine expression in moDC and moMφs which is in line with clinical case studies 81 (Fig 4 in II, Fig 9 and 10 in III). Consistent with our result Zhao 298 and colleagues reported that H5N1 and H7N9 viruses can efficiently infect human moMφs (Fig 8 A in III) and the expression of pro-inflammatory cytokines are higher in H5N1 than in H7N9 virus-infected cells (Fig 9 in III). The severe cytokine storm is associated with severe influenza infection with markedly higher serum and lung levels of IFNs, TNFs, interleukins and chemokines 81, 299 and it is only rarely observed in seasonal and other mild influenza virus infections 300. This is in line with our results on cytokine expression by HPAI H5N1 virus. However, it is unlikely that the cytokine storm is caused solely as a consequence of multi-basic cleavage site of HA protein, since it is also associ-
ated with the pandemic H1N1 1918 virus infection. Infection experiments with a recombinant virus that consists of HA and NA from PR8 and internal genes from HPAI H5N1 led to enhanced production of cytokines and IFNs, indicating that the internal genes affect more to the emergence of cytokine storm than the surface glycoproteins. Especially polymerase genes of H5N1 virus have been reported to have an impact on cytokine gene expression.

![Figure 7](image)

Figure 7. Inflammatory cytokine gene expression in moDCs infected with avian isolates of H5N2, H7N3 and H9N2 viruses. moDCs were infected with a human isolate of seasonal influenza virus A/Beijing/353/1989 (H3N2) or with avian isolates of avian influenza virus A/mallard/Finland/13748/07 (H5N2), A/teal/Finland/9201/10 (H7N3), A/mallard/Finland/13384/10 (H9N2) or A/teal/Finland/10529/10 (H9N2) with MOI values of 0.02 for 24 h. Virus-infected cells were collected at different time points and cells from four donors were pooled and total cellular RNA was isolated. CXCL10, TNF-α and IL-1β gene expression was analysed by qRT-PCR. The values were normalized to 18S rRNA and presented as relative gene expression in relation to an RNA sample obtained from uninfected control moDCs. Statistical differences cannot be assessed, because samples from different donors were pooled.

We have shown that HPAI H5N1 virus is able to start an infection and spread to the whole cell culture even when starting at extremely low virus dose (Fig. 1, 5 and 6 in III). At the same time, the H5N1 virus induced a massive IFN response (Fig 2 in II). The infected cells produce IFNs, which should induce antiviral protein expression in autocrine and paracrine manner. In order to elucidate why H5N1 virus is able to spread so efficiently from few infectious particles, we decided to investigate whether the cytokine gene expression was induced with low multiplicity of infection in human moMψs and moDCs. The cells were infected with MOI values of 10, 1, 0.1 and 0.01 and it was evident that at 24 h after infection even with MOI 0.01 the H5N1/04 virus was able to induce massive gene expression of IFN-λ1, IFN-β, IFN-α1, CXCL10, CCL5 and TNF-α in moMψs (Fig 9 in III) and moDCs (Fig 10 in III). With MOI 0.01 in H5N1/04 infected moMψs (Fig 1 B in III) and moDCs (Fig 1 D in III) the proportion of infected cells is only a few percent at a 6 h time point, but it is almost 100% at a 24 h time point. This indicates that H5N1/04 virus is able to spread irrespective of induced cytokine expression. However, it has been
shown that avian and human isolates of H5N1 viruses are sensitive to interferons. So IFNs induced by the virus infection should restrict the spread of the virus. Thus, it may be that HPAI H5N1 virus is able to spread even faster than IFN responses are induced and thus ISGs can not restrict the spread of the virus. The study by Matthaei and colleagues shows that avian isolates of H5N1 induce strong IFN responses, which attenuate virus replication while NS1 of human-isolated H5N1 virus suppress IFN production enhancing the virus replication.

NS1 protein is known to inhibit IFN production by inhibiting IRF3 and NF-κB transcription factors but this feature can vary between different virus subtypes and strains. A very recent study by Wand and colleagues indicates that NS1 of the A/Anhui/1/2013 (H7N9) virus, with the amino acid residue S212, suppresses host RIG-I dependent IFN responses which could be the mechanistic explanation also to our observation of impaired IRF3 activation and subsequent lack in IFN mRNA and protein expression in H7N9 virus-infected cells. In addition to inhibiting transcription factors, NS1 is able to inhibit the processing and translation of IFN mRNA resulting in impaired IFN production. NS1 is shown to bind to the U6 snRNA, CPSF30 or PABII, which inhibits pre-mRNA splicing and polyadenylation. In addition to NS1 protein, which is a well-known IFN antagonist, IAVs have also other mechanisms to suppress host innate immune responses. Many of the influenza A virus strains encode a full-length PB1-F2 protein and PB1-F2 protein has been shown to bind to MAVS and thus inhibits the production of IFN. NS1 protein of IAV can also inhibit IFN production by binding to PKR, which prevents PKR activation and thus inhibits PKR functions to activate IRF3. NP protein has also been shown to bind to a human chaperone Hsp40 which inhibits PKR-mediated IRF3 phosphorylation and IFN production. A study by Yi and colleagues shows that PA protein of IAV, like H5N1 and H7N9 viruses, interacts with IRF3 leading to inhibition of IFN-β production. Thus, there may be multiple factors which could explain our results on attenuated IFN and P-IRF3 expression in H7N9 virus-infected cells.

### 4.5.3 Expression of antiviral proteins by avian influenza viruses

IFNs induce the expression of multiple antiviral proteins, such as MxA. We have shown that infection with avian and human isolates of AIV in human immune cells leads to the expression of IFN genes, except for H7N9-infected cells in which IFN responses were impaired. Next, we set out to investigate, whether IFN gene expression induced by AIV infection lead to the production of antiviral proteins. We noticed that MxA protein was expressed at similar levels in H5N2, H7N3, H9N2/529, H9N2/384 and H3N2 -infected moDC (Figure 8) which correlates well with IFN gene expression (Figure 6). Surprisingly, H9N2/384 virus-infected cells showed the highest MxA protein expression, although IFN gene expression was lowest in these samples.
Results and Discussion

Figure 8. Antiviral MxA protein expression in moDCs infected with avian isolates of H5N2, H7N3 and H9N2 viruses. moDCs were infected with a human isolate of seasonal influenza virus A/Beijing/353/1989 (H3N2) or with avian isolates of avian influenza virus A/mallard/Finland/13748/07 (H5N2), A/teal/Finland/9201/10 (H7N3), A/mallard/Finland/13384/10 (H9N2) or A/teal/Finland/10529/10 (H9N2) with MOI values of 0.02 for 24 h. Cellular protein lysates were collected at different time points and samples from four donors were pooled. The antiviral protein Mxa expression was analysed by Western blotting. GAPDH protein expression was analysed to control equal loading of the samples.

We obtained very similar results from moDCs infected with the human isolate of H9N2 virus: the expression of antiviral protein ISG15, IFITM3 and MxA were equal in H3N2 and H9N2 -infected moDCs (Fig 6 in I) which indicates that LPAI avian influenza viruses induce similar antiviral responses as seasonal influenza viruses. It was surprising that Mxa protein expression was almost at the same level in H7N9 virus-infected cells as in H5N1 or H3N2 virus-infected cells (Fig 3C in II and Fig 11 in III), although IFN gene expression was impaired in H7N9 virus infection (Fig 2 in II). However, it has been shown, that the expression of MxA is very sensitive and a direct marker for IFN induction, since already less than 10 IU/ml of IFN-α can induce MxA protein expression 236. Unexpectedly, we noticed that ISG protein expression, like MxA and IFITM3, were at a similar level in H5N1 and in H3N2 virus-infected cells (Fig 3 in II), which is contradictory for the strong induction of IFN genes in H5N1-infected cells. The inhibition of host protein synthesis during influenza virus infection is called a “host shutoff”. By this mechanism virus can escape from host antiviral responses and promote virus replication and spread. In addition to blocking cellular mRNA processing by NS1 protein, one proposed mechanism behind the host shutoff is host mRNA degradation by the PA-X protein 28, 29, 315, 316. Unlike other non-essential proteins of IAVs, PA-X is encoded by most of IAV strains 317. It has been shown that PA-X decreases the pathogenicity of the 1918 H1N1 virus and HPAI H5N1 virus in mice by modulating host gene expression 28, 29.

4.5.4 Binding of NS1 of H5N1 virus to the importin α-isoforms

The transport of viral RNA into nucleus for viral replication occurs via importin α-isoforms and it has been shown that interactions between viral PB2 and NP proteins and certain importin α-isoforms are one determinant of the host range318, 319. In addition, interactions between importin α-isoforms and viral proteins might also have an impact on host interferon signaling and viral evasion of antiviral defence. Previous studies have shown that STAT1 binds to importin-α5 but not to importin-α759. However, a study by Reid and colleagues showed that importin -α5, -α6 and -α7 (also known as karyopherin α1, α5, and α6, respectively) can interact with tyrosine-phosphorylated STAT1 320. Ebola viral VP24 protein has been shown to bind to the importin-α5, -α6 and -α7 and thus prevent the nuclear accumulation of STAT1 impairing the interferon-
Results and Discussion

We noticed that H5N1/04 induces MxA expression at similar levels as the H7N9 virus (Fig 3 in II and Fig 11 in III) in spite of the interferon gene expression being higher in H5N1/04 infected cells (Fig 2 in II and Fig 9 and 10 in III). IAV NS1 is known to be a multifunctional protein, which can inhibit both IFN production and ISG protein expression despite the differences between different IAV strains. Therefore, we studied whether the NS1 of H5N1 virus inhibits the nuclear localization of STAT1 in the same way as Ebola viral V24 does it by binding to importin-isoforms.

To investigate the JAK-STAT-signalling pathway in H5N1 virus infection, we studied interactions between the NS1 of H5N1 virus and importin-isoforms by an importin binding assay. It was evident that the binding of H3N2 virus NS1 was weak for all human importin-isoforms (α1, -α3, -α4, -α5, -α6 and -α7) (Figure 9). Also, H5N1 virus NS1 binding to importin-α3 and-α5 was weak (Figure 9), which indicates that H5N1 NS1 can not inhibit STAT1 nuclear localization by binding to importin-α5. Interestingly, H5N1 NS1 bound to a greater extent with importin-α1, -α4, -α6 and -α7 (Figure 9). Based on the study by Reid and colleagues, which indicates that Ebola virus VP24 can bind to the importin-α6 and -α7 and therefore inhibit IFN signaling, it might be that the H5N1 virus NS1 can also inhibit the nuclear translocation of STAT1 to some extent. This inhibition, still, can not be complete since some ISG protein expression was evident in H5N1-infected cells (Fig 3 C in II and Fig 11 in III). This result indicates that STAT2 is phosphorylated in H5N1 infection (Fig 11 in III) but the signaling pathway might be disturbed after that because ISG protein expression was at same level in H3N2 and H5N1 virus-infected cells (Fig 3 in II and Fig 11 in III) despite strong IFN expression (Fig 2 in II and Fig 9 and 10 in III) and efficient replication and spread of the H5N1 virus (Fig 1 in II and Fig I and II in III). There is still some inconsistency with our result and the study by Jia and colleagues where the authors showed that transfected H5N1 NS1 reduces tyrosine phosphorylation of STAT1, STAT2 and STAT3 induced by IFNs, and inhibits the nuclear translocation of STAT2.

Figure 9. NS1 protein of highly pathogenic avian influenza H5N1 and seasonal influenza H3N2 viruses bind to importin-α1, -α3, -α4, -α5, -α6 and -α7-isoforms. NS1 protein of A/Vietnam/1203/2004 (H5N1) or A/Udorn/1972 (H3N2) viruses were in vitro translated and 35S-labeled and allowed to bind to GST-importin-α1, -α3, -α4, -α5, -α6 and -α7. Importin-bound proteins were separated by SDS-PAGE. 35S-labeled NS1 proteins were visualized by autoradiograph. Coomassie blue staining was used to visualize the equal amount of Sepharose-
immobilized GST-importins. I abbreviation refers to input of *in vitro* translated NS1 without GST binding. C abbreviation refers to sepharose matrix bound *in vitro* translated NS1 without GST binding. G abbreviation refers to sepharose-immobilized GST.

### 4.6 Sensitivity of the avian influenza viruses to interferons

For IAV control strategy, vaccination against IAV remains a challenge due to antigenic drift and shift among seasonal influenza and unpredicted occurrence of new influenza A virus subtypes. Thus, in addition to the prevention of infections by vaccination, also antiviral drugs are needed to treat IAV infections. Currently there are three kinds of antiviral drugs: M2 ion channel blockers (amantadine and rimantadine), NA inhibitors (oseltamivir, zanamivir and peramivir) and in the clinical trials polymerase inhibitor (favipiravir)\(^\text{323, 324}\). However, there are some obstacles in the use of antiviral drugs. Effectivity may be limited and virus resistance to drugs has appeared among seasonal influenza but also in avian influenza viruses isolated from humans who have been treated by antivirals\(^\text{123, 143, 325-328}\). IFNs serve as the first line of antiviral defense and clearance of IAV infection is mainly dependent on the antiviral actions of IFNs\(^\text{329}\). The treatment of the patient with IFNs has been proposed to inhibit virus spread and promote viral clearance\(^\text{329-331}\). The advantages are that antiviral actions of IFNs are not strain specific and thus far there is no unquestionable evidence that IAV virus could adopt resistance against IFNs *in vivo*, however, *in vitro* IFN-resistant IAV strains have emerged\(^332\). Seo and colleagues\(^333\) have reported that HPAI H5N1 viruses would not be sensitive to the antiviral actions of IFN, but their study is highly contradictory with other studies\(^296, 304, 334-336\) and has been disagreed later by Ngunjiri and colleagues\(^304\). To investigate whether the human isolates of avian origin H7N9 and H9N2 viruses are sensitive to the antiviral actions of type I IFNs, we pre-treated moDCs with IFN-α- or IFN-β for 16 h before virus infections. Viral M1 mRNA expression decreased remarkably in IFN-pretreated cells, indicating that both H9N2 (Fig 7 in I) and H7N9 (Fig 6 in II) viruses were extremely sensitive to the antiviral actions of IFNs. However, there could be some limitations of using IFNs as antiviral drugs against influenza. Studies indicate that the timing of IFN treatment is essential, the therapeutic window is short and although later treatment could restrict the viral spread, it can also enhance the side effects of IFNs leading to increased cell deaths\(^331, 337, 338\).
5. Conclusions and future perspectives

Avian influenza viruses are widespread around the world among birds and some AIVs are able to infect humans with high mortality rates. In addition to direct transmission from avian species to humans that results in sporadic human infection, AIVs can contribute to the evolution of seasonal influenza viruses with unpredictable consequences. It is most likely that also next pandemic influenza strain will contain genes from avian influenza viruses, so it will be extremely important to investigate avian influenza infections in human cell models.

In this thesis avian influenza virus infections in human primary macrophages and dendritic cells were studied. Virus replication, spreading and innate immune responses induced by avian influenza viruses were studied. Low pathogenic strains were used, isolated from human patients or from Finnish wild birds and human isolates of highly pathogenic H5N1 strains. It was evident that both human and avian isolates of low pathogenic avian influenza viruses were able to infect human cells and to induce similar innate immune responses as seasonal influenza viruses. In these studies, we have shown that H5N1 viruses were able to start a productive infection from extremely low virus amounts and spread efficiently to the whole cell culture in spite of strong cytokine responses in human macrophages and dendritic cells. A proposed model for HPAI H5N1 or seasonal influenza virus infection is presented in Figure 10. Further studies are needed to investigate whether this is a feature of all highly pathogenic avian influenza viruses, including the recently emerged HPAI H7N9 virus, or whether it is limited to the H5 subtype. In contrast to excessive cytokine responses induced by H5N1 viruses, the H7N9 virus induced relatively weak antiviral innate immune responses. Our results indicate that both the H5N1 and the H7N9 virus can avoid innate immunity but by different mechanisms. The publication appended to this thesis (II) on innate immune responses induced by H7N9 virus was among the first publications regarding the novel H7N9 virus and innate immunity. Alongside the scientific work of this thesis study, this work contributed to the development of safe working practices and methodology at the biosafety level 3 laboratory in THL and thus improved the preparedness for emerging virus threats in Finland.
Conclusions and future perspectives

Recent outbreaks by novel avian influenza subtypes, such as those of H7N9 and H5N6 viruses, has shown that there will definitely be more human infections with avian influenza viruses also in the future. Recently, highly pathogenic avian influenza viruses were detected also in the Nordic countries, which indicates that global preparedness is necessary for human infections with these viruses. Although no human infection with HPAI H5N8 virus has so far been detected, the likelihood of such an event cannot be excluded. Thus, an appealing subject for future work could be to investigate the replication, spreading and virus-induced innate immune responses by these recent HPAI strains in human cells.

In addition to the essential proteins of IAV, multiple new virus-specific accessory proteins have been identified. The PB1-F2 and PA-X have recently been the most actively studied assessor proteins, but also other non-essential proteins, PB2-S1, PB2-$\Delta$, PB1-N40, M42, NS3, PA-N155 or PA-N182, may have an impact on host immune responses. Studies indicate that at least PB2-S1 and PB2-$\Delta$ can modulate host immune responses, but definitely further investigations are needed on the interactions between these newly identified viral proteins and the host.
References


89. Centers for Disease Control and Prevention. Avian Influenza A (H7N2) in Cats in Animal Shelters in NY; One Human Infection. **2018** (2016).


94. WHO. Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness; *WHO*, 1-10 (2018).


100. Lindh, E. Avian Influenza and Newcastle Disease viruses in Finland. (2015).


105. Centers for Disease Control and Prevention. Summary of Influenza Risk Assessment Tool (IRAT) Results. **2018**.


Influenza A viruses are one of the most significant respiratory pathogens to humans. Avian influenza H7N9, H5N1, H7N7 and H9N2 viruses have caused infections in humans with very high mortality. In the present study, viral replication and the mechanisms of activation of innate immune responses induced by avian influenza viruses have been characterized in human immune cells. Human monocyte-derived macrophages and dendritic cells have been used as cell models, since they play a central role in host resistance against viral infections. This study provides new information on how avian influenza viruses replicate in primary human cells and how host antiviral responses are activated.