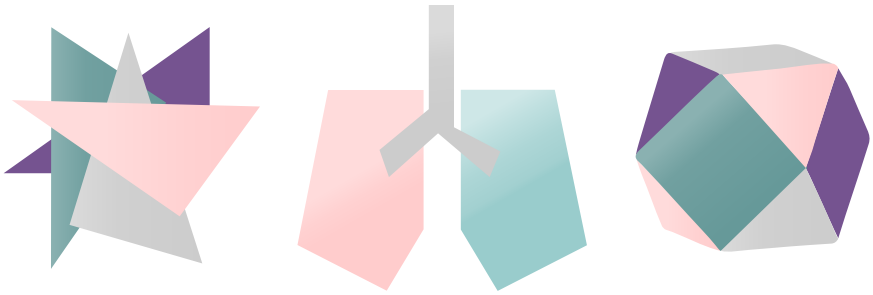
Thesis for doctoral degree (Ph.D.)
2016

Human dendritic cells in blood and airways during respiratory viral infection



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HUMAN DENDRITIC CELLS IN BLOOD AND AIRWAYS DURING RESPIRATORY VIRAL INFECTION

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Stockholm 2016

Published by Karolinska Institutet.
Printed by E-Print AB, 2016.

ISBN 978-91-7676-373-5

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

Publicly defended in Rolf Luft Auditorium, L1:00
Karolinska University Hospital, Solna

Friday October 21st 2016 14.00

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You might as well question why we breathe.

If we stop breathing, we'll die.

If we stop fighting our enemies,

the world will die.

Victor Laszlo in Casablanca

ABSTRACT

The air we inhale contains oxygen necessary for life, but also potentially harmful microorganisms, toxins and allergens. This presents an important immunological dilemma: how can our lungs quickly and selectively eliminate harmful agents without inflicting damage on the delicate tissues of the lungs? We have thus evolved a network of cells involved in immune surveillance, made up of dendritic cells (DCs), monocytes and macrophages. Together, these mononuclear phagocytes sample the lungs and airways for presence of foreign pathogens such as viruses or bacteria. Recognition of pathogenic patterns – for instance the genetic material of viruses or the lipid membrane of bacteria – triggers a cascade of events in these immune cells. They produce inflammatory mediators to signal that a source of danger has been detected, and to contain the infection while awaiting the arrival of other immune cells. DCs migrate to lymphoid organs where they present antigens to naïve T cells, thus shaping the generation of protective and adaptive immunity. Much of what we know of how our immune system functions come from studies in murine models.

In this thesis, we focus our attention on human DCs. Using super resolution microscopy, we assessed the early trafficking events that take place upon internalisation of influenza A virus (IAV) by human DCs. We report that IAV trafficked via early and late endosomes in DCs, similar to epithelial cells, but with more delayed kinetics. Next, we investigated whether maturation of monocyte-derived versus *bona fide* DCs affects their susceptibility to IAV infection. Indeed, the two subsets of DCs are inherently different in their ability to respond to pathogenic signals by producing antiviral mediators, which protect them from IAV infection. The accessibility of human blood has improved our understanding of human DCs. However, immune cells residing at mucosal barriers are our first line of defence against respiratory viruses. Increasing data suggest that there is tissue-specific regulation of immune cells due to factors present in the local microenvironment. Hence, we performed bronchoscopies on healthy subjects and hantavirus-infected patients to characterise DCs residing in the airways and bronchial mucosal tissue. We identified several subsets of respiratory DCs at steady state, alongside alveolar macrophages and monocyte-derived cells. During acute hantavirus disease, DCs and monocytes were depleted from circulation, whereas the lungs were infiltrated with monocytes and DCs.

Collectively, our findings reveal the heterogeneity of human DCs in their response to respiratory viruses, depending on their origin and anatomical location. A deeper understanding of the complex interplay between respiratory viruses and human DCs reveals how DCs contribute to immunity or pathogenesis. This knowledge may help us develop better preventive and therapeutic strategies by targeting or modulating DCs to achieve favourable immune responses.

LIST OF SCIENTIFIC PAPERS

I. **Faezzah Baharom**, Oliver S Thomas, Rico Lepzien, Ira Mellman, Cécile Chalouni and Anna Smed Sörensen.

Visualization of early influenza A virus trafficking in human dendritic cells using STED microscopy.

Manuscript

II. **Faezzah Baharom**, Saskia Thomas, Andrea Bieder, Maria Hellmér, Julia Volz, Kerrie J Sandgren, Gerald McInerney, Gunilla B Karlsson Hedestam, Ira Mellman and Anna Smed Sörensen.

Protection of human myeloid dendritic cell subsets against influenza A virus infection is differentially regulated upon TLR stimulation.

J Immunol, 2015 May, 194(9):4422-30

III. **Faezzah Baharom**, Saskia Thomas, Gregory Rankin, Rico Lepzien, Jamshid Pourazar, Annelie F Behndig, Clas Ahlm, Anders Blomberg and Anna Smed Sörensen.

Dendritic cells and monocytes with distinct inflammatory responses reside in lung mucosa of healthy humans.

J Immunol, 2016 June, 196(11):4498-509

IV. Saskia Scholz*, **Faezzah Baharom***, Gregory Rankin, Kimia T Maleki, Shawon Gupta, Sindhu Vangeti, Magnus Evander, Jamshid Pourazar, Andrea Discacciati, Jonas Höijer, Matteo Bottai, Niklas Björkström, Johan Rasmuson, Hans-Gustaf Ljunggren, Anders Blomberg, Jonas Klingström, Clas Ahlm and Anna Smed Sörensen. **equal contribution*

Massive depletion of monocytes and dendritic cells in human peripheral blood during acute hantavirus infection.

Manuscript

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PREFACE

This thesis is a documentation of research conducted in support of my doctoral degree (PhD). In the past four years, my colleagues and I have dedicated our time and effort in investigating the role of human dendritic cells, an important player in our immune system, in defending us against respiratory viruses such as influenza virus and hantavirus. The thesis can be broadly divided into two sections:

In the first section, I will provide an overview into the field of human immunology, by introducing basic concepts of immunity and inflammation that is hopefully accessible to all readers. I will then define the general aim of this thesis and the specific aims of the four studies included. Finally, I will explore our existing knowledge of the topic by delving deeper into our understanding of dendritic cells and also the close relationship with monocytes and macrophages, with details on their history, origins and roles. An overview of the respiratory viruses responsible for causing diseases will also be presented, with details on their molecular structure and life cycle and the immune responses mounted against the viruses.

In the second section, I will describe the materials and methods used throughout the papers included in the thesis. I will then highlight the key findings of each study and provide an analysis of their implications in the context of existing literature. Finally, I will summarise the main conclusions of this thesis and speculate the future directions of where the studies may continue moving forward, followed by reprints of the original papers.

Faezzah Baharom *September 2016, Stockholm*

LIST OF ABBREVIATIONS

APC	antigen-presenting cell
BATF3	basic leucine zipper ATF-like transcription factor 3
BDCA	blood dendritic cell antigen
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
cDC	conventional DC
CDP	committed DC progenitor
cMop	common monocyte progenitor
CMP	common myeloid progenitor
CSF	colony stimulating factor
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
FLT3	Fms-like tyrosine kinase 3
GM-CSF	granulocyte macrophage colony stimulating factor
HA	haemagglutinin
HPS	hantavirus pulmonary syndrome
HFRS	haemorrhagic fever with renal syndrome
HSC	haematopoietic stem cells
HTNV	Hantaan virus
IAV	influenza A virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
ISG	interferon-stimulated genes
LPS	lipopolysaccharide
MDC	myeloid dendritic cell

MDDC	monocyte-derived dendritic cell
MDP	monocyte-macrophage DC progenitor
MHC	major histocompatibility complex
MNP	mononuclear phagocytes
MxA	myxovirus resistance gene A
MyD88	myeloid differentiation primary response 88
NA	neuraminidase
NK	natural killer
NP	nucleoprotein
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
poly(I:C)	polyinosinic:polycytidylic acid
PRR	pathogen recognition receptor
PUUV	Puumala virus
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
SNV	Sin Nombre Virus
Th	T helper cell
TGF-β	transforming growth factor β
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β

1 INTRODUCTION

Immunology is the study of our body's immune system, evolved to protect us against various threats. The origin of these threats can be foreign, such as invading bacteria and viruses called pathogens, or self, such as cancers or damaged cells from injury. In the late 18th century, Edward Jenner demonstrated the concept of immunity; getting cowpox, a mild disease, can be protective against the more deadly smallpox[1]. This laid the foundation for modern vaccination, where healthy individuals are inoculated with weakened pathogens to prevent disease. Seminal studies by Louis Pasteur and Robert Koch further established the "germ theory": specific microorganisms cause infectious diseases[2,3]. To protect ourselves against disease-causing pathogens, our body is trained to mount an immune response. This is a highly coordinated process that can be divided into 4 main tasks: 1) identify the threat, 2) eliminate the threat, 3) avoid collateral damage to harmless bystanders, and 4) remember the threat.

The human respiratory tract is organised like a tree with a trachea that branches into airways terminating in millions of vascularised alveoli, the site of gas exchange. The total surface area of healthy human lungs is approximately 90 m^2; a massive area exposed to the external environment[4]. Hence, the lungs need to be poised to respond to invading pathogens. The airways can filter out virus particles in inhaled air via nasal hairs and mucous layers that trap the virus particles thus preventing virus binding and attachment to host cells. However when the viruses penetrate the mucous layer, immune cells need to be able to detect the breach in physical defence and retaliate against the invading pathogens (Figure 1).

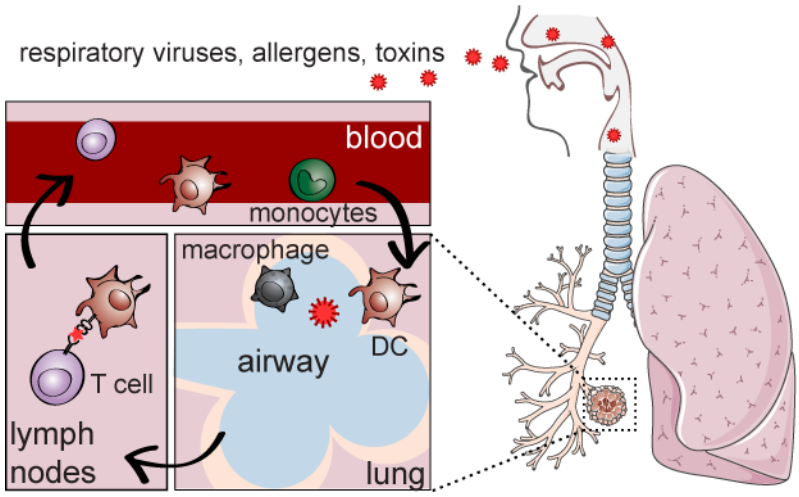

Figure 1. Initiation of immune responses in the lungs against respiratory threats. Innate immune cells such as dendritic cells (DCs) and alveolar macrophages reside in the airways to sample incoming pathogens, allergens or toxins. During inflammation, more monocytes and DCs infiltrate the lungs from circulation. Mature DCs migrate to draining lymph nodes via the afferent lymphatic vessels where they present antigens to naïve T cells. Antigen-specific T cells exit via the efferent lymphatic vessels and home to the site of infection. Lung illustration modified from Servier Medical Art.

Viruses are small, obligate parasites that are entirely dependent on their host cell for survival[5,6]. They carry their own genetic material, but cannot replicate without using the machinery of their host[7]. Influenza A virus (IAV) and hantavirus are the main threats that are in focus in this thesis due to their ability to cause disease and even death in humans. IAV causes the seasonal flu, or the occasional but more severe pandemic flu. Hantaviruses can cause haemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS) depending on the viral strain. The respiratory tract acts as an entry point of these viruses into their human host. IAV infection typically manifests within the upper airways, whereas hantaviruses first replicate in the lungs but can then enter the blood circulation and be detected in various other organs.

The innate immune response is readily available to detect and respond to a wide range of pathogens without developing long lasting memory[8]. Innate immune cells include monocytes, macrophages and dendritic cells (DCs), key players of our innate immunity that will be the focus of this thesis, but also granulocytes, mast cells and natural killer (NK) cells. These cells recognise pathogen-associated molecular patterns (PAMPs) via receptors such as Toll-like receptors (TLRs). Activation of TLRs trigger the production of specific proteins called cytokines and chemokines, and chemical factors such as histamines and prostaglandins, which mediate inflammation. While individual innate immune cells play different roles, they can all contribute to inflammation, clinically recognised as the development of fever, pain, swelling and ache. Inflammation is an important response to an infection, as it triggers the permeability of blood vessels so that more immune cells can enter the site of infection from the blood. However, a dysregulated immune response can lead to exaggerated inflammation that is harmful to surrounding, healthy cells.

In contrast, the adaptive immune response is specific and long lasting[9]. The key players in our adaptive immunity include B cells, responsible for generating antibodies against specific pathogens, and T cells, subdivided into $CD4^+$ T helper cells (Th) or $CD8^+$ T cells that develop into cytotoxic T lymphocyte (CTLs), killing infected cells directly. As professional antigen presenting cells (APCs), DCs that patrol the peripheral tissues acquire antigens (e.g. small fragments of a pathogen) and present them to B and T cells on receptors called major histocompatibility complex (MHC). This specific interaction between an antigen-MHC complex and the B cell or T cell receptor, gives effector cells the licence to produce antibodies or attack infected cells. CTLs can also be harmful if not carefully regulated, hence negative feedback and immunosuppressive mediators are an important aspect of how our immune system can function properly.

Now, 220 years after Edward Jenner experimented on a healthy eight-year-old boy to discover smallpox vaccine, stricter ethical guidelines have been put in place for conducting modern day research, in order to protect human volunteers from abuse.

The Declaration of Helsinki established in 1964 requires that human research be based on results from laboratory animals[10]. As a consequence, the focus of immunological research has shifted to using inbred mouse models. This led to a wider range of tools to manipulate different aspects of the immune system, resulting in many important discoveries. Among others, DCs were discovered in the spleen of mice with the unique ability to activate naïve T cells[11]. Important fate mapping studies in mice have also dissected the origin of different immune cell populations[12]. However, a reliance on mouse models to study the immune response against infectious diseases may have skewed our understanding of human immune responses that are ultimately most critical in developing effective human vaccines. Misinformation can also be dangerous, as we have learnt from the anti-CD28 clinical trials on six healthy human volunteers, resulting in massive cytokine storms and multi-organ failure that was not predicted in animal studies[13]. This reaffirms the need for more translational studies in humans to overcome the discrepancies between the immune system of animal models and of humans. Although mechanistic insights may be limited in human immunology studies, they can complement important findings in mice in a more correlative manner.

2 AIMS OF THESIS

The general aim of this thesis was to better understand how DCs, equipped to detect, capture and present viruses to other immune cells, are involved during respiratory viral infections in humans. The specific objectives were as follows:

- To study the early trafficking events of IAV upon internalisation by human DCs, using super resolution imaging **(Paper I)**,

- To assess how prior exposure to pathogenic signals induces maturation and affects the susceptibility of different subsets of human DCs to infection by IAV **(Paper II)**,

- To characterise the phenotype and function of monocytes and DCs in blood and airways at steady state **(Paper III)**,

- To investigate the impact of hantavirus infection on monocytes and DCs in blood and lung mucosal tissue during acute and convalescent phases of disease in hantavirus-infected patients compared to healthy controls **(Paper IV)**.

3 DENDRITIC CELLS, MONOCYTES & MACROPHAGES

3.1 BRIEF HISTORY OF MONONUCLEAR PHAGOCYTES

3.1.1 Discovery of mononuclear phagocytes

Mononuclear phagocytes (MNPs) refer to an umbrella term including DCs, monocytes and macrophages. They form a heterogeneous population of cells that excel in taking up antigens for destruction, or for processing and presentation to initiate and regulate immune responses[14]. The capacity of DCs to regulate immune responses have made them attractive targets for vaccination, cancer immunotherapy, antiviral therapy and treatment of autoimmune/inflammatory diseases[15-17].

The term mononuclear phagocytes was first coined in the 1960s by van Furth, referring to both circulating monocytes and tissue macrophages[18], as opposed to the other group of polymorphonuclear phagocytes (granulocytes)[19], but their history dates further back. In the 1880s, the concept of phagocytosis (from ancient Greek, meaning “to devour”) was established by the Nobel Laureate Elie Metchnikoff, who described the ability of macrophages to engulf foreign entities as a defence mechanism[20]. Following labelling studies using radioactive thymidine, monocytes were defined as precursors of macrophages circulating in blood as has been extensively studied by van Furth and others. In 1973, the Nobel Laureate Ralph Steinman discovered a novel type of “dendritic-shaped cell that can process and present antigen to activate naïve T cells" in the spleen of mice, calling them DCs in his seminal papers[11,21-23]. DCs then joined monocytes and macrophages as another member of the mononuclear phagocyte system.

3.1.2 Relationship between DCs, monocytes and macrophages

More recently, there has been a paradigm shift in our understanding of the relationship between DCs, monocytes and macrophages. For decades since their discovery, DCs and macrophages were thought to be functional variations of monocytes. This was supported by the ease in which monocytes can be skewed to behave like DCs or macrophages *in vitro*[24-27], depending on the culture conditions, and also *in vivo* during inflammation[28-31].

However, more careful lineage studies in mice have identified haematopoietic precursors to DCs (called committed DC progenitors, CDPs) that are distinct from monocytes (Figure 2)[32-34]. Monocytes are derived from a different progenitor (called common monocyte progenitor, cMop)[35]. These observations in mice have also been confirmed in humans following the identification of DC precursors in circulation, cord blood and bone marrow[36,37]. Another paradigm-shifting discovery is that tissue-resident macrophages are not exclusively derived from circulating monocytes, as has been the dogma following van Furth’s findings in the 1960s. Instead, tissue-

resident macrophages can develop from embryonic precursors such as yolk sac macrophages or fetal liver monocytes[12,38-44]. In short, there is mounting evidence to suggest that monocytes, DCs and macrophages are not developmental progressions from one cell type to another, but instead originate from distinct precursors.

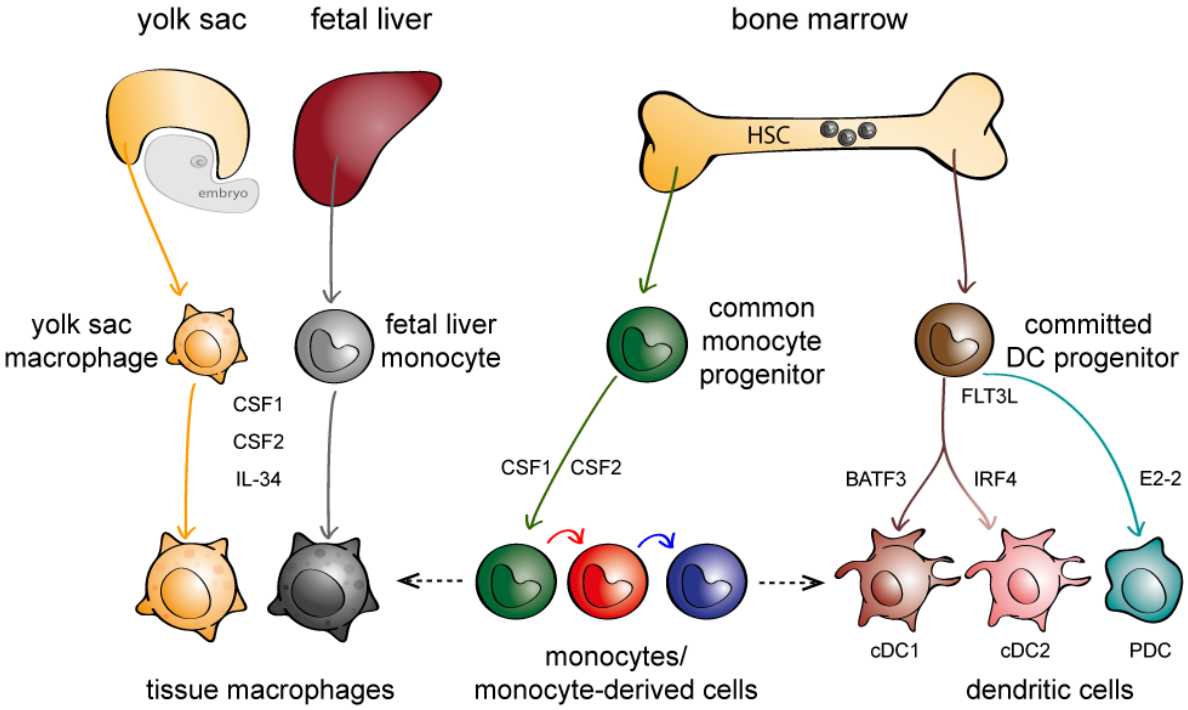

Figure 2. The embryonic and haematopoietic development of mononuclear phagocytes. Monocytes, DCs and macrophages originate from distinct lineages, with monocytes and DCs originating from haematopoietic stem cell (HSC) precursors, whereas some tissue macrophages have an embryonic origin. They are also differentially dependent on various growth factors and express different transcription factors critical to their development.

The matter is complicated by the plasticity of monocytes that can acquire different functional properties shared by macrophages and DCs, depending on the inflammatory environment[45,46]. Identification of cell types based purely on expression of surface markers or functional specialisation presents a challenge as several different populations share the same receptors, and subsets can acquire or lose functional capacities during inflammation[47]. Beyond semantics, the definition of cell populations is important for interpretation and translation of findings between different groups, especially when specific functional attributes are assigned to distinct populations. A shift towards complementing phenotypic identification with transcriptional profiling has allowed a better separation of DCs, monocytes and macrophages, including a better alignment of cells across tissues and species. The important contributions of individual cell populations can then be carefully elucidated, to improve our understanding of immunopathogenesis or to be able to design better vaccine/therapeutic strategies targeting specific MNPs.

3.2 SUBSETS AND FUNCTIONS

3.2.1 Ontogeny

The ontogeny of DCs refers to both the origin from their precursors, and the development pathways resulting in functional DCs. Classification of cell populations based on their ontogeny has been proposed by several experts in the field as a consistent and robust way to distinguish *bona fide* DCs from monocytes and macrophages, allowing us to understand their specific roles during health and disease[46].

Experiments in murine models relying on adoptive transfer of specific precursors to irradiated animals have defined DCs as a separate haematopoietic lineage[34,48]. Briefly, their lineage begins in the bone marrow from haematopoietic stem cells (HSCs) in the following sequential order of increasingly restricted progenitors: common myeloid progenitor (CMPs), monocyte-macrophage DC progenitors (MDPs), common DC progenitor, and finally PDCs or pre-classical DCs (cDCs) that can differentiate to either cDC1 ($CD141^+$ MDCs in humans) or cDC2 ($CD1c^+$ MDCs in humans). Maintenance of DC development is linked to their expression of Fms-like tyrosine kinase 3 (FLT3) and their ability to respond to FLT3 ligand[49,50].

In parallel, monocytes diverge at the MDP stage where they form common monocyte progenitors (cMop) with the potential to develop into monocytes and monocyte-derived macrophages, relying on the cytokine CSF-1. As briefly discussed before, the origin of tissue-resident macrophages is more heterogeneous, as they can arise from yolk sac macrophages, fetal liver monocytes or bone marrow monocytes, depending on the tissue[51].

Translation of these findings to human DC ontogeny has been difficult, but a combination of comparative transcriptomics analyses, clinical observations and *in vitro* culture models support the observations in mice. Cross-species transcriptomic studies suggest that PDCs, $CD1c^+$ DCs and $CD141^+$ DCs represent *bona fide* DCs as they are homologous to well-defined mice PDCs, $CD11b^+$ DCs and $CD8^+$/$CD103^+$ DCs respectively[52-59]. Patients undergoing haematopoietic stem cell transplantation have been valuable to study the kinetics and turnover of DCs in tissues[60]. Further, patients lacking *GATA2* and *IRF8* are deficient for all DC subsets in their blood, suggesting a common precursor that is missing[61,62]. Finally, several recent studies have advanced the DC field by mapping human myeloid precursors that closely resemble those found in mice bone marrow and blood[36,37] (Figure 3). In human cord blood and bone marrow, human granulocyte-monocyte-DC progenitors (hGMP), human monocyte-DC progenitors (hMDP) and human common DC progenitors were identified, with increasingly restricted capacity to generate specific cell populations[37]. In blood, a migratory precursor giving rise to only $CD1c^+$ and $CD141^+$ MDCs were identified[36].

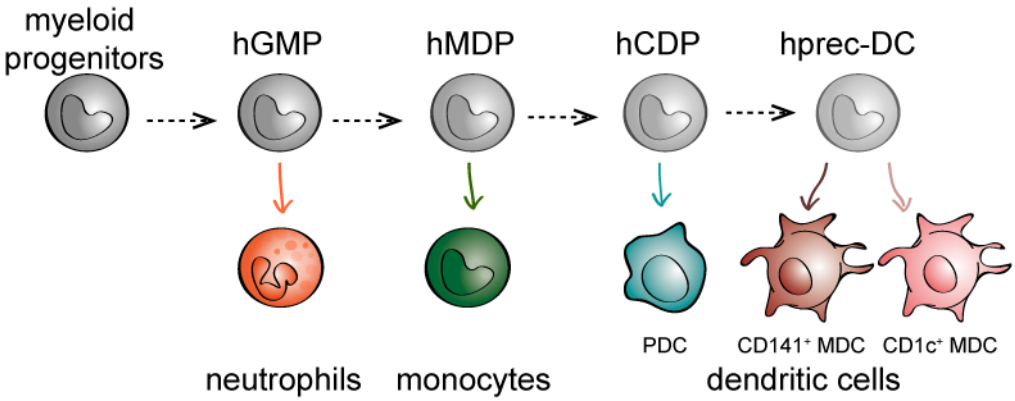

Figure 3. Schematic displaying the sequential origin of human DCs from myeloid progenitors. *In vitro* culture models recapitulate *in vivo* DC hematopoiesis employing progenitors from human cord blood and bone marrow; human granulocyte-monocyte-DC progenitor (hGMP), human monocyte-DC progenitor (hMDP), human common DC progenitor (hCDP) and human migratory precursor (hprec-DC).

3.2.2 Cell surface markers

Defining DCs based on ontogeny presents a challenge in humans, in the absence of amendable genetic studies to deplete entire lineages. Hence, cell surface markers continue to be a reliable source of information for classification of DCs, monocytes and macrophages based on their phenotype, preferably supported by transcriptomic and functional analyses. Recent comparative studies have attempted to unify the MNP populations between mice and humans. The cell surface markers used to identify and sort out individual populations of MNPs are summarised in Figure 4. A common gating strategy used by our group and several others to identify DCs in blood and tissue is by first gating on all haematopoietic cells ($CD45^+$), excluding all lineage cells (monocytes, B cells, T cells, NK cells and neutrophils) and then gating on cells expressing the MHC class II molecule, $HLA\text{-}DR^+$ cells to identify DCs[63,64]. CD11c can be used to distinguish myeloid DCs from plasmacytoid DCs. Aside from peripheral blood, all three populations have also been identified in human bone marrow, skin, gut, lungs, liver, spleen, lymph nodes and tonsils[52,55,64-68]. However, the precise phenotype of DCs in human tissue, such as the lungs, continues to be investigated and debated upon. The most studied tissue in humans is the skin[69,70]. Most recently, Guilliams *et al.* propose a framework to standardise the identification of DCs in human tissues at steady state and during inflammation[71].

In human blood, three populations of monocytes have been described based on their differential expression of CD14 and CD16: classical monocytes, intermediate monocytes and non-classical monocytes[72]. In mice, monocytes under non-inflamed conditions can extravasate constitutively into tissues while retaining their monocytic character[41]. It is still unclear if, in humans, all three monocyte populations present in blood can extravasate into tissues, and whether they remain undifferentiated, or differentiate into macrophages/DCs. In the human airways, $CD14^+CD16^+$ cells are a major population of MNPs, forming a third of all $HLA\text{-}DR^+lineage^-$ cells, excluding

alveolar macrophages[64]. In peripheral tissues, phenotypic analysis is complicated by the presence of monocyte-derived cells with overlapping cell surface markers that exhibit features of DCs or macrophages. $CD14^+$ cells in the skin have previously been thought to be an additional DC population, but recent transcriptomic and functional analysis suggested that these cells are distinct from the DC lineage, instead resembling monocyte-derived macrophages[73]. Similar careful characterisation of $CD14^+$ cells described in human lungs[64,65], would be valuable to understand whether these are tissue monocytes, monocyte-derived DCs or monocyte-derived macrophages. A better definition of DCs, monocytes and macrophage populations will also help to translate important findings across different research groups.

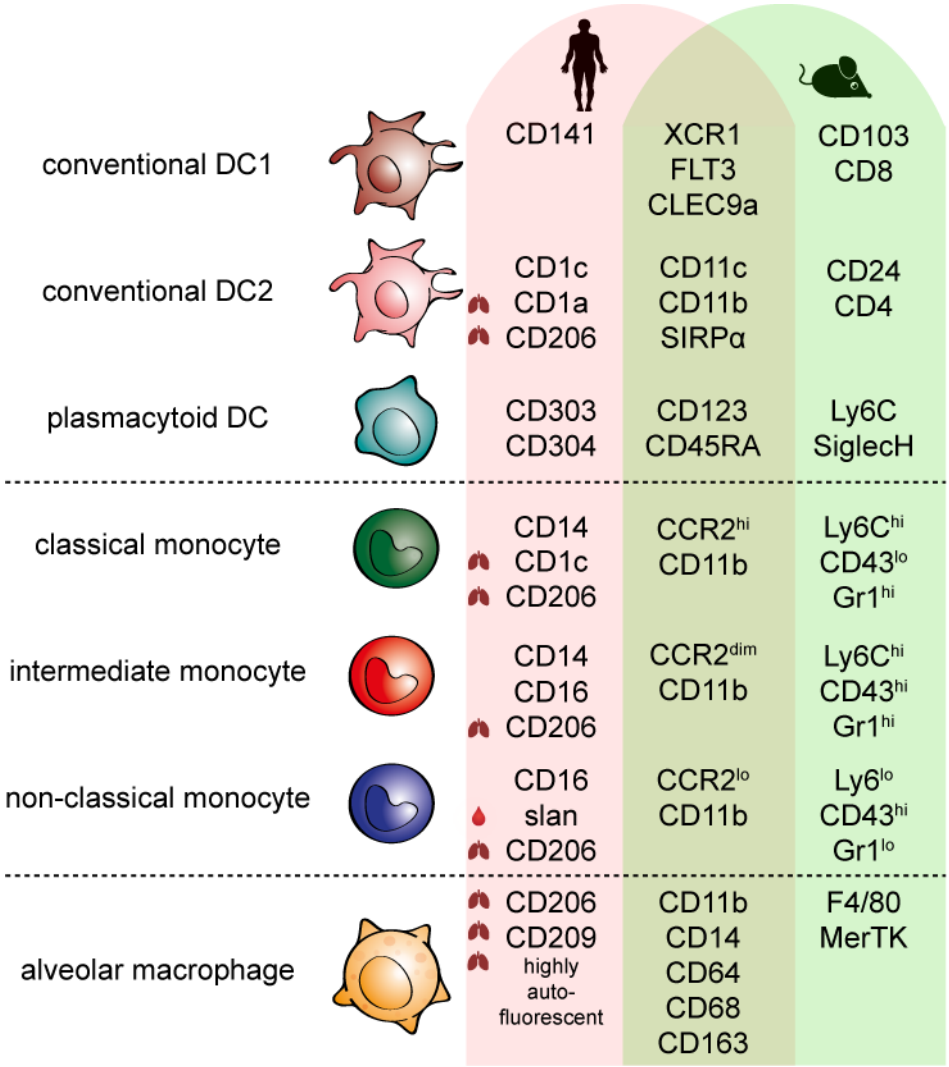

Figure 4. Phenotype of human MNPs and their homologues in mice. Human MNPs (left column) in blood have been well characterised, while tissue MNPs are still being carefully investigated. Distinct markers for lung or blood MNPs are indicated by lung or blood symbols. Homologues in mice are indicated in the right column whereas conserved markers expressed by both species are indicated in the middle column.

3.2.3 Functional properties

The variety of MNPs play distinct but overlapping roles in the induction/regulation of immunity, including antigen uptake, processing and presentation, and cytokine production[74].

3.2.3.1 Antigen capture by endocytosis

Endocytosis is a constitutive process performed by all cells for the uptake of a wide variety of molecules, such as metals, metabolites and vitamins from the external environment, necessary for cell growth and health[75]. There are two routes of endocytosis: phagocytosis (cell eating) and pinocytosis (cell drinking). Briefly, phagocytosis refers to the internalisation of particles larger than 0.5 μm, by the formation of pseudopod extensions from the cell's plasma membrane to engulf the bound particle[76]. Pinocytosis refers to the uptake of small particles together with the extracellular fluid, and can occur via clathrin-mediated endocytosis, caveolae, or macropinocytosis[77].

In order to survey the local microenvironment, macrophages, monocytes and immature DCs are adept at taking up antigens from their surroundings[78-80]. There are numerous endocytic receptors on MNPs that mediate endocytosis, including mannose receptor (CD206), DEC-205 (CD205), Fc receptors, complement receptors and scavenger receptors[81-86]. Once taken up by MNPs, antigens are sorted into different cytoplasmic compartments called endosomes[87]. Early endosomes are the first structures where endocytic vesicles are targeted to[88]. Here, receptor-ligand complexes are dissociated due to the mildly acidic environment (pH 6.0 – 6.8), so that receptors can be recycled back onto the cell surface[75]. Next, the cargo in early endosomes can be transported to late endosomes by the migration of vesicles to the perinuclear cytoplasm where they fuse with late endosomes, and finally lysosomes for degradation. Stimulation via TLRs induces a maturation programme in DCs that is characterised by downregulation of phagocytosis and macropinocytosis[82,89,90]. However, there is an initial burst in macropinocytosis prior to downregulation of the endocytic machinery[91]. Also, receptor-mediated endocytosis persists in mature DCs[92].

3.2.3.2 Recognition of pathogenic signals induces maturation of DCs

The concept of innate immune recognition was first theorised by Charles Janeway in 1989, thus pioneering studies in innate immunity[93]. The eventual discovery of TLRs by Nobel laureates Jules Hoffman and Bruce Beutler and the subsequent elucidation of the TLR signalling pathway have been instrumental in our understanding of how innate immune cells recognise structurally conserved features of pathogens[94,95]. This adds a layer of sophistication to the innate immune system in the ability to rapidly discern if a given antigen that has been endocytosed is harmless or harmful. The strategic subcellular compartmentalisation of TLRs also helps to distinguish between

self and foreign antigens; TLRs involved in recognition of nucleic acids (TLR3, TLR7, TLR8, TLR9) are localised within endo-lysosomal compartments whereas other TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) are on the cell surface. Different subsets of human MNPs express distinct repertoires of TLRs, which contribute to their functional specialisation[96]. Further, the tissue specificity of MNPs can also determine their TLR expression, as exemplified in DC populations from human blood and skin[97].

Upon TLR stimulation, MNPs undergo a cascade of events promoting inflammation and their own activation[98]. Especially for DCs, the maturation programme upregulates distinctive genes and signalling pathways, allowing migration to draining lymph nodes and efficient activation of T cells[47,99]. This revolves around the three signals required for activation, clonal expansion and differentiation of T cells; signal 1 comprises antigen-specific interaction between peptide-MHC complex and the TCR, signal 2 is delivered by co-stimulatory signals that either promote or inhibit survival of T cells, and signal 3 consists of cytokines that can polarise the differentiation of T cells into specific subsets of effector T cells[100,101]. TLR stimulation controls the generation of peptide-MHC complexes by regulating the translation and traffic of MHC molecules onto the cell surface[102,103]. Signals through TLRs also result in the enhanced expression of co-stimulatory molecules, often used as a measure of DC maturation[104,105]. Co-stimulatory signals include CD80 and CD86 molecules that bind to CD28 on T cells thus inducing production of IL-2, which promotes survival of T cells. Both CD80 and CD86 can also shut down T cell activation by binding to cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which inhibits T cell activation. CTLA-4 is upregulated on the cell surface upon T cell activation[106]. Other activating co-stimulatory molecules include CD83 and CD40, whereas OX40 ligand, programmed death ligand 1 (PD-L1) and 2 (PD-L2) are inhibitory. Cytokines produced by DCs can also promote the differentiation of activated $CD4^+$ T cells into various effector Th cells: Th1, Th2, Th17 and Tregs being the most well-characterised populations[107] (Figure 5).

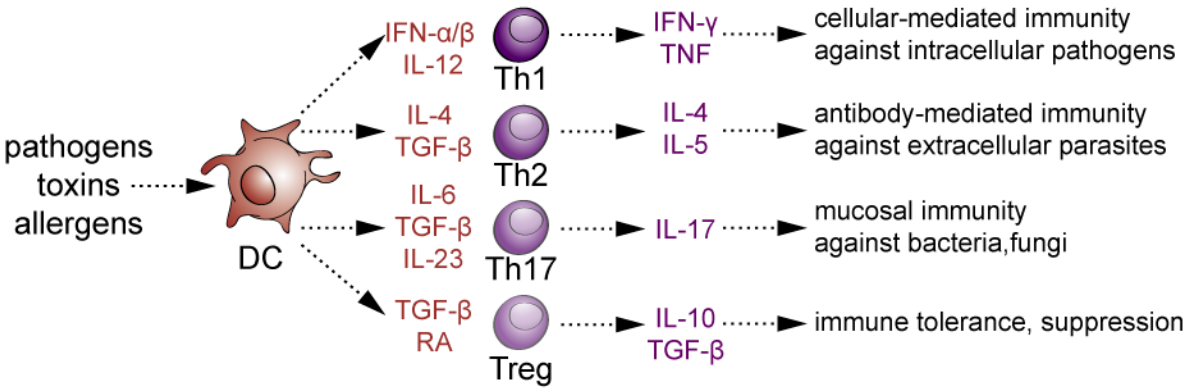

Figure 5. Orchestration of T cell polarisation by MNP-derived cytokine milieu. Recognition of PAMPs by MNPs promotes release of specific cytokines that can initiate differentiation of naïve $CD4^+$ T cells into subsets with distinct effector programmes.

Production of type I interferons (IFN) such as IFN-α and IFN-β, and interleukin (IL) 12 induces Th1 cells, which promote cellular immunity against intracellular pathogens by increasing killing efficacy of macrophages and CTLs, while production of IL-4 and transforming growth factor β (TGF-β) promote Th2 responses, supporting humoral immunity against extracellular pathogens. Production of IL-6, TGF-β and IL-23 promote Th17 cells that are important in pathogen clearance at mucosal surfaces, including extracellular bacteria and fungi. TGF-β and retinoic acid (RA) promotes regulatory T cells (Treg) that are important in the maintenance of tolerance against self antigens[108]. TLR activation of monocytes triggers their differentiation into monocyte-derived DCs or monocyte-derived macrophages[109].

3.2.3.3 Antigen processing and presentation to T cells

DCs excel at antigen processing, as illustrated by their specialised endocytic machinery[110]. Lysosomes contain hydrolytic enzymes that are active in an acidic environment (pH 4.5 – 5.0)[111]. The lysosomal system of DCs has an attenuated proteolytic capacity in order to preserve antigenic peptides that can be efficiently used for antigen presentation, distinct from macrophages that can rapidly degrade internalised proteins to amino acids[112]. The eventual outcome of antigen presentation is determined by whether the antigen is displayed on MHC classes I or II, which bind to their cognate T cell receptors (TCRs) and to CD8 or CD4 co-receptors on T cells respectively (Figure 6).

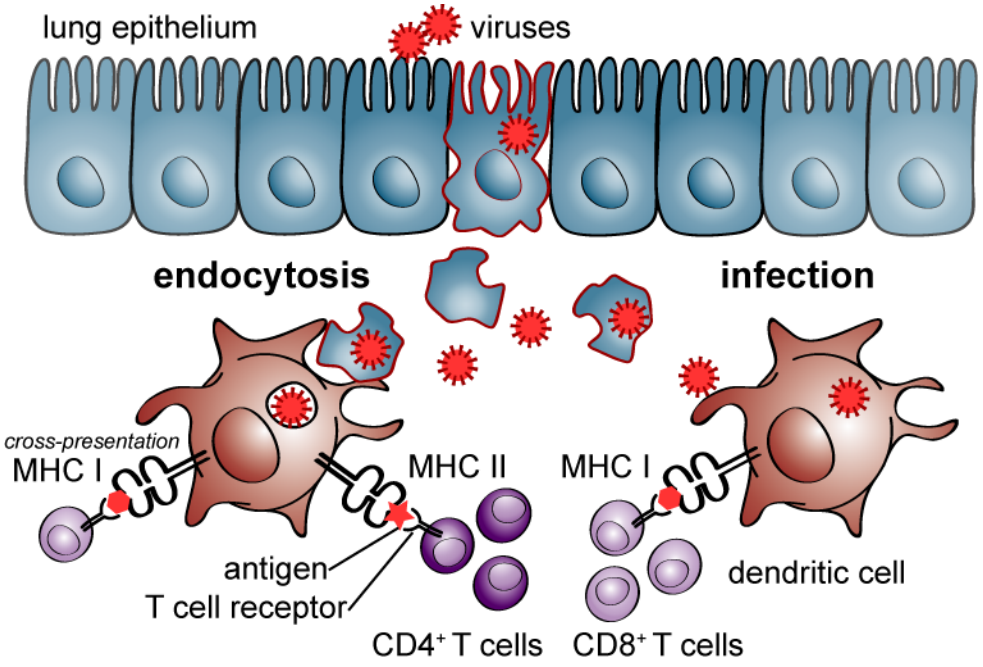

Figure 6. Antigen presentation via MHC I and MHC II. DCs patrolling the lungs may encounter exogenous viral antigen by endocytosing cellular debris containing viruses, or they may acquire endogenous viral antigens when they become infected directly. An additional mechanism, cross-presentation, allows exogenous antigens to be presented on MHC I.

Peptides are generated from proteins synthesised (endogenous antigens) or acquired (exogenous) by the cell. Endogenous antigens can include both self antigens and viral antigens produced as a consequence of viral infection. Endogenous antigens are predominantly presented on MHC I, whereas exogenous antigens are displayed on MHC II. Classically, activation of $CD4^+$ T cells is optimised for elimination of extracellular pathogens by promoting antibody responses, whereas activation of $CD8^+$ T cells produces CTLs that can eliminate intracellular pathogens[14,113]. An additional mechanism, called cross-presentation, allows DCs that are not directly infected to also activate CTLs by presenting extracellular antigens from other infected cells on MHC I molecules to $CD8^+$ T cells, developing into effector CTLs[114]. DCs are described to be superior to monocytes and macrophages in being the principal activators of naïve T cells[115].

In immature DCs, lysosomes are enriched in MHC class II molecules, which upon maturation can be readily redistributed to peripherally-located vesicles[116]. Newly synthesised MHC II molecules bind a protein termed the invariant chain (Ii), which targets the MHC II to endosomal-lysosomal antigen-processing compartments containing antigenic peptides. The cleavage of Ii by proteolytic enzymes in these compartments results in a small fragment called class II-associated invariant chain peptide (CLIP) that remains in the MHC II peptide-binding groove. Upon removal of CLIP, an antigenic peptide is loaded and the peptide-MHC II complex traffics to the cell surface where they can be presented to $CD4^+$ T cells[110].

MHC I-restricted antigen presentation occurs via a different mechanism, as MHC I molecules do not accumulate in the lysosomes of DCs[117]. Classic MHC I presentation occurs when cytosolic proteins undergo proteasomal proteolysis and the resulting peptides are translocated into the lumen of the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). Here, peptides can be loaded on MHC I molecules that are in the ER. For cross-presentation, two pathways have been described: vacuolar or cytosolic. The vacuolar pathway is independent of proteasomal degradation and TAP activity, instead relying on degradation via endosomal or phagosomal proteases such as cathepsin S[118,119]. Peptides are loaded on MHC I within the endosomal compartments. The cytosolic pathway requires internalised proteins to be translocated into the cytoplasm and targeted to proteasomes, similar to the classical MHC I pathway[120].

3.2.3.4 Division of labour between MNPs

In human studies, blood MNPs have been the predominant source of cells for functional analysis. Here, the functional specialisations of distinct human MNP subsets will be discussed.

$CD141^+$ MDCs are characterised by high expression of TLR3, and respond to poly(I:C) by producing pro-inflammatory cytokines TNF, IL-6, IFN-β, CXCL10 and IL-

12p70[58]. $CD141^+$ MDCs are also known for their superior capacity to cross-present antigens[56,58,59,121]. Necrotic cell-derived and soluble antigens are cross-presented via CLEC9A (also known as DNGR-1)[122]. In mice, $CD8^+/CD103^+$ DCs (homologues of $CD141^+$ MDCs) are unique in their cross-presentation capacity with their specialised endocytic machinery being well suited for that function (high pH limiting degradation and high export to cytosol). However, when investigating lymphoid tissue-resident DCs from human tonsils, $CD1c^+$ MDCs and PDCs are also capable of cross-presentation depending on the source of antigen, especially with TLR stimulation and $CD4^+$ T cell help[67,68].

$CD1c^+$ MDCs can respond to a wide range of bacteria- and virus-derived antigens as they express high levels of TLRs 1, 2, 4, 5 and 8[97,123]. Upon stimulation, $CD1c^+$ MDCs can produce TNF, IL-1β and IL-10[123]. In addition to their wide TLR repertoire, $CD1c^+$ MDCs also express several CD1 glycoproteins, such as CD1a, CD1c and CD1d, allowing them to present lipid antigens[124]. $CD1c^+$ MDCs are extremely versatile, as they can induce Th1, Th2 and Th17 polarisation depending on the antigen and stimulation[55,125,126]. When antigens are targeted to early endosomes of $CD1c^+$ MDCs, they are equally proficient at cross-presenting antigens as the $CD141^+$ MDCs[127]. This is related to enhanced degradation when antigens are targeted to late compartments[128].

PDCs are known for their ability to produce large amounts of type I IFN upon viral infection[129]. They express TLRs 7 and 9 in their endosomal compartments, thus allowing them to detect single-stranded RNA and unmethylated CpG sequences in DNA molecules respectively, of potentially viral origin[130]. PDCs can also induce Th1 or Th2 polarisation[131], cross-present antigens[119], and induce tolerance by activating Tregs[132].

Classical monocytes and intermediate monocytes are superior at phagocytosis compared to non-classical monocytes, perhaps related to the expression of CD14, a co-receptor for LPS[133,134]. In response to TLR stimulations, all monocyte subsets produce cytokines such as TNF, IL-1β and IL-6 upon TLR stimulation, although there are conflicting reports on whether intermediate monocytes[134,135] or non-classical monocytes[136,137] are the most potent responders. All monocyte populations are able to stimulate naïve T cells in an allogeneic mixed lymphocyte reaction[134]. For activation of memory T cell responses, antigen presentation by human monocytes is impaired in the presence of LPS[134,138], due to the presence of IL-10 produced upon stimulation of monocytes. Interestingly, both intermediate monocytes and non-classical monocytes are expanded in blood during infection and inflammation[139]. Inflammatory monocyte-derived cells have been described in synovial and ascites fluid sharing gene signatures with *in vitro*-generated monocyte-derived DCs[140].

Human alveolar macrophages are long-lived, highly phagocytic cells that line the airways in close contact with the respiratory epithelium[141]. Compared to monocytes

and DCs, alveolar macrophages are large with a mean diameter of 25 μm and highly granular[142]. They promote tolerance and prevent inflammatory responses to harmless antigens by producing immunosuppressive prostaglandins and TGF-β, which suppresses T cell activation[143,144].

4 IMMUNE RESPONSES TO RESPIRATORY VIRUSES

The respiratory tract is constantly exposed to potential threats in the air. Pathogens such as influenza viruses and rhinoviruses are easily transmitted when people cough or sneeze, thus causing typical respiratory infections such as the flu or the common cold. Another virus that can infect via the airways is hantavirus – when humans come in contact with aerosolised virus-contaminated rodent droppings. Both influenza virus and hantavirus infections require a robust yet well-regulated immune response to eliminate the viruses without causing collateral damage to the delicate tissues of the lungs.

4.1 INFLUENZA A VIRUS

Influenza viruses causing the flu continue to be a public health concern due to annual death and debilitation rates, and the potential to cause severe pandemics. According to the World Health Organization (WHO), the seasonal flu can lead to 3–5 million infections and up to 500 000 deaths every year[145]. The understanding of human influenza immunopathology has been limited, but experimental animal models have improved our knowledge of the complex mechanisms surrounding influenza infection.

4.1.1 Brief history of influenza viruses

Influenza virus was first identified in 1878 as causing an animal disease affecting poultry in northern Italy[146,147]. In 1889, influenza virus entered the human population, resulting in the first recorded pandemic (Table 1), killing approximately 1 million people worldwide[148]. In 1918, an even more devastating pandemic flu ensued. Despite its name, the Spanish flu resulted in massive deaths not only in Spain, but also worldwide. Reports of the flu was censored at that time to maintain morale during the war[149]. Most deaths occurred among youths who succumbed to bacterial pneumonia, a secondary infection after the flu. Nevertheless, some patients also died soon after the onset of symptoms with massive haemorrhaging or fluid in the lungs. Based on autopsies performed on Spanish flu victims, the primary cause of death was pneumonia and respiratory failure[150].

Name of pandemic	Date	Deaths	Subtype involved
Russian Flu	1889–1892	1 million	H3N8 or H2N2
Spanish Flu	1918–1920	20–100 million	H1N1
Asian Flu	1957–1958	1–1.5 million	H2N2
Hong Kong Flu	1968–1969	0.75–1 million	H3N2
Swine Flu	2009–2010	18 000 – 284 500	H1N1/09

Table 1. Known influenza pandemics throughout history.

Pandemics stem from the transmission of novel viral strains from animal reservoirs into the human population. This occurs by reassortment of viral genome from two

distinct strains of influenza, termed “antigenic shift”. The first pandemic of the 21st century happened in 2009 when an H1N1 virus of swine origin entered the human population[151]. Transmission of avian strains occurs sporadically, albeit in a dead end fashion due to the limited ability of the virus to be transmitted between humans[152,153]. The highly pathogenic H7N7 affecting the Netherlands in 2003 or the H7N9 in China in 2013 were consequences of exposure to infected poultry on farms. While antigenic shifts occur occasionally resulting in pandemics, small changes in the genes of influenza viruses arise continuously. These changes, termed “antigenic drift”, accumulate over time as a result of viral variants escaping immune pressure. This necessitates the annual update of influenza vaccines, in order to review the composition of vaccines to keep up with evolving viruses[145].

4.1.2 Clinical signs and symptoms

Influenza virus infection may lead to three syndromes: a respiratory illness, a primary viral pneumonia, or a secondary bacterial pneumonia[154] (Figure 7). Uncomplicated cases of influenza are characterised by a sudden onset of respiratory illness, including fever, headache, general discomfort, muscle ache, sore throat and runny nose[155]. The illness resolves after 3-7 days for most people, with cough persisting for more than 2 weeks[155]. In severe cases, primary viral pneumonia is common, also frequently a cause of death[156]. Secondary bacterial pneumonia is estimated to occur in up to 30% of fatal cases[157], with respiratory failure being the cause of death.

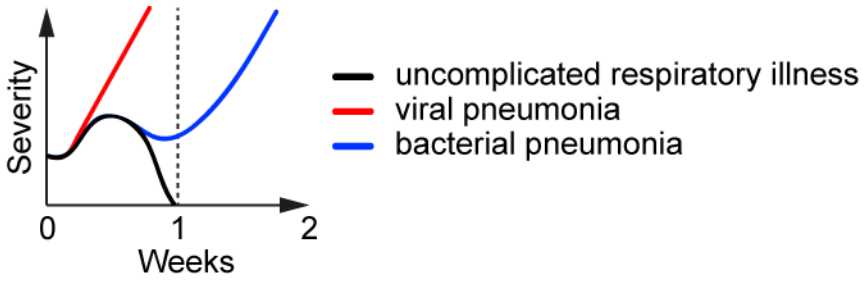

Figure 7. Model of influenza virus syndromes. After 1-2 days of incubation, three syndromes of varying severity may occur. Viral pneumonia has a rapid onset of 1 day post infection. Uncomplicated illness peaks after 3-5 days and subsides within a week. However, if a secondary bacterial infection occurs, recovery is impeded and symptoms progressively worsen.

4.1.3 Genome organization, structure and replication

Influenza viruses belong to the *Orthomyxoviridae* family of negative-sense single-stranded RNA viruses, consisting of eight genome segments (Figure 8). Influenza viruses are classified into three types: A, B and C. Influenza A and B viruses are responsible for causing seasonal epidemics, whereas influenza C infections cause a mild respiratory illness.

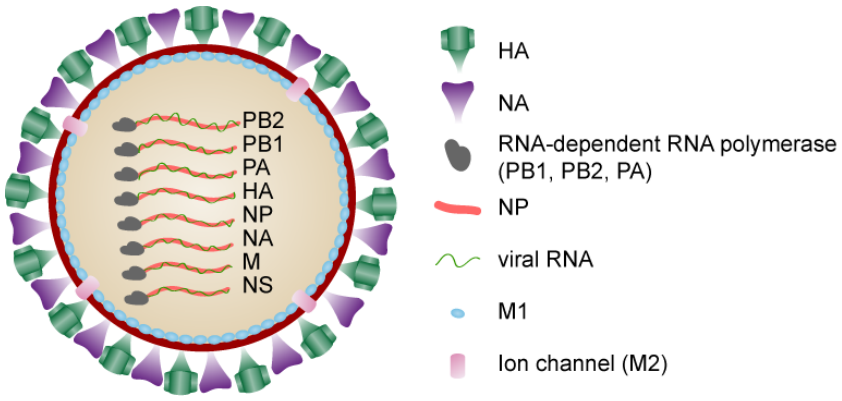

Figure 8. Influenza structure, genes and proteins. Two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), and an ion channel M2 are embedded in the viral envelope, derived from the host cell plasma membrane. Three polymerase proteins (PB1, PB2, PA) are associated with viral RNA bound to nucleoprotein (NP), packaged inside the virion. Matrix protein M1 forms a shell underneath the viral envelope.

The focus of this thesis is on IAV, the most abundant and pathogenic influenza virus to infect humans. The eight RNA segments encode for up to 16 proteins; multiple proteins may be expressed due to alternative splicing of the same RNA fragment, but not all influenza viruses express all 16 proteins[158] (Table 2).

Gene segment	Protein	Function
1	Polymerase PB2	Component of RNA-dependent RNA polymerase
2	Polymerase PB1 N40 PB1-F2	Component of RNA-dependent RNA polymerase Unknown function Pro-apoptotic
3	Polymerase PA PA-X PA-N155 PA-N182	Component of RNA-dependent RNA polymerase Represses cellular gene expression Unknown function Unknown function
4	Haemagglutinin HA	Binds sialic acids on host cell receptors
5	Nucleoprotein NP	Binds viral RNA and other proteins, nuclear import
6	Neuraminidase NA	Cleaves off sialic acids from cell surfaces
7	Matrix protein M1 Matrix protein M2 Matrix protein M42	Matrix protein Ion channel protein Ion channel protein
8	Non-structural protein NS1 Non-structural protein NS2	Antiviral response antagonist Nuclear export protein

Table 2. Influenza A virus RNA segments and proteins encoded.

Each virion is roughly spherical, but sometimes filamentous, and measures approximately 80-120 nm in diameter[159]. The viral envelope is made of a lipid membrane embedded with glycoproteins haemagglutinin (HA) and neuraminidase (NA), and M2 proteins that form ion channels across the membrane. M1 proteins form a shell underneath the lipid membrane. Proteins PB1, PB2 and PA form the RNA-dependent RNA polymerase. Each viral RNA is tightly bound around many copies of nucleoprotein (NP). The viral RNAs, NP molecules, and RNA polymerase together form the viral ribonucleoprotein (vRNP).

Infection is initiated when IAV particles enter and bind to cells in the upper respiratory tract epithelium. In humans, HA recognises host cell receptors, both glycoproteins and glycolipids, containing sialyloligosaccharides terminated by *N*-acetyl sialic acid linked to galactose with an α2,6 linkage, as opposed to HA on avian viruses which prefer sialic acids with α2,3 linkage[160]. Epithelial cells in the human trachea contain these sialic acid residues[161]. Nevertheless, infection with avian flu in humans occur as there are cells in the lower respiratory tract in humans that contain α2,3-linked sialic acids[162].

Extensive studies combining microscopy and biochemical methods have elucidated the influenza virus life cycle, summarised in Figure 9[158,163,164]. After the virus is attached via binding of HA to sialic acid, virus particles are internalised via endocytosis[165-168]. Lakadamyali *et al.* described by visualising individual viruses in real-time in living cells, that influenza progresses to the nucleus in three distinct stages[169]. Firstly, the endocytic vesicles are transported to early endosomes via an actin-dependent manner. Secondly, virus-containing early endosomes are rapidly transported towards the perinuclear region via dynein-mediated movement on microtubules. Here, acidification of endosomes takes place. Finally, the acidic environment (pH ~5) triggers conformational changes of HA resulting in fusion of viral and host cell membranes[170-172]. The ion channel M2 further acidifies the internal compartment of the virus, allowing vRNPs to dissociate from M1. The uncoated vRNPs are then released into the cytoplasm, and enter the nucleus of the host cell via nuclear localisation signals on NP[173].

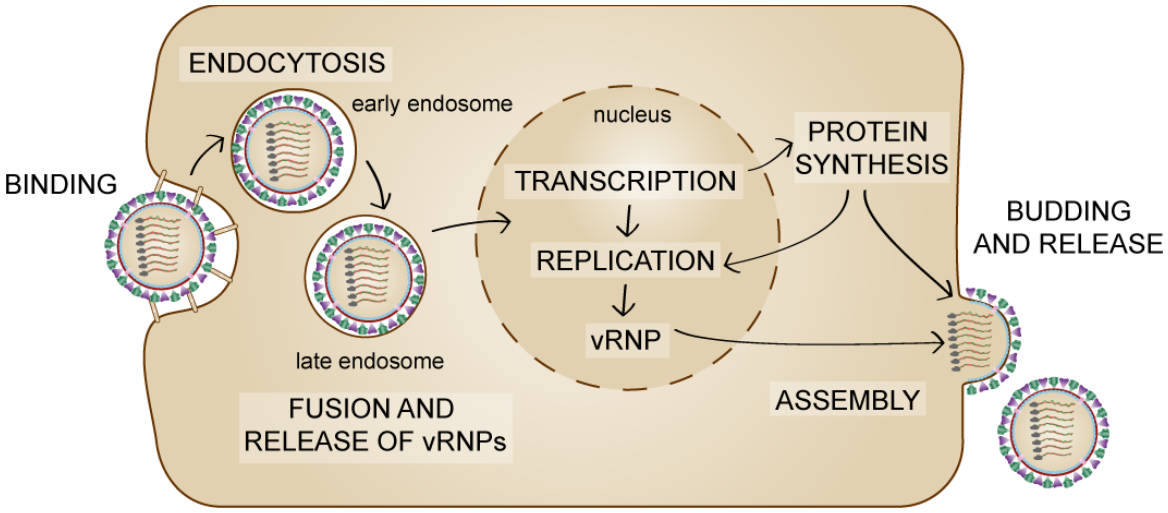

Figure 9. Influenza virus life cycle and replication. Upon binding to sialic acid residues on host cells, a virion is endocytosed and targeted to early endosomes, followed by late endosomes where the low pH allows fusion and penetration of the viral genome into the cytoplasm. vRNPs are transported into the nucleus where the negative-sense RNA acts as a template for mRNA to be translated to viral proteins in the cytoplasm, or cRNA for synthesis of new viral genome. Assembly takes place at the plasma membrane where new virions eventually bud off.

Influenza viruses are one of the few RNA viruses to undergo replication in the nucleus[174]. The negative-sense viral RNA is transcribed to positive-sense RNA for

two purposes: viral messenger RNA (mRNA) for protein synthesis, and viral complementary RNA (cRNA) for synthesis of more negative-sense RNA as the genomic content of new virions[175]. Viral mRNA are capped on the 5' end from the host's RNA is a process called cap snatching. Capped viral mRNA are exported to the cytoplasm for viral protein synthesis using the host cell's ribosomal machinery. Newly synthesised viral proteins such an NP and the RNA polymerase complex get imported into the nucleus where they associate with the newly synthesised vRNA.

Prior to viral assembly, HA proteins (HA_0) need to undergo cleavage to be processed into two subunits (HA_1 and HA_2). This is a necessary step as HA_2 contains the fusion peptide required for entry into the next cell. The protease mediating cleavage in humans is thought to be tryptase produced by club cells (previously called Clara cells) residing in the respiratory tract[176]. This limits the spread of influenza infection in humans as uncleaved HA remain non-infectious. Nevertheless, HA of avian strains may be cleaved by other proteases, resulting in a more extensive pattern of infection, with H5N1 being detected in multiple organs[177].

Finally, the individual components assemble at specialised areas of the plasma membrane, rich in lipid rafts, with M1 being central to this interaction. Viral budding occurs via the action of NA, a sialidase, that can remove sialic acids from the surface of host cells[175]. This is so that newly released virus particles do not immediately re-bind to their receptors, but instead are released into the extracellular space where they may infect a neighbouring cell. This has been exploited to the development of NA inhibitors as antiviral drugs (e.g. sialic acid analogues, Zanamivir and Oseltamivir).

4.1.4 Immune responses to IAV

In this thesis, key studies from murine studies are briefly discussed, with a greater focus on the existing literature on influenza infection of humans. Both the innate and adaptive arms of the immune system play important roles in viral control and eventual clearance[178,179]. Coincidentally, an exaggerated innate immune response may also contribute to disease, including acute respiratory distress syndrome (ARDS), suggesting the importance of a tightly regulated and well-orchestrated immune response to the infection.

Upon penetration of the mucosal layer, the first innate barrier against infection, IAV can enter a variety of cells in the respiratory tract, including ciliated epithelial cells, type I and type II alveolar cells, and immune cells[180,181]. Equipped with virus-sensing receptors, respiratory epithelial cells respond by secreting various cytokines and chemokines such as type I IFNs, IL-6, TNF and IL-8 to limit the initial viral replication, and to trigger the recruitment of other immune cells[182]. Although pro-inflammatory cytokines are necessary for initiation of immunity against influenza virus, an aggressive cytokine storm may also exacerbate the disease by causing injury to the

delicate tissues of the lungs. In the Spanish flu pandemic of 1918, the inflammatory environment due to increased recruitment of leukocytes may have contributed to increased morbidity[183]. Local and systemic increase in cytokine levels during infection has been reported in influenza-infected patients and also in participants of human challenge studies[184-186]. In the 2009 H1N1 pandemic, a dysregulated cytokine response correlated to more severe disease[187]. Interestingly, although IAV infection eventually leads to cell death due to apoptosis/necrosis or clearance by effector immune cells, club cells can survive IAV infection and maintain heightened levels of interferon stimulated genes (ISGs) for a prolonged time after virus has been cleared, thus driving non-specific immunity against subsequent viral infections[188,189].

The innate response limits viral replication while the adaptive response is developing. This is initiated by tissue-resident alveolar macrophages and DCs residing in the respiratory tract that patrol and probe the surroundings for presence of microbial components. Upon release of inflammatory mediators, more neutrophils and monocytes infiltrate into the site of infection. As highly phagocytic cells, they are important for clearance of infected and apoptotic cells[190,191].

In influenza-infected mice, both myeloid DC subsets and PDCs with an activated phenotype accumulate in the trachea and lung interstitial tissue[192-194]. Monocyte-derived DCs also infiltrate the lungs[195,196]. DCs secrete pro-inflammatory cytokines and chemokines and migrate to draining lymph nodes to induce adaptive immune responses[192-194,197,198]. Distinct subsets of DCs differ in their capacities to induce $CD8^+$ cytotoxic T lymphocytes (CTLs), with $CD103^+$ MDCs (cDC1) identified as the key subset presenting antigen to drive the proliferation of lung-homing flu-specific $CD8^+$ T cells[197,199,200]. Confirming the important role of DCs in viral control, depletion of DCs in mice before challenge with influenza resulted in higher viral load in the lungs and increased mortality[192,194,201]. Similarly in humans, DCs have been reported to accumulate in the nasal mucosa, whereas numbers in peripheral blood are reduced[202-204]. *In vitro* studies of human DC subsets suggest that infection with influenza viruses induce upregulation of MHC molecules, co-stimulatory molecules and chemokine receptors[205-210]. A potential consequence of viral infection of DCs is impaired function, as blood $CD1c^+$ DCs (cDC2) infected with IAV resulted in poorer expansion of flu-specific $CD8^+$ T cells[210].

In mouse models, depletion of alveolar macrophages has shown contrasting results, possibly dependent on the virus strain and dose used. Depletion of alveolar macrophages did not affect severity of disease upon lethal PR8 infection, but caused uncontrolled viral titres when challenged with H1N1 pandemic isolate[211]. In humans, IAV infection of alveolar macrophages does not cause excessive TNF production[212].

In mice, CCR2-dependent recruitment of monocytes to the lungs is critical to both the innate immune response against influenza by production of pro-inflammatory cytokines, and also by enhancing influenza-specific T cell responses[213,214]. In

humans, increased numbers of monocytes are found in the nasal mucosa[203,204,215] and in peripheral blood of influenza-infected patients[203,204,215-217].

Neutrophils have been shown to be important for controlling viral replication in mouse models[218]. Depletion of neutrophils resulted in increased viral titres in the lungs[219]. In humans, addition of activated neutrophils to a culture of bronchoalveolar lavage (BAL) cells *in vitro* improves viral clearance[220]. In contrast, *in vitro* infection of human neutrophils induces apoptosis[221]. The specific role of neutrophils during influenza infection in humans *in vivo* remains unclear.

NK cells accumulate in the lungs of mice challenged with IAV, with an activated phenotype that can lyse infected cells via the action of granzyme B and perforin[222-224]. To confirm the importance of NK cells, depletion of NK cells resulted in delayed viral clearance[222]. In influenza-infected patients, infection resulted in a transient deficiency of circulating NK cells[225-228]. Although this may reflect recruitment of NK cells to the site of infection, investigations in fatal cases of IAV infections revealed low numbers or absence of NK cells in the lungs[228,229].

The adaptive immune response, both humoral and cellular immunity, act at different time points to limit disease caused by an acute respiratory viral infection. Cellular immunity via the action of CTLs is important for eliminating virus-infected cells thus reducing disease severity. Humoral immunity through B cells and antibodies represent the major mechanism for prevention of re-infection. The induction of durable and effective immunological memory ensures protection from re-infections.

Flu-specific antibodies have long been used as a correlate of protection. Since the 1960s, experimental human challenges have demonstrated that lasting immunity correlated with antibody levels[230]. Priming of naïve B cells is thought to occur when DCs that have acquired viral antigens from the respiratory tract transport them to lymphoid tissues; draining lymph nodes or nasal/bronchial-associated lymphoid tissue[231,232]. Short-lived plasmablasts are formed at this stage, that can go on to become antibody-producing long-lived plasma cells[233]. In parallel, memory B cells are also formed that can rapidly develop into plasmablasts in subsequent re-infections. As influenza infections are primarily restricted to the respiratory tract, mucosal IgA plays an important role in preventing infection. IgA-secreting B cells are preferentially generated in bronchial-associated lymphoid tissues[234]. IgA knockout mice are poorly protected against influenza[235]. Human studies have mainly focused on B cell responses in the peripheral blood. During natural infection, IAV-specific plasmablasts can be readily detected 7-10 days after symptom onset[236,237]. Formation of bronchial-associated lymphoid tissue has been difficult to demonstrate in humans[238]. Nevertheless, protective humoral responses continue to be a benchmark for development of vaccine-induced immunity.

Primary T cell responses are also generated in lymphoid organs, upon delivery of viral antigens from the lungs by DCs. Both $CD4^+$ and $CD8^+$ T cells play important roles in adaptive immune responses to influenza[239]. $CD4^+$ T cells have a wide repertoire of functions, providing help for both $CD8^+$ T cells and also B cells. In human influenza infection models, pre-existing memory $CD4^+$ T cells provided protection against influenza challenge[240]. Influenza is typically associated with Th1 responses, involving production of IFN-γ, TNF and IL-2[241]. Importantly, memory $CD4^+$ T cells remain in lung tissue to provide rapid and optimal responses to secondary influenza infections. In the lymph nodes, DCs imprint $CD4^+$ T cells with chemokine receptors and integrins that allows them to home to the lung tissue and remain there[107]. This was shown in mice to be mediated by expression of CCR4 on T cells, driven by lung DCs[242]. CCR4 binds to CCL5 (also known as RANTES) expressed by epithelial cells in the lungs during infection[243].

Viral clearance during influenza infection requires the activation of CTLs[244]. Furthermore, generating IAV-specific $CD8^+$ T cells is an attractive vaccine strategy, as they are predominantly directed towards conserved internal proteins, hence providing broader cross-reactive protection against distinct strains of IAV[245]. As mentioned earlier, IAV-specific T cell responses are initiated in the draining lymph nodes. However, recent advances in mouse models have indicated that upon arrival to the IAV-infected lungs, the local lung environments can shape the differentiation of T cells into effector cells[246]. CTLs downregulate their ability to secrete IFN-γ in the lungs, to avoid causing collateral damage[247]. They can also produce IL-10, which functions to inhibit excessive pulmonary inflammation[248]. Lung DCs can also provide additional survival and proliferative signals by interacting with $CD8^+$ T cells[249,250]. Indeed, depletion of lung MNPs reduced CTL proliferation[192]. In humans, the importance of $CD8^+$ T cells in protecting against influenza have been confirmed in an extensive study involving 342 participants with natural infection[251]. The IAV-specific $CD8^+$ T cell responses are long-lived[252]. Further, IAV-specific memory $CD8^+$ T cells are more frequent in the lungs than in blood or spleen, with a resident memory T cell phenotype (expressing CD69 and CD103)[253-255].

4.2 HANTAVIRUS

Hantaviruses pathogenic to humans are rodent borne, but do not cause pathogenesis in their natural hosts[256]. Transmission to humans via aerosolised excreta may lead to HFRS or HPS depending on the viral species[257]. As an emerging infectious disease, hantavirus infections represent a threat to public health worldwide. The annual incidence of reported hantavirus infections varies depending on geographical location: approximately 250 cases in the Americas, 3000 in the Nordic countries and almost 40 000 in China[258].

4.2.1 Brief history of hantaviruses

An important outbreak of hantavirus occurred during the Korean war in 1950s, when more than 3000 United Nation soldiers presented with a febrile illness characterised by acute renal failure[259,260]. Prior to that, similar symptoms had been reported in China in 960 AD, among French troops in 1915, in Sweden in 1930s, and in East Siberia in 1940s, without an understanding of the causative agents. Eventually, the discoveries of Hantaan virus (HTNV) in Asia and Puumala virus (PUUV) in Europe shed light on the aetiology of HFRS. In 1993, a mysterious outbreak of a pulmonary illness occurred in “The Four Corners” of United States that led to the discovery of Sin Nombre virus (SNV), and other hantaviruses endemic to the Americas. Infections caused by SNV and other American hantaviruses were categorised as HPS due to severe pulmonary failure associated with the virus infection[261].

4.2.2 Clinical syndromes

Viruses from Eurasia can cause HFRS whereas viruses from the Americas may lead to HPS. However, mounting evidence suggests a more diffuse pattern of symptoms, as patients infected with PUUV may also present with pulmonary symptoms to the extent of fatality due to respiratory failure[262-264]. A common hallmark of both syndromes is increased vascular leakage leading to low blood pressure, dilation of blood vessels and acute loss of thrombocytes. In the case of HFRS, vascular beds in the kidneys are most affected, whereas in HPS, pulmonary capillaries are compromised[265].

Patients with HFRS experience an abrupt fever followed by headache, nausea, vomiting and abdominal pain[266]. Kidney problems begin to manifest with back pain and tenderness, including oliguria or low urine output and excessive protein or blood in urine. Elevated levels of creatinine in blood are indicative of an acute kidney failure. In the second week of illness, patients transition to polyuria or excessive urine output as the kidneys improve in function. HFRS patients typically recover after two weeks. Most fatalities associated with HFRS occur during the hypotensive shock stage[267,268], with a case fatality rate of 0.1-10%[257].

The clinical manifestations of HPS range from mild oxygen deficiency in the blood, fluid accumulation in the lungs, to respiratory failure[269]. Patients typically exhibit respiratory manifestations such as coughing and shortness of breath, with up to 60% of cases requiring mechanical ventilation[270]. Other symptoms are comparable to HFRS, such as fever, low platelet counts, increased creatinine levels and protein in urine[271-274]. Rapid progression of HPS may lead to death within hours after the onset of cardiopulmonary phase[275], with up to 40% case fatality rate[257].

4.2.3 Genome organization, structure and replication

Hantaviruses belong to the *Bunyaviridae* family of negative-sense single-stranded enveloped RNA viruses, with three genome segments (Figure 10). Each segment, called small, medium and large contains an open reading frame encoding the nucleocapsid (N) protein, the glycoprotein precursor that matures into Gn and Gc, and the RNA-dependent RNA polymerase (RdRp), respectively. The morphology and structure of hantaviruses have been elucidated by electron microscopy[276-278]. Hantaviruses are described as round particles of 120-160 nm in diameter[278]. Each virion comprises of a lipid envelope covered with spikes (made up of Gc and Gn) that protrude out of the membrane. Within the virion, the viral RNA segments are encapsidated by N proteins. Critical to hantavirus transmission, the virions are stable at room temperature for 10 days and even longer at 4°C to -20°C[279,280].

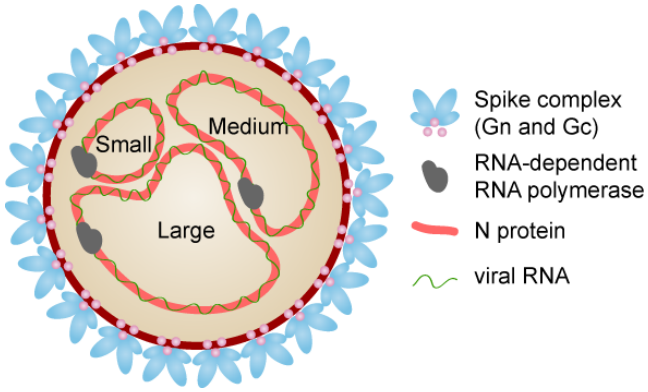

Figure 10. Hantavirus structure, genes and proteins. Spike complexes comprising two surface glycoproteins, Gn and Gc, are embedded in the viral envelope, derived from the host cell plasma membrane. Each RNA polymerase is associated with viral RNA bound to nucleocapsid proteins, packaged inside the virion.

Hantaviruses replicate in the vascular endothelium[281]. As such, endothelial cell lines have been relied on for *in vitro* studies investigating the molecular mechanisms of viral entry and replication, summarised in Figure 11[265]. Host cell integrins, that promote adhesion of cells to the adhesion matrix, mediate hantavirus entry into cells, but this has not yet been confirmed *in vivo*. Interestingly, pathogenic and non-pathogenic strains of hantaviruses utilise distinct integrins for entry, αVβ3 and α5β1 respectively[282]. After binding a receptor on the surface of a host cell, the hantavirus is taken up by the cell by various methods of endocytosis[257,283]. Similar to influenza viruses, hantaviruses are trafficked to early and late endosomes where the low pH triggers a change in the conformation of the Gc glycoprotein, allowing binding of the Gc fusion loop to the endosomal membrane, eventually leading to fusion of viral and cellular membranes[257]. The viral genome is then released into the cytoplasm where viral replication can begin. Transcription of viral RNA begins at the ER-Golgi intermediate compartment (ERGIC). Nascent virions assemble and bud into the cis-Golgi where they are transported to the plasma membrane for release via exocytosis.

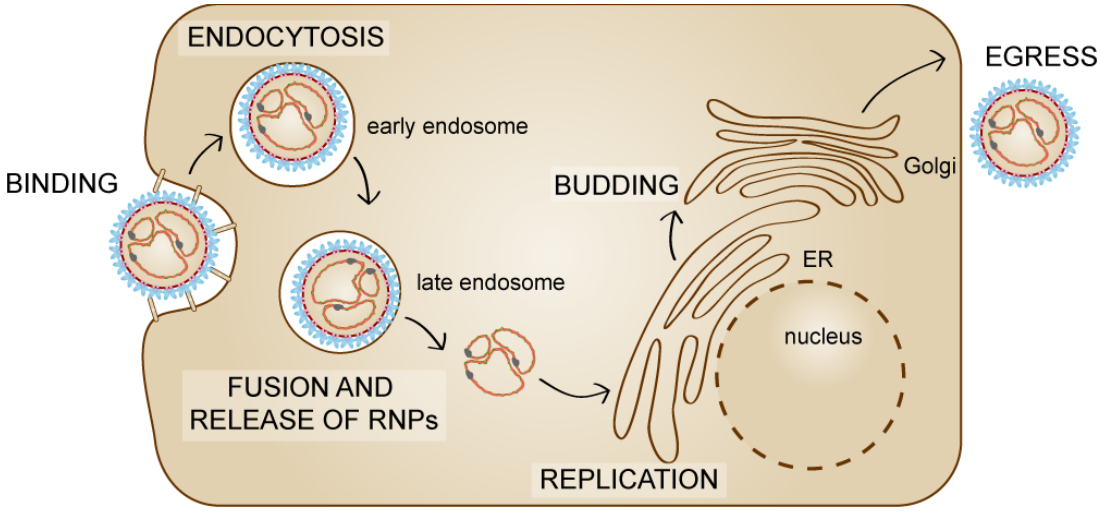

Figure 11. Hantavirus life cycle and replication. Upon binding to integrins on host cells, incoming virions are endocytosed and targetted to early endosomes, followed by late endosomes where the low pH allows fusion and penetration of the viral genome into the cytoplasm. Transcription of viral RNA begins at ERGIC. New virions assembly and bud into the cis-Golgi where they are targeted to the plasma membranes via recycling endosomes.

4.2.4 Immune responses to hantaviruses

The immune response to hantavirus infection is of relevance to study as the pathogenesis of hantavirus has been suggested to be immune mediated[284]. The exact sequence of events beginning from inhalation of hantaviruses in the lungs to systemic viral dissemination has not yet been elucidated *in vivo*. Nevertheless, *ex vivo* assessment of viral RNA or protein in patients, combined with *in vitro* studies indicate that in the lungs, hantavirus may result in infection of lung endothelial cells, epithelial cells or immune cells present in the lungs such as alveolar macrophages or DCs[263,285-288]. Recognition of vRNA by PRRs triggers a cascade of signalling pathways leading to production of type I IFNs and other pro-inflammatory cytokines[289]. The precise PRR responsible for recognition of genomic RNA has yet to be identified. Hantaviruses are relatively poor inducers of type I IFNs, but the ISG MxA has been detected in endothelial cells following hantavirus infection, colocalising with the hantavirus N protein[290-292]. These cytokines are important in limiting viral spread, via direct effects of type I IFNs but also the recruitment of other innate immune cells such as NK cells to the site of infection[263,293,294]. Monocytes, macrophages and lymphocytes may also respond by producing pro-inflammatory cytokines such as TNF, IL-1 and IL-6. Indeed, high levels of cytokines have been found in the plasma, urine and tissues of hantavirus patients[295-298]. Human blood and monocyte-derived DCs from healthy volunteers are also susceptible to hantavirus infection *in vitro*[299]. After replication in endothelial cells, virions are released into peripheral blood circulation, and hantavirus RNA can be detected in patients for diagnosis[300,301]. Hantaviruses can be disseminated to other organs, including the kidneys and bone marrow[302,303].

Activation of the adaptive immune system can be categorised by humoral and cellular immune responses to hantavirus. Elevated levels of total and virus-specific IgA, IgE, IgM and IgG have been reported in patients with acute HFRS[304]. The virus-specific antibodies persist in sera of patients, providing long-lasting immunity against hantavirus infections[305,306]. Neutralising antibodies recognise epitopes on the glycoproteins Gc and Gn, with a high level of cross-reactivity towards different hantaviruses[304]. A significant association between low virus-specific IgG responses and more severe disease indicates an important role of humoral immune responses in managing hantavirus pathogenesis[307]. In contrast, high numbers of virus-specific CTLs appear to be correlated with disease severity. $CD8^+$ T cells play a central role in the cellular immune response against hantavirus. High numbers of $CD8^+$ T cells in blood and BAL have been associated with severe HPS and HFRS requiring mechanical ventilation or oxygen treatment[308,309]. In the lungs of patients who died from HPS and HFRS, increased numbers of $CD8^+$ T cells producing TNF, IL-2 and IFN-γ were detected[310]. Surprisingly, hantavirus-infected endothelial cells are protected from CTL-mediated induction of apoptosis[311]. Further investigations are required to understand why CTLs remain activated in hantavirus-infected patients, despite an impaired ability to eliminate infected cells.

5 MATERIALS AND METHODS

A brief description of the central methods and underlying principles are outlined in this chapter. Detailed information of materials and methods can be obtained in the original papers (**I–IV**).

5.1 BRONCHOSCOPY

In **papers III** and **IV**, bronchoscopies were performed to obtain lung specimens from healthy volunteers and hantavirus-infected HFRS patients who provided written and oral consent. It is a procedure done by a team of trained nurses and a respiratory physician. Participants were first treated with oral and intravenous anaesthetics approximately 30 minutes before the bronchoscopy. A flexible bronchoscope was inserted into the airways through the mouth, allowing visualisation of the inside of the airways. Topical anaesthesia was sprayed onto the distal trachea and bronchi via the bronchoscope. Using fenestrated forceps, endobronchial biopsies (EBB) were taken from the main carina and the main bronchial divisions. From the contralateral side, bronchial wash (BW) of 2 x 20 mL saline solution, followed by BAL of 3 x 60 mL were taken to sample the airways.

5.2 PROCESSING OF BLOOD, BW, BAL AND EBB

For **papers I** and **II**, buffy coats, the fraction of whole blood after density centrifugation that is enriched in leukocytes, were used from healthy blood donors at Karolinska University Hospital. Buffy coats were diluted in PBS at a ratio of 1:1, layered over a Ficoll gradient, and centrifuged at 1000 g for 25 minutes with no brake to obtain peripheral blood mononuclear cells (PBMCs). For studies **III** and **IV**, peripheral blood was collected in CPT tubes (BD) already containing Ficoll solution, and centrifuged according to manufacturer's instructions. Ficoll is a synthetic, high molecular weight polymer of sucrose and epichlorohydrin[312]. Differential migration of cells during centrifugation through the density gradient results in the formation of layers: red blood cells aggregate and settle on the bottom, followed by denser granulocytes that can migrate through the Ficoll gradient, the Ficoll layer, an interface of PBMCs and finally the plasma layer. PBMCs consisting of monocytes, lymphocytes and platelets are not dense enough to migrate through the Ficoll layer.

In **papers III**, BW and BAL were kept on ice immediately after collection, and centrifuged at 400 g for 15 minutes at 4°C to separate the fluid from the cells. Intact biopsy specimens were washed and incubated in 1,4-Dithiothreitol (DTT) to remove mucous and enzymatically digested with collagenase and DNase to obtain single cells for flow cytometry. In parallel, several EBB were fixed in acetone and embedded in glycol methylacrylate (GMA) resin for tissue sectioning and immunohistochemical analysis. To assess whether the enzymatic digestion might cleave off, alter or interfere with antibody binding, PBMCs expressing similar

markers were treated with the digestive enzymes and stained with the same antibody panel used for flow cytometry. Additionally, tissue sections were also stained with specific antibodies by immunohistochemistry to confirm that epitopes were not lost upon enzymatic digestion.

All single cells from peripheral blood, BW, BAL and digested EBB were counted manually using Trypan Blue exclusion to measure viability. Cells were subsequently stained with antibodies for flow cytometric analysis.

5.3 ISOLATION AND GENERATION OF DCs

In **papers I** and **II**, MDDCs were generated by culturing enriched monocytes, obtained by RosetteSep monocyte enrichment (StemCell Technologies), at a concentration of 0.5 x 10^6 cells per mL of R10, RPMI-1640 cell culture medium containing 10% fetal bovine serum (FBS), containing recombinant human GM-CSF (40 ng/mL) and IL-4 (40 ng/mL) (both Peprotech) for six days.

In **paper II**, blood $CD1c^+$ MDC and PDCs were isolated by magnetic isolation using anti-CD1c (BDCA-1) or anti-CD304 (BDCA-4) antibodies conjugated to MACS microbeads, (Miltenyi Biotec) beginning from enriched monocytes and PBMCs respectively. $CD1c^+$ MDCs were cultured in R10 with 2 ng/ml GM-CSF whereas PDCs were cultured in R10 with 1 ng/ml IL-3.

5.4 STIMULATION AND *IN VITRO* INFECTION OF DCs

In **paper I**, MDDCs were adhered on Alcian-blue coated coverslips and exposed to IAV/X31 (derived from influenza A/Aichi/2/68; H3N2) at a multiplicity of infection (MOI) of 25 (as assessed by MDCK plaque assays) for 60 minutes at 4°C to allow virus particles to bind to the surface of cells. Infection was allowed to proceed at 37°C with cells being washed and fixed between 0-30 minutes, to capture the early events of viral entry. The pulse at 4°C ensures a more synchronised process of viral entry by eliminating the variation in how long it takes for each virus particle to settle on the cells by gravity.

In **paper II**, MDDCs and $CD1c^+$ MDCs were stimulated overnight with a panel of purified TLR ligands: Polyinosinic:polycytidylic acid (poly(I:C)), lipopolysaccharide (LPS), and 3M019 (7/8L). poly(I:C) resembles double stranded RNA and binds TLR3 to simulate a viral infection. LPS is an endotoxin found in the outer membrane of Gram-negative bacteria. 3M019 is an imidazoquinoline compound, a synthetic molecule resembling a nucleic acid base that binds to TLR7/8. Supernatants and cell lysates were harvested after the overnight stimulation for RNA and protein analyses. In parallel, stimulated cells were exposed to IAV/X31 at an MOI of 0.6 for 24 hours and cells were then analysed by flow cytometry.

In **paper III**, bulk PBMCs and BAL cells were stimulated with the TLR ligands for 3 hours with brefeldin A (BFA) added after 30 minutes to prevent release of cytokines, so that production of TNF can be assessed by intracellular staining using flow cytometry. BFA is an antibiotic produced by fungal organisms that works by inhibiting protein transport from the ER to the Golgi apparatus, resulting in accumulation of newly produced proteins in the ER.

5.5 FLOW CYTOMETRY

Different flow cytometers have been used in this thesis: BD FACSCanto II in **paper I**, BD FACSCalibur in **paper II**, BD LSRII and BD LSRFortessa in **papers III** and **IV**. Additionally, cells were sorted on a MoFlo XDP (Beckman Coulter) for RNA analysis in **paper II**.

Flow cytometry is a technology combining fluidics, optics and electronics to allow single cells labelled with fluorescence-conjugated antibodies to flow in a stream of fluid through a beam of light[313]. The illuminated particles emit light at specific wavelengths that can be detected by the electronics of the instrument, thus measuring relative fluorescence intensities. Additionally, relative size and granularity/internal complexity can also be measured.

Cells were stained with a panel of antibodies conjugated with different fluorochromes targeting different cell surface receptors that may be expressed by specific cell types. For intracellular staining, cells were first fixed with paraformaldehyde followed by permeabilisation with saponin, and staining with antibodies against intracellular proteins such as transcription factors or cytokines. Paraformaldehyde forms intra-molecular cross-links to preserve the morphology of cells prior to formation of holes in the membrane to allow the antibodies to gain access to the intracellular proteins[314]. Saponin forms holes by selectively removing cholesterol in the plasma membrane[315].

5.6 MICROSCOPY

Similar to flow cytometry, the core principle behind fluorescence microscopy is immunofluorescence; cells were stained with fluorescence-conjugated antibodies binding to specific host cell proteins or viral proteins. The microscope then illuminates the specimens at specific wavelengths to visualise multiple proteins. Unlike flow cytometry, microscopy provides a greater degree of subcellular spatial understanding. Confocal microscopy is advantageous over regular epifluorescence microscopy with the addition of a pinhole to eliminate out-of-focus light[316]. This enables the collection of multiple slices of images at different depths so that there can be a 3 dimensional (3D) reconstruction of the entire volume of a cell. Stimulated Emission Depletion (STED) microscopy further enhances the resolution of images by adding a second laser of lower energy overlapping the excitation spot in a doughnut-shaped beam[317]. This additional stimulation sends excited fluorophores back to their

ground state; in a non-fluorescent state. This improves the resolution of images by distinguishing two close molecules within the range of 30-80 nm apart, as compared to the 250 nm resolution of a regular confocal microscope.

In **paper I**, confocal images were acquired on a Zeiss LSM700 using a 63x objective and STED images were acquired on a Leica SP8 STED 3X platform using a 100x white light, with a numerical aperture 1.4 oil immersion objective.

In **papers III** and **IV**, sections of EBB embedded in GMA were stained with antibodies against specific proteins, but instead of a fluorescence molecule, a biotinylated secondary antibody was added, allowing visualisation by adding of a strepatividin-peroxidase complex that can be imaged by a light microscope. For cellular analysis, positively-stained, nucleated cells were counted within the bronchial submucosa and intact epithelium, excluding areas of smooth muscle, glands, large blood vessels and mismatched or damaged tissue.

5.7 RNA AND PROTEIN ANALYSES

Proteins secreted by cells that were harvested in supernatants were analysed using commercially available ELISAs to detect pro-inflammatory cytokines such as TNF, IL-6 and IFN-α for **papers II** and **IV**. Additionally, a type I IFN bioassay was developed in-house to detect small but biologically active levels of type I IFNs in **paper II**. Briefly, type I IFNs were detected indirectly by measuring the extent to which adding supernatants potentially containing type I IFNs can rescue a cell line from cell death upon challenge with Semliki Forest Virus (SFV).

Proteins produced by cells were harvested in cell lysates and analysed by Western blots in **paper II**. Proteins were first separated by molecular weight using sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. ISGs including ISG15 and MxA were detected by standard immunoblotting using peroxidase-conjugated secondary antibodies.

In **paper II**, total RNA from sorted cells was extracted using Qiagen RNeasy kit according to manufacturer's instructions. cDNA was synthesised using a cDNA reverse transcription kit. 48 TaqMan gene expression assays were pre-configured on a microfluidic card with 384 wells. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a QuantStudio 7 flex system and gene quantities were measured relative to an endogenous housekeeping gene.

6 RESULTS AND DISCUSSION

This thesis was initially built upon a key finding by Smed Sörensen *et al.* describing how human blood CD1c$^+$ MDCs are susceptible to IAV infection, and that infected DCs have an impaired capacity to present IAV antigens on MHC I to CD8$^+$ T cells, in contrast to DCs exposed to heat-inactivated IAV[210]. Hence, **papers I** and **II** were designed to follow up on the published observation by visualising where IAV traffics to upon entry into human DCs using super resolution microscopy (**paper I**), and by assessing whether mature DCs are also susceptible to IAV infection (**paper II**). However, the limitation of *in vitro* studies using blood DCs prompted us to investigate lung DCs that may be more relevant in the context of respiratory virus infection. We first characterised lung MNPs at steady state (**paper III**) and observed differences in subpopulations of monocytes and DCs in blood and lungs. Using this knowledge, we next investigated how hantavirus infection affects MNP subsets in blood and lungs of acute HFRS patients (**paper IV**). Key findings are highlighted in this section, followed by a general discussion on how the data fits into the existing literature. Detailed information can be obtained in the original papers (**I–IV**).

6.1 SUBCELLULAR TRAFFICKING OF IAV IN HUMAN DCs (PAPER I)

DCs are highly reliant on the endocytic machinery in order to sample the surrounding for presence of pathogens. Regulation of endocytic pH in DCs ensures that there is a balance between the destructive capacity of proteolytic enzymes and the ability to conserve antigenic peptides[318,319]. Like many viruses, IAV exploits this endocytic machinery in order to gain access into target cells[320]. Studies investigating the events following uptake and subcellular trafficking of IAV have mostly used animal or human cell lines such as MDCK cells and A549 cells[166,321]. Upon binding and internalisation, virions are delivered to early endosomes characterized by proteins such as EEA1 and Rab5. The virus then progresses to late endosomes or lysosomes, expressing Lamp1 and Rab7, where the lower pH allows membrane fusion and release of viral RNP into the cytoplasm. Although DCs have been shown to be susceptible to IAV infection, it has yet to be investigated whether the virus traffics in a similar manner in these cells with a unique endocytic machinery. We took advantage of STED imaging to generate valuable insight into the nanoscale organisation of viral and cellular proteins[317].

In this study, *in vitro*-generated MDDCs were pulsed with IAV at an MOI of 25 for 1 hour at 4°C, and replication was allowed to proceed after washing off excess virus by incubating the cells at 37°C. The cells were then fixed after 0, 5, 10, 15 and 30 min to track the spatiotemporal localisation of IAV in different endosomal compartments. Cells were then stained with antibodies against IAV NP, HLA-DR and EEA1 or LAMP1 to identify early endosomes or late endosomes/lysosomes respectively. Using STED microscopy with deconvolution, we were able to improve

the resolution significantly. We measured the degree of spatial coincidence in the deconvolved STED images by using an automated analysis programme where colocalisation was defined by an overlap of 10% between the fluorescent signals of IAV NP (parent) and EEA1 or LAMP1 (child). We found that the peak of IAV NP colocalising with EEA1^{+} early endosomes occurred 5 minutes after infection, whereas IAV NP colocalises with LAMP1^{+} late endosomes/lysosomes at a maximum after 15 minutes (Figure 12). Compared to epithelial cells where fusion of IAV occurs after 8 minutes, subcellular trafficking of IAV in human DCs follows a more delayed kinetics, in line with preservation of antigens for presentation to T cells.

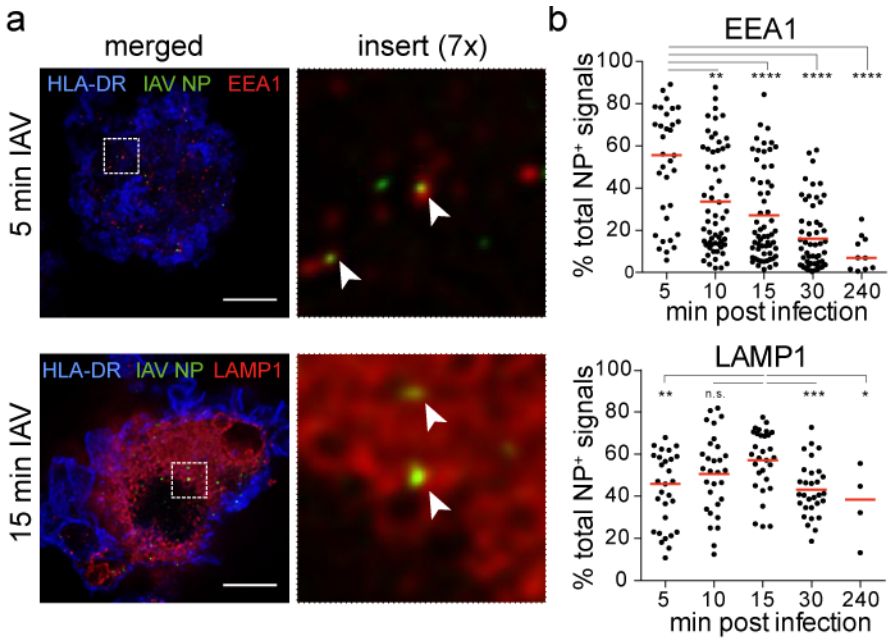

Figure 12. IAV traffics to early and late endosomes in human MDDCs. (a) STED images of a human MDDC exposed to IAV for 5 min (top panel) and stained with antibodies against IAV NP (green), HLA-DR (blue) and EEA1 (red), or IAV for 15 min (bottom panel) and stained with antibodies against IAV NP (green). Scale bar, 5 µm. **(b)** Bar graphs summarise the percentages of total IAV NP^{+} signals that colocalise with EEA1 (top panel) or LAMP1 (bottom panel) with median values indicated by a red line. Statistical differences were assessed using an unpaired *t* test: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ n.s., not significant.

The novel method of investigating viral trafficking in human DCs by three-colour STED microscopy provides a platform for further investigations. Continuous efforts are focused on dissecting the machinery of how IAV infection in DCs impairs antigen presentation on MHC I, whereas exposure to heat-inactivated virus permits efficient presentation of antigens to CD8 T cells[210]. By immuno-labelling proteins involved in the MHC I processing machinery, differences between DCs exposed to replicating or heat-inactivated virus could be compared to uncover possible mechanisms of impairment.

6.2 DIFFERENTIAL SUSCEPTIBILITY OF MDDCs AND CD1c$^+$ MDCs TO IAV (PAPER II)

In vitro-generated MDDCs are frequently used in studies investigating human DCs, due to the rarity of primary human DCs. In this study, we investigated the role of TLR-induced DC maturation in modulating the susceptibility of MDDCs to IAV infection and in parallel compared with primary CD1c$^+$ MDCs. This is to simulate how in the lungs, blood monocytes and DCs infiltrate the lungs upon induction of an inflammatory state. DCs may have received other stimuli and undergone maturation prior to encountering IAV. Mature DCs can continue to capture, process, and present antigens via receptor-mediated endocytosis[92]. Thus, it is of relevance to understand how mature DCs are affected by IAV infection.

In this published study, we showed that both MDDCs and CD1c$^+$ MDCs were differentially susceptible to IAV infection, depending on how the cells were stimulated (Figure 13a). MDDCs infected with IAV for 24 hours after being stimulated overnight with LPS or poly(I:C) showed a significantly reduced infection frequency compared to unstimulated MDDCs. In contrast, CD1c$^+$ MDCs infected with IAV after stimulation with poly(I:C) and 7/8L were less susceptible to infection compared to unstimulated CD1c$^+$ MDCs.

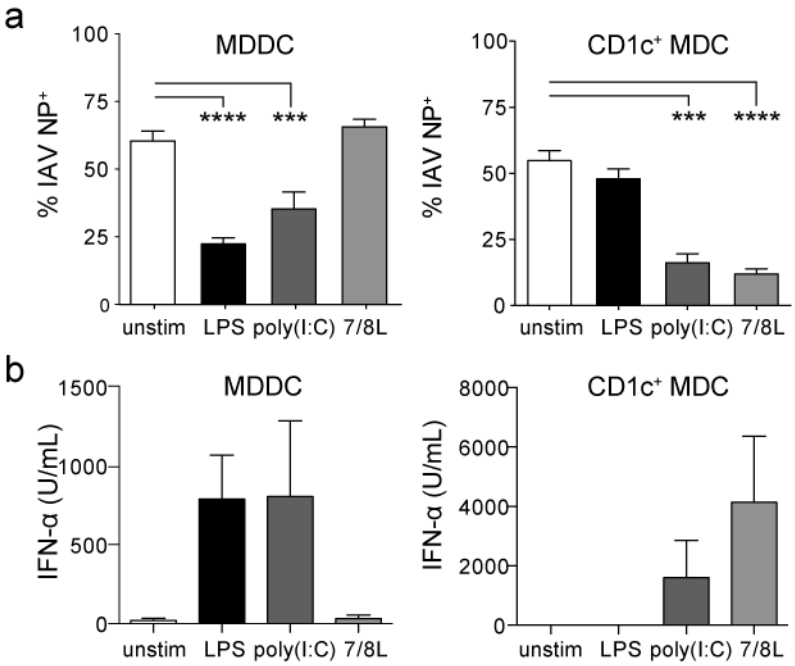

Figure 13. Mature MDDCs and CD1c$^+$ MDCs are differentially susceptible to IAV. (a) Bar graphs show mean±SEM percentage of IAV NP$^+$ MDDCs (left panel) or CD1c$^+$ MDCs (right panel) (*n* = 26). **(b)** Bar graphs show mean±SEM levels of biologically active type I IFNs in supernatants collected from MDDCs (left panel) or CD1c$^+$ MDCs (right panel) (*n* = 11).

As IAV is sensitive to type I IFN-mediated responses, we investigated whether the reduced susceptibility to infection could be related to the differential capacity of DCs to produce type I IFNs upon stimulation with TLR ligands. Indeed, MDDCs stimulated with LPS or poly(I:C) produced detectable levels of biologically active IFN-

α unlike unstimulated or 7/8L-stimulated MDDCs (Figure 13b). On the other hand, $CD1c^+$ MDCs stimulated with poly(I:C) or 7/8L produced higher levels of IFN-α than unstimulated or LPS-stimulated $CD1c^+$ MDCs. This observation was further confirmed with experiments using a cocktail of blocking antibodies against IFN-α, IFN-β and the IFN receptor, which reversed the protective effect against IAV conferred on mature DCs.

To understand why MDDCs and $CD1c^+$ MDCs responded differently to TLR ligands at the molecular level, we performed qRT-PCR on pure, sorted populations of MDDCs and $CD1c^+$ MDCs. Strikingly, MDDCs and $CD1c^+$ MDCs exhibited different mRNA expression levels of adaptor proteins *TRIF* and *MyD88*. MDDCs expressed markedly higher levels of *TRIF* compared to MDCs, whereas $CD1c^+$ MDCs expressed higher levels of *MyD88* than MDDCs. Higher TRIF expression in MDDCs could explain the differences we observed with LPS stimulation, because the LPS/TLR4/TRIF axis is responsible for inducing type I IFN[98]. Similarly, higher MyD88 expression in $CD1c^+$ MDCs could explain the difference we observed with 7/8L stimulation, as TLR7/8 utilises the MyD88 adaptor molecule for its downstream effects, including induction of type I IFNs[98]. The differences at RNA level were functionally confirmed using TRIF siRNA on MDDCs, which depleted the RNA levels of TRIF, resulting in LPS- or poly(I:C)-stimulated MDDCs to be equally susceptible to IAV as unstimulated MDDCs. Using a peptide inhibitor specific to MyD88 on $CD1c^+$ MDCs also resulted in lower levels of *IFN-β* produced. The findings in this study underline the inherent, genetic differences between *in vitro*-generated MDDCs and *bona fide* $CD1c^+$ MDCs that influence their ability to respond to danger signals and produce type I IFNs. Thus, the predominance of a specific population at the site of infection, e.g. inflammatory MDDCs that have been reported *in vivo* in humans, may alter the outcome of how well these DCs can go on to activate T cells, as infected DCs are less efficient at cross-presentation[210].

6.3 CHARACTERISATION OF DCs IN HUMAN LUNGS AT STEADY STATE (PAPER III)

Studies in mouse models illustrate that DCs play distinct roles depending on tissue location, as the local microenvironment in the lungs may fine-tune the functionality of DCs[322]. Lung DCs in mice have been demonstrated to be important in resolving respiratory viral infections but have also been implicated in lung immunopathology[323-325]. However, the translation of these findings to humans has been limited due to the inaccessibility of human lung tissue coupled with the rarity of DCs in tissue. Pioneering studies relied on morphology and expression of single markers such as HLA-DR and CD11c using immunohistochemistry. However, the identification of DCs in tissue is complicated by presence of macrophages and monocyte-derived cells expressing similar markers. Thus, to carefully characterise populations of monocytes and DCs in the lungs at steady state, we performed

bronchoscopies on healthy, non-smoking individuals from which we obtained mucosal tissue (EBB), as well as two sequential lavages sampling the proximal and distal airways (BW and BAL respectively).

In this published study, we performed multi-colour flow cytometry on PBMCs, BW, BAL and EBB cells from 20 healthy subjects. In addition to a majority population of highly autofluorescent alveolar macrophages in the airways, we identified six additional populations of MNPs in the proximal and distal airways (Figure 14a). Unlike in blood, the airways were enriched with $CD14^{+}CD16^{+}$ monocyte-derived cells. We were unable to ascertain whether these cells are monocyte-derived macrophages, as they did not upregulate CD163, the macrophage scavenger receptor. Further, we expected them to downregulate CCR2, as has been shown in monocyte-derived macrophages in the lungs of rhesus macaques[326] but this was also not the case. To further investigate the identity of monocytes in the airways, we stained for other typical macrophage markers, but could not detect an upregulation of CD64 or CD68 on monocyte populations in the airways as compared to blood (unpublished data). In enzymatically-digested EBBs, we could only identify classical monocytes, $CD1c^{+}$ MDCs, $CD141^{+}$ MDCs and PDCs.

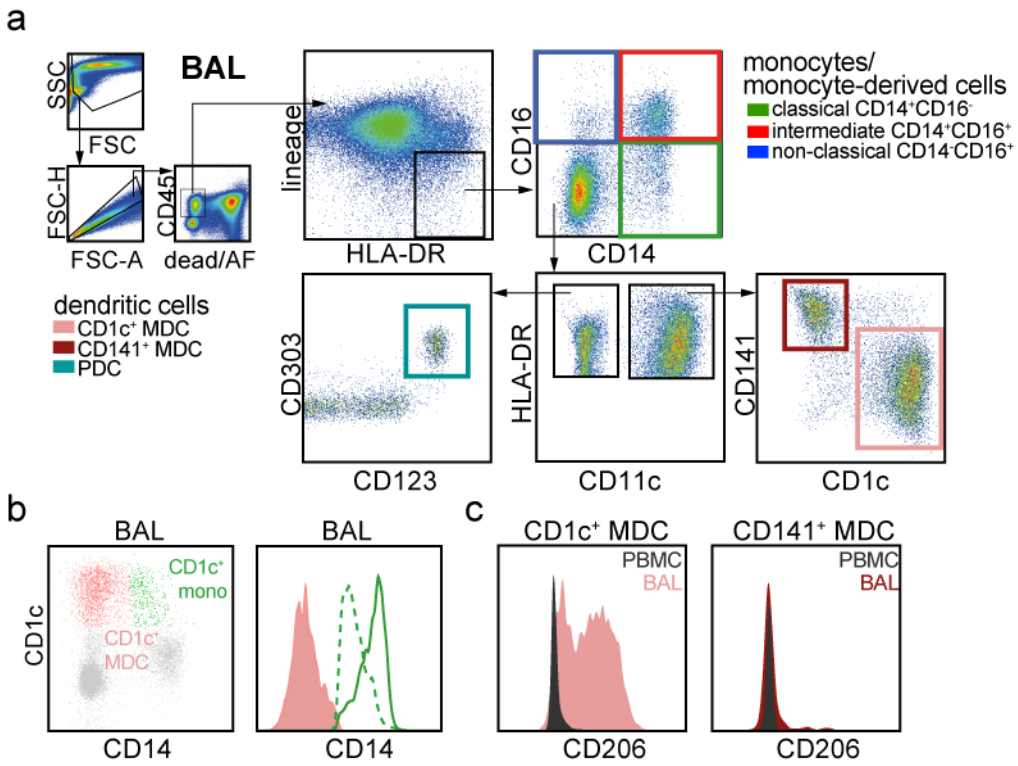

Figure 14. Identification of lung MNPs in BAL. (a) Flow cytometry plots show the gating strategy to identify monocyte populations and DC populations in the lungs. One representative donor is shown out of 19 healthy subjects. **(b)** Dot plot of CD14 against CD1c (left panel) depicts $CD1c^{+}$ $CD14^{+}$ cells (green dots) and $CD1c^{+}$ MDCs (coral dots) that were backgated to the parent gate consisting of total $HLA\text{-}DR^{+}lin^{-}$ cells (gray dots). Histogram (right panel) depicts CD14 expression on $CD1c^{+}$ MDC (coral), $CD1c^{+}$ $CD14^{+}$ monocytes (dashed green line) and $CD1c^{-}$ $CD14^{+}$ monocytes (green line) (n=19). **(c)** Histograms show CD206 expression on $CD1c^{+}$ MDC (left panel) and $CD141^{+}$ MDC (right panel) in PBMCs (grey) and BAL (coral or maroon).

Unlike blood MNPs, the populations identified in the lungs also express different phenotypic markers. For instance, a subpopulation of $CD14^+CD16^-$ classical monocytes in the airways upregulated CD1c, perhaps indicative of their differentiation into DCs[140] (Figure 14b). Our functional data suggest that upon stimulation with TLR ligands, the $CD1c^+$ monocyte-derived cells responded in a similar fashion to $CD14^+$ monocytes than to $CD1c^+$ MDCs (data not shown). However, the possibility that they may be $CD1c^+$ MDCs that have upregulated CD14 cannot be completely excluded. Further transcriptomics analysis of sorted cell populations may be necessary to elucidate this. $CD1c^+$ MDCs in BAL also express mannose receptor (CD206), unlike their blood counterparts, providing an additional endocytic receptor for antigen sensing in the lungs (Figure 14c).

$CD1c^+$ MDCs in BW and BAL also expressed a semi-mature phenotype, with higher expression of CD86 and CCR7 compared to blood $CD1c^+$ MDCs. This could indicate that MNPs lining the airways have already encountered self antigens or harmless particles, and perhaps responded in a tolerogenic manner, as the subjects included in this study were healthy and had no signs of airway inflammation. In contrast, all MNPs identified in the mucosal biopsies were relatively immature. Finally, we investigated whether respiratory MNPs could respond in an inflammatory manner if challenged with pathogens. The induction of a cytokine storm resulting in severe inflammation in the lungs has also been observed in human lungs during pandemic influenza[184]. To simulate a microbial exposure in *ex vivo* cultures, we stimulated bulk populations of PBMCs and BAL cells with a panel of TLR ligands for TLR3, TLR4 and TLR7/8 to mimic bacterial or viral challenge. Blood monocytes were superior in producing the pro-inflammatory cytokine TNF compared to BAL monocytes. However, $CD1c^+$ MDCs in BAL were better at producing TNF than blood $CD1c^+$ MDCs. This further illustrates that lung MNPs are distinct from blood MNPs due to local signals from the microenvironment. In conclusion, we have characterised lung monocytes and DCs, to provide a clearer baseline for future studies investigating the role of lung MNPs in pathological conditions.

6.4 LOSS OF DCs IN PERIPHERAL BLOOD OF ACUTE HFRS PATIENTS (PAPER IV)

An exaggerated immune response has been suggested to contribute to pathogenesis of hantavirus infections, as the viruses do not cause direct cytopathic effects. In particular, an expansion of effector cells such as NK cells and CTLs in the blood and lungs has been implicated to attack endothelial cells and cause capillary leakage, a hallmark of hantavirus pathogenesis[262,294,309]. DCs and monocytes can regulate the activation of other immune cells, yet little is known on the role of monocytes and DCs during hantavirus infection. In this study, we collected longitudinal blood samples from HFRS patients confirmed to be infected with PUUV,

the endemic hantavirus strain in Sweden. Additionally, we collected lung biopsies, embedded in GMA for tissue sectioning and immunohistochemical analysis.

Strikingly, there was a pronounced loss of blood monocytes and especially DCs during the acute phase of disease (Figure 15a). The numbers of blood MNPs was inversely correlated to viral load detected in plasma, as the cell numbers returned to normal values during the convalescence phase of disease when virus could no longer be detected by PCR. The low numbers of MNPs in blood during acute HFRS could be explained by cell death or cell migration. As NK cells and CTLs are activated in the blood during hantavirus infections[263,294], this immune-mediated cytotoxicity may inadvertently kill off monocytes and DCs in the blood.

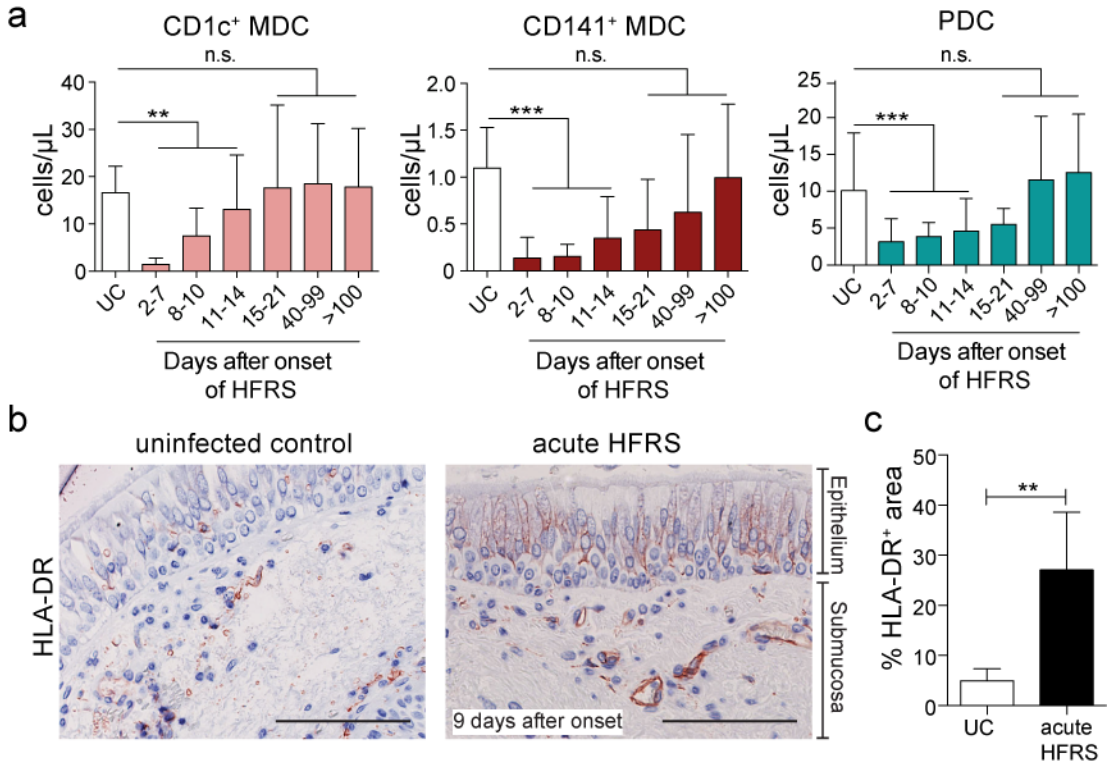

Figure 15. Massive loss of blood DCs in acute HFRS patients concurrent with infiltration of HLA-DR$^+$ cells in the lungs. (a) Bar graphs depict mean±SD absolute cell numbers of DC subsets in blood of HFRS patients (coloured bars) compared to uninfected controls (white bars). Differences in mean absolute number of MDCs were assessed using Poisson regression. **(b)** Representative images of endobronchial biopsies revealing high numbers of HLA-DR$^+$ cells in HFRS patients (n=5), as compared to uninfected controls (n=5) are shown. Specific staining is shown in red and cell nuclei counterstained with hematoxylin in blue. Visualization was performed using immunohistochemistry, and percentage of positive stained area was quantified. Scale bar, 100 µm. **(c)** Bar graph summarizes mean±SD percentage of HLA-DR stained area of all subjects.

The alternative hypothesis is that blood monocytes and DCs have migrated out of circulation to lymphoid tissues or peripheral tissues. Indeed, the few monocytes and DCs still present in blood during acute HFRS upregulated CCR7, a lymph node-homing chemokine receptor for DCs, also important for tissue-homing in monocytes[327]. In tissue sections of lung biopsies taken during the acute phase of disease, we detected higher levels of HLA-DR, CD11c and CD123, as compared to biopsies from uninfected controls, indicating an infiltration of MNPs into the lungs

(Figure 15b). The increase in HLA-DR$^+$ staining in lung sections from acute HFRS was statistically significant compared to uninfected controls (Figure 15c). However, since the lung sections were limited to staining with single markers, it is also possible that other cells such as T cells or NK cells that can upregulate HLA-DR upon activation, thus contributing to the increased number of HLA-DR$^+$ cells observed. An evaluation of similar markers by multi-colour flow cytometry on digested lung biopsies or BAL cells as done in **paper III** would provide a more complete depiction on the identities of cell infiltrates into the lungs of hantavirus-infected patients. Nevertheless, an influx of monocyte-derived cells and DCs in the lungs might explain the reported activated phenotype of CD8$^+$ T cells in the lungs during acute HFRS, correlating to respiratory symptoms in patients[263,309]. By mapping the movement of monocytes and DCs during an acute viral disease in humans, we learn more about the potential roles of specific subsets based on their tissue locations during the course of disease.

7 CONCLUSIONS

"Savoir pour prévoir, afin de pouvoir" – Auguste Comte

The studies in this thesis were designed to improve our understanding of how DCs, key players in modulating our immune response, are involved during respiratory viral infections in humans. A vast majority of our current understanding of the interaction between DCs and viruses is derived from studies in animal models, leaving a big gap of knowledge to be filled where human immunology is concerned. Furthermore, most human immune studies have focused on cells in circulation. Immunologists are shifting their attention towards tissue specificity, as immune cells residing in peripheral tissues have distinct and locally important traits than those in circulation[328-330]. Working with rare immune cells in human blood and lungs prove to be challenging, with limitations on what can be done with the few cells that can be obtained. Basic research using human immune cells, even if at first necessarily descriptive, is essential in generating important insights on whether similar monocyte and DC populations that have been described in mice also exist in humans, both at steady state and in disease. By extending our body of knowledge on how our human immune system functions, we can begin to translate important findings in mice to eventually developing novel strategies against respiratory viruses such as influenza and hantavirus that cause severe disease in humans.

In summary, the work presented in this thesis support the following four general conclusions:

(1) IAV traffics in human DCs via early and late endosomes, similar to what has been documented in epithelial cells, albeit at a more delayed pace (**paper I**).
(2) DCs derived from monocytes are inherently different from *bona fide* DCs, illustrated by their distinct capacity to produce protective antivirals upon recognition of pathogenic signals (**paper II**).
(3) Monocytes and DCs are patrolling our airways and lung tissue during healthy conditions, and they have slightly different phenotypes and functions than their blood counterparts (**paper III**).
(4) During acute disease, there are fewer monocytes and DCs circulating in the blood of hantavirus-infected patients, as they may be migrating towards the lungs where the virus first arrives, or lymph nodes where they can activate other adaptive immune cells (**paper IV**).

8 FUTURE DIRECTIONS

Based on the current findings presented in this thesis, there are still many unanswered questions that we can continue to explore.

Using the platform that we have established in **paper I** for performing three-colour STED microscopy, we can investigate the mechanism for impairment of antigen presentation in IAV-infected DCs by visualising IAV proteins relative to components of the host cell machinery. A key hypothesis is that defective ribosomal products (DRiPs), a side product of newly synthesised viral proteins, may become sequestered in DC aggresome-like induced structures (DALIS), thus limiting the amount viral antigen available on MHC I[331,332]. Hence, the observed impairment can potentially be overcome if DCs are allowed to be infected for a longer time, as DALIS structures are transient and dissociate after 24 hours. The delay in antigen presentation may be a strategy by DCs to conserve viral antigens until they complete their maturation process and have arrived in draining lymph nodes.

Following up on the findings of **paper II**, we can investigate whether mature DCs that are still susceptible to IAV infection could also have an impaired capacity to present antigen. The maturation programme may override the viruses' ability to disrupt efficient antigen presentation. As monocyte-derived cells such as TNF/iNOS-producing DCs (TIP-DCs) have been described to infiltrate the lungs during IAV infection in mice[214], it may be worth assessing whether viral replication takes place in human MDDCs *in vivo* and if so, whether these cells retain their ability to activate $CD8^+$ T cells.

Our characterisation of lung MNPs in **paper III** opened up Pandora's box as we contemplate the division of labour between the subsets of monocyte-derived cells and DCs residing in the lungs at steady state. On-going discussions are focused on how these cells lining the airways squeeze through the epithelial barrier and whether they can cross back to migrate to draining lymph nodes. Another interesting aspect of capturing a snapshot of all cells at one time point in the lungs, is how we can distinguish the lung-resident cells versus newly infiltrating cells, as there will be a mixed population of cells especially during inflammation.

Finally, we would like to elucidate the mechanism for the loss of MNPs in the blood of acute HFRS patients described in **paper IV**, by continuing on *in vitro* infection experiments where we can include more chemokine receptors and integrins that can reveal whether the cells are migrating towards specific tissues, such as the lungs or the kidneys. In future HFRS patients, we can sort out individual populations of lung MNPs and perform functional experiments and transcriptomics analysis to gain even more information on these cells. Additionally, urine from patients may be collected to investigate if monocytes and DCs infiltrate the kidneys during acute disease.

The study of the human immune system, particularly in the context of infectious diseases, has its own unique challenges but can have an important impact in the development of novel preventive and treatment strategies that can be applied to society. While we have learnt a lot from mouse studies, there are obvious limitations in terms of their evolutionary distance from humans. As described by David Masopust, laboratory mice are bred in such clean environments that they no longer resemble the human immune system, because humans do not live in sterile environments[333]. Bridging the gap between advanced mouse studies and human clinical trials requires a deeper understanding of how human immune cells behave and function at a basic level, both in health and disease. In order to achieve this, more collaborations between scientists and surgeons with access to precious human tissues/organs need to be established and prioritised. To paraphrase the French philosopher Auguste Comte, by expanding our wealth of knowledge, we can predict how our enemies may launch their attack against us, and this gives us the artillery to protect ourselves and prevent a repeat of devastating events.

9 ACKNOWLEDGEMENTS

Thank you to colleagues, friends and family who have been a part of my journey, you have all in one way or another been instrumental in getting me here:

Anna Smed Sörensen, my supervisor. Thank you for this opportunity, you have trusted me from the beginning and given me so much freedom to grow as an independent scientist, while still looking out for me when I needed it. I have learnt so much from you, not only on how to be a good scientist, but more importantly, how to be a good person.

Gunilla Karlsson Hedestam, my co-supervisor, you are the most passionate scientist I know. I enjoy our scientific discussions. You are critical of the work I do, because anything less would not be worth our effort. Thank you for making me better.

Ira Mellman, my co-supervisor, thank you for giving me the opportunity to perform one of my studies at Genentech. In my short interaction with you, I have learnt that one can be a brilliant scientist and also have a great sense of humour.

Florent Ginhoux, my mentor, I hope I get to work with you in the coming years – the science you do is exciting and inspiring! My take home message as your mentee is to work hard and party hard!

Karin Loré, thank you for all the scientific feedback and life advice. You are a role model on how to be a nurturing supervisor.

Karolinska Institutet, the Department of Microbiology, Tumor & Cell Biology, and the Department of Medicine, Solna, in particular **Kristina Broliden** and **Marianne van Hage**, thank you for providing an excellent scientific infrastructure.

All **co-authors**, thank you for working with me in the lab, and the many hours of editing manuscripts: **Cécile Chalouni** for teaching me all I know about STED, **Oliver Thomas** who wrote the Python script in paper I. **Andrea Bieder**, **Maria Hellmér** and **Julia Volz** who processed blood and tonsils with me, **Kerrie Sandgren** who helped me optimise my DC cultures, **Gerald McInerney** for our discussions on flu and interferons in paper II. **Anders Blomberg** for our ongoing collaboration and for performing the bronchoscopies, together with **Annelie Behndig** and all the **research nurses**, **Gregory Rankin** for taking care of us in Umeå, and for all the IHC work in papers III and IV, **Jamshid Pourazar** for handling the bronchoscopy samples so meticulously. **Clas Ahlm**, **Hans-Gustaf Ljunggren**, **Jonas Klingström**, **Niklas Björkström**, **Johan Rasmuson** and **Magnus Evander** for all the HFRS patient samples, and your expertise on hantaviruses and immunology, **Andrea Discacciati, Jonas Höijer** and **Matteo Bottai** for help with statistical modelling, **Kimia Maleki**, **Shawon Gupta** and **Sindhu Vangeti** for our continuous efforts in paper IV.

Special mention to the people I have worked most closely with: **Saskia**, for a while it was just the two of us and I have enjoyed every part of it, especially our travel adventures. I think we make a great team! **Rico**, your scientific input has always been refreshing, and I hope you'll never lose that energy. Thank you for keeping me sane and motivated these past months! You are the person I trust completely.

KL-ASS lab past and present (also known affectionately as Groggy Frog): **Saskia**, **Rico**, **Jens**, **Sindhu**, **Thomas, Julia**, **Oliver**, **Adeline**, **Kevin**, **Liz**, **Frank**, **Gustaf**, **Ang**, **Maria**, **Mona**, **Elena**, **Avery**, **Sebastian**, **Tyler**, **Sandra, Frederika**. I am happy that we have such an inclusive and collaborative environment. You make going to work every morning easy, and hanging out after work even easier!

Everyone in the **Unit of Immunology and Allergy**, I have learnt so much about allergies, exosomes, mast cells, urinary bladder cancer, T regs and chronic kidney disease, thanks to you! I only wish we had more movie nights together.

Everyone at **MTC** where I started, especially **Paola**, **Ganesh**, **Marjon**, **Martina**, **Lotta**, **Nestor**, **Sharesta**, **Christopher**, **Gerry**, **Marc**, **Thomas**, **Bastian**, **Kai**, **Jonathan**, **Kerrie**, **Emily**, **Gabriel**, **Flavio**. You are all such good, dedicated scientists! You were the support system I needed when Anna was in France, so thank you.

Other people I have discussed science with, **Liv Eidsmo**, **Stanley Cheuk**, **Elisa Martini, Susanna Bächle, Annelie Tjernlund, Bence Rethi. Connla Edwards** for all your help with the LSM 700. And all the intelligent, fun people I have met at retreats and conferences (and breweries).

Dr Adrian Loo, my biology tutor from Raffles Junior College. The letter you wrote for me was the first vote of confidence I needed to believe that I was good enough.

Till familjen Eriksson, särskilt **Linda**, **Håkan**, **Wille**, **Radka**, min sötaste **Magda** & **Richard**, ni var som en extra familj för mig – jag önskar er massor av kärlek! Och **Richard**, tack för att du tror på mig.

My friends in Singapore especially **Nicole,** and those in Sweden, especially **Nurzian, Andreas, Yaty, Hana, Nadia, Mariam, Didi, Tina – aka Makciks in Stockholm, Jimmie, Sharon, Rachel, Emelie**. Thank you for the company/food/babies/cats.

My family, **Mama**, **Ayah**, **Faiz**, **Along**, **Abg Ayim** & **Afiq**, terima kasih kerana selalu doakan Adik dan datang temankan Adik masa Adik sakit atau sedih. 9638 km rasa macam jauh, tapi semua orang dekat dalam hati Adik.

Finally, to all funding agencies, the 278 healthy blood donors, 17 hantavirus patients and 32 healthy subjects who braved a bronchoscopy – thank you for making this thesis possible!

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