Alveolar Macrophages Initiate Lung Innate Immune Responses

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Abstract

Alveolar macrophages (AMs) are one of the first cells to respond to inhaled pathogens. The RIG-I-like receptors (RLRs) are critical for recognition of respiratory syncytial virus (RSV), a common cause of lower respiratory tract infections. Upon detection of the virus, RLRs signal via MAVS to induce the synthesis of pro-inflammatory mediators, including type-I-interferons (IFNs), which trigger and shape antiviral responses and protect cells from infection. AMs are among the first immune cells to encounter RSV and the main producers of type I IFNs. The ability of AMs to restrict viral replication places AMs as ideal sensors of RSV infections and important initiators of immune responses in the lung. Whether IFNs act to prevent AMs from serving as vehicles for viral replication remains unclear. To answer this question, AMs from MAVS (*Mavs*$^{-/-}$) or type I IFN receptor (*Ifnar1*$^{-/-}$) deficient mice were exposed to RSV *ex vivo* and *in vivo*. Wildtype (wt) AMs but not *Mavs*$^{-/-}$ or *Ifnar1*$^{-/-}$ AMs produced inflammatory mediators in response to RSV. Furthermore, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs accumulated more RSV proteins compared to wt, however the infection was abortive, demonstrating that induction of pro-inflammatory mediators from AMs upon RSV infection, but not the viral restriction, depends on RLR-MAVS and IFNAR signalling.

Infants are vulnerable to severe RSV infection, which may require hospitalisation, and which is associated with asthma and wheezing in adolescence. Upon RSV infection, neonatal mice lack an enhanced innate response compared to adults. In order to understand this characteristic and examine potential adjuvants that can boost an immune response, neonates were exposed to various pattern recognition receptor ligands agonists (R848, imiquimod, CpG, poly(I:C) and LPS). When compared to adults, the neonates responded strongly to R848 and not to the other stimuli, highlighting the importance of TLR7 signalling in neonates. Improving the understanding of this response and the cell types responsible can open avenues to using ligands as potential vaccine adjuvants to RSV.

Statement of contribution

I hereby certify that all the work in this thesis is my own.

Spyridon Makris 2017.

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Abbreviations

A

ACK	Ammonium chloride potassium
AIM-2	Absent in melanoma 2
AKT1	RAC-α serine/threonine-protein kinase
ALRs	AIM 2-like receptors
ALRI	Acute lower respiratory infection
AMs	Alveolar macrophages
ASC	Apoptosis-associated speck-like protein containing a CARD
ATI	Type I alveolar epithelial cells
ATII	Type II alveolar epithelial cells
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate

B

B lymphocytes	B cells
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BFA	Brefeldin A
BLP	Bacterial lipopeptides
BM	Bone marrow
BSA	Bovine Serum Albumin

C

cAMP	Cyclic adenosine monophosphate
CARD	Caspases activation and recruitment domain
CCL2 (MCP-1)	C-C motif ligand 2 (monocyte chemoatractic protein 1)
CCL3 (MIP1α)	C-C motif ligand 3 (macrophage inflammatory protein 1α)
cDCs	Conventional dendritic cells
cDNA	Complementary DNA
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP synthase
CDP	Common dendritic cell progenitor
CLR	C-type lectin receptors
cMoP	Common monocyte precursor
COPD	Chronic obstructive pulmonary disease
CREB	cAMP responsive-element-binding protein
CSF	Colony-stimulating factor
CSFR	Colony-stimulating factor receptor
CXCL1 (KC)	C-X-C motif ligand 1
CXCL10 (IP-10)	C-X-C motif ligand 10

D

DAB	3,3'-diaminobenzinide
DAI	DNA-dependent activator of IRFs
DAMPs	Damage-associated molecular patterns
DAPI	4'6-diamidino-2-phenylindole
DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I (bovine pancreas
DR5	Death receptor 5
ds	Double-stranded

E

ECs	Epithelial cells
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMPs	Erythro-myeloid progenitors
ER	Endoplasmic reticulum

F

F	Fusion glycoprotein
FACS buffer	Staining buffer
Fc Block	Rat IgG2b anti-mouse CD16/CD32 receptor antibody
FCS	Foetal calf serum
FI	Formalin-inactivated
FFU	Focus forming unit

G

g	Grams
g	Gravity
G protein	Glycoprotein
GAS	IFN-γ-activated site
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor

H

HBD	Human-β-defensin
HEp-2	Human larynx epidermoid carcinoma cells
HGF	Hepatocyte growth factor
hrs	Hours
HSCs	Haematopoietic stem cells

I

IAV	Influenza A virus
IBs	Inclusion bodies

IFI16	IFN-γ inducible protein 16
IFITM	IFN-inducible transmembrane proteins
IFNs	Interferons
IFNAR	Type I IFN receptor
Ig	Immunoglobulin
IκB	Inhibitors of kappa B
IKK	IκB kinases
IL	Interleukin
i.n.	Intranasal
IRAK	IL-1 receptor-associated kinases
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF	ISG factor
ISREs	IFN-stimulated response elements

J

JAK	Janus kinase

L

L	RSV polymerase
LBP	LPS-binding protein
LCMV	Lymphocytic choriomenengitis
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharides
LRR	Leucine rich region
LRT	Lower respiratory tract
LRTI	Lower respiratory tract infection

M

M	RSV matrix protein
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signalling protein
MDA5	Melanoma differentiation-associated protein 5
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
min	minutes
ml	Millilitre
µl	Microlitre
MLN	Mediastinal lymph nodes
MNPs	Mononuclear phagocytes
moDCs	Monocyte-derived dendritic cells
MOI	Multiplicity of infection
MyD88	Myeloid differentiation factor-88
Mx1	Myxovirus resistance 1

N

N	RSV nucleoprotein
NF-κB	Nuclear factor kappa beta
NK cells	Natural killer cells
NDV	Newcastle disease virus
NETs	Neutrophil extracellular traps
NLR	NOD-like receptors
NOD	Nucleotide binding oligomerisation domain
NP	Nucleoprotin
NS	Nonstructural proteins

P

P	Phosphoprotein
PAP	Pulmonary alveolar proteinosis
PAMPs	Pathogen-associated structural
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDCs	Plasmacytoid DCs
PDL1	Program cell death 1
PI3K	Phosphoinositide 3-kinase
PIAS	Protein inhibitors of activated STAT
PKR	Protein kinase R
pMACs	Macrophage precursors
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
PVM	Pneumovirus of mice

Q

qPCR	Quantitative polymerase chain reaction

R

R848	Resiquimod
RIG-I	Retinoic acid-inducible gene 1
RIP	Receptor-interacting kinase
RLRs	Retinoic acid-inducible gene 1 receptors
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT	Room temperature

S

SBE	Smad binding elements
SEM	Standard error of the mean
SH	RSV small hydrophobic (protein/gene)
SLE	Systemic lupus erythromatosus
SOCS	Suppressor of cytokine proteins

SP	Surfactant protein
ss	Single-stranded
STAT	Signal transducer and activator of transcription
STING	Stimulator of IFN genes
SUMO	Small ubiquitin-like modifier

T

T cells	T lymphocytes
TAK	TGF-β activated kinase
TANK	TRAF family associated NF-κB activator
TBK	TANK-binding kinase-1
Tfh	T follicular helper cells
TGF	Transforming growth factor
Th	T helper cells
Th1	Type 1 helper
Th2	Type 2 helper
TIR	Toll-interleukin 1 receptor domain
TIRAP	TIR domain-containing adaptors
TLRs	Toll like receptors
TMB	3,3'5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TRADD	TNF receptor type I DEATH domain-associated protein
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
Tregs	Regulator T cells
TREM	Triggering receptor expressed on myeloid cells
T_{RM}	Tissue resident memory cells
TRIF	TIR domain-containing adaptor IFN-β
TRIM	Tripartite motif
TYK	Tyrosine kinase

U

USP	Ubiquitin carboxyl-terminal hydrolase

V

VLP	Virus like particles
VSV	Vesicular stomatitis virus

W

wt	Wildtype

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1 Introduction

1.1 Respiratory Tract

Mucosal surfaces such as those found in the intestine and the lungs are the most common targets for invading pathogens. The surface area of the human lung is approximately 70m^2 and its main function is gas exchange (Kopf et al., 2014). The lung is constantly in direct contact with the environment. From birth onwards, maintaining homeostasis is critical for health and disease. Its breakdown may cause chronic inflammatory disorders such as asthma or Crohn's disease in the lung and intestine respectively (Hammad & Lambrecht, 2008; Mowat, 2003). Therefore, it is important for the lung to maintain a 'constant' state where tissue repair, surfactant secretion, and a balance between anti- and pro-inflammatory cytokines occurs, thus allowing gaseous exchange to occur. The organ must also be able to discriminate between harmless antigens and initiate immune responses to pathogens, as the balance between a pro- and anti-inflammatory immune responses in the lung is critical for the outcome of many respiratory infections.

The respiratory tract is divided into the upper and the lower airways, each of which has multiple distinct functions. The lung can be characterised by the mucosal tissue which is composed of ciliated epithelial cells and goblet cells in the upper airways. The function of goblet cells is to secrete mucus which acts as a barrier for pathogens and particles that may be harmful for the lower airway (P. G. Holt et al., 2008). The mucus produced by goblet cells can trap many inhaled particles, and together with the propulsion of ciliated epithelial cells, can protect the lower airways from harmful substances. The mucus also contains immunoglobulin (Ig) A and antimicrobial proteins which are important for targeting potentially harmful pathogens. The lower airway is divided into the bronchus, bronchioles and alveoli. The alveoli increase the surface area for gas exchange to occur. The alveoli are ideal for gas exchange as they are composed of a thin, single layer of epithelial cells which are in close proximity to blood vessels (P. G. Holt et al., 2008). A vital feature of the airways is the presence of immune cells that are constantly surveying the airways for potential harmful substances.

As the lung is one of the few mucosal barriers that in places is composed of a single cell line, it is important to maintain a balance which allows the gas exchange to occur whilst protecting from opportunistic pathogens. Therefore, the close interactions between the innate immune cells and the airway epithelial cells are of great importance. One characteristic of the lower airway is the absence of ciliated cells. In fact, the alveoli are composed of two types of epithelial cells: type I and type II alveolar epithelial cells (ATI and ATII cells respectively). The ATI cells make up as much as 98% of the surface area of the airway alveoli and their roles include maintaining structural integrity and gas exchange (Hussell & Bell, 2014). ATII cells have multiple roles in maintaining homeostasis by producing many important growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF). ATII cells also secrete surfactants which have multiple roles such as protecting the airways from hypertension during gas exchange and controlling the activation and phagocytic activity of AMs. The interaction between ATII cells and AMs is very important during all stages of infection of the lower airways. For example, an important protein expressed on the surface of ATII cells is CD200 which interacts with the CD200R on AMs and is believed to 'set' the activation threshold (Hussell & Bell, 2014).

Mononuclear phagocytes (MNPs) have an important role in the protection from pathogens. MNPs are of greater importance in mucosal tissue, where the control of exacerbated-responses is central to tissue function and survival. There are three main types of MNPs and these include dendritic cells (DCs), macrophages and monocytes. The main function of these cells is to protect the airway by initiating the immune response against pathogens, removing cellular debris and repairing the tissue after the threat has been eliminated (C. L. Scott et al., 2014). All these events are tightly controlled in order to minimise the possibility of structural damage to the organ.

AMs are referred to as 'gatekeepers' of the lower airways. They constantly survey their surroundings for potential invading pathogens whilst maintaining a specific activation threshold through their close interaction with ATII cells (Hussell & Bell, 2014). The main challenge, for these otherwise active cells, is the distinction between invading pathogens and non-invasive antigens or commensal bacteria. In order to make this distinction AMs possess a series of receptors and signalling pathways (see section 1.1.4).

Another type of innate immune cells that are important for the recognition of invading pathogens are DCs. The airway DCs are located within the epithelial cell compartment, unlike other mucosal surfaces such as the small intestine where DCs reside beneath the microvasculature (Hammad & Lambrecht, 2008; Worbs et al., 2016). In both tissues however, DC projections through the epithelium are important for the surveillance of the lumen (Mildner & Jung, 2014; Worbs et al., 2016). Upon pathogen-associated molecular pattern (PAMP) activation, DCs upregulate CCR7 (interacts with CCL21) and migrate to the draining lymph nodes where antigen presentation to T cells and B cells occurs. For presentation to T cells, DCs upregulate major histocompatibility complex (MHC) class I (MHC-I) or MHC-II to present antigens to $CD8^+$ or $CD4^+$ T cells respectively (Durai & Murphy, 2016; Mildner & Jung, 2014; Worbs et al., 2016).

1.1.1 Dendritic cells and monocytes

The development of MNPs is currently being revisited and knowledge regarding the ontogeny of these cells is evolving. Monocytes and DCs originate from haematopoietic stem cells (HSCs) whereas tissue resident macrophages are of embryonic origin (Guilliams et al., 2014; C. L. Scott et al., 2014) (Fig 1.1). The HSCs in the bone marrow (BM) require Flt3L in order to differentiate into a common DC progenitor (CDP) (Onai et al., 2006). The expression or non-expression of the E2-2 transcription factor gives rise to pre-plasmacytoid DCs (pDCs) or pre-conventional DCs (cDCs) which migrate from the BM toward the lung and other tissues (Ghosh et al., 2010). As they translocate towards the lung, the pre-cDCs initiate their maturation to cDCs that express either CD11b or CD103 (C. L. Scott et al., 2014). The expression of these receptors is dependent on interferon (IFN) regulatory factor (IRF) 4 and Batf3 expression respectively (Bachem et al., 2010; Persson et al., 2013; Schlitzer et al., 2013).

Monocytes originate from the common monocyte precursor (cMoP) in the BM. The development of monocytes is dependent on colony-stimulating factor (CSF)-1 (CSF-1) (Cecchini et al., 1994; Greter et al., 2012; Wiktor-Jedrzejczak et al., 1996). Monocytes are known as classical/inflammatory ($Ly6C^{hi}$) or non-classical/patrolling ($Ly6C^{lo}$) which reside in tissue and the vascular compartment respectively. One role of the $Ly6C^{lo}$ monocytes is to patrol the vascular wall (Carlin et al., 2013).

Despite having a minimal role in peripheral tissue during steady state conditions, the Ly6C^{hi} monocytes can rapidly travel to inflamed tissue. Activated monocytes downregulate Ly6C and have multiple roles including phagocytosis, antigen presentation, angiogenesis and secretion of mediators that drive the inflammation or resolution of the response (Cheong et al., 2010; Ellis et al., 2015; León et al., 2007; C. L. Scott et al., 2014). Depending on the phenotype and surface markers expressed after being terminally differentiated, these Ly6C^{lo} cells can be termed monocyte-derived DCs (moDCs), monocyte-derived macrophages, inflammatory DCs or inflammatory monocytes (Durai & Murphy, 2016; C. L. Scott et al., 2014). Inflammatory monocytes can acquire characteristics that are similar to macrophages. The differentiated monocytes can also acquire features similar to DCs as they have DC-like cellular projections, express CD11c and can migrate to the lymph node where antigen presentation to T cells occurs via the MHC-II (León et al., 2007; Plantinga et al., 2013). Recent findings suggest that a subtype of monocytes can enter the resting lymph nodes. Their phenotype plasticity and the lack of evidence as to the transcription factors required for the differentiation of these cells limits further characterisation (Jakubzick et al., 2013; C. L. Scott et al., 2014).

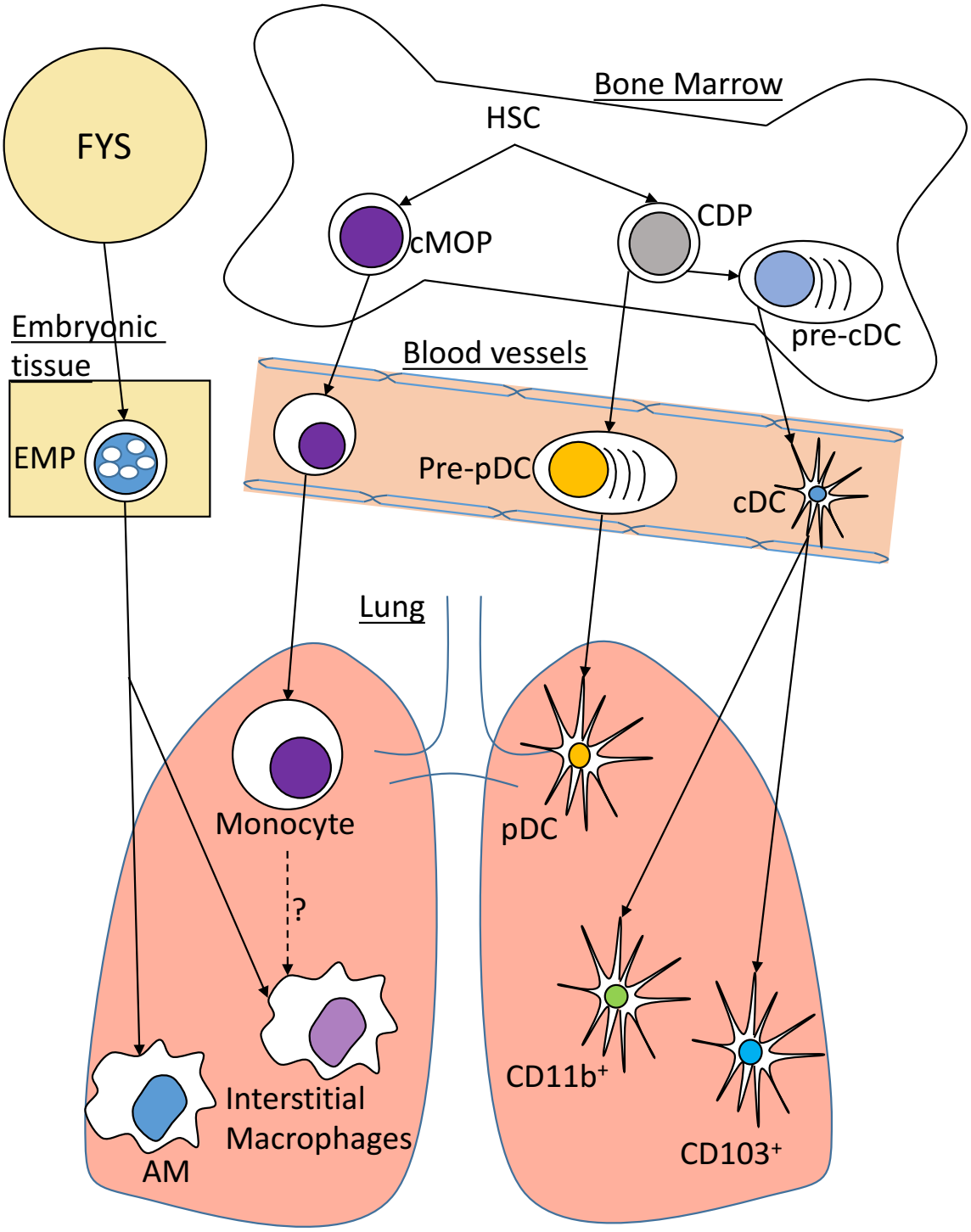


Figure 1.1 Mononuclear phagocyte ontogeny. Dendritic cells (DCs) originate from adult haematopoietic stem cells (HSCs) which initially differentiate into a common DC progenitor (CDP). The CDP cells differentiate into pre-plasmacytoid DCs (pDCs) and migrate through to the tissue via the blood vessels. Upon entering the tissue, they become pDCs. CDPs can also differentiate into pre-conventional DCs (cDCs) in the bone marrow which become cDCs as they migrate to tissue where they express CD103 or CD11b in an IRF4- or Batf3-dependent manner respectively. HSCs also differentiate into a common monocyte precursor (cMOP)

which differentiates into monocytes upon exposure to CSF-1 (M-CSF). Tissue resident macrophages such as alveolar macrophages (AMs) develop from the foetal yolk sac (FYS) precursors known as erythro-myeloid precursors (EMPs) which colonised embryonic tissue and differentiate into tissue resident macrophages at the time of birth. AMs are believed to replenish in situ and are dependent on GM-CSF; whereas interstitial macrophage population might be replenished by monocytes.

1.1.1.1 Monocytes and DCs in the lung

In the resting state (lack of inflammation), the lung DCs can be subdivided into three main populations: $CD103^+$ cDCs, $CD11b^+$ cDCs and pDCs (See Figure 1.1) (Guilliams et al., 2013b). Specifically, $CD103^+$ cDCs are $CD103^+CD11b^{lo}CD11c^{hi}CX3CR1^-$ and belong to the CD8α-type cDCs; $CD11b^+$ cDCs belong to the CD11b-type cDCs and are $CD103^-CD11b^{hi}CD11c^{hi}CX3CR1^{int}SIRP1\alpha^+CD64^-$ whereas pDCs are $CD103^-CD11b^{lo}CD11c^{int}CX3CR1^{int}$Siglec-$H^+Ly6C^+$ (Guilliams et al., 2010; Guilliams et al., 2013b; Heath & Carbone, 2009). In addition, upon inflammation, moDCs ($CD64^{hi}CD11b^{hi}CD11c^{hi}Mar1^+SIRP1\alpha^+CX3CR1^{int}$) are also present in the lungs (Goritzka et al., 2015; Heath & Carbone, 2009).

The cDCs compartmentalise in various areas of the lung tissue. $CD103^+$ cDCs are located within the epithelial layer and form cellular protrusions into the airway where they can sample the environment (Sung et al., 2006). In the resting state, pulmonary $CD103^+$ cDCs preferentially acquire apoptotic cell-associated antigen and migrate to the lymph nodes where they prime $CD8^+$ T cells via MHC-I (Desch et al., 2011). The $CD11b^+$ cDCs cells mainly reside in the lamina propria or the basolateral space beneath the epithelium, and survey the airway through the epithelial tight junctions and the zonula occludens proteins that connect the epithelial cells (Hammad & Lambrecht, 2008; Lambrecht & Hammad, 2012). $CD11b^+$ cDCs and pDCs are also present in the parenchyma. Recently a transcriptional repressor of the BTB-ZF family, ztbt46 (BTBD4), has been identified to be shared between $CD103^+$ and $CD11b^+$ cDCs (Satpathy et al., 2012). This marker (zbtb46) is not involved in the development of cDCs but rather acts as a negative regulator during toll-like receptor (TLR) signalling and has the

potential to be used as target for depleting cDCs (Guilliams et al., 2013b; Meredith et al., 2012). Viral/microbial-insults, allergens, pollutants or TLR ligands can cause moDCs recruitment to the parenchyma and the airways (see section 1.1.1) (Cheong et al., 2010; León et al., 2007). As the nomenclature of the lung DCs is still being redefined, the best markers to use in order to distinguish moDCs from the cDCs are the expression of CD64 and MAR-1 (Guilliams et al., 2013b; Vermaelen & Pauwels, 2004).

1.1.2 Development of tissue resident macrophages

There are two types of macrophages in the lung. These include AMs, which reside in the alveoli (further described in section 1.1.2.1) and interstitial macrophages which reside in the lung interstitium. For decades, the dogma was that circulating monocytes replenish the population of tissue resident macrophages (van Furth et al., 1972). This work was justified by labelling monocytes with thymidine to study their kinetics and was further confirmed by studying monocyte differentiation *ex vivo* (van Furth & Cohn, 1968). Lung interstitial macrophages express $CD11b^{int}CD64^{hi}MerTK^{hi}MHCII^{hi}CD11c^{lo\text{-}int}$ and parabiosis experiments indicated that they are of yolk sac and adult HSC derived origin (Schulz et al., 2012). However, data from mouse models describing the origin of interstitial macrophages is controversial and believed to be affected by the microbiota of various animal facilities (Jakubzick et al., 2013; C. L. Scott et al., 2014).

A new dogma regarding the development of macrophages has evolved from various independent studies (Perdiguero & Geissmann, 2016). The current dogma is that tissue resident macrophages arise from erythro-myeloid progenitors (EMPs) that colonise the foetal liver and give rise to macrophage precursors (Guilliams et al., 2013a; Mass et al., 2016). These precursors colonise all tissue of the embryo in a CX3CR1-dependent manner and their final differentiation depends on tissue-specific stimuli and transcription factors (Amit et al., 2015; Mass et al., 2016). Tissue resident macrophages are believed to be maintained by *in situ* proliferation with minimal contribution by circulating monocytes (Guilliams et al., 2013a; Hashimoto et al., 2013; Schulz et al., 2012). The main exceptions appear to be in intestinal, dermal, heart and pancreatic macrophages (Ginhoux & Guilliams, 2016) where the intestinal yolk sac and foetal-derived macrophages are outcompeted by bone marrow derived

macrophages (C. L. Scott et al., 2014). The embryonic precursor cells also reside in the intestinal mucosa and undergo *in situ* differentiation after birth. Around the time of weaning, driven strongly by the microbiota, and during adult life these cells are replaced by a CCR2-dependent influx of circulating Ly6C^{hi} monocytes (C. C. Bain et al., 2013). A similar dogma is arising for replenishment of the AMs in the lung after infection. However, this work is ongoing and still controversial as most animal models lack the dynamic for long term studies that would relate to humans (Perdiguero & Geissmann, 2016).

A specific way of depleting tissue resident macrophages is currently unavailable and therefore the contribution of circulating monocytes in replenishing the original population of tissue resident macrophages is difficult to assess. By identifying the Kupffer cell-specific *Clec4* gene, these cells were successfully depleted and it was shown that there was a specific niche located in the liver that a certain number of cells can occupy (C. L. Scott et al., 2016). Recruited monocytes are believed to have features that would be disadvantageous after infection and therefore the original population of foetal derived macrophages would be able to outcompete them (van de Laar et al., 2016). A limitation in studying the development of macrophages in the murine model is that it does not permit the study of tissue resident macrophages over long periods of time (Amit et al., 2015). Although minimal, there appears to be an increased contribution of HSCs in adult tissue resident macrophages after infection and with increasing age (Perdiguero & Geissmann, 2016). The development and replenishment of tissue resident macrophages is a constantly evolving area and many ongoing studies are focusing on how these cells develop, encounter pathogens and replenish.

1.1.2.1 Alveolar Macrophages

The differentiation of tissue resident macrophages is driven by c-Myb-independent factors located in the tissue (Perdiguero & Geissmann, 2016). Current lack of fate-mapping approaches in mouse models limits the number of studies that can provide more detailed tracking of transcription factors involved in the differentiation of tissue resident macrophages (Perdiguero & Geissmann, 2016). GM-CSF is abundant within the lung tissue and is the driving force of AM differentiation from pMacs, their proliferation, and is also important for their maintenance, survival and their distinct phenotype (Guilliams et al., 2013a; Y. Shibata et al.,

2001). The vital characteristics of AMs during homeostasis and infection are driven by the transcription factor PU.1 and mediated by GM-CSF (Y. Shibata et al., 2001). In addition to the upregulation of *Csf1r*, the transcription factor PPARγ has been shown to be critical for the differentiation of AMs after birth and also for maintaining an anti-inflammatory profile during homeostasis and the resolution of inflammation (Gautier et al., 2012; Mass et al., 2016). Murine and human studies have shown that a decrease in the presence of GM-CSF or lack of the receptor (CSFR) on AMs can cause pulmonary alveolar proteinosis (PAP) and increased morbidity following infection (Lieschke et al., 1994; Perdiguero & Geissmann, 2016; C. Schneider et al., 2014a; Y. Shibata et al., 2001). In mice with PAP, the transplantation of AMs expressing CSFR was effective as a long-lasting therapy (Happle et al., 2014).

1.1.2.1.1 Steady state alveolar macrophages

AMs reside within the airway lumen and are constantly exposed to the challenges that the lungs encounter (Hussell & Bell, 2014). The presence of AMs is vital throughout life, as they are involved in the development of the airway tissue, initiating innate responses and resolving inflammation (Ginhoux & Guilliams, 2016; Hussell & Bell, 2014). The role and importance of AMs during the steady state and their pivotal role in the maintenance of homeostasis is of equal importance as their role during inflammation. Surfactants are constantly secreted by ATII cells and are abundant in the fluid that lines epithelial cells in the alveoli (Khoor et al., 1993). One role of surfactants is to decrease the tension of the alveoli when breathing (Hussell & Bell, 2014). The steady state AMs catabolise surfactants in order to protect the lower airways from obstruction and the surfactant proteins (SP)-A and SP-D have a role in their regulation. This interaction with surfactants induces an anti-inflammatory state in AMs as SP-A binds to SIRPα which causes the inhibition of C1 (essential for complement activation) and blocks the binding of TLR ligands and co-receptors (Watford et al., 2001; Yamada et al., 2006).

There are a series of mechanisms in the lower airways that regulate the activation state of AMs. Other than secretion of surfactants, ATII cells also express CD200 which binds to the CD200R on AMs and is critical in setting the activation threshold for AMs as it interferes with the induction of inflammatory cytokines (Jiang-Shieh et al., 2010; Snelgrove et al., 2008). In

fact, this interaction and the increase of the activation threshold after the resolution of disease may make the lungs more prone to opportunistic secondary infections (Ghoneim et al., 2013; Hussell & Bell, 2014; Snelgrove et al., 2008). AMs are highly phagocytic and possess pattern recognition receptors (PRRs) that can initiate immune responses (Hussell & Bell, 2014). It is therefore critical for AMs to be controlled from becoming activated by the commensal bacteria present in the lower airways. Mannose receptors on AMs are able to distinguish unopsonised bacteria and control the activation of TLR4 (J. Zhang et al., 2005a). Further to this, studies in mice and humans have shown the importance of the triggering receptor expressed on myeloid cells (TREM) family vital in controlling the activation of TLR4 on AMs (X. Gao et al., 2013). Overall, the lung has developed a series of mechanisms to control the activation threshold of AMs and prevent unnecessary inflammation

1.1.2.1.2 Alveolar macrophages during inflammation

The localisation of AMs in the airway lumen positions them as ideal sensors of pathogens and strong initiators of inflammatory responses (Makris et al., 2016). Therefore, whilst AMs are critical for maintaining homeostasis, they are also vital in initiating inflammatory responses, thus making them the 'masters of contradictory function' (Hussell & Bell, 2014). There is increasing evidence that AMs have a very active role during respiratory infections. Murine models of infection have shown that AMs are the main producers of type I IFNs after Newcastle disease virus (NDV) and respiratory syncytial virus (RSV – see section 1.4.4.2; (Goritzka et al., 2015; Kumagai et al., 2007)). It is speculated that the breakdown of the regulatory mechanisms, as the epithelium is destroyed during infection, contributes to the activation of AMs (Hussell & Bell, 2014). Depletion of AMs using clodronate liposome treatment suggested that they are involved in the secretion of an array of inflammatory mediators after respiratory infections (Kolli et al., 2014; Pribul et al., 2008; Tate et al., 2010; Tumpey et al., 2005). The main limitation with depleting AMs using clodronate treatment is that other phagocytic cells such as DCs and interstitial macrophages can also be depleted and thus the precise role of AMs might be misinterpreted (Benoit et al., 2006). Furthermore, the depletion of AMs using this method causes the increase of cellular debris which could contribute to many of the symptoms observed (Machado-Aranda et al., 2014). In addition, the depletion of AMs results in an induced recruitment of neutrophils, natural killer (NK) cells

and monocytes that can influence the outcome of infection independently of the lack of AMs (Kolli et al., 2014; Machado-Aranda et al., 2014; Pribul et al., 2008).

1.1.3 The airway microbiome

For decades it was believed that the lower airways were a sterile environment and the lung was initially excluded from the human microbiome study (Dickson et al., 2014). Recent findings utilising RNA 16S sequencing contradict this and an array of microbes that constitute the microbiome of the lung has been discovered. Humans are hosts to more than a trillion bacterial cells (S. R. Gill et al., 2006). Neonates are considered sterile until the time of birth and their tissue is immediately colonised by microbes derived initially from the mother and subsequently from the environment (Fanaro et al., 2003). This interplay is very important for shaping the lung environment and studies have shown that dysbiosis of commensal lung bacteria, similarly to the intestine, is associated with chronic lung conditions such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Budden et al., 2016; Gollwitzer et al., 2014; Marsland & Gollwitzer, 2014). Thus, on all mucosal surfaces, the interplay between the immune cells and the microbiome, virome and mycobiome is a critical balance between health and disease (Marsland & Gollwitzer, 2014). Furthermore, although the microbiome is relatively unique at different mucosal sites, emerging evidence suggests that during disease or after exposure to pollutants, stimuli or cigarette smoke, the commensals might migrate between the gut and lung; thus, adding a further dimension to the critical balance between immune tolerance and exuberant response in the lung (Budden et al., 2016).

1.2 Pattern Recognition Receptors

The notion that the innate immune response was non-specific vanished after the discovery of toll-like receptors and the pattern recognition theory proposed by Charles Janeway in 1989 (C A Janeway, 1989). Multiple cell types, including leukocytes and structural cells, can detect conserved components of foreign pathogens known as pathogen-associated molecular patterns (PAMPs) in various cellular compartments. Recognition of PAMPs by germline-encoded pattern-recognition receptors initiate an intracellular signalling cascade which causes the translocation of transcription factors to the nucleus and the initiation of innate immune responses. The location of PRRs within the cells and their signalling pathways reduce the prospect of activation to self-antigens (Brubaker et al., 2015). There are multiple subgroups of PRRs which include cell membrane and cytosolic receptors. The group of membrane bound receptors are composed of TLRs and C-type lectin receptors (CLRs). There are 10 functional TLRs in humans and 13 in mice which are bound to the cell- or endosomal-membrane (Brubaker et al., 2015; Kawai & Akira, 2011; T. Li et al., 2012). There are multiple cytosolic PRRs such as the retinoic acid-inducible gene 1 (RIG)-I-like receptors (RLRs), nucleotide binding oligomerisation domain (NOD)-like receptors (NLRs) and absent in melanoma (AIM) 2-like receptors (ALRs). Furthermore, cyclic GMP-AMP synthase (cGAS) was discovered as a cytosolic DNA sensor that activates type I IFNs (Sun et al., 2013). One difference between the two groups of PRRs is that TLRs located on the cell or endosomal membranes survey the extracellular space; whereas activation of the cytosolic PRRs occurs when the pathogen gains access to the cytosol (Brubaker et al., 2015).

1.2.1 Cell membrane PRR signalling

A group of TLRs are bound to the outer cell membrane and, together with their co-receptors, identify structural components of invading pathogens. These TLRs include TLR-1, -2, -4, -5 and -6, each of which detect different components of pathogens (Table 1-1). TLRs are type I transmembrane proteins with an ectodomain composed of leucine rich region which is believed to give the specificity for the recognised PAMPs (Kobe & Kajava, 2001; O'Neill & Dinarello, 2000). Upon recognition of pathogens by the ectodomain, the transmembrane and

cytosolic intracellular toll-interleukin (IL)-1 (IL-1) receptor (TIR) domain initiate a signalling cascade. The recognition of various pathogenic components and the activation of TLRs is dependent on the formation of homo- or hetero-dimers and, in some cases, the interaction with other co-receptors (Ozinsky et al., 2000). These combinations, which add to the repertoire of components recognised by TLRs, include the requirement of co-receptors such as CD14 or CD18, or TLR2 dimerization with TLR1 or 6 (Hoebe et al., 2005; Manukyan et al., 2005). The cellular activation induced by membrane bound TLRs initiates by recruiting TIR domain-containing adaptors (TIRAP) and myeloid differentiation factor-88 (MyD88). This activates a signalling cascade which causes the translocation of the nuclear factor kappa beta (NF-κB) to the nucleus and the transcription of inflammatory cytokines (Brubaker et al., 2015; Kawai & Akira, 2011) (Fig. 1.2).

Table 1-1 Pathogenic components recognised by cell membrane bound TLRs. TLR-1, -2 and -6 can form dimers or trimers PAMPs. Table derived from (Brubaker et al., 2015).

Receptors	Pathogenic components
TLR2/TLR1	*E. coli*-based synthetic lipopeptides Pam3CSK4
TLR2/TLR6	*E. coli*-based synthetic lipopeptides Pam3CSK4
TLR2/TLR6	*Mycoplasma fermentas*-derived lipopeptide MALP-2
TLR2/TLR6	*Mycoplasma salivarium*-based lipopeptide FSL-1
TLR2/TLR1	Human and mouse cytomegolvirus
TLR2/TLR1	Herpes simplex virus
TLR2/TLR1/TLR6	Herpes simplex virus, endogenous ligands
TLR4	Lipopolysaccharide*
TLR5	Flagellum

*lipopolysaccharide is the main ligand for TLR4, however a several exogenous and endogenous ligands can activate members of the LPS-multireceptor complex (Brubaker et al., 2015).

TLR4 is one the most extensively studied receptors partially due to its importance during septic shock (Brubaker et al., 2015; Poltorak et al., 1998). Two main characteristics that make TLR4 signalling unique involve how PAMPs are recognised as well as the signalling pathway utilised upon activation. The detection of lipopolysaccharides (LPS) by TLR4 requires the LPS-binding protein (LBP) complex composed of CD14 and MD-2. CD14 exists in a soluble form

and its main role is to bind to LPS on the wall of bacteria and to transfer it to the TLR4-MD-2 complex (Kawasaki et al., 2000). This causes the dimerisation of TLR4 and the initiation of the signalling cascade previously described (Fig 1.2). An example of the LBP complex sensitivity is that a single bacterium can provide enough LPS monomers to activate 1000 macrophages via TLR4 signalling (Gioannini & Weiss, 2007).

The second characteristic of TLR4 signalling is that it can also get activated via the endosomal pathway. Specifically, some forms of CD14 are anchored in cholesterol-rich areas of the cellular membrane and upon binding to LPS cause the recruitment of TLR4 to these regions and form lipid rafts. This initiates endocytosis and signalling via the endosomal signalling pathways which requires the adaptor proteins TIR domain-containing adaptor IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). This results in the IRF3-mediated production of IFN-β (Brubaker et al., 2015; Kawai & Akira, 2011) (Fig 1.2).

1.2.2 Endosomal PRR signalling

There are three TLRs that are bound to the endosomal membrane and especially expressed by professional phagocytes (Brubaker et al., 2015). These include TLR-3, -7 and -9 which all detect different forms of nucleic acids. From an evolutionary perspective, the location of the endosomal TLRs adds additional regulatory steps to the PAMPs recognised since this protects from TLR activation of self-nucleic acids. TLR3 recognises dsRNA which initiates the TRIF-dependent signalling cascade (unlike TLR4, TLR3 does not require TRAM) (Brubaker et al., 2015). The other two endosomal TLRs include TLR-7/8 and -9 which recognise GU-rich single-stranded (ss) RNA and unmethylated CpG DNA respectively (Kawai & Akira, 2011). The activation through these receptors requires MyD88 and can lead to the induction of type I IFNs/chemokines or inflammatory mediators. The induction of type I IFNs is mediated via IKK-α and IRF7; whereas inflammatory mediators are induced by a signalling complex formed of an IL-1 receptor-associated kinases (IRAK) complex, IKK complex and TAK1. The endosomal TLRs are of special importance when it comes to antiviral immune responses (Fig 1.2) and furthermore, the production of type I IFNs is especially important in the antiviral responses (see section 1.3) (Kawai & Akira, 2010; 2011; Kerkmann et al., 2003).

Unlike the other endosomal TLRs, TLR3 activation does not occur through the adaptor protein MyD88 but instead occurs via TRIF. Specifically, TRIF signalling occurs through inhibitor of kappa-B (IκB) kinases (IKKs), tumour necrosis factor (TNF) receptor-associated factor (TRAF) family associated NF-κB activator (TANK)-binding kinase-1 (TBK1) and IKK-ε. This causes the activation of IRF3 which in turn induces the expression of IFN-β (Gürtler & Bowie, 2013). The activation of TLR3 can also induce the production of inflammatory mediators via TRIF activating a complex formed by TRAF-6, TNF receptor type I DEATH domain-associated protein (TRADD), Pellino-1 and the receptor-interacting kinase (RIP)-1. This causes the activation of NF-κB- and the mitogen-activated protein kinase (MAPK)-pathways which are mediated by the IKK complex and transforming growth factor (TGF) beta activated kinase (TAK)-1 (Fig 1.2) (Brubaker et al., 2015; Gürtler & Bowie, 2013; Kawai & Akira, 2011).

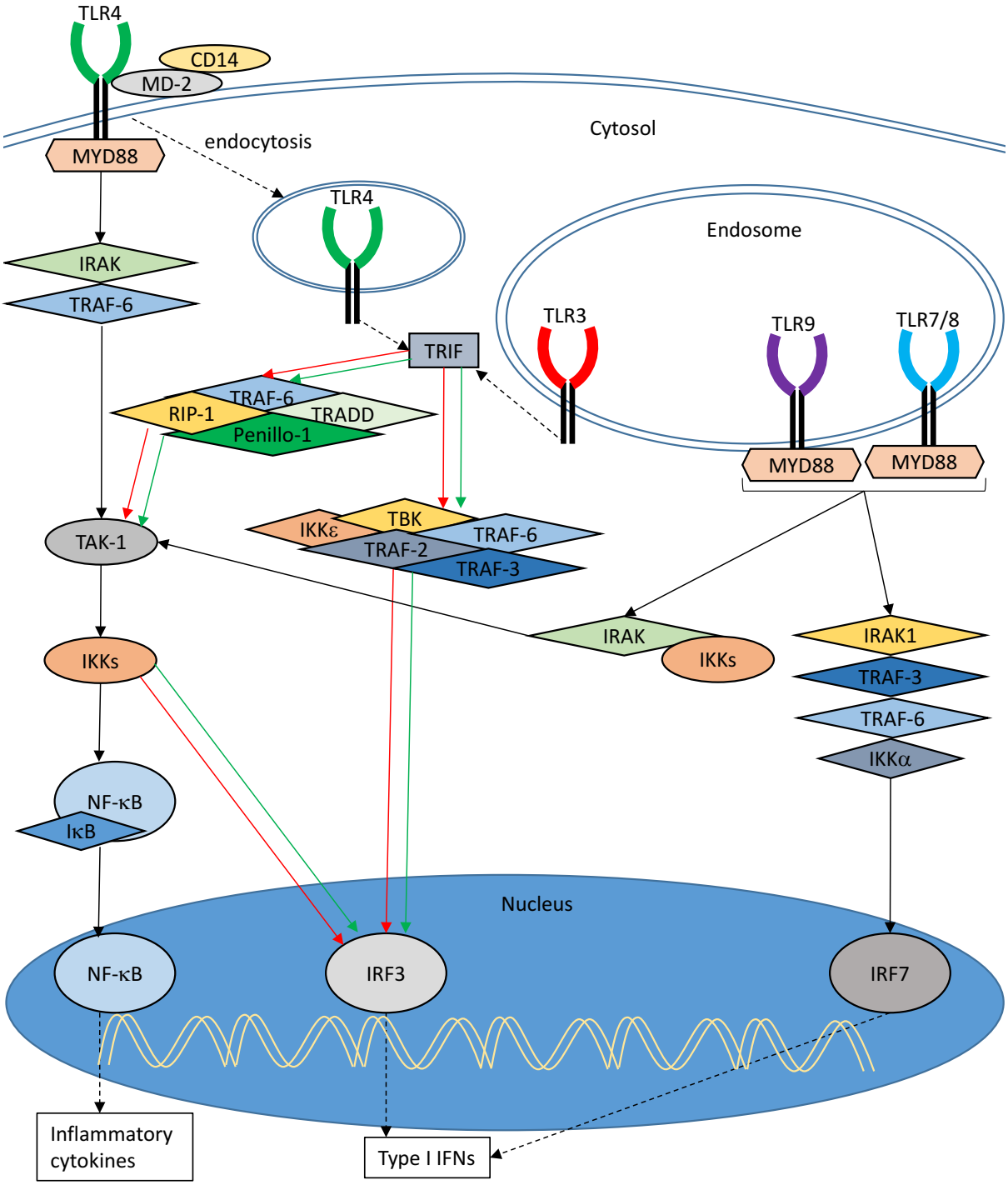


Figure 1.2 TLR signalling pathways involved in viral recognition. Surface bound TLR4 signals through the adaptor protein MyD88 to induce the activation of the IRAK/TRAF-6/TAK-1 complex. This causes the activation of the IKK complex (IKKs) which leads to the translocation of NF-κB into the nucleus and the secretion of inflammatory cytokines. TLR3 signals via the adaptor protein TRIF and activation of the TRAF-6/TRADD/RIP-1/Penillo-1/TBK complex which can either lead to the secretion of inflammatory cytokines directed by NF-κB or the production of type I IFNs directed by IRF3. TLR4 can also use this signalling pathway after

translocating to cholesterol-rich areas of the membrane that form lipid rafts. The activation of IRF3 and production of type I IFNs by TLR3 can also occur directly by activation initiated by TRIF and followed by the TBK/IKKε/TRAF-2/TRAF-3/TRAF-6 complex. TLR7 (also TLR8 in humans) and TLR9 use the adaptor protein MyD88. This leads to the activation of an IRAK/IKKs/TAK-1 complex which causes the NF-κB directed production of inflammatory cytokines. The MyD88-mediated signalling via TLR7 and TLR9 can also activate the IRAK1/TRAF-3/TRAF-6/IKKα complex which activates IRF7 and initiates the production of type I IFNs.

1.2.3 PAMP detection and signalling by cytosolic PRRs

In addition to membrane-bound TLRs, PAMPs are also detected by a series of cytosolic PRRs. RLRs are one of the most important set of receptors that detect viral RNA in the cytosol and initiate the antiviral response by inducing type I IFNs. There are three receptors in the RLRs family: RIG-I, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Loo & Gale, 2011). Structurally, RIG-I and MDA5 share many similarities as they are both composed of two N-terminal caspases activation and recruitment domains (CARDs). The CARDs are essential for the downstream signalling that initiates once they undergo conformational changes (Loo & Gale, 2011). Furthermore, the two receptors also share a C-terminal domain and a central DExD/H-box RNA helicase domain which can hydrolyse adenosine triphosphate (ATP) (Gürtler & Bowie, 2013). The LGP2 lacks the CARD domains but possesses an RNA binding domain. Although the LGP2 can bind to RNA its involvement in viral detection is yet to be proven. Two suggested roles for LGP2 are its involvement in the regulation of MDA5 signalling or that it is a negative regulator of RIG-I (Bruns & Horvath, 2012; 2015; Bruns et al., 2013) (Fig 1.3).

The cytosolic receptors RIG-I and MDA5 recognise dsDNA or ssRNA from many viruses. Due to their localisation within the cytoplasm, these receptors are constantly exposed to host nucleic acid and it is therefore important to distinguish host and pathogen nucleic acids for activation to occur. For this reason, the receptors can detect features that are common in many viral genomes and viral replication intermediates (Brubaker et al., 2015). Such features

include binding to small uncapped 5'-tri- or diphosphate RNA, long dsRNA and viral-genome-specific sequences such as poly-uridine regions (Goubau et al., 2013; Goubau et al., 2014; Hornung et al., 2006; Loo & Gale, 2011; Pichlmair et al., 2006). In addition, synthetic dsRNA poly(I:C) has proven that RIG-I can recognise smaller fragments; whereas MDA5 detects high-molecular-weight poly(I:C) fragments (Goubau et al., 2013; Kato et al., 2008). These observations were also confirmed using blunt-end dsRNA fragments that were as short as 24 base pairs and were detected by RIG-I (Marques et al., 2006).

Although RIG-I and MDA5 are specialised in detecting RNA viruses, they are also able to detect DNA viruses and bacterial pathogens after cytosolic DNA is converted to RNA by RNA Pol-III (a DNA cytosolic 'sensor') which can convert the B-form DNA to dsRNA which can then activate RIG-I (Broz & Monack, 2013; Y.-H. Chiu et al., 2009). The activation of RIG-I or MDA5 induces a conformational change from the resting to the active state. The binding of nucleic acids causes a filamentous oligomerisation which releases the CARD domains from autoregulation. This open confirmation leads to oligomerisation and CARD-mediated interaction with adaptor protein molecule mitochondrial signalling protein (MAVS) (I. Scott, 2010) (Fig 1.3). MAVS is bound to the mitochondria, mitochondria-associated membranes and peroxisome membranes (Brubaker et al., 2015; Seth et al., 2005). It consists of an N-terminal CARD which is homologous to the CARDs of RIG-I and MDA5 (Loo & Gale, 2011). Upon activation, the RLR activate MAVS where signalling molecules accumulate to form a signalosome which leads to the production of type I IFNs, inflammatory cytokines and chemokines (Dixit et al., 2010; Öhman et al., 2009). The key factors involved in the signalosome include IRF3, IRF7 and NF-κB (Kawai & Akira, 2009; Loo & Gale, 2011). The IRF3 and IRF7 transcription factors require phosphorylation IKKε or TBK1 in order to drive gene transcription; whereas NF-κB requires the IKK complex-mediated phosphorylation (Fig 1.3) (Loo & Gale, 2011). However, RLR signalling can also bypass MAVS and initiate an inflammatory response by secretion of IL-1β and IL-18 by associating with apoptosis-associated speck-like protein containing a CARD (ASC) in order to activate the inflammasome in a caspase-1-dependent response (Poeck et al., 2010).

However, there are several receptors that can detect DNA in the cytoplasm and lead to the induction of type I IFNs. The mechanism behind how some of these receptors produce IFNs remains elusive. Two of the first DNA-binding receptors identified include the DNA-dependent activator of IRFs (DAI) and AIM2. Despite initially being shown to drive type I IFN production *ex vivo*, further characterisation of DAI indicated that it is important for the detection of murine cytomegalovirus and driving necrosis rather than inducing type I IFNs (Upton et al., 2012). AIM2 has been shown to be involved in the assembly of inflammasomes however it does not have a role in the production of type I IFNs (Broz & Monack, 2013). This gap in the identification of the DNA sensing receptor that induces type I IFN production by cytosolic PRRs has led to the identification of other receptors and signalling pathways. The stimulator of IFN genes (STING) protein in the cytoplasm activates IRF3 and drives the production of type I IFNs. Overall studies have shown that STING, which is bound to the endoplasmic reticulum (ER), is important as an adaptor protein (Burdette et al., 2011) and binds the secondary messenger cyclic GMP-AMP (cGAMP) to induce type I IFNs (Brubaker et al., 2015; Burdette et al., 2011). Cytosolic DNA is recognised by cGAS which catalyses the synthesis of cGAMP and induces the activation of IRF3 and the production of type I IFNs in a STING dependent manner (Sun et al., 2013; J. Wu et al., 2013). Other proteins that may be involved in DNA recognition include DExD/H-box helicase (DDX41), PYHIN family proteins (including IFN-γ inducible protein 16 (IFI16)) (Broz & Monack, 2013; Paludan & Bowie, 2013). However, the precise role of these proteins in viral DNA recognition is not yet fully elucidated.

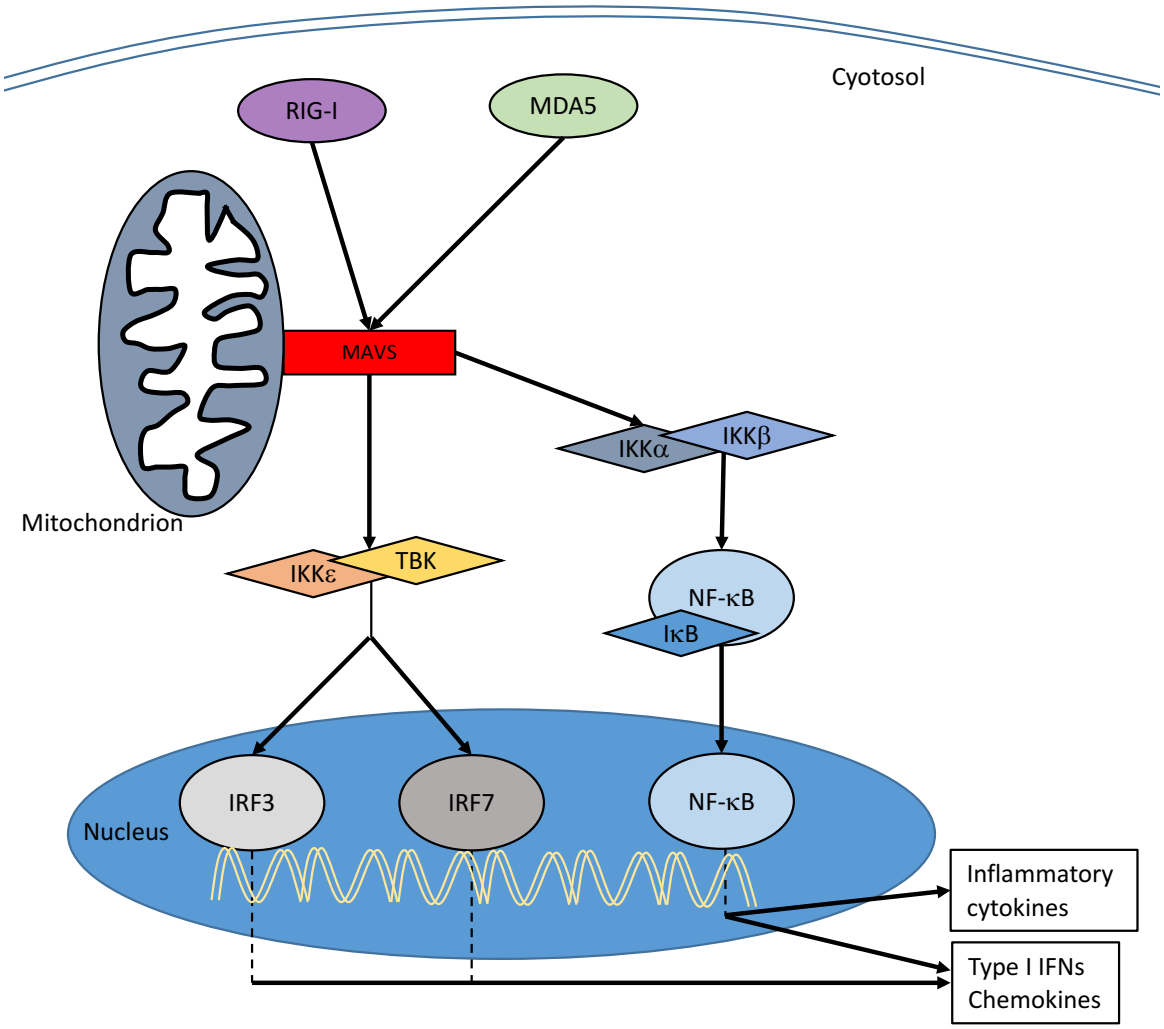


Figure 1.3 Cytosolic pattern recognition receptor signalling. Upon binding to their respective ligands, RIG-I and MDA5 relocate to the MAVS adaptor protein. This causes the phosphorylation of IKKε/TBK to activate IRF3 and IRF7 which initiates the secretion of type I IFNs and chemokines. MAVS can also cause the phosphorylation of IKKα/IKKβ complex and the translocation of NF-κB to the nucleus which initiates the secretion of inflammatory cytokines.

1.2.4 Recognition of DAMPs during inflammation

The recognition of PAMPs induces the production of many inflammatory cytokines. Extensive inflammatory responses however also require a second type of signal that is acquired from damage-associated molecular patterns (DAMPs). These types of molecules are often endogenous molecules that are produced by damaged cells (Escamilla-Tilch et al., 2013). DAMPs are recognised by approximately 100 receptors including PRRs (Escamilla-Tilch et al., 2013; Zelenay & Reis e Sousa, 2013). It is believed that PAMPs and DAMPs act in synergy to drive the course of inflammation and that this synergy is important especially, for the production and secretion of members of the IL-1 family of cytokines (Carta et al., 2013).

Cytokines such as IL-1β require two signals for their secretion (Carta et al, 2013). The first signal via TLRs and trigger the expression and synthesis of pro-IL-1β whereas the second signal is provided by endogenous molecules such as ATP which is an extracellular DAMP. The presence of ATP is especially important for the secretion of IL-1β as it drives inflammasome activation (Piccini et al., 2008). The inflammasome activation results in caspase activation, which cleaves the pro-IL-1β into its mature form (Guo et al., 2015, Howard et al., 1991). The requirement of two such signals for the maturation and secretion of IL-1β varies between different types of cells, however, it is strictly required in murine macrophages (Carta et al., 2013; Piccini et al., 2008). The secretion of IL-1β can occur via vesicular routes driven by the inflammasome or via non-vesicular routes (Carta et al., 2013). The non-vesicular routes of secretion can occur in macrophages where a caspase-1-mediated pyroptotic death is driven by the activation of the NLRP3 or NLRP4 inflammasome cascade (Jacobs & Damania, 2012, Guo et al., 2015). Infection with influenza, RSV or measles can cause cell death which provides the activation signal for the NLRP3 inflammasome and leads to the production of IL-1β during infection (Jacobs & Damania, 2012).

Mediators such as IL-1α and IL-33 are classified as both cytokines and DAMPs (Carta et al., 2013). These two mediators accumulate in epithelial and endothelial cells and are secreted during stress conditions. Specifically, IL-1α release is mediated by S100A13 in both human U937 and NIH 3T3 cells, whereas IL-33 release from epithelial cells is triggered by ATP, which acts as a sensor for allergens (Kouzaki et al., 2011, Mandinova et al., 2003). As these two

mediators are constitutively produced they are not always classified as DAMPs as they are not only produced by dying cells (Carta et al., 2013). However, other DAMPs such as high mobility group nucleosome binding domain 1 (HMGB1) can also undergo regulated release by cells dependent on the caspase-1 and inflammasome (Keller et al., 2008). The importance of synergy between PAMPs and DAMPs is illustrated by the dependence of LPS on HMGB1 to act as an adjuvant (Zelenay & Reis e Sousa, 2013, Yang et al., 2012). RSV infection has also been shown to trigger release of HMGB1 by epithelial cells (Hosakote et al., 2016).

The induction of inflammation by DAMPs is an evolutionary conserved mechanism (Zelenay & Reis e Sousa, 2013). The interplay between PAMPs and DAMPs is especially important for driving the innate immune responses to cytopathic pathogens and can shape the type of adaptive immune response that is elicited (Zelenay & Reis e Sousa, 2013). Further to the interplay between these two pathways, individual DAMPs can induce different types of cellular responses that are important for the clearance of infection, maintaining homeostasis or for restructuring/remodelling of tissue (Zelenay & Reis e Sousa, 2013). The knowledge of how DAMPs drive immune responses is currently expanding and it is interesting to consider how various DAMPs affect different cells to shape these responses.

1.3 The viral interference phenomenon

The viral interference phenomenon was first described in plant viruses in the 1930's (González-Navajas et al., 2012). A study using heat-inactivated influenza virus in 1957 identified IFNs as the proteins responsible for viral interference (Isaacs & Lindenmann, 1987). There are three families of IFNs that are classified as type I, type II or type III IFNs. There are 16 members of type I IFNs, 13 of which are IFN-α subtypes in humans (14 in mice), IFN-β, IFN-ε, IFN-κ and IFN-ω (de Weerd & Nguyen, 2012; van Pesch & Renauld, 2004). The second family of IFNs are type II IFNs which consists of IFN-γ. Type III IFNs consist of IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). All three families of IFNs can induce antiviral responses and activation of IFN-stimulated genes (ISGs), however the morphology of type III IFNs is homologous to IL-22, member of the IL-10 family (Gad et al., 2009; González-Navajas et al., 2012). The expression of the IFN-λ receptor (IFNλR1 is restricted (Gad et al., 2009)). Studies of influenza virus infection suggested that epithelial cells carry functional IFNλR1 (Mordstein et al., 2008). Most cell types including fibroblasts, epithelial cells and leukocytes can produce type I IFNs (González-Navajas et al., 2012). The recognition of PAMPs by various PRRs in these cells (see section 1.2) initiates signalling cascades that activate transcription factors IRF3, IRF7 and NF-κB which translocate to the nucleus and initiate the secretion of IFN-α and IFN-β. This causes the production and secretion of more type I IFNs and other cytokines and the initiation of an antiviral state in the cell. Since all nucleated cells express the type I IFN receptor (IFNAR) this means that secreted type I IFNs can act in an autocrine or paracrine manner to induce an antiviral state in both the activated and the neighbouring cells (Pestka et al., 2004; Sadler & Williams, 2008). Furthermore, it also leads to the activation and secretion of inflammatory mediators by neighbouring cells such as macrophages, DCs or pDCs (González-Navajas et al., 2012). The type I IFNs may also shape the type of adaptive T cell responses that develop after infection (Durbin et al., 2000).

1.3.1 Type I IFN signalling

The IFNAR is a heterodimeric receptor made up the subunits IFNAR1 and IFNAR2 (Honda & Taniguchi, 2006). The c-termini of IFNAR1 and IFNAR2 are associated with the tyrosine kinase

2 (TYK2) and Janus kinase 1 (JAK1) respectively. The activation of the receptor transduces the phosphorylation of JAK1 and TYK2 by tyrosine phosphorylation ((Platanias, 2005) Fig 1.4). This initiates a signalling cascade composed of proteins of the signal transducer and activator of transcription (STAT) family. The STAT1 and STAT2 proteins are activated upon JAK1 phosphorylation, dimerise and, together with IRF9, form the ISG factor 3 (ISGF3) complex (Darnell et al., 1994; W. M. Schneider et al., 2014b). This complex translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) in ISG promoters and initiate gene transcription (Fig 1.4) (Aaronson & Horvath, 2002; Schindler et al., 1992; Stark & Darnell, 2012).

Signalling through IFNAR can also occur independently of IRF9 recruitment through STAT1 homodimers that can bind to IFN-γ-activated site (GAS) and then to ISG promoters (Platanias, 2005). Both pathways initiate transcription which promotes the induction of a range of pro-inflammatory mediators and enhance the antiviral state (Schoggins et al., 2011). The JAK-TYK signalling pathway can also promote signalling pathways independently of STAT signalling. One such pathway includes MAPKs, which are important for signals regulating important cellular functions such as gene transcription, post-transcription, apoptosis and cell-cycle progression (Platanias, 2005). Specifically, the p38 signalling cascade after IFN-stimulation drives transcription of genes that are important for inducing the antiviral effects of type I IFNs and are regulated by ISREs and GAS (G. Li et al., 2004; Platanias, 2005; Uddin et al., 2000). Further to MAPK, the type I IFN signalling can also activate the phosphoinositide 3-kinase (PI3K) signalling pathway which mediates gene transcription of ISGs (Kaur et al., 2008; Platanias, 2005) (Fig 1.4).

The activation of PI3K occurs in a type I-IFN-dependent manner downstream of JAKs. Type I IFNs cause the phosphorylation of insulin-receptor substrate (IRS) protein IRS1, not STATs, and activation of PI3K signalling (Platanias, 2005). The phosphorylation of PI3K causes the activation of the RAC-α serine/threonine-protein kinase (AKT1)/cAMP responsive-element-binding protein (CREB) (Platanias, 2005). This signalling pathway can bind to smad binding elements (SBE) and is believed to be important for transcription of genes controlling cellular survival and secretion of inflammatory genes. PI3K can have a dual role in regulating apoptosis. The activation of AKT1 downstream of PI3K can have anti- or pro-apoptotic signals

in cell specific context (Platanias, 2005; Ruuth et al., 2001; Vanhaesebroeck et al., 2012; K. Wang et al., 2003). The activation of PI3K by IFN-α was shown to induce apoptosis in tumour cells in a signalling pathway dependent on phosphorylation of mTOR (Thyrell et al., 2004). Activation of the PI3K-AKT1 signalling by type I IFNs can induce cell survival by inducing NF-κB activation (K. Wang et al., 2003; C. H. Yang et al., 2001). In a PI3K-dependent manner IFN-β can inhibit early neutrophil apoptosis (K. Wang et al., 2003); whereas IFN-α was shown to inhibit spontaneous apoptosis of primary B-lymphocytes (Ruuth et al., 2001). Stimulation of human pDCs with endosomal TLR ligands showed that PI3K is critical for the translocation of IRF-7 to the nucleus and production of type I IFNs (Guiducci et al., 2008).

Type I IFNs cause the upregulation of ISGs which have a multitude of roles including enhancing the production of inflammatory mediators, enhancing cell communication, interference with the pathogen life cycle and induction of apoptosis in infected cells (González-Navajas et al., 2012; Platanias, 2005; Sadler & Williams, 2008; W. M. Schneider et al., 2014b). Equally as important are the negative regulators which are required to restore cellular homeostasis. The type I IFN response is tightly controlled by a series of mechanisms that are dependent on cell-intrinsic factors, ISG-mediated proteins and miRNA. The activity of STAT proteins can be regulated by protein inhibitors of activated STAT (PIAS) (Horvath & Matunis, 2003). The mechanism of action of PIAS is not fully clarified, however it can act on STATs independently or can enhance the attachment of small ubiquitin-like modifier (SUMO) to STAT1 and thus affect the induction of ISGs (W. M. Schneider et al., 2014b). The suppressor of cytokine proteins (SOCS) are important for the regulation of IFN-mediated signalling early in the IFN response (Kubo et al., 2003). SOCS proteins (SOCS1, SOCS3) bind directly to phosphorylated residues that are present on IFNAR or JAK proteins directly inhibiting the STAT signalling pathway (Yoshimura, 2006). Furthermore, SOCS proteins contain a SOCS box domain on their C-terminus initiating protein recruitment and causing ubiquitination of the receptor or proteasome degradation (Fenner et al., 2006; Kamura et al., 2004). A very important ISG for the regulation of type I IFN signalling is the ubiquitin carboxyl-terminal hydrolase protein, USP18, which displaces JAK1 from IFNAR2 (Ivashkiv & Donlin, 2014; Sarasin-Filipowicz et al., 2008). Thus, signalling via the type I IFN receptor activates a positive feedback loop which induces a series of ISGs and inflammatory factors which drive the immune response. In

parallel, this signalling is tightly controlled with cell intrinsic properties and ISGs whose role is to supress type I IFN signalling.

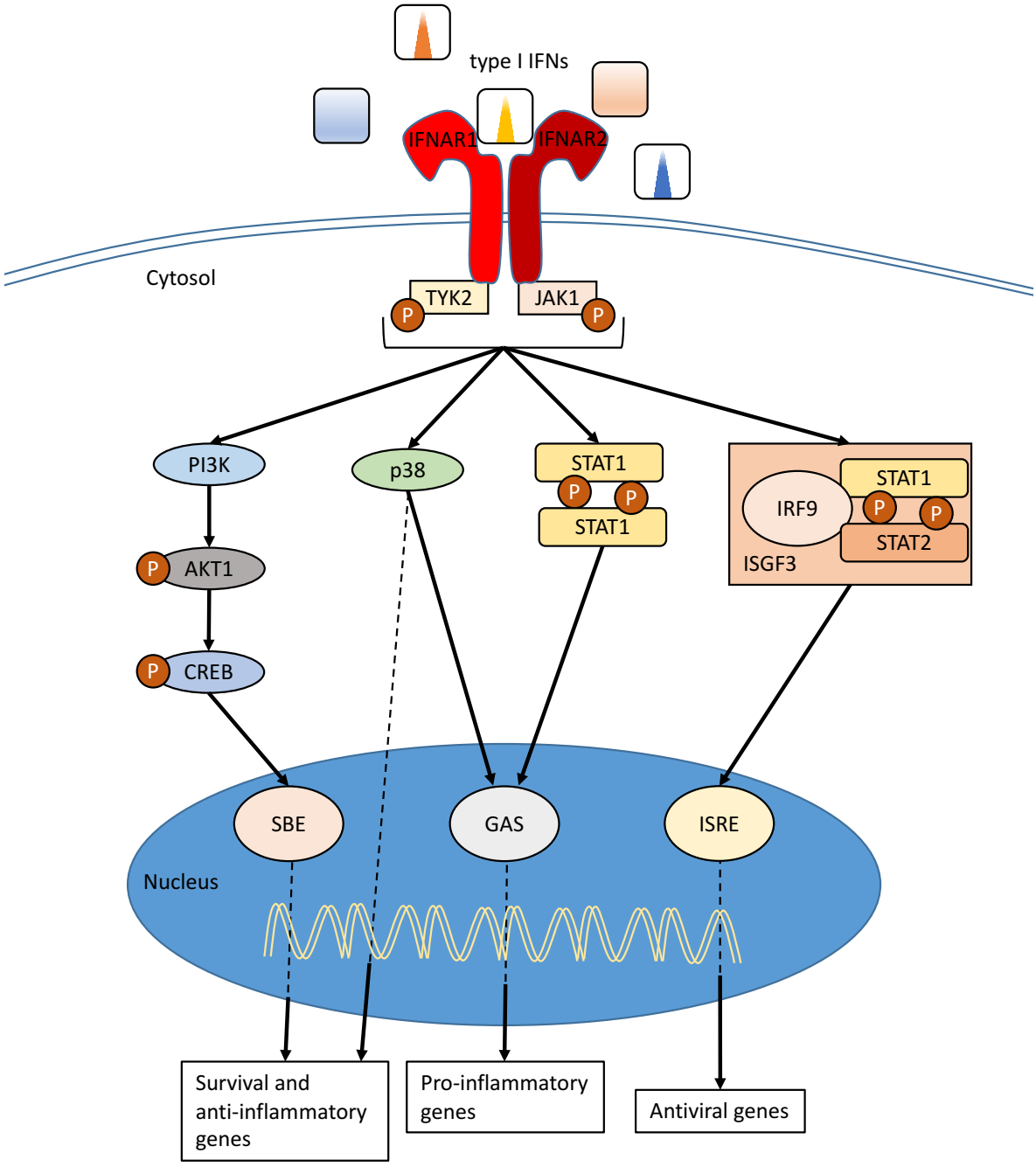


Figure 1.4 Type I IFN signalling. Type I IFNs signal via IFNAR in an autocrine or paracrine manner. IFNAR signalling induces antiviral, pro-inflammatory, survival and anti-inflammatory genes. Activation of ISGF3 induces the expression of ISRE and the antiviral genes; whereas signalling via the STAT1 homodimer activates GAS which induces the expression of pro-inflammatory genes. IFNAR can also induce survival and anti-inflammatory genes

independently of STAT signalling through the p38 and PI3K led signalling pathways. The phosphorylation of PI3K activates the RAC-α serine/threonine-protein kinase (AKT1)/cAMP responsive-element-binding protein (CREB) that can bind to smad binding elements (SBE) and control cell survival and expression of inflammatory genes.

1.3.2 Type I IFNs and viral infections

IFNs interfere with the viral life cycle directly or indirectly. The upregulation of ISGs is one of the most important characteristics of type I IFN signalling. Other than enhancing or regulating the type I IFN-mediated response, the ISGs can directly target the viral life cycle. A successful viral cycle is when a virion attaches, gains entry into the cell, replicates and a new virion is assembled and exits (W. M. Schneider et al., 2014b). ISGs have evolved to target each of the key steps in the viral life cycle. These include myxovirus resistance 1 (Mx1), which inhibits viral entry. The Mx1 oligomerises and forms ring structures which are important during infection by influenza A viruses (S. Gao et al., 2010). The viral entry is also controlled by IFN-inducible transmembrane (IFITM) proteins and their importance has been shown in multitude of viral infections (I.-C. Huang et al., 2011). Finally, the tripartite motif (TRIM) proteins can inhibit viral entry by binding to the viral nuceolcapsid (Ozato et al., 2008). Viral replication is also targeted by a series of ISGs including IFIT, protein kinase R (PKR) and OAS/RNaseL (A. Chakrabarti et al., 2011; Diamond & Farzan, 2013; Ivashkiv & Donlin, 2014; Sadler & Williams, 2008). PKR also has a role in inducing apoptosis of infected cells (Thapa et al., 2013). The post translational modifications of viruses are targeted by ISG15, which is one of the most upregulated ISGs (C. Zhao et al., 2013a). Finally, the viral release can be targeted by ISGs such as viperin (induced by JAK-STAT or IRF1/3 signalling) and tetherin. Specifically, viperin interferes with the lipid raft formation during viral replication whereas tetherin inhibits viral budding (Perez-Caballero et al., 2009; X. Wang et al., 2007).

The effect of type I IFNs can either be inhibitory or stimulatory (Urban, 2011).Type I IFN signalling can also drive the immune response to a number of pathogens and affect the state of immune cells. The role of IFN signalling can also drive the immune response to several pathogens and affect the state of immune cells. Type I IFNs are crucial for all steps of the

immune responses directed against viral infections as the production of type I IFNs early after infection occurs in order to limit the viral replication and to stimulate an effective humoral or cellular adaptive immune response (Trinchieri, 2010). Furthermore, type I IFNs have been shown to drive a multitude of events in DCs including the differentiation of precursors and their activation (McNab et al., 2015). Upon activation, DCs present and cross-present antigens, express co-stimulatory molecules and promote chemokine expression that allows them to migrate to the lymph nodes where T and B cell activation occurs (Tomoki Ito et al., 2001; Le Bon et al., 2003; Rouzaut et al., 2010; Spadaro et al., 2012). $CD4^+$ and $CD8^+$ T cells may also be affected by type I IFNs directly during various infections. The effect of type I IFNs on $CD4^+$ T cells include: increased cellular survival and mediation of B cell clonal expansion after lymphocytic choriomeningitis virus (LCMV) (Le Bon et al., 2006; McNab et al., 2015). After infection with influenza virus, type I IFNs can promote the activation and antibody responses directly on B cells localised in the respiratory tract (Coro et al., 2006). In LCMV and vesicular stomatitis virus (VSV), infections drive the survival and clonal expansion of the $CD8^+$ T cell pool in a type I IFN-dependent manner (Kolumam et al., 2005; Marrack et al., 1999). Furthermore, the memory $CD8^+$ T cell pool is also affected by type I IFNs (Kolumam et al., 2005; Thompson et al., 2006).

In many diseases, the role of type I IFNs has been debated as to whether it is a friend or a foe (Trinchieri, 2010). Like all immune responses, a balance is required and launching a response is as important as the ability to dampen it. An imbalance of the type I IFN response can influence the cellular response during infection. This can be either a chronic immunosuppression or an acute immunopathology that is driven by type I IFNs. After influenza virus or LCMV infection type I IFNs can induce the secretion IL-10 and the expression of program cell death 1 ligand 1 (PDL1) (Willette & Flaño, 2013; E. B. Wilson et al., 2013). Together, this can lead to suppression of the ability of T cells to initiate an antiviral response and thus they fail to clear the infection (Brincks et al., 2011). In severe influenza virus infections, epithelial damage occurs by type I IFN-driven upregulation of TNF-related apoptosis-inducing ligand (TRAIL) expression on pDCs and inflammatory monocytes (Chaperot et al., 2006; Fujikura et al., 2013; Högner et al., 2013; McNab et al., 2015). This ‘death’ ligand binds to the death receptor 5 (DR5) on epithelial cells, the expression of which is also upregulated by type I IFNs. Thus, the TRAIL-expressing inflammatory monocytes and

macrophages cause the death of the epithelial cells which in turn increases morbidity or mortality (Davidson et al., 2014; Herold et al., 2008; McNab et al., 2015) .

1.3.3 Type I IFNs during bacterial and fungal infections

1.3.3.1 Bacterial lung infections

The type I IFNs have long been established for their antiviral effects however, increasing studies suggest an important role during bacterial and fungal infections (Honda & Taniguchi, 2006; Müller et al., 1994; Trinchieri, 2010). Studies of patients infected with *Mycobacterium tuberculosis* (Mtb) had an upregulation in the expression of type I IFN genes (Berry et al., 2010; Ottenhoff et al., 2012). The role of type I IFNs during respiratory bacterial infections has been extensively studied in *Ifnar1*$^{-/-}$ mice. Type I IFNs do not appear to have a direct role in controlling bacterial and fungal infections, but rather a role in regulating and modulating the overall immune responses. During *Staphylococcus aureus* infection the lack of TLR9 and IFNAR signalling in mice showed enhanced clearance (Parker & Prince, 2012). This study showed that *S. aureus* can exploit TLR9 mediated type I IFN signalling in DCs as a mechanism for enhanced infection (Parker & Prince, 2012). A *Chlamydia muridarum* study in IFNAR-deficient mice suggested that type I IFNs promote apoptosis of macrophages which increases bacterial burden, cytopathology and susceptibility (Qiu et al., 2008). Type I IFNs can also modulate the phagocytosis functions of cells such as neutrophils and increased immunopathology in mice exposed to Mtb. Finally, type I IFNs were important for restricting spread of *Streptococcus pneumoniae* from the lung (LeMessurier et al., 2013). Specifically, mice lacking IFNAR signalling had an increased bacteraemia and bacterial presence in the lung that was rescued by administration of IFN-β (LeMessurier et al., 2013). Overall, type I IFNs exert an increased inflammatory response which may vary depending on the type of bacterial infection. The data would suggest that, during lung bacterial infections, type I IFNs are part of the inflammatory response and might initiate immune responses.

1.3.3.2 Fungal lung infections

The usage of IFNAR-deficient mice characterised the role of type I IFNs during fungal infections. Specifically during infection with *Pneumocystis murina,* the lack of IFNAR signalling led to an influx of eosinophils in the lung and increased changes to the bronchial epithelium (Meissner et al., 2005). Furthermore, there was a delay in the clearance of *P. murina* in these mice which led to fibrosis and increased IgE responses which indicated allergic sensitisation (Meissner et al., 2005). The IFNAR-deficient mice have also been used to study the role of type I IFNs during *Cryptococcus neoformans* infection. Specifically, type I IFNs can regulate the early host defence as lack of IFNAR1 led to improved clearance and higher levels of T cell activity and mucus secretion (Sato et al., 2015). Furthermore it was suggested that type I IFNs are important for polarising the immune response during *C. neoformans* (Biondo et al., 2008). Overall, during *P. murina* and *C. neoformans* infections, type I IFNs appeared to have an important role in modulating the phenotype of the immune response which could be especially important for driving asthma. In comparison to viral and bacterial infections, evidence of the role of type I IFNs during fungal respiratory infections indicate its importance in regulating the immune responses.

1.4 Respiratory syncytial virus (RSV)

RSV is the leading cause of lower respiratory tract infections in infants and immunocompromised adults (Falsey et al., 2005; Hall et al., 2001; Nair et al., 2010). Children under the age of 5 are especially prone to acute lower respiratory infection (ALRI) due to RSV infection. Worldwide estimates in 2005 stated that there were 33.8 million new episodes of RSV in children under 5-years of age and primarily in developing countries. Of these, 3.4 million required hospitalisation and 66,000-199,000 children died from RSV-related ALRI, with 99% of these deaths occurring in developing countries (Nair et al., 2010).

The virus was first identified in chimpanzees in 1956 (J. A. Morris et al., 2016) however, there are currently no vaccines available or effective treatments. Many attempts to develop a vaccine have been shadowed by the trial of the formalin-inactivated (FI) RSV vaccine, which was administered to children in the 1960's. The results from this trial were disastrous, as upon natural infection the vaccinated children developed severe morbidity which resulted in two deaths (Kapikian et al., 1969; Y. J. Kim et al., 2014). Only prophylactic treatment is available for high-risk groups such as early-born infants, in the form of the monoclonal antibody Palivizumab. Although effective to some extent, the price and timing of administering this drug remains problematic (D. Wang et al., 2011).

1.4.1 RSV structure, replication and genome

RSV is a non-segmented, negative-sense ssRNA virus belonging to the Pneumoviridae family, Pneumonovirus genus and pneumonovirinae subfamily (Afonso et al., 2016; P. L. Collins & Karron, 2013). Two subtypes of RSV have been identified- subgroup A and subgroup B, whose main differences are located on the surface glycoprotein (G protein), fusion glycoprotein (F), nucleoprotein (NP) and phosphoprotein (P) (L. J. Anderson et al., 1985; Sullender, 2000). Although simultaneous circulation of both subgroups of RSV occurs, only one subgroup predominates during outbreaks (Mufson et al., 1987). The RSV genome is composed of 10 genes that encode for 11 proteins (Fig 1.5). These proteins can be divided into two main groups: the envelope proteins and the nucleocapsid proteins.

There are three envelope proteins that are located on the surface of the virus and, together, make the glycoprotein spikes. The G protein is an attachment protein that can either be bound to the virion surface or soluble (P. L. Collins & Karron, 2013). The F protein has multiple roles during the RSV replication cycle. It is an attachment protein and can also direct the viral penetration by fusion of the virion envelope and the host cell plasma membrane/or pinosomal membranes (Hallak et al., 2000; Krzyzaniak et al., 2013; McLellan et al., 2011; Tayyari et al., 2011). The RSV F protein also has an important role during the final stages of the viral cycle as it drives filament formation, budding of new viral particles and syncytia formation. The absence of the F protein in virus like particle (VLP) models causes abortive viral replication (Shaikh et al., 2012; Teng & Collins, 1998). The small hydrophobic (SH) protein is the third member of the glycoprotein spikes. The SH protein is a transmembrane protein with multiple forms, the role of which have not been fully elucidated. Studies in bacteria and artificial membranes show that SH may have a role in modifying membrane permeability via viroporins as well as an importance during virion budding and cellular apoptosis (Carter et al., 2010; P. L. Collins & Karron, 2013). The matrix (M) protein is located in the inner section of the glycoprotein spikes. The structure of the M protein can provide stability to the virion by linking the membrane with the nucleocapsid (Ghildyal et al., 2006; Ghildyal et al., 2002; Money et al., 2009). The two main roles of the M protein are to organise the virion in order for budding to occur and to silence viral RNA synthesis during the assembly/packaging of new virus particles (Ghildyal et al., 2003).

The nucleocapsid of RSV is composed of five proteins. The nucleoprotein (N) is bound along the entire RNA genome providing it with stability and making it RNase-resistant; thus, protecting it from cellular intrinsic factors (Ruigrok et al., 2011). During viral replication, inclusion bodies (IBs) are formed and are present in the cytosol when viral assembly initiates. In a permissive cell, where all viral proteins and mechanisms are intact, IBs are present only at early stages of the viral cycle (Lindquist, Utley, & Crowe, 2010). The role of IBs is important for the packaging of new virus particles as they translocate to the host cell membrane on filaments whose formation is dependent on the F protein (Baviskar et al., 2013; Mehedi et al., 2016). Furthermore, IBs have also been suggested to antagonise the innate immune response mediated by MDA5 and MAVS (Lifland et al., 2012). The ability to detect distinct IBs in the cell cytosol and the lack of filaments is an indication that viral replication is abortive (Baviskar et

al., 2013). The most abundant protein in the IBs is the RSV N protein, as it binds to the viral RNA (Mitra et al., 2012). The RSV P protein is a multifunctional adapter that is important in viral replication as it mediates interactions between the nucleocapsid-polymerase complex. Furthermore, it is important in dissociating the M protein from the nucleocapsid during the early stages of infection (Asenjo et al., 2008). The large polymerase (L) protein provides the polymerase catalytic domains which are vital for viral replication to occur (P. L. Collins & Karron, 2013).

The N, P and L proteins together with the matrix M2-1/M2-2 proteins are essential for successful viral replication to occur. The M2-1/M2-2 proteins have a role during the transcription of the viral genome and during viral assembly/packaging (P. L. Collins, Hill, & Cristina, 1996). The M2 gene is composed of two overlapping open reading frames that make up the M2-1 and M2-2 proteins. The synthesis of viral mRNA is significantly high at early stages after viral entry as this increases the number of proteins that are translated and thus are more likely to evade intrinsic immune recognition (Kuo et al., 1996). One of the most important events for successful viral replication is immune evasion. The two non-structural (NS) proteins, NS1 and NS2 are important in interfering with the induction and signalling of type I and type III IFNs (Borchers et al., 2013; Lo et al., 2005; Spann et al., 2004). The NS proteins may also activate pro-survival signalling pathways of infected cells (Bitko et al., 2007). Furthermore, there is evidence that the NS2 protein promotes shedding of epithelial cells and distal airway obstruction in a hamster model of infection (Liesman et al., 2014). Taken together, the effects of NS proteins are to increase the survival of the host cell whilst decreasing the type I IFN responses, allowing the viral cycle to be completed and maximised.

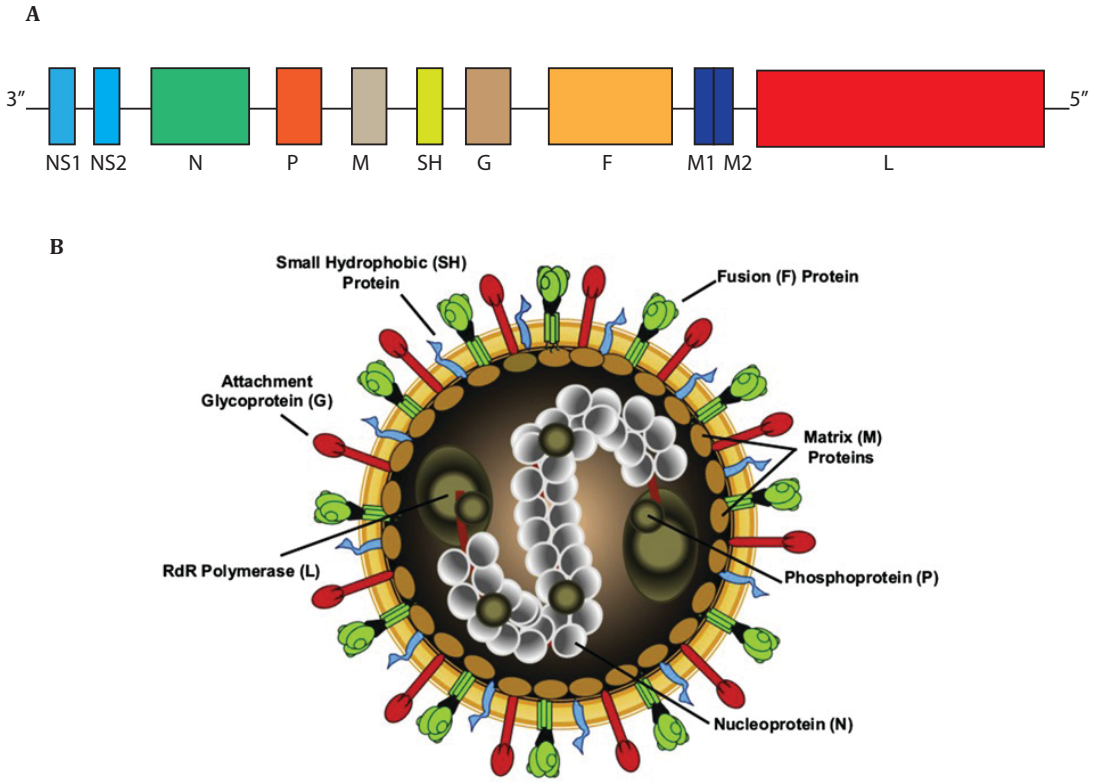


Figure 1.5 RSV genome and schematic of RSV structure. (A) The RSV genome in a 3”-5” direction and (B) the RSV structure: non-structural proteins (NS-1 and NS-2) (are not in the virion but expressed in the host cell), nucleoprotein (N), Phosphoprotein (P), Matrix (M) protein, small hydrophobic (SH) protein, glycoprotein (G), Fusion (F) protein, M1/M2 and Polymerase (L). RSV structure adapted from (Lay et al., 2013).

1.4.2 Pathogenesis, clinical manifestation and treatment after RSV infection

Infection by RSV can occur through inoculation of the nose or eyes by direct contact or aerosols (P. L. Collins & Graham, 2008). The virus has an incubation period of 4-5 days in the nasopharynx before symptoms initiate. In individuals prone to RSV infection there is fever correlating with the peak in viral shedding especially between 3-7 days post infection (Openshaw & Tregoning, 2005). The peak of viral shedding, which is approximately when the adaptive immune response is activated, is associated with otitis media, rhinorrhoea, lower respiratory tract infections (LRTI) and coughing (Karron et al., 1997; Openshaw & Tregoning, 2005).

Healthy adults infected by RSV experience a self-limited, upper respiratory tract infection with symptoms including a sore throat and rhinorrhoea. The most threatening form of RSV infection is in the lower respiratory tract (LRT). The LRT is involved in 15-50% of infants and young children with primary RSV infections and of these, 1-3% require hospitalisation. Although pneumonia has also been associated with LRTI, the most common form is bronchiolitis especially in 2-6-month-old infants (Borchers et al., 2013). LRTI in infants is associated with wheeze and asthma-like symptoms during adolescence (Blanken et al., 2013; Rossi & Colin, 2015). All children are infected by RSV at least once and 30-75% are reinfected by the age of two (Borchers et al., 2013). The subsequent exposures to RSV result in milder symptoms. Although symptoms in the adult population range from mild to asymptomatic, 25% may experience tracheobronchitis, bronchitis and wheezing (Hall et al., 2001).

The risk factors that are associated with severe RSV infection and hospitalisation requirement can be either due to host factors or viral factors. The most common host factors associated with hospitalisations are preterm births, chronic lung disease of prematurity and congenital heart disease (Borchers et al., 2013). Preterm birth is associated with the highest mortality and morbidity due to RSV. This can be further subcategorised in preterm infants with a very low weight or immunodeficiencies such as Down syndrome, interstitial lung disease, liver disease or inborn errors in metabolism (Hjuler et al., 2012; Kristensen et al., 2009). However, more than 50% of the infants hospitalised due to RSV are previously healthy. The most common risk factors for these children include young age, siblings, attending day-care (infant or siblings), exposure to tobacco smoke in the environment or through maternal smoking during pregnancy and lactation (Borchers et al., 2013; P. L. Collins & Graham, 2008; R. C. Welliver, 2003).

The disease caused by RSV infection is not only due to the virus but also due to immune responses directed towards it. Viral infection causes airway inflammation and the secretion of pro-inflammatory cytokines and chemokines which leads to infiltration of cells, necrosis and sloughing of the respiratory epithelium which can lead to bronchiolitis (Everard et al., 1994; J. E. Johnson et al., 2006). An airway hyperreactivity can cause increased mucus production by goblet cells; whereas an aberrant immune response can induce increased

cellular infiltration and damage to the epithelial barrier (Everard et al., 1994; Habibi & Openshaw, 2012; J. E. Johnson et al., 2006). Taken together, these events cause narrowing of the airway lumen and reduced gas exchange. Clinically this leads to atelectasis, hyperinflammation, wheeze and even death (Habibi & Openshaw, 2012; R. C. Welliver, 2003).

Despite increased understanding of RSV infection and the cellular mechanisms involved in its pathogenicity, there are currently limited methods of prophylactic treatment. The most effective compound used against RSV is Palivizumab and this is only used prophylactically for high-risk infants. Similarly, ribavirin is a common antiviral drug available against RSV however its high cost reduces its usage. Palivizumab is a monoclonal antibody that targets the F protein of RSV; whereas ribavirin is a purine analogue targeting RSV replication and is primarily used to treat children with severe bronchiolitis (DeVincenzo, 2000; Hruska et al., 1982; Lay et al., 2013; D. Wang et al., 2011). The significance of developing strategies to reduce the viral load or to skew the immune response during RSV infection is gaining more focus (Habibi & Openshaw, 2012).

1.4.3 Innate immune responses against RSV

After infection, the host must keep inflammation regulated in order to prevent detrimental effects to gas exchange and tissue integrity whilst the virus is eradicated. The innate immune response is important for maintaining the lung functions whilst clearing infections. Upon infection, RSV is recognised at various stages of its life cycle by PRRs and this induces an antiviral response led by the secretion of type I IFNs and inflammatory cytokines (Table 1.2) and chemokines (Table 1.3) (Borchers et al., 2013). The pro-inflammatory mediators produced during the innate response affect the viral control and cytopathology associated with RSV (Borchers et al., 2013).

Table 1-2 Pro-inflammatory cytokines involved in the innate response during RSV infection (Borchers et al., 2013). Mediators are listed by their protein name, respective receptors and general function.

Mediator	Receptor	Function	References
IL-1α	IL-1F1 to IL-1F10	Pro-inflammatory response	(Akira et al., 1990; Dinarello, 2005)
IL-1β	IL-1F1 to IL-1F10	Pro-inflammatory response	(Akira et al., 1990; Dinarello, 2005)
IL-2	IL-2R	Proliferation of T cells and Tregs	(Ma et al., 2006)
IL-4	IL-4R	Production of IgE and class switching of B cells, eosinophil survival	(Bentley et al., 2012; Yamaguchi et al., 1988)
IL-5	IL-5R	Eosinophil migration, tissue localisation/function and apoptosis blocking	(Bentley et al., 2012)
IL-6	IL-6R	Pro-inflammatory	(Akira et al., 1990)
IL-7	IL-7R	B and T cell development	(Ma et al., 2006)
IL-10	IL-10R1 and IL10R2	Immunoregulation	(Asadullah et al., 2003)
IL12p40/ p70	IL-12R-b1 and IL-12R-b2	Proliferation and differentiation with IL-2, generation of Th1 cells	(Heinzel et al., 1997; Hunter, 2005)
IL-13	IL-4Rα, IL-13Rα1 and IL-13Rα2	IgE switching, immunoregulation and regulation of mucus production, inflammation, fibrosis, remodelling	(Wynn, 2003)
IL-15	IL-15R	NK cell and memory CD8+ T cell homeostasis	(Ma et al., 2006)
IL-17	IL-17R	Pro-inflammatory responses, Th17 proliferation	(Gaffen et al., 2014; Valeri & Raffatellu, 2016)
IL-18	IL-18Rα and IL-18Rβ	Porinflammatory response and IFN-γ inducing	(Dinarello, 2002)
IL-22	L-10R2, IL-22R1	Tissue homeostasis, repair and wound healing	(Valeri & Raffatellu, 2016)
IL-23A	IL-23R	Pro-inflammatory responses, Th17 proliferation	(Gaffen et al., 2014; Hunter, 2005)
IL-27	Gp130 and WSX1	Regulating adaptive immune responses	(Hunter, 2005)
IL-33	ST2 and IL-R accessory protein	Th2 cytokines, mast cells, eosinophils and basophils	(Liew et al., 2010)

IFN-α	IFNAR	Antiviral	(McNab et al., 2015)
IFN-β	IFNAR	Antiviral	(McNab et al., 2015)
IFN-γ	IFNGR	Antiviral, IL-4-induced class switching and inhibits cell proliferation	(Sadler & Williams, 2008)
IFN-λ	IFNLR	Antiviral	(Sadler & Williams, 2008)
MIF	CXCR2 and CXCR4	Induce pro-inflammatory activity by macrophages, activation of T cells	(Bernhagen et al., 2007; Nishihira et al., 2003)
MMP9	-	Bone healing, angiogenesis in bone growth, vascular remodelling	(Löffek, Schilling, & Franzke, 2011)
MMP12	-	Macrophage proteolysis	(Löffek et al., 2011)
MMP13	-	Bone remodelling, MMP8 expression	(Löffek et al., 2011)
SP-A	SIRP1α, SP-R210	Immunoregulation, infections, tissue tension	(J. R. Wright, 2005)
SP-C	S1P4, S1P5	Immunoregulation, infections, tissue tension	(J. R. Wright, 2005)
SP-D	SIRP1α, SP-R210	Immunoregulation, infections, tissue tension	(J. R. Wright, 2005)
TNF-α	TNFRSF	Cytotoxic and pro-inflammatory	(Akira et al., 1990)

Endosomal-bound TLR3 and TLR7 (TLR8 also in humans) can detect the RSV dsRNA intermediate and the single-stranded genome respectively (P. Liu et al., 2007; S. Morris et al., 2011). TLR3 functions primarily on epithelial cells and although it does not affect RSV replication it reduces IL-13 secretion and mucus production, therefore skewing the adaptive immune response away from a type 2 phenotype (Rudd et al., 2005). The role of TLR7 is especially important in pDCs as they drive $CD8^+$ T cell responses and the production of type I IFNs after pneumovirus infection of mice (Davidson et al., 2011). Using knockout mice, TLR7 signalling has also been associated with the control of IL-17-asscociated mucus production by regulating IL-23 (Lukacs et al., 2010). TLR4 can recognise the RSV F protein of the viral glycoprotein spike however, the response from this interaction is controversial. Studies state that detection of the F RSV protein leads to activation NF-κB (Casola et al., 2002; Kurt-Jones et al., 2000). The TLR4 genotype and environmental LPS have been associated with type 2 response polarisation during RSV bronchiolitis in infants (Caballero et al., 2015). In contrast, other studies indicate that TLR4 plays no role in RSV detection (Ehl et al., 2004). Finally, TLR2

and TLR6 signalling appear to be important in the control of viral replication *in vivo* and TLR2 interactions with RSV promoted the migration of neutrophils and DCs into the lung (Bowen et al., 2009).

Table 1-3 Chemokines involved in innate immune response during RSV infection (Borchers et al., 2013). The mediators are listed with their protein name, respective receptors and general function. Information adapted from (Griffith et al., 2014).

Mediator	Receptor	Function
CCL2	CCR2	Inflammatory monocyte trafficking
CCL3	CXCR2	Neutrophil trafficking
CCL4	-	Procoagulant
CCL5	CCR1, CCR3, CCR5	Neutrophil trafficking
CCL7	CXCR2	Neutrophil trafficking
CCL12	CXCR4	Bone marrow homing
CCL17	CCR4	Th2 migration/responses, Treg homing
CCL20	CCR6, CCR7	T cell and DC lymph node homing
CCL22	CCR4	Th2 migration/response, Treg homing
CXCL1	CXCR2	Neutrophil trafficking
CXCL3	CXCR2	Neutrophil trafficking
CXCL8	CXCR1, CXCR2	Neutrophil trafficking
CXCL9	CXCR3	Th1 response, Th1, CD8 and NK cell trafficking
CXCL10	CXCR3	Th1 response, Th1, CD8 and NK cell trafficking
CXCL11	CXCR3	Th1 response, Th1, CD8 and NK cell trafficking
CXCL16	CXCR6	NKT and innate lymphoid cell migration/survival
XCL1	XCR1	Cross presentation by $CD8^+$ DCs
CX3CL1	CX3CR1	NK, monocyte and T cell migration

Cells can also recognise RSV through cytosolic RLRs. NOD2 and RIG-I can detect the ssRNA genome; whereas PKR and MDA5 recognise the dsRNA intermediate (Marr et al., 2014). Although RSV can be detected by many PRRs, *Mavs*$^{-/-}$ mice show that that viral detection through RIG-I and MDA-5 is associated with induction of type I IFNs and decreased viral load and morbidity (Bhoj et al., 2008; Demoor et al., 2012; Goritzka et al., 2015; P. Liu et al., 2007). Studies using *Mavs*$^{-/-}$ mice however suggested that even when lacking an optimum innate response to RSV, the cytotoxic T cell response and IFN-γ production were intact (Bhoj et al., 2008; Demoor et al., 2012; Goritzka et al., 2015).

The secretion of type I IFNs after RSV infection in humans can decrease the viral load, morbidity and inflammation (R. Janssen et al., 2007; Miyake & Kaisho, 2014). Mouse models provide evidence that type I IFNs drive, via IFNAR, a reduction in viral load, the production of pro-inflammatory cytokines and CD8^{+} T cell activation after RSV infection (Goritzka et al., 2014; T. R. Johnson et al., 2005). The addition of recombinant IFN-α was sufficient for the production of inflammatory mediators in the lung indicating the importance of type I IFNs in driving the innate response in the lung (Goritzka et al., 2014). One possible source of type I IFNs after RSV infection is believed to be pDCs as they express IRF7 and can secrete high amounts of type I IFNs during virus infections (Finke et al., 2005; Hornung et al., 2004; Kawai & Akira, 2011; Schijf et al., 2013). Studies performed *in vivo* and *ex vivo* focusing on the pDC contribution of type I IFNs during RSV infection are controversial (Goritzka et al., 2015; Guerrero-Plata et al., 2009). Increasing evidence suggest that AMs have an active role in respiratory infections. *Ifna6*$^{gfp/+}$ reporter mice have shown that AMs produce type I IFNs after NDV and that they are the main producers of type I IFNs during RSV infection (Goritzka et al., 2015; Kumagai et al., 2007).

However, RSV possesses the NS1 and NS2 proteins which interfere with type I IFN production and signalling (Bitko et al., 2005; Boyapalle et al., 2012; Ling et al., 2009; Schlender et al., 2000; W. Zhang et al., 2005b). The RSV NS2 protein can affect the RIG-I induced production of type I IFNs by binding to its CARD terminal and inhibiting its interaction with MAVS (Ling et al., 2009). Studies in A549 cells infected with RSV showed that the viral NS1 protein can co-localise with MAVS at the early stage of infection and interfere with the RIG-I-MAVS interaction (Boyapalle et al., 2012). In addition, the NS1 protein can affect the activation of

IKKε which is a regulator of IRF3 (Swedan et al., 2009). RSV NS2 protein inhibits the type I IFN signalling pathway via the JAK/STAT signalling pathway (Swedan et al., 2009; van Drunen Littel-van den Hurk & Watkiss, 2012). The lack of type I IFNs or STAT signalling after RSV infection also decreased the cytokines and chemokines secreted by epithelial cells (Ioannidis et al., 2012; Villenave et al., 2012). The production of type I IFNs can also be dampened by IBs where the RSV N protein can antagonise signalling via MAVS and/or MDA5 (Lifland et al., 2012). The effects of RSV are multifactorial and depend on the virus, on the host factors and types of immune responses that are elicited. To understand the pathogenesis of RSV it is important to focus on the interplay between a multitude of immune cells that are activated during infection (Fig 1.6).

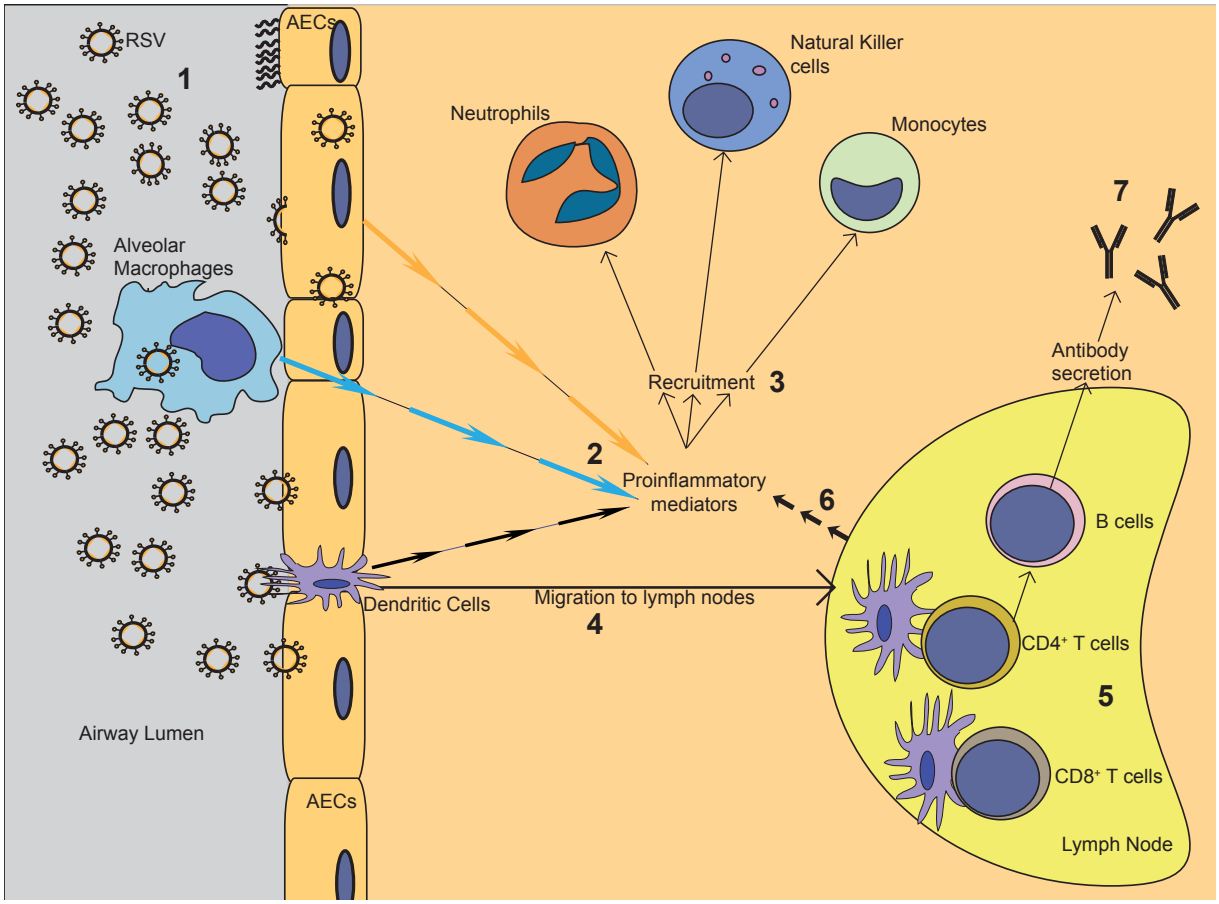


Figure 1.6 Overview of a primary immune response during RSV infection. (1) RSV infects airway epithelial cells (AECs) and encounters alveolar macrophages and dendritic cells in the airways. (2) This causes the production of inflammatory mediators which (3) initiate the recruitment of neutrophils, NK cells and monocytes to the site of infection. (4) Dendritic cells sample the airway for RSV antigens and upon maturation migrate to the lymph nodes where

(5) they present antigens to T and B cells. This leads to (6) the production of mediators, T cell recruitment to the lung and to (7) the secretion of antibodies by B cells.

1.4.3.1 Epithelial cells

Epithelial cells (ECs) are the main target of RSV (Borchers et al., 2013; L. Zhang et al., 2002). The receptor that RSV uses to infect ECs remains elusive although it has been suggested that the F RSV protein may bind to nucleolin; whereas the G protein may use CX3CR1 (Choi et al., 2010; Harcourt et al., 2006; S. M. Johnson et al., 2015; Tayyari et al., 2011). The budding of the virus occurs from the apical side of the ECs and newly formed viral particles spread by infecting neighbouring cells directly or through ciliary motion (Villenave et al., 2012). Features that are common in severe infant cases and have been reproduced in *ex vivo* EC models include: sloughing, apoptosis, mucus production and production of inflammatory mediators (Borchers et al., 2013; Tristram et al., 1998). The contribution of ECs to the responses against RSV has primarily been studied *ex vivo* using immortalised cell lines, which showed that they can produce an array of high amounts of cytokines and chemokines such as CCL3, CCL2, CCL5, CCL11, CXCL8, CXCL9, CXCL10, CX3CL1, IL-1β, IL-2R, TNF-α, IL-6 and IFN-α (Borchers et al., 2013; Ioannidis et al., 2012; Tabarani et al., 2013; Villenave et al., 2012). Nasopharyngeal washes of children visiting the hospital after RSV infection further suggest the presence of biomarkers of severe RSV disease which include IL-1β, IL1-RA, IL-7, the epidermal growth factor (EGF) and the hepatocyte growth factor (HGF) (Tabarani et al., 2013). The production of IFN-α by ECs is debated and seems to be dependent on the cell line used (Tayyari et al., 2011; Villenave et al., 2012). The usage of primary ECs which retain more characteristics of the donor and their natural differentiation indicate that, in fact, the ECs might not be responsible for all mediators previously described and that the interplay with other immune cells might be crucial for mounting an efficient immune response (Fonceca & Smyth, 2012). RSV is thought to primarily infect ciliated epithelial cells, although RSV has been detected in cells of the lower airways of deceased patients (J. E. Johnson et al., 2006; P. F. Wright et al., 1996).

1.4.3.2 Alveolar macrophages

AMs are one of the first cells, together with ECs, to encounter RSV in the lower airways (Fig 1.6). The interaction between AMs and ECs during infection is especially important when the innate immune response is initiated and driven by the mediators these cells produce (Goritzka et al., 2014; 2015; Miyairi & DeVincenzo, 2008). The importance of AMs during infection have been suggested *in vivo* and *ex vivo* through a range of animal models and depletion studies. Using the New Zealand black mice, which have a deficiency in macrophage function, similar histopathologic changes occurred as those previously observed in infants with fatal cases of primary RSV infection (Reed et al., 2008). Such changes included airway occlusion and accumulation of infected cells, apoptotic cells and cellular debris (J. E. Johnson et al., 2006). This would suggest that AMs are important in keeping the airway clear from the damage caused by the infection. However, there is increasing evidence that AMs have a more active role in the initiation of the innate response during RSV infection.

AMs are the main source of type I IFNs after RSV infection (Goritzka et al., 2015). The role of type I IFN production by AMs is critical as it induces the production of inflammatory mediators including TNF-α and IL-6 (Kolli et al., 2014). The broncholaveolar lavage (BAL) of macrophage-depleted mice infected with RSV showed a decreased presence of TNF-α, IL-6, CCL3, IFN-α, IFN-β, IFN-γ and IL-1β; whereas there were increased levels of IL-12p40, GM-CSF and G-CSF (Becker et al., 1991; Kolli et al., 2014; Pribul et al., 2008). In all cases, the lack or partial lack of AMs or type I IFNs was not associated with mortality. However, there was an increase in weight loss, viral load and a decrease in NK recruitment. Furthermore, there appeared to be an increase in recruitment of neutrophils, DCs and activated $CD4^+$ and $CD8^+$ T cells (Demoor et al., 2012; Goritzka et al., 2015; Kolli et al., 2014; Pribul et al., 2008). *Ex vivo* studies of human AMs suggest that AMs are infected by RSV and can produce TNF-α, IL-6, IL-10 and CCL5 (Franke-Ullmann et al., 1995; Panuska et al., 1995; Stadnyk et al., 1997).

Further to how AMs direct the innate immune response during RSV infection, it is critical to understand how they interact with the virus. The findings from various studies are contradictory regarding whether or not AMs are infected by RSV. Especially earlier studies of

primary AMs from humans and mice suggested that the cells are actively infected by RSV; whereas other studies suggested that they are not permissive to infection (Becker et al., 1991; Cirino et al., 1993; Franke-Ullmann et al., 1995; Panuska et al., 1990; Ravi et al., 2013). The large variations in protocols previously used to study AMs after RSV infection may be the reason for many conflicting results generated regarding their inflammatory profile and permissibility to RSV. As the plasticity of AMs is immense it is important to assess their role *in vivo* and to study them *ex vivo* immediately after their isolation.

1.4.3.3 Dendritic Cells

DCs play a critical role in antigen presentation and initiation of the adaptive immune response (see section 1.1.1.1) (Fig 1.6). Upon exposure to RSV, lung DCs upregulate MHC-II, CD80, CD86, CD40, CD83 and ICAM-1. These markers are crucial for the maturation of DCs and for antigen presentation (Guerrero-Plata et al., 2009; Luongo et al., 2008; Boogaard et al., 2007; Schwarze, 2008; Smit et al., 2006). During RSV infection of mice, there is an early influx of pDCs and cDCs into the lungs and the draining lymph node (Guerrero-Plata et al., 2009; Smit et al., 2006; H. Wang et al., 2006). A similar influx was observed in the nasal mucosa of RSV infected children (M. A. Gill et al., 2005). The kinetics and persistence of the DC subsets in the lungs after RSV infection remains controversial (Borchers et al., 2013). Some studies showed that pDCs in the lung increased early after RSV infection and remained elevated throughout the inflammatory and the resolution phase of infection, which was up to 30 days post infection (Beyer et al., 2004; Smit et al., 2006; H. Wang et al., 2006). In contrast, another study showed that pDCs in the lung were only present up to 10 days post infection and that cDCs were present between days 7 and 18 post infection (Guerrero-Plata et al., 2009). There has also been controversy regarding pDCs as a major source of IFN-α. Recent findings using *Ifna6*$^{gfp/+}$ reporter mice show that AMs, not pDCs, are the source of type I IFNs during RSV infection (Goritzka et al., 2015). Depletion of pDCs caused an increase in RSV viral load, inflammation and airway hyperresponsiveness which were rescued when activated pDCs were transferred into the lung (Smit et al., 2006; H. Wang et al., 2006). These findings suggest that pDCs have an important regulating role during RSV infection by reducing inflammation and airway hyperresponsiveness independently of type I IFN production.

As professional antigen presenting cells, the role of cDCs during RSV infection is to process and present antigen and to induce the polarisation of the T cell responses (Borchers et al., 2013). Upon antigen detection cDCs migrate to the lymph nodes by upregulating CCR7 expression (Mildner & Jung, 2014). Upon reaching the lymph nodes, cDCs can cause polarisation of T helper cells via MHC I or MHC II peptide presentation. Antigen presentation also depends on specific maturation markers such as CD40, CD80 and CD86 (Boogaard et al., 2007). In order to drive full T helper cell polarisation, cDCs must provide an MHC peptide, costimulatory signals within the B7 family members and instructing cytokines (Mildner & Jung, 2014; Spörri & Sousa, 2005). The interplay between pDCs and cDCs seems to be important for the outcome of pulmonary infection (Smit et al., 2006). By expanding cDCs and pDCs using Flt3L (increases number of DCs), there was a reduction in airway hyperreactivity, type II helper (Th2) cytokines, inflammation and mucus; whereas there was an increase in type I helper (Th1) cytokines (Beyer et al., 2004; Remot et al., 2016; Smit et al., 2006).

There are two main subsets of cDCs in the lung that are characterised by the expression of the integrins CD11b ($CD11b^+$ cDCs) and CD103 ($CD103^+$ cDCs) (Lambrecht & Hammad, 2012). $CD103^+$ cDCs are in the basolateral layer of the epithelium and are separated from the airway by a single cell layer (Lambrecht & Hammad, 2012). These cells form projections through the epithelial barrier for sampling the airway. $CD11b^+$ cDCs are situated in the lamina propria, below the basal membrane, and express CX3CR1 and SIRPα (Hammad & Lambrecht, 2008; Lambrecht & Hammad, 2012). During influenza virus infection, $CD103^+$ cDCs can sample the lung and migrate to the mediastinal lymph nodes (MLNs) and present antigen to T cells (GeurtsvanKessel et al., 2008; Helft et al., 2012). This is followed by an influx of $CD11b^+$ cDCs into the airways which then also migrate to the MLNs (GeurtsvanKessel et al., 2008; Lambrecht & Hammad, 2012). $CD103^+$ cDCs are specialised in the uptake of apoptotic cells and cross-presenting viral antigen to $CD8^+$ T cells (GeurtsvanKessel et al., 2008; Helft et al., 2012); whereas $CD11b^+$ cDCs are specialised in producing high amounts of cytokines and presenting soluble antigens to $CD4^+$ T cells (Beaty et al., 2007). Altogether, this suggests that during respiratory infection a balance in the DC responses mediates priming of T cells.

1.4.3.4 Natural killer cells

NK cells are major producers of granzyme B and IFN-γ early after infection and are important for controlling the virus (Borchers et al., 2013; Harker et al., 2010; Topham & Hewitt, 2009). In mice, NK cells are the most abundant producers of IFN-γ and their influx peaks at day 4 post infection (Hussell & Openshaw, 1998; Openshaw & Tregoning, 2005). Furthermore, NK cells appear to shape the type of T cell response as low numbers of NK cells caused lung eosinophilia during RSV infection (Hussell & Openshaw, 1998). The crucial interplay between innate cells during RSV infection is demonstrated in a study with NK cell deficiency. Specifically, the depletion of NK cells caused a reduction in IFN-γ and an upregulation of notch ligand Jagged1 on DCs which was induced by EC derived IL-25 (Kaiko et al., 2010). This drove a Th2 response and subsequent allergic lung disease. Furthermore, when neonatal mice were infected with RSV, the NK cell response limited the antibody responses (Tregoning et al., 2013). Interestingly, children and infants admitted to hospital with LRTI or bronchiolitis requiring ventilation appeared to have a significant decrease in the number of NK cells present in the airways compared to non-ventilated children (De Weerd & Twilhaar, 1998; T. P. Welliver et al., 2007).

1.4.3.5 Neutrophils

Neutrophils are one of the first cells recruited into the lung during RSV infection (Borchers et al., 2013; Goritzka et al., 2014; 2015). Their role however is not fully elucidated as they might have a role in viral control and/or in inducing lung pathology (Geerdink et al., 2015). The neutrophils can remove RSV by phagocytosis or by the formation of neutrophil extracellular traps (NETs), which can capture viral and microbial agents (Funchal et al., 2015). Furthermore, neutrophils can also produce oxygen radicals and proteolytic enzymes which cause damage to ECs (Miyamoto et al., 2003; S. Z. Wang et al., 1998). Samples from children with RSV infection and severe RSV bronchiolitis showed a significant influx of neutrophils into the airways (Everard et al., 1994; McNamara et al., 2003). As neutrophils are so abundant throughout the lung during RSV infection, it is crucial that their role is characterised further.

1.4.3.6 Monocytes

The recruitment of monocytes during RSV infection is dependent on type I IFNs (Goritzka et al., 2015). This recruitment is driven by CCR2, expressed by monocytes, and its ligand CCL2 and CCL7 (Ginhoux & Jung, 2014). Monocytes are involved in controlling RSV but their precise role during RSV infection is not fully understood (Goritzka et al., 2015). By inducing the recruitment of monocytes in *Mavs*$^{-/-}$ mice using recombinant CCL2, there was a decrease in morbidity and viral load during RSV infection (Goritzka et al., 2015). *Ex vivo* studies of monocytes have suggested a role of monocytes in containing RSV infection and potentially polarising immune responses (Soukup & Becker, 2003). Furthermore, monocytes can control viral infection by killing infected epithelial cells via TRAIL signalling (Ellis et al., 2015; Herold et al., 2008). The activation of monocytes during RSV infection has been associated with the secretion of IL-6, TNF-α and IL-1β (Schijf et al., 2013; Takeuchi et al., 1998). Overall, the monocytes might be involved in the activation of other immune cells and directly or indirectly interfering with the RSV infection and associated cytopathology. However, increased levels of CCL2 in nasal secretion and BAL of children with RSV bronchiolitis also highlights the importance of maintaining a balanced response in the lung tissue (McNamara et al., 2005).

1.4.4 Adaptive immune responses

The adaptive immune response against RSV is driven by T and B cells. Upon infection, antigen presenting cells such as DCs migrate to the draining lymph nodes where antigen presentation to T and B cells occurs (Fig 1.6). A main feature of RSV is that the healthy adult population is reinfected throughout lifetime. This could indicate that naturally occurring memory responses are short-lived and with limited efficiency; however, they might still be sufficient to protect from LRTI.

1.4.4.1 T cell responses

The clearance of RSV is initially driven by AMs, monocytes and NK cells however this role is overtaken by $CD8^+$ T cells later during the infection (Fig 1.6). The T cell response during RSV

infection is essential for the final viral clearance to occur (Johansson, 2016). During primary RSV infection in mice, the peak of the adaptive immune response correlates with the peak of the T cell response (Graham et al., 1991; Johansson, 2016). Approximately 40% of the $CD8^+$ T cells recruited to the mouse lung are specific for the M2 protein (Jun Chang & Braciale, 2002). Depletion studies indicated that $CD8^+$ T cells are indispensable in viral clearance (Graham et al., 1991).

The data in humans is controversial as the presence of activated $CD4^+$ and $CD8^+$ T cells correlates with more severe disease in humans (Alwan et al., 1992; Openshaw & Chiu, 2013; Walsh et al., 2013). A study of immunocompromised children undergoing chemotherapy, thus lacking T cell responses, showed increased viral shed and disease severity after RSV infection, which was prolonged for several months in contrast to 'healthy' children that showed disease symptoms and viral shed for 7 to 21 days (Hall et al., 1986).

T cells can differentiate into various subsets which are characterised by the major cytokines produced. Naïve $CD4^+$ T cells can differentiate into a various of T helper (Th) cells including Th1, Th2, Th17, Th9, Th22, T follicular helper (Tfh) and regulatory T cells (Tregs) (Kara et al., 2014; Luckheeram et al., 2012). Each Th cell subset has important roles during infection and can shape the phenotype of the immune response (Bluestone et al., 2009). Antigen presentation for $CD4^+$ T cells occurs through MHC-II, whereas $CD8^+$ T cells are primed by antigen presented on MHC-I. Upon activation, naïve or memory T cells proliferate and home to the lung tissue. Mice that were depleted of $CD4^+$ and/or $CD8^+$ T cells showed higher lung viral load however no severe morbidity was reported (Graham et al., 1991). Interestingly, although both T cell types appeared to drive pathology, $CD8^+$ T cells had a more dominant role (Graham et al., 1991; Openshaw & Askonas, 1988). Th1 cells can also influence the cytotoxic response caused by $CD8^+$ T cells; whereas Tregs can supress this activation through secretion of IL-10, TGF-β, perforin and granzyme B. During viral infection, the $CD8^+$ T cells can interfere with the epithelial cell infection or supress the viral spread; whereas Th1 responses can interfere with the viral spread through the secretion of IFN-γ, TNF and IL-2 (C. Chiu & Openshaw, 2015). There are three types of memory T cells: central memory, T_{RM} and effector memory $CD8^+$ T cells which are important for the clearance of infection at the lung mucosa (Heidema et al., 2004; Purwar et al., 2011; Y. Zhao et al., 2013b). The T_{RM} cells remain in the

mucosa after infection and recognise conserved internal viral proteins and, therefore, become sensory cells and early effector cells when pathogens are re-encountered (C. Chiu & Openshaw, 2015; Jozwik et al., 2015; Wilkinson et al., 2012). The presence of RSV-specific T_{RM} $CD8^+$ T cells in adult human lungs has been shown to be protective (Jozwik et al., 2015). The presence of memory T cells in the lung after RSV has been cleared also indicates an importance in enhanced protection upon reinfection but the longevity of the response is not clear.

RSV has evolved mechanisms to evade detection, dampen the innate immune response and interfere with $CD8^+$ T cell activity. Mouse studies showed that lung RSV-specific $CD8^+$ T cells had impaired IFN-γ production and cytolytic ability compared to spleen $CD8^+$ T cells (Jun Chang & Braciale, 2002). Furthermore, to protect from tissue damage there was an upregulation of PD-/PD-L1 which might also be utilised by RSV (Telcian et al., 2011). Specifically, co-cultures of epithelial cells and $CD8^+$ T cells showed that PD-L1 on RSV-infected epithelial cells drives $CD8^+$ T cell proliferation, activation and antiviral functions. In contrast, the lack of PD-L1 decreased viral load and enhanced IFN-γ, IL-2 and granzyme B production (Telcian et al., 2011). The importance of T cells in shortening the disease severity and clearing the virus has been shown in several animal and human studies. However, there might be a dual role of T cells during RSV infection as they drive many features of the immunopathology associated with RSV. Although mice depleted of T cells had a higher viral load, they appeared to have a reduced weight loss especially after $CD8^+$ T cell depletion (Graham et al., 1991; Openshaw & Askonas, 1988).

The failure of the FI-RSV vaccination proved that the vaccine skewed the immune response towards a Th2 phenotype that was characterised by eosinophilia. The interplay between antigen presentation and Th/$CD8^+$ T cells is critical in skewing the immune response towards an antiviral Th1 or an asthma-like Th2/Th17 response, as the latter is an important factor in the immunopathology (Caballero et al., 2015; Legg et al., 2003). The T cell responses against RSV must be regulated. To minimise immunopathology and tissue damage, so to dampen their cytotoxic profile, T cells also secrete IL-10 (Borchers et al., 2013; Loebbermann et al., 2012; Openshaw & Chiu, 2013; Ouyang et al., 2012). Furthermore, the recruitment of Tregs is important in controlling T cell inflammation during RSV infection. The lack of Tregs in mice

infected with RSV appeared to drive a higher degree of inflammation and promoted a Th2 phenotype (Durant et al., 2013; D. C. P. Lee et al., 2010; Loebbermann et al., 2012; Loebbermann et al., 2013). Altogether, the T cell responses can aid in the final clearance of RSV but the balance between the magnitude, phenotype and response period appear to be equally responsible for RSV induced lung pathology as the virus itself.

1.4.4.2 Humoral responses

B cells are also activated by APCs with help from Th cells such as Tfh cells. During viral infections memory or naïve B cells differentiate into plasmablasts. Together with long-lived plasma cells, plasmablasts secrete antibodies that can neutralise the virus, promote phagocytosis, increase cytotoxicity and interfere with viral entry (C. Chiu & Openshaw, 2015). Antibodies specific to RSV F proteins are abundant in adults, yet reinfections occur throughout life (Hall et al., 1991). The reason for the short half-life of RSV antibodies is not established however, this can be the reason behind the lack of vaccines and protection from recurrent infections (C. Chiu & Openshaw, 2015; Falsey et al., 2009).

During viral infections and with the help of Tfh cells, B cells differentiate from antibody-secreting to memory cells. Furthermore, there is a class-switch from IgM to IgG and IgA, which is followed by secretion of virus-specific antibodies. The antibodies are critical in neutralising the virus, blocking its entry into cells, fixing complement, promoting phagocytosis and allowing antibody-dependent cytotoxicity (C. Chiu & Openshaw, 2015). After infection, subsets of plasma cells differentiate into long-lived plasmablasts and migrate to the respiratory tract and bone marrow (C. Chiu & Openshaw, 2015). The most dominant RSV proteins that contain epitopes for binding to neutralising antibodies are F and G (Borchers et al., 2013). When humoral responses are absent, viral clearance is intact however, upon reinfection there was an increased disease severity, inflammation and viral replication (Graham et al., 1991).

In RSV infected infants, mucosal IgG (maternally derived) levels correlate better with RSV clearance and decreased CXCL10 compared to plasma IgG; whereas in studies of RSV challenged healthy adults, nasal IgA levels correlated with better protection (Habibi et al.,

2015; Vissers et al., 2016). An important difference between adults and infants is that the infant IgG is maternally derived (Johansson, 2016); whereas IgA must be made by the host and is therefore more prevalent in adults. Despite being protective, the serum antibody decay in adults after RSV infection is rapid and therefore protection is short lived (Falsey et al., 2009). This characteristic of the humoral response might, in part, be responsible for the lifelong susceptibility and recurrent infection caused by RSV. This phenomenon adds to the complexity of RSV vaccine development. As the target groups for RSV vaccination would be infants, children, pregnant women and the elderly, the maturity of the immune responses must also be considered for each group (L. J. Anderson, 2013; Guvenel et al., 2014). There is a notion that multiple vaccines must be developed to cover the groups that are most prone to develop severe RSV (Dudas & Karron, 1998; Jorquera & Anderson, 2015). Specifically, since maternal antibodies are the best correlate of protection, by vaccinating pregnant women maternal antibodies can be transferred to the infant that will be protective during the early stages of infancy (Lambert et al., 2014; Saso & Kampmann, 2016). In addition, the administration of an RSV vaccine together with an adjuvant might skew a beneficial neonatal immune response and overcome the immature/supressed immune state of infants (Dudas & Karron, 1998; Jorquera & Anderson, 2015).

1.5 Neonatal immunity

Respiratory infections such as RSV are a major burden for infants (Borchers et al., 2013; Glezen et al., 1986; Nair et al., 2010). In order to study the infection and to develop potential vaccines and treatment options, animal models have been utilised. Although the models have provided indispensable insight into the dynamics, mechanisms and immunopathology associated with RSV, there are many limitations as to how these apply to the human infant. Approximately 77% of the PubMed articles on RSV are performed on mice and only 2.5% of these use neonatal mice (Cormier et al., 2010).

As in man, primary RSV infection in mice indicated a type-I response that is characterised by NK cells, Th1 cells and CTLs which produce IFN-γ (see section 1.4.4.1) (Johansson, 2016; Lambert et al., 2014). In infants, there is an imbalance between the Th1 and Th2 cytokine ratio. Specifically, in the nose of infants, higher levels of IL-4 and diminished IFN-γ levels have been observed (Aberle et al., 1999; Kristjansson et al., 2005; Legg et al., 2003; Semple et al., 2007). Infants with such a phenotype in the BAL or PBMCs developed a more severe RSV infection (Aberle et al., 1999; Semple et al., 2007). In fact, mouse models suggested that T cells in the lung of neonates appeared to express limited functionality (Torow et al., 2017). There is a lower number of T cells in the neonatal lung compared to adults, and this population of T cell is primarily composed of naïve cells which, upon stimulation, present an intrinsic bias towards a Th2 profile (Roux et al., 2011; Torow et al., 2017). The neonatal T cells do not appear to be defective however, the lung environment does not allow efficient priming to occur (Qureshi et al., 2001). In fact, Tregs and $CD11b^+$ cDCs are the only cells upregulated in the neonatal lung and this coincides with the colonisation of tissue by the microbiota (Torow et al., 2017). Thus, as development of the lung may influence chronic conditions, the neonatal lungs might require specific stimuli for an optimum immune response.

1.5.1 Neonatal immunity and the lung

The adaptive immune response of neonates has been described as having many defects so newborns rely primarily on the innate immune responses (Levy, 2007). The relevance of the

innate immune response in neonates is emerging as studies of viral and bacterial infections increase. The production of cytokines and chemokines is dependent on the invading pathogen and this can drive different types of adaptive immune responses. Exposure to TLR agonists early in life skews toward a Th1 response which can be protective against allergy and atopic disease (Vercelli, 2006; Yamaguchi et al., 2012).

The cellular composition of neonatal lungs differs from that of adults. There is evidence that there are increased numbers of Tregs present in the lung of neonates which are important for dampening Th1 responses (Godfrey et al., 2005; Gollwitzer et al., 2014; Z.-X. Zhang et al., 2000). The usage of TLR agonists has been shown to change this phenotype, as TLR7/8 signalling leads to IL-6 production by DCs and Treg activation which skews the response toward a Th1 response (Levy, 2007; Pasare & Medzhitov, 2003; Peng et al., 2005). Overall, the effects of TLR-mediated responses are conflicting. Many studies in mononuclear cells from neonatal cord blood show that, compared to adult monocytes, they produce less IL-1α, IL-1β, TNF-α, IL-18, and IL-12p70 after exposure to TLR ligands. However, after exposure to *S. aureus* or group B streptococcal microbes, they can produce equal or higher amounts of IL-6 and IL-10 (Kollmann et al., 2009; La Pine et al., 2003; Upham et al., 2002). In contrast, *ex vivo* studies of pDCs and monocytes suggested that neonates can produce similar levels of cytokines as adults after TLR stimulation, however there appears to be skewing toward a Th2/Th17 response (Kollmann et al., 2009). This suggests a reduced capacity of neonatal innate cells to deal with intracellular pathogens which require a Th1 response such as RSV.

In addition to the differences in the TLR signalling, the composition of immune cells between adults and neonates also varies. Neutrophils in neonates are suggested to have both quantitative and qualitative defects (Levy, 2007). The small neutrophil pool and reduced number of precursors causes a diminished number of neutrophils infiltrating the lungs after bacterial infections (Christensen, 1989). Furthermore, the neonatal neutrophils appear to have impaired chemotaxis, rolling adhesion and transmigration (D. C. Anderson et al., 1991; R. Carr & Huizinga, 2000). The impaired recruitment of neutrophils might be due, in part, to antigen presenting cells of neonates which produce high amounts of IL-6, potentially inhibiting neutrophil migration (Angelone et al., 2006; Levy, 2007; Schultz et al., 2002).

The function of DC cell subsets is also impaired in neonates. Firstly, the DC system in mouse neonates is immature and *in vitro* studies showed decreased IL-12 p35 gene transcription factor which is associated with impaired LPS-induced production of type I IFNs (Levy, 2007). Studies with poly(I:C) and CpG indicate that neonates have an impaired production of type I IFNs by pDCs; whereas neonatal monocyte-derived DCs have impaired IFN-β production after LPS exposure (Aksoy et al., 2007). Finally, the antigen presentation induced by neonatal DCs was impaired as there was a reduction in the costimulatory molecules CD80 and CD86 expression by DCs and a reduced TNF production following phagocytosis of necrotic cells (Levy, 2007; Wong et al., 2005). The role of IL-6 has also been vastly highlighted in neonates as it is induced after birth and is believed to be a driving force for mobilising mucosal, epithelial and blood anti-infective proteins and peptides to protect from initial microbial colonisation (Levy, 2007). Overall, understanding the strengths and weaknesses of the neonatal immune responses could potentially be beneficial towards skewing the antiviral response toward a more beneficial phenotype during infection.

1.5.1.1 Using TLR ligands as adjuvants

The expression and signalling through TLRs is especially important during the development of the foetal and neonatal lung (Kollmann et al., 2009; Kollmann et al., 2012; Kramer & Jobe, 2005). Studies regarding the full development of TLR signalling in neonates are insufficient. A number of studies have focused on the expression of TLR2 and TLR4 in the lung which, around the time of birth, are almost undetectable and increases several folds with age (Harju et al., 2001). Evidence regarding the expression and the signalling pathways of TLR2 and TLR4 in neonates remain conflicting. Some studies indicated that there was impairment of these receptors after LPS administration into the airways of rats. This impairment affected the inflammatory responses and the recruitment of leukocytes for approximately 4 weeks (Martin et al., 1995). Primary human epidermal cells were shown to enhance IL-1 and human-β-defensin (HBD)-2 after LPS exposure and especially after exposure to monocyte-derived cells (L. Liu, Roberts, & Ganz, 2003). HBD-2 is an antimicrobial peptide and studies suggest that its production in the neonatal lung is dependent on MyD88 signalling (Levy, 2007).

Stimulation with bacterial lipopeptides (BLP), LPS and imiquimod caused a decreased stimulation in neonatal cord blood cells compared to adult cells (Levy et al., 2004). In contrast, resiquimod (R848), which is a congener of the synthetic imidazoquinoline (imiquimod), induced the production of TNF-α in neonatal blood monocytes (Levy et al., 2004). The compound has no direct antiviral or anti-proliferative effects however, it stimulates innate immune responses by inducing cytokine production by monocytes, macrophages and DCs (Jurk et al., 2002; Levy et al., 2006b). Studies as to why there are differences in the secretion of cytokines after TLR stimulation suggested that neonatal cord blood monocytes contain 20-fold more intracellular cyclic AMP (cAMP) which acts directly or indirectly on PKA to supress the production of TNF (Levy et al., 2006a). The precise effect of TLR ligands, such as R848, and the potential cells involved in responses in the neonatal lung needs to be better characterised.

1.5.2 Neonatal immune responses to RSV

As the neonatal immune response is different from that of adults, the age when RSV infection occurs plays a critical role in the pathogenesis (Cormier et al., 2010; Lambert et al., 2014; P. Wu et al., 2008). Infants under the age of 6 months are especially susceptible to RSV infection and disease severity (Boyce et al., 2000). Various studies in Scandinavia and The Tucson Children's Respiratory study suggested that children with a RSV infection at a young age (even mild) were more likely to be prone to wheeze and asthma up to 20 years of age (Piippo-Savolainen et al., 2004; Sigurs et al., 2010; Stein et al., 1999). Similar results have also been reproduced in the mouse model. Mice under 7 days old exposed to RSV developed airway remodelling in addition to Th2 cytokines and cellular responses upon reinfection (Becnel et al., 2005; Culley et al., 2002; Dakhama et al., 2005; You et al., 2006). Quite interestingly, the innate response in mouse neonates to RSV is almost undetectable (Culley et al., 2002). There appears to be a correlation between how young the neonate is when first exposed to RSV and the disease severity upon reinfection (Culley et al., 2002). These studies also showed that, upon RSV or allergen stimulation, the mice that were primed as neonates had severe asthma-like responses (Becnel et al., 2005; Culley et al., 2002; Dakhama et al., 2005; Plotnicky et al., 2003; You et al., 2006). There is evidence of innate cells directing pathogenic responses during RSV infection and that the addition of specific cytokines can alter the immune response upon

reinfection (Harker et al., 2010; Harker et al., 2014). In humans, evaluation of associations of *Tlr* gene polymorphism and lung function 5-7 years after RSV-induced bronchiolitis showed that *Tlr7* polymorphism was associated with impaired lung function (Lauhkonen et al., 2016). A better understanding of the innate immune response against RSV could provide insight for more successful vaccination.

1.5.2.1 Development of RSV vaccines in neonates

The first months of birth are crucial for the final development of the lung as well as the establishment of the immune cells that are localised in the tissue. When assessing the maturation of the neonatal lung, a physiological and immunological perspective must be considered. The development of the lung is tightly regulated and one of the last events before birth is the development of the alveoli, a process which continues until 8 years of age (Botas et al., 1998; Grenache & Gronowski, 2006). This also highlights how respiratory infections at early age can have long lasting effects on the lung. The need to understand the neonatal immune responses is therefore critical in order to develop vaccine candidates or use of adjuvants in order to skew the immune responses towards a protective immune response.

Following the success with the FI-polio vaccine, the FI-RSV vaccine was developed for 2-9-month-old infants in 1966 and 1967. Upon natural infection, vaccinated children developed more severe symptoms and 80% required hospitalisation whilst two succumbed to the infection due to the resulting immunopathology. The main clinical symptoms of these children were rhinitis, bronchiolitis and pneumonia, and the histopathology of the children who died showed a severe infiltration of monocytes and eosinophils (Kapikian et al., 1969; W. K. Kim et al., 1968b). Similarly to the FI-RSV vaccinated children, studies in mice vaccinated with FI-RSV showed extensive pathology, eosinophilia, elevated IL-4, IL-5 IL-10 and IL-13 levels upon reinfection (Openshaw & Tregoning, 2005; Plotnicky et al., 2003).

Furthermore, studies of the vaccinated children suggested that the FI-RSV vaccine induced poor neutralising antibodies. Mouse studies suggested that the viral proteins have a diminished ability to stimulate TLRs after formalin treatment (Delgado et al., 2009). There also appeared to be increased levels of Tregs following administration of FI-RSV

(Loebbermann et al., 2013). The limitations of the FI-RSV vaccine in infants and in mice highlight the obstacles that need to be surpassed in order to successfully administer an RSV vaccine to children. Many current research studies focus on developing RSV vaccines that can increase the generation of protective antibodies. This can be done directly or passively. Specifically, some studies are focusing on immunising women in their third trimester of pregnancy in order to boost the anti-RSV antibody titres in newborns (Munoz et al., 2003). Multiple RSV studies are focusing on generating vaccines where there is an antibody mediated response directed toward the pre-fusion viral F-protein (Lambert et al., 2014).

The administration and effect of RSV vaccines in neonates are challenging and the state of the neonatal immune response is a limiting factor. There are two main disadvantages with vaccine development in neonates. The first is that the antibodies passively received from the mother can interfere with potential vaccines (Lambert et al., 2014). Specifically, the maternal antibodies can have an effect on antigenic load, or mask specific epitopes that decrease the generation of antibodies (Lambert et al., 2014; Siegrist et al., 1998a; Siegrist et al., 1998b). The second limitation is the immaturity of the immune responses in neonates. This means that, even in scenarios where the maternal antibodies are not a limiting factor, the neonatal responses are weak and polarised (Siegrist et al., 1998a; Siegrist et al., 1998b). This 'immaturity' of the humoral responses in neonates can also be shown in the administration of a measles vaccine in 6 month old infants (Gans et al., 1998). Despite the diminished presence of neutralising antibodies as infants, sufficient protection was elicited 5-10 years after primary measles immunisation especially after administration of subsequent 'booster' doses (Gans et al., 2013). This suggests that, with a vaccine that elicits the optimal target and immune response, neonatal vaccination is feasible. Another solution for developing an RSV vaccine in neonates is to administer specific TLR agonists as adjuvants that would increase the immune response toward the vaccine (Mifsud et al., 2014). To do so, further understanding of TLR signalling in neonates is required in order to induce an optimum response toward an RSV vaccine and thus overcome the limitation of vaccine administration.

2 Hypothesis and Aims

2.1 Hypothesis

i) Alveolar macrophages are necessary for initiating inflammatory responses during RSV infection.

ii) The neonatal lung responds differently to innate stimulation compared to the adult lung.

1.1 Aims

i) Establish how alveolar macrophages are activated by RSV and what inflammatory mediators they produce.

ii) Determine if alveolar macrophages are infected by RSV.

iii) Evaluate neonatal lung responses to TLR stimulation and how they compare to the adult lung.

iv) Determine whether the right adjuvant can elicit a response in the neonatal lung.

3 Materials

3.1 Mice

Wildtype (wt) C57BL/6 mice were purchased from Charles River or Harlan (UK). Type I interferon α/β receptor knockout (*Ifnar1*$^{-/-}$) mice (obtained from C Reis e Sousa, The Francis Crick Institute, UK) and *Ifna6*^{gfp} and mitochondrial antiviral-signalling protein knockout (*Mavs*$^{-/-}$) (obtained from S. Akira, Japan) were screened to ensure the genotype was maintained. All mice were bred and maintained in specific pathogen-free conditions. For experiments mice were gender matched and aged 8-12 weeks old for adult experiments or 4-6 days old for neonates. All animal experiments were reviewed and approved by the Animal Welfare and Ethical Review Board (AWERB) within Imperial College London and approved by the UK Home office in accordance with the Animals (Scientific Procedures) Act 1986 and the ARRIVE guidelines.

3.2 Consumables

Name	Provider
24-well tissue culture plates	Thermo Scientific, DE, USA
96-well ELISA microplates	Microlon®, GER;
96-well plates (V-bottom or culture)	Costar, New York, NY, USA
175 cm^2 culture flasks	Cell Star, Frichenhausen, GER
C-Tubes	Miltenyi Biotec, Cologne, GER
Cell Strainers Nylon (100 μm)	BD Bioscience, Oxford, UK
CryoTube™ Vials	Thermo Scientific, Wilmington, DE, USA
Cytospin Slides	Tharmac, Waldsoms, GER
ELISA MaxiSorp plates	Nunc, Roskilde, DEN; GBO, GER
Falcon Tubes (15 and 50 ml)	Fisher Scientific, PA, USA
Low adherence ultra-long round bottom polystyrene 96-well plates	Corning, New York, NY, USA
MicroAmp Optical Adhesive Film	Applied Biosystems, Paisley, UK
MultiCount 10™	ImmuneSystems, Paignton, UK
Natural thin walled tubes (0.2 ml)	Bioquote Limited, York, UK
Phase Lock Gel eppendorfs	VWR, Leicestershire, UK
Shandon Filter Cards	Thermo Scientific, Wilmington, DE, USA

3.3 Kits and Reagents

Name	Provider
Ambion® DNA-free™ kit	Applied Biosystems, Paisley, UK
BD CompBeads	BD Bioscience, Oxford, UK
BD Cytofix™	BD Bioscience, Oxford, UK
BSA	Sigma-Aldrich, Dorset, UK
Collagenase D	Roche, Products Limited, Welwyn Garden City, UK
Complete, Mini, EDTA-free Protease Inhibitor cocktail tablets	Roche Products Limited, Welwyn Garden City, UK
Dulbecco's Modified Eagle Medium	PAA, Pasching, Austria
DAB Substrate (plaque assay)	Sigma-Aldrich, Dorset, UK
DAPI	Thermo Scientific, Wilmington, DE, USA
Desoxyribonuclease I (bovine pancreas)	Sigma-Aldrich, Dorset, UK
ELISA grade streptavidin	Biosource, Paisley, UK
ELISA kits (CXCL1, CXCL10, CCL2, CCL3, IFN-γ, IFN-λ, TNF-α)	R&D systems, Minneapolis, MN, USA
Extravidin-peroxidase	Sigma-Aldrich, Dorset, UK
EZ-ECL	Biological Industries, Cromwell, CT, USA
Foetal Calf Serum	Gibco, Paisley, UK
High Capacity RNA-to-cDNA kit	Applied Biosystems, Paisley, UK
Hoechst 3358, Pentahydrate (bis-Benzimide)	Thermo Scientific, Wilmington, DE, USA
Human IFN alpha Matched Antibody Paris	Affymetrix, CA, USA
Imiquimod (5 mg)	InvivoGen, San Diego, CA, USA
Intracellular staining perm wash buffer (x10)	Biolegend, Cambridge, UK
L-Glutamine	Gibco, Paisley, UK
Lipopolysaccharide	Invivogen, CA, USA
Live/Dead® Fixable Aqua Dead Stain	Life Technologies, Paisley, UK
Luminex™ Cytokine Mouse Magnetic 20-Plex	Invitrogen, Paisley, UK
MagicMark™XP Western protein standard	Invitrogen, Paisley, UK
Mouse Procarta 2-plex (IFNalpha.beta)	eBioscience, Hatfield, UK
Nuclease-free water	Promega, Southampton, UK
NuPage 4-12% Bis-Triz Gel (1.0 mm x10)	Novex, Wilmington, DE, USA
NuPage buffer	Novex, Wilmington, DE, USA
Phosphate buffered saline	PAA, Pasching, Austria
Pierce™ BCA protein assay kit	Thermo Scientific, Wilmington, DE, USA
Penicillin-Streptomycin	PAA, Pasching, Austria
poly(I:C) (5 mg)	InvivoGen, San Diego, CA, USA
ProLong® Gold Antifade Mountant	Thermo Scientific, Wilmington, DE, USA
PVDF filter paper 0.45 pore size	Novex, Wilmington, DE, USA
QuantiTec Probe PCR kit	Qiagen, Crawley, UK

Resiquimod (5 mg)	InvivoGen, San Diego, CA, USA
Rat IgG2b anti-mouse CD16/CD32	BD Biosciences, Oxford, UK
RNase AWAY	Molecular BioProducts, San Diego, CA, USA
RNase-Free DNase set	Qiagen, Crawley, UK
RNAeasy Mini Kit	Qiagen, Crawley, UK
RNAeasy Micro Kit	Qiagen, Crawley, UK
RT^2 PreAMP cDNA synthesis kit	Qiagen, Crawley, UK
RT^2 Profiler PCR arrays- antiviral response (PAMM-122Z)	Qiagen, Crawley, UK
RT^2 Profiler PCR arrays- mouse cytokines (PAMM-150Z)	Qiagen, Crawley, UK
Streptavidin-HRP	Biosource, Paisley, UK
Sytox Blue Dead Cell Stain	Life Technologies, Paisley, UK
TMB single solution	Invitrogen, Paisley, UK
Tris-Glycine Transfer Buffer	Novex, Wilmington, DE, USA
Trizol® Reagent	Invitrogen, Paisley, UK
Trypan Blue	Sigma-Aldrich, Dorset, UK
Trypsin-EDTA	Sigma-Aldrich, Dorset, UK
QuantiTec® Probe PCR kit	Qiagen, Crawley, UK
Quick-Diff staining kit	Reagena LTD, Toivala, FI

3.4 Instruments

Name	Provider
Aria III-U sorter	BD Bioscience, Franklin Lake, NJ, USA
BD LSRII	BD Bioscience, Franklin Lake, NJ, USA
BD FortessaLSR	BD Bioscience, Franklin Lake, NJ, USA
BD FortessaLSR-SORP	BD Bioscience, Franklin Lake, NJ, USA
BioPlex 200	Bio-Rad Laboratories, UK
Cytospin III Cytocentrifuge	Cytospins Thermo, Shandon, UK
Eppendorf centrifuge 5810R (Rotor-A-4-81)	Eppendorf, Cambridge, UK
7500 Fast Real-Time PCR system	Applied Biosystems, Paisley, UK
Fusion RX	Viber Lourmat, Eberhardzell, GER
GeneAmp® PCR system 9700	Applied Biosystems, Paisley, UK
GentleMACS dissociator	Miltenyi Biotec, Surrey, UK
Heraeus Megafuge 40R (Rotor75003807)	Thermo Scientific, Wilmington, DE, USA
Herra Safe Microbiological Safety Cabinet	Thermo Scientific, Wilmington, DE, USA
NanoDrop 1000 Spectrophotometer	Thermo Scientific, Wilmington, DE, USA
PowerPac HC 250V	Biorad, Hercules, CA, USA
Sorvall legend Micro 21	Thermo Scientific, Wilmington, DE, USA
SPECTRAMax plus S384	Molecular Devices, Berkshire, UK
Tissue Culture Incubator	Binder Inc., Bohemia, NY, USA
Tissue Lyser LT	Qiagen, Crawley, UK
Ultrasonic Water Bath- XUBA3	Asynt, Cambridge, UK
Zeiss confocal LSM-510	Zeiss, Jena, GER

3.5 Chemicals

Name	Provider
Bromophenol blue	Sigma-Aldrich, Dorset, UK
Chloroform	VWR, Leicestershire, UK
Ethanol (96%)	VWR, Leicestershire, UK
Hydrogen Peroxidase 30% (w/w)	Sigma-Aldrich, Dorset, UK
Isopropanol (100%)	VWR, Leicestershire, UK
Methanol (99.5%)	VWR, Leicestershire, UK
β-Mercaptoethanol	Sigma-Aldrich, Dorset, UK
Paraformaldehyde	Sigma-Aldrich, Dorset, UK
PBS tablets	Sigma-Aldrich, Dorset, UK
Sulphuric acid	Sigma-Aldrich, Dorset, UK
Triton X-100	Sigma-Aldrich, Dorset, UK
Tween 20	Sigma-Aldrich, Dorset, UK

3.5.1 Anaesthetics

Name	Provider
Isofluorane	Centaur, Somerset, UK
Pentobarbitone	Centaur, Somerset, UK

3.6 Software

Name	Provider
Adobe Illustrator	Adobe Systems Europe Limited, Berkshire, UK
BioPlex Manger v6.1	Bio-Rad Laboratories, UK
DIVA	BD Bioscience, Paisley, UK
GraphPad Prism v6.0	Graph Pad software, La Jolla, CA, USA
7500 Fast Systems SDS v1.4	Applied Biosystems, Paisley, UK
Fiji	ImageJ, NIH, Bathesda, MD, USA
Flowjo v10	Tree Star, Ashland, OR, USA
Fusion FX7	Viber Lourmat, Eberhardzell, GER
RT^2 Profiler PCR Arrays data analysis v3.5	Qiage, Crawley, UK
SoftMax Pro v5.2	Molecular Devices, Berkshire, UK

3.7 Media, solutions and buffers

Table 3-1 Contents of media, solutions and buffers

Name	Contents	Final concentration
ACK buffer	Distilled water	-
	$KHCO_3$	0.15 mM
	Na_2EDTA	0.1 mM
	NH_4CL	0.15 mM
BAL media	EDTA	0.5 mM
	PBS	1x
cDMEM	DMEM (500ml)	1x
	L-glutamine	2 mM
	Penicillin-Streptomycin	100 U/ml
	Heat-inactivated FCS	10%
Extraction media primary AMs (*ex vivo*)	EDTA	0.25 mM
	PBS	1x
FACS buffer	BSA	1%
	EDTA	0.05 mM
	NaN_3	0.005%

4 Methods

4.1 Growing respiratory syncytial virus (RSV)

Plaque purified human RSV A2 was originally obtained from the American Type Culture Collection (ATCC). Prior to infection 1 x 10^7 human larynx epidermoid carcinoma cells (HEp2) cells were seeded in 175 cm^2 flasks (Cell Star, GER) in complete Dulbecco's Modified Eagle Medium (cDMEM) (Table 3-1) and incubated overnight (all incubations were performed at 37°C, 5% CO_2). After expanding to a confluence of 70% the cells were washed with serum-free DMEM and infected with RSV at a multiplicity of infection (MOI) of 0.1 in 4 ml serum-free DMEM for two hours (hrs). The flasks were rotated at a 90° angle every 15 minutes (min) in order to spread the infection throughout the monolayer equally. After the incubation 26 ml of DMEM + 10% FCS was added and the cells were incubated for 24 hrs. The concentration of FCS was reduced to 2% by removing 25 ml of medium from the flasks, spinning it at 500 x *g* for 5 min at 4°C and resuspending the pellet in 25 ml of serum-free medium. The resuspened suspension was transferred to its respective flask. Incubation followed for a further 24-48 hrs in which the cytopathic effect caused by the virus was assessed regularly. Once 80% of the cells had detached, all cells were removed using a cell scraper and sonicated for 2min in a cold-water bath. After centrifugation (300 x *g* for 5 min at 4°C) the supernatant was aliquoted into cryovials and immediately snap-frozen in liquid nitrogen.

4.2 Immunoplaque assay

4.2.1 Viral stock quantification

2.5 x 10^4 HEp2 cells/well in antibiotic-free cDMEM were seeded into a flat bottom 96-well plate and incubated at 37°C, 5% CO_2 for up to 24 hrs (or until 90% confluency was achieved). The cells were washed with PBS and the virus stock was serially diluted in serum-free DMEM with an initial dilution of 1:200 (total volume 50 µl). All dilutions were performed in duplicate. Diluted virus was added to the monolayers and the cells were incubated for 2 hrs on a shaker before 150 µl of pyruvate-free DMEM + 2% FCS was added and incubated for 16-20 hrs at

37°C, 5% CO_2. The medium was expelled and the cell monolayer was washed with 100 µl PBS and fixed for 20 min at room temperature (RT) by adding 100 µl absolute methanol containing 2% hydrogen peroxide. Cells were then washed twice with 200 µl of PBS/1% BSA and were kept at 4°C overnight. Biotinylated anti-RSV (AbD serotec, UK), diluted 1:500 in PBS/1% BSA, was added to each monolayer (100 µl/well) and incubated for 2 hrs at RT. The plate was washed before adding 100 µl of extravidin peroxidase (1:500 in PBS/1%BSA; Sigma-Aldrich, UK) for 2 hrs. After washing the plate 5 times, 50 µl of DAB (3,3'-diaminobenzidine; Sigma-Aldrich, UK) substrate was added to each well and observed for 10-20 min or until the desired staining was achieved. The reaction was stopped by washing the plate twice with 200 µl PBS/1% BSA followed by a delicate washing step with H_2O. The monolayer was then air-dried before the stained cells (plaques) were counted using a light microscope. The focus forming units (FFU)/ml were calculated using the following equation:

$$\frac{stained\ cells\ counted\ x\ dilution}{volume\ added}$$

4.2.2 Assessing viral titre

4.2.2.1 RSV infections *in vivo*

The weight of the tissue was determined before the tissue was homogenised in 300 µl DMEM using a tissue lyser (TissueLyser LT, Qiagen, UK). The homogenate was spun for 1 min at 15000 x *g* and the supernatant was serial diluted in DMEM and the viral load was quantified as described in 4.2.1.

4.2.2.2 RSV exposure *ex vivo*

Cell supernatants were serial diluted and transferred to the HEp2 cell monolayer and the viral titre was quantified as described in 4.2.1.

4.2.2.3 Ultraviolet inactive RSV

For ultraviolet inactive RSV (UV-RSV) the virus was placed in a 12-well plate on ice and then exposed to UV light for 2 min in a CX-2000 UV crosslinker (UVP, USA).

4.3 Murine Model

Experiments were performed on 8-12 week old (adults, average weight 20 g) or on 4-6-day old (neonates, average weight 4 g) mice. When receiving virus or ligands intranasally (i.n.) mice were lightly anaesthetised using isoflurane (Centaur, Somerset, UK). Mice were euthanized at the desired timepoints by intraperitoneal injection of pentobarbitone (Centaur, Somerset, UK); 100 μl for adults and 50 μl for neonates.

4.3.1 Infection with RSV

During a brief anaesthesia, adult mice were infected i.n. with 1 x 10^6 FFU of RSV in a volume of 100 μl.

4.3.2 Exposure to toll-like receptor (TLR) ligands

Adult mice received TLR ligands i.n. in a volume of 100 μl; whereas neonates received them in a 20 μl volume. The ligands and concentrations used are presented in Table 4-1.

Table 4- 1 Amount of TLR agonists administered during *in vivo* challenges

Agonist name	Receptor target	Concentration/g body weight
CL264 Rhodamine	TLR7	1.25 μg
CpG	TLR9	1.25 μg
imiquimod	TLR7	2.5 μg
LPS	TLR4	500 ng
poly(I:C)*	TLR3	2.5 μg
R848	TLR7	2.5 μg

*poly(I:C) can also activate the RLR signalling pathways.

4.3.3 Tissue and cell collection

4.3.3.1 Harvest and processing of lung tissue

The lungs were perfused with PBS in order to flush the blood out of the lungs. The superior, middle and inferior lung right lobes were placed into cryovials and immediately snap frozen in liquid nitrogen. The left lung and post-caval lobe were placed into C-tubes (Miltenyi Biotec, GER) containing 2.5 ml cDMEM. For experiments where intracellular cytokine staining (ICS), the cDMEM contained 5 ng/ml Brefeldin A (BFA, Sigma-Aldrich, Dorset, UK), DNase (30 μg/ml, Sigma-Aldrich, Dorset UK) and 2.5 ml of Collagenase D (1 mg/ml, Roche, W. Sussex, UK) and the lung tissue was processed with the gentleMACS dissociator according to the manufacturer's instructions (Miltenyi Biotec, GER). Further steps of ICS staining for innate cells contained 20 μg/ml of GolgiStop (containing monensin, BD Biosciences, UK) was used instead of Brefaldin A. After an incubation of 1 hr (37°C, 5% CO_2 on a shaker) the tissue was further homogenised using the gentleMACS dissociator. The lung cells were washed with 5 ml PBS followed by a 5min incubation with ACK buffer (Table 3-1) at RT. Finally, 5 ml of cDMEM was added to each sample and were centrifuged at 300 x *g* for 5 min at 4°C. The pellet was then resuspended in 3 ml of PBS and filtered through a 100 μm strainer. Cells were enumerated using trypan blue (see section 4.4) and resuspended in FACS Buffer (Table 3-1).

4.3.3.2 Serum collection

After euthanasia blood was collected from the femoral or carotid arteries. Approximately 200 μl of blood was collected from each mouse and placed at 4°C overnight. The clotted blood was removed and the remaining serum was aliquoted and stored at -80°C.

4.3.3.3 Bronchoalveolar lavage (BAL) collection and processing

At each endpoint the trachea was exposed and a small incision was made using a scalpel. The lungs with flushed 3 times with the same 1 ml of BAL media (Table 3-1) passing through a 2

ml syringe with tubing attached. For neonates the lungs were flushed with 250 μl BAL media using a 1 ml syringe with tubing attached. The BAL was spun at 3500 x *g* for 5 min and the supernatant (BALF) was collected and stored at -80°C. The cell pellet was resuspended in 100 μl of ACK buffer for 2 min and the then 400 μl of cDMEM was added (150 μl for neonates). The cells were enumerated on counting slides using trypan blue and then spun at 3500 x *g* for 5 min before being resuspended in 500 μl of media (250 μl for neonates) and used for differential cell counts on cytospin slides (see section 4.4).

4.3.3.3.1 Extracting primary alveolar macrophages (AMs) ex vivo

More than 98% of cells in naïve mice are AMs (Goritzka et al., 2014). The trachea was exposed and a small incision was made using a scalpel. The lungs were filled with 1 ml of ice cold BAL media for 5 min before being flushed 3 times with the same fluid. This process was repeated a second time and the collected BAL was pooled from several mice. The cells were spun at 300 x *g* for 10 min at 4°C and then resuspended in ACK for 2 min before receiving 2 ml of cDMEM (volumes representative of 5-mouse batches). The cells were enumerated using counting slides and trypan blue (see section 4.4). After being spun for 10 min at 300 x *g* at 4°C the cells were resuspended with cDMEM achieving a concentration of 1.25 x 10^6 cells/ml. The cells were seeded into flat bottom 96-well plates (1.25 x 10^5 cells/well) and incubated at 37°C, 5% CO_2 for 2 hrs. The cells were washed three times with 200 μl of cDMEM before being exposed to various conditions (Table 4-2) for 21hrs.

Table 4-2 Dosage or concentrations primary alveolar macrophages were exposed to *ex vivo*

Name	Concentration/*Dosage*
CpG	1 ng/ml
LPS	1 ng/ml
PR8	*MOI2*
R848	1 ng/ml
RSV	*MOI 2*
UV-PR8	*MOI 2*
UV-RSV	*MOI 2*

4.4 Total and differential leukocyte count

The total live cell count for lung and BAL isolated cells was performed manually using 0.1% Trypan Blue. For differential leukocyte cell counts 100 µl of cell suspension from processed BALF was added to 100 µl of medium in a cytofunnel attached to a glass slide. The cells were then transferred to a cytocentrifuge (Cytospin III Cytocentrifuge, Thermo Scientific, USA) and spun at 400 rpm for 5 min. The slides were then left to dry overnight and then fixed and stained using the Quick-Diff staining kit (Reagena, Finland). A total of 300 cells were counted using a light microscope providing the proportion of macrophage, lymphocyte, neutrophil and eosinophil populations.

4.5 Flow cytometry analysis

4.5.1 Flow cytometric analysis of lung leukocyte populations

At least 2 x 10^6 cells/well were seeded in a round-bottom 96-well plate, centrifuged for 2 min at 700 x *g* at 4°C and washed using staining buffer (FACS buffer) (Table 3-1). The cells were incubated for 20 min with a purified rat IgG2b anti-mouse CD16/CD32 receptor antibody (Fc Block; 1:200 in FACS Buffer, BD Bioscience, UK). The cells were then washed and surface markers were stained with fluorochrome-conjugated antibodies in the dark for 30 min at 4°C (Table 4-3). Cells were washed with PBS before being incubated with 100 µl LIVE/DEAD® Fixable Aqua Dead Stain (1:1000 in PBS; Life Technologies, UK) for 30 min at 4°C in the dark. Cells were washed twice and resuspended in FACS buffer and acquisition was followed on BD LSR Fortessa using the FACSDiVa software (BD Bioscience, USA).

The gates were set using FMO controls and gating strategy is shown in Fig 4.1 and 4.2. Briefly, the cells were gated on non-debris followed by selection of singlets and live cells. All cell gates were set using an FMO control gated on the CD45+ leukocytes. Two gating strategies were used either to define the innate cell (section 4.5.1.1) or T cell (section 4.5.1.2) subsets.

Table 4-3 Antibodies for flow cytometry surface stain

Antigen	Clone	Fluorochrome	Isotype	Supplier
CD3	145-2C11	PE-Cy7	Rat IgG2b,χ	BD Pharmingen, Oxford, UK
CD4	GK1.5	APC-Cy7	Rat IgG2b,χ	BD Pharmingen, Oxford, UK
CD8α	53-6.7	AF700	Rat IgG2a,χ	BD Pharmingen, Oxford, UK
CD11b	M1/70	AF700	Rat IgG2b,κ	eBioscience, Hatfield, UK
CD11c	HL3	PE-CF594	Armenian Hamster IgG1,λ2	BD Pharmingen, Oxford, UK
CD19	1D3	FITC	Rat IgG2a,κ	eBioscience, Hatfield, UK
CD45	30F11	eFluor780	Rat IgG2b,κ	eBioscience, Hatfield, UK
CD45R (B220)	RA3-6B2	eFluor450	Rat IgG2a,κ	eBioscience, Hatfield, UK
CD64 (FcγRI)	X54-5/7.1	APC	Rat IgG2b,κ	eBioscience, Hatfield, UK
CD103	2E7	PerCP/Cy5.5	Armenian Hamster IgG1,λ2	Biolegend, Cambridge, UK
CD335 (NKp46)	29A1.4	eFluor450	Rat IgG2a,κ	eBioscience, Hatfield, UK
Ly6C	HK1.4	eFluor450	Rat IgG2c,κ	eBioscience, Hatfield, UK
Ly6G	1A8	BV570	Rat IgG2a,κ	BioLegend, Cambridge, UK
Siglec F	E50-2440	PE	Rat (LOU) IgG2a,κ	BD Pharmingen, Oxford, UK
Siglec H	eBio440c	eBio440c	Rat IgG2b	eBioscience, Hatfield, UK

4.5.1.1 Gating strategy for innate cells

For innate cell gating the neutrophils were selected by the expression of Ly6G whereas all non-neutrophils gated on the expression of siglec-F and CD11c (Fig 4.1-1). This distinguished AMs ($CD11c^{+}SigF^{+}$) or eosinophils ($SigF^{+}CD11c^{-}$) (Fig 4.1-2). All siglec-F^{-} cells on $CD64^{hi}CD11b^{hi}$ populations followed by Ly6C and CD11c expression (Fig 4.1-3). This defined $CD64^{hi}CD11b^{-}Ly6C^{hi}$ (inflammatory monocytes) or for $CD64^{hi}CD11b^{-}Ly6c^{-}CD11c^{+}$ (moDCs) (Fig 4.1-3). The $siglecF^{-}CD11c^{+}$ cells (Fig 4.1-2) were gated and $CD103^{+}$ DCs and $CD11b^{+}Ly6C^{-}CD64^{+}$ DCs (CD11b DCs) were defined (Fig 4.1-4). For intracellular staining (ICS) 5 ng/ml Brefeldin A was included in the harvest medium; whereas 20 μg/ml of GolgiStop (containing monensin, BD Bioscience, UK) was included in every step of staining (see sections 4.3.3.1, 4.3.3.3). Each population was further assessed for the expression of IL-6 (see section 4.5.2).

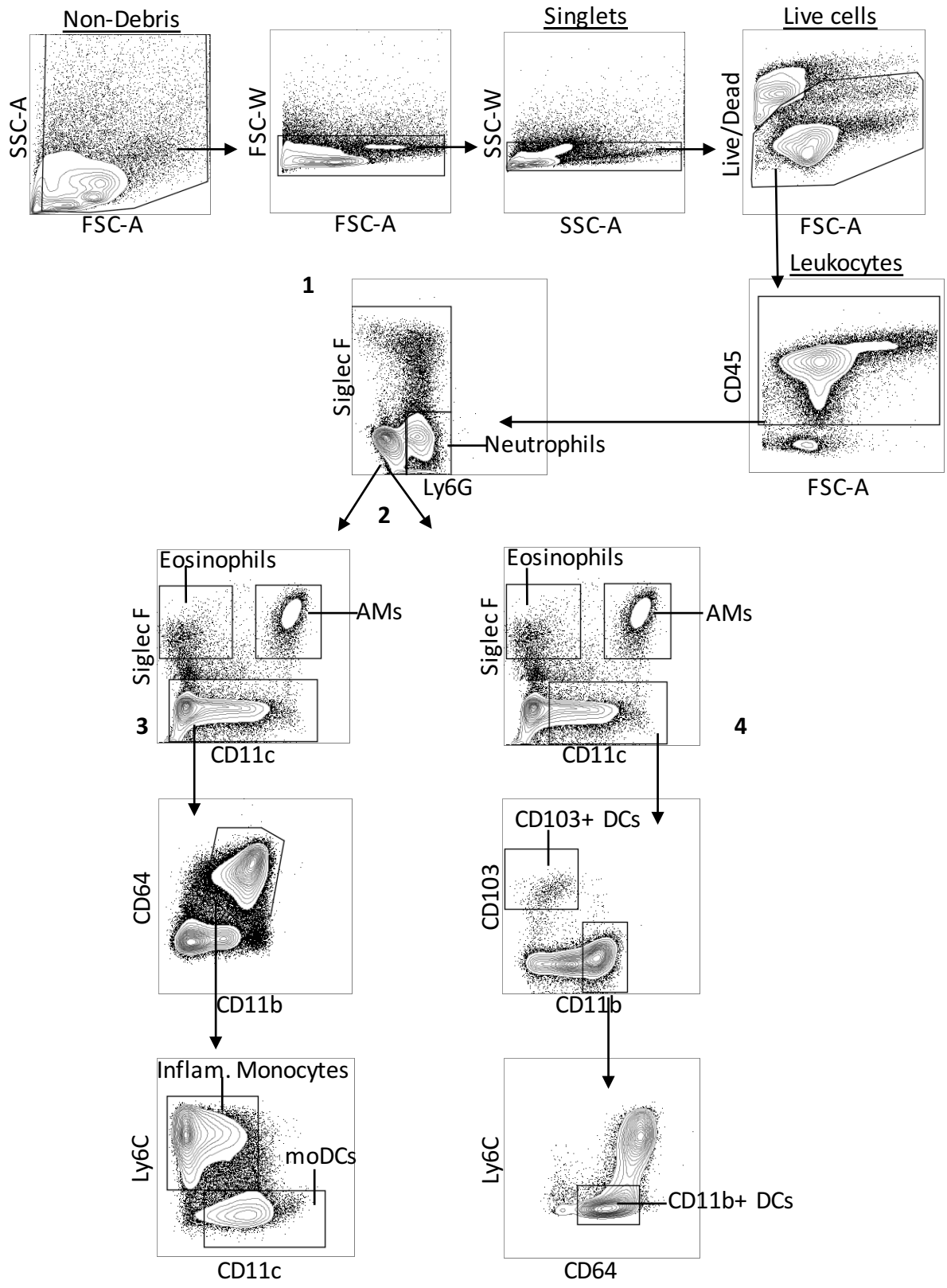


Figure 4.1 Gating strategy for innate cell surface markers. Lung and BAL cells were gated to remove debris, doublets using the forward and side scatter area and width (FSC and SSC-A or -W). Dead cells were removed using a viability dye and the cells and all $CD45^{+}$ leukocytes

populations were subsequently characterised. (1) Neutrophils where selected using the Ly6G marker. All 'non-neutrophil' cells were then gated on the expression of siglec-F or CD11c. (2) Alveolar macrophages (AMs) were discriminated as $CD11c^{hi}sigF^{hi}$ and eosinophils as $siglecF^{hi}CD11c^{-}$. (3) All siglec-F^{-} cells were then gated for $CD64^{hi}CD11b^{-}Ly6C^{hi}$ (inflammatory monocytes) or for $CD64^{hi}CD11b^{-}Ly6c^{-}CD11c^{+}$ (moDCs). (4) The $siglecF^{-}CD11c^{+}$ cells were selected and $CD103^{+}$ DCs and $CD11b^{+}Ly6C^{-}CD64^{+}$ DCs (CD11b DCs) were identified. For intracellular staining each population was individual assessed for cytokine expression.

4.5.1.2 Gating strategy for T cells

The $CD45^{+}$ leukocytes were gated on $CD19^{-}$ cells in order to exclude B cells. All selected cells were then gated on the expression of CD3 marker. The $CD3^{+}$ cells were then gated on the expression of CD4 and CD8 in order to define $CD4^{+}$ and $CD8^{+}$ T cells respectively. All gates were set using FMO controls. For ICS the $CD4^{+}$ and $CD8^{+}$ T cells were assessed on the expression of IFN-γ and IL-4 (see section 4.5.2).

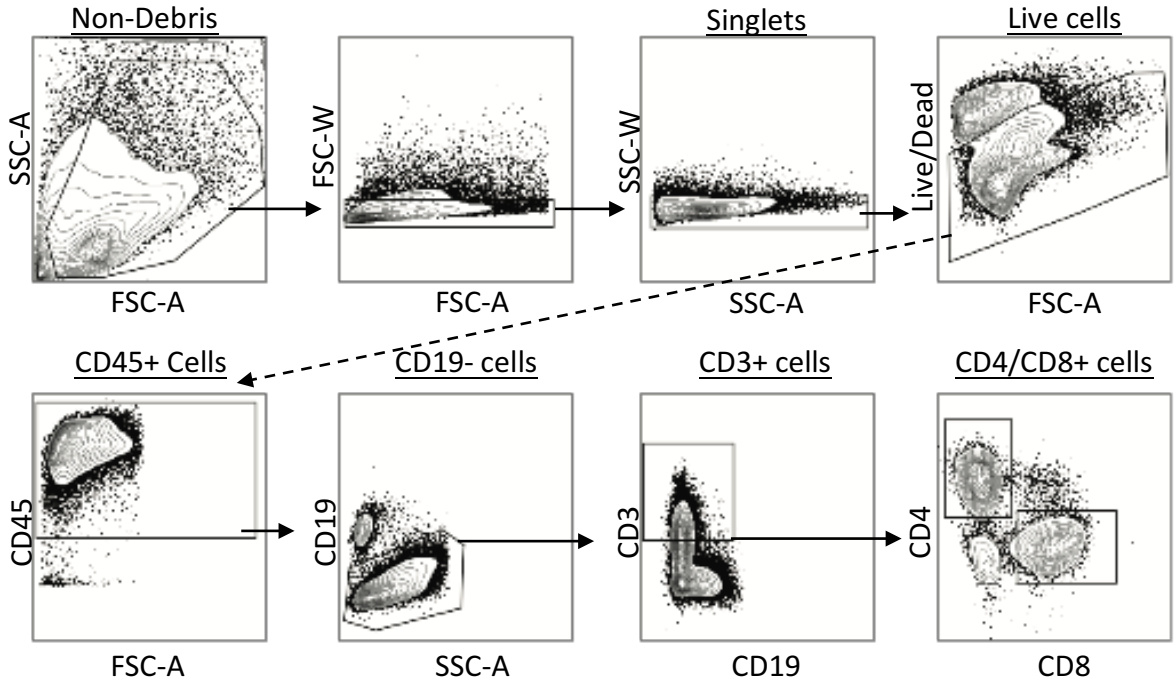


Figure 4.2 Gating strategy for T cell surface markers. Lung and BAL cells were gated to remove debris, doublets using the forward and side scatter area and width (FSC- and SSC-A or -W). Dead cells were removed using a viability dye and the cells and all $CD45^{+}$ leukocytes

populations were subsequently characterised. The $CD19^-$ cells were then gated in order to remove neutrophils and the $CD3^+$ cells were selected. The $CD3^+$ T cells were then distinguished by the expression of CD4 or CD8 receptors. For intracellular staining the populations were individual assessed for cytokine expression.

4.5.2 Flow cytometric analysis of intracellular cytokines

Before intracellular staining the cells were stained for surface markers as described in section 4.4.1. and were fixed in BD Cytofix/Cytoperm (BD Bioscience, UK). Intracellular staining (ICS, Table 4-4) was performed in permeabilisation buffer containing Fc Block for 60 min at 4°C in the dark. The cells were washed with permeabilisation buffer (Biolegend, UK), stained for intracellular markers and resuspended in FACS buffer. Cells were acquired on BD LSR Fortessa using FACSDiVa software (BD Bioscience, UK). The same gating strategy as section 4.4.1. was used for ICS however every population was assessed for the presence of the targeted cytokines.

Table 4-4 Antibodies for intracellular stain

Antigen	Clone	Fluorochrome	Isotype	Supplier
IL-1β	NJTEN3	PerCp-eFluor710	Rat IgG1,κ	eBioscience, Hatfield, UK
IL-4	11B11	BV605	Rat IgG1,κ	Biolegend, Cambridge, UK
IL-6	MP-520F3	APC	Rat IgG1,κ	Biolegend, Cambridge, UK
IL-10	JESS-16E3	BV421	Rat IgG2b,κ	Biolegend, Cambridge, UK
IFN-γ	XMG1.2	BV711	Rat IgG1,κ	BD Pharmingen, Oxford, UK
TNF-α	MP-XT22	BV650	Rat IgG1,κ	Biolegend, Cambridge, UK

4.5.2.1 ICS for primary AMs *ex vivo*

After exposure to various conditions for 6 hrs, 20 μg/ml of GolgiStop was added to the cultures for an additional 14 hrs (see 4.3.3.3.1), AMs were washed with PBS and then resuspended with cold extraction media (Table 3.7) to remove them from the culture plates. After a 5 min incubation at RT the cells were vigorously pipetted and transferred into a 15 ml Falcon tube (Fisher Scientific, USA). The wells were further washed with extraction media. The cells were enumerated and staining for surface marker and ICS proceeded (see 4.4.1 and 4.4.2). For each step of the staining methods 20 μg/ml GolgiStop was included. The surface antibodies used for studying primary AMs are shown in Table 4-5.

Table 4-5 Antibodies used for fluorescent-activated cell sorting

Antigen	Clone	Fluorochrome	Isotype	Supplier
CD11c	HL3	PE-CF594	Armenian Hamster IgG1,λ2	BD Pharmingen, Oxford, UK
Ly6G	1A8	BV570	Rat IgG2a,κ	BioLegend, Cambridge, UK
Siglec F	eBio440c	eBio440c	Rat IgG2b	eBioscience, Hatfield, UK

4.5.2.2 T cell flow cytometry staining

BAL and lung single cell suspensions were transferred to 96-well plate (see 4.3.3). The cells were exposed to 100 ng/ml of phorbol myristate acetate (PMA) and 1 μg/ml of ionomycin with brefeldin for 1 hr (37°C, 5% CO_2). The cells were then processed as described in sections 4.5.1 and 4.5.2. T cells were gated as shown in Fig 4.2 and the surface antibodies used are described in Table 4-3. Approximately 2.5 x 10^5 $CD45^+$ cells were collected and the $CD4^+$ and $CD8^+$ T cell populations were then assessed for the expression of IFN-γ and IL-4 (see section 4.5.1.2 and Table 4-4).

4.6 Fluorescent-activated cell sorting (FACS)

BAL cells were acquired from wt mice (described in 4.3.3.3) and pooled for each desired group. The cells were enumerated and transferred into a round-bottom 96-well plate. Cells were incubated with FACS buffer containing Fc Block (1:200; BD Biosciences UK) for 20 mins at 4°C. Cells were washed with FACS buffer and incubated with fluorochrome-conjugated antibodies against the cell surface markers for 45 min at 4°C (see Table 4-5). The cells were washed twice with FACS buffer and 1 μl of Hoechst 3558, Pentahydrate (Live/dead antibody; Thermo Scientific, USA) immediately before running them on BD FACSAria III using FACSDiVa software (BD Biosciences, USA). The cells were sorted into AMs and neutrophils according to the gating strategy in Fig 4.3.

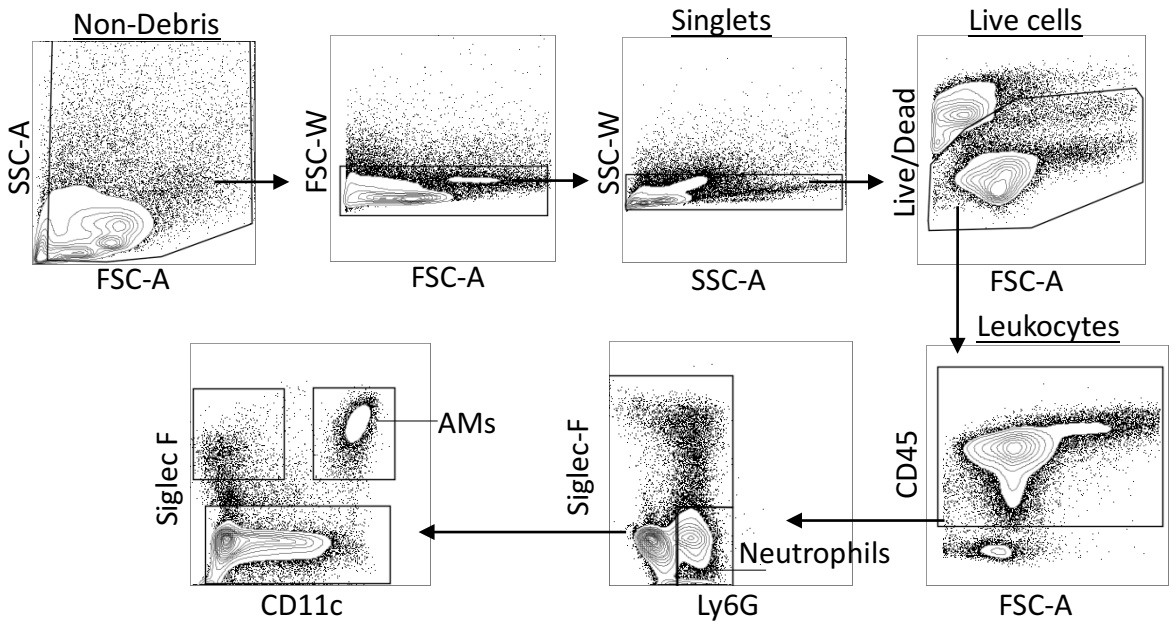


Figure 4.3 Gating strategy for fluorescent-activated cell sorting of alveolar macrophages. All debris, doublets and dead cells were excluded. The remaining cells were gated on the expression of CD45 to define leukocytes. The neutrophils were defined as Ly6G+. All non-neutrophils were gated on the expression of siglec-F and CD11c. The AMs (sigFhiCD11chi) were sorted. All gates were set using FMO controls and the purity of the sorted AMs was over 98%.

4.7 Measurement of inflammatory mediators

4.7.1 RNA extraction from AMs and animal tissue

The primary AMs exposed to stimuli *ex vivo* were resuspended in Trizol™ Reagent and transferred into Phase Lock Gel eppendorfs (VWR, UK). The RNA was then extracted using phenol-chloroform extraction (see sections 4.6.1.1).
The frozen lung lobes were homogenised using a tissue lyser (TissueLyser LT, Qiagen, UK) and RNA was extracted using the RNeasy kit (Qiagen, UK) according the manufacturer's instructions including a DNA digestion step.

4.7.1.1 Phenol-chloroform extraction

200 μl of chloroform was added to the Phase Lock Gel eppendorfs (VWR, UK) and shaken vigorously for 15 seconds (s) and left at RT for 10 min. The tubes were then then centrifuged for 15 min at 4°C at 12000 x *g*. The RNA-containing aqueous phase was transferred into a new eppendorf tube and incubated for 10 min at RT in 800 μl of Isopropanol (VWR, UK). The tubes were briefly rotated 10 times and then centrifuged for 10 min at 4°C at 12000 x *g*. The supernatant was disposed and the RNA was resuspended in 1 ml of cooled ethanol for 5 min at RT. The RNA was centrifuged for 5 min at 4°C at 7500 x *g*, resuspended in water and then DNase treated (see section 4.6.1.1.1.).

4.7.1.1.1 DNase treatment

The RNA from primary AMs that was extracted using phenol-chloroform were DNase treated using the Ambion® DNA-free™ Kit (Applied Biosystems, UK) following the manufacturer's instructions. The treated RNA was the quantified and transcribed (see section 4.6.3).

4.7.2 RNA extraction from sorted cells

Sorted AMs were resuspended in 1 ml of Trizol™ Reagent, transferred into Phase Lock Gel eppendorfs (VWR, UK) and 200 μl of chlorophorm was added. The cells were vigorously shaken for 15 s and incubated for 10 min at RT before being centrifuged for 15 min at 4°C at 12000 x *g*. The RNA-containing aqueous phase was processed using the RNeasy Mini kit (Qiagen, UK) including a DNA digestion step.

4.7.2.1 RNA quantification and RNA to complementary DNA (cDNA) conversion

The RNA yields from lung tissue, primary AMs and sorted AMs was determined using a Nanodrop 100 spectrophotometer. 1 μg or 0.5 μg of RNA from the lung tissue or primary AMs was reverse transcribed using a High Capacity RNA-to-cDNA kit according to manufacturer's instructions (Applied Biosystems, UK).
RNA from sorted AMs was converted using the RT^2 Pofiler PCR Array Handbook following the manufacturer's instructions.

4.7.3 Quantitative polymerase chain reaction (qPCR)

The quantifications of mRNA levels from lung tissue or AMs was performed using the primers and probes listed in Table 4-6. The probes were tagged with a double-dye (FAM-fluorophor, TAMRA-quencher). The gene quantifications were determined using the reaction mix shown in Table 4-7, 4-8, 4-9 and relative expression using the reaction mix shown in Table 4-10. All results were normalised to the housekeeping gene *Gapdh* to eliminate any differences in tissue, starting amount or enzymatic activity. *Gapdh* was used as an internal control for all reactions and its probe was tagged with VIC. When performing copy number/absolute quantification (Table 4-11) an internal plasmid standard was used to determine the copy number of the gene of interest. To determine the relative quantification of the gene of interest the expression of the gene was normalised to *Gapdh*. This was done by determining the ΔCt (Ct=cycle threshold) between the target gene and *Gapdh* for each sample and then calculating the 2^-ΔCt. The samples were analysed using the 7500 Fast System SDS software.

Table 4-6 Sequences primers and probes used for qPCRs

Primer/Probe	Sequence (5'-3')	Supplier
L-gene forward	GAACTCAGTGTAGGTAGAATGTTTGCA	Invitrogen, UK
L-gene reverse	TTCAGCTATCATTTTCTCTGCCAAT	Invitrogen, UK
L-gene probe	TTTGAACCTGTCTGAACATTCCCGGTT	Eurofins, GER
N-gene forward	CATCCAGCAAATACACCATCCA	Invitrogen, UK
N-gene reverse	TTCTGCACATCATAATTAGGAGTATCAA	Invitrogen, UK
N-gene probe	CGGAGCACAGGAGAT	Eurofins, GER
RSV positive strand	CCCCACTTTATAGATGTTTTTGTTCA	Invitrogen, UK
RSV negative strand	TCCTGCAAAAATCCCTTCAACT	Invitrogen, UK
***Tnfa* forward**	CATCTTCTCAAAATTCGAGTGACAA	Invitrogen, UK
***Tnfa* reverse**	TGGGAGTAGACAAGGTACAACCC	Invitrogen, UK
***Tnfa* probe**	CACGTCGTAGCAAAC	Eurofins, GER
***Ifnb* forward**	CCATCATGAACAACAGGTGGAT	Invitrogen, UK
***Ifnb* reverse**	GAGAGGGCTGTGGTGGAGAA	Invitrogen, UK
***Ifnb* probe**	CTCCACGTGCGTCCTGCTGTG	Eurofins, GER
***Oas1a* forward**	TCCTGGGTCATGTTAATACTTCCA	Invitrogen, UK
***Oas1a* reverse**	CCCCAGGGAGGTACATTCCT	Invitrogen, UK
***Oas1a* probe**	CAAGCCTGATCCCAGAATCTATGCCATC	Eurofins, GER
***Rsad2* forward**	CGAAGACATGAATGAACACATCAA	Invitrogen, UK
***Rsad2* reverse**	AATTAGGAGGCACTGGAAAACCT	Invitrogen, UK
***Rsad2* probe**	CCAGCGCACAGGGCTCAGGG	Eurofins, GER
***Eif2ak2* forward**	CGGAACATCCTCTAGCGTTGTC	Invitrogen, UK
***Eif2ak2* reverse**	GGGAAACACCATTACTTGTCATAGAC	Invitrogen, UK
***Eif2ak2* probe**	AGCTGCTGGAAAAGCCACTGA	Eurofins, GER
***Ifng* forward**	TCAAGTGGCATAGATGTGGAAGAA	Invitrogen, UK
***Ifng* reverse**	TGGCTCTGCAGGATTTTCATG	Invitrogen, UK
***Ifng* probe**	TCACCATCCTTTTGCCAGTT	Eurofins, GER
***Ifnl2/3* forward**	AAAGGATTGCCACATTGCTC	Invitrogen, UK
***Ifnl2/3* reverse**	TCAAGCAGCCTCTTCTCGAT	Invitrogen, UK
***Ifnl2/3* probe**	TCCCCAAAAGAGCTGCAGGC	Eurofins, GER
Ccl2, Ccl3, Csf2, Cxcl1, Cxcl9, Cxcl10, Ifna6, Ifna5, il1a, il1b, il2, il4,il5, il10, il1240/p70, il13, il17, Vegfa	Not revealed by supplier	Applied Biosystems, UK

Table 4-7 Reaction mix composition for absolute quantification of RSV L gene

Reagent	Volume/weight
Master Mix	6.25 µl
Forward primer (5 µM)	1.00 µM
Reverse primer (5 µM)	0.33 µM
Probe (5 µM)	0.2 µM
Nuclease-free water	x µl
Total	11.50 µl- (Master Mix + Forward primer + Reverse primer + Probe)
cDNA	1.00 µl

Table 4-8 Reaction mix composition for absolute quantification of RSV N gene

Reagent	Volume/weight
Master Mix	6.25 µl
Forward primer (5 µM)	0.49 mM
Reverse primer (5 µM)	0.49 mM
Probe (10 µM)	0.22 mM
Nuclease-free water	x µl
Total	11.50 µl- (Master Mix + Forward primer + Reverse primer + Probe)
cDNA	1.00 µl

Table 4-9 Reaction mix composition for absolute quantification of cytokines and interferons stimulated genes

Reagent	Volume/weight
Master Mix	6.25 µl
Forward primer (5 µM)	1.00 mM
Reverse primer (5 µM)	1.00 mM
Probe (5 µM)	0.20 mM
Nuclease-free water	x µl
Total	11.50 µl- (Master Mix + Forward primer + Reverse primer + Probe)
cDNA	1.00 µl

Table 4-10 Reaction mix composition for relative expression

Reagent	Volume (µl/well)
Master Mix	6.25
Target PP	0.63
Nuclease-free water	4.62
Total	11.50
cDNA	1.00

Table 4-11 qPCR thermal profile

Time (min)	Temperature (°C)	Cycle (number)
2	50	1
10	90	2
0.25	95	45
1	60	

4.7.4 RT^2 qPCR arrays

The quantification of mRNA levels in sorted AMs from infected or naïve mice was performed following the manufacturer's instructions in the Real-Time PCR for RT^2 Profiler PCR Array protocol for formats A, C, D, E, F and G (Antiviral PAMM-150Z and chemokines/cytokines PAMM-122Z, Qiagen, UK).

4.8 Measurement of inflammatory mediators

4.8.1 Enzyme-linked Immunosorbent Assay (ELISA)

Protein concentrations from BALF and the supernatant from primary AMs exposed to innate stimuli *ex vivo* were measured in 96-well ELISA microplates (Microlon®, Germany). Measurement of mediators where ELISA kits were commercially available (CCL2, CCL3, CXCL1, CXCL10, GM-CSF, IFN-γ, and TNF-α) was performed following the manufacturer's instructions (R&D systems, UK). For measurement of cyclic adenosine monophosphate (cAMP), the lung tissue and BALF were processed and assessed according to the manufacturer's instructions (R&D systems, UK). For IFN-α and IL-6 measurement (Table 4-12 and 4-13 respectively), plates were coated with 50μl of capture antibody diluted in coating buffer (0.1M $NaHCO_3$, pH 8.2) and incubated overnight at 4°C. The plate was washed 3 times with washing buffer (1x PBS, 0.05% Tween20) and blocked (1x PBS, 1% BSA) for 2 hrs at RT. The sample and standards (50 μl/well) were added and incubated for 2 hrs at RT. The plate was washed 5 times, and incubated for 2 hrs at RT with a biotinylated antibody. The plate was washed and incubated for 30 min with extra-avidin peroxidase (Sigma-Aldrich, UK). TMB substrate (Life Technologies, UK) was added to the plate (100 μl/well) and colour development was stopped using the ELISA stop-solution (1M H_2SO_4). The optical density was read at 450 nm on a SpectraMax Plus plate reader and concentration of the proteins was calculated from the standard curve using the SoftMax Pro software (Molecular Devices, USA).

Table 4-12 Antibodies and standard for IFN-α ELISA

Antibody	Clone	Isotype	Concentration	Supplier
Capture	F18	Rat IgG_1	1 μg/ml	Cambridge Bioscience, Cambridge, UK
Primary Detection	Polyclonal rabbit anti-IFN-α	-	0.2 μg/ml	PBL Biomedical Laboratories, PA, USA
Secondary Detection	Biotin-polyclonal mouse anti-rabbit	Mouse IgG	1:1000	Jackson ImmunoResearch, PA, USA

Standard	Recombinant IFN-α	-	$4x10^5$ units/ml	Cambridge Bioscience, Cambridge, UK

Table 4-13 Antibodies and standard for IL-6 ELISA

Antibody	Clone	Isotype	Concentration	Supplier
Capture	MP5-20F3	Rat IgG_1	4 μg/ml	BD Bioscience, Oxford, UK
Primary Detection	Biotin-MP5-32C11	Rat IgG_2	0.2 μg/ml	BD Bioscience, Oxford, UK
Standard	Recombinant IL-6	-	100 ng/ml	BD Bioscience, Oxford, UK

4.8.2 Multiplex

Cytokines in BALF and in supernatant from primary AMs stimulated *ex vivo* were measured using the ProcartaPlex mouse IFN-α and IFN-β assay (Affymetrix, USA), and the Cytokine Mouse magnetic 20-plex (Invitrogen, UK) following the manufacturer's manual. Luminex plates were analysed using the BioPlex 200 and BioPlex manager software v6.1 (Bio-Rad Laboratories, UK).

4.8.3 Measurement of C1q

The presence of C1q was measured in the lung, BAL and serum. Readout of C1q levels was performed by Marina Botto's laboratory (Imperial College London, Faculty of Medicine, Hammersmith Campus) following previously published methodology for C1q detection (Carlucci et al., 2016; Naito et al., 2012).

4.9 Assessing intracellular proteins

4.9.1 Western blots

Lysates from 8 x 10^5 primary AMs that were exposed for 20 hrs to RSV or UV-RSV *ex vivo* were extracted using RIPA buffer (Sigma-Aldrich, UK) supplemented with cOmplete Protease Inhibitors (Mini; Roche, UK). The cells were resuspended in RIPA buffer and placed on a rotator at 4°C for 30 min and then centrifuged for 10 min at 4°C at 12000 x *g*. Quantification of the protein in the supernatant was performed using the Peirce™ BCA protein assay kit (Thermo Scientific, USA) following the manufacturer's manual. Proteins were added onto a 10-lane 4-12% Nis-Tris gel (Novex, USA) at 5 μg/lane at a 1:1 dilution with Laemmli buffer containing bromophenol blue (Sigma-Aldrich, UK). The gels were run in running buffer (NugePage MES SDS (Novex, USA)) and transferred to a polyvinylidene difluoride filter paper sandwich with 0.45-μm pore membrane (Novex, USA) using Tris-glycine transferase (Novex, USA). The membranes were blocked for 1 hr (1x TBS, 5% BSA and 0.01% Tween20) at RT and then incubated with a goat-anti-RSV antibody (1:2000 in blocking buffer; (Abcam, UK)) or Direct-Blot™ HRP anti-β-actin (1:1000 in blocking buffer (BioLegend, UK)) at 4°C. For the anti-RSV membrane, a secondary anti-goat HRP antibody (1:5000 in blocking buffer (Jackson ImmunoResearch, USA)) was added for 1 hr at RT before the being developed using EZ-ECL (Biological Industries, USA). The membrane was read using a Fusion FX reader (Vilber Lourmat, Germany) and analysis performed using the Vilber Lourmat software program.

4.9.2 Fluorescence microscopy

Primary AMs were seeded in 24-well-flat bottom plates (ThermoScientific, USA) containing 13 mm borosilicate glass cover slips (VWR, UK) and treated as described in section 4.3.3.3.1. After 21 hrs the cells were washed with PBS and fixed with 4% PFA (Sigma-Aldrich, UK) for 10 min at RT and then blocked with 3% BSA in Triton X/ PBS for a further 10 min at RT. The cells were stained with a polyclonal anti-RSV (1:200 (Abcam, UK)) or a monoclonal anti-RSV N (1:300 (Abcam, UK) and anti-Lamp-1 (1:300 (Abcam, UK) antibody for 1.5 hrs in the dark at RT. The slides were then stained with species specific secondary antibodies conjugated to

Alexa Fluor 488 or Alexa Fluor 568 (1:1000 (Invitrogen, UK)) for 45 min in the dark at RT. The coverslips were then mounted on glass slides with ProLong® Gold Anti-fade Mountant DAPI (Thermofisher Scientific, USA). The Zeiss confocal laser-scanning microscope (LSM)-510 on a 100x/1.4 Plan-apochromat oil lens was used for obtaining images of the cells. All analysis was performed on Fiji, an open-source image processing software (ImageJ, USA).

4.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (Graphpad Software Inc., USA). Two group comparisons were performed using either an unpaired, two-tailed Student's t test One-way ANOVA with Tukey's post hoc test was used for to compare multiple groups. For time-course experiments a two-way ANOVA with Bonferroni's post hoc test was performed. For all tests $P<0.05$ was considered significant. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ND: not detected.

5 Results

5.1 Alveolar macrophages produce inflammatory mediators after RSV exposure *ex vivo*

Type I IFNs act against invading viruses by increasing the antiviral state of infected and uninfected cells and activating immune cells. Protection of mice infected with RSV has been shown to be dependent on type I IFNs (Goritzka et al., 2014; 2015). Importantly MAVS and IFNAR1 signalling appear to drive the production of type IFNs after RSV infection *in vivo*. Using an *Ifna6*$^{gfp/+}$ reporter mouse model it has been shown that AMs are the main producers of type I IFNs *in vivo* during RSV infection (Goritzka et al., 2015). Furthermore, this production of type I IFNs is also crucial for driving the inflammatory response in the lung (Goritzka et al., 2014). Which inflammatory cytokine, other than the type I IFNs, and chemokines AMs produce after exposure to RSV has not been fully characterised. To study their inflammatory profile, primary AMs were studied after RSV exposure *ex vivo*.

5.1.1 Production of inflammatory mediators is dependent on the dose of RSV

To study AMs *ex vivo,* primary AMs were acquired from adult (8-12 week) C57BL/6 (wt) mice. The extracted primary AMs were counted and seeded for 2 hrs before being exposed to RSV at various MOIs for 20 hrs. Analysis of the inflammatory mediators indicated that primary AMs respond to RSV in an MOI dependent manner (Fig 5.1). Cytokine and chemokine analysis using Luminex indicated that AMs produced IFN-α, IFN-β, CXCL10, TNF-α, IL-6 and CCL3 after exposure to RSV (Fig 5.1a). The production of these inflammatory mediators was also confirmed by gene expression analysis (Fig 5.1b). Furthermore, exposure to UV-RSV did not induce the production of these inflammatory mediators, indicating that untreated RSV is required to initiate the response to RSV by AMs. A separate virus titration experiment running to an MOI of 20 suggested that the plateau for protein production after RSV infection was reached at RSV MOI 3 (data not shown) and therefore for subsequent experiments RSV MOI 2 was used.

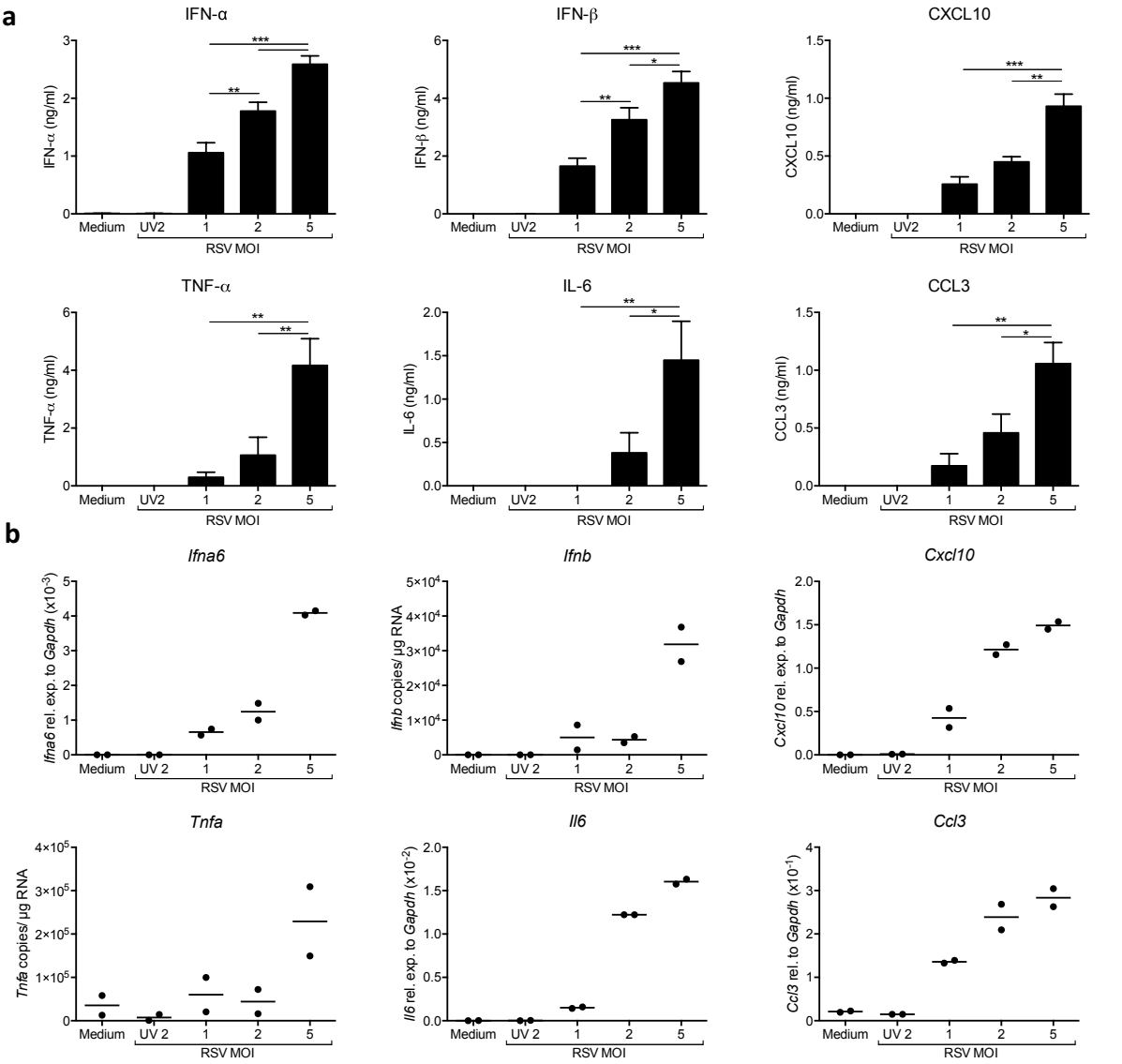


Figure 5.1 Primary AMs respond to RSV in an MOI dependent manner. Primary AMs were isolated from adult C57BL/6 mice and 1.25 x 10^5 cells were exposed to medium, ultraviolet-inactivated RSV at an MOI of 2 (UV2), and RSV at MOIs of 1, 2 and 5 for 20 hrs. Protein secretion in the culture supernatant for (a) IFN-α, IFN-β, CXCL10, TNF-α, IL-6 and CCL3 was detected by Luminex. The gene expression for (b) *Ifna6*, *Ifnb*, *Cxcl10*, *Tnfa*, *Il6* and *Ccl3* was quantified using qPCR. The data for (a) protein production are shown as $\pm$ SEM of 4-9 individual wells pooled from 2-3 experiments. Statistical significance of the differences between the indicated groups was determined by one-way ANOVA with Tukey's post hoc test. *p<0.05; **p<0.01; ***p<0.001. For (b) gene expression analysis each dot represents one individual experiment with cells pooled from 2-3 individual cultures per stimulation. The horizontal bar depicts the mean data from two individual experiments.

In order to assess which time point to study AMs after RSV exposure *ex vivo,* a time course experiment was designed. Specifically, wt primary AMs were exposed to RSV MOI 2 and the protein production (Fig 5.2a) and gene expression (Fig 5.2b) for various inflammatory mediators was studied at 6, 12, 18 and 24 hrs post exposure. The results indicated that 20 hrs post RSV exposure was an ideal time point for measuring protein production and the gene expression for most of the chosen inflammatory mediators and therefore this time point was used for subsequent experiments. Other time course experiments running up to 96 hrs post RSV exposure (data not shown) showed that there was no increase in inflammatory mediators at later time points.

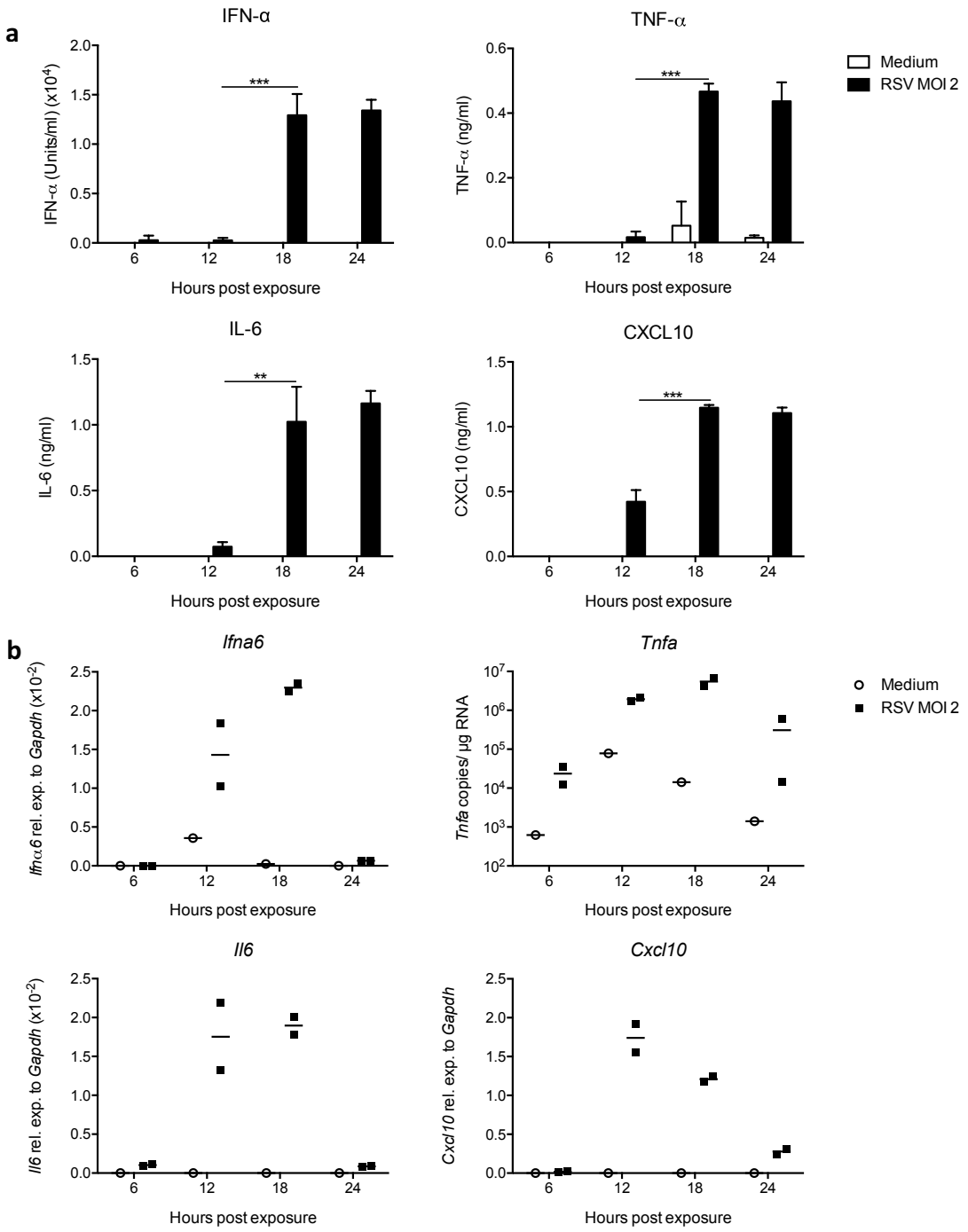


Figure 5.2 Primary AMs protein production and gene expression for most inflammatory mediators peak at 18-24 hours post RSV exposure. Primary AMs were obtained from C57BL/6 (wt) mice and 1.25 x 10^5 of AMs were exposed to medium or RSV MOI 2 for 6, 12, 18 and 24 hrs. Protein production for (a) IFN-α, TNF-α, IL-6 and CXCL10 was determined by ELISA. Gene expression for (b) *Ifna6*, *Tnfa*, *Il6* and *Cxcl10* was determined by qPCR. The data for (a) protein production are shown as ± SEM of 4-6 individual cultures pooled and representative of two experiments. Statistical significance of the differences between the indicated groups

was determined by two-way ANOVA with Bonferroni's post hoc test. **p<0.01; ***p<0.001. For (b) gene expression analysis each dot represents one individual experiment with cells pooled from 2-3 individual cultures per stimulation. The horizontal bar depicts the mean data from two individual experiments.

5.1.2 AMs produce inflammatory cytokines after RSV exposure *ex vivo*

The presence of inflammatory mediators produced by AMs was determined using a 20-plex Luminex platform (see 4.7.2 and Fig 5.1a). Many cytokines and chemokines were not produced by AM and not detected by the Luminex or qPCR (Table 5-1). These include IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL12p40/70, IL-13, IL-17, IL-18, IFN-λ, IFN-γ, and CXCL1. To confirm these findings, many of the mediators were also assessed in the supernatant by ELISA. Interestingly, mediators that have been shown to be important during RSV infection such as IL-1α, IL-1β, CXCL1 and CCL2 (Goritzka et al., 2014) were not produced by AMs (Table 1). Although there was a low expression of the respective genes for these mediators, protein production was absent. This would indicate that although AMs responded to RSV with the production of many innate inflammatory mediators (IFN-α, IFN-β, CXCL10, TNF-α, IL-6 and CCL3), they were not the driving force behind the production of all inflammatory mediators.

Table 5-1 Inflammatory mediators not detectable using Luminex and ELISA, or partially detectable by qPCR after primary AMs being exposed to RSV MOI 2 for 20 hours *ex vivo*. Data are representative of at least two experiments.

Mediator	Assay		
	Luminex	ELISA	Gene expression
IL-1α	-		+
IL-1β	-	-	+
IL-2	-		-
IL-4	-		-
IL-5	-		-
IL-10	-		-
IL12p40/ p70	-		-
IL-13	-		-
IL-17	-		-
IL-18			-
IFN-λ			-
IFN-γ	-		-
CXCL1	-	-	+
CCL2	-	-	+
CXCL9	-		+
GM-CSF	-		-
VEGF	-		-

5.1.3 The production of inflammatory mediators by AMs is dependent on type I IFNs

It was previously shown that the production of inflammatory mediators during RSV infection was dependent on the production of type I IFNs (Goritzka et al., 2014; 2015). Specifically, production of type I IFNs was dependent on viral recognition via RLR-MAVS signalling pathway; whereas enhancement of this activation and further production of inflammatory mediators was dependent on signalling via the IFNAR receptor (Fig 1.4 (Goritzka et al., 2014)). To examine the role of these signalling pathways in the capacity of AMs to respond to RSV the *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ murine models were utilised. Primary AMs were extracted as

previously described (see 4.3.3.3.1) and 1.25 x 10^5 cells were exposed to medium and UV-RSV or RSV, both at an MOI 2. AMs deficient in MAVS and IFNAR1 signalling were unable to produce IFN-α, IFN-β, CXCL10, TNF-α or IL-6 when exposed to RSV (Fig 5.3). To prove that this lack of cytokine production by *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ primary AMs was specific for RSV and not to an intrinsic incapacity of these cells to produce cytokines, the cells were exposed to the innate stimuli LPS or R848 and TNF-α and IL-6 were detected by ELISA (Fig 5.4a). The design and optimisation of an experimental protocol for intracellular staining of AMs was also used to measure the expression of TNF-α and IL-6 after exposure to LPS or R848 (Fig 5.4b). These findings suggested that the lack of protein production in the MAVS- and IFNAR-deficient AMs was specific to RSV and not due to an intrinsic inability of the AMs to produce these cytokines.

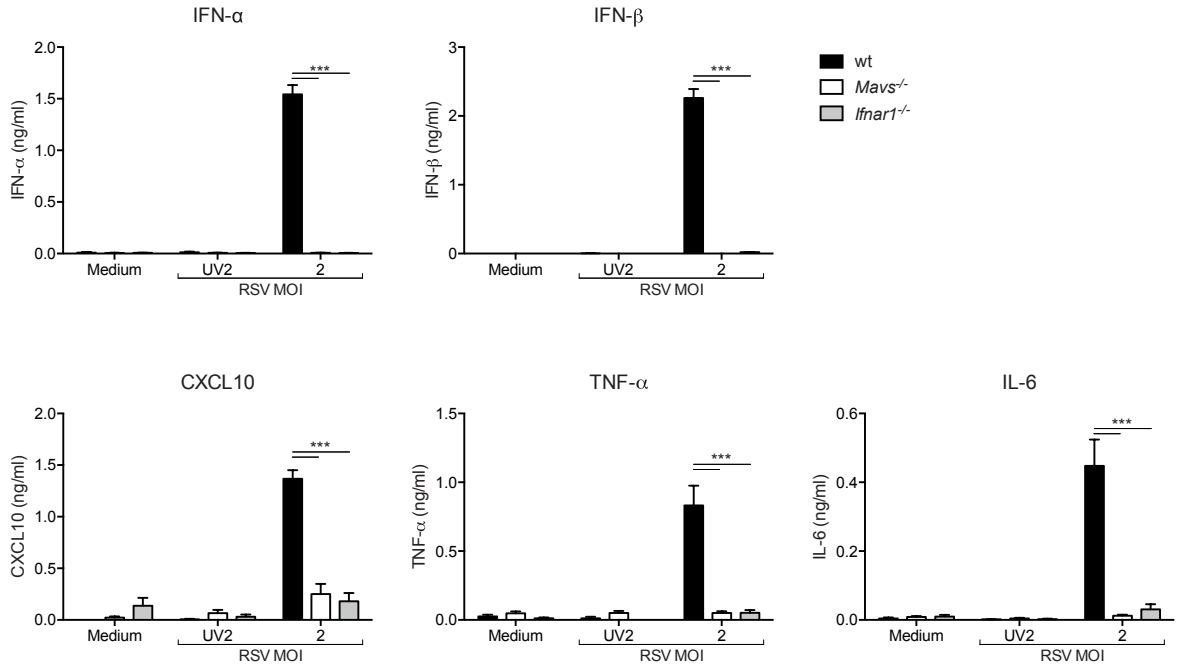
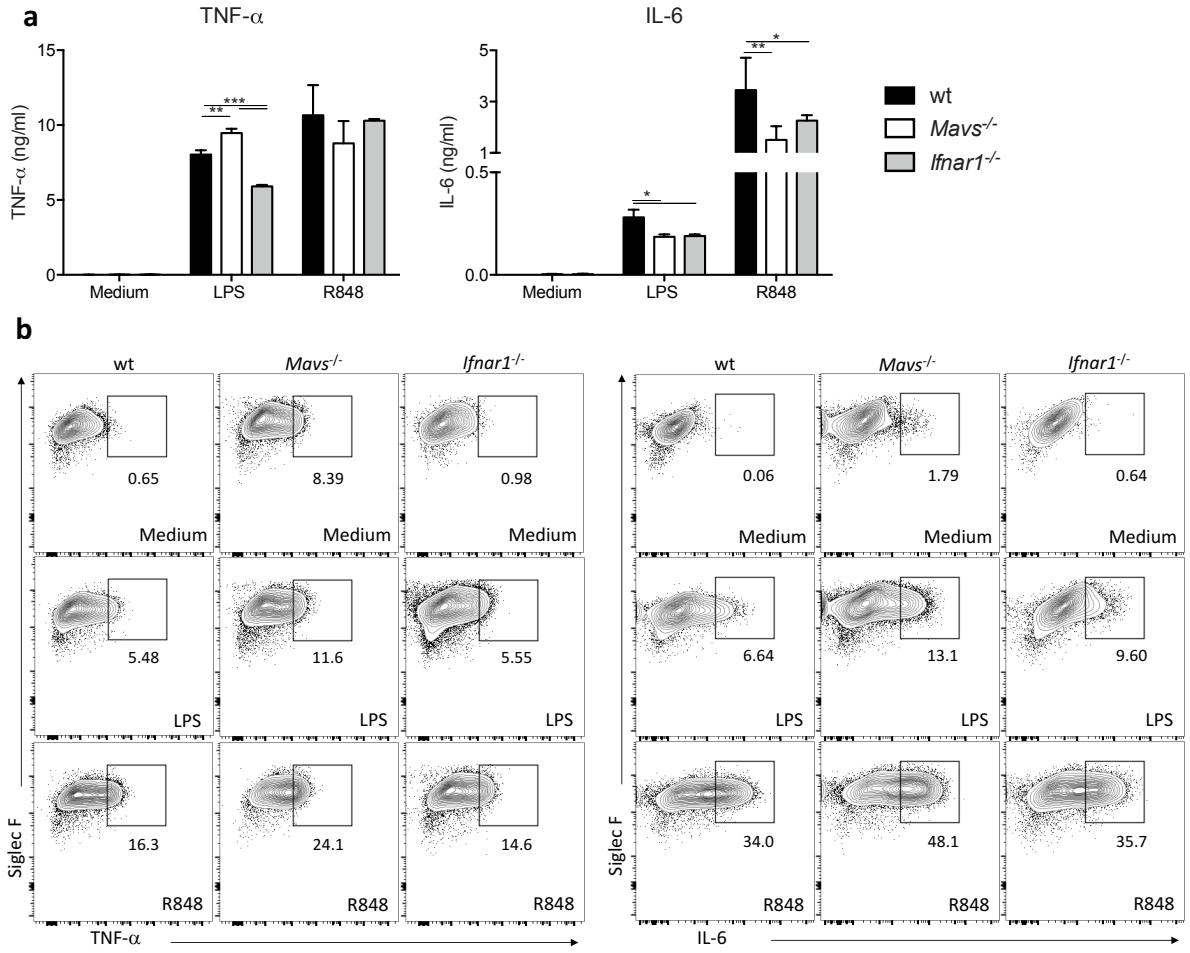


Figure 5.3 The production of inflammatory mediators by primary AMs after RSV exposure is driven by type I IFN production and signalling. Primary AMs extracted from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ were exposed medium, or MOI of 2 of UV-inactivated (UV2) or RSV (2) for 20 hrs. Production of IFN-α and IFN-β in the culture supernatant were determined by Luminex and CXCL10, TNF-α and IL-6 were detected via ELISA. The data are shown as mean ± SEM of 4-15 cultures per stimulation pooled from 3-6 experiments. Statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test *p<0.05; **p<0.01; ***p<0.001.



c

	Medium		LPS		R848	
	IL-6 (%)	TNF-α (%)	IL-6 (%)	TNF-α (%)	IL-6 (%)	TNF-α (%)
wt	0.055 (.004)	0.625 (0.04)	6.245 (0.56)	5.155 (0.46)	32.7 (1.84)	15.55 (1.06)
Mavs$^{-/-}$	1.685 (0.15)	6.59 (2.55)	12.6 (0.71)	13.35 (2.48)	48.3 (0.28)	24.65 (0.78)
Ifnar1$^{-/-}$	0.635 (0.007)	1.1 (0.17)	7.83 (2.5)	5.495 (0.08)	40.9 (7.4)	14.7 (0.14)

Figure 5.4 The production of TNF-α and IL-6 in primary AMs after TLR activation occurs regardless of MAVS and IFNAR signalling. Primary AMs extracted from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ were exposed to medium, 1 ng/ml of LPS or R848 for 20 hrs *ex vivo*. (a) Production of TNF-α and IL-6 in the culture supernatants was measured by ELISA. (b) Flow cytometry was utilised in order to assess the production of TNF-α and IL-6 by intracellular staining (ICS). Gating strategy shown in Fig 4.1 and ICS was performed on AMs. (c) Table represents the mean percentage (+/- SEM) of IL-6^{+} and TNF-α^{+} AMs. (a) Data shown are 4-8 cultures per stimulation pooled from 2-4 independent experiments. Statistical significance of the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test *p<0.05; **p<0.01; ***p<0.001. (b) Flow cytometry plots are indicative of the average phenotype of

the AMs from 2-4 independent experiments. (c) Percentage shown are from 3 individual cultures per stimulation and representative 3 independent experiments.

5.1.4 Discussion

AMs are increasingly recognised as having an important role during respiratory infections. Murine models have shown that AMs are the main producers of type I IFNs *in vivo* after RSV and NDV infection (Goritzka et al., 2015; Kumagai et al., 2007). Furthermore, *ex vivo* exposure of human and ovine AMs to RSV suggested that they can produce an array of inflammatory mediators (Becker et al., 1991; Fach et al., 2010; Kolli et al., 2014; Panuska et al., 1995). The main limitation in the field is that there is no precise method to study the role of AMs *in vivo*. Furthermore, one study investigated the role of macrophages after RSV exposure but most of the data in this study are on pulmonary macrophages (Ravi et al., 2013). Clodronate-loaded liposomes have been extensively utilised in order to deplete/study AMs in different disease models *in vivo* (Benoit et al., 2006; Pribul et al., 2008; Tate et al., 2010). Treatment with clodronate liposomes depletes AMs, as well as Gr-1^{int}/Ly-6C^{high} monocytes and GR-1^{-}/Ly-6C^{-} monocytes (Jenkins et al., 2011). These monocytes are precursors to F4/80^{low} inflammatory macrophages and tissue resident macrophages respectively (Auffray et al., 2009; Jakubzick et al., 2013; Sunderkotter et al., 2004). Taken together these findings suggested that treatment with clodronate-loaded liposomes does not solely deplete AMs but other cells such as pulmonary macrophages and DCs may also be affected. Therefore, the precise role of AMs during RSV infection is not fully elucidated.

Protection against RSV infection *in vivo* was driven by type I IFNs and AMs were shown to be the main producers of these cytokines (Goritzka et al., 2014; 2015). To establish a model for studying the precise role of AMs during RSV infection, murine primary AMs were utilised. Cytospin counts and flow cytometry have shown that over 98% of the recovered cells in the bronchoalveolar lavage (BAL) of naïve wt mice are AMs (Goritzka et al., 2015). The primary objective was to establish an RSV model for studying AMs *ex vivo* to allow determination of inflammatory mediators produced by AMs and how this is regulated by type I IFNs.

To begin addressing these questions the time point and RSV concentration had to be determined. Time course experiments and RSV titration experiments showed that the ideal time point for studying protein production and gene expression for AMs was 20 hrs; whereas the ideal MOI of RSV for sub-optimal stimulation was MOI of 2. Primary AMs produced inflammatory mediators such as IFN-α, IFN-β, CXCL10, TNF-α, IL-6 and CCL3. This suggested the importance of AMs in initiating the pro-inflammatory response when encountering RSV. By performing a 20-plex Luminex, ELISAs and qPCRs a better profile for the mediators AMs produced after RSV exposure was established. Surprisingly AMs exposed to RSV *ex vivo* did not produce CXCL1, CCL2 and IL-1β ; mediators that have been shown to be important for controlling RSV infection *in vivo* (Borchers et al., 2013; Goritzka et al., 2015). The lack of mediators such IL-1β, that has been associated with RSV clearance and macrophage activation (Brochers et al., 2013; Goritka et al., 2015; Hussel & Bell, 2013), can signify the absence of a second signal that would drive the activation of the inflammasome cascade (see section 1.2.3, Carta et al., 2013). Thus, after being exposed to RSV *ex vivo*, primary AMs responded by producing pro-inflammatory mediators that are crucial during the innate response (Table 1-1 and 1-2). These mediators contribute significantly to enhancing the pro-inflammatory environment of the lung. Several polymorphisms in innate immune genes including those related to IFNs have been related to severity after RSV infection of infants (Awomoyi et al., 2007; R. Janssen et al., 2007; Siezen et al., 2009). As the production of these mediators is linked to the presence of type I IFNs, understanding the source during infection is important (Bhoj et al., 2008; Crotta et al., 2013; Goritzka et al., 2014; 2015).

The severity of disease after many viral infections has been linked to deficiencies in genes controlling the IFN system and type I IFN signalling (Dupuis et al., 2003; R. Janssen et al., 2007; Sykes & Johnston, 2008). Type I IFNs signal in an autocrine or paracrine fashion via the IFNAR and this induces over 300 IFN stimulated genes (ISGs) (Der et al., 1998) (Fig 1.4). Lack of signalling via MAVS or IFNAR1 made mice more susceptible to RSV infection with a characteristic increased morbidity, viral load and lack of cytokine production (Bhoj et al., 2008; Demoor et al., 2012; Goritzka et al., 2014; 2015). These studies showed that the control of viral infection and the production of many key mediators was dependent on detection of RSV by RLRs (signalling via MAVS) and the enhancement of the response via IFNAR signalling. Although, the importance of MAVS and IFNAR signalling has been established *in vivo*, the role

of this signalling in AMs after RSV exposure still needs to be elucidated. AMs orchestrate the innate immune response to RSV through recruitment of antiviral monocytes. Moreover, the influx of neutrophils, and $CD4^+$ and $CD8^+$ T cells was similar in the absence of type I IFNs (Bhoj et al., 2008; Goritzka et al., 2014; 2015). The role of MAVS and IFNAR signalling after AMs were exposed to RSV *ex vivo* was studied using the primary AMs from *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice. Results showed that the production of IFN-α, IFN-β, CXCL10, TNF-α and IL-6 by primary AMs after RSV exposure was dependent on type I IFN production (*Mavs*$^{-/-}$) and signalling (*Ifnar1*$^{-/-}$). *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ primary AMs were also exposed to LPS (TLR4 agonist) and R848 (TLR7 agonist) to show, that they could respond to another stimulus independent of type I IFNs and, that the previously mentioned unresponsiveness was not due to cell-intrinsic effects. Protein was measured in the cell culture supernatants and showed that MAVS-deficient cells produce more TNF-α after LPS stimulation; whereas both *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs had diminished production of IL-6. Using these stimulations, the ICS protocol for studying AMs *ex vivo* also provided similar results. Type I IFNs are important for enhancing the pro-inflammatory responses and the lack of MAVS or IFNAR signalling diminishes the capacity of the cells to respond RSV (Goritzka et al., 2014). Collectively these data show that AMs produce pro-inflammatory cytokines after RSV exposure and that the production of these cytokines is dependent on type I IFNs.

5.2 RSV replication in alveolar macrophages is abortive

AM reside in the airway lumen and have an important role in homeostasis and during infection (Hussell & Bell, 2014). It has previously been shown that human AMs are not susceptible to RSV infection when compared to cells from nasal or bronchial brushings (Becker et al., 1992). Study of murine lung macrophages also suggested that AMs are not infected by RSV (Ravi et al., 2013). Type I IFN production and signalling has been suggested to enhance protection from a series of respiratory pathogens including RSV (Bender et al., 2015; Hastings et al., 2015; McNab et al., 2015; Sadler & Williams, 2008) and the presence of type I IFNs after RSV infection in mice reduced the viral load and morbidity associated with infection (Goritzka et al., 2014; 2015). Considering that MAVS- and IFNAR1-deficient primary AMs lack production of many inflammatory mediators, including type I IFNs; this study attempted to determine if the lack of IFNs by AMs makes them susceptible to RSV infection.

5.2.1 Increased viral load in MAVS- and IFNAR1-deficient AMs

The recognition of RSV, via the RLR signalling pathways, causes the induction of type I IFNs, which signal via IFNAR to initiate the upregulation of over 300 ISGs ((Der et al., 1998) Fig 1.3 and 1.4). During RSV infection ISGs such as OAS1a, viperin and PKR have multiple roles including interfering with viral replication or disrupting budding of viral particles (Behera et al., 2002; Corn Minor et al., 2010; McGillivary et al., 2013). For example, PKR enhance the anti-inflammatory state and regulate signalling pathways such as NF-κB, MAPK, STAT-1 and IRFs (Garcia et al., 2006; Goh et al., 2000; McGillivary et al., 2013). When infected with RSV *in vivo*, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice had an enhanced morbidity, viral load, absence of inflammatory mediators and ISGs (Goritzka et al., 2014; 2015). After primary AMs were exposed to RSV *ex vivo*, there was an absence of expression of OAS1a, viperin and PKR (Fig 5.5). These results indicated that in the type I IFN deficient cells the induction of ISGs were lacking and the control of the virus may therefore be inefficient.

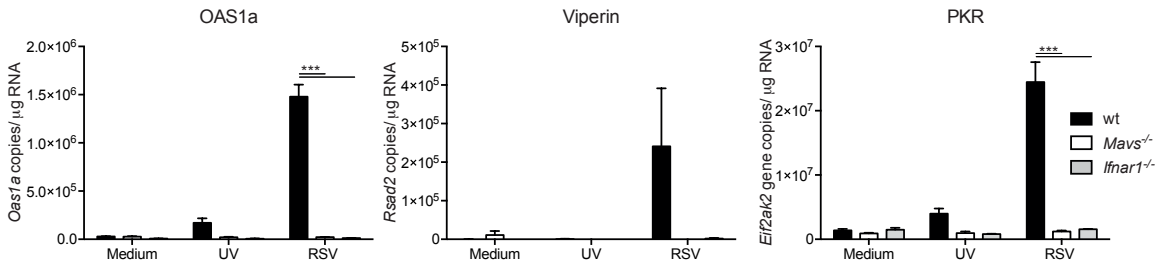


Figure 5.5 Primary AMs deficient in MAVS and IFNAR signalling lack the expression of ISGs important during RSV infection. Primary AMs from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were exposed to medium, UV-inactivated RSV (UV) or RSV, both at an MOI of 2 for 20 hrs *ex vivo*. The expression of the ISGs; OAS1a, viperin and PKR was determined by qPCR. The data are shown as mean ± SEM of 3-4 individual RNA samples pooled from 3-4 independent experiments. Statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test ***p<0.001.

To examine whether the absence of type I IFNs and ISGs renders *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs more susceptible to RSV replication the RSV L and N gene copy were quantified (Fig 5.6a and b). Primary AMs lacking MAVS or IFNAR signalling had a slightly increased expression of L gene and a significantly higher expression of N gene copies when compared to wt AMs. In fact, the viral genes in wt AMs did not increase when compared to the viral gene copy number present in the AMs exposed to UV-RSV. The viral load in primary AMs was compared to a mouse epithelial cell line (LA4). As RSV has been shown to replicate in epithelial cells, the LA4 cell line would provide a more susceptible cell type (Bannister et al., 2010; Loebbermann et al., 2012). When compared to AMs, LA4 cells had a 100-1000-fold increase in the expression of L and N gene copies 48 hrs after infection (Fig 5.6a and b). The quantification of the viral load in the culture supernatant was also performed using immunoplaque assay however no plaques could be detected from any of the AMs (data not shown). To assess if viral replication initiates in AMs, the expression of positive- and negative-sense RSV strand was measured. Using an RSV-strand specific qPCR assay (Bannister et al., 2010), primary AMs did not appear to have a substantial increase in any of the RSV strands, although there was a significant increase in the positive RSV strand in the IFNAR1-deficient cells (Fig 5.6c). Interestingly, the replication found in primary AMs was not comparable to the more susceptible LA4 cells where both negative and positive RSV strands were readily detected.

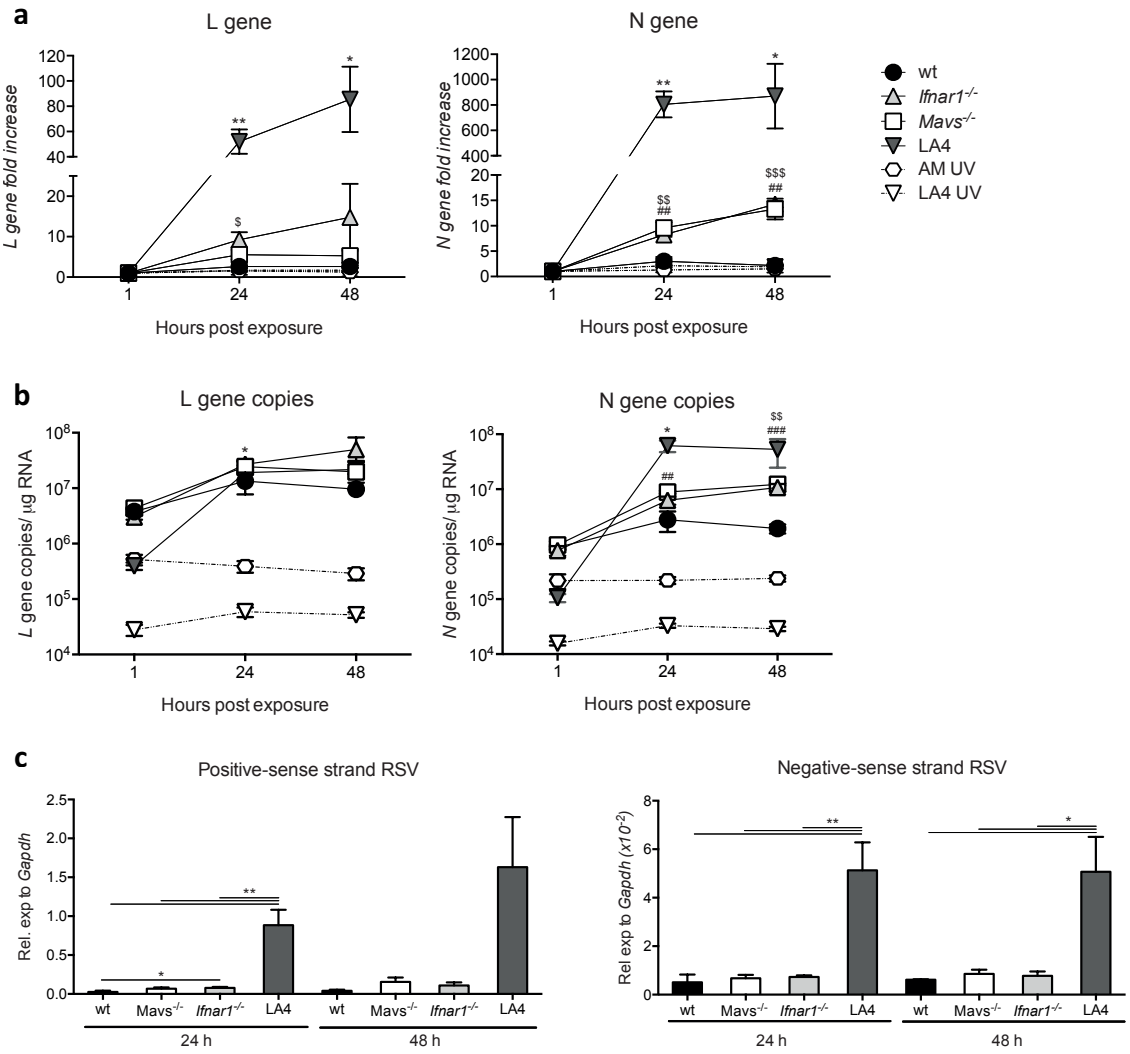


Figure 5.6 RSV replication in primary AMs is unsuccessful regardless of the production of type I IFNs. AMs from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice and mouse epithelial cells (LA4) were exposed to RSV or UV-inactivated RSV (AM UV or LA4 UV) at an MOI of 2 for 1, 24 and 48 hrs. L and N gene were quantified by qPCR and the expression is shown as (a) fold increase over the copies present at 1 hr post-exposure or (b) gene copy number. (c) Positive- and negative-sense strand RSV was detected using qPCR. The data are shown as mean ± SEM of 3-4 individual RNA samples pooled from 3 independent experiments and (c) 4-6 individual RNA samples pooled form 4 independent experiments. Statistical significance of differences between indicated groups was determined by (a,b) two-way ANOVA with Bonferroni's post hoc test or (c) two-tailed, unpaired Student's *t* test. For data in (a,b) the '*' represents differences between LA4 and wt AMs, '$' between wt and *Ifnar1*$^{-/-}$ AMs and '#' between wt and *Mavs*$^{-/-}$ AMs. *p<0.05; **p<0.01; ***p<0.001.

5.2.2 Higher levels of RSV proteins in *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ primary AMs

To determine if the lack of type I IFNs would alter the presence and localisation of RSV proteins, AMs were assessed using fluorescence microscopy. When stained with a polyclonal anti-RSV antibody, the RSV exposed wt AMs appeared to have a similar appearance as the UV exposed cells (Fig 5.7). However, the *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs had a higher presence of RSV proteins both on the surface and in the cytoplasm (Fig 5.7- RSV panel). Areas within the cytoplasm of the MAVS and IFNAR1 deficient AMs where the density of RSV proteins is more condensed would suggest the presence of inclusion bodies (IBs). The presence of IBs would indicate that viral replication was abortive. In permissive cells, the IBs are present in the cytosol when viral assembly initiates and are not easily detectable (Lindquist et al., 2010). Cells that are permissive to RSV infection, such as HEp2 cells, have filament formations that are abundant in the viral F protein (Bajorek et al., 2014; Mehedi et al., 2016). The F protein is important for filament formations that facilitate the translocation of the assembling viral particles toward the cell membrane where budding occurs (Mehedi et al., 2016). To further elucidate the dynamics of RSV replication, the cells were also stained with an RSV anti-F antibody (Fig 5.8). The importance of the F protein in the viral life cycle has been assessed in VLP models that lack the F protein (Baviskar et al., 2013; Shaikh et al., 2012; Teng & Collins, 1998). The lack of F protein caused abortive replication as the assembling virion could not translocate to the cell membrane; instead a lack of filament formation and a presence of larger IBs in the cytosol of cells were observed (Baviskar et al., 2013; Shaikh et al., 2012; Teng & Collins, 1998). The presence of IBs would suggest that viral replication was abortive and that the assembly of the virus cannot translocate toward the membrane. In preliminary experiments, AMs were stained with RSV anti-F and although the viral staining was not of the optimum quality, the AMs from all genotypes had a similar staining pattern of and no filaments were present (Fig 5.8). Overall these findings suggested that the combination of the lack of filaments in AMs, and the presence of IBs, is indicative of abortive replication.

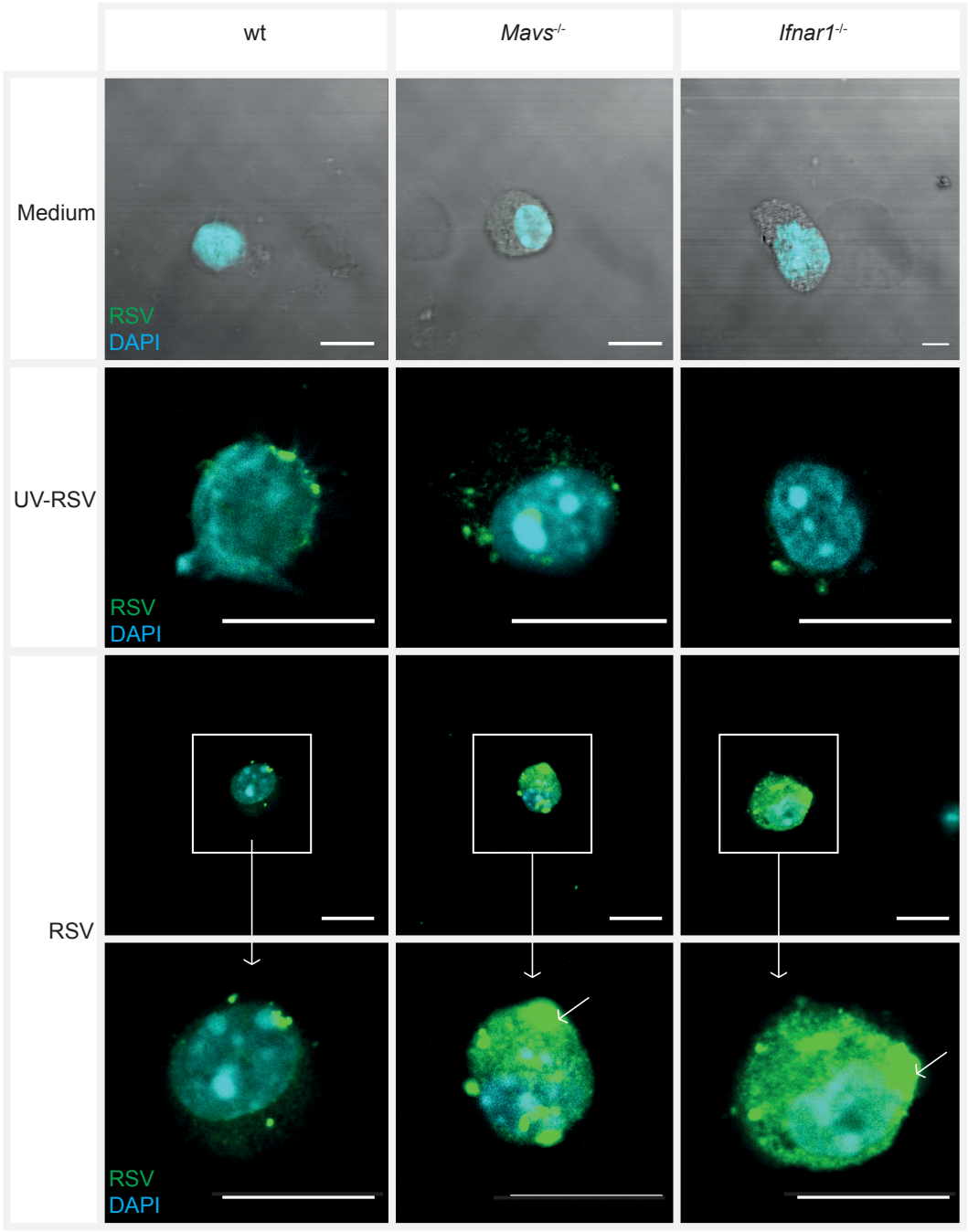


Figure 5.7 Control of RSV protein production is dependent on type I IFN signalling. Primary AMs from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were exposed to medium, UV-inactivated RSV (UV-RSV) and RSV both at an MOI of 2 for 21 hrs. The cells were stained with a polyclonal anti-RSV antibody [green] and DAPI [blue]. Images were acquired on a 100x/1.4 Plan-Apochromat oil lens by line averaging. Anti-RSV (Alexa Fluor 488) was acquired on the 488 nm laser and DAPI was acquired on violet 405 nm laser. RSV exposed cells were analysed and inclusion-like bodies are indicated with white arrows. The scale bar represents 10 μm. The images represent

the majority of AMs from at least 4 independent experiments. Analysis of the images was performed on Fiji, an open-source imaging processing software (ImageJ)

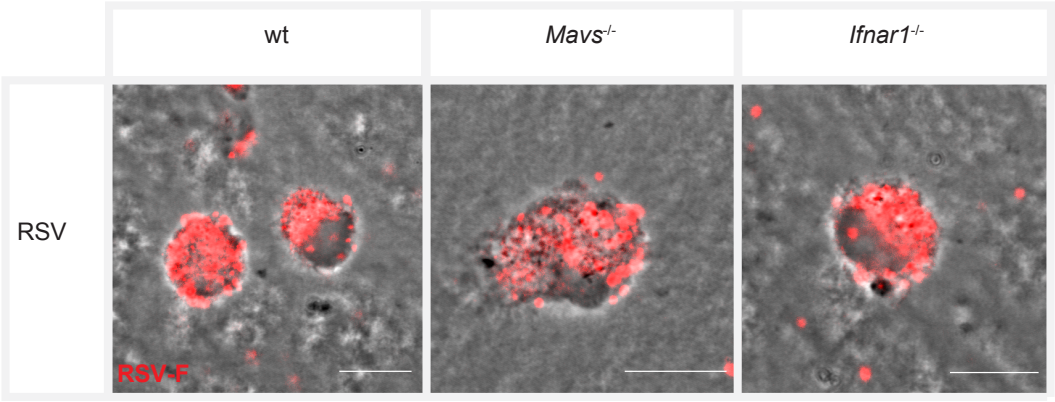


Figure 5.8 Presence of RSV F protein is independent of type I IFN signalling. Primary AMs from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were exposed to RSV at an MOI of 2 for 24hrs. The cells were stained with a monoclonal RSV anti-F antibody [red]. Images were acquired on a 100x/1.4 Plan-Apochromat oil lens by line averaging. RSV anti-F (Alexa Fluor 568) was acquired on 548 nm laser. The scale bar represents 10 μm. The images represent the majority of AMs from one experiment. All images were acquired as monochromes and converted to composite images using the Fiji software program (ImageJ).

5.2.3 Presence of inclusion bodies in primary AMs

The most abundant RSV protein present in IBs is the RSV N protein (Mitra et al., 2012). The significance of IBs is that their detection indicates that viral replication is abortive. Specifically, when the viral genome is present in the host cytoplasm it is immediately coated by the N protein in order to maintain stability and to protect the viral genome from degradation or recognition by host factors. During viral budding, the IBs initiate a brief transient aggregation of proteins before formation and release of viral particles. The detection of these protein aggregations in the cytoplasm of cells would therefore suggest abortive replication. The presence of IBs was assessed using fluorescence microscopy by staining for RSV anti-N protein (Fig 5.9a). Furthermore, in order to elucidate the possibility that the viral presence is not due to phagocytosis, an antibody against the phagolysosome marker LAMP-1 was used. The AMs

deficient in IFNAR and MAVS signalling had an increased presence of viral N protein and distinct IBs were present in their cytoplasm (Fig 5.9a). Intensity analysis of co-localised areas confirmed that the N staining was not in the phagolysosomes and indicates the presence of IBs (Fig 5.9b). Using western blotting, the presence of viral proteins in the primary AMs was characterised. Cell lysates from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ primary AMs previously exposed to RSV or UV-RSV for 20 hrs were assessed for the presence of RSV proteins using the polyclonal anti-RSV antibody. The MAVS and IFNAR1-deficeint primary AMs had an increased presence of viral proteins, and there was especially an increase of the N protein levels in these cells (Fig 5.10). Taken together this indicated that viral replication in AMs in the absence of type I IFNs was more advanced, albeit, abortive.

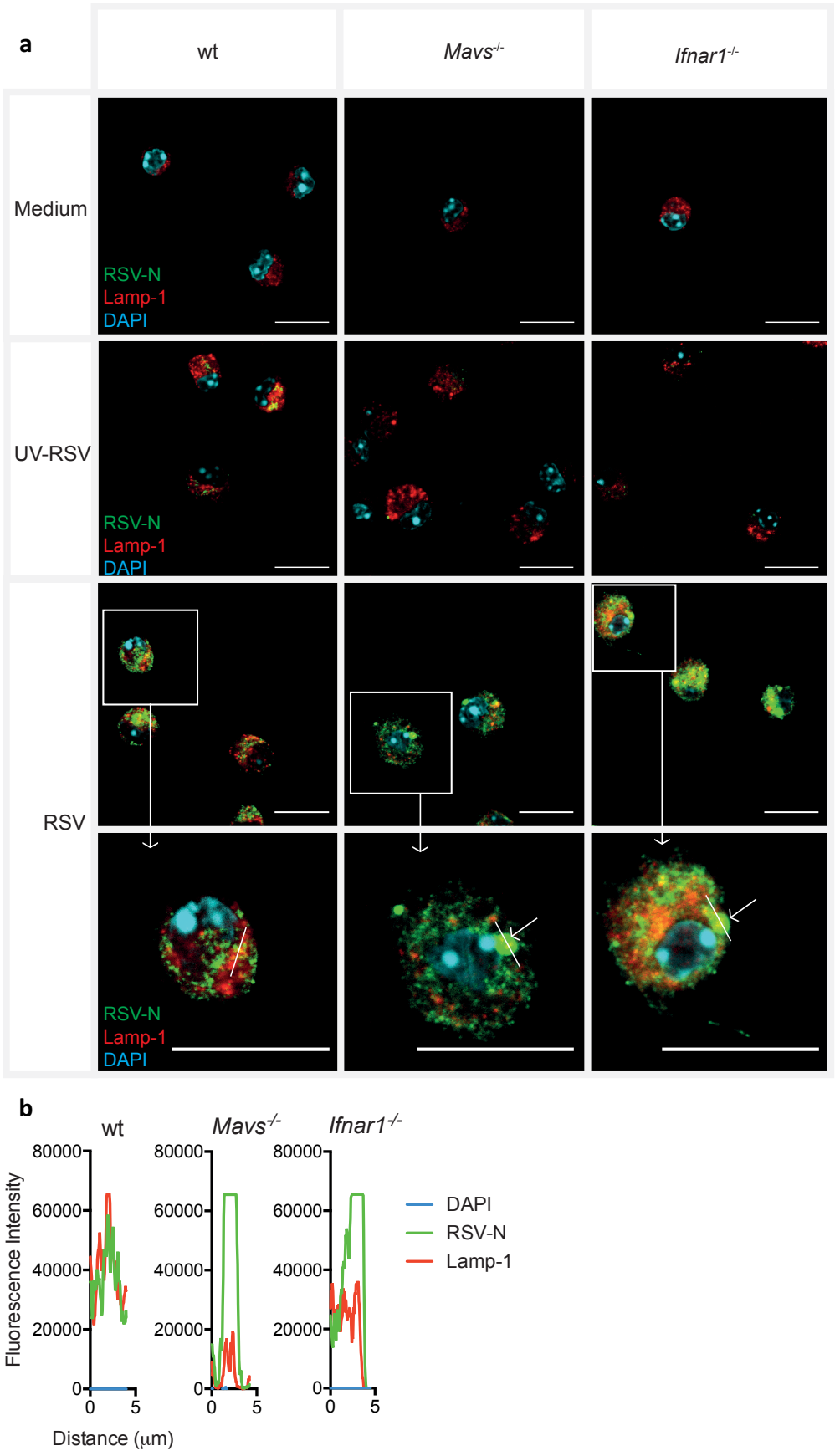


Figure 5.9 Inclusion bodies are present in AMs lacking MAVS and IFNAR1 signalling. Primary AMs from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were exposed to medium, UV-inactivated RSV (UV-

RSV) or RSV MOI 2 for 21 hrs. The cells were fixed and stained with RSV-anti-N [green], anti-LAMP-1 [red] and DAPI blue and examined by fluorescence microscopy. Images were acquired on a 100x/1.4 Plan-Apochromat oil lens by line averaging. RSV-anti-N (Alexa 488) was acquired on the 488 nm laser, anti-LAMP-1 (Alexa Fluor 568) was on 548 nm laser and DAPI on the violet 405 nm laser. (a) Images are representative of medium, UV-RSV and RSV exposed primary AMs with inclusion bodies indicated with a white arrow. The scale bars represent 10 μm. All images were acquired as monochromes and converted to composite images using the Fiji software program (ImageJ). (b) The graphs are indicative of the pixel intensity of RSV-N, LAMP-1 and DAPI along the white line on the monographs. Images are representing the appearance of the majority of cells from three independent experiments.

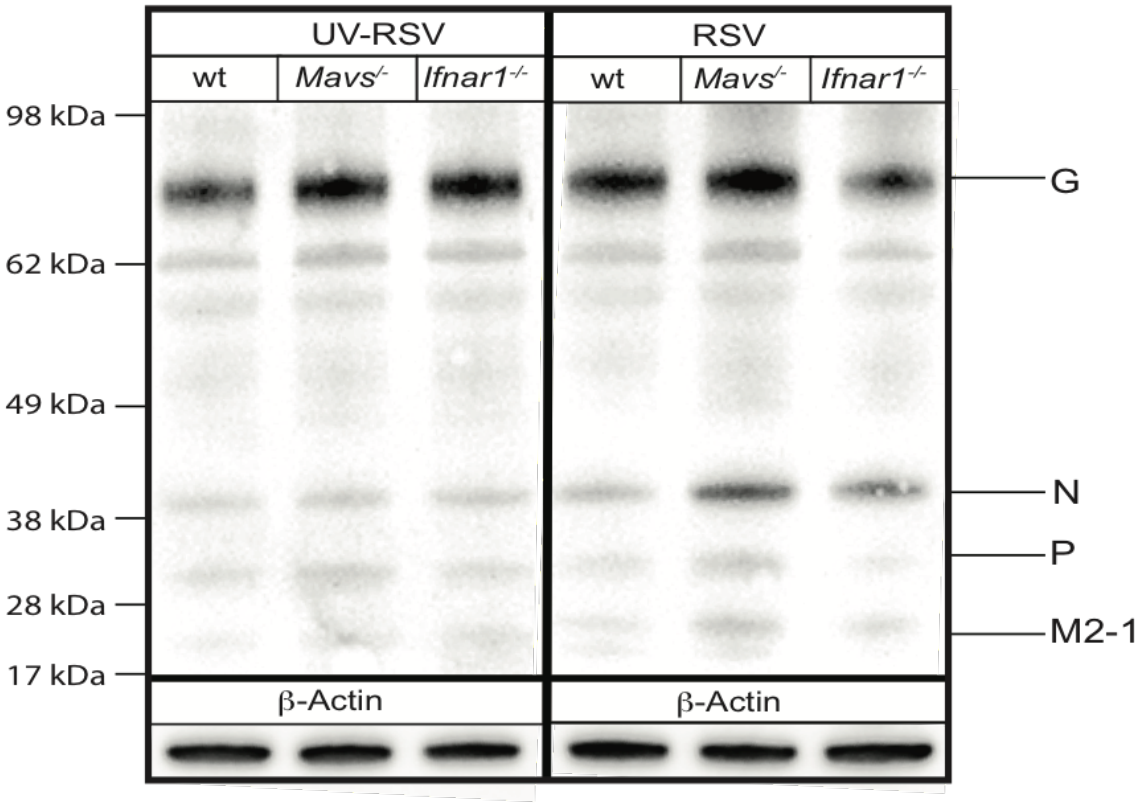


Figure 5.10 MAVS- and IFNAR1-deficient AMs contain higher levels of RSV N protein. Primary AMs were extracted from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice and exposed to UV-inactivated RSV (UV-RSV) or RSV both at an MOI of 2 for 21 hrs. The cell lysate was obtained using RIPA buffer and the protein content was analysed by western blots. The blots were exposed to a polyclonal anti-RSV antibody or to an anti-β-actin antibody. The protein standard

is indicated to the left of the blot whereas the detected RSV proteins are indicated to the right of the blots. The blots are representative of at least 3 experiments.

5.2.4 Discussion

The localisation of AMs positions them as ideal sensors of invading pathogens and initiators of inflammatory responses in the lung. The production of inflammatory mediators by primary AMs after RSV exposure is dependent on MAVS and IFNAR1 signalling (see section 5.1). As AMs are one of the first immune cells to encounter RSV in the airways, elucidating the viral interaction with these cells is of equal importance as understanding their inflammatory profile. Furthermore, how MAVS and IFNAR signalling affects how AMs interact with RSV was also determined.

For the first time using intracellular flow cytometry staining the *ex vivo* inflammatory profile of AMs was confirmed after stimulation with the innate stimuli R848 and LPS. Furthermore, the production of IL-6 and TNF-α by *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ primary AMs after R848 or LPS stimulation indicated that the lack of inflammatory mediators by these cells during RSV exposure is specific to the virus and not due to an intrinsic signalling deficiency. Mice deficient in type I IFN signalling are more susceptible to RSV infection, whilst primary AMs from these mice lack the production of inflammatory mediators (Goritzka et al., 2014). It was hypothesised that the unresponsiveness of *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs to RSV would suggest that these cells were more permissive to RSV replication. This hypothesis was further supported by the absence of the ISGs such as viperin, OAS-1 and PKR in the *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ primary AMs after RSV exposure.

A trend toward increased expression of RSV L and N genes was observed in the *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ primary AMs compared to wt AMs. RSV was previously shown to be capable of replicating in mouse lung and LA4 cells (Bannister et al., 2010). However, when comparing the presence of positive and negative RSV strands, viral replication did not occur in any AMs (from any of the genotypes). In terms of understanding the interactions of AMs with RSV the limitation with using qPCRs is that it detects the total RSV genome and not the presence of

proteins. An increased presence of RSV proteins in the MAVS- and IFNAR1-deficient AMs was observed using fluorescence microscopy. Despite the increase of viral proteins in the cytoplasm of these cells, the detection of IBs and the absence of filaments indicated that RSV underwent abortive replication. The presence of IBs may be due to many reasons, including, the lack of filament formation and/or of important host cell factors (Jeffree et al., 2007; Makris et al., 2016; Santangelo & Bao, 2007). Interestingly, N protein is most abundant in IBs and its interaction with RSV M protein is important for the budding of the viral ribonucleoproteins (RNPs) (Mitra et al., 2012). Therefore, in addition to cellular factors, lack of proteins important for viral transport may also be the reason for abortive replication in AMs.

Western blots confirmed an increased presence of RSV N protein in *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs, which would suggest that viral replication and protein production initiates. With these data, the reason that RSV replication in AMs is abortive cannot be elucidated. One speculation could be that there is arrested replication either at the assembly or budding stage of the viral life cycle. Furthermore, unique characteristics of AMs might make them non-permissive to RSV infection. Such characteristics include the high phagocytic activity of AMs or differences in the presence of various cellular factors. In fact, a number of cellular proteins, such as Rab11 pathway proteins, caveolin, actin related protein 2 (ARP2), RhoA and Coflin 1, are important for RSV replication (Brock et al., 2003; Gower et al., 2005; Kipper et al., 2015; Mehedi et al., 2016; Utley et al., 2008). RSV required Rab11 to bud off polarised epithelial cells (Brock et al., 2003); whereas the caveolin reduced RSV production in infected cells (Kipper et al., 2015). The ARP2 and viral F proteins facilitated the production and spread of RSV in HEp2 cells (Mehedi et al., 2016). Furthermore, RhoA signalling was important for inducing syncytium formation and the filamentous appearance of RSV in infected HEp2 cells (Gower et al., 2005). Analysis of the filaments from permissive cells using mass spectroscopy have suggested the presence of actin isoforms, coflin 1 and filament-1 (Bitko et al., 2003; Gower et al., 2005; Santangelo & Bao, 2007). Despite the presence of these proteins, their role in viral replication and differences in their presence is unknown (Mitra et al., 2012). HSP90 and mitochondrial proteins are crucial for successful RSV infection to occur and differences in the presence of these proteins may block a productive viral replication (Brock et al., 2003; Kipper et al., 2015; Radhakrishnan et al., 2010; Utley et al., 2008). The lipid composition of AMs might also be a

reason that RSV infection in these cells is abortive. The lipid content in epithelial cell membranes and lipid rafts drives the release of infectious virions, whereas actin is primarily important at the budding/release phase (Burke et al., 1998; Te-Hung Chang et al., 2012; Jeffree et al., 2003; McCurdy & Graham, 2003; Mitra et al., 2012). Although one can only speculate regarding the mechanism by which AMs control RSV replication, it is important to highlight that although AMs are one of the first cell types to encounter RSV, they are not productively infected by the virus

5.3 RSV encountered by AMs *in vivo*

The inflammatory mediators that AMs produce and how they interact with RSV were determined *ex vivo* (see sections 5.1, 5.2) (Makris et al., 2016). The precise interaction between RSV and AMs after infection *in vivo* remains to be elucidated. AMs are tightly regulated by various receptors in the lung which set their activation threshold and it is possible that removing them from their environment their interaction with RSV will change. Therefore, the *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice models were utilised to study the infectivity and inflammatory profile of AMs *in vivo*.

5.3.1 Presence of viral proteins in AMs exposed to RSV *in vivo*

Replication of RSV in primary AMs is abortive *ex vivo* (Fig 5.7, 5.8, 5.9). To confirm these findings *in vivo*, mice from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ genotypes were infected with RSV intranasally. The viral load was higher in RSV-infected *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice compared to wt mice (Goritzka et al., 2014; 2015). As AMs are one of the first immune cells to encounter the virus, the cells were studied at 18 hrs post infection. Specifically, cells were acquired from the BAL of mice and allowed to adhere to coverslips for 2 hrs before being stained and analysed using fluorescence microscopy. When stained with a polyclonal anti-RSV antibody, the MAVS- and IFNAR-deficient AMs had higher levels of viral proteins present. Furthermore, IB-like structures were present in the cytoplasm of the AMs derived from the type I IFN deficient mice (Fig 5.11) confirming the results from *ex vivo* exposed AMs (Fig 5.9)

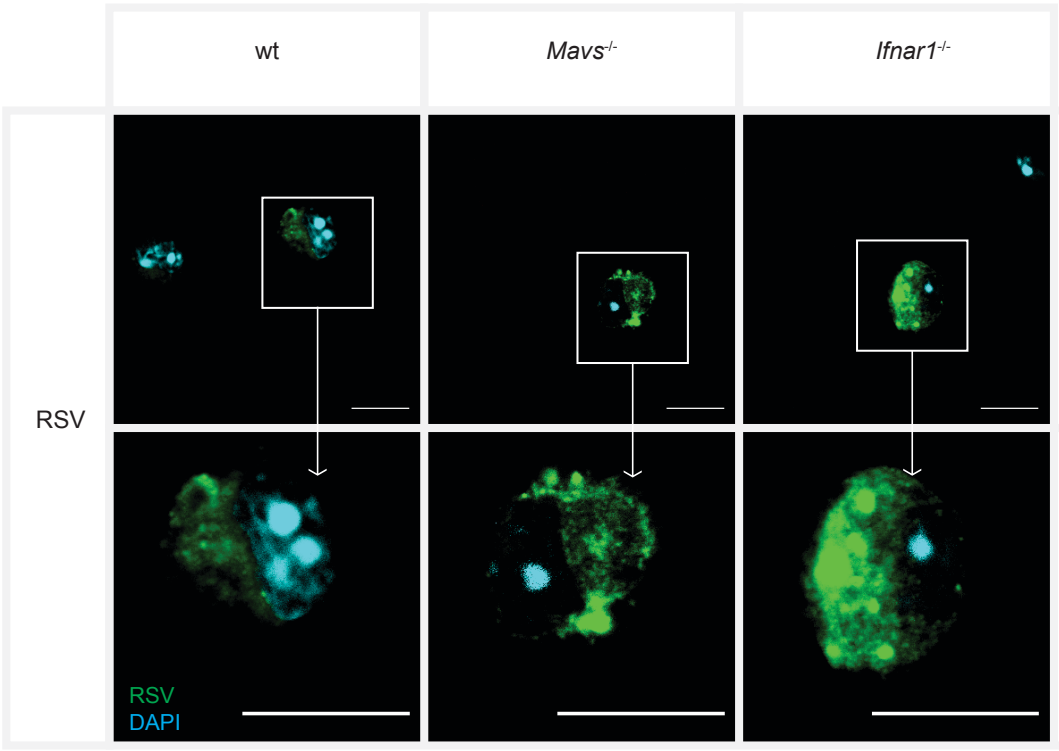


Figure 5.11 RSV proteins are abundantly present in *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs after encountering RSV *in vivo*. Mice from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ genotypes were intranasally infected with 1 x 10^6 FFU of RSV and 18 hrs post infection primary AMs were extracted via BAL. The cells were allowed to adhere to a plastic coverslip for 2 hrs before being fixed and stained with a polyclonal anti-RSV antibody [green] and DAPI [blue]. To ensure purity cells were also examined by cytospin slides. The cells were stained with a polyclonal anti-RSV antibody [green] and DAPI [blue]. Images were acquired on a 100x/1.4 Plan-Apochromat oil lens by line averaging. Anti-RSV (Alexa Fluor 488) was acquired on the 488 nm laser and DAPI was acquired on violet 405 nm laser. The scale bar represents 10 μm. The images represent the majority of AMs from 2 independent experiments.

5.3.2 Inclusion body presence in AMs exposed to RSV *in vivo*

The RSV N protein is most abundant in IBs the detection of which is a confirmation of abortive viral replication (Jeffree et al., 2007; Mitra et al., 2012; Santangelo & Bao, 2007). To confirm that the more distinct stained areas present in the cytoplasm (Fig 5.11) of the *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs were IBs, the cells were stained for anti-RSV N. The phagolysosome marker

LAMP-1 was also stained in order to eliminate the possibility that the RSV present was due to phagocytosis. At 18 hrs after *in vivo* infection higher amounts of RSV N protein were detected in *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs compared to wt AMs (Fig 5.12). Furthermore, the areas of more distinct RSV N staining may suggest the presence of IBs (Fig 5.12 bottom panel) in *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs. Overall these findings suggested that viral replication in AMs that encountered RSV *in vivo* may be abortive.

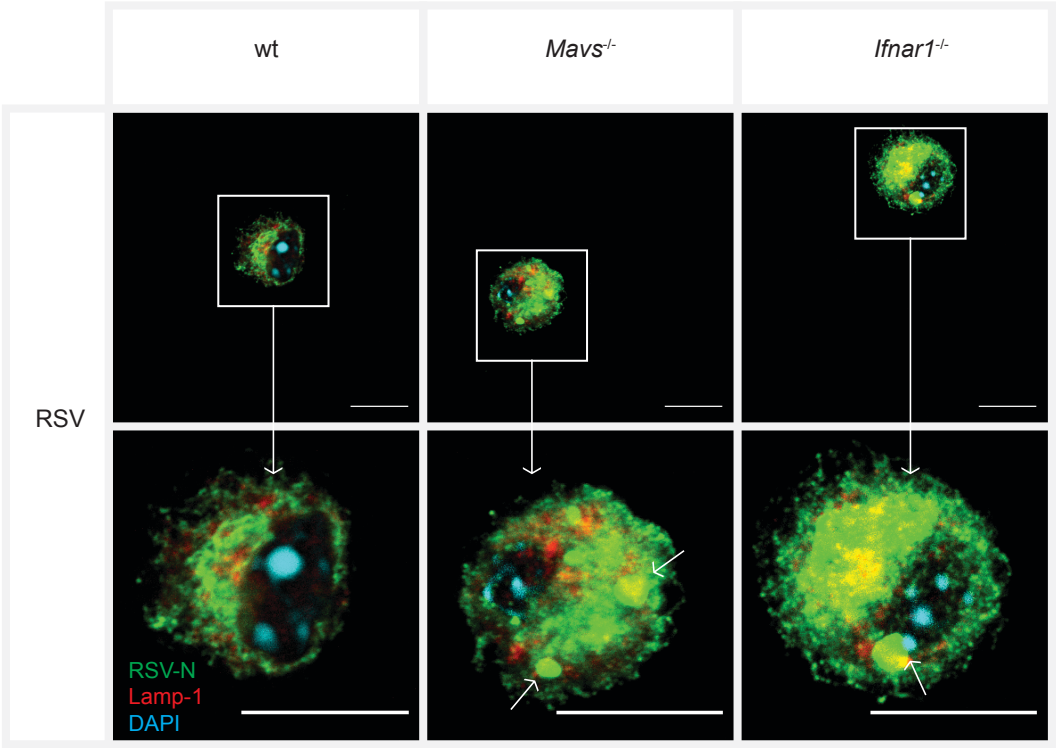


Figure 5.12 *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs that encountered RSV *in vivo* contain IBs in their cytoplasm. Wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were intranasally infected with 1x10^{6} FFU of RSV and 18 hrs post infection primary AMs were extracted from the BAL. The cells were fixed stained for RSV N protein [green], LAMP-1 [red] and DAPI [blue]. Images were acquired on a 100x/1.4 Plan-Apochromat oil lens by line averaging. RSV-anti-N (Alexa 488) was acquired on the 488 nm laser, anti-LAMP-1 (Alexa Fluor 568) was on 548 nm laser and DAPI on the violet 405 nm laser. The scale bar represents 10 µm. The images represent the majority of AMs from 2 independent experiments.

5.3.3 Inflammatory profile of AMs after RSV exposure *in vivo*

Previous studies suggested that AMs are responsible for production of inflammatory mediators during RSV infection (Benoit et al., 2006; Makris et al., 2016; Pribul et al., 2008; Tate et al., 2010). In order to provide a more extensive inflammatory profile of AMs after RSV exposure, mice were intranasally infected with RSV and at 12 hrs post-exposure the BAL was collected and the AM population was sorted using FACS (gating strategy in Fig 4.3). The expression of various genes by AMs was assessed using the RT^2 Mouse antiviral response or the mouse cytokine/chemokine arrays (Qiagen, UK).

5.3.3.1 Cytokine expression by AM exposed to RSV *in vivo*

RSV infection induced the secretion of an array of pro-inflammatory cytokines that drive the immune response and disease severity (Table 1-1) (Borchers et al., 2013; Hornsleth et al., 2001). AMs that encountered RSV *in vivo* had a similar inflammatory profile as *ex vivo* exposed primary AMs (Fig 5.1, (Makris et al., 2016)) as they expressed type I IFNs, CXCL10, TNF-α, IL-6 and CCL3 (Fig 5.13). Furthermore, in AMs that encountered RSV i*n vivo*, the expression of inflammatory cytokines such as IL-1α and IL-1β is significantly higher in AMs from RSV infected mice compared to those infected with PBS (Fig 5.14). AMs also expressed other mediators such as IL-1RA, IL-15, IL-27 and TRAIL (Fig 5.14).

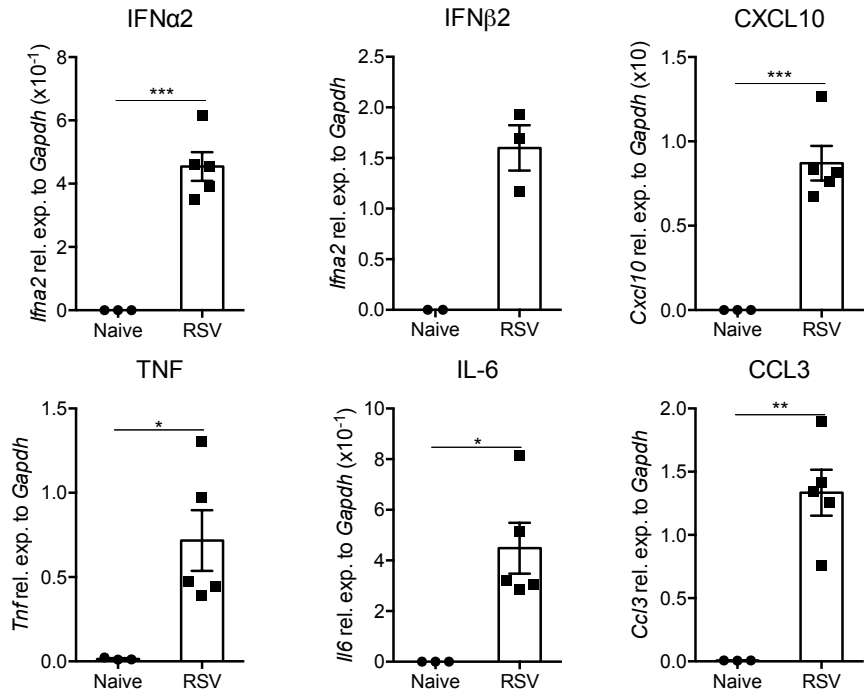


Figure 5.13 AMs exposed to RSV for 12 hours *in vivo* express identical inflammatory mediators as ex vivo exposed primary AMs. Wt mice were intranasally infected with RSV (1 x 10^6 FFU) or PBS and were then sacrificed 12 hrs post exposure. The AMs were sorted from the BAL and the expression of antiviral and cytokine/chemokine genes were assessed using the RT^2 array platform (Qiagen, UK). Data are shown as $\pm$ SEM and each point represents RNA pooled from individual sorts of 9-15 mice in RSV infected groups and 5-10 mice in naïve groups. The statistics IFNα2, CXCL10, TNF, IL-6 and CCL3 were performed due to an overlap between the RT^2 antiviral and cytokine/chemokine arrays which provided a sufficient number of data points. Statistical significance between indicated groups was determined by two-tailed, unpaired Student's *t* test. *p<0.05; **p<0.01; ***p<0.001.

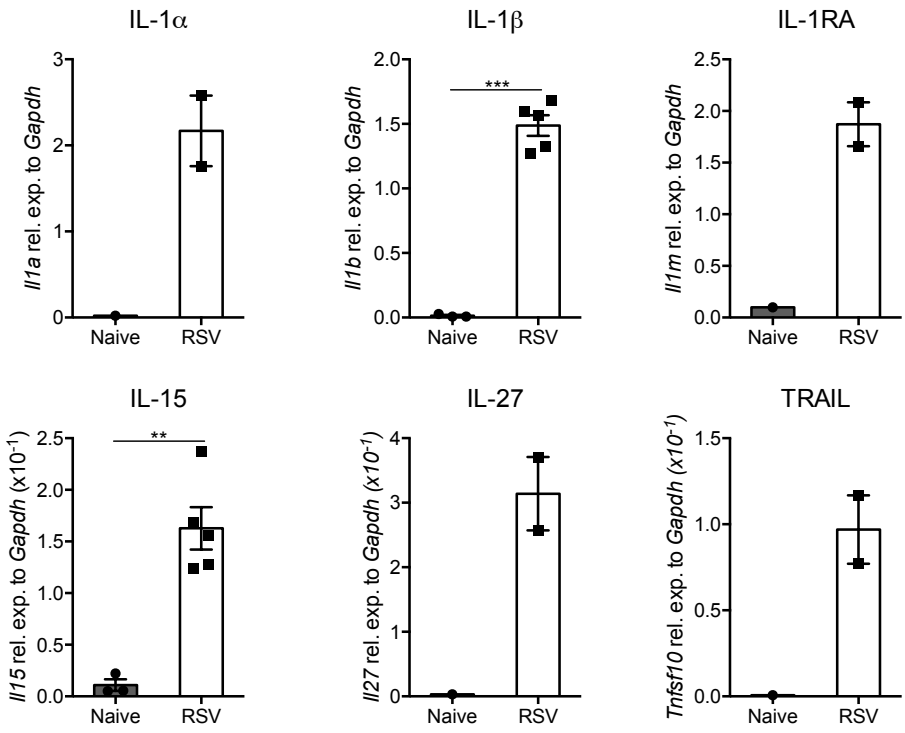


Figure 5.14 AMs exposed to RSV for 12 hours *in vivo* express pro-inflammatory cytokines. Wt mice were intranasally infected with RSV (1 x 10^6 FFU) or PBS and were then sacrificed 12 hrs post exposure. The AMs were sorted from the BAL and the expression of antiviral and cytokine/chemokine genes were assessed using the RT^2 array platform (Qiagen, UK). Data are shown as ± SEM and each point represents RNA pooled from individual sorts of 9-15 mice in RSV infected groups and 5-10 mice in naïve groups. The statistics for IL-1β and IL-15 were performed due to an overlap between the RT^2 antiviral and cytokine/chemokine arrays which provided a sufficient number of data points from independent experiments. Statistical significance between indicated groups was determined by two-tailed, unpaired Student's *t* test. **p<0.01; ***p<0.001.

5.3.3.2 Chemokine expression by AMs exposed to RSV *in vivo*

Further to the expression of inflammatory cytokines, the sorted AMs also expressed an array of important chemokines. The expression of CCL2 by AMs was detected after RSV exposure (Fig 5.15) and was also detected on RNA level in the *ex vivo* exposed AMs (Table 5-1). In addition, AMs expressed genes for chemokines such as CCL4, CCL5, CCL7, CCL12, CCL17, CXCL1, CXCL3, CXCL9, CXCL11 and CXCL16 (Fig 5.15). This vast array of chemokines expressed

by AMs would support previous studies speculating their involvement in the recruitment of other immune cells such as neutrophils, monocytes and T cells (Hussell & Bell, 2014).

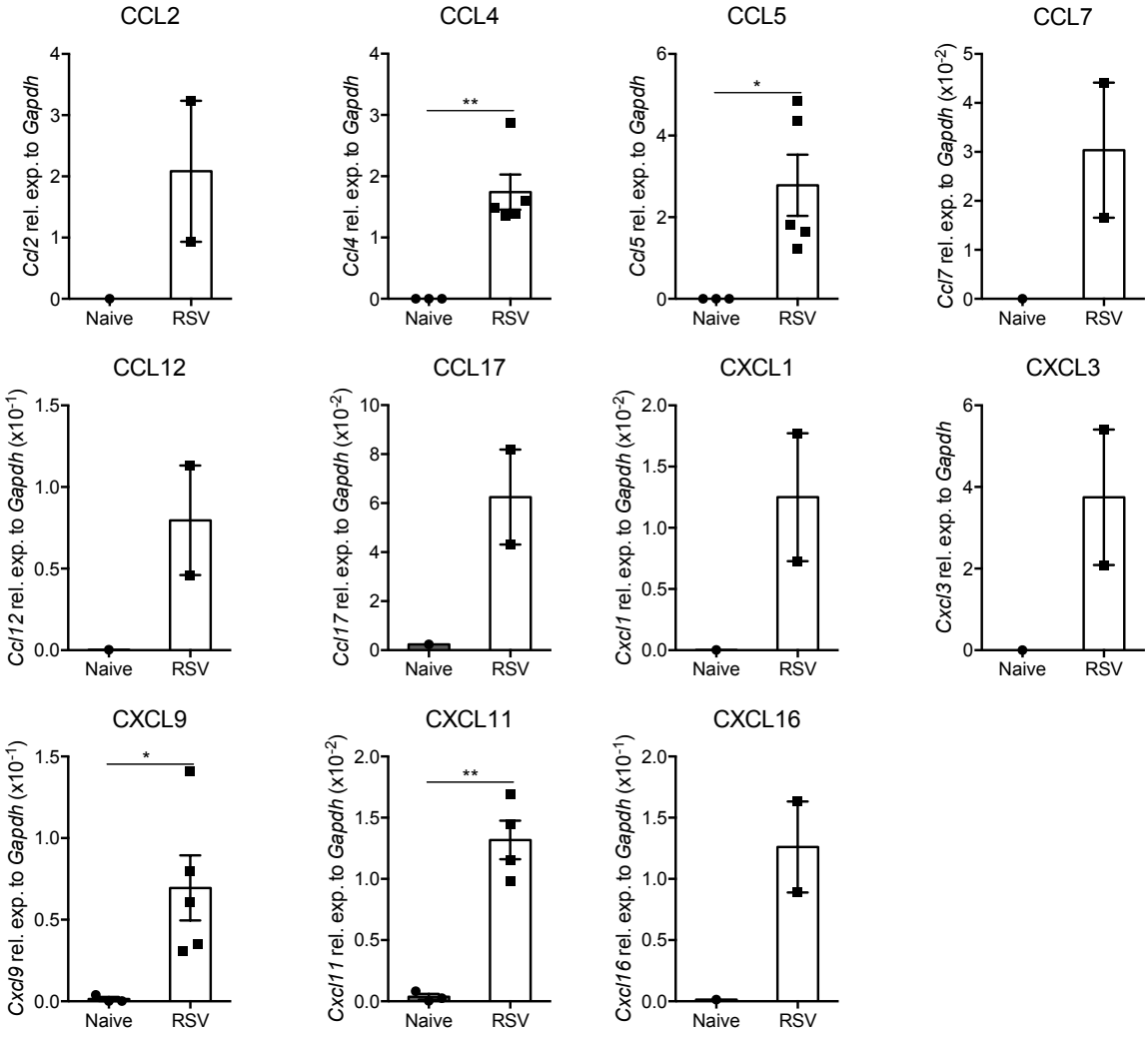


Figure 5.15 *In vivo* expression of chemokines by AMs 12 hours after exposure to RSV. Wt mice were intranasally infected with RSV (1 x 10^6 FFU) or PBS and were then sacrificed 12 hrs post exposure. The AMs were sorted from the BAL and the expression of antiviral and cytokine/chemokine genes were assessed using the RT^2 array platform (Qiagen, UK). Data are shown as ± SEM and each point represents RNA pooled from individual sorts of 9-15 mice in RSV infected groups and 5-10 mice in naïve groups. The statistics for CCL4, CCL5, CXCL9 and CXCL11 were possible because the same chemokines were detected on both RT^2 antiviral and cytokine/chemokine arrays which provided a sufficient number of data points from separate experiments. Statistical significance between indicated groups was determined by two-tailed, unpaired Student's *t* test. *p<0.05; **p<0.01.

5.3.3.3 Type I IFN signalling in AMs after *in vivo* exposure to RSV

AMs have been shown to be the main source of type I IFNs after RSV infection. Furthermore, the inflammatory profile of AMs appears to be driven by recognition of RSV via RIG-I/MDA5 and type I IFN production/signalling (Goritzka et al., 2015). Gene expression in AMs sorted from wt mice that were exposed to RSV *in vivo* supported the notion that AMs are a major source of type I IFNs and they expressed IFNα2 and IFNβ2 after RSV exposure (Fig 5.13). The recognition of RSV by AMs, and the type I IFN signalling, drive the expression of a series of ISGs and signalling pathways (Fig 1.2). The expression of IRF7 and its upstream signalling intermediate TANK are both induced by RSV in AMs. The ISGs, OAS2 and ISG15 are also induced after RSV exposure (Fig 5.16). Finally, STAT1 expression is also upregulated, which could suggest the activation of the IFNAR signalling pathway (Fig 1.4 and Fig 5.16).

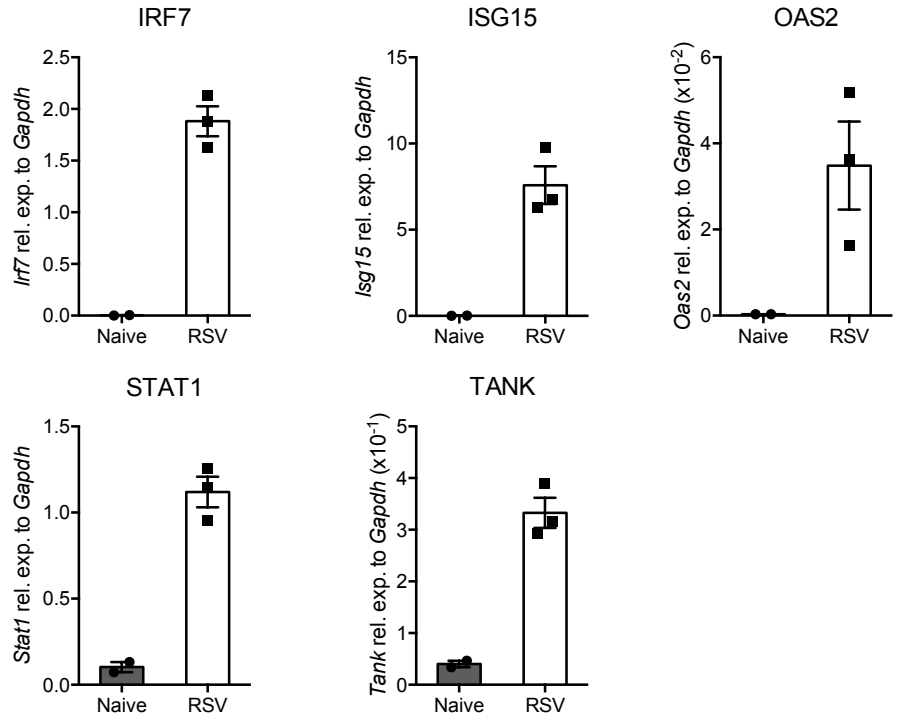


Figure 5.16 Type I IFNs, ISG and type I IFN-signalling are upregulated in AMs after a 12 hour exposure to RSV *in vivo*. Wt mice were intranasally infected with RSV (1 x 10^6 FFU) and were then sacrificed 12 hrs post exposure. The AMs were sorted from the BAL the expression of antiviral cytokines and chemokines was assessed using the RT^2 array platform (Qiagen, UK). Data are shown as $\pm$ SEM and each point represents RNA pooled from individual sorts of 9-15 mice in RSV infected groups and 5-10 mice in naïve groups.

5.3.3.4 Expression of PRRs, signalling molecules and growth factors in AMs exposed to RSV *in vivo*

The recognition of RSV by PRRs and their respective signalling pathways is the driving force of the innate immune response (T. H. Kim & Lee, 2014). RSV induced the expression of AIM2, MDA5, MX1, RIG-I, RLR-3 expression in AMs after RSV infection. All of these are important for the recognition of invading pathogens in the cytosol (Fig 5.17). Furthermore, the expression of endosomal TLR3 and IκBα (important for signalling via TRIF (Fig 1.2)) were upregulated. Finally, AMs appeared to express various co-stimulatory receptors and growth factors after infection. These receptors include CD40 and CD86 (Fig 5.18). The expression of macrophage growth factor, CSF1, and the platelet factor, PF4, appear to also be induced in AMs after exposure to RSV (Fig 5.18).

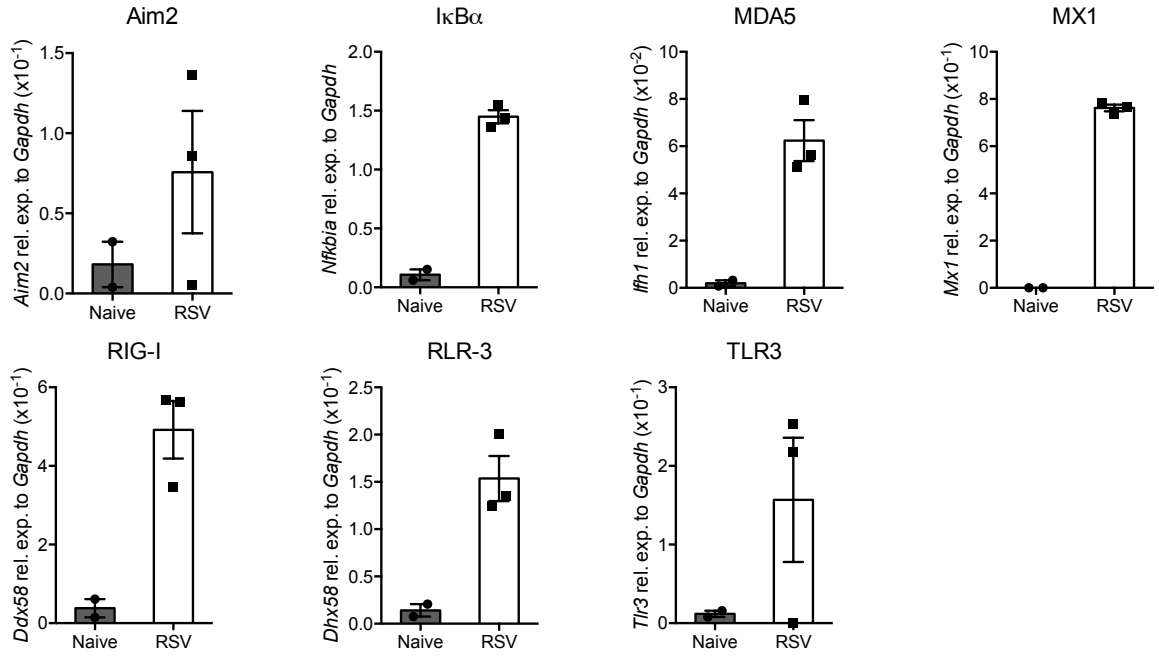


Figure 5.17 PRR upregulation in AMs after exposure to RSV for 12 hours in vivo. Wt mice were infected with RSV (1 x 10^6 FFU) and were then sacrificed 12 hrs post exposure. The AMs were sorted from the BAL the expression of antivrial cytokines and chemokines was assessed using the RT^2 array platform (Qiagen, UK). Data are shown as ± SEM and each point represents RNA pooled from individual sorts of 9-15 mice in RSV infected groups and 5-10 mice in naïve groups. The expression of genes was assessed using the RT^2 antiviral array platform.

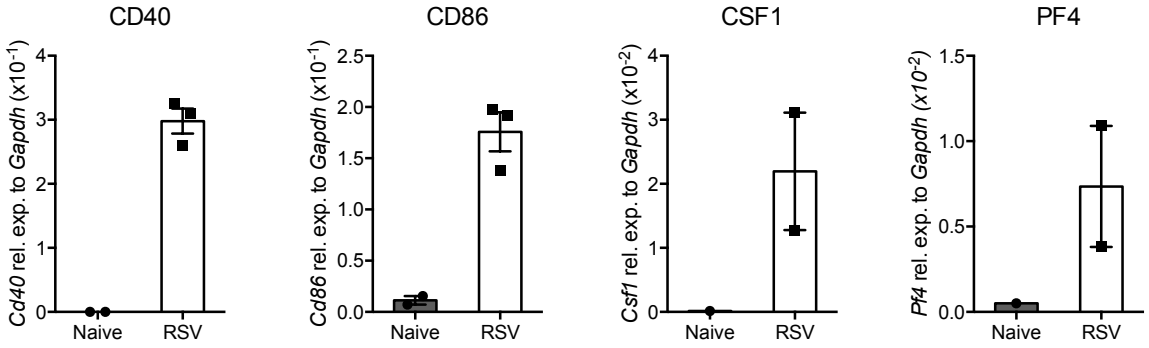


Figure 5.18 Upregulated co-stimulatory receptor and growth factor expression in AMs exposed to RSV for 12 hours *in vivo*. Wt mice were infected with RSV (1 x 10^6 FFU) and were then sacrificed 12 hrs post exposure. The AMs were sorted from the BAL the expression of antiviral cytokines and chemokines was assessed using the RT^2 array platform (Qiagen, UK). Data are shown as ± SEM and each point represents RNA pooled from individual sorts of 9-15 mice in RSV infected groups and 5-10 mice in naïve groups. The expression of CD40 was assessed using the RT^2 antiviral array platform whereas the CD70, CD86, CSF1 and PF4 were assessed using the RT^2 mouse cytokine/chemokine array platform.

5.3.4 Discussion

Together with epithelial cells, AMs are one of the first cells to encounter viral infections in the airways. Therefore, it is vital to understand what their role is in initiating and regulating the immune response and how these cells interact with respiratory viruses such as RSV. The *in vivo* interaction between AMs and RSV has not been extensively studied. The usage of fluorescence microscopy provided a detailed idea regarding the interaction between AMs and RSV and the role MAVS and IFNAR signalling have in it. Previous data from this study convincingly showed that RSV replication in AMs is abortive *ex vivo* and it was vital to confirm these findings *in vivo*. RSV has been shown to efficiently replicate in the mouse lung (Bannister et al., 2010; Loebbermann et al., 2012) and therefore AMs were studied in the lung, where there are more cellular interactions occurring. There appeared to be a higher intensity in N staining for the wt AMs when compared to the *ex vivo* exposed primary AMs. This might be due to the fact that more RSV proteins are stuck to the surface of the cells as more viral particles are present since RSV infection is successful in mouse epithelial cells (Bannister et

al., 2010; Loebbermann et al., 2012). It was therefore important to study AMs in their 'natural' environment. It would be interesting to also study the presence of RSV proteins in AMs at later timepoints. Similarly, to the *ex vivo* data, AMs that encountered RSV *in vivo* appeared to control RSV better when MAVS and IFNAR1 signalling was intact. Furthermore, IBs in the cytosol of the MAVS- and IFNAR1-deficient AMs were observed. As this is the first time that the current fluorescence microscopy methodology was used to visualise IBs in AMs that encountered RSV *in vivo*, further repetitions of the experiment are required before conclusively stating that IBs are in fact present in these cells.

Furthermore, studies have suggested that AMs can produce many inflammatory mediators that are important during RSV infection such as CCL3, IL-1β, IL-6, IFN-γ and TNF-α (Table 1-1 and 1-2 for extensive list of RSV mediators and general functions; (Benoit et al., 2006; Pribul et al., 2008; Tate et al., 2010)). By studying AMs *ex vivo* their contribution to the production of many of these mediators was confirmed (see Section 5.1 and (Makris et al., 2016)). One limitation with previous studies of AMs was that they were depleted using clodronate liposomes or stimulated *ex vivo*. This depletion treatment is considered harsh and additional cells to AMs may be affected, which may influence the interaction between other immune cells (Benoit et al., 2006; Pribul et al., 2008; Tate et al., 2010). In this study, wt mice were infected with RSV intranasally and the AMs were then sorted using FACS. The extracted RNA was studied using cytokine/chemokine and antiviral gene arrays (Qiagen, UK). This provided a precise tool to assess the mediators that AMs contribute during RSV infection. The IFN-α2 and IFN-β2 expression was upregulated after RSV infection. These results confirm the notion that AMs are a major source of type I IFNs during RSV infection.

A multitude of inflammatory chemokines were induced after RSV infection including CXCL10, CCL2 and CCL3. These mediators have also been shown to be upregulated in infants with RSV bronchiolitis (McNamara et al., 2005). The patterns observed in these infants suggested that the source of these chemokines may vary at different stages of disease as the RSV infection expands to the lower airway and cellular recruitment, especially of neutrophils, occurs (McNamara et al., 2005). AMs that encountered RSV *in vivo* had a similar inflammatory profile as the primary AMs that were exposed to RSV *ex vivo* (Table 5-2). Mediators such as CCL2 have been shown to be important for the recruitment of monocytes, to the lungs, which are

important for controlling the viral load (Goritzka et al., 2015). The expression of monocyte chemoattractants CCL17 and CCL12 was also upregulated in AMs that encountered RSV *in vivo*. Both of these mediators signal via CCR2 and were also shown to be upregulated in bone marrow-derived macrophages exposed to herpes simplex virus type 1 (Conrady et al., 2013). Finally, CCL3 was upregulated in AMs both *ex vivo* and *in vivo* and has been associated with more severe forms of RSV bronchiolitis in infants (Garofalo et al., 2001).

Infants with RSV bronchiolitis have large influx of neutrophils into the airways that might be driven by RSV or cell death (McNamara et al., 2003; Sheeran et al., 1999). The presence of neutrophils, combined with the expression of CXCL1 and CXCL8, in the airway during RSV-induced bronchiolitis has been associated with a more severe phenotype (Everard et al., 1994; P. K. Smith et al., 2001). AMs had an increased expression of CXCL1 after exposure to RSV *in vivo*, a chemokine important for the recruitment of neutrophils, during RSV infection and have been detected in infants with severe RSV-induced disease (Hornsleth et al., 2001; Kolaczkowska & Kubes, 2013). Understanding the source of neutrophil chemoattractants and how this influences RSV disease severity would be important for managing the more severe cases of RSV disease, especially in infants.

Table 5-2 Inflammatory mediators that are not detectable using Luminex or ELISA, but detectable qPCR, after primary AMs being exposed to RSV MOI 2 for 20 hrs *ex vivo* or wt mice were infected with RSV (1 x 10^6 FFU) and were then sacrificed 12 hrs post exposure and AMs were sorted by FACS. Expression of the genes was assessed by RT^2 mouse antiviral and cytokine/chemokine profile arrays (PAMM-150Z and PAMM 122Z Qiagen, UK). Data are representative of at least two experiments.

	Assays			
Mediator	***Ex vivo***			***In vivo***
	Luminex	ELISA	Gene expression	RT^2 array
IL-1α	-		+	+
IL-1β	-	-	+	+
IL-2	-		-	-
IL-4	-		-	-
IL-5	-		-	-
IL-10	-		-	-
IL12p40/ p70	-		-	+/-
IL-13	-		-	-
IL-17	-		-	-
IL-18			-	-
IFN-λ			-	-
IFN-γ	-		-	+
CXCL1	-	-	+	+
CCL2	-	-	+	+
CXCL9	-		+	+
GM-CSF	-		-	+
VEGF	-		-	-

Furthermore, results from the chemokines expressed by AMs after encountering RSV *in vivo* suggested their involvement in T cell recruitment. This notion has previously been described with contradictory results, and their role in antigen presentation and activation of lung resident T cells is not fully elucidated (Hussell & Bell, 2014). The expression of CXCL10, CXCL9 and CXCL11 might suggest that AMs interact with T cells during RSV infection. No T cells would be recruited into the lung 12 hrs after RSV infection, however AMs have been shown to interact with populations of tissue resident T cells (Soroosh et al., 2013). Interestingly, all three chemokines are ligands to CXCR3 which is expressed on activated T cells, DCs, epithelial cells, NK cells and fibroblasts (Lindell et al., 2008; Van Raemdonck et al., 2015). AMs from mice are poor antigen presenting cells although a small number can transport antigens to the lung-draining lymph nodes. This is characterised by a weak expression CD40 and CD86, co-stimulatory molecules that are important during antigen presentation to T cells during respiratory infections (Bartz et al., 2003; Y. Liu et al., 1997). The reduced levels of CD86 in human AMs is believed to be an indication that they are poor antigen presenting cells (Blumenthal et al., 2001; Hussell & Bell, 2014).

The production of chemokines and pro-inflammatory cytokines after RSV infection is driven by type I IFNs. To initiate their activation, AMs recognise RSV through various PRR and their respective signalling pathways. The data indicated that there is a strong expression of the cytosolic PRR signalling pathways including RIG-I, MDA5 and RLR-3. Furthermore, there appeared to be an increased expression of TLR3 which recognises dsRNA in the endosome (Alexopoulou et al., 2001). Finally, the induced expression of STAT1 and various ISGs can support the importance of type I IFN signalling in AMs during RSV infection. In addition, AMs that encountered RSV *in vivo* also upregulated an array of cytokines. Disease severity has been in bronchiolitis associated with the presence of IL-1, IL-6, IL10 and TNF-α (Borchers et al., 2013).

Overall, results from the arrays performed on AMs support previous *ex vivo* findings which characterise them as an important source of type I IFNs and inflammatory mediators. However, one must be objective when interpreting such results. Although they provide evidence of the involvement of AMs in the response to RSV, and are confirming previous studies, they are not directly compared to other lung cells which may contribute to the

cytokine production and therefore the dominant cytokine/chemokine-producing cell has not been evaluated for each mediator. For example, CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL11, CXCL10, IL-6, TNF-α and IFN-α have been believed to be produced by epithelial and other structural cells (Borchers et al., 2013; Ioannidis et al., 2012; Tabarani et al., 2013; Villenave et al., 2012). This study confirmed findings of Goritzka et al., that type I IFNs are in fact produced by AMs and provided evidence that AMs contribute to viral recognition and induction of important pro-inflammatory cytokines (Table 5-2).

5.4 Neonates respond to PRR ligands exposure *in vivo*

Infants and young children are especially vulnerable to RSV infection. Most of the vaccine development programs have been overshadowed by the formalin-inactivated-RSV (FI-RSV) vaccination failure in the 1960's which caused more severe disease/symptoms when vaccinated children were naturally infected RSV (H. W. Kim et al., 1968a; W. K. Kim et al., 1968b). As a result, there is need to develop an effective vaccination regime against RSV. Neonatal mice, infected with RSV, do not initiate major immune responses during primary infection (Culley et al., 2002). However, during a secondary infection there are characteristics associated with asthma and a more severe disease that is Th2-biased (Lambert et al., 2014). The innate immune responses in neonates is believe to be important for driving the adaptive responses (Harker et al., 2014). Therefore, how the lung responds to different innate stimuli was compared in neonates and adults.

5.4.1 Neonatal response to R848 is more potent than adults

To assess differences in the TLR responses the presence of neutrophils and IL-6 in the BAL were assessed after exposure to ligands such as CpG, poly(I:C), LPS, R848 and imiquimod (Fig 5.19). Due to the limitation in the number of pups and the time of the project, full time course experiments could not be performed for each ligand. Cell composition in the BAL was assessed by cytospin counts (Fig 5.19a). The percentage of neutrophils in adults and neonates was very low at baseline but after challenge with CpG, poly(I:C), and LPS, neonates and adults responded in a similar way recruiting the same frequency of neutrophils (Fig. 5.19a). In contrast, imiquimod and R848 induced an influx of neutrophils in the neonates which was absent in adults (Fig 5.19a). TLR4 stimulation with LPS appeared to drive the highest influx of neutrophils in both adults and neonates. Adult and neonatal mice had similar expression of TLRs in the lung (data not shown, (Kollmann et al., 2009)). Exposure to CpG, poly(I:C) or LPS did not induce IL-6 production; whereas R848 and not imiquimod (despite both being ligands to TLR7), induced the production of IL-6 in the BAL of neonates (Fig 5.19b). These findings suggest more a potent signalling in neonates when the TLR7 signalling pathway is activated by R848.

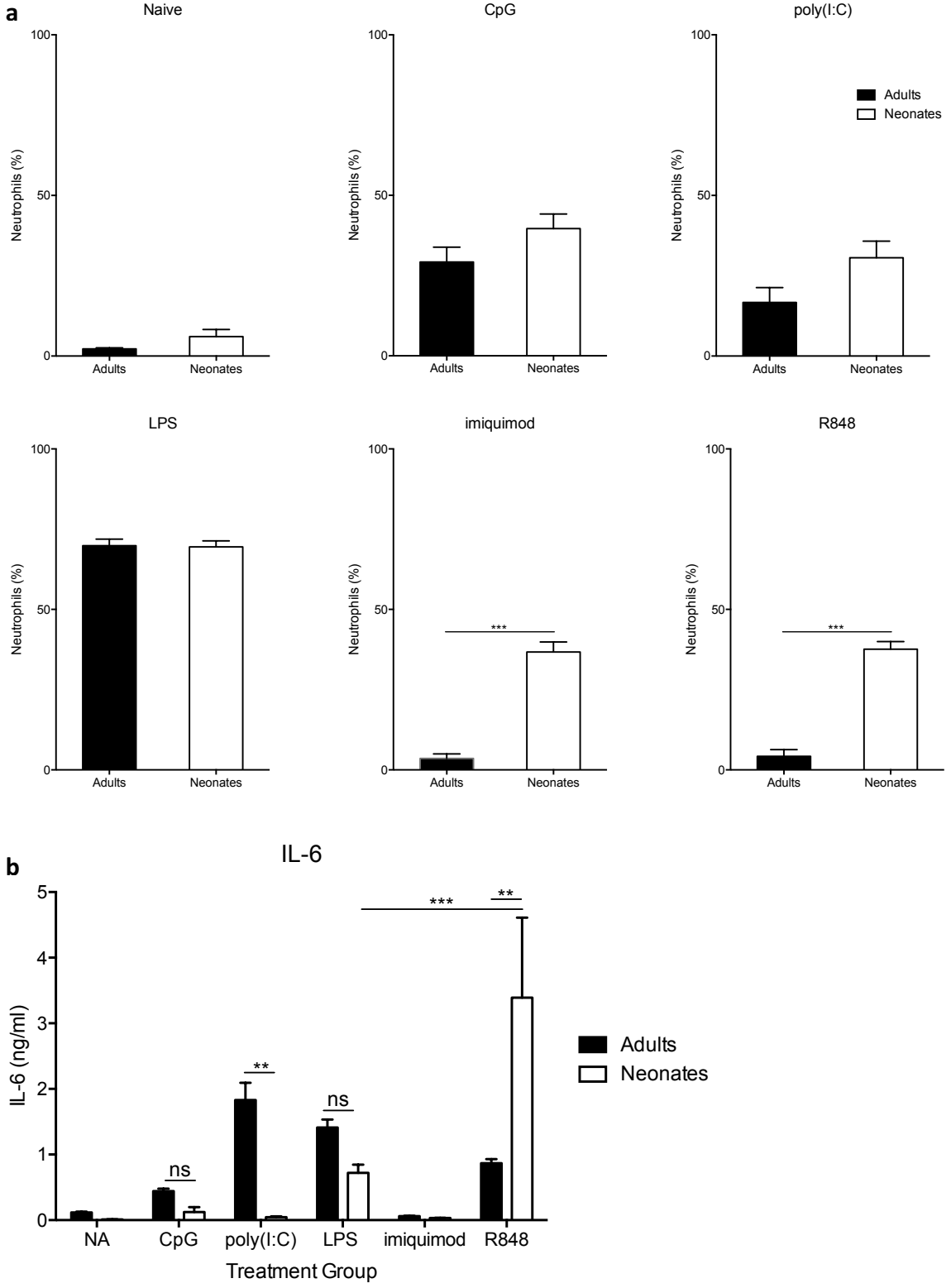


Figure 5.19 Neonates challenged with TLR7 ligands have a potent neutrophil influx however only R848 induced IL-6 production. Adult (8-10 weeks old) or neonatal (4-6 days old) C57BL/6 mice were intranasally exposed to PBS (NA), CpG (1 μg/g), poly(I:C) (2.5 μg/g), LPS (500 ng/g), imiquimod (2.5 μg/g) and R848 (2.5 μg/g) for 12 hrs. (a) The percentage of neutrophils in the

BAL of the mice was assessed by H&E staining of cytospin slides. (b) Production of IL-6 in the BALF of challenged mice was assessed by ELISA. Data are shown as ± SEM of (a) n=10-13 mice polled from 3 experiments and (b) and n=3-4 mice representative of at least 3 experiments. Statistics were determined by (a) two-tailed, unpaired Student's *t* test or (b) one-way ANOVA multiple with Tukey's post hoc test. ns, no statistical significance **p<0.01; ***p<0.001.

5.4.1.1 Neutrophil recruitment and production of inflammatory mediators in neonates are driven by R848

Previous unpublished findings in our laboratory and ligand comparison experiments (Fig 5.19) suggested that neonates respond differently to R848 exposure compared to adults with a profound production of IL-6 in the airways. The dynamics of these observations were studied in more detail. Neonatal (4-6-day-old) and adult (8-10-week-old) mice were exposed intranasally to R848 (2.5 μg/g of body weight) and the presence of inflammatory mediators and the cell composition of the airways was assessed at 6, 12 and 24 hrs post exposure. The neonates had higher gene expression of the neutrophil chemoattractant CXCL1 at 6 hrs post exposure compared to adults (Fig 5.20a). In relation, a higher percentage of neutrophils in the airways was observed at 6, 12 and 24 hrs post exposure in neonates compared to adults (Fig 5.20b). Neonates also produced more IL-6 at 6 and 12 hrs post exposure; whereas more TNF-α was observed at 6 hrs compared to the adults (Fig 5.20c). Gene expression analysis in the lung tissue confirmed the trend of increased of TNF-α in the neonatal group (data not shown). Taken together these data suggest that after intranasal R848 exposure, neonates elicit a more active innate response in the lungs compared to adults.

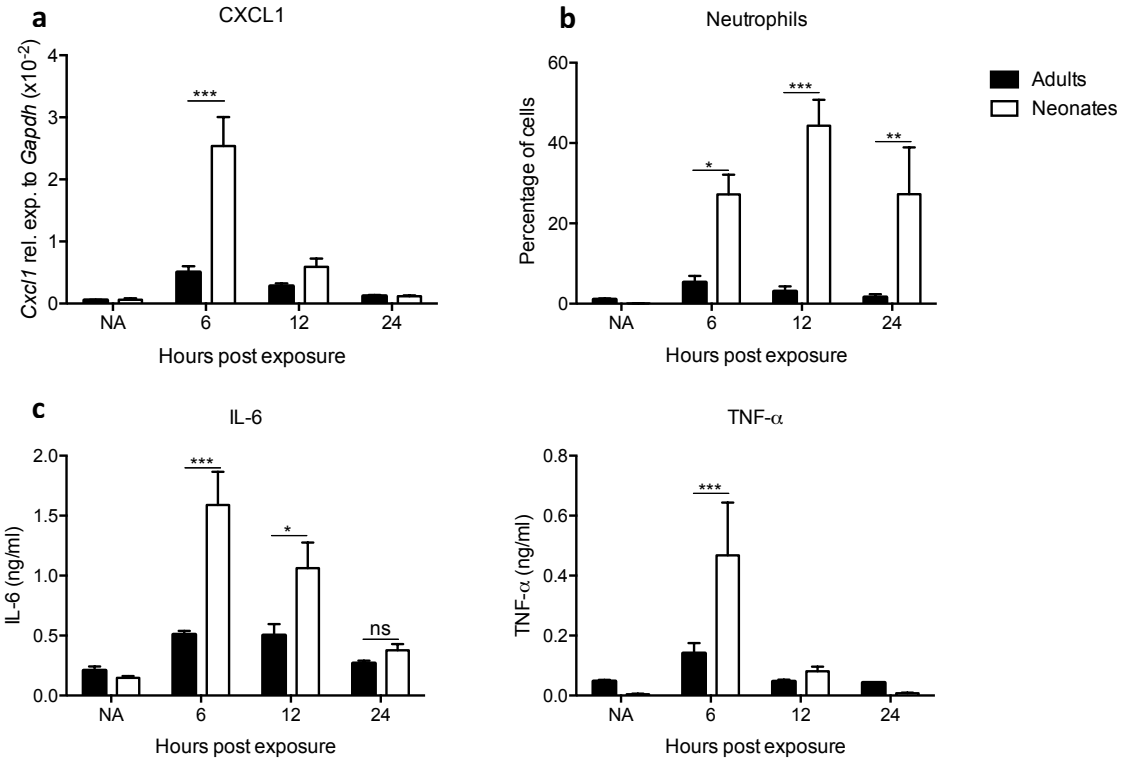


Figure 5.20 R848 induced more neutrophil recruitment and secretion of IL-6 and TNF-α in the airways of neonatal mice than adult mice. Neonatal and adult C57BL/6 mice were exposed to R848 intranasally at 2.5 μg/g of body weight or to PBS (NA). (a) The expression of *Cxcl1* in the lung tissue was measured by qPCR. (b) The percentage of neutrophils in the BAL was calculated on H&E stained cytospin slides. (c) The production of IL-6 and TNF-α in the BALF was measured by ELISA. Data are shown as mean ± SEM of 3-6 mice per group and representative of 3 experiments. Statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test. *p<0.05; **p<0.01; ***p<0.001.

5.4.1.2 AMs and inflammatory monocytes are the source of IL-6 in neonates after R848 exposure

Neonates were more potent responders to TLR7 stimulation than adults (Fig 5.18) and R848 induced the production of inflammatory mediators in neonates (Fig 5.18, 5.19). The limitation with using ICS as a method to study innate cells is that the protocols and antibodies are primarily designed for adaptive cell assays. Therefore, the ICS flow cytometry methodology,

previously used *ex vivo,* was adjusted to study the source of IL-6 in neonatal mice exposed to R848. Adult and neonatal mice were intranasally exposed to R848 (2.5 μg/g of body weight) for 12 hrs. The lung cells were then analysed using flow cytometry after staining for various populations of immune cells (Fig 4.1). Each population of cells was analysed for the presence of IL-6. Only AMs (Fig 5.21a) and inflammatory monocytes (Fig 5.21b) appeared to have a higher percentage and number of IL-6-expressing cells in the neonates compared to the adults. Preliminary ICS data confirmed that adults responded to CpG and poly(I:C); whereas neonates did not (data not shown). As these figures represent one experiment there is only one mouse representing the naïve group. However, after exposure to R848 these data suggest that the neonatal response to R848 is more potent than in adults and that the source of IL-6 appeared to be AMs and inflammatory monocytes.

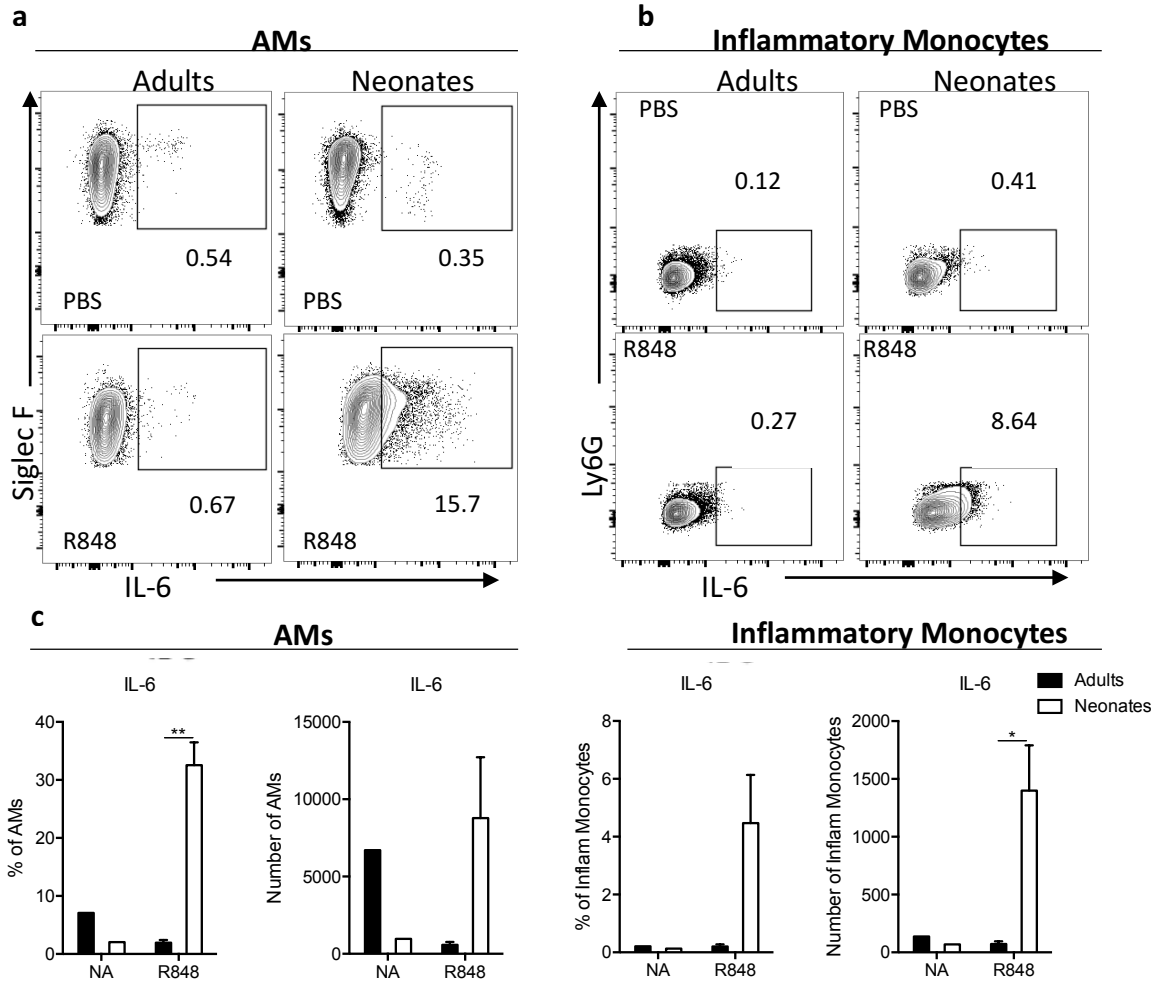


Figure 5.21 AMs and inflammatory monocytes are the source of IL-6 in neonates after R848 exposure. Neonatal and adult C57BL/6 mice were exposed to R848 intranasally at 2.5 μg/g of body weight or to PBS (NA). The cell populations (Fig 4.1) were analysed by flow cytometry

and the expression of IL-6 was assessed by ICS. The representative IL-6 gating is shown for (a) AMs and (b) inflammatory monocytes ($CD11c^{-}CD11b^{hi}CD64^{hi}Ly6C^{hi}$) together with (c) the percentage and number of IL-6-positive cells for each defined cell population. Data are shown as ± SEM of 3 mice for R848- and 1 mouse for PBS-exposure. Statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test. *p<0.05; **p<0.01.

5.4.1.3 Assessing R848 as an adjuvant

The main outcome of the RSV vaccination trial in the 1960s was that when the children got natural exposure to RSV they had severe lung pathology. Similarly, mice primed with RSV as neonates develop more severe pathology to RSV upon a secondary challenge. The most profound feature of this secondary infection was a Th2 dominated response with higher levels of $CD4^{+}IL\text{-}4^{+}$ T cells. Furthermore, there appeared to be higher levels of neutrophils and eosinophils in the BAL in these mice (Culley et al., 2002). As neonates innate responses to RSV are minimal adjuvants can be used in order to boost response. Pre-exposure to CpG in mice was shown to protect against the delayed effects of neonatal RSV infection and that the correct adjuvant can elicit a protective response in neonates (Yamaguchi et al., 2012). TLR7 adjuvants co-delivered with RSV F-trimers increased the Th1 response and protection in adult mice during RSV infection (Francica et al., 2016). To assess if R848 can be used as a potential vaccine adjuvant against RSV, and to determine if it can elicit a protective response upon reinfection, mice were primed together with RSV and R848 and were infected with RSV 42 days later.

5.4.1.4 Exposure to R848 or RSV + R848 does not affect weight gain of neonates

Neonatal mice were primed with PBS, RSV (3.2×10^5 FFU) or R848 (2.5 μg/g)+ RSV (3.2×10^5 FFU) 4-5 days after birth. Adult mice aged 7-8 weeks were primed with RSV (1.5×10^6 FFU). The weight was measured for 10 consecutive days (Fig 5.22). Previous data have shown that neonates exposed to RSV still gain weight albeit at a slower slope (Culley et al., 2002). Priming

neonates with R848 + RSV or RSV did not appear to affect the weight gain in the neonates significantly when compared to the PBS group.

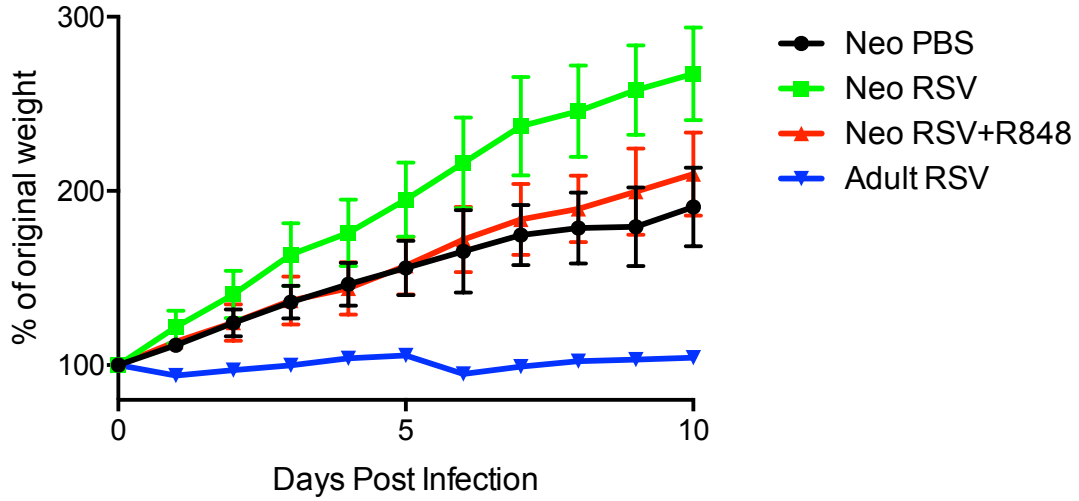


Figure 5.22 Priming neonates with RSV and R848 does not affect weight gain. Neonatal mice were intranasally challenged with PBS [black], RSV (3.2 x 10^5 FFU) [green] or R848 (2.5 μg/g) + RSV (3.2 x 10^5 FFU) [red] and adult mice were intranasally challenged with RSV (1.5 x 10^6 FFU) [blue]. The weight of the mice was monitored for 10 days and plotted as the percentage of the original weight at day 0. Data are shown as mean ± SEM of the weight of 4-8 mice per group. No statistical significance between the neonatal groups was observed using 2-way ANOVA with Bonferroni's post hoc test.

5.4.1.5 Immune responses of neonatally primed mice rechallenged with RSV

All groups, including a "naive" adult group were intranasally re-challenged with 1.5 x 10^6 FFU RSV 42 days after priming and the samples were collected 4 days after the secondary infection since this is the time point that where viral load increases and there is an influx of T cells and production of IFN-γ and IL-4 during secondary infections (Harker et al., 2014). The R848 + RSV mice presented with a rough appearance with scruffy fur and weight lost close to 20% of their original weight during secondary challenge (data not shown). Furthermore, they had a higher percentage and number of neutrophils present in the BAL when compared to all other groups (Fig 5.23). Neonates that were primed with RSV had a significantly higher percentage of neutrophils compared to the PBS challenged neonatal group and RSV primed adults; however

not higher than the "naïve" adult group. These data would suggest that the priming of neonates with R848 + RSV alters the composition of the BAL cells during secondary infection.

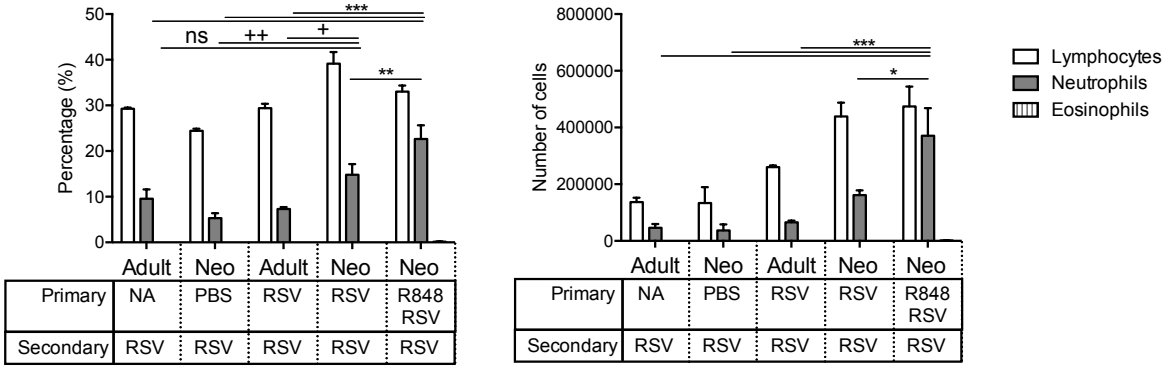


Figure 5.23 Neonatal priming with R848 + RSV induces neutrophilia after secondary challenge with RSV. Mice were challenged as neonates with PBS, RSV or R848 + RSV; whereas adults were either naïve (NA) or challenged with RSV (See section 5.6.1). After 42 days all groups were infected with RSV and 4 days later the cells present in the BAL was enumerated using H&E stained cytospin slides. Data are shown as mean ± SEM of the percentage or number of cells for lymphocytes, neutrophils or eosinophils for 4-8 mice per group. Statistical significance for the differences between the indicated groups was determined by one-way ANOVA with Tukey post hoc test ('*' symbol for RSV+R848 primed and '+' for RSV primed comparisons). ns, no statistical significance; *p<0.05; **p<0.01; ***p<0.001.

5.4.1.6 T cell responses are not significantly altered between RSV and R848 primed mice

In order to estimate the type of immune response induced by priming neonates and adults with RSV or R848 the production of IFN-γ, granzyme B and IL-4 was measured in the BALF. The results showed a higher production of IFN-γ in the R848 + RSV group compared to all other groups (Fig 5.24). The presence of granzyme B (Fig 5.24) and the serum antibodies levels (data not shown) appeared to be identical in all re-challenged groups regardless of their age during the primary challenge. There was no detectable IL-4 present in the BALF of any of the groups (data not shown). Collectively these results suggest that there might be a bias in type 1 responses in the R848 + RSV group; however, in contrast to previous studies in BALB/c mice, the RSV primed neonates also produced some IFN-γ ,although not statistically significant

(Culley et al., 2002). The priming with R848 and RSV appeared to further enhance the production of IFN-γ.

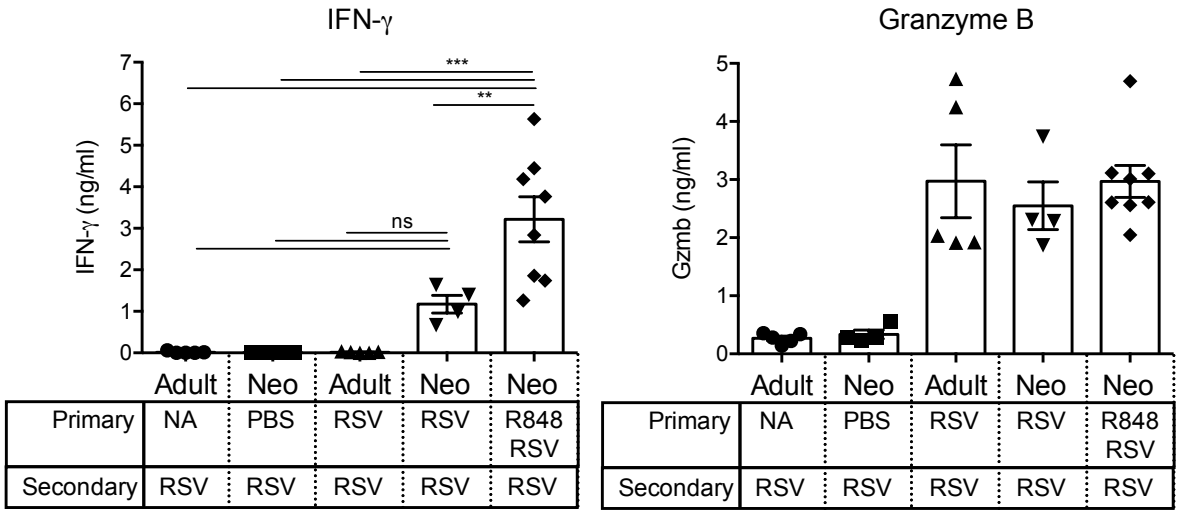


Figure 5.24 Neonates primed with R848 + RSV secrete more IFN-γ after secondary challenge with RSV. Mice were challenged as neonates with PBS, RSV or R848 + RSV; whereas adults were either naïve (NA) or challenged with RSV (See section 5.6.1). After 42 days all groups were infected with RSV and 4 days later the presence of IFN-γ and granzyme B was measured in the BALF by ELISA. Each point on the graphs represents an individual mouse and data are shown as mean ± SEM of 4-8 mice per group and statistical significance for the differences between the indicated groups were determined by one-way ANOVA with Tukey post hoc test. ns, no statistical significance; *p<0.05; **p<0.01; ***p<0.001.

The main feature of neonatal BALB/c mice primed with RSV is that they have a Th2-biased response after secondary infections with an elevated IL-4 and delayed IFN-γ production which potentially leads to increased disease severity (Adkins, 2009; Culley et al., 2002). This is a characteristic which is absent/weak in the C57BL/6 model (See section 5.4.4, (R. Janssen et al., 2007; Tregoning et al., 2010; Watanabe et al., 2004)) where a resistance to infection and Th1 bias is expected during RSV infection. The adaptive immune response was assessed in the lung and BAL of mice that were primed with RSV or R848 + RSV using flow cytometry. The cells were gated as shown in Figure 4.2. Although not statistically significant, there appeared to be a trend toward an increased number of leukocytes in the lung of mice that were primed with either RSV or R848 + RSV as neonates (Fig 5.25a). When comparing the frequencies of

the $CD4^+$ and $CD8^+$ T cells there were no significant differences in the between the challenged group in the lung or BAL (Fig 5.25b). Only the neonatal RSV and R848+RSV primed groups had increased numbers of $CD8^+$ T cells in the lung compared to the RSV primed adults (Fig 5.25c). Overall, these findings suggested that although there was a trend for a higher cellular influx in the R848 + RSV group, there were no major differences between the percentage and number of T cells, when compared to the neonates primed with RSV.

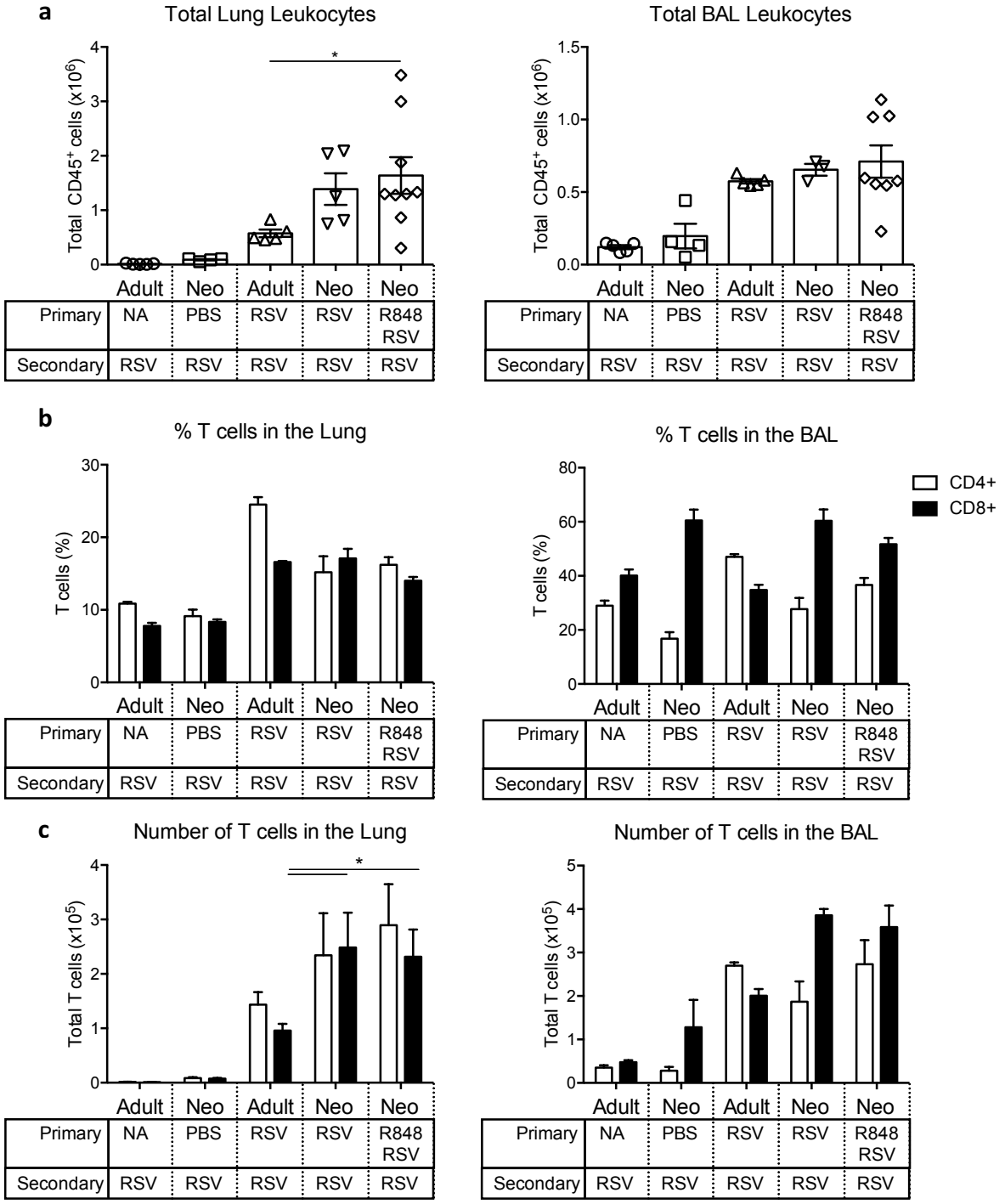


Figure 5.25 Influx of $CD4^+$ and $CD8^+$ T cells is similar regardless of priming. Mice were challenged as neonates with PBS, RSV or R848 + RSV; whereas adults were either naïve (NA) or challenged with RSV (See section 5.6.1). After 42 days all groups were infected with RSV and 4 days later the lung and BAL T cell populations were assessed by flow cytometry. Cells were gated on non-debris, singlet, live cells and CD45 was used as a leukocyte marker. The cells were then gated on $CD19^-CD3^+$ cells and the T cell population were analysed by the expression of CD4 or CD8. (a) The number of T cells was calculated from $CD45^+CD19^-$ cell

populations. (b) The percentage and (c) number of $CD4^+$ and $CD8^+$ T cells were calculated from the $CD3^+$ T cells. All gates were placed using respective FMOs. Each point on the graphs represents an individual mouse and data are shown as mean ± SEM of 4-8 mice per group and statistical significance for the differences between the indicated groups were determined by one-way ANOVA with Tukey post hoc test. *$p<0.05$.

5.4.1.7 There is no evidence of increased Th1 skewing in $CD4^+$ T cells

In order to distinguish if priming with R848 + RSV skewed to a Th1 response after secondary infection to RSV, ICS was utilised to identify IL-4 and IFN-γ expressing $CD4^+$ T cells after PMA and ionomycin treatment. When comparing the production of IL-4 and IFN-γ in the $CD4^+$ populations there were no major differences between the primed groups (Fig 5.26a). Percentage or numbers of IFN-γ^+ cells in the lung and BAL appeared to be at similar levels with a significantly higher expression in the lung of RSV primed adult group (Fig 5.26b, c). The frequencies of IL-4^+ T cells were very low and therefore were excluded from being evaluated (data not shown).

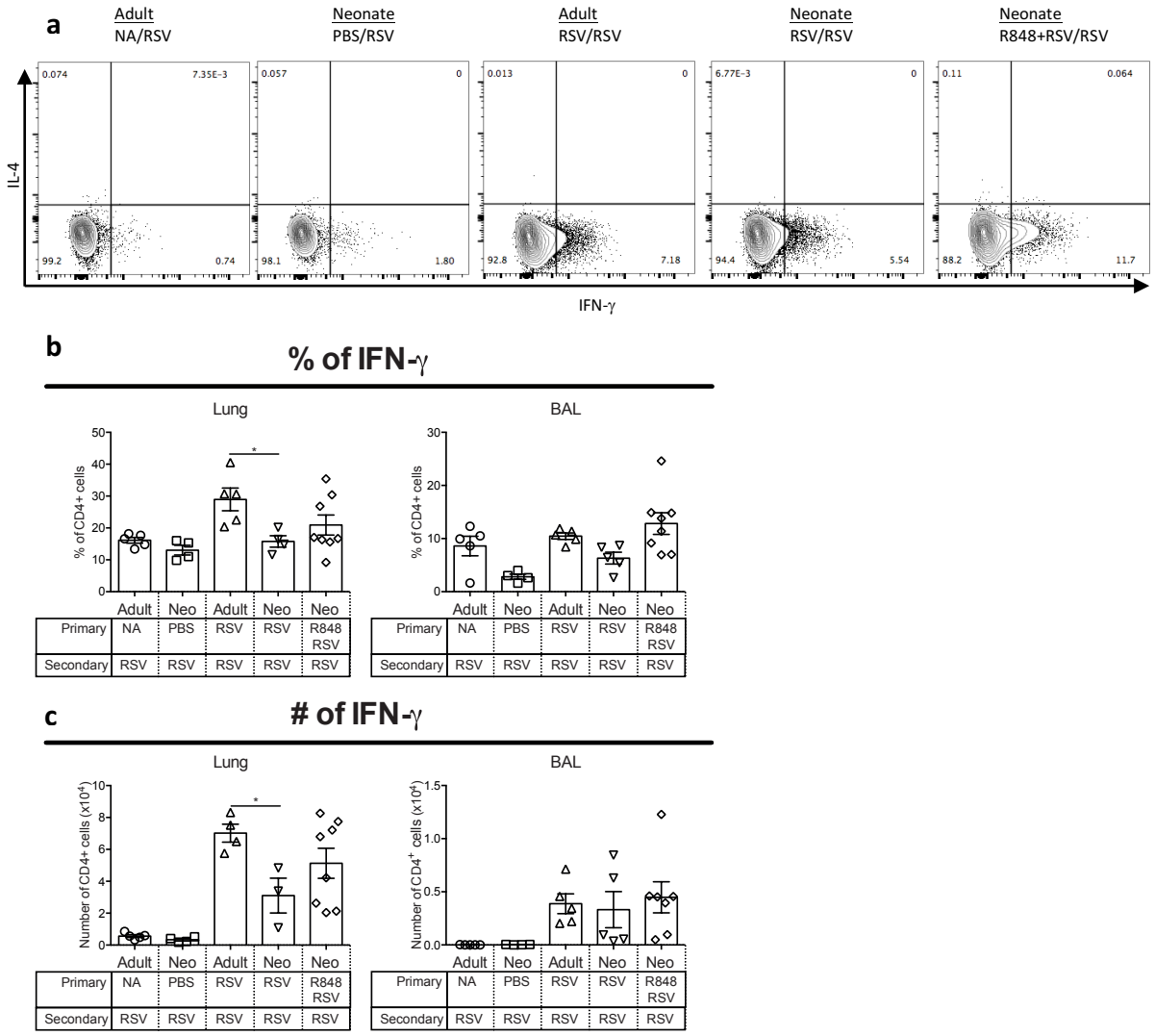


Figure 5.26 Proportion and numbers of IFN-γ$^+$ CD4$^+$ T cells is similar in all primed groups. The CD3$^+$ cells (Fig. 5.24) were assessed for the production of IL-4 and IFN-γ. The production of the cytokines was enhanced by treatment with PMA-ionomycin. (a) Representative gating on lung CD4$^+$ T cells where gates were positioned based on FMOs respective for each cytokine. CD4$^+$ T cells in the BAL and lung were assessed for the expression of (b) the percentage and (c) number of IFN-γ expressing cells. The percentage of the cells was calculated from the total CD4$^+$ population whereas the number was calculated from the total CD45$^+$ cells. The data are shown as mean ± SEM of 4-8 mice per group and statistical significance for the differences between the indicated groups were determined by one-way ANOVA with Tukey post hoc test. *p<0.05.

5.4.1.8 Possibly more IFN-γ in CD8$^+$ T cells after neonatal priming using R848 and RSV

The proportion of IL-4 and IFN-γ expressing CD8$^+$ T cells was also assessed by ICS and flow cytometry. There was no significant induction of IL-4 in the lung CD8$^+$ T cells; however, there was an induction of IFN-γ (Fig. 5.27). When evaluating the CD8$^+$ T cells in the lung there was a statistically significant increase in the percentage of IFN-γ$^+$ cells in the R848 + RSV primed group when compared to either adult- or neonate-RSV primed groups; however, no difference was observed in the BAL CD8$^+$ T cells (Fig. 5.27b). There was a similar trend when determining the total cell number of CD8$^+$IFN-γ$^+$ T cells in the lung; however, there was no statistical signifiance between the RSV and R848 + RSV primed groups (Fig. 5.27c). In addition, the neonatal primed groups had a trend toward an increased number of cells expressing IFN-γ in the BAL (Fig 5.27c). Finally, there was no presence of IL-4$^+$ CD8$^+$ T cells in the BAL or lung of any of the groups.

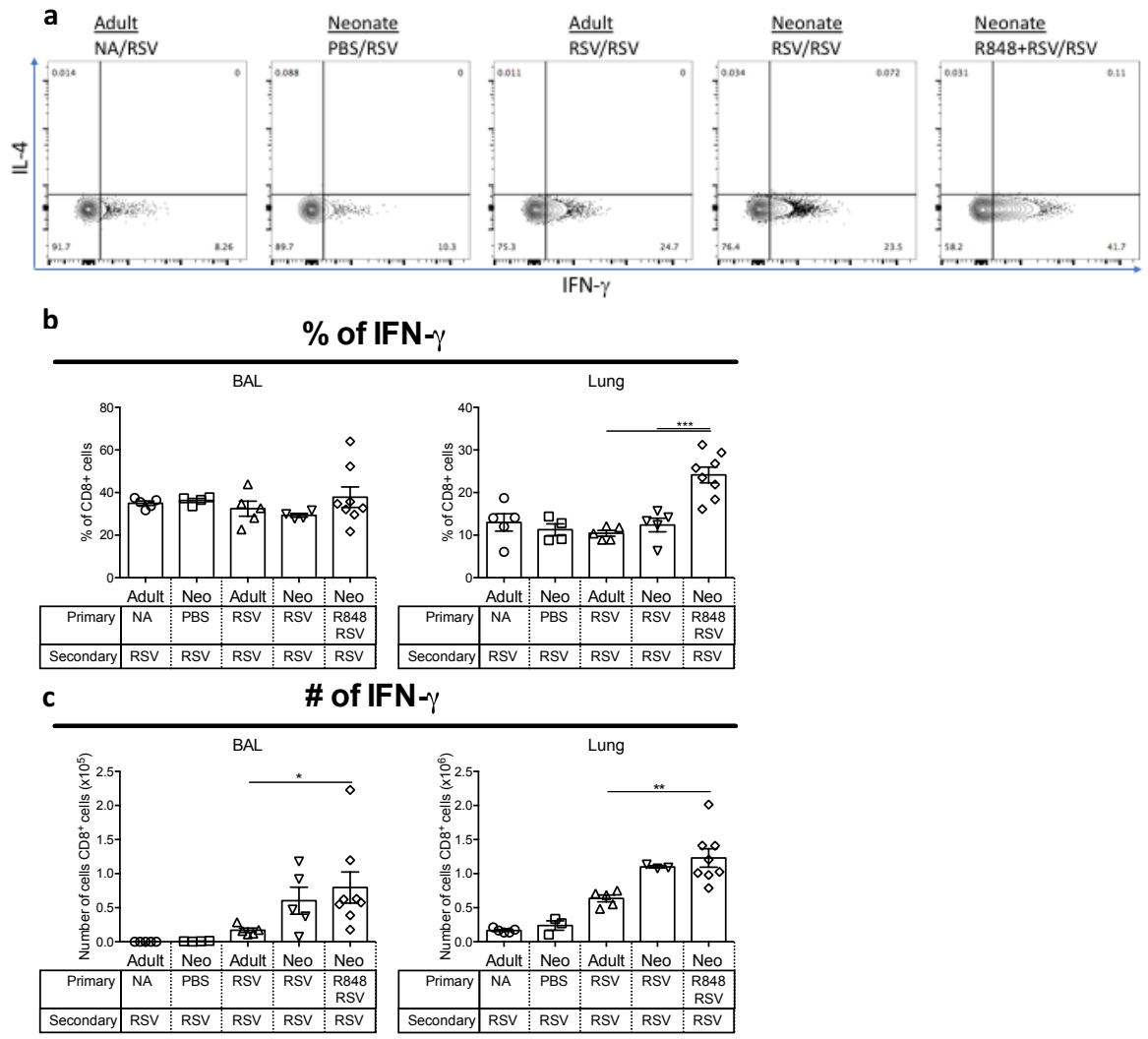


Figure 5.27 Frequency and number of IFN-γ$^+$ CD8$^+$ T cells is increased in the R848+RSV primed mice. The CD3$^+$ T cells (Fig. 5.22) were assessed for the production of IL-4 and IFN-γ. The production of the cytokines was enhanced by treatment with PMA-ionomycin. (a) Representative gating on lung CD8$^+$ T cells where gates were positioned based on FMOs respective for each cytokine. The (b) percentage and (c) number of CD8$^+$ T cells expression of IFN-γ was assessed in the BAL and lung. The percentage of the cells is from the CD3$^+$ population whereas the number is from the percentage of CD45$^+$ leukocytes. Each point on the graphs represents an individual mouse and data are shown as mean ± SEM of 4-8 samples per group and statistical significance for the differences between the indicated groups were determined by one-way ANOVA with Tukey post hoc test. *p<0.05; **p<0.01; ***p<0.001.

5.4.2 Steady state differences that favour R848 signalling in neonates

The neonatal response to R848 was different to that of adult mice. Furthermore, and although preliminary, there was no evidence that priming neonates with R848 would skew the response to a more protective response during re-infection with RSV. In order to identify why the adult and neonatal response to innate stimulation and RSV infection varies to such an extent the gene expression of selected proteins important in the lung were measured in the lungs of naïve adult and neonatal mice.

Factors that are important for development, regulation and lung structure were investigated and compared between adults and neonates. CX3CR1 is known to be important for the recruitment/development of various immune cells such as monocytes and DCs (Auffray et al., 2009; Schulz et al., 2012; Unkel et al., 2012) and is a receptor RSV used to infect epithelial cells (Chirkova et al., 2015; S. M. Johnson et al., 2015). No significant difference was apparent in the expression of CX3CR1 in the lung between adult and neonatal mice, although there was a trend toward higher expression in the adults (Fig 5.28a).

AMs were the main source of IL-6 in neonates after R848 exposure (Fig 5.21). The regulation of AMs was assessed by looking at the CD200R and CD200 levels in the lung. The interplay between the CD200R expression on AMs and CD200 on epithelial cells is important for determining the activation threshold of AMs (Hussell et al., 2001). Whilst no difference was observed in the expression of CD200, the adult lung had a higher expression of CD200R1 when compared to neonates (Fig 5.28a). Surfactants have multiple roles in the lung including tension retention and restraining the activation of AMs (Hussell & Bell, 2014; W. J. Janssen et al., 2008). Interestingly, adult mice expressed significantly higher levels of SP-A, SP-C and SP-D when compared to the neonates (Fig 5.28b). Furthermore, metalloproteinases (MMPs) are proteolytic enzymes that have multiple roles in the lung, during tissue development and remodelling (Greenlee et al., 2007; Woessner, 1991). Adult lungs expressed higher levels of MMP9, MMP12 and slightly higher MMP13 compared to neonates (Fig 5.28c).

Furthermore, gene expression of mediators important for maintaining homeostasis and lung tissue development/maintenance were measured. Cyclooxyganse (COX)-1 and COX-2 are

important enzymes for the synthesis of prostaglandins and can affect multiple parameters of the lung including surfactant secretion, AM activity and mucus secretion (Radi et al., 2010; Tamaoki et al., 1992). Neonatal lung expression of COX-2 and Muc5ac were also lower compared to adults (Fig 5.29a). Migration inhibitory factor (MIF) is important for driving growth factors that enhance lung tissue maturation; whereas IL-22 is important for lung repair and its presence can also be linked to neonatal hyperoxia (Fig 5.29b (Kevill et al., 2008; Pociask et al., 2013; Reilly et al., 2015)). There were no difference in the expression of these two factors between neonates and adults. Furthermore, the expression of TLRs (TLR-3, -4, -7) between adults and neonates were identical (data not shown). Since the neonates showed an increased IL-6 production after TLR7 stimulation (Fig 5.19, 5.20) the expression of UNC93B1 and AP-3 was also determined. These proteins are important for the production and transport of TLR7. Adult lungs had a significantly higher expression of UNC93B1 and a trend toward a higher expression of AP-3 (Fig 5.29c). Collectively, comparison of the baseline expression of these genes does not produce evidence of a determining factor that makes the neonatal response to R848 different. However, the gene expression of many important lung factors was lower in neonates.

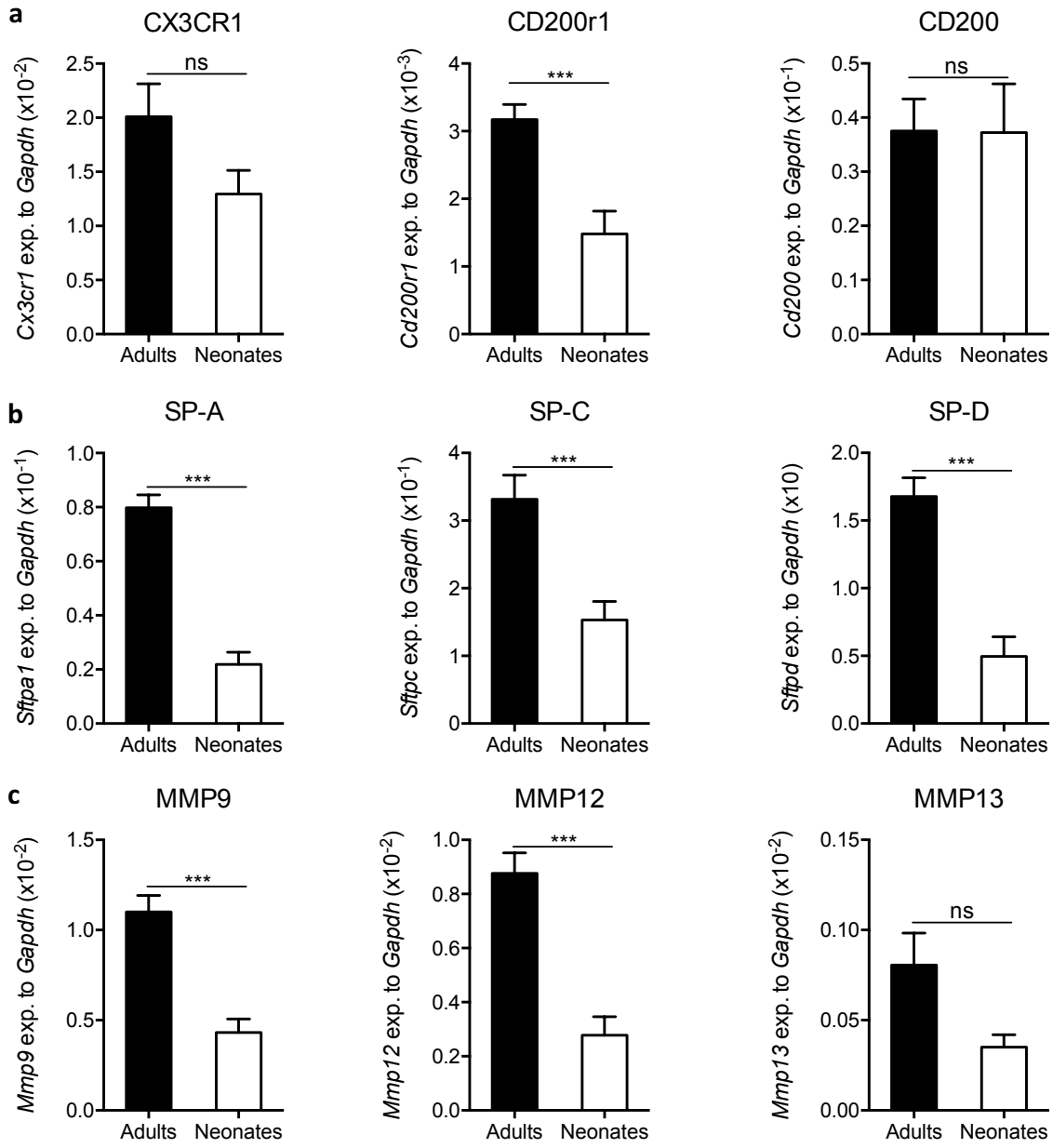


Figure 5.28 Neonates have diminished expression of regulatory receptors, surfactants and MMPs in the lungs at steady state. The lungs of naïve neonatal and adult C57BL/6 mice were collected and the gene expression of (a) CX3CR1, CD200r1, CD200, (b) SP-A, SP-C, SP-D, (c) MMP9, MMP12 and MMP13 were determined by qPCR. The data are shown as mean ± SEM of 5-10 mice pooled from two independent experiments and the statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test. ns, no statistical significance; ***p<0.001.

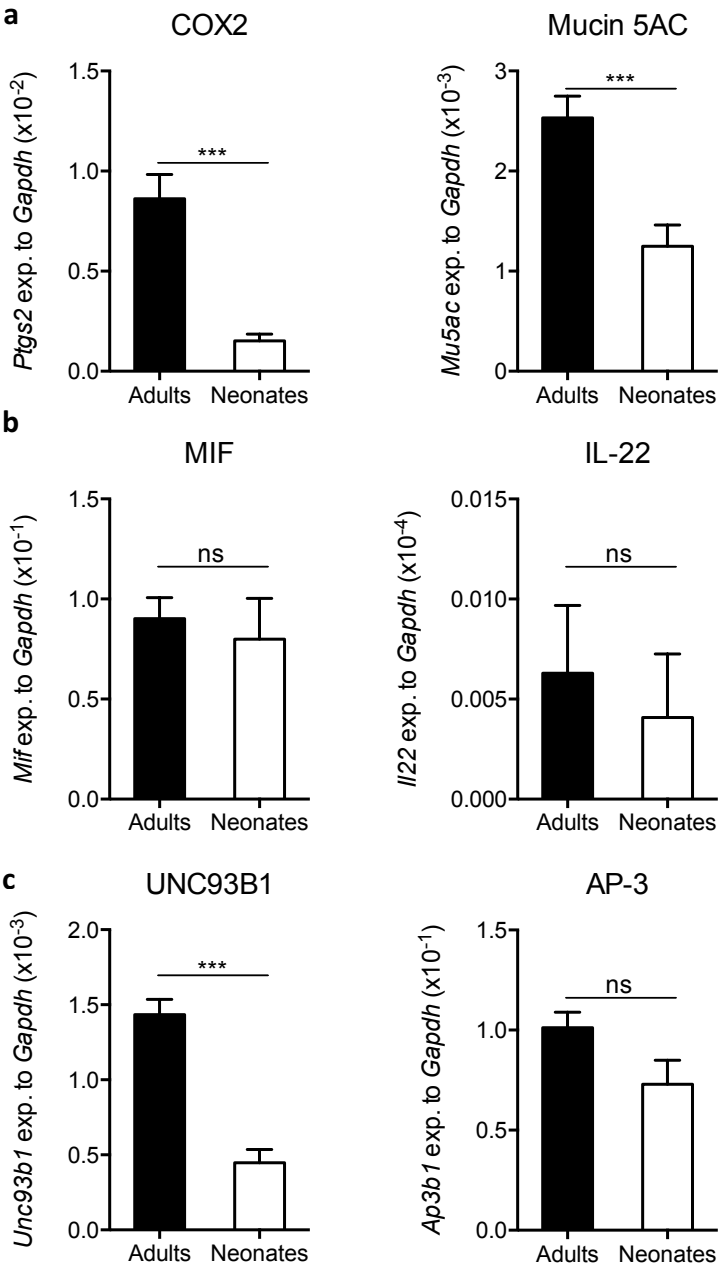


Figure 5.29 Neonates have a diminished expression of genes involved in tissue development, mucus production and TLR7 cleavage. Naive lungs of neonatal and adult C57BL/6 mice were collected and the gene expression of (a) COX2, Muc5AC, (b) Unc93B1, AP-3, (c) MIF and IL-22 were determined by qPCR. The data are shown as mean ± SEM of 5-10 mice pooled from two independent experiments and the statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test. ns, no statistical significance; ***p<0.001.

5.4.2.1 The adenosine system and the neonatal response to R848

Signalling via TLR7 has been shown to be affected by platelet factors and the adenosine system. Furthermore, the complement component C1q has been shown to regulate responses to TLR7 stimulation (Carlucci et al., 2016). The serum, lung lysate and BALF were therefore examined in naïve mice for the presence of C1q. Results suggested that there were significantly higher levels in the adult serum compared to neonates (Fig 5.30). However, there were no differences in the levels of C1q in the lung lysate and no C1q was detectable in the BALF of either neonates or adults (Fig 5.30).

The adenosine system in neonates has multiple roles such as lung tissue development and regulating TLR signalling (Levy, 2007; Levy et al., 2006a; Linden, 2005). Furthermore, it has been implicated in inhibiting TLR-mediated TNF-α production by human PBMCs and cord blood monocytes (Levy et al., 2006a). Adenosine induces the generation of cAMP by binding to the A3 adenosine receptors (Haskó & Cronstein, 2004). When comparing the presence of cAMP protein in the lung there was approximately 3 ng/ml and 8 ng/ml in the neonatal lung and BAL respectively; whereas adults did not produce any significant cAMP (Fig 5.31). Collectively, these results suggested that differences in the adenosine system might contribute to the neonatal sensitivity to R848.

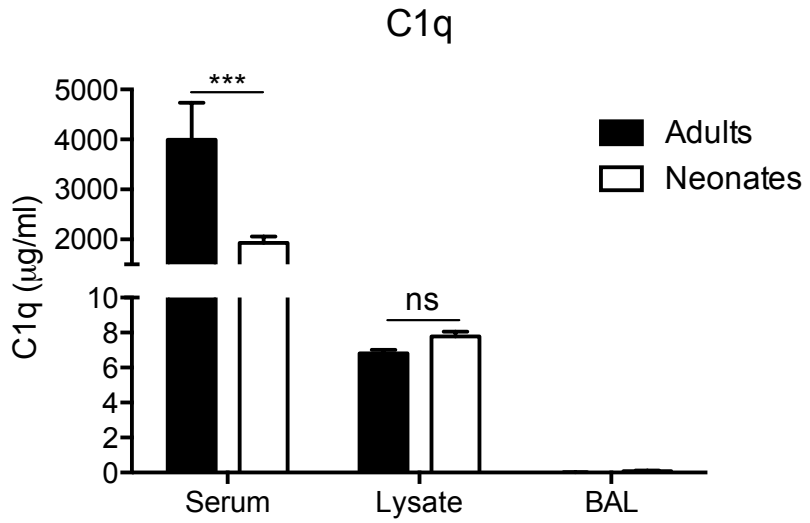


Figure 5.30 Levels of C1q are not different in neonates versus adults. The serum, lung tissue and BAL were collected from naïve adult and neonatal C57BL/6 mice. The presence of C1q was measured in the serum, lung lysate and BAL by ELISA (ELISA performed by Maria Botto's lab, see section 4.8.3). The data are shown as mean ± SEM of a total of 6 samples pooled from 2 experiments and the statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test. ns, no statistical significance; ***p<0.001.

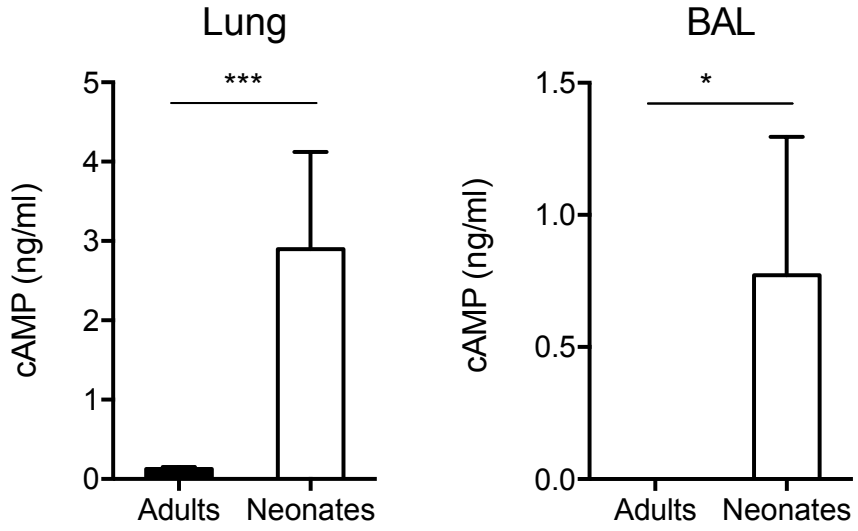


Figure 5.31 Neonates have increased production of cAMP in lung and BAL. The lungs of naïve neonatal and adult C57BL/6 mice were collected. The presence of cAMP protein was measured in the (a) lung and (b) BALF by ELISA. The data are shown as mean ± SEM of 9

samples pooled from three independent experiments and the statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test. *p<0.05; ***p<0.001.

5.4.3 Discussion

The development of the lung especially at infancy can affect the health of the organ in the long-term. RSV is one of the main causes of pulmonary morbidity during infancy requiring hospitalisation (Alexander-Miller, 2014; Bancalari, 2012; P. L. Collins & Graham, 2008; Nair et al., 2010) and have long term effects resulting in wheeze and asthma (Borchers et al., 2013; Glezen et al., 1986; Nair et al., 2010). The vaccine development has been hampered by the FI-RSV failure in the 1960s. Since then, the vast majority of studies on RSV infection have focused on adult animal models. Despite being beneficial for understanding many crucial cellular interactions during infection, these models fail to provide indications as to why the neonatal immune response to RSV varies. Interestingly, primary RSV infection in neonatal mice does not induce significant immune responses. Using the BALB/c mouse model it was shown that mice that were primed with RSV as neonates had a more severe pathology upon reinfection as adults which was characterised by a strong Th2 response (Culley et al., 2002). In this study, multiple TLR agonists were assessed in the neonatal and adult C57BL/6 mice with the objective of skewing the neonatal response during reinfection with RSV.

Studies using human PBMCs and cord blood monocytes to assess differences in the cellular responses to various TLR agonists found that although neonates had a diminished capacity of producing TNF-α after exposure to multiple TLR ligands, they retained this ability when exposed to R848 (Levy et al., 2004). This despite that neonatal and adult cells have no difference in the overall expression of TLRs or other functionally related molecules (Levy et al., 2004). Furthermore, studies in APCs of newborns showed that R848 skewed toward a Th1 immune response, which is impaired at birth (Adkins, 2009; Levy et al., 2006b). Intranasal administration of a high dose of CpG, poly(I:C) and LPS caused a similar neutrophil influx into the airways of adult and neonates. In contrast, the TLR7 agonists imiquimod and R848 induced a neutrophil influx only in the neonatal airways. These findings suggested differences

in either the sensitivity or presence of certain innate immune cells of neonates sensitive to TLR7 agonists. Other than its importance during viral infection, neutrophil influx is also associated with lung inflammation/injury and the activation of myeloperoxidase (MPO) may affect the surfactant production in the lung (Bancalari, 2012). When comparing the production of IL-6, only R848, and not imiquimod, induced its production in the neonatal mice and to much higher levels compared to adults. One reason for this might be that R848 has an ethanol and ethoxymethyl groups that make it more soluble in aqueous solvents than imiquimod (Levy et al., 2004).

As R848 was the only ligand to induce the production of IL-6, the dynamics of its actions were studied in more detail through a time course. The gene expression of CXCL1 at 6 hours was associated with neutrophil influx in the neonatal airway starting at 6 hrs post exposure and peaking at 12 hrs. The neutrophil levels were high even at 24 hrs post exposure in the neonates; whereas the influx in adults was minimal. The production of IL-6 and TNF-α was also measured in the BALF and only neonates produced significant levels of IL-6 at 6- and 12-hrs post exposure; whereas TNF-α was only present at 6 hrs post exposure. The main limitation of these studies is that the number of neonates are limited and the BALF recovered from a neonatal mouse is 140-180 μl, in contrast to the 1 ml from an adult, thus limiting the number of assays that can be performed.

In order to study differences in the cellular responses after exposure of adult and neonatal mice to different TLR ligands, flow cytometry was utilised. In addition, the integration and optimisation of ICS into the flow cytometry panel was also attempted. Results indicated that two of the main sources of IL-6 in the neonatal lungs were AMs and inflammatory monocytes. These results are preliminary, and more naïve controls and detailed analysis of the cellular composition of the mice are required in order to conclusively summarise these observations. However, when comparing the adults and neonates exposed to R848, there was a clear production of IL-6 in the AMs and inflammatory monocytes of neonates which was absent in adult mice. Overall, the *in vivo* exposure of lungs to R848 had two effects: 1) neonate immune cells were stimulated by the ligand, 2) adults did not respond to the ligand in a similar manner.

The possibility of using R848 as a potential adjuvant for intranasal administration was also assessed. Previous re-challenge studies of RSV were primarily performed in BALB/c mice, where a strong induction of Th2 cells and cytopathology was observed upon reinfection with RSV (Culley et al., 2002; Lambert et al., 2014). Previous attempts to use TLR ligands in the neonatal RSV model exposed the mouse to the ligand before administration of RSV. By exposing neonates to CpG before RSV infection mice were protected during secondary exposure as adults with a reduction in viral load and increased Th1 responses (Yamaguchi et al., 2012). The main objective of this study was in the first instance to see if administering R848 with RSV simultaneously would skew the immune response in neonates and in the second instance to use it in an FI-RSV vaccination model (Openshaw et al., 2001; Waris et al., 1997). The primary infection did not show any significant differences between the neonates exposed to RSV, PBS or RSV + R848. Although the observations were only limited to weight curves; the results indicated a similar weight gain in all groups. After the mice were re-infected with RSV 42 days after the primary exposure the results that were especially interesting focused on the groups that were exposed as neonates to RSV or R848 + RSV. No differences were observed in the cellular influx into the BAL of these mice, although there was a trend toward higher neutrophils present in the BAL of the R848 + RSV group. However, there was statistically higher levels of IFN-γ present in the BALF of neonates of mice that were primed with R848 + RSV.

The expression of IFN-γ and IL-4 in T cells was assessed by ICS. The data did not indicate differences in the skewing of the T cells responses in the different groups as similar levels of IFN-γ were detected; whereas small numbers of IL-4^+ $CD4^+$ T cells were detected to provide objective data. There was a higher percentage of $CD8^+$ T cells expressing IFN-γ in the lung of the R848 + RSV primed group compared to the RSV primed adults. When quantifying the number of $CD8^+$ T cells that were IFN-γ^+, there was no significant differences between the RSV and R848 + RSV neonatal primed groups, however, there was a weak trend to higher numbers in the R848 + RSV group. Overall, these results did not provide evidence that R848 can be used as an adjuvant in this model or in its current form of administration since if anything an extremely enhanced type 1 response was detected. There were two major limitations of this re-challenge study. Quite importantly, as the experiment was highly dependent on the

number of pups available and the cost for maintaining the mice was high, the experiment was only performed once. Furthermore, additional control groups would be required such as adults that were primed with PBS. This study was performed on C57BL/6 neonates. The skewing of the immune responses varies in different murine models (Watanabe et al., 2004) and evidence indicates a stronger Th1 skewing and more resistance to RSV infection in C57BL/6 mice compared to BALB/c mice (Jessen et al., 2011; Tregoning et al., 2010). Therefore, in future experiments BALB/c mice should also be utilised.

Levy and associates, showed that R848 is unique in inducing the production of TNF in cord blood monocytes. Their study also assessed the basal levels of TLR1-10 and associated adaptor proteins and found no significant differences between adult and newborn monocytes (Levy et al., 2004). Additional studies were conducted to elucidate the mechanism behind why the cord blood monocytes responded differently to TLR7 and a skewing towards a Th1 response by antigen presenting cells was observed (Levy et al., 2006a). An attempt was made to answer this question in the mouse lung. Previous studies of rat neonatal AMs suggested that they selectively produced LBP (P. T. Lee et al., 2012). These results suggested that AMs, in rat neonates, may provide a compensatory mechanism for the deficiency in specific neonatal immune responses during this developmental period (P. T. Lee et al., 2012). The gene expression of various genes that are involved in lung development, tissue homeostasis and function of AMs were measured in the lung tissue of naïve adult and neonates. Considering that AMs were one of the main cell types to express IL-6 in neonates after R848 exposure it is interesting to speculate as to why neonates respond differently after R848 stimulation comparing baseline differences of various genes. CD200R is present on AMs whereas CD200 is expressed on ATII cells. The interaction between this receptor and its ligand is one of the main factors that sets the activation threshold for AMs and controls homeostasis (Hussell & Bell, 2014; Snelgrove et al., 2008). The higher expression of CD200R in the lung of adult mice, whilst CD200 is similar to neonates, might suggest that the adult AMs are more tightly regulated. However, at this point only speculations can be made, as the expression of these genes is in the whole lung and not specific to AMs.

If one was to speculate further using the regulation of AMs as a potential mechanism behind why neonates respond differently to R848 the expression of the surfactant proteins is

especially interesting. Surfactants have multiple roles, including lung development, reducing tissue stress, alveoli tension and regulating the activation of AMs. Surfactant replacement in the 1980s has significantly improved the survival of preterm infants (Bancalari, 2012; Fujiwara et al., 1990). AMs are especially important in removing excessive surfactants from the airways during homeostasis. The lack of AMs, leads to the pulmonary proteinosis (PAP) which is characterised by the high concentration of proteins and debris in the airways (C. Schneider et al., 2014a). Furthermore, SP-A and SP-D can regulate the induction of inflammatory cytokines by AMs (Haczku, 2008; Hussell & Bell, 2014). Specifically, the interaction of these surfactants with SIRPα on AMs induces RHOA which, in turn, decreases the phagocytosis capacity of these cells (Barclay & Brown, 2006). Furthermore, surfactants can also interact with TLR6 or TLR4 which in turn blocks the activation of NF-κB (Hussell & Bell, 2014). Taking all this into account, the observation that neonates express lower levels of surfactants at baseline may provide preliminary evidence of a lower activation threshold for the neonatal AMs and the increased phagocytosis might encourage the internalisation of R848 into the endosome. However, this does not provide an explanation as to why this phenomenon is specific to R848 and not the other TLR ligands.

Since at steady state there were no differences in the expression of TLR7, and the other TLRs, between adult and neonatal mice, the expression of proteins involved in the function and transport of TLR7 to the endosome were assessed. TLR7 and TLR9 follow a similar transport pathway from the endoplasmic reticulum to the Golgi. The main difference is that TLR9 is cleaved on the cell membrane and is then internalised in the endosome in its functional form (Pelka et al., 2016). In contrast TLR7 utilises UNC93B1 and is transported into the endosome by AP-4. Another protein, AP-3, can ucleave UNC93B1 (B. L. Lee et al., 2013; Pelka et al., 2016). Thus, the expression of these proteins was examined, in order to distinguish if a non-functional form of TLR7 is present in adults. Whilst the expression of UCB93B1 was higher in adults, the AP-3 expression was similar. Thus, no speculations could be made on differences in these pathways as UNC93B1 is also involved in retention of TLR9 from the cell membrane into the endosome. Complement is produced by ATII cells and is very important during the development of the neonatal lung (Bancalari, 2012). Furthermore, the complement system is important during homeostasis but can also assist with phagocytosis of pathogens. In a murine systemic lupus erythematosus (SLE) model C1q is believed to play a controversial role as

complement can drive tissue injury, yet lack of C1q was associated with development of SLE. Evidence suggested that C1q modulates the response to TLR7 stimulation by macrophages (Carlucci et al., 2016). Quantifying C1q in lung tissue, BAL and serum did not provide evidence that this was a potential mechanism related to the sensitivity of neonates specifically to R848 as it was similar in adults and neonates.

The adenosine system has multiple roles during health and disease. It is important for the healthy development of the lung and at early ages affects the secretion of surfactants in the lung (Fitzgerald et al., 2007). Levy and colleagues also examined differences in the adenosine system as a mechanism associated with the increased sensitivity of cord blood monocytes toward R848 stimulation. Their findings suggested that the adenosine system selectively inhibits the TLR-mediated TNF-α production except for TLR7 stimulation (Levy et al., 2006a). Furthermore, their study showed high levels of the secondary messenger cAMP in the cord blood which was associated with the inhibited TLR-mediated TNF-α production especially after stimulation of TLR2 and exposure to microbial particles. However, there was no change in the response to R848. The transfer of infant serum onto adult monocytes and their exposure to TLR ligands suggested that the adenosine system selectively inhibits TLR1, 2, 3, 4 and not TLR7 signalling in infants (Levy et al., 2006a). Therefore, the lung tissue and the BALF of adult and neonatal mice were examined at baseline for the presence of cAMP. Results suggested significantly higher levels of cAMP in both the lung and the BALF of neonates compared to adults. One may speculate that this is an important feature in the neonatal response to R848 (see section 6). Taken together, results suggested that R848 is a potential adjuvant candidate, however further confirmation regarding its mechanism of action and utilisation of additional animal models is required.

6 Conclusions

RSV is the leading cause of lower respiratory tract infections in infants, the immunocompromised and elderly. The murine model is an indispensable tool in understanding the immunopathology caused by RSV and to study the immune responses mounted against the virus (P. L. Collins & Melero, 2011; Cormier et al., 2010; Openshaw & Tregoning, 2005). The common dogma regarding RSV infection has been that airway epithelial cells (AECs) were targeted and were the host of the virus and also the producers of an array of inflammatory chemokines and cytokines such as type I IFNs (Table 1-1 and 1-2) (Borchers et al., 2013; Garofalo et al., 2001; Openshaw & Tregoning, 2005). More recent studies show an increasingly important role of AMs and DCs in shaping the innate immune response during RSV infection (Bhoj et al., 2008; Goritzka et al., 2015; Makris et al., 2016; Ravi et al., 2013). As there is currently no vaccine available against RSV and the prophylactic treatment (Palivizumab) is administered only to high-risk groups such as premature born infants at only certain periods of the year and, as it is an expensive treatment, only in the developed world (D. Wang et al., 2011).

For decades, the focus of research has been directed toward understanding how the adaptive immune response profile enhances pathology during RSV infection. Findings suggested that a Th2-biased response is sometimes detected and shown to be detrimental during RSV infection (Caballero et al., 2015; Durant et al., 2013; D. C. P. Lee et al., 2010; Legg et al., 2003; Loebbermann et al., 2012; 2013). Understanding how this response initiates and the cells involved in shaping the immune profile associated with the RSV immunopathology is essential for the development of vaccinations and better treatment regimes. Recent findings that AMs are the main source of type I IFNs during RSV infection (Caballero et al., 2015; Goritzka et al., 2014; 2015; Legg et al., 2003). In addition to AECs, which are the main target and host of RSV, AMs are one of the first cells to encounter invading pathogens in the lower airway. Early studies of human AMs *ex vivo* demonstrated that they retain the ability to produce type I IFNs after stimulation with influenza or other TLR ligands (Nugent & Pesanti, 1983). The localisation of AMs positions them as gatekeepers of the lower airways and, together with DCs and structural cells, can drive the immune response against invading pathogens (Borchers

et al., 2013; González-Navajas et al., 2012; Openshaw & Tregoning, 2005). The focus of the current study was to provide a clear inflammatory profile of AMs and more information about how AMs interact with RSV.

A characteristic that is shared between severe cases of influenza infection and RSV is the excessive inflammatory response (Jewell et al., 2007; McNamara et al., 2005). Therefore, understanding the contribution of each cell type in relevance to the overall response is vital. Studies of RSV and NDV have shown that AMs are the main producers of type I IFNs (Goritzka et al., 2015; Kumagai et al., 2007). During influenza infection pDCs and epithelial cells appear to be potent producers of type I IFNs (Diebold et al., 2004; Jewell et al., 2007). Furthermore, type III IFNs (composed of IFN-lamda1 (λ1), -λ2, and λ3 or IL-29, IL-28A and IL-28B respectively) produced by epithelial cells are important for controlling influenza virus infection (Mordstein et al., 2010; Okabayashi et al., 2011). Interestingly, type III IFNs are absent during RSV infection (Goritzka et al., 2015; Mordstein et al., 2010). The *Mavs*$^{-/-}$ mouse model showed that the immune response to RSV were initiated upon RLR recognition; whereas lack of IFNAR also caused enhanced cytopathology and lack of pro-inflammatory cytokines (Bhoj et al., 2008; Demoor et al., 2012; Goritzka et al., 2014; 2015). The current study focused on the importance of AMs in orchestrating the immune response during RSV infection. In addition to confirming that AMs are potent producers of type I IFNs, the current study also showed that the production of type I IFNs and other pro-inflammatory cytokines is dependent on MAVS and IFNAR signalling. As type I IFNs drive the production of other inflammatory mediators during RSV infection (Table 1-1 and 1-2), a better understanding of how AMs encounter RSV was sought.

AMs do not appear to be the main source of type I IFNs after influenza virus infection, although they do contribute (Helft et al., 2012; Jewell et al., 2007). Instead, pDCs, which are very efficient in endosomal TLR7 signalling are believed to contribute to the production of type I IFNs (García-Sastre & Biron, 2006; Jewell et al., 2007). In fact, detection of influenza virus by this pathway does not require viral replication (Iwasaki & Pillai, 2014). Furthermore, it is suggested that activation of AMs occurs by uptake of infected/dying cells during infection; whereas pDCs can become activated by influenza virus directly (Högner et al., 2013; Iwasaki & Pillai, 2014). Studies of AMs exposed to influenza virus *ex vivo* suggested that they are not

potent producers of TNF-α (van Riel et al., 2011). *Ex vivo* and *in vivo* studies of pDCs during influenza virus infection showed that they were potent inducers of type I IFNs even in the absence of IFNAR signalling (Barchet et al., 2002; Jewell et al., 2007; Prakash & Smith, 2005); whereas similar studies of cDCs and fibroblasts suggest that viral infection and IFNAR signalling is essential for IFN production (Diebold et al., 2004; Kato et al., 2008; Kumagai et al., 2007).

Pathology caused by respiratory infections is a delicate balance between excessive and restrained inflammation. Mice that lack CD200 and were infected with influenza virus showed increased mortality, increased production of nitric oxide and cellular influx into the lung despite a reduced viral load (Snelgrove et al., 2008). These results indicate the important role of tolerance during influenza virus infection where the role of AMs in removing cellular debris is equally important to the immune response directed against the virus. Others suggested that AMs produce type I IFNs very early during influenza virus infection and this response is enhanced by pDCs, which are indispensable for the adaptive immune responses (GeurtsvanKessel et al., 2008; Guilliams et al., 2013b; Wolf et al., 2009). Furthermore, studies have also suggested that over 90% of AMs are depleted during influenza virus infection, with the remaining cells having necrotic phenotype (Ghoneim et al., 2013).

AMs reside in the airway lumen, positioning them in a strategic location as they are constantly exposed to external stimuli, microbial pathogens and the microbiota (Hussell & Bell, 2014). Previous studies have attempted to provide a more elaborate picture regarding the inflammatory profile of AMs during RSV infection and how they interact with the virus (Ravi et al., 2013). Murine studies *in vivo* primarily used the clodronate liposome depletion method to investigate the effect of depleting AMs (Kolli et al., 2014; Pribul et al., 2008; Tate et al., 2010; Tumpey et al., 2005). Despite providing evidence that the lack of macrophages decreases the levels of IL-6, TNF-α, CCL3 and IFN-α the depletion method could not exclude the possibility that cells other than AMs were also affected (Benoit et al., 2006; Machado-Aranda et al., 2014). Furthermore, depletion of AMs, which are responsible for maintaining homeostasis and removing cellular debris, would change the lung environment before infection. Murine AMs have also previously been studied *ex vivo*, however, there were some limitations with this study (Ravi et al., 2013). For example, Ravi et al used very elegant

microscopy analysis to study the viral interactions and arrays to provide an inflammatory profile of lung macrophages. However, the cells studied were pulmonary macrophages and not exclusively AMs. By optimisation of the BAL extraction technique the yield of extracted AMs was increased 3-fold and allowing a more extensive study of AMs *ex vivo*. This allowed us to evaluate which mediators AMs produce after RSV infection. Studies using mouse and human AMs have suggested that viral replication in macrophages is abortive (Cirino et al., 1993; Ravi et al., 2013). This project confirmed these findings in wt AMs and attempted to elucidate the role of type I IFNs in this interaction.

Findings confirmed that AMs are the main producers of type I IFNs. Furthermore, it was shown that these cells contributed to the production of key pro-inflammatory mediators that were previously associated with epithelial cells (Table 1-1, 1-2). Such mediators included type I IFNs, CXCL10, TNF-α, IL-6 and CCL3 (Fig 6.1). Past studies on human AMs also suggested that they produce pro-inflammatory cytokines including IL-6, TNF and IL-8 (Becker et al., 1991). In this project, primary AMs from *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were studied to elucidate the effect of type I IFNs on the production of cytokines, recognition of the virus and the cellular permissibility to RSV infection. The lack of viral recognition via MAVS (lack of type I IFN production) and the lack of the IFNAR receptor signalling rendered the ability of AMs to produce inflammatory cytokines. Type I IFNs induce an antiviral state to infected and neighbouring cells by binding to IFNAR and inducing a JAK-STAT signalling cascade causing the induction of ISGs (for detailed descriptions of IFNAR signalling and ISGs see section 1.3.2). Findings showed an absence of ISGs in the MAVS- and IFNAR1-deficient AMs, yet despite not responding to/or recognising the virus through these pathways, viral replication was abortive (Fig 6.1). Studies have suggested that differences in the nature of RSV replication can partially be due to species-specific host cell-viral interactions (Bannister et al., 2010). The optimal host for RSV replication are human epithelial cells, although replication can occur in mice and in mouse epithelial cells such as LA4 cells (Bannister et al., 2010; Loebbermann et al., 2012). Sorting AMs from mice infected with RSV *in vivo* confirmed the inflammatory profile of AMs that were exposed to RSV *ex vivo*. Although preliminary, fluorescence microscopy results suggested that RSV infection of AMs *in vivo* is abortive, which is in line with findings in human AMs exposed to RSV (Cirino et al., 1993).

The importance of AMs, in both steady state and infection, can be portrayed in patients suffering from PAP (see section 1.1.2.1). These individuals can either have defective CSFR or develop autoantibodies against GM-CSF, which induces a defective activation and development of AMs (Kitamura et al., 1999; Suzuki et al., 2008). This makes these patients develop an accumulation of protein and debris in the airways (C. Schneider et al., 2014a; Trapnell et al., 2009) and they are more susceptible to microbial and viral infections (Seymour & Presneill, 2012). Using a mouse model for PAP it was shown that following influenza virus infection, mice lacking AMs (due to GM-CSF or CSFR deficiency) suffered of respiratory failure and enhanced morbidity (C. Schneider et al., 2014a). Thus, the essential role of AMs orchestrating responses throughout development, homeostasis, disease and resolution is important for shaping health and the microenvironment of the lung.

Further to what is produced by AMs, an interesting aspect from this study are mediators, that are important during RSV infection (Table 1-2 and 1-2), and which AMs do not or only weakly produced (Table 5-1). Chemokines such as CXCL1, CCL2 and CXCL9 did not appear to be produced by AMs, albeit their genes were expressed after RSV infection. Furthermore, IL-1α or IL-1β proteins were not detected in supernatants from AMs exposed to RSV, however, there some expression of their respective genes, especially *in vivo* could be detected. One must not exclude the limitations in current assays with recognising alternative forms of these cytokines or that the lack of secondary signals that drive their secretion are absent primarily *ex vivo* (Edye et al., 2015). DAMPs are present in the airway during RSV infection as airway obstruction occurs through the accumulation of mucus, dead/dying cells and inflammatory cells (Johansson 2016). Furthermore, recent findings suggested that RSV triggers the release of DAMPs such as HMGB1 and S100A9 (see section 1.2.4 and Johansson 2016, Hosakote et al., 2016, Foronjy et al., 2016). Overall, the results suggested that AMs can produce many proinflammatory cytokines during RSV. This selective involvement of AMs in producing some of the important mediators during infection indicates the important cellular interplay that occurs during RSV infection of the lungs. Findings from our laboratory have suggested that structural cells are the main source of chemokines (unpublished work in thesis from F. Kausar, 2016). The importance of the results observed in AMs after encountering RSV needs to be compared to structural cells and other immune cells in parallel to evaluate the major sources of these mediators during infection.

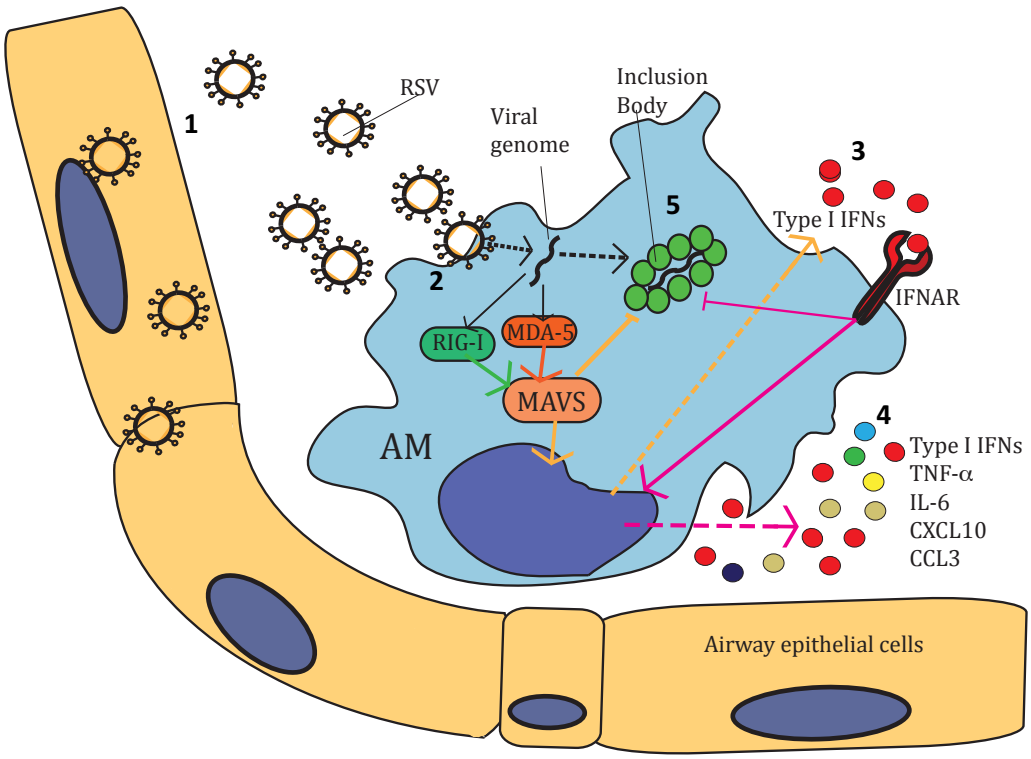


Figure 6.1 Interaction of AMs and RSV in the lower airways. (1) RSV enters the lower airways and primarily targets airway epithelial cells (AECs) and encounters alveolar macrophages (AMs). (2) Upon entry into the AMs the viral genome is recognised by RLRs and signals via MAVS in order to induce the production of type I IFNs. (3) The type I IFNs signal via the IFNAR and the JAK-STAT signalling pathways which causes the induction of interferon-stimulated genes (ISGs) and (4) inflammatory mediators such as additional type I IFNs, TNF-α, IL-6, CXCL10 and CCL3. (5) In the absence of MAVS or IFNAR signalling, there is no type I IFNs produced, however, the replication of RSV is abortive and inclusion bodies are present.

Maintaining the delicate balance between excessive inflammation and under-responsiveness is important in all stages of immune development. Many diseases are not driven by the pathology caused directly by viral infection, but rather an exuberant immune response (Iwasaki & Pillai, 2014; Openshaw & Tregoning, 2005). For example, during fatal influenza infection cases, one of the causes of death is not the structural damage caused by the infection but neutrophil influx which by the process of NETosis increases the levels of microbes in the lung (Drescher & Bai, 2013; Iwasaki & Medzhitov, 2015; Pillai et al., 2016).

Nowhere is the maintenance of this balance more crucial than in the lung of neonates. The neonatal lung starts with a volume capacity of 200 ml at the time of birth and has 2.4 x 10^7 alveoli and by reaching adulthood this maximises at about 5,500 mls and 3 x 10^8 alveoli (Dunnill, 1962; Ochs et al., 2004). Even by the age of two the development that occurs, in a structural level, is rapid, with the number of alveoli increasing to approximately 1.6 x 10^8 alveoli (Ochs et al., 2004). The immune cells of neonates are very tightly regulated as adverse responses at early age can lead to long lasting affects (Botas et al., 1998; Grenache & Gronowski, 2006).

RSV is a major burden especially in infants under the age of 2, with infections in the first 6-months of life associated with increased morbidity (Borchers et al., 2013; Glezen et al., 1986; Nair et al., 2010). Considering that a mouse at the age of 7 days represents a human infant up to 6 months old (Cormier et al., 2010); the murine model was used in this study to examine differences in responses of neonates to various TLR ligands and to study differences in the baseline level of various genes crucial for lung development (Cormier et al., 2010). Surfactants have very important roles in the lung during development, homeostasis, disease and resolution (Bancalari, 2012; Botas et al., 1998; Grenache & Gronowski, 2006). Abnormality in the surfactant levels in the young leads to defective lung development and limited alveolarisation (Bancalari, 2012; Husain et al., 1998). SP-A and SP-D are also important for the regulation of AM activity by interfering with TLR2/TLR4 binding to respective ligands and their co-receptors (Haczku, 2008; Hussell & Bell, 2014; Sano et al., 1999; Yamada et al., 2006). MMPs have multiple roles in lung development and homeostasis. MMP-13 in mice has been associated with DC function and inhibiting T cell activation (Bartmann et al., 2016). In addition, the activation of MMPs, as well as the control of their activity, is important for controlling collagenase breakdown and surfactant secretion (S. Chakrabarti & Patel, 2009). Together with CD200 expressed on ATII, surfactants are important for controlling the activation threshold of AMs (Hussell & Bell, 2014).

In addition to lung development, shortly after birth the lung tissue is colonised by microbial species; whilst the maternal antibody levels decrease (Gallacher & Kotecha, 2016). The regulation of TLR signalling is therefore crucial, as exuberant response to microbial species

that occupy the microbial niche or to harmless environmental antigens would be devastating. AMs are one of the first cells to encounter microbes, and their activation must therefore be regulated. Quite interestingly, in this study although the levels of CD200 were similar between adults and neonates, the levels of CD200R in neonatal lungs was decreased. This combined with the decrease in surfactant levels would suggest that the activation threshold of neonates would be lower than in adults and that AMs would be more active. In contrast, the lungs of neonates stimulated by TLR3, TLR4 or TLR9 ligands induced neutrophil influx; whereas TLR7 ligands increased neutrophil influx and production of IL-6 and TNF-α only in neonates compared to adults. This would suggest a conserved evolutionary regulation of the TLR sensitivity and perhaps the signalling pathways that restrains AMs from mounting an excessive immune response to microbes that would use these pathways (Kollmann et al., 2012; Levy et al., 2006b). The endosomal TLR7 is vital for recognising ssRNA and its importance has been shown during influenza virus infection (Akira & Takeda, 2004; Diebold et al., 2004). Regulating the activation of TLR7 is important when distinguishing necrotic tissue, bacteria and self-RNA (Karikó et al., 2005). This would especially be important during the developmental phase of the lung where an exuberant response could affect the long-term functionality of the lung and responses to secondary infections (Bancalari, 2012). Findings suggested that neutrophil influx was similar between R848 and imiquimod; however latter did not induce the production of IL-6 or TNF-α. Considering that R848 is a congener of imiquimod, and that both stimulate TLR7 it was surprising that only R848 is capable of inducing a cytokine response. Similar results however were observed between the two ligands when cord blood monocytes were stimulated to R848 or imiquimod (Levy et al., 2004).

The interplay between the immune response and growth factors are crucial for driving the development of the neonatal lung (Bancalari, 2012). Studies on pDCs from human infants that were exposed to R848 *ex vivo* suggested differences in the potency of these cells in producing IFN-α between males and females (Cho et al., 2013; Seillet et al., 2012; J. P. Wang et al., 2012). Specifically, during a developmental period known as 'mini-puberty' where the level of sex hormones is elevated, the female pDCs produced more IFN-α after R848 stimulation, and the levels were decreased when androgens were administered (J. P. Wang et al., 2012). This sensitivity of the pDCs is believed to be a reason why female infants have a survival benefit

to males. In the current study, there was no way of distinguishing if the mice utilised were male or females. However, these findings further support that stimulation of innate immune cells during the developmental period is an extremely crucial interplay between pathogens, immune cells, growth factors and hormones.

Following on, the adenosine system has also been suggested to regulate the responses of neonatal cells against different TLR stimuli. Specifically, neonatal monocytes have increased sensitivity of the adenosine A3 receptor which induces the secondary messenger cAMP (Levy et al., 2006a). This in turn selectively inhibits specific TLR signalling (Levy et al., 2006a). The study by Levy and colleagues suggest that the adenosine system skewing the TLR7 response might be important during the perinatal period for modulating the innate and adaptive responses. Interestingly, the adenosine system is also crucial for lung development. Specifically, mice lacking the A3 receptor developed normally in utero but died from respiratory failure within 1 hour after birth (Ban et al., 2007; Bancalari, 2012; Fitzgerald et al., 2007). The histological analysis of these mice showed that there was an inhibited development of the lung tissue and that this was linked to decreased secretion of SP-A, -B, -C and -D (Fitzgerald et al., 2007). Thus, these findings present a link between the adenosine system, surfactant production and the development of the neonatal lung tissue and immune responses.

Although preliminary, results from this study suggest that the levels of cAMP, and therefore A3 receptor signalling, are elevated in neonatal lung and BAL. This may allow speculation that the adenosine system drives the enhanced TLR7 mediated signalling observed after R848 administration in the neonates. Interestingly however, the surfactant levels are decreased at baseline when compared to the adult lung. As the gene expression arrays are a representation of the complete lung these results do not necessarily represent what occurs in the alveoli of the mice. The increased activation of AMs observed by ICS allows the speculation that the combination of increased adenosine system signalling in combination with decreased activation threshold of the cells might permit the response to R848. Further experiments are required to understand the mechanism of R848 stimulation of the neonatal lung (see Section 7). TLR7/8-targetting adjuvants have been extensively used as a cream for the protection of external genital warts, superficial basal cell carcinoma and actinic keratosis (Vasilakos &

Tomai, 2014). By co-delivering RSV F trimers with an TLR7/8 adjuvant there was an increase Th1 response and protection during intranasal RSV infection adult mice (Francica et al., 2016). Furthermore, R848 appears to decrease the severity of asthma by decreasing eosinophils and Th2 responses when administered in an adult OVA mouse model (Jirmo et al., 2016; Pham Van et al., 2011; Quarcoo et al., 2004). When put into perspective, TLR7 administration to neonates results in a different response, compared to adults, and there is skewing towards a Th1 immune response (Levy, 2007; Levy et al., 2004; Levy et al., 2006b). Better understanding of the structural, developmental and immune profile of the neonatal lung can potentially allow better preventative care during RSV infection.

7 Future work

AMs in context with other immune cells

This study focused on the contribution of AMs to the innate response against RSV and the role of type I IFNs in driving it. In the first instance, primary AMs were extracted from wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice and studied *ex vivo*. Furthermore, by FACS the AMs were isolated and gene expression arrays were performed for antiviral genes. Results from AMs exposed to RSV *in vivo* confirmed the *ex vivo* observations. The main limitation when studying any specific type of cell in solitude is that the data might not be placed in perspective with the overall immune response. For example, previous unpublished data from our group indicate that structural cells also contribute to the production of many mediators although they are mainly chemokines (Kausar, 2016). The limitation in the field is that there is no method to genetically, deplete AMs. In order to provide a comprehensive idea of the involvement of AMs during the innate phase of RSV infection, AMs must be sorted together with DCs and structural cells over various timepoints in the first 24 hours after RSV infection *in vivo*. The sorted cells can then be analysed for gene expression and thus provide a more complete comparison of the precise role of AMs in the general response.

Role of AMs during the resolution of phase

In addition to being important during homeostasis and orchestrating the immune response during RSV infection, AMs are central during the resolution phase of respiratory infections (Hussell & Bell, 2014). There is limited information regarding what role AMs have during the transition from the inflammatory to the resolution state during RSV infection. One main characteristic of AMs is their increased plasticity which does not allow them to be easily categorised into the macrophages activated states of M1 or M2 and are therefore referred to as the 'masters of contradictory function' (Hussell & Bell, 2014). The profile of AMs during the resolution phase cannot be studied *ex vivo* as this would require long periods of time in culture that would alter their phenotype. Furthermore, the lack of interaction with other immune and structural cells would not provide a comprehensive representation of their role.

A question that can be asked in future work regarding the AMs could be focused on their transitional role from driving the immune response to a more resolution profile at later stages of RSV infection. Mice should therefore be infected with RSV and the AMs should be sorted at specific time points to see the changes in cytokine levels. Specific time points that would be interesting to follow the change in the role of AMs would be between 2-4, and then, 8-10 days post infection. The first phase represents the decrease in many pro-inflammatory mediators involved in the innate response to RSV; whereas the latter is the recovery phase after RSV infection (Goritzka et al., 2015). This would also allow the study of AMs during phases where a significant influx of cells such as neutrophils, monocytes, NK cells and T cells are present to the lung. Studies have suggested that after influenza virus infection there is an upregulation of CD200R and IL-10 by AMs (Snelgrove et al., 2008). Furthermore, it would be interesting to sort other immune cells and especially epithelial cells and see if there are fluctuations in CD200, which can set the activation threshold of AMs, or other regulatory genes. These results could provide an insight on the role of AMs in clearing airways from debris after RSV infection or if they are involved in the possible susceptibility of high risk groups to secondary opportunistic infections (van der Sluijs et al., 2004).

Differences in the uptake/phagocytosis of AMs during RSV and other respiratory infections

The common dogma regarding RSV infection was that the virus enters cells by endocytosis (Kahn et al., 1999; Srinivasakumar et al., 1991). Recent studies have suggested that RSV can also use micro- or macro-pinocytosis and, upon entry, the replication cycle of the virus is pH dependent (Krzyzaniak et al., 2013; Mercer & Helenius, 2008). These findings raise questions regarding the interaction between AMs and RSV. Entering AMs via phagocytosis could potentially be disastrous for the virus as it may enter endosomes with a less favourable pH. The potential reason that the RSV viral cycle is abortive in AMs could be distinguished by identifying the entry route RSV uses to enter the cytosol.

Firstly, using fluorescence microscopy and staining of the cellular membrane of macrophages we could confirm that the RSV proteins detected are indeed present intracellularly. This can be done by using plasma membrane stains and then by z-stack analysis distinguishing the location of the IBs in regards to the cell membrane. Furthermore, the phagocytic ability of

AMs and how that is affected by RSV exposure would provide insight as to how their activity is altered during infection. The usage of FITC-labelled synthetic beads of different sizes would allow the visualisation of phagocytosis by macrophages by flow cytometry and ICS (Alvarez & Casadevall, 2006; Y. Shibata et al., 2001; Smythies et al., 2005). This technique would permit the distinction of differences in the phagocytic abilities between wt, *Mavs*$^{-/-}$ and *Ifnar1*$^{-/-}$ AMs. Furthermore, by exposing AMs to the FITC-beads after RSV infection, changes in the phagocytic capacity of AMs can be observed. The same techniques could also be applied to other respiratory viruses, such as influenza virus, in order to distinguish how differences in how AMs interact with different pathogens.

Comprehensive inflammatory profile of neonatal innate responses to R848

Using fluorescence microscopy, the location of R848 should be identified. This could be done by a rhodamine-labelled TLR7/8 agonist and would distinguish if R848 directly activates cells that produce the observed mediators, or if these mediators are secreted by indirect activation. In order to provide a comprehensive inflammatory profile of the neonatal response to R848 in the first instance the BALF must be tested in a multiplex platform. A limitation with the neonatal BAL flushing is that very small volume of fluid is extracted and variation in the recovery of fluid is common. Therefore testing an array of cytokines by ELISAs is impossible. By using Luminex very small volumes can provide an array of data and provide a more elaborate idea of the precise role of R848 in inducing inflammatory mediators. This could be applied on all timepoints (6, 12 and 24 hrs post-exposure) and a comparison between the other TLR ligands should also be considered. Due to the limitation in the number of litters available, a full comparison between all TLR ligands at all timepoints was infeasible. Although this study focused on the differences between adults and neonates after R848 exposure, a comparison of the other ligands would also be interesting.

R848 as a vaccine adjuvant against RSV

The current study attempted to assess R848 as a potential adjuvant for initiating the neonatal innate response to RSV and eliciting a protective secondary response. In the first instance, an inactive form of RSV might decrease the results observed in the RSV primed neonates. There

are two potential vaccination pathways that could be used together with R848: FI-RSV or RSV virosomes (Stegmann et al., 2010). Immunisation, of mice and cotton rats, with virosomes produced from RSV envelopes containing a lipopeptide adjuvant induced: neutralising antibodies, IFN-γ and a balanced T cell response to RSV (Stegmann et al., 2010). This approach can cause a reduction in the cellular influx observed in the RSV primed neonates in the current study. An important aspect that must be considered when assessing potential vaccines against RSV in neonates are differences in the murine models (Tregoning et al., 2010). Therefore, a rechallenge experiments should also be performed in BALB/c mice, as they are more prone to RSV infection and show a type 2 response upon reinfection.

The role of the adenosine system

The adenosine system appears to be one of the most spectacular differences between adults and neonates at baseline in the lung tissue and the BAL. This in part could explain the sensitivity of neonates to signal via TLR7 after R848 exposure (Levy et al., 2004; Levy et al., 2006a). The study of this system in more detail would be interesting both *in vivo* and *ex vivo*. The ICS data indicated that AMs are one of the sources of IL-6 after R848 exposure. If these cells are also the main target of R848 it would be interesting to transfer adult AMs into medium derived from the neonatal BAL flush (and vice versa) and add LPS, poly(I:C) or CpG. This could replicate experiments performed by Levi et al where serum was transferred from neonatal cord blood onto adult monocytes (Levy et al., 2006a). Potentially, the increased cAMP levels in the BAL of the neonates would decrease the sensitivity of adult AMs to specific ligands whilst increasing to R848. A similar experiment, although requiring optimisation could be considered *in vivo*. The BALF of neonatal mice can be transferred to adults intranasally together with the exposure to TLR ligands. This might increase the sensitivity of adults to R848. Finally, a more feasible model in both models in the first instance would be to use A3 agonists to block the adenosine signalling (Gallo-Rodriguez et al., 2002; Jacobson, 1998; Levy et al., 2006a). By doing so in neonates this could potentially decrease the sensitivity to R848 and/or increase the sensitivity toward the other TLR ligands.

8 References

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