
Regulation of virus induced inflammation by type I interferons

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Abstract

Type I interferons (IFNs) are the first line of defence against viral pathogens. They play a crucial role in activating antiviral responses to limit viral replication and viral spread. Human respiratory syncytial virus (RSV) can cause severe lung inflammation and this has been associated with polymorphisms in several innate immune response genes, including many that control the IFN system. In order to elucidate the importance of type I IFNs in regulating local inflammatory responses in the lung, their role in pulmonary immune responses to RSV infection was assessed in mice lacking the type I IFN receptor (*Ifnar1*^{-/-}). Levels of proinflammatory cytokines and chemokines were markedly reduced during RSV infection. Similar results were obtained when *Ifnar1*^{-/-} mice were challenged with non-infectious innate stimuli such as Toll-like receptor agonists. It was further shown that the administration of recombinant IFN- α together with innate stimuli was sufficient to potentiate the proinflammatory cytokine production.

Exploiting a reporter mouse strain expressing GFP under the control of the *Ifna6* promoter, alveolar macrophages (AMs) were identified as the predominant source of type I IFNs during RSV infection. This type I IFN production solely relied on signalling through the cytosolic RIG-I-like receptors (RLRs), as AMs lacking MAVS, the adaptor downstream of RLRs, were unable to produce type I IFNs. Furthermore, *Mavs*^{-/-} mice showed compromised production of proinflammatory cytokines and chemokines, and as a consequence had an increased viral burden and RSV-induced pathology. Interestingly, *Mavs*^{-/-} mice had a marked deficiency in the production of monocyte chemoattractants and recruitment of inflammatory monocytes (infMo). The administration of recombinant CCL2 during RSV infection permitted monocyte recruitment into the lung of *Mavs*^{-/-} mice and mediated an unexpected antiviral activity and reduced disease severity.

This work emphasises a role for type I IFNs in antiviral immune responses by driving early proinflammatory cytokine responses and recruitment of antiviral monocytes, a yet underappreciated novel cell type responsible for dampening RSV-disease burden *in vivo*.

Statement of Contribution

I herewith certify that all laboratory work presented in this dissertation is my own work.

Michelle Goritzka 2015

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I. Abbreviations

A

Ab	Antibody
ADV	Adenovirus
AIM2	Absent in melanoma 2
AM	Alveolar macrophages
APC	antigen-presenting cell
APC conjugate	Allophycocyanin
Areg	Amphiregulin
ASC	Apoptosis-associated speck-like protein containing a carboxy-terminal CARD

B

BAL	bronchoalveolar lavage
BALF	BAL fluid
BATF3	Basic leucine zipper ATF-like 3 transcription factor
BM	bone marrow
BMDC	bone marrow derived dendritic cells
BSA	Albumin, from bovine serum

C

CARD	Caspase activation and recruitment domains
CCL12 (MCP-5)	C-C motif ligand 12 (monocyte chemotactic protein 5)
CCL2 (MCP-1)	C-C motif ligand 2 (monocyte chemotactic protein 1)
CCL3 (MIP-1 α)	C-C motif ligand 3 (macrophage inflammatory protein 1 α)
CCL4 (MIP-1 β)	C-C motif ligand 4 (macrophage inflammatory protein 1 β)

CCL5 (RANTES)	C-C motif ligand 5 (regulated on activation, normal T cell expressed and secreted)
CCL7 (MCP-3)	C-C motif ligand 7 (monocyte-specific chemokine 3)
CD	cluster of differentiation
cDC	conventional dendritic cell
cDNA	complementary DNA
CDP	common DC progenitor
cGAS	guanosin-monophosphate adenine-monophosphate (cyclic GMP-AMP or cGAMP) synthase
CLR	C-type lectin receptors
cMoP	common monocyte precursor
CMV	Cytomegalovirus
CO ₂	carbon dioxide
COX-2	Prostaglandin-endoperoxide synthase 2
CpG	cytosine-phosphate-guanine
CTD	C-terminal domain
CTL	cytotoxic T lymphocyte
CX3CR1	Fraktalkine receptor
CXCL1 (KC)	C-X-C motif ligand 1
CXCL10 (IP-10)	C-X-C motif ligand 10 (interferon gamma-induced protein 10)
CXCL11 (ITAC)	C-X-C motif ligand 11 (interferon-inducible T cell alpha chemoattractant)
CXCL8 (IL-8)	C-X-C motif ligand 9 (interleukin 8)
CXCL9 (MIG)	C-X-C motif ligand 9 (monokine induced by gamma interferon)

D

DAB	3,3'-diaminobenzidine
DAI	DNA-dependent activator of IFN-regulatory factors

DC	Dendritic cells
DENV	Dengue virus
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
E	
EBV	Epstein-Barr virus
EC	epithelial cells
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ER	endoplasmic reticulum
F	
FACS	fluorescent-activated cell sorting
FAM	6-carboxyfluorescein
FCS	foetal calf serum
FFU	focus forming units
FGF	Fibroblast growth factors
FI-RSV	formalin-inactivated RSV
Flt3L	Fms-related tyrosine kinase 3 ligand
G	
g	gram
<i>g</i>	gravity
G-CSF (CSF3)	Granulocyte colony-stimulating factor (colony stimulating factor 3)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Interferon gamma stimulated elements
GFP	Green Fluorescent Protein

GM-CSF (CSF2)	Granulocyte-macrophage colony-stimulating factor (colony stimulating factor 2)
H	
H&E stain	Hematoxylin and Eosin stain
H ₂ SO ₄	Sulphuric acid
HEp-2	Human larynx epidermoid carcinoma cells
HGF	Hepatocyte growth factor
hr(s)	hour(s)
HSC	Haematopoietic stem cell
HSV	Herpes simplex virus
I	
i.n.	intra nasal
i.p.	intra peritoneal
Id2	inhibitor of DNA protein 2
IFI16	Interferon gamma-inducible protein 16
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IFNAR	IFN- α/β Receptor
Ig	immunoglobulin
I κ B	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor)
IKK	I-kappa B kinase
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
ILC	innate lymphoid cells
infMo	inflammatory monocytes
iNOS	inducible Nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISG	Interferon-stimulated genes
ISGF	Interferon-stimulated gene factor

ISRE	Interferon-stimulated response element
J	
JAK	Janus kinase
JEV	Japanese encephalitis virus
K	
KHCO ₃	Potassium bicarbonate
KO	knock out
L	
<i>L.m.</i>	<i>Listeria monocytogenes</i>
L	litre
LCMV	Lymphocytic choriomeningitis virus
LGP2	Laboratory of genetics and physiology 2
LN	lymph node
LPS	lipopolysaccharide
LRTI	lower respiratory tract infection
Ly6C/6G	lymphocyte antigen 6C/6G
M	
<i>m.t.</i>	<i>Mycobacterium tuberculosis</i>
M	Molar
M-CSF (CSF1)	Macrophage colony-stimulating factor (colony stimulating factor 1)
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signalling protein
MCMV	Mouse cytomegalovirus
MDA5	Melanoma differentiation associated gene 5
mDC	myeloid DC
MerTK	Tyrosine-protein kinase Mer precursor

MDP	monocyte-macrophage DC progenitor
mg	milligram
MHC	Major histocompatibility complex
min	minute
miRNA	micro RNA
ml	millilitre
mM	millimolar
MNV	Murine Norovirus
moDC	monocyte-derived DC
MOI	multiplicity of infection
MPS	mononuclear phagocyte system
mRNA	messenger RNA
mW	milliwatt
MX-1	Interferon-induced GTP-binding protein Mx1
MyD88	Myeloid differentiation primary response 88
µg	microgram
µm	micrometre
µM	micromolar
N	
Na ₂ EDTA	Ethylenediaminetetraacetic acid disodium salt dehydrate
NaHCO ₃	Sodium bicarbonate
NaN ₃	Sodium azide
NDV	Newcastle disease virus
NEMO (IKK-γ)	NF-kappa-B essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NH ₄ Cl	Ammonium chloride
NFIL3	Nuclear factor IL-3 regulated
NIK	NF-kappa-B induced kinase

NK	Natural killer cells
NLR	NOD-like receptors
NLRP3	NLR family, pyrin domain containing 3
nm	nanometre
NOD	Nucleotide-binding oligomerisation domain
NS-protein	non-structural protein
NZB mice	New-Zealand Black mice

O

OAS1	2'-5'-oligoadenylate synthetase 1
------	-----------------------------------

P

p.i.	post infection
PAMP	Pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	plasmacytoid dendritic cell
PDCA-1	plasmacytoid dendritic cell antigen 1
PE	Phycoerythrin
PGE2	Prostaglandin E2
PI3K	Phosphatidyl-inositol-3-kinase
PKB	Protein kinase B
PKR	Protein kinase R
PolIII	RNA-polymerase III
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
PVM	Pneumovirus of mouse

Q

qPCR	quantitative real-time polymerase chain reaction
------	--

R

R848	Resiquimod
rCCL2	recombinant CCL2
rIFN	recombinant interferon
RIG-I	Retinoic acid inducible gene I
RIP-1	Receptor-interacting kinase 1
RLR	RIG-I-like receptors
RNA	ribonucleic acid
RSV	Respiratory syncytial virus
RT	room temperature

S

sec	second
SeV	Sendai virus
SIRP- α	Signal-regulatory protein alpha
SNP	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes

T

TAK1	Transforming growth factor β activated kinase-1
TAMRA	Tetramethylrhodamine
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	TANK-binding kinase 1
TGF	Transforming growth factor
Th cell	T helper cell
Tip-DCs	TNF-iNOS-producing DC
TIR	Toll/IL-1 receptor

TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF	TNF receptor associated factor
TRAIL	Tumor necrosis factor-related apoptosis-induced ligand
TRIF	TIR-domain-containing adapter-induced interferon- β
TSLP	Thymic stromal lymphopoietin
TYK	tyrosine kinase
<i>T. gondii</i>	<i>Toxoplasma gondii</i>

U

U	units
UK	United Kingdom
URTI	upper respiratory tract infection
USA	United States of America
USP18	Ubiquitin carboxy-terminal hydrolase 18
UV	ultraviolet

V

VEGF	vascular endothelial growth factor
VSV	Vesicular stomatitis virus
VV	Vaccinia virus

W

WNV	West Nile virus
wt	wild-type

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1. Introduction

1.1. The mononuclear phagocyte system (MPS)

The mononuclear phagocytes system (MPS) consists of a group of cells that play a critical role as major effectors of the innate immunity. As sentinels in barrier tissues of the body like the intestine, skin, or lung they coordinate appropriate responses to a wide range of antigens, pathogens and commensals (Scott et al., 2014). These cells are involved in tolerance or protective immunity to minimise tissue damage and guarantee survival of the organism. The MPS is composed of dendritic cells (DCs), macrophages and monocytes. It was originally thought that the ultimate fate of monocytes is to replenish the tissue-resident mononuclear phagocyte pool under steady state conditions (Van Furth and Cohn, 1968). However this concept is revisited by new findings: (i) most tissue resident macrophages originate from embryonic progenitors (Ginhoux et al., 2010; Guilliams et al., 2013b; Hashimoto et al., 2013; Schulz et al., 2012; Yona et al., 2013), (ii) DCs arise from a dedicated common DC progenitor (CDP), and (iii) monocytes originate from a lineage committed precursor cells – the common monocyte precursor (cMoP) (Fogg et al., 2006; Hettinger et al., 2013; Liu et al., 2009; Varol et al., 2007), (Figure 1-1).

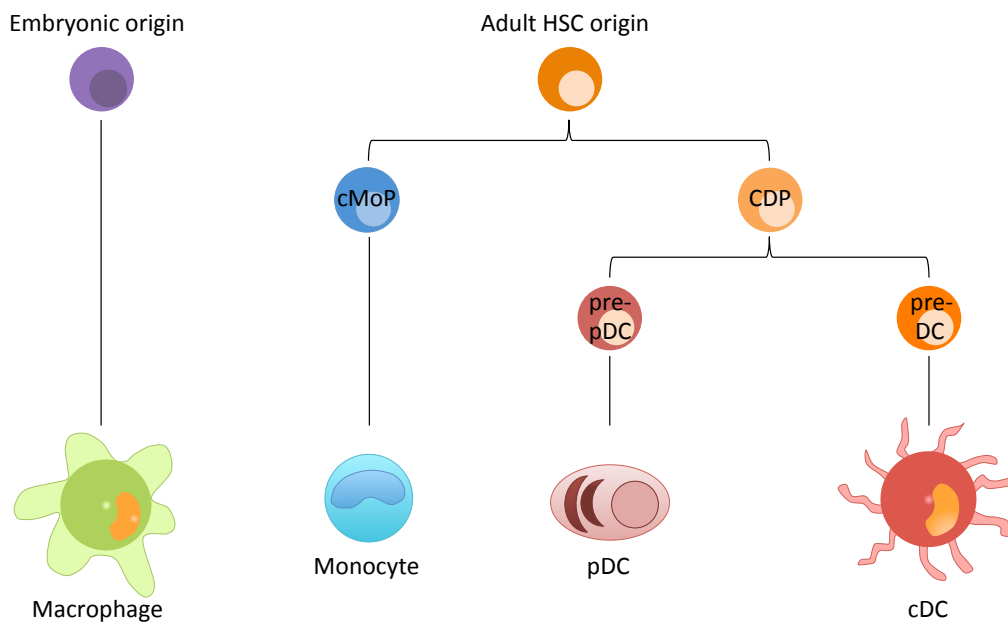


Figure 1-1 Ontogeny of the mononuclear phagocyte system. Cells of the mononuclear phagocyte system arise from embryonic or adult bone marrow cells. HSC=haematopoietic stem cell; cMoP=common monocyte progenitor; CDP=common dendritic cell progenitor; pDC=plasmacytoid dendritic cell; cDC=conventional dendritic cell.

1.1.1. Monocytes: Origin and fate

Monocytes are a population of mononuclear cells of hematopoietic origin with bean shaped nuclei. The growth factor monocyte-stimulating factor 1 (M-CSF or CSF-1) is essential for monocyte development, since mice that are deficient in CSF-1 or its receptor develop severe monocytopenia (Dai et al., 2002). In steady state they circulate in the blood and bone marrow and do not show proliferation. The blood circulating half-life of mouse monocytes is about 22 hours, whereas in humans it can be three times longer (Muller, 2001; Van Furth and Cohn, 1968). Around half of the monocytes leave the circulation each day. Under inflammatory conditions, the influx of monocytes from the bone marrow into the blood and from the circulation into the inflamed tissue is two times higher than under steady state conditions (Van Furth et al., 1973).

The lack of proliferation in peripheral blood alongside with rare numbers made it difficult to study monocyte fate and heterogeneity. The development of a reporter mouse system expressing green-fluorescent protein (GFP) under the control of the

fractalkine receptor (CX3CR1), a highly expressed marker on mouse peripheral blood monocytes, served as a great tool to study monocyte development and dynamics (Jung et al., 2000). Two subsets of circulating murine monocytes with different morphologically, phenotypically, and functionally characteristics were identified: first, the CX3CR1^{lo}CCR2^{hi}CD62L^{hi}Gr1(Ly6G/C)^{hi} (further named Ly6C^{hi} monocytes) subset is short lived and termed 'inflammatory subset' due to their affinity to home to inflamed tissue (Geissmann et al., 2003); second, the long lived CX3CR1^{hi}CCR2^{lo}CD62L^{lo}Gr1(Ly6G/C)^{lo} (further named Ly6C^{lo} monocytes) subset (Geissmann et al., 2003) which continuously patrols the vascular system to survey endothelial cell integrity (Auffray et al., 2007). Several independent studies have suggested that Ly6C^{hi} monocytes are obligatory precursors for Ly6C^{lo} blood monocytes, with monocyte conversion occurring in the BM (Sunderkötter et al., 2004; Varol et al., 2007; Yona et al., 2013). These observations might also suggest that Ly6C^{lo} monocytes terminally differentiate into blood macrophages.

In transendothelial migration assays, monocytes, in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4), showed potential to differentiate into macrophages and DCs *in vitro* (Randolph et al., 1998; Sallusto and Lanzavecchia, 1994). With the development of new techniques to assess monocyte fate *in vivo* it was demonstrated that, with a few exemptions, most tissue-resident mononuclear phagocytes are maintained independent of recruited blood monocytes in the steady state. Indeed, under homeostatic conditions lymphoid DCs and non-lymphoid tissue DCs, including CD103⁺ lamina propria DCs, lung CD103⁺ DCs, skin CD11b⁺ DCs and pDCs exclusively arise from CDP and pre-DCs precursors (Bogunovic et al., 2009; Edelson et al., 2010; Ginhoux et al., 2009; Liu et al., 2009; Tamoutounour et al., 2013; Varol et al., 2007; 2009). Only a small proportion of tissue DCs is dependent on continuous monocyte input. CX3CR1^{hi} DCs in the gut are replenished by Ly6C^{hi} monocytes under the control of CSF-1R ligand (Bogunovic et al., 2009). CD11b⁺ lung DCs are most likely of monocytic origin due to their phenotypic similarities in expressing monocyte and macrophage markers such as F4/80, SIRP- α , CX3CR1 and CSF-1R (Plantinga et al., 2010). Recently it was shown that monocytes are not excluded from peripheral tissue during the steady state. In the lung, skin and lymph nodes (LN) undifferentiated monocytes have been found to contribute to antigen transport to the draining LN (Jakubzick et al., 2013). In some cases transcriptomic features of these

monocytes resemble CD11b⁺ DCs (Tamoutounour et al., 2013). In case of tissue macrophages, experimental approaches demonstrated that most tissue-resident macrophage populations do not rely on continuous replenishment from BM precursors. Fate mapping experiments using Cre-lox-P-based models and parabiosis experiments gave evidence that liver Kupffer cells, skin Langerhans cells and dermal CD64^{hi} macrophages, brain microglia cells, alveolar, splenic, and peritoneal macrophages populate the peripheral tissues prior to birth and are locally self-maintained independent of monocytes in steady state (Ajami et al., 2007; Guillems et al., 2013b; Hashimoto et al., 2013; Hoeffel et al., 2012; Schulz et al., 2012; Tamoutounour et al., 2013; Yona et al., 2013). Therefore, monocytes are a distinct subset of the MPS that are not committed to develop into DCs or macrophages by default under steady state conditions.

In humans, three different subsets of peripheral blood monocytes are defined by their surface antigen expression of CD14 and CD16: ‘classical’ monocytes are characterized by CD14^{hi}CD16^{lo} expression, ‘non-classical’ monocytes express CD14^{lo}CD16^{hi} and a third monocyte subset can be phenotypically characterized as CD14^{hi}CD16^{hi} (Grage-Griebenow et al., 2001; Passlick et al., 1989; Segura and Amigorena, 2013).

1.1.1.1. Inflammatory monocytes

It was clearly demonstrated that under homeostatic conditions blood monocytes play an inferior role in peripheral tissue (see section 1.1.1). However, under inflammatory conditions Ly6C^{hi} monocytes can be rapidly mobilized into injured tissue and are important in shaping the immune response by the release of mediators or modulating cellular responses. They are termed monocyte-derived DCs (moDCs), inflammatory DCs, inflammatory monocytes or macrophages, on the basis of phenotypic characteristics and functional specialisation they obtain after extravasation. Monocyte-derived cells can show DC-like features as expression of major histocompatibility complex (MHC)-class II and CD11c, and have the capacity to present antigens for T-cell priming (León et al., 2007). But unlike DCs, their development highly depends on the macrophage growth factor CSF-1 (also M-CSF) (Greter et al., 2012). Indeed, inflammatory monocytes can display macrophage characteristics as they express CD64, MerTK or F4/80 and have

phagocytic activities (Bain et al., 2012; Hammad et al., 2010; Plantinga et al., 2013). These characteristics are difficult to assess *in vivo* and are non-exclusive. The nomenclature used in published data is therefore arbitrary and it remains unclear if a distinct set of transcription factors and molecules determines the fate of monocyte-derived cells or if they are highly plastic and get skewed by tissue specific micro-environmental factors (Scott et al., 2014).

The recruitment of monocyte-derived inflammatory cells during inflammatory processes has been described for various peripheral tissues such as the intestine, lung, genital tract, skin, spleen and brain (Ajami et al., 2011; Iijima et al., 2010; Naik et al., 2006; Plantinga et al., 2013; Tamoutounour et al., 2013; 2012). In response to inflammatory signals various cell types produce the chemokines CCL2, CCL7, and CCL12 resulting in the recruitment of CCR2 receptor expressing Ly6C^{hi} monocytes into inflamed tissue (Geissmann et al., 2003; Shi and Pamer, 2011). Several studies in infectious diseases have described a role of monocyte-derived cells in antimicrobial defence. For example, during bacterial infections with *L. monocytogenes* (*L.m*) inflammatory monocyte-derived cells produce high levels of tumor necrosis factor α (TNF- α) and inducible nitric oxide synthase (iNOS) to contribute to innate defence in the spleen. These characteristics together with CD11c expression, gave them the name TIP-DCs (TNF- and iNOS- producing cells) (Serbina et al., 2003). Similar cells have been identified in the lamina propria of the intestine after infection with the intracellular protozoa *T. gondii* (Dunay et al., 2008; Robben et al., 2005). Mice deficient in CCR2 or CCL2 succumb from *T. gondii* infection, caused by uncontrolled pathogen replication, neutrophil mediated-pathology and excessive tissue necrosis (Dunay et al., 2008; Robben et al., 2005). Protection from lethal *T. gondii* infection by Ly6C^{hi} monocytes is mediated through the release of the lipid mediator prostaglandin E2 (PGE2) (Grainger et al., 2013). Although these monocyte-derived cells produce TNF- α and iNOS they lack CD11c surface expression, which distinguishes them from the TIP-DCs described in *L.m* infection (Dunay et al., 2008). These differences in cellular phenotype are possibly attributable to the distinct inflammatory environment induced by these two pathogens or the specific tissue environment. Additionally, control of pathogen replication and neutrophil activation monocyte-derived inflammatory cells induce protective Th1 T cell responses in protozoal infection and *M. tuberculosis* (*M.t*) (León et al., 2007; Peters et al., 2004). Inflammatory monocytes are also important in shaping the immune response to

viral pathogens. For example, they are recruited to the brain or cornea to enhance clearance of west nile virus (WNV) (Lim et al., 2011) or Herpes simplex virus type 1 (HSV-1) (Conrady et al., 2013), respectively, and in models using mouse cytomegalovirus (MCMV) and vaccinia virus (VV) infection Ly6C^{hi} monocytes elicit their antiviral effect through type I IFN production (Barbalat et al., 2009). In the vaginal tract inflammatory monocytes re-stimulated the secretion of IFN- γ by effector Th1 cells to support HSV-type 2 (HSV-2) clearance, but they appear dispensable for initial CD4⁺ T cell priming (Iijima et al., 2010). Additionally, Ly6C^{hi} monocytes have been reported to induce memory CD8⁺ T cell responses in HSV-1 infection (Wakim et al., 2008).

Inflammatory monocytes have also been identified in humans in various inflammatory conditions, such as atopic dermatitis, psoriasis, and rheumatoid arthritis. They express HLA-DR, CD11c, BDCA1, CD1a, CD206, CD172a, CD14, CD11b, M-CSFR1 and Fc ϵ R1 (Segura and Amigorena, 2013). Inflammatory monocytes obtained from inflammatory fluids are distinct from macrophages and steady-state DCs, and transcriptomic similarities with *in vitro* cultured monocyte-derived DCs, suggested that these inflammatory monocytes derive from blood monocytes, similar to mouse (Segura et al., 2013).

1.1.2. Mononuclear phagocytes of the lung

The lung is continuously exposed to foreign antigens such as microbes, pollen or environmental dust. Different subsets of DCs and macrophages reside in the lung to surveil the tissue environment and respond to potential threats. Pulmonary DCs can be divided into three distinct populations depending on distinct surface marker expression: conventional DCs (cDCs) can be divided into two main subsets CD103⁺ DCs (CD103⁺CD11c^{hi}CD11b^{lo}CX3CR1⁻) and CD11b⁺ DCs (CD103⁻CD11c^{hi}CD11b^{hi}CX3CR1^{int} DCs). The third pulmonary DC subtype are CD11c^{int} plasmacytoid DCs (pDCs) (Guilliams et al., 2013a; Mildner and Jung, 2014). In case of macrophages, two different subtypes can be described in the lung: alveolar macrophages (AMs; CD64^{hi}Siglec-F^{hi}CD11c^{hi}CD11b^{lo}MHCII^{lo}) and interstitial macrophages (CD11c^{lo}CD11b^{hi}MHCII^{hi}). Additionally to DCs and macrophages, undifferentiated tissue monocytes in steady state

and monocyte-derived cells during inflammation complete the different monocytic phagocyte population residing the lung (Jakubzick et al., 2013; Shi and Pamer, 2011).

1.1.2.1. Alveolar macrophages

Alveolar macrophages (AM) populate the lung alveoli shortly after birth originating from foetal liver monocytes (Guilliams et al., 2013b). AMs self maintain locally by *in situ* proliferation with minimal contribution from monocytes in steady state (Hashimoto et al., 2013). During pulmonary infections inflammatory monocytes are recruited into the tissue, but they are poorly maintained in the lung as they were eventually outcompeted by resident embryonic AMs after resolution of inflammation (Hashimoto et al., 2013). Only when the local repopulation capacity of AMs was severely depleted due to infection or radiation, recolonisation was in favour of recruited monocytes (Guilliams et al., 2013b; Landsman and Jung, 2007). CSF-2 (GM-CSF) is crucial for AM homeostasis and promotes AM-mediated surfactant clearance. Deficiencies in Csf-2 or its receptor (Csf-2R), leads to a condition called proteinosis, where surfactant accumulates in the alveoli and aggravates gas exchange (Suzuki et al., 2014). AMs have high phagocytic activity and are equipped with various pattern recognition receptors (PRRs) to provide them with the ability to clear apoptotic cells, residual cellular debris, and to participate in immune responses (Hussell and Bell, 2014). AMs are described to be the main producers of type I interferons (IFNs) in murine infection with Newcastle disease virus (NDV) and *in vitro* infections with Vesicular stomatitis virus (VSV) (Kumagai et al., 2007) and influenza virus infection (Helft et al., 2012). Type I IFNs are known for their antiviral properties, which put AMs into a superior role in initiating antiviral immune responses in the lung. For more extensive description of IFNs, please refer to section 1.3 in this thesis.

1.1.2.2. Dendritic cell subsets and monocyte-derived inflammatory cells

Conventional DCs (cDCs) are professional antigen-presenting cells that link the innate and the adaptive immune system. They constantly surveil the tissue to capture foreign antigens and allergens (Mildner and Jung, 2014). Upon PRR-mediated pathogen sensing,

cDCs undergo a maturation process, which involves upregulation of CCR7, to enable them to migrate to the lymph node (LN), and upregulation of co-stimulatory molecules such as CD80, CD86 and CD40 to enhance T cell and B cell activation. Once in the LN they present endogenous and exogenous captured antigen, on major histocompatibility complex I (MHC-I) or MHC-II molecules, to CD8⁺ T cells and CD4⁺ T cells, respectively, in order to initiate adaptive immune responses (Mildner and Jung, 2014). In addition to PRRs, cDC maturation can be modulated by the release of mediators from other cells (Asselin-Paturel et al., 2005; Honda et al., 2003; Hoshino et al., 2002; Pang et al., 2013; Yadava et al., 2013). Type I IFNs, triggered by viral infection, are potent activators of DCs (Honda et al., 2003). Thymic stromal lymphopoietin (TSLP) has been shown to be important for CD11b^{hi} DCs of monocytic origin to re-stimulate CD8⁺ effector T cells in the lung (Yadava et al., 2013), and IL-1 and IL-18, produced by epithelial cells upon inflammasome stimulation, promote cDC maturation (Pang et al., 2013). Depending on the cytokine and chemokine cocktail secreted by cDCs during DC-T cell-interactions, T cells differentiate into lineages including Th1, Th2, Th9, Th17 or T-regulatory (Treg) T cells.

CD103⁺ DCs exclusively arise from pre-DCs under the control of Fms-related tyrosine kinase 3 ligand (Flt3L), inhibitor of DNA protein 2 (Id2), basic leucine zipper ATF-like 3 transcription factor (BATF3), interferon regulatory factor 8 (IRF8), and nuclear factor IL-3 regulated (NFIL3) (Mildner and Jung, 2014). They can directly sample antigens from the airway lumen by extending their dendrites, as they are located within the epithelial layer (Hammad and Lambrecht, 2008). Intraepithelial CD103⁺ DCs have been shown to express tight junction proteins zonula-2, claudin-1, and claudin-7 resulting in antigen sampling from the airway lumen without disrupting the epithelial cell barrier (Sung et al., 2006). CD103⁺ DCs are the main migratory subset during infection and their superior ability to unconventionally present exogenous non-cytosolic antigens via MHC-I molecules (cross-presentation) designates them as potent inducers of CD8⁺ T effector cell responses in the LN against viruses (Helft et al., 2012; Kim et al., 2014; Molteni et al., 2011). Additionally, CD103⁺ DCs are the only lung DC subset with the capacity to cross-present antigens acquired from dead cells that succumb during infection (Desch et al., 2011). Depletion of CD103⁺ DCs prior to influenza virus infection led to severe illness, reduced viral clearance and diminished virus-specific CD8⁺ T cell responses,

emphasising a nonredundant role for CD103⁺ DCs in antiviral immunity (GeurtsvanKessel et al., 2008).

CD11b⁺ DCs reside in lamina propria below the basement membrane and are the most abundant DC subtype in the lung and their development depends on CSF1, suggestive of a monocytic origin (Plantinga et al., 2010). During influenza virus infection they predominantly present viral antigens on MHC-II molecules to induce virus-specific CD4⁺ T cell responses (Kim and Braciale, 2009). In addition, CD11b⁺ DCs exhibit the capacity to prime and re-stimulate effector Th1 cells in the lung and are reported to be the main producers of chemokine and cytokines in inflammation (Beaty et al., 2007; Plantinga et al., 2013). Recently, migrating CD11b⁺ DCs were shown to induce memory CD8⁺ T cells in primary infections (Kim et al., 2014). Hence, CD103⁺ DCs and CD11b⁺ DCs, as the two major subsets in the lung, trigger the activation of CD4⁺ and CD8⁺ T cell responses to ensure viral clearance.

pDCs arise from the common DC progenitor (CDP), dependent on Flt3L signalling and the transcription factor E2-2 for their development (Mildner and Jung, 2014). Constitutive expression of IRF7 enables pDCs to rapidly secrete type I IFNs during viral infections. The role of pDCs in pulmonary infections is not clear. Depletion of pDCs during sublethal influenza virus infection indicated a role for pDCs in early T cell accumulation in the airways, yet an accountable effect on viral clearance and virus-specific CD8⁺ T cell activation was absent (Wolf et al., 2009). However, in lethal influenza virus infection they enhance mortality by eliminating CD8⁺ T cells (Langlois and Legge, 2010). Also during pulmonary infections with RSV pDCs play a regulatory function on effector T cell responses (Smit et al., 2008; Wang et al., 2006). Other non-pulmonary viral infections show a role for pDCs in supporting cDC-mediated T cell priming (Yoneyama et al., 2005), and the accumulation of virus-specific T cells and NK cells (Swiecki et al., 2010).

Ly6C^{hi} monocytes have been suggested as the predominant cause of disease in influenza virus infection (Aldridge et al., 2009; Davidson et al., 2014; Dawson et al., 2000; Herold et al., 2008; Lin et al., 2008). However, removing Ly6C^{hi} cells had no beneficial effect on disease outcome, as it diminished CD8⁺ T cells responses and numbers in the lung but notably not CD8⁺ T cell numbers in the LN. Indeed, it was shown that Ly6C^{hi} monocytes directly promote T cell responses through local antigen

presentation in the lung (Aldridge et al., 2009). Interestingly, by partially diminishing the recruitment of Ly6C^{hi} monocytes, the potential lethal consequence of these cells was reduced without compromising viral clearance by CD8⁺ T cells. In house dust mite (HDM) induced allergic inflammation, Ly6C^{hi} monocytes can drive Th2 inflammatory responses by promoting the recruitment of eosinophils and T cells into the airways (Plantinga et al., 2013). Thus, the recruitment of Ly6C^{hi} inflammatory monocytes into the inflamed lung can promote protective immunity but at the same time have detrimental consequences for the host.

1.2. Pattern recognition receptors (PRRs)

Pattern Recognition Receptors (PRRs) are germ-line encoded receptors of the innate immune system to detect invading pathogens and eliciting an appropriate immune response by triggering distinct signalling cascades. PRR molecules recognise conserved molecular structures known as pathogen-associated patterns (PAMPs) and are located at different compartments of the cell to ensure the sensing of pathogens. Following recognition an immediate response activates a signalling cascade involving transcription factors that regulate the gene expression of cytokines and chemokines important to initiate immune responses. Several different classes of PRRs have been discovered; membrane bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), cytoplasmic retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and DNA-sensors (Kawai and Akira, 2011).

1.2.1. Detection of viral nucleic acids by endosomal PRRs

The first family of PRRs that was discovered were membrane-bound TLRs that detect PAMPs in the extracellular milieu or within endosomes (Kawai and Akira, 2011). TLRs are type I transmembrane proteins, which consist of a leucine-rich repeat ectodomain mediating recognition of PAMPs and facing towards the extracellular space or endosome lumen. The transmembrane domain, and the intracellular Toll-interleukin 1 (IL-1)

receptor (TIR) domain are required for signal transduction. To date, 10 and 13 functional TLRs are identified in human and mice, respectively (Kawai and Akira, 2011; Li and Chen, 2012). Nucleic acid sensing TLRs are located within intracellular compartments with TLR3 detecting dsRNA and the double-stranded synthetic analogue Polyinosinic:polycytidylic acid (PolyI:C). TLR7 and 8 detect ssRNA and the derivate resiquimod (R848), and TLR9 binds CpG DNA (Table 1-1).

TLR3 signalling is dependent on the adaptor protein TIR domain-containing adapter inducing IFN- β (TRIF) and activates IRF3, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways. For the induction of type I IFN expression, TRIF co-localises with activated TLR3 and further recruits TANK-binding kinase 1 (TBK1), I-kappa B kinase (IKK)- ϵ , and TNF receptor associated factor 2 (TRAF2), TRAF3 and TRAF6 leading to the phosphorylation of the transcription factor IRF3 and the consequent induction of type I IFNs. In order to induce proinflammatory cytokines, TRIF assembles a signalling complex including TRAF6, TNF receptor type I DEATH domain-associated protein (TRADD), Pellino-1, and receptor-interacting kinase 1 (RIP-1) that activates transforming growth factor β activated kinase-1 (TAK1), which in turn activates NF- κ B and MAPK pathways (Figure 1-2).

The endosomal TLRs TLR7, 8 and 9 signal through the myeloid differentiation factor-88 (MyD88). Downstream pathway activation is mediated through NF- κ B, which requires a signalling complex comprised of IL-1 receptor-associated kinases (IRAK)-1, IRAK2, IRAK4 and IRAK-M, the IKK complex (IKK- α , IKK- β and IKK- γ also NK-kappa-B essential modulator (NEMO)) and TAK1. Type I IFN production requires IKK- α and is IRF7 mediated, a mechanism particularly important in pDCs (for deeper review refer to (Gürtler and Bowie, 2013; Kawai and Akira, 2010)), (Figure 1-2)

Other intracellular TLRs not involved in viral sensing are TLR11, a non-functional pseudogene in humans that detects profilin from *T. gondii* in rodent infection (Yarovinsky et al., 2005), and TLR12 and 13, that are completely absent in the human genome. At the moment, the role of TLR12 in mice is unknown, but TLR13 was shown to be important in bacterial defence (Li and Chen, 2012) (Table 1-1).

Table 1-1 Endosomal PPRs sensing nucleic acids

(adapted by (Kawai and Akira, 2010; Li and Chen, 2012; Yarovinsky et al., 2005))

PRR	Ligand	Pathogen
TLR3	dsRNA	dsRNA virus (reovirus), ssRNA virus (RSV, EMCV, WNV) DNA virus (MCMV, HSV-1)
TLR7/8	ssRNA	ssRNA virus (VSV, influenza A virus, HIV-1), endogenous retrovirus, Group B Streptococcus
TLR9	Unmethylated CpG DNA	DNA virus (MCMV, HSV-1/2), <i>P. falciparum</i> -generated hemozoin
murine TLR11	profilin	<i>T. gondii</i>
murine TLR12	unknown	unknown
murine TLR13	23S ribosomal RNA	gram neg. and gram pos. bacteria

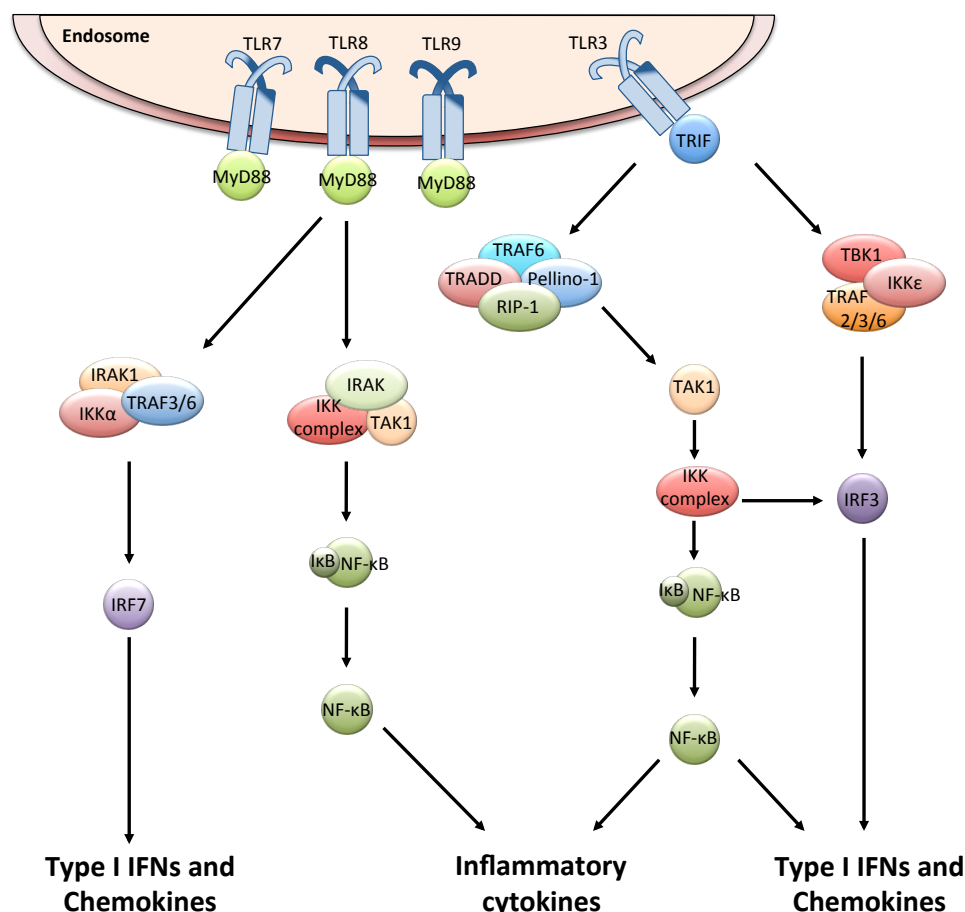


Figure 1-2 Endosomal nucleic acid sensing by PRRs leading to the activation of MyD88 and TRIF dependent pathways to upregulate type I IFNs, inflammatory cytokines and chemokines through IRFs and NF-κB transcription factors.

1.2.2. Detection of viral nucleic acids by cytosolic PRRs

Viral RNA that reaches the cytosol by endosomal escape, fusion of the virus with the membrane or as replication intermediate is sensed by RLRs consisting of three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Table 1-2). RIG-I and MDA5 are in possession of a DExD/H-box RNA-helicase domain, which binds to dsRNA and hydrolyses ATP, N-terminal tandem caspase activation and recruitment domains (CARD), which are essential for mediating downstream signalling, and finally a C-terminal domain (CTD). Although LGP2 is encoding the RNA-binding domain it lacks expression of the CARD domains, and is therefore not involved in signalling upon sensing of nucleic acids. Instead recent finding suggest a role for LGP2 in assisting MDA5-mediated signalling

(Bruns et al., 2014). RIG-I and MDA5 recognise non-self dsRNA and ssRNA from a range of different viruses (Goubau et al., 2013). Located in the cytosol, they are surrounded by host RNA and defined self versus non-self discrimination is crucial to avoid autoimmunity. The ligand specificity is highly dependent on the sequence length of the dsRNA molecule (Kato et al., 2008). Additionally, RIG-I has the property to recognise pathogen-specific motifs absent in host RNAs. The C-terminal domain of RIG-I specifically binds to short uncapped 5'-triphosphate (5'-ppp) ssRNA and 5'-diphosphate (5'-pp) bearing base-paired RNA molecules, a RNA motif known to be present in some viral genomes and very distinct from the 5'-capped host RNAs (Goubau et al., 2014; Hornung et al., 2006; Pichlmair et al., 2006). For both receptors binding of dsRNA or ssRNA leads to filamentous oligomerisation along the foreign RNA which brings the CARD domains into proximity and allows downstream signalling through the adaptor molecule mitochondrial antiviral signalling protein (MAVS) (Peisley et al., 2011; 2013; Wu et al., 2013a). MAVS possesses a N-terminal CARD domain and a C-terminal transmembrane domain that anchors MAVS to the mitochondrial membrane (Seth et al., 2005). Following recruitment of activated RLRs to the mitochondria, CARD-CARD interaction between RLR and the adaptor promotes MAVS polymerisation (Hou et al., 2011). MAVS filaments further recruit signalling molecules (involving the IKK complex, TBK1 and several TRAF proteins) and lead to the activation of NF- κ B and IRF3 and IRF7, resulting in gene expression of various antiviral cytokines including type I IFNs (Liu et al., 2013) (Figure 1-3). Recently a study revealed that MAVS additionally resides at the peroxisome membrane in human and mouse (Dixit et al., 2010). Altogether, these structures elicit the appropriate antiviral immune response. RIG-I can additionally induce proinflammatory responses independently of MAVS by binding to apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), which acts as an adaptor leading to inflammasome-mediated maturation of IL-1 β and IL-18 (Poeck et al., 2010).

Infection with DNA bearing viruses might lead to the release of DNA into the cytosol and similar to RNA sensors several DNA sensors have been described to bind cytosolic DNA (Table 1-2). The first sensor described was DNA-dependent activator of IFN-regulatory factors (DAI), which was shown to induce type I IFNs during *in vitro* infections of fibroblast with either HSV-1 or cytomegalovirus (CMV). However, the *in vivo* role for DAI remains unclear (Ishii et al., 2008). Another DNA sensor, RNA

Polymerase III (PolIII), was discovered to transform DNA into a 5'-ppp RNA, the ligand for RIG-I activation, yet the physiological role of PolIII remains elusive (Paludan and Bowie, 2013). Other proteins belonging to the PYHIN (IFI16; interferon gamma-inducible protein 16, and AIM2; absent in melanoma 2) or DExD/H-box helicase (DDX) protein families were suggested likely DNA sensors, but their specific role *in vivo* is unclear (for a extensive review about DNA sensing refer to (Paludan and Bowie, 2013)). The discovery of the adaptor protein stimulator of interferon genes (STING) identified a pathway crucial for the recognition of foreign dsDNA *in vivo*. Located at the endoplasmic reticulum (ER), cytosolic dsDNA activates STING to recruit and activate IKK and TBK1, which in turn activate the downstream transcription factors NF- κ B and IRF3, resulting in the production of type I IFNs. Recently, several groups identified the DNA sensor leading to the downstream activation of STING. The guanosin-monophosphate adenine-monophosphate (cyclic GMP-AMP or cGAMP) synthase (cGAS) was identified to bind dsDNA and catalyse the synthesis of the second messenger cGAMP from ATP and GTP. CGAMP binding to STING in turn activates the signalling cascade leading to type I IFN production (Ablasser et al., 2013a; Sun et al., 2013; Wu et al., 2013b). Interestingly, synthesised cGAMP is transferred through gap junctions from infected cell to neighbouring cells to spread antiviral immunity (Ablasser et al., 2013b).

Table 1-2 Cytosolic PRRs sensing nucleic acids

(adapted by (Gürtler and Bowie, 2013; Li et al., 2013))

PRR	Ligand	Pathogen
RIG-I	Blunt-end	DNA virus (Rhabdoviridae; VSV, Hepadnaviridae; EBV)
	dsRNA	ssRNA virus (Orthomyxoviridae; influenza virus,
	5'ppp and 5'pp ssRNA	Paramyxoviridae; NDV, RSV, Flaviviridae; WNV, DENV, JEV) dsRNA (reovirus)
MDA5	dsRNA	DNA virus (MHV, VV)
		ssRNA (Flaviviridae; WNV, DENV, Caliciviridae; MNV, Picornaviridae; EMCV)
		dsRNA (reovirus)

PRR	Ligand	Pathogen
cGAS	dsDNA	HSV-1 retroviruses
DAI	dsDNA	HSV-1
PolIII	AT-rich dsDNA	HSV-1, ADV, EBV
IFI16	dsDNA	HSV-1
AIM2	dsDNA	VV
DHX9	CpG-B	HSV-1
DHX36	CpG-A	HSV-1
DDX41	dsDNA	HSV-1
DDX60	dsDNA, ss/dsRNA	VSV, HSV-1, poliovirus, SeV
DDX3	dsRNA	VSV

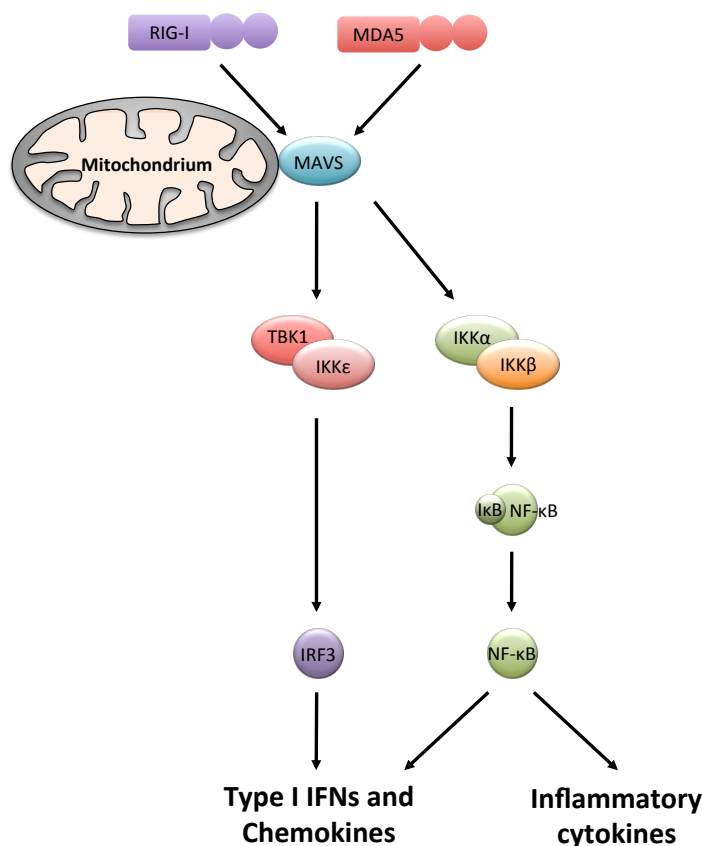


Figure 1-3 Cytosolic nucleic acid sensing by RIG-I and MDA5 leading to the activation of MAVS dependent pathways to upregulate type I IFNs, inflammatory cytokines and chemokines through IRF3 and NF- κ B transcription factors.

1.3. Type I interferons (IFNs) in antiviral immune responses

Interferons (IFNs) were discovered in the 1950s and represent a family of cytokines, which induce robust antiviral and immunomodulatory responses to interfere with virus replication and spread (Durbin et al., 2013; González-Navajas et al., 2012; Swiecki and Colonna, 2011). IFNs can be classified into 3 main subclasses, type I, type II and type III IFNs. Type I IFNs consist of several IFN- α isoforms (13 in human and 14 in mice) IFN- β , IFN- ϵ , IFN- κ , and IFN- ω . In contrast to type I IFNs, type II IFNs include only one member, which is designated IFN- γ . The most recently discovered group of IFNs are the type III IFNs, including IFN- λ 1 (IL-29; non-functional pseudogene in mice), IFN- λ 2 (IL-28A), IFN- λ 3 (IL-28B) and the very recently described human IFN- λ 4 (Egli et al., 2014; Prokunina-Olsson et al., 2013). Sensing of viral PAMPs and transcriptional activation via

IRF3 and IRF7 (highly expressed only in pDCs) leads to the secretion of early type I IFNs (IFN- α 4 and IFN- β) by infected cells and activates antiviral pathways with extensive effect on the immune response. First, they elicit an antimicrobial state in infected and neighbouring uninfected cells. Second, type I IFN modulates the immune response by promoting antigen presentation, cytokine production, DC and NK cell activity and macrophage function. Third, they activate the adaptive immune response by enhancing T cell effector function and antibody production. Overall, the antiviral and immune stimulatory potential of type I IFNs is required for the effective clearance of acute viral infections (Durbin et al., 2013; González-Navajas et al., 2012; Swiecki and Colonna, 2011).

1.3.1. Type I IFN signalling

Type I IFNs bind to the heterodimeric transmembrane IFN- α/β receptor (IFNAR), which is composed of the two subunits IFNAR1 and IFNAR2. Each of these subunits is bound intracellularly to a member of the janus kinase (JAK) family. The IFNAR1 subunit is associated with tyrosine kinase 2 (TYK2) and IFNAR2 is associated to JAK1. Ligand binding leads to dimerization of the IFNAR subunits followed by autophosphorylation and activation of JAKs. JAKs in turn phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. Phosphorylated STAT1-STAT2 dimerise and translocate into the nucleus (Darnell et al., 1994). Together with IRF9 they form the multiprotein complex IFN-stimulated gene factor 3 (ISGF3), which binds specific DNA elements called ISRE (IFN-stimulated response elements) and induce gene transcription from these loci (Levy et al., 1989; 1988; Schindler et al., 1992). Other STAT complexes that are induced by type I IFNs bind other types of elements known as IFN- γ activated sequences (GAS) (Platanias, 2005) (Figure 1-4). The canonical JAK-STAT pathway of IFN signalling is known to stimulate the transcription of hundreds of genes, known as IFN-stimulated genes (ISGs), which mediate various biological responses (Larner et al., 1986; 1984; Schoggins et al., 2011). Additionally, other non-canonical pathways have been identified to be essential for antiviral responses, with MAPKs important for transcriptional regulation of ISGs (Uddin et al., 1999), and phosphatidyl-inositol-3-kinase (PI3K)-mediated regulation of pro- or anti-apoptotic pathways and mRNA

translation (Uddin et al., 1995), (for a deeper review on type I IFN signalling refer to (Platanias, 2005)). During an immune response, type I IFN production and signalling are tightly regulated by feedback mechanisms. The production of type I IFNs is mediated by a positive feedback-loop, with IFN- β and IFN- α 4 stimulating the expression of the ISG IRF7, which regulates the expression of all IFN- α isotypes (Marié et al., 1998). Amplification of the primary signal is achieved by upregulation of IRF9 and STAT1, which are ISGs themselves fostering further type I IFN signalling. Mechanisms to attenuate type I IFN responses include the downregulation of IFNAR, the expression of negative regulators such as suppressor of cytokine signalling 1 (SOCS1), SOCS2 (Fenner et al., 2006), ubiquitin carboxy-terminal hydrolase 18 (USP18) (Sarasin-Filipowicz et al., 2009), ISG15 (Zhang et al., 2014), the induction of miRNAs, and the activation of non-canonical NF- κ B signalling (Jin et al., 2014), (for a deeper review on the regulation of type I IFN responses refer to (Ivashkiv and Donlin, 2014)).

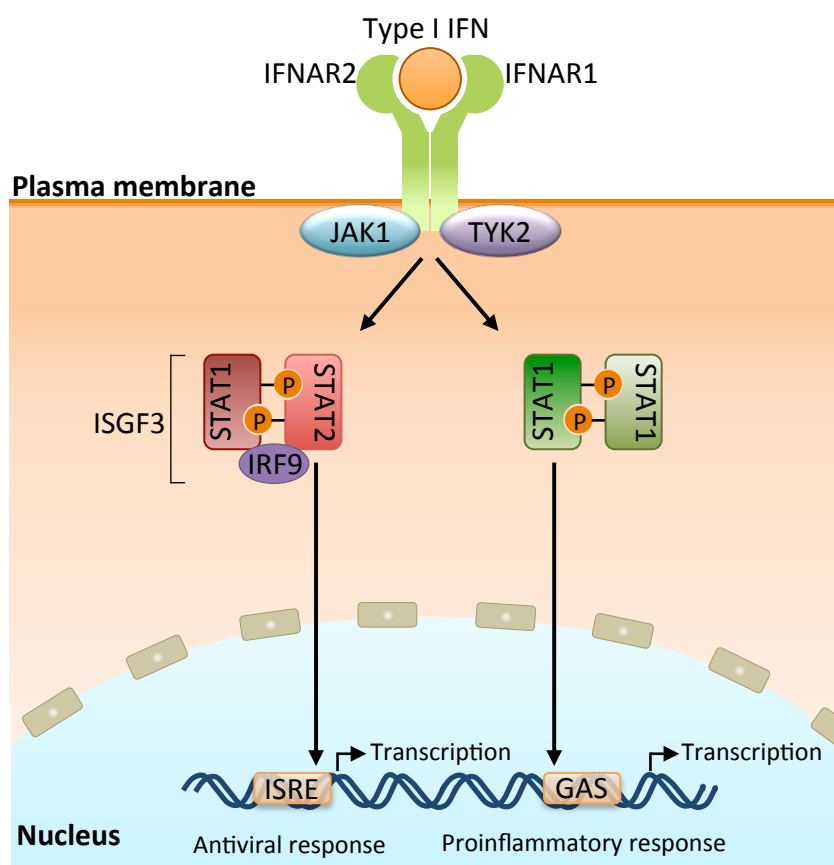


Figure 1-4 The canonical type I IFN signalling pathway. Engagement of the IFN- α/β receptor (IFNAR; composed of IFNAR1 and IFNAR2) leads to the activation of receptor associated janus kinases (JAK1 and TYK2), following phosphorylation, dimerization and nuclear translocation of signalling transducer and activation of transcription (STAT) proteins. The interferon stimulated gene factor 3 (ISGF3; composed of

STAT1, STAT2 and interferon regulatory factor 9 (IRF9)) binds to interferon stimulated response element (ISRE) to activate response genes, many of which elicit an antiviral state in cells. The STAT1 homodimer binds to gamma interferon activated sequences (GAS) to activate inflammatory response genes.

1.3.2. Type I IFNs in viral infections

Type I IFNs are rapidly produced in viral infections and contribute to the initial control of viral replication before effective humoral immunity and adaptive cellular responses are generated to clear the virus. Type I IFNs generate an antiviral state in cells that counters successful viral infection through the IFNAR. IFNAR signalling activates the transcription of a cascade of proteins that directly interfere with viral replication and viral spread. This includes proteins that inhibit virus entry (e.g. Mx1, IFITM proteins, TRIM proteins), modulate membrane lipids to circumvent viral release (e.g. Viperin, Tetherin), induce apoptosis of infected cells (e.g. protein kinase R (PKR)), regulate transcriptional (e.g. Viperin) and post-transcriptional (e.g. OAS/RNaseL, PKR) mechanism, and post-translational events (e.g. ISG15) (Helbig and Beard, 2014; Schneider et al., 2014; Thapa et al., 2013). In addition to the ISG-mediated effects, type I IFNs modulate cell viability and function (e.g. apoptosis, inhibiting cell death, differentiation, migration, proliferation) to support antiviral defence (Mattei et al., 2010). Type I IFNs are known to enhance immune responses especially by promoting DC responses. They have been shown to increase antigen-presentation and migratory capacities of DCs, to induce CD4⁺ T cell responses and promote cross-presentation of peptides from apoptotic cells to cross-prime CD8⁺ T cells and increase survival of antigen-bearing DCs (Asselin-Paturel et al., 2005; Honda et al., 2003; Le Bon et al., 2003; Longhi et al., 2009; Lorenzi et al., 2011; Montoya et al., 2002). Type I IFNs have been shown to impact on adaptive cellular responses as timing and magnitude of type I IFN production modulates the clonal expansion and memory formation of CD8⁺ T cells during primary lymphocytic choriomeningitis virus (LCMV) infections (Kolumam et al., 2005; Wang et al., 2012). Type I IFNs regulate CD4⁺ T cells responses during primary responses by promoting clonal expansion in the early phase upon activation, following contraction of CD4⁺ T cells later to restore normal numbers (Dondi et al., 2004). Additionally to T cell expansion, type I IFNs have been shown to promote T effector cell function due to the dependence on IFNAR mediated STAT1 signalling for the cytolytic

activity of memory CD8⁺ T cells during recall responses with respiratory viruses (Kohlmeier et al., 2010).

Despite the essential role for type I IFNs in activating the immune response to successfully combat viral infection and to guarantee survival of the organism they can have deleterious effect on the host. High magnitudes of type I IFN production as a consequence of influenza virus infection causes excessive inflammation with increased morbidity and mortality during acute infection (Davidson et al., 2014). This negative effect of excessive type I IFN abundance is mediated by recruited inflammatory monocytes that have previously been shown to correlate with disease severity (Aldridge et al., 2009; Davidson et al., 2014; Herold et al., 2008). Furthermore, the induction of type I IFNs during influenza virus infection attenuates chemokines important for neutrophil recruitment, which can promote secondary bacterial pneumonia (Shahangian et al., 2009). In addition, in acute LCMV infections type I IFNs elicit potent anti-viral activities, yet persistent type I IFN signalling is associated with immunosuppression ultimately promoting viral replication and disease progression in chronic LCMV infections (Teijaro et al., 2013; Wilson et al., 2013).

1.4. Human respiratory syncytial virus (RSV)

Human respiratory syncytial virus (RSV) is an enveloped, non-segmented, negative-sense, single-stranded RNA virus of the family *Paramyxoviridae* and the genus *Pneumovirus*. RSV causes respiratory tract infection and was first discovered in chimpanzee (BLOUNT et al., 1956) and recovered from infants with lower respiratory tract infection in 1957 (CHANOCK et al., 1957). There is a single RSV serotype with two antigenic subgroups, A and B. Its viral genome encodes 10 genes, which give rise to 11 proteins, as the M2 RNA contains two overlapping open reading frames yielding the matrix proteins M2-1 and M2-2 (Figure 1-5).

The viral envelop contains three proteins: i) the major surface glycoprotein G, which is involved in attachment and exists in membrane-bound and soluble form, ii) the small hydrophobic (SH) protein, which functions as a viroporin to modify membrane permeability, and iii) the fusion protein F, essential for regulating virus fusion with the cell membrane and virus entry. The nucleocapsid structure is made of five proteins: i)

the nucleocapsid protein N, involved in replication and transcription, ii) two non-structural proteins (NS1 and NS2), with mainly immunosuppressive function (see section 1.4.3), iii) the phosphoprotein P, an important co-factor in RNA replication, iv) the large (L) protein, necessary for viral replication, and v) the matrix proteins M2-1/M2-2, involved in virus assembly (Figure 1-5). The RSV virion is an irregular spherical particle with 100-to 300 nm diameter or it can form up to 100 μ m long filaments with a diameter of up to 200 nm. RSV enters host cells either through direct interaction of viral F protein with cellular nucleolin on the apical side or the endosomal membrane after clathrin-mediated endocytosis (Borchers et al., 2013; Collins and Karron; Tayyari et al., 2011). The replication occurs in the cytoplasm with detectable mRNA sequences and expression of viral proteins starting from 4 to 6 hrs post infection (p.i.) and functional virus progeny at 12 hrs p.i. (Borchers et al., 2013; Collins and Karron).

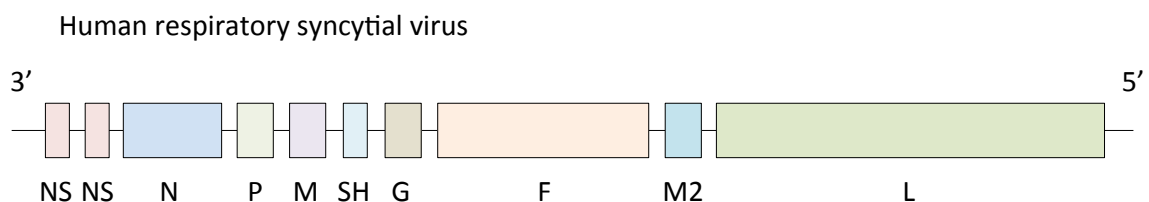


Figure 1-5 The genome of RSV. NS=non structural protein, N=nucleoprotein, P=phosphoprotein, M=matrix protein, SH=small hydrophobic protein, G=glycoprotein, F=fusion protein, M2=matrix protein, L=large protein

1.4.1. Pathogenesis and pathology

RSV infections show a distinct seasonality in temperate countries; the onset of infection starts during late autumn or early winter on the northern hemisphere, peaking between mid-December and early February, and ending late spring. Humans are the only host of RSV and transmission occurs through direct contact with infected individuals or through larger droplet aerosols. Three to five days after inoculation, the first symptoms will manifest ranging from mild upper respiratory tract infection (URTI; Rhinorrhea, cough, low grade fever) to severe lower respiratory tract infections (LRTI). RSV is the most common cause of LRTI in children and a major cause of respiratory disease dependent hospitalisation (Borchers et al., 2013; Collins and Graham, 2008).

The common forms of severe childhood LRTI are bronchiolitis and viral pneumonia. Around ~15-50 % of infants and young children develop lower respiratory symptoms after primary RSV infection, with 1-3 % admitted to hospital. Amongst these ~5-10 % require intensive care. By the age of 2 most children will have experienced at least one RSV infection (Borchers et al., 2013). During the first year of life, reinfection is frequent and occurs throughout life with symptoms either absent in older children and adults or limited to upper respiratory tract symptoms, indicating increasing protective immunity. However, elderly and individuals with underlying pulmonary or cardiac disease, or with immunosuppression, have a significantly increased risk of developing severe LRTI with RSV (Borchers et al., 2013; Collins and Melero, 2011). The global burden of RSV-associated LRTI is estimated to 33.8 million new cases worldwide in children younger than 5 years, of which 3.4 million required hospital admission through severe LRTI manifestation (Nair et al., 2010). It was estimated that 66,000-199,000 children died due to RSV infection worldwide, with most of the deaths in severe cases occurring in the first 2 years of life and 99 % of death occurring in developing countries (Nair et al., 2010). Risk factors for the development of RSV-associated LRTI include preterm birth, congenital heart disease, chronic lung disease of prematurity, and other underlying medical conditions. Hospitalisation after RSV infection amongst previously healthy children is associated with male sex, day-care attendance, older siblings, exposure to tobacco smoke during gestation or environmental cigarette smoke (Borchers et al., 2013). Additionally, genetic polymorphisms in innate response genes have been associated with severe RSV-associated LRTI (Awomoyi et al., 2007; Drysdale et al., 2014; Janssen et al., 2007; Tal et al., 2004; Tulic et al., 2007). In severe cases the pathology of RSV infections results from lung damage and airway obstruction induced by robust infiltration of inflammatory cells, increased mucus production from goblet cells, and the cytopathic effect of RSV on epithelial cells leading to shedding of the airway epithelial cells into the infected airways (Everard et al., 1994; Johnson et al., 2006). The consequences are general narrowing of the airways, reduced gas exchange rates, and in extreme cases respiratory failure and death (Habibi and Openshaw, 2012). Early RSV-associated severe LRTI in infancy is linked with an increased chance of developing asthma and wheeze later in life (Blanken et al., 2013), with an estimated 30-40 % of severe LRTI children developing asthma-like symptoms (Rossi and Colin, 2014).

Currently, there is a lack of effective immunotherapies for RSV-associated LRTI and LRTI treatment only involves supportive care. There is one exception as in infants with prematurity or underlying diseases the administration of Palivizumab (MedImmune), a RSV F-neutralising antibody, is used for a prophylactic protection from RSV-induced severe disease. However, Palivizumab elicits no long term protection against RSV (Collins and Melero, 2011).

1.4.2. Sensing of RSV by the innate immune system

Viral sensing is usually a multi-stimuli response mediated by TLRs and RLRs (see section 1.2) A number of TLRs have been implicated in RSV recognition including TLR2, TLR3, TLR4 and TLR7 (Haeberle et al., 2002; Haynes et al., 2001; Kurt-Jones et al., 2000; Lukacs et al., 2010; Murawski et al., 2009; Rudd et al., 2005; 2006; Segovia et al., 2012). TLR2 is expressed on the cell surface as a heterodimer complex with either TLR1 or TLR6 (Kawai and Akira, 2010). *In vivo* infections with RSV suggest that TLR2 signalling activates the antiviral immune response by promoting the production of inflammatory cytokines and chemokines, which in turn supports neutrophil recruitment and DC activation to control the early viral replication in the lung (Murawski et al., 2009). Segovia *et al.* (2012) show that TLR2-MyD88 signalling mediates the expression of pro-IL-1 β . Following NLR family, pyrin domain containing 3 (NLRP3) inflammasome activation, caspase-1 proteolytic activity mediates the generation of biologically active mature IL-1 β (Segovia et al., 2012).

A second cell surface receptor described in RSV sensing is TLR4. TLR4 has been shown to be particularly important for innate immune activation in response to lipopolysaccharide (LPS) from Gram-negative bacteria (Akira et al., 2006), but several studies have indicated a role for TLR4 signalling in RSV disease. The early activation of NF- κ B pathways was shown to depend on TLR4 recognition of RSV F protein (Haeberle et al., 2002). Mice deficient in TLR4 showed decreased IL-12 secretion, reduced recruitment of NK cells and CD14⁺ cells, impaired NK cells cytotoxicity, and delayed viral clearance (Haynes et al., 2001; Kurt-Jones et al., 2000). These data are challenged by a study from Ehl *et al.* (2004), where TLR4 deficiency was unimportant in RSV infection, since no impact on the previously stated immune responses was observed nor an effect on

viral clearance or RSV-specific T cell responses (Ehl et al., 2004). However, single nucleotide polymorphisms (SNPs) in the ectodomain of TLR4 have been associated with an increased risk for RSV bronchiolitis (Awomoyi et al., 2007; Caballero et al., 2015; Tal et al., 2004) and human bronchial epithelial cells transfected with cDNA encoding a known TLR4 SNPs show reduced NF- κ B activation and reduced cytokine secretion despite unchanged TLR4 protein levels (Tulic et al., 2007).

RSV sensing by endosomal TLR3 triggers the TRIF dependent gene expression of chemokines *in vitro* with no effect on viral clearance (Rudd et al., 2005). Similarly in mice TLR3 signalling rather influences the development of the appropriate immune response to RSV than clearing the virus (Rudd et al., 2006). The absence of TLR3 leads to predominantly Th2-driven responses characterised by increased IL-13 and IL-5 levels and enhanced mucus production. Deletion of TLR7 increases immunopathology by the induction of IL-4, IL-13 and IL-17, which are all cytokines involved in mucus production (Lukacs et al., 2010). This is in part driven by DCs, which shape the immune response by increasing secretion of IL-23, which in turn stimulates T cells to produce IL-17. In infections with pneumovirus of mouse (PVM), the rodent equivalent to RSV, TLR7 is important for type I IFN production by pDCs and CD8⁺ T cell responses (Davidson et al., 2011).

The replication of RSV occurs in the cytosol, indicating a role for cytosolic RLRs in sensing of RSV infection. RSV detection by RIG-I in airway epithelial cells induces the activation of NF- κ B and IRF3, and the transcription of CCL5, CXCL10 and IFN- β by infected cells (Liu et al., 2007), whereas MDA5 was critical to sustain antiviral responses (Grandvaux et al., 2014). In nasal washes from children with bronchiolitis, substantial changes in PRR transcripts, with increased RIG-I, MDA5, TLR7, and TLR8 expression, were detected and RIG-I expression positively correlated with the viral load (Scagnolari et al., 2009). *In vivo* data from mice deficient in the RLR adaptor MAVS suggest a crucial role of RLR sensing for the induction of innate immune responses, but CD8⁺ T cell development was not affected (Bhoj et al., 2008; Demoor et al., 2012). However, the distinct pulmonary cell population mediating RSV sensing and the subsequent initiation of antiviral host immune responses *in vivo* remains elusive.

1.4.3. Type I IFN responses in RSV infection

RSV infection can lead to the induction of type I IFNs *in vivo* and *in vitro* (Bhoj et al., 2008; Demoor et al., 2012; Hornung et al., 2004; Jewell et al., 2007). Pre-treatment of mice with IFN- α reduced viral replication, inflammation and disease severity, demonstrating the sensitivity of RSV to type I IFNs (Guerrero-Plata et al., 2005). Mice lacking the type I IFN receptor IFNAR (*Ifnar1*^{-/-}) show reduced numbers of CD8⁺ T cells indicating a role for type I IFNs in CTL survival or priming (Johnson et al., 2005), but overall these mice show no reduced virus control (Johnson et al., 2005; Mordstein et al., 2010).

Viruses have evolved mechanism to evade host immune responses by inhibiting the type I IFN response at different levels. The RSV genome encodes for the two non-structural proteins NS1 and NS2, both interfering with the induction of IFNs and IFN signalling (Borchers et al., 2013). NS1 inhibits the production of both type I and III IFNs (Spann et al., 2004) by circumventing the interaction (e.g. RIG-I/MAVS) (Boyapalle et al., 2012; Ling et al., 2009) and activation of signalling molecules (e.g. IKK ϵ , TRAF3, IRF3) (Bossert et al., 2003; Swedan et al., 2009), and regulating gene expression of miRNAs related to IFN responses (Thornburg et al., 2012). The RSV NS2 protein interrupts the IFNAR signalling by inducing the degradation of STAT2 thereby inhibiting these autocrine responses in the infected cell (Ramaswamy et al., 2006; Swedan et al., 2009). The antagonising effect of RSV-NS proteins will be most efficient in epithelial cells, the primary target of RSV infection and replication (Johnson et al., 2006; Villenave et al., 2012; Wright et al., 1997; Zhang et al., 2002). Indeed, RSV infected primary human epithelial cells have been shown to be poor inducers of type I IFNs (Ioannidis et al., 2012; Okabayashi et al., 2011; Villenave et al., 2012). Thus, other pulmonary innate immune cells will be important in initiation of primary immune responses to RSV.

PDCs constitutively express high levels of the transcription factor IRF7, which is IFNAR dependent in most other cell types. PDCs are therefore capable of producing high amounts of type I IFNs upon direct sensing of viruses. The exact role of pDCs in type I IFNs secretion during RSV infections is at the moment controversial. Infection of human pDCs with the RSV Long strain leads to a robust secretion of IFN- α , whereas infection with RSV A2 strain markedly attenuated the production of IFN- α from pDCs (Hornung et

al., 2004; Schijf et al., 2013; Schlender et al., 2005). This is in contrast to a study from Guerrero-Plata *et al.* (2006), where RSV A2 was shown as a potent inducer of IFN- α in human pDCs (Guerrero-Plata et al., 2006). Different multiplicity of infection (MOI) and purification methods of RSV used in the respective studies might explain these differences in pDC stimulation. Similar to human pDCs, *in vitro* RSV infected murine bone marrow derived pDCs abundantly secreted IFN- α (Boogaard et al., 2007; Wang et al., 2006), yet there are conflicting data about the contribution of pDCs in type I IFN production *in vivo* (Jewell et al., 2007; Smit et al., 2006).

Alveolar macrophages (AMs) were previously shown to be the primary type I IFN producers during pulmonary infection with NDV (Kumagai et al., 2007). Depletion of AMs prior to RSV infection led to a complete abrogation of type I IFN production in the lung, but whether type I IFN production is directly or indirectly mediated by AMs during RSV infections needs to be examined (Kolli et al., 2014; Pribul et al., 2008).

To date, the identity of exact PRRs that lead to type I IFN production is not known. Several studies indicate a role for sensing of RSV RNA and type I IFN production through cytosolic RLRs and their adaptor MAVS (Bhoj et al., 2008; Demoor et al., 2012; Marr et al., 2014). PDCs isolated from adult peripheral blood mononuclear cells (PBMC) donors exposed to RSV produce type I IFNs in a RIG-I dependent manner (Marr et al., 2014) and mice lacking functional MAVS expression have no detectable induction of IFN- α or IFN- β (Bhoj et al., 2008). Furthermore, the production of proinflammatory cytokines was attenuated in these mice. Thus, RLR sensing must play an essential role in initiating innate immune responses during RSV infection (Bhoj et al., 2008).

Taken together, type I IFN production during RSV infection can be mediated by cytosolic RLRs in various pulmonary cell types. How individual cells respond to RSV and their cross talk with other cell subsets in order to modulate type I IFN responses requires further investigation.

1.4.4. Cellular responses to RSV

1.4.4.1. Epithelial cells

Pulmonary epithelial cells (EC) line the lung airways at the interface between the host tissue and the environment. They are directly exposed to invading pathogens, allergens and pollutants and will be the first line of defence against foreign antigens. The primary targets of RSV for the production of progeny are ECs as shown in primary EC culture and histology of fatal RSV cases (Guo-Parke et al., 2013; Johnson et al., 2006; Villenave et al., 2012; Wright et al., 1997; Zhang et al., 2002). RSV F protein interacts with cellular nucleolin on the apical side of predominantly ciliated ECs to enable virus entry and replication. Newly generated virus sheds from the apical side and re-infects neighbouring cells facilitated by ciliary motility (Guo-Parke et al., 2013; Villenave et al., 2012; Zhang et al., 2002). Data from fatal RSV cases found RSV antigen even in pneumocytes of the alveoli (Johnson et al., 2006; Wright et al., 1997). Upon infection, ECs produce an array of chemokines and cytokines to modulate the immune response. Cytokines identified in *in vitro* cultured ECs were chemokines like CCL2 (MCP-1), CCL3 (MIP-1 α), CCL5 (RANTES), CXCL1 (KC)/CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (ITAC), growth factors including G-CSF and M-CSF, but also the proinflammatory cytokine IL-6. All of them contribute in general to leukocyte recruitment and probably injury of the airways (Ioannidis et al., 2012; Miller et al., 2004; Olszewska-Pazdrak et al., 1998; Tristram et al., 1998; Villenave et al., 2011). Many of the described cytokines and chemokines are found in nasal aspirates or BAL fluid of children with severe RSV LRTI where they correlate with disease severity (Choi et al., 2010; McNamara et al., 2005; Tabarani et al., 2013). Recently, novel biomarkers associated with severe illness were identified in nasal washes of RSV LRTI: IL-1 β , interleukin-1 receptor antagonist (IL-1RA), IL-7, epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (Tabarani et al., 2013).

1.4.4.2. Dendritic cells

Pulmonary dendritic cells are the major antigen-presenting cells in the lung and play a central role in the induction of RSV specific T and B cell responses. RSV is sensed by

murine and human DCs *in vitro* (Boogaard et al., 2007; Guerrero-Plata et al., 2006; Le Nouën et al., 2009; Munir et al., 2008; Rudd et al., 2007). Upon RSV recognition DCs undergo a maturation processes characterised by the upregulation of MHC-II molecules, the T cell co-stimulatory markers CD80, CD86 and CD40, the maturation marker CD83 and the signalling receptors CD38 and CD54 (ICAM-1) (Boogaard et al., 2007; Guerrero-Plata et al., 2006; Gupta et al., 2013; Le Nouën et al., 2009; Munir et al., 2008). Additionally, the production of a wide range of proinflammatory cytokines (IFN- γ , TNF- α , IL-6, IL-1- α , IL-1 β , IL-12), chemokines (CXCL10, CCL2, CCL3, CCL4, CCL5, CXCL8), and anti-inflammatory cytokines (IL-10, IL-1RA) was described for RSV infected human DCs, but no production of antiviral type I and III IFNs (Bartz et al., 2002; Guerrero-Plata et al., 2006; Gupta et al., 2013; Le Nouën et al., 2009; Munir et al., 2008). Several *in vitro* studies showed that despite the upregulation of maturation markers RSV exposed DCs inefficiently induce T cell activation and proliferation (Guerrero-Plata et al., 2006; Gupta et al., 2013). Soluble mediators secreted by DCs are responsible for the impaired T cell stimulation (de Graaff et al., 2005). Increased levels of transforming growth factor (TGF)- β , IL-1RA (de Graaff et al., 2005), IFN- α and IFN- λ were detectable in the supernatant from RSV-infected human monocyte derived DCs {Chi:2006dm}. Blocking of the individual cytokines had no obvious effect, but simultaneous blocking of the IFNAR2 and IL28R subverted the suppressive effect on T cell activation (Chi et al., 2006). Impairment in T cell activation is not predominantly mediated by soluble factors. Gonzalez *et al.* (2008) showed that CD4⁺ T cells, when cultured with RSV infected DCs, failed to polarise their Golgi to the surface, a mechanism needed to form the immune synapse to assure T cell activation and function (González et al., 2008). This is supported by recently published data, where establishing of the immune synapse between antigen-presenting cells (APC) and naïve CD4⁺ T cells was aggravated by RSV N protein localised on the surface of infected cells (Céspedes et al., 2014). Therefore RSV is able to inhibit T effector cell activation by mediating the release of soluble mediators and interrupting APC and T cell interactions. Whether RSV also interferes with the priming of T cells *in vivo* is unclear, especially since T cell responses were associated with disease severity in mouse models of RSV (Alwan et al., 1992; Cannon et al., 1988; Graham et al., 1991).

A clearer picture about the kinetics and function of DCs was established utilizing *in vivo* RSV infections. In mice infected with RSV immature DCs mature by upregulation of MHC-II molecules and co-stimulatory molecules. It is unknown whether DC activation

requires direct recognition of RSV PAMPs or if the presence of inflammatory cytokines is sufficient to induce maturation (Guerrero-Plata et al., 2009; Lukens et al., 2009; Rudd et al., 2007). Migratory CD103⁺ DCs and CD11b⁺ DCs in the LN contain viral RNA and present antigens on MHC-I and MHC-II molecules for T cell stimulation. Both DC subsets show comparable capacities to stimulate IFN- γ secretion by CD4⁺ and CD8⁺ T cells (Lukens et al., 2009). However, RSV can render the migratory capacity of DCs *in vitro* by inhibiting the expression of the migratory receptor CCR7 (Le Nouën et al., 2011). During ongoing inflammation newly recruited CD11b⁺ DCs, most likely of monocytic origin, migrate into the tissue to support local immune responses (Guerrero-Plata et al., 2009; Lukens et al., 2009). Similar to CD103⁺ DCs and CD11b⁺ DCs, RSV infection induces maturation and migration of pDCs to the LN (Wang et al., 2006). Their role in the initiation of T cell responses remains elusive.

However, several studies have highlighted a role for pDCs in promoting the appropriate response to RSV. Depletion of pDCs enhances mucus production, increases airway hyperreactivity (AHR) and inflammation, and attenuated viral clearance. Additionally, lung T cells from depleted mice displayed increased transcripts for the Th2 cytokines IL-4, IL-5 and IL-13, and the Th1 cytokine IFN- γ (Smit et al., 2006; Wang et al., 2006). To substantiate the role of pDCs in reducing RSV pathology, pDCs were expanded in the lung by administration of Flt3L. This scenario promoted anti-viral responses by reducing Th2 cytokine and in turn increasing beneficial Th1 responses. Thus, mucus production and inflammation were significantly reduced (Smit et al., 2008). This effect was completely reversed by depleting pDCs after RSV infection, indicating a regulatory effect of pDCs on effector T cell response either direct or indirectly through CD103⁺ or CD11b⁺ DC function. The direct influence on RSV specific T cell responses could be mediated by type I IFNs, however if pDCs are a source of IFNs *in vivo* is still under debate (Jewell et al., 2007; Smit et al., 2006). Whereas pDCs inhibit Th2 T cell responses during RSV infection CD11b⁺ DCs promote the development of Th2 responses. Preventing the recruitment of CD11b⁺ DCs significantly reduces airway inflammation and alters lung CD4⁺ T cell responses by reducing the Th2 cytokines IL-4 and IL-13 (Kallal et al., 2010). An imbalance in DC subpopulations was also associated with severe RSV-associated LRTI. At the time of admission, children with severe RSV-LRTI had elevated levels of myeloid DCs (mDCs) and decreased levels of pDCs in peripheral blood. Three months after discharge, the mDC/pDC ratio was still increased in RSV LRTI children compared to

healthy controls (Weng et al., 2014), which increases susceptibility to asthma later in life (Silver et al., 2009). In summary, a balance between the different pulmonary DC subsets is crucial for the development of an adequate immune response to RSV infection.

1.4.4.3. Neutrophils

Neutrophils are early detectable in the BAL during murine RSV infection (Durant et al., 2014; Jafri et al., 2004; Stokes et al., 2011). Diminished type I IFN responses increases the presence of neutrophils in the lung, suggesting a role for IFNs in neutrophil accumulation (Bhoj et al., 2008; Demoor et al., 2012; Durbin et al., 2002; Johnson et al., 2005). In ventilated children with severe RSV illness neutrophils were the prominent cell type infiltrating the airways (Everard et al., 1994; Heidema et al., 2007; Lukens et al., 2010; McNamara et al., 2003). These neutrophils can potentially enhance damage to the respiratory epithelium in RSV infection (Wang et al., 1998).

1.4.4.4. Alveolar macrophages

Together with epithelial cells alveolar macrophages (AMs) are one of the first immune cells encountering RSV infection. Strategically placed in the airway lumen of the lower respiratory tract they express a variety of proinflammatory cytokines and chemokines during RSV infection. Both human and mouse AMs are permissive to RSV infection *in vitro* and secrete cytokines including IL-6, IL-8, IL-10 IL-12p40, TNF- α , the cytokines and growth factors G-CSF, GM-CSF, and chemokines such as CCL4, and CCL5 (Becker et al., 1991; Demoor et al., 2012; Kolli et al., 2014; Panuska et al., 1995). Viral replication and shedding are restricted in AMs, which suggest that AMs play an important protective role against RSV infection and are not involved in viral spreading (Becker et al., 1992; Cirino et al., 1993; Franke-Ullmann et al., 1995; Kolli et al., 2014; Ravi et al., 2013). In mice, AM ablation caused a marked reduction of the early cytokines TNF- α , IL-6, IFN- α , IFN- β , IFN- γ , CCL3, and CCL5 whereas an increase in IL12p40, IL-1 α , IL-1 β , IL-17A, GM-CSF, and G-CSF secretion was detectable in BAL fluid of AM depleted mice (Kolli et al., 2014; Pribul et al., 2008). Significantly more neutrophils and

inflammatory dendritic cells were present in the lung of these AM depleted mice, however, NK cell recruitment and activation was diminished. All of this lead to an inefficient viral clearance due to AM deficiency (Kolli et al., 2014; Pribul et al., 2008). Surprisingly, the depletion of AMs had no effect on RSV-specific T cell responses or disease severity (Pribul et al., 2008). The effect of impaired macrophage function during RSV was studied in New-Zealand Black (NZB) mice, a strain with macrophages poor in recognition and uptake of apoptotic cells (Reed et al., 2008). During RSV infection the failure of AMs in the uptake of infectious virions increases epithelial cell infection, which in turn results in enhanced secretion of cytokines from ECs and EC death. In addition, the diminished AM-mediated clearance of apoptotic ECs causes airway occlusion and increases mortality (Reed et al., 2008). This very much alike to severe infant LRTI where airway obstruction is attributed to epithelial and inflammatory cell debris (Johnson et al., 2006). Deficiencies in macrophage function and responses might increase the susceptibility to RSV in infants. Indeed, macrophages obtained from infants below the age of 2 show several functional deficiencies such as reduced phagocytosis and cytokine release compared to macrophages from children 2 years of age and older (Grigg et al., 1999). Furthermore, another study showed that alternatively activated AMs are involved in controlling RSV induced inflammation and contribute to resolution (Shirey et al., 2013). Taken together, these data suggest that AMs play a critical role in reducing RSV viral load and immunopathology.

1.4.4.5. Natural killer cells

Natural Killer (NK) cells are cytotoxic lymphocytes of the innate immune system and are newly classified as innate lymphoid cells type 1 (ILC1) (Gasteiger and Rudensky, 2014). They are involved in antiviral responses and tumour surveillance, and modulate immune responses through direct cytotoxicity and the secretion of cytokines particularly IFN- γ (Gasteiger and Rudensky, 2014). NK cells are a major source of IFN- γ during RSV infection and prevent from Th2-induced immunopathology by promoting Th1 T cell development and IFN- γ production (Hussell and Openshaw, 2000; Kaiko et al., 2010). Strong cellular responses to RSV mediated by NK cells might come at an expense of the humoral response. As one of the major producer of IFN- γ they potentially

antagonise neonatal responses by attenuating the production of RSV specific antibodies in a mouse model (Tregoning et al., 2013). Nevertheless, clinical data from hospitalised children show inverse correlation of NK cell numbers and disease severity, with great reduction in numbers in ventilated children. (De Weerd et al., 1998) and in fatal cases NK cells were absent (Welliver et al., 2007).

1.4.4.6. T lymphocytes

T cells play an important role in adaptive immune responses. After encountering the cognate antigen on professional APCs they can differentiate into different subtypes, depending on the context of antigen presentation and the cytokines secreted by the APC. CD8⁺ T cells recognise peptide-MHC-I (pMHC-I) complexes and CD4⁺ T cells pMHC-II. CD4⁺ T cells can develop into different T helper (Th) cell subtypes; Th1, Th2, Th9, Th17 and T regulatory cells (Treg; for more extensive review please refer to (Bluestone et al., 2009; Dong, 2010; Hirahara et al., 2013; O'Shea and Paul, 2010)). Both CD4⁺ and CD8⁺ T cells are important to resolve RSV infection from the lung and are associated with immunopathology (Graham et al., 1991)⁹. Upon depletion of CD4⁺ or CD8⁺ T cells, RSV illness was remarkably reduced and completely absent when both subsets were depleted, but at the cost of prolonged viral replication (Graham et al., 1991). However, transferred CD8⁺ effector T cells were able to clear the persistent infection in immunodeficient mice. Similarly, in immunocompetent mice the rate of viral clearance and pathology was proportional to the number of transferred CTLs (Cannon et al., 1988). Transfer of CD4⁺ T cells alone augmented RSV disease and was characterised by an eosinophilic influx into the airways (Alwan et al., 1992). The data from murine models suggests that the immunopathology is driven in part by lymphocytes rather than virus induced cytopathology alone (Alwan et al., 1992; Graham et al., 1991). The mode of RSV antigen presented by DCs determines different T cell responses following live virus challenge. Sensitisation of BALB/c mice with vaccinia virus expressing RSV G protein concurrently induced Th1 and a dominant Th2 driven immune responses characterised by lung eosinophilia. Whereas sensitisation with RSV F protein resulted in Th1 and CD8⁺ T cell response with mononuclear cell infiltrate (Culley et al., 2006; Openshaw et al., 1992; Spender et al., 1998; Srikiatkachorn et al., 1999). Interestingly, by coupling RSV-

G to a dominant CD8⁺, class I restricted epitope (RSV-M2), eosinophilia was prevented (Srikiatkachorn and Braciale, 1997). These data support the role of CD8⁺ T cells in down-regulating CD4⁺ Th2 like effector responses. RSV infections in children are suggested to be predominantly Th2 instead of antiviral Th1 responses. Some studies support this finding by reporting elevated Th2 cytokines in severe paediatric RSV cases, whereas other studies identified predominantly Th1 or mixed T cell responses (Garofalo et al., 2001; Lee et al., 2007; Legg et al., 2003; Mobbs et al., 2002). Data from fatal cases provide opposing results about the role of T cell subsets. Lung autopsies analysed by Welliver *et al.* (2008) were completely devoid of CD8⁺ T cells whereas in a second study by Johnson *et al.* (2006) CD8⁺ T cells outnumbered CD4⁺ T cells (Johnson et al., 2006; Welliver et al., 2008). In hospitalised children with RSV bronchiolitis, CD8⁺ T cell levels in the blood are inversely correlated with disease severity (De Weerd et al., 1998) which is contrary to clinical data from another study that claims CD8⁺ T cell activity was highest during convalescence. From the time of events, the authors conclude that RSV disease is caused by a marked influx of neutrophils, rather than CD8⁺ T cells (Lukens et al., 2010). Although, it is difficult to draw conclusions from peripheral blood, where several factors including timing and sampling can bias the obtained results in RSV infection, the data from both clinical studies suggest that an imbalance in Th1/Th2 responses may account for RSV disease.

1.4.5. Humoral responses

The main antibody subclasses eliciting protection from RSV are IgA and IgG (Collins and Graham, 2008). IgA is important for the early defence at mucosal surfaces when the virus enters the airways to infect the lining of epithelial cells. Particularly secretory IgA mediates protection of the upper respiratory tract where IgG responses are limited. IgG responses are described to predominantly generate protection in the lower respiratory tract (Collins and Graham, 2008). Protective immunity to RSV is to be considered as short-lived, based on the fact that strong reinfections with the same strain of RSV can occur throughout life (Collins and Graham, 2008). However, RSV itself has developed strategies to evade the immune system. The dominant neutralising antibody responses are generated against the RSV F and G protein (Borchers et al., 2013). Especially G is

highly glycosylated to circumvent antibody recognition (García-Beato and Melero, 2000; Palomo et al., 2000). RSV G protein additionally exists in two forms: i) a membrane bound full-length protein and ii) a truncated form called secretory G (sG), which can be secreted from infected cells. It is thought that RSV sG acts as a decoy for RSV-specific antibodies (Bukreyev et al., 2008).

In summary, recognition of RSV leads to a cascade of cytokine and chemokine expression that stimulates innate and adaptive immune responses to limit viral replication. An inappropriate release of pro-inflammatory cytokines leads to the recruitment of inflammatory cells that amplify the immune responses and together with the cytopathic effect of RSV on airway epithelial cells enhances lung damage. Additionally, the antagonising effect of RSV on innate and adaptive immune functions will contribute to disease severity. Overall a balanced inflammatory immune response is necessary to guarantee viral clearance and to minimise damage to the host.

2. Hypothesis and aims

Hypothesis

Type IFN production in the lung is crucial for the initiation of inflammatory responses to control viral load and protect from severe lung disease during respiratory syncytial virus infections.

Aims

1. Determine the role of type I IFN signalling in inflammatory responses in the mouse model of RSV infection.
2. Identify the pulmonary cell types secreting type I IFNs in response to RSV infection.
3. Elucidate which sensing pathway recognises RSV infection and induces type I IFN secretion.
4. Evaluate the role of type I IFNs in regulating RSV-specific immune responses.

3. Material

3.1. Mice

C57BL/6 mice were purchased from Harlan or Charles River, UK. Type I interferon receptor deficient mice (*Ifnar1*^{-/-}) were obtained from Cancer Research UK (CRUK). *Ifna6*^{gfp/+} mice and *Ifna6*^{gfp/+} mice deficient in MyD88/TRIF or MAVS were obtained from S. Akira, Japan. The coding sequence of the *Ifna6* gene was replaced by the GFP coding sequence (*Ifna6*^{gfp/+}) (Kumagai et al., 2007). Because these mice are heterozygote for the *Ifna6gfp* gene, mice were continuously screened to maintain the genotype, either in house or by Transnetyx Inc. (Cordova, TN, USA). For transgenic *Ifna6gfp/+* mice littermates were used as controls. All mice were bred and maintained in pathogen-free conditions under UK Home Office guidelines and ARRIVE guidelines were followed. Gender and age-matched mice aged 7-12 weeks were used for each experiment. The group of naïve mice or PBS treated mice was always a mix of the appropriate strains used in the experiments.

3.2. Chemicals

Chloroform	VWR, Leicestershire, UK
Ethanol	VWR, Leicestershire, UK
Isopropanol	VWR, Leicestershire, UK
β-Mercaptoethanol	Sigma-Aldrich, Dorset, UK
PBS tablets	Sigma-Aldrich, Dorset, UK
Sulphuric acid	Sigma-Aldrich, Dorset, UK
Tween 20	Sigma-Aldrich, Dorset, UK

3.3. Reagents and kits

Albumin ELISA Quantitation Set	Bethyl Laboratories Inc., Montgomery, TX, USA
Ambion® DNA-free™ Kit	Applied Biosystems, Paisley, UK

Aqua fixable dye	Invitrogen, Paisley, UK
BD CompBeads	BD Bioscience, Oxford, UK
BD Cytotfix™	BD Bioscience, Oxford, UK
Chemokine Mouse Magnetic 20-plex	Life Technology, Paisley, UK
Collagenase D	Roche Products Limited, Welwyn Garden City, UK
DAB substrate for plaque assay	Sigma-Aldrich, Dorset, UK
Desoxyribonuclease I from bovine pancreas	Sigma Aldrich, Dorset, UK
ELISA Grade Streptavidin	Biosource, Paisley, UK
ELISA kits (CCL2, CXCL1, CXCL10, IL-6, IFN- γ , IFN- λ)	R&D Systems, Minneapolis, MN, USA
Extravidin-peroxidase	Sigma-Aldrich, Dorset, UK
High Capacity RNA-to-cDNA Kit	Applied Biosystems, Paisley, UK
ProcartaPlex Mouse IFN-alpha3/IFN-beta 20 Plex Assay	Affymetrix, Inc., Santa Clara, CA, USA
Prostaglandin E2 EIA Kit	Cayman Chemical, Cambridge, UK
Complete, Mini, EDTA-free Protease Inhibitor	
Cocktail Tablets	Roche, Welwyn Garden City, UK
RNase AWAY	Molecular BioProducts, San Diego, CA, USA
RNase-Free DNase Set	Qiagen, Crawley, UK
RNAesy Mini Kit	Qiagen, Crawley, UK
RNAesy Micro Kit	Qiagen, Crawley, UK
Nuclease-free water	Promega, Southampton, UK
TMB Single Solution	Invitrogen, Paisley, UK
Trizol® Reagent	Invitrogen, Paisley, UK
QuantiTec® Probe PCR Kit	Qiagen, Crawley, UK

Quick-Diff Kit

Reagentia Ltd., Toivola, FI

Anaesthetics

Isoflurane and Pentobarbitone

Centaur, Somerset, U

3.4. Consumables

ELISA MaxiSorp™ plates

Nunc, Roskilde, Denmark

Cell strainers Nylon

BD Bioscience, Oxford, UK

CryoTube™ Vials
USA

Thermo Scientific, Wilmington, DE,

gentleMACS™ tubes

Miltenyi Biotec, Surrey, UK

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 tubes

VWR, Leicestershire, UK

RNAeasy columns

Qiagen, Crawley, UK

Shandon filter cards
USA

Thermo Scientific, Wilmington, DE,

Slides for cytopsin

Tharmac, Waldsoms, Germany

3.5. Instruments

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

Cytospin III Cytocentrifuge

Cytospins Thermo Shandon

Eppendorf centrifuge 5810R (Rotor A-4-81)

Eppendorf, Cambridge, UK

7500 Fast Real-Time PCR system

Applied Biosystems, Paisley, UK

Gen Amp PCR System 9700

Applied Biosystems, Paisley, UK

gentleMACS dissociator	Miltenyi Biotec, Surrey, UK
Heraeus Megafuge 40R (Rotor 75003807)	Thermo Scientific, Wilmington, DE, USA
Hera Safe Microbiological Safety Cabinet	Thermo Scientific, Wilmington, DE, USA
Incubator	Binder Inc., Bohemia, NY, USA
NanoDrop 1000 Spectrophotometer	Thermo Scientific, Wilmington, DE, USA
Sorvall legend Micro 21	Thermo Scientific, Wilmington, DE, USA
SPECTRAMax Plus S384	Molecular Devices, Berkshire, UK
TissueLyser LT	Qiagen, Crawley, UK

3.6. Software

Adobe Illustrator	Adobe Systems Europe Ltd, Berkshire, UK
DIVA	BD Bioscience, Paisley, UK
GraphPad Prism v6.0	Graph Pad software, La Jolla, CA, USA
7500 Fast System SDS v1.4	Applied Biosystems, Paisley, UK
FlowJo v9.7	Tree Star, Ashland, OR, USA
SoftMax Pro v5.2	Molecular Devices, Birkshire, UK

4. Methods

4.1. Growing of RSV stock

Plaque purified human RSV A2 was cultured in HEp-2 cells in complete Dulbecco's modified Eagle medium (DMEM) (Table 4-1). Before infection 10^7 HEp-2 cells were seeded into a 175 cm² flask in 30 ml complete media and left overnight to grow to approximately 80 % confluence at 37°C and 5 % CO₂. Next day media was removed and

cells were washed gently with 10 ml of serum-free DMEM. The cells were infected with RSV (MOI 0.1) in 4 ml serum-free media. As control 4 ml of serum-free media alone were added to a flask. Infected cells were incubated for 2 hrs at 37°C and 5 % CO₂ and rotated 90° every 15 min to counteract any uneven surfaces in the incubator. After the incubation 30 ml of DMEM+10 % FCS were added and the flasks were left for 24 hrs in the incubator. In order to reduce the FCS to 2 %, 25 ml of media were removed, spun down at 500 x *g* for 5 min at 4 °C and after removing the supernatant 25 ml of serum-free media was added to the pellet. The resuspended pellet was added back to the flask and the cytopathic effect of the virus was assessed regularly. Cells were harvested with a cell scraper when 80 % were detached and transferred into a 50 ml falcon tube and sonicated for 2 min in a water bath. After spinning for 5 min at 500 x *g* at 4 °C the virus culture supernatant was aliquoted into cryo vials and rapidly frozen down in liquid nitrogen.

Table 4-1 Composition of complete media

Reagent	Weight/Volume	Volume	Supplier
DMEM		500 ml	PAA, Pasching,
L-glutamin (100x)	2 mM	5 ml	Gibco, Paisley, UK
FCS	10 %	50 ml	Source Bioscience,
Penicillin-	100 U/ml	5 ml	PAA, Pasching,
Streptomycin (100x)			Austria

4.2. Immunoplaque assay

4.2.1. Assessing RSV titres in viral stock

Immunoplaque assays were used to determine the virus titre. 2.5×10^4 HEp-2 cells/well were seeded into flat bottom 96-well plate in complete DMEM and incubated at 37 °C and 5 % CO₂ for 20-24 hrs to grow cells until confluent. When confluent, media was expelled from the plates and cells were washed with PBS. The virus stock was serially diluted in serum-free media starting with 200x dilution. 50 µl of the virus dilutions were added as duplicates on the cells and incubated for 2 hrs at 37 °C and 5 % CO₂ on a shaker. Then, 150 µl of pyruvate-free DMEM+2 % FCS were added to each well and the plate was further incubated for 16-20 hrs. Media was then expelled and cells were fixed

for 20 min in methanol containing 2 % hydrogen peroxide. Afterwards cells were washed with 1 % BSA in PBS (PBS/BSA) followed adding of 100 µl of biotinylated anti-RSV antibody (1:500 in PBS/BSA) (AbD serotec, Kidlington, UK) and incubated for 1 hr at room temperature. After washing twice with PBS/BSA, 100 µl of extravidin-peroxidase (1:500 in PBS/BSA) was added and incubated for 30 min in the dark. Cells were then washed three times with PBS/BSA and 50 µl of DAB (3,3'-diaminobenzidine) substrate was applied. The plate was incubated in the dark until the plaques acquired the desired coloration. After stopping the reaction by washing the plate with PBS, plaques were ready for counting by light microscopy.

Focus forming units (FFU)/ml were calculated as: number of plaques x dilution factor x 20 (for 1 ml virus stock)

4.2.2. Assessing RSV titres in animal tissue

After weighing, fresh lung tissue was homogenised in 300 µl DMEM using a tissue lyser (TissueLyser LT, Qiagen, Crawley, UK). Samples were spun at full speed for 30 sec and the supernatant was serially diluted in DMEM. The plaque assay was further performed as described in 4.2.1.

Focus forming units (FFU)/ml were calculated as: number of plaques x dilution factor x 6 (for 300 µl homogenate)

4.3. Working with animals

All work was approved and licensed appropriately by the U.K. Home Office and the animal ethic committee of Imperial College London. Euthanasia of animals was performed by intraperitoneal injection of 100 µl pentobarbitone.

4.3.1. Infection of mice with RSV

Mice were lightly anaesthetised with isoflurane and subsequently intranasally (i.n.) infected with 2×10^6 focus forming units (FFU) of RSV in 100 µl total volume (virus stock: 2×10^7 FFU/ml).

4.3.2. Challenge of mice with TLR agonists, recombinant proteins and antibody treatment

Intranasal installation of TLR agonist was performed in 100 µl of each in the concentrations stated in Table 4-2.

Table 4-2 Concentration of TLR agonists used for animal work

TLR agonist*	Concentration/g body weight
CpG	1.25 µg
PolyI:C	3.5 µg
R848	3.5 µg
LPS	500 ng

* purchased from InvivoGen, San Diego, CA, USA

Lung i.n. installation of recombinant proteins; 500 ng/mouse of IFN-α11 (Miltenyi Biotech) and 25 µg/mouse of rCCL2 (PeproTech) was performed 6 hrs post infection (p.i.). For weight loss experiments, rCCL2 25 µg/mouse was given 6, 18 and 48 hrs p.i.. The anti-CCR2 antibody MC21 or IgG2b isotype control (BD Bioscience) was administered intraperitoneally (i.p.) at 20 µg/mouse 6 hrs before infection and then daily during infection.

4.3.3. Collection of cells and tissue

4.3.3.1. Bronchoalveolar lavage (BAL)

At different time points after RSV infection mice were sacrificed and BAL was performed using a 2 ml syringe with attached tubing. The tracheae were exposed and an incision using a scalpel was made. The tubing was carefully inserted and 1 ml of BAL media (0.5 mM EDTA in PBS) was injected to flush the lungs. Flushing the lung was performed three times, using the same liquid, before the fluid was recovered. The obtained BAL fluid (BALF) was spun down for 5 min at 3,500 x *g* and the supernatant was stored at -80 °C. The remaining cell pellet was resuspended in 100 µl ACK buffer (Table 4-3) to lyse red blood cells. After 2 min, 400 µl complete DMEM was added and samples were spun down in a tabletop centrifuge for 5 min at 3,500 x *g*. The pellet was resuspended in 500

µl complete media and an aliquot was stained with trypan blue and counted using counting slides (MultiCount 10™, ImmuneSystems, Paignton, UK).

Table 4-3 Composition of ACK buffer

Reagent	Concentration	Supplier
NH ₄ CL	0.15 mM	Sigma-Aldrich, Dorset, UK
KHCO ₃	1 mM	Sigma-Aldrich, Dorset, UK
Na ₂ EDTA	0.1 mM	Sigma-Aldrich, Dorset, UK
distilled water		
Sterile filtered (0.22 µm)		

4.3.3.2. Harvest and adoptive transfer of primary alveolar macrophages (AMs)

Primary alveolar macrophages (AMS) were harvested by flushing the lung 3 times with BAL media (0.5 mM EDTA in PBS). This procedure was repeated twice. Following spinning of pooled AMs for 10 min, 500 *g* at 4°C, cells were counted and washed again. The final cell concentration was adjusted with PBS to allow intranasal administration of 4-5x10⁵ AMs per mouse.

4.3.3.3. Harvest of lung tissue

Perfusion of the lung was performed by injecting PBS into the right heart ventricle. The right upper, middle and lower lung lobe were taken and immediately snap frozen in liquid nitrogen. The rest of the lung was used for flow cytometry and placed into a C-tube (Miltenyi Biotec) containing 2.5 ml complete DMEM. To obtain single cell suspension, 2.5 ml of Collagenase (1 mg/ml, Table 4-4) and DNase I (30µg/ml, Sigma Aldrich) were added to the lungs and processed with a gentleMACS dissociator according to manufacturer's protocol (Miltenyi Biotec, Surrey, UK). After 1 hr of incubation at 37 °C lung tissue was further homogenised on the gentleMACS dissociator, 5 ml of FACS-buffer (Table 4-5) were added and samples were spun for 5 min, 500 x *g* at 4 °C. The cell pellet was washed in 5 ml PBS and incubated for 5 min in 5 ml ACK buffer for red blood cell lysis. Then, 5 ml of complete media was added and the samples were

centrifuged for 5 min, 500 x *g* at 4 °C, resuspended in 1 ml PBS and filtered through a 100 µm cell strainer.

Table 4-4 Reconstitution of Collagenase D

Reagent	Weight/volume	Supplier
Collagenase D	1 mg/ml	Roche, West Sussex, UK
FCS	10 %	Source Bioscience,
PBS	Dissolved in 1x	PAA, Pasching, Austria

Table 4-5 Composition of FACS-buffer

Reagent	Weight/Volume	Supplier
BSA	1 %	Sigma-Aldrich, Dorset, UK
EDTA	0.05 mM	Sigma-Aldrich, Dorset, UK
NaN ₃	0.005 %	Sigma-Aldrich, Dorset, UK
PBS	Dissolved in 1x	PAA, Pasching, Austria

4.4. Total and differential leukocyte count

Following isolation of cells from the lung or BAL, total cell count was performed manually. Cells were mixed with 0.1 % trypan blue in the appropriate dilution and 10 µl were transferred into a haemocytometer and counted under a light microscope. For different cell counts of cells, 100 µl of the cell suspension were transferred onto a class slide using a cytopspin centrifuge for 5 min at 400 rpm. After air-drying the cells, slides were fixed and stained using Quick-Diff stain kit. Cells were counted using a light microscope. For differential cell count of BAL, 300 cells/slide were counted and differentiated into macrophages, lymphocytes, neutrophils and eosinophils.

4.5. Flow cytometric analysis

4.5.1. Flow cytometry analysis of cell surface marker expression and tetramer staining

A minimum of 2×10^6 lung cells per 96 plate well were washed with PBS and incubated for 30 min at 4 °C with Live/dead Aqua fixable dye (1:1000 in PBS) (Invitrogen, Paisley, UK). FACS-buffer was added and cells were spun down for 2 min, 700 x *g* at 4°C, followed by incubation for 20 min with a purified rat IgG2b anti-mouse CD16/CD32 receptor antibody (1:200 in FACS buffer; BD Bioscience). After washing the cells were stained with fluorochrome-conjugated antibodies against surface markers for 25 min at 4 °C in the dark (Table 4-6). After two washing steps with FACS-buffer, samples were measured on a standard Becton Dickinson LSRII equipped with 50mW 405nm, 50mW 488nm, 20mW 633nm lasers and a ND1.0 filter in front of the FSC photodiode. Acquisition was set to 500,000 live, single cells when pDC staining was included, otherwise 250,000 live, and single cells were acquired. PMT voltages were adjusted after standardized CST checks minimizing the spectral overlap to increase data precision. Data were analysed using FACSDiva software. Alexa Fluor 647-conjugated M₁₈₇₋₁₉₅ tetramers (H-2D^b/ NAITNAKII) were obtained from the NIH Tetramer Core Facility (Emory University Atlanta, GA, USA). M Tetramer staining was performed following Fc-block and prior to surface staining for 30 min at RT in FACS buffer. Following surface staining for 25 min at 4 °C in the dark (Table 4-6) cells were fixed for 30 min in BD Cytofix/Cytoperm. Samples were measured on a standard Becton Dickinson FortessaLSR equipped with 50mW 405nm, 50mW 488nm, 50mW 561nm, 20mW 633nm lasers and a ND1.0 filter in front of the FSC photodiode. Acquisition was set to 250,000 single live cells. PMT voltages were adjusted after standardized CST checks minimizing the spectral overlap to increase data precision. Data were analysed using FACSDiva software.

4.5.2. Flow cytometry analysis of intracellular cytokines

For intracellular staining single cell suspensions were stained for surface marker expression as described in 4.5.1 and fixed in in BD Cytofix/Cytoperm. Intracellular cytokine staining (Table 4-6) was performed in the presence of purified rat IgG2b anti-mouse CD16/CD32 receptor antibody in permeabilisation buffer (BD) for 60 min at 4°C. Samples were measured on a Becton Dickinson FortessaLSR-SORP equipped with 20mW

355nm, 50mW 405nm, 50mW 488nm, 50mW 561nm, 20mW 633nm lasers and a ND1.0 filter in front of the FSC photodiode. Acquisition was set to 250,000 single live cells. PMT voltages were adjusted after standardized CST checks minimizing the spectral overlap to increase data precision. Data were analysed using FACSDiva software.

Table 4-6 Antibodies used for flow cytometry

Antigen	Fluorochrom	Clone	Isotype	Supplier
CD11b	AF700	M1/70	Rat IgG2b, κ	eBioscience, Hatfield, UK
CD11c	PE-CF594	HL3	Armenian Hamster IgG1, λ 2	BD Pharmingen, Oxford, UK
CD45	eFluor780	30-F11	Rat IgG2b, κ	eBioscience, Hatfield, UK
CD45R (B220)	eFluor450	RA3-6B2	Rat IgG2a, κ	eBioscience, Hatfield, UK
CD64 (Fc γ RI)	APC	X54-5/7.1	Mouse IgG2b, κ	eBioscience, Hatfield, UK
CD103	PerCP/Cy5.5	2E7	Armenian Hamster IgG	BioLegend, Cambridge, UK
Siglec H	eFluor660	eBio440c	Rat IgG2b	eBioscience, Hatfield, UK
Ly6C	eFluor450	HK1.4	Rat IgG2c, κ	eBioscience, Hatfield, UK
Ly6G	BV570	1A8	Rat IgG2a, κ	BioLegend, Cambridge, UK
Siglec-F	PE	E50-2440	Rat (LOU) IgG2a, κ	BD Pharmingen, Oxford, UK
CD3	PE-Cy7	145-2C11	Rat IgG2b, χ	BD Pharmingen, Oxford, UK
CD19	FITC	1D3	Rat IgG2a, κ	eBioscience, Hatfield, UK

Antigen	Fluorochrom	Clone	Isotype	Supplier
CD4	APC-Cy7	GK1.5	Rat IgG2b, χ	BD Pharmingen, Oxford, UK
CD8a	AF700	53-6.7	Rat IgG2a, χ	BD Pharmingen, Oxford, UK
CD335 (NKp46)	eFluor450	29A1.4	Rat IgG2a, κ	eBioscience, Hatfield, UK
CD253 (TRAIL)	PE	N2B2	Rat IgG2a, κ	eBioscience, Hatfield, UK
Intracellular staining				
CD3e	APC-eFluor780	145-2C11	Armenian Hamster IgG	eBioscience, Hatfield, UK
CD19	APC-eFluor780	eBio1D3	Rat IgG2a, κ	eBioscience, Hatfield, UK
Ly6G	APC-Cy7	1A8	Rat IgG2a, κ	BioLegend, Cambridge, UK
IL-6	FITC	MP-520F3	Rat IgG1, κ	eBioscience, Hatfield, UK
TNF- α	BV650	MP-XT22	Rat IgG1, κ	BioLegend, Cambridge, UK
IL-1 β	PerCp-eFluor710	NJTEN3	Rat IgG1, κ	eBioscience, Hatfield, UK
IL-10	BV421	JESS-16E3	Rat IgG2b, κ	BioLegend, Cambridge, UK
IFN- γ	BV605	XMG1.2	Rat IgG1, κ	BioLegend, Cambridge, UK

4.6. Fluorescent-activated cell sorting (FACS)

For the purification of lung cells, single cell suspension (described in 4.3.3.3) was incubated for 20 min with a purified rat IgG2b anti-mouse CD16/CD32 receptor antibody (1:200 in MACS buffer containing 30 μ g/ml DNaseI). After washing for 8 min at 500 x *g* a CD11c enrichment of single lung cells was performed. Cells were incubated with anti-CD11c microbeads (N418, Miltenyi Biotec) for 15 min on a shaker and afterwards positively selected on an autoMACS Pro Separator (Miltenyi Biotech Ltd,

Bisley, UK) using running mode “possel_s”. The positive selected cell fraction was forwarded to surface staining as described in 4.5. In some cases the negative fraction was further processed to enrich for CD11c^{int} plasmacytoid dendritic cells (pDC) using Anti-PDCA1 microbeads (Miltenyi Biotec). The enrichment was performed as described for the CD11c enrichment and obtained cells were stained for cell surface markers. 30µm pre-filtered samples were sorted using a standard Becton Dickinson ArialIII equipped with 50mW 405nm, 50mW 488nm, 50mW 561nm, 20mW 633nm lasers and a ND1.5 filter in front of the FSC photodiode, a nozzle size of 100 µm and the corresponding BD FACSFlow sheath pressure of 20 psi, matched with a transducer frequency of 29 kHz. Input pressure was adjusted to ensure that every 5th to 6th drop was populated by an event. FACS sorted cells were cultured over night (see 4.6.1) or stored in Trizol to perform quantitative RT-PCR analysis.

4.6.1. *In vitro* stimulation of FACS purified innate cells

Purified FACS sorted cells were seeded at 2×10^5 cells/well plate in complete media. In some cases, cells were stimulated with LPS (1 µg/ml, Invivogen, San Diego, CA, USA). After 18 hrs incubation at 37°C supernatants were assessed for protein or prostaglandin production and cells were lysed in Trizol® to further assess RNA expression levels of different cytokines.

4.7. Chemokine and Cytokine expression

4.7.1. RNA extraction from animal tissue

4.7.1.1. Phenol-chloroform extraction

One lung lobe was homogenized in 350 µl Trizol® reagent using a tissue lyser (TissueLyser LT, Qiagen, Crawley, UK). 200 µl of chloroform was added and after 15 sec of vigorous shaking samples were incubated for 10 min at RT, following spinning down at $12,000 \times g$ for 15 min at 4 °C. RNA remaining in the upper aqueous phase was transferred into a fresh tube and 800 µl isopropanol was added and left for 10 min at RT. Samples were inverted and spun for 10 min at $12,000 \times g$ and 4 °C. The RNA precipitate was washed twice with 1 ml of chilled 70 % ethanol and spun at $5,000 \times g$ for 5 min at 4 °C. Finally, the isolated RNA was resuspended in 50 µl Nuclease-free water.

4.7.1.1.1. DNase treatment of Phenol-chloroform extracted RNA

Removal of DNA from Trizol extracted RNA was carried out using the Ambion® DNA-free™ Kit (Applied Biosystems, Paisley, UK). Isolated RNA (45 µl) was transferred into a 0.75 µl reaction tube and 0.1 volume of 10x DNase Buffer I and 1 µl rDNase I was added and mixed gently. After incubation at 37 °C for 30 min 0.1 volume of DNase inactivation reagent was added, mixed well and incubated for 2 min with occasionally mixing. Samples were centrifuged at 10,000 x *g* for 1.5 min and RNA was transferred into a fresh tube. RNA yields were determined by using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

4.7.1.2. Column purification

Lungs were homogenised as described in 4.7.1.1. Total RNA was isolated using RNeasy Mini Kit including a DNase step according to manufacturer's instructions (Qiagen). RNA yields were determined by using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

4.7.2. RNA extraction from FACS purified cells

Sorted cells in 1 ml of Trizol were disrupted using a vortex. Following transfer into Phase Lock gel tubes (VWR) 200 µl of chloroform was added and after 15 sec of vigorous shaking samples were spun 12,000 *g* for 15 min. The aqueous phase was transferred into a new tube. RNA for >100,000 cells was isolated using using RNeasy Mini Kit including a DNase step according to manufacturer's instructions (Qiagen). RNA for <100,000 cells was isolated using using RNeasy Micro Kit including a DNase step according to manufacturer's instructions (Qiagen).

4.7.3. Conversion of RNA into complementary DNA (cDNA)

For lung samples, one microgram of RNA and for sorted cells 9 µl of RNA sample was reverse-transcribed using a High Capacity RNA-to-cDNA kit according to manufacturer's instructions (Applied Biosystems).

4.7.4. Quantification of mRNA levels using quantitative Polymerase-chain-reaction (qPCR)

To quantify mRNA levels in lung tissue, qPCR reactions were performed using the primers and probes listed in Table 4-7 together with a polymerase-containing mastermix and the target cDNA. In order to measure the amount of target in the sample a measurable signal will be generated, which is proportional to the amount of amplified product. In this case double-dye labelled probes were used (FAM-fluorophor, TAMRA-quencher). The composition of the reaction master mix for each gene of interest was dependent on absolute (Table 4-8, Table 4-9) and relative quantification (Table 4-10). The thermal profile for each run is displayed in Table 4-11. To exclude misinterpretation of the results, due to variable amount of starting material, enzymatic efficiency and differences in tissue the obtained results were normalised using *Gapdh* as a housekeeping-gene. For absolute quantification, the exact number of copies of the gene of interest was calculated using an internal plasmid standard. For relative quantification, the expression of the gene of interest in a sample was expressed relatively to another gene (*Gapdh*). First, the ΔC_t (C_t =cycle threshold) between the target gene and *Gapdh* for each sample was calculated, following the calculation of $2^{-\Delta C_t}$. Analyses were performed using 7500 Fast System SDS Software.

Table 4-7 Sequences of primer and probes for quantitative PCR analysis of RSV L and N gene and murine cytokines and ISGs

Primer* / Probe**	Sequence (5'-3')
L gene forward (fwd)	GAACTCAGTGTAGGTAGAATGTTTGCA
L gene reverse (rev)	TTCAGCTATCATTTTCTCTGCCAAT
L gene probe	TTTGAACCTGTCTGAACATTCCCGGTT
N gene fwd	CATCCAGCAAATACACCATCCA
N gene rev	TTCTGCACATCATAATTAGGAGTATCAA
N gene probe	CGGAGCACAGGAGAT
IFN- β fwd	CCATCATGAACAACAGGTGGAT
IFN- β rev	GAGAGGGCTGTGGTGGAGAA
IFN- β probe	CTCCACGCTGCGTTCCTGCTGTG
IFN- $\lambda 2/3$ fwd	AAAGGATTGCCACATTGCTC
IFN- $\lambda 2/3$ rev	TCAAGCAGCCTCTTCTCGAT

Primer*/ Probe**	Sequence (5'-3')
IFN- λ 2/3 probe	TCCCCAAAAGAGCTGCAGGC
IFN- γ fwd	TCAAGTGGCATAGATGTGGAAGAA
IFN- γ rev	TGGCTCTGCAGGATTTTCATG
IFN- γ probe	TCACCATCCTTTTGCCAGTT
TNF- α fwd	CATCTTCTCAAAATTCGAGTGACAA
TNF- α rev	TGGGAGTAGACAAGGTACAACCC
TNF- α probe	CACGTCTGTAGCAAAC
OAS1a fwd	TCCTGGGTCATGTTAATACTTCCA
OAS1a rev	CCCCAGGGAGGTACATTCCT
OAS1a probe	CAAGCCTGATCCCAGAATCTATGCCATC
PKR fwd	CGGAACATCCTCTAGCGTTGTC
PKR rev	GGGAAACACCATTACTTGTCATAGAC
PKR probe	AGCTGCTGGAAAAGCCACTGA
Viperin fwd	CGAAGACATGAATGAACACATCAA
Viperin rev	AATTAGGAGGCACTGGAAAACCT
Viperin probe	CCAGCGCACAGGGCTCAGGG
Target primer/probe***	
IL-1, IL-1 β , Ptg2, CCL2, CCL7, CCL12, CXCL1,	Not revealed by supplier

* Primer sequences were generated by Invitrogen, Paisley, UK; ** probes were synthesised by Eurofin mwg operon, Ebersberg, Germany; *** target primer/probe-sets were obtained from Applied Biosystems, Paisley, UK.

Table 4-8 Composition of reaction mix for absolute quantification of viral L gene and murine cytokines and ISGs using qPCR

Reaction mix	Weight/Volume L gene	Weight/Volume Cytokines and ISGs
MasterMix	6.25 µl	6.25 µl
Forward primer (5 µM)	1 µM	1 µM
Reverse primer (5 µM)	0.33 µM	1 µM
Probe (5 µM)	0.2 µM	0.2 µM
Nuclease-free water	x µl	x µl
Total volume/well	11.5 µl	11.5 µl
cDNA	1 µl	1 µl

Table 4-9 Composition of reaction mix for absolute quantification of viral N gene using qPCR

Reaction mix	Weight/Volume for N gene
MasterMix	6.25 µl
Forward primer (5 µM)	0.49 µM
Reverse primer (5 µM)	0.49 µM
Probe (10 µM)	0.22 µM
Nuclease-free water	x µl
Total volume/well	11.5 µl
cDNA	1 µl

Table 4-10 Composition of reaction mix for relative quantification of murine cytokines, using qPCR

Reaction mix	Volume
MasterMix	6.25 µl
Target primer/probe-set*	0.625 µl
Nuclease-free water	4.625 µl
Total volume/well	11.5 µl
cDNA	1 µl

*Target primer/probe-sets were obtained from Applied Biosystems, Paisley, UK

Table 4-11 Thermal profile for qPCR run

Cycle number	Cycle set-up
1 cycle	50 °C for 2 min
1 cycle	95 °C for 10 min
45 cycle	95 °C for 15 sec, 60 °C for 1min

4.7.5. Preparation of lung homogenate

After determining the weight a lung lobe was homogenised in 350 µl protease inhibitor buffer (1 tablet in 10 ml PBS, Roche, Welwyn Garden City, UK) using a tissue lyser (TissueLyser LT, Qiagen, Crawley, UK). Homogenate was spun for 10 min at 10,000 x *g* at 4 °C and supernatant was transferred into a fresh reaction tubes containing 650 µl protease inhibitor buffer.

4.7.6. Enzyme-linked Immunosorbent Assay (ELISA)

To determine cytokine concentrations in BALF or lung homogenate ELISAs were performed using 96 well ELISA MaxiSorp™ plates (Nunc, Roskilde, Denmark). Commercial available ELISAs (R&D Systems, Minneapolis, MN, USA; Bethyl Laboratories Inc., Montgomery, TX, USA) were performed according to manufacturer's instructions. For other ELISAs (IL-6 and IFN- α), plates were coated with capture antibody in coating buffer (0.1 M NaHCO₃, pH 8.2) and left over night at 4 °C. After washing the plate 3 times with washing buffer (0.05% Tween20 in PBS) the plate was blocked (2.5 % FCS, 0.02% NaN₃ in PBS) for 2 hrs at RT. Afterwards 50 µl of sample or standard were added as duplicates and incubated for 2 hrs at room temperature. A biotinylated detection antibody was incubated for 2 hrs, following 1 hr incubation with extravidin peroxidase and TMB substrate (Table 4-12). Colour development was blocked with stop solution (1M H₂SO₄) and optical densities were read at 450 nm. Cytokine concentration was determined from the standard curve using SoftMax Pro software.

Table 4-12 Antibodies and Standards used for in-house ELISAs

Reagents		End Concentration	Company
<i>IL-6 ELISA</i>			
Capture-Ab	MP5-20F3	4 µg/ml	BD Pharmingen, Oxford, UK
Detection-Ab	MP5-32C11 biotinylated	1 µg/ml	BD Pharmingen, Oxford, UK
Standard	Recombinant IL-6		BD Pharmingen, Oxford, UK
<i>IFN-α ELISA</i>			
Capture-Ab	F18 Hycult	1 µg/ml	Cambridge Bioscience, Cambridge, UK
1 st Detection-Ab	Rabbit-Anti-IFN-α	0.2 µg/ml	PBL Biomedical Laboratories, Piscataway, NJ, USA
2 nd Detection-Ab	Mouse-Anti-Rabbit biotinylated	1:1000	Jackson ImmunoResearch, Suffolk, UK
Standard	Recombinant IFN-α		Cambridge Bioscience, Cambridge, UK

4.7.7. Multiplex assays

ProcartaPlex IFN-alpha 3/IFN-beta 2-plex assay (Affymetrix, Inc., Santa Clara, CA, USA) and Cytokine Mouse magnetic 20-plex (Invitrogen, Paisley, UK) were performed according to manufacturer's manual using lung homogenate or BAL fluid.

4.7.8. Prostaglandin E2 ELISA

Prostaglandin E2 metabolite EIA Kit (Cayman Chemical, Cambridge, UK) was performed according to manufacturer's manual using lung homogenate.

4.7.9. Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 5 (GraphPad Software Inc.). Two-group comparisons were performed using unpaired, two-tailed Student's t-test. One-way ANOVA with Tukey's post hoc test was used to compare multiple groups, and two-way ANOVA with Bonferroni's post hoc test was used for time-kinetic comparison of weight loss. For all tests a value of $P < 0.05$ was considered as significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ND, not detectable.

5. Results

5.1. Type I interferon signalling amplifies pro-inflammatory cytokine production in a model of respiratory syncytial virus (RSV) infection

Type I IFNs are crucial mediators of antiviral and immunomodulatory responses to prevent viral replication and spread. Their extensive effect on the immune responses ranges from the activation of an antiviral state in infected and neighbouring cells to the stimulation of innate immune cells and enhancing the adaptive immune response.

Lack of type I IFN signalling in IFNAR-deficient mice increases the susceptibility to many viruses (Davidson et al., 2014; McNab et al., 2015; Müller et al., 1994). In contrast, the immunomodulatory role of type I IFNs can worsen the outcome of bacterial infections and renders IFNAR-deficient mice more resistant (Trinchieri, 2010). The specific role for signalling through IFNAR in RSV infections and their impact on inflammatory responses is not clear. In order to explore the role of type I IFNs in the early inflammatory response to RSV infection mice deficient in the type I IFN receptor (*Ifnar1*^{-/-}) IFNAR were intranasally infected with RSV.

5.1.1. Lack of IFNAR signalling abrogates type I, II and III IFN responses in RSV infected mice

To assess the *in vivo* role of type I IFN signalling in RSV induced inflammation and pathology, wild-type (wt; C57BL/6) and IFNAR1 deficient mice (*Ifnar1*^{-/-}) were intranasally infected with 2x10⁶ FFU of RSV. The role of type I IFN signalling in controlling RSV infection was examined through quantification of viral L gene copy numbers in the lung of wt and *Ifnar1*^{-/-} mice during the course of infection. A significantly higher viral load was detected in *Ifnar1*^{-/-} mice throughout all time points, spanning from 8 hrs post infection (p.i.) up to day 14 p.i.. By day 14 p.i. it was evident that *Ifnar1*^{-/-} mice had a delayed viral clearance, because all 12 infected *Ifnar1*^{-/-} mice still had detectable levels of viral L gene copies compared to the wt mice, where 11 of 12 mice had cleared the virus (Figure 5-1 A). The increased viral burden in the lungs of *Ifnar1*^{-/-} mice was associated with an increased

weight loss, which is a measure for RSV induced disease severity in the mouse model (Pribul et al., 2008). Both wt and *Ifnar1*^{-/-} mice started to lose weight by day 5 p.i., but *Ifnar1*^{-/-} mice showed significant more weight loss and a slower recovery in comparison to infected wt mice (Figure 5-1 B).

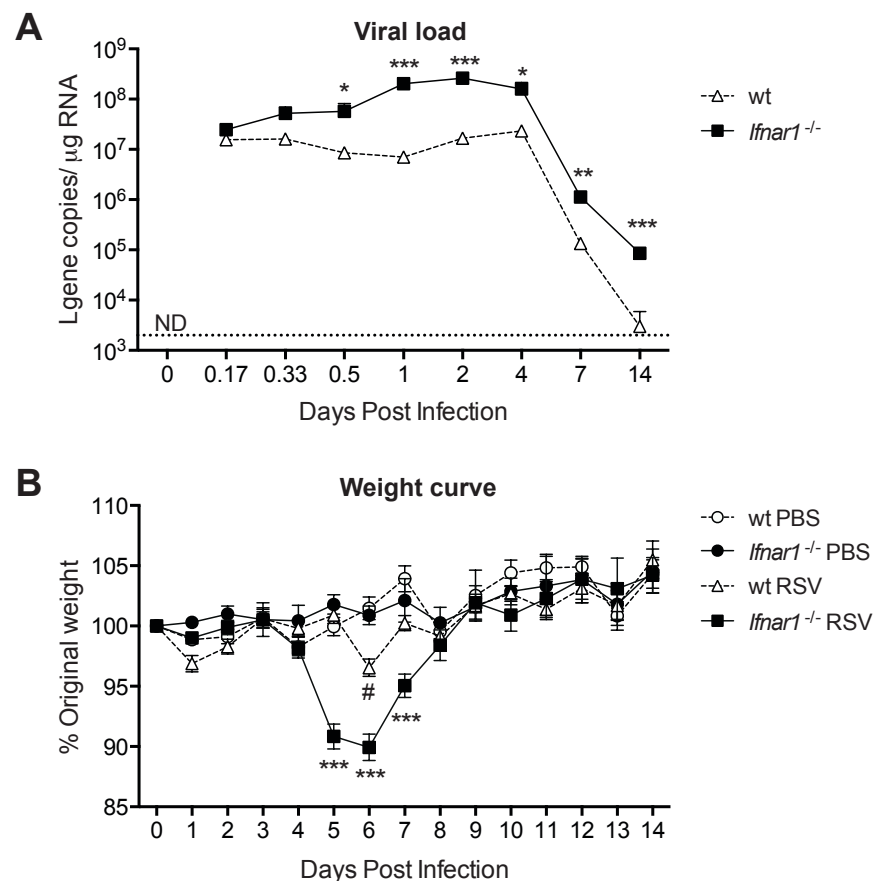


Figure 5-1 Viral load and weight loss after RSV infection. C57BL/6 wt and *Ifnar1*^{-/-} mice were infected intranasally with 2x10⁶ FFU of RSV. A) Lung levels of RSV L gene were quantified at different time points post RSV infection. Total RNA was isolated from lung tissue and, after conversion into complementary DNA (cDNA), copy numbers of RSV L gene were determined using quantitative PCR (qPCR). Dotted lines represent the detection limit, ND, not detectable. Data are pooled from 3 individual experiments with 3-5 mice per group. B) Weight was monitored daily and plotted as a percentage of the respective initial weight at t=0. Statistically significant differences comparing uninfected mice with infected wt mice are represented by '#'; comparing infected wt mice and infected *Ifnar1*^{-/-} mice represented by '*'. Data shown are representative of three individual experiments with 3 to 5 mice per group. Results are presented as mean ± SEM. Statistical significance of differences between groups was determined by A) two-tailed, unpaired Student's t-test, and B) 2-Way ANOVA with Bonferroni's post hoc test. ***, p≤0.001; **, p≤0.01; *, p≤0.05. #, p≤0.05.

Type I IFN responses in RSV infected mice were then determined. A transient production of IFN- α protein in bronchoalveolar lavage (BAL) fluid was detectable by 8 hrs p.i., peaking at 12 hrs, and undetectable after 48 hrs p.i. (Figure 5-2 A). A similar induction was observed for IFN- β mRNA levels (Figure 5-2 B). No IFN- α protein expression was detectable in the BAL fluid of *Ifnar1*^{-/-} mice, but interestingly at 4 hrs p.i., the induction of IFN- β mRNA expression was detectable in *Ifnar1*^{-/-} mice. However, in the lungs of wt mice IFN- β mRNA levels increased around 100 fold at later time points, which is in agreement with the known autocrine and paracrine positive-feedback loop for type I IFN production (Figure 5-2 B) (Honda and Taniguchi, 2006). It was speculated that other subtypes of the IFN family (type II or type III IFNs) could play a compensatory role for the lack of type I IFN expression after RSV inoculation. In the *Ifnar1*^{-/-} mice, IFN- γ mRNA and protein expression was abrogated compared to wt mice, where mRNA and protein peaked at 12 hrs and 18 hrs p.i., respectively (Figure 5-2 C). The type III IFN (IFN- λ) was undetectable in BAL fluid of *Ifnar1*^{-/-} mice, although mRNA levels were increased in the lung tissue of these mice (Figure 5-2 D).

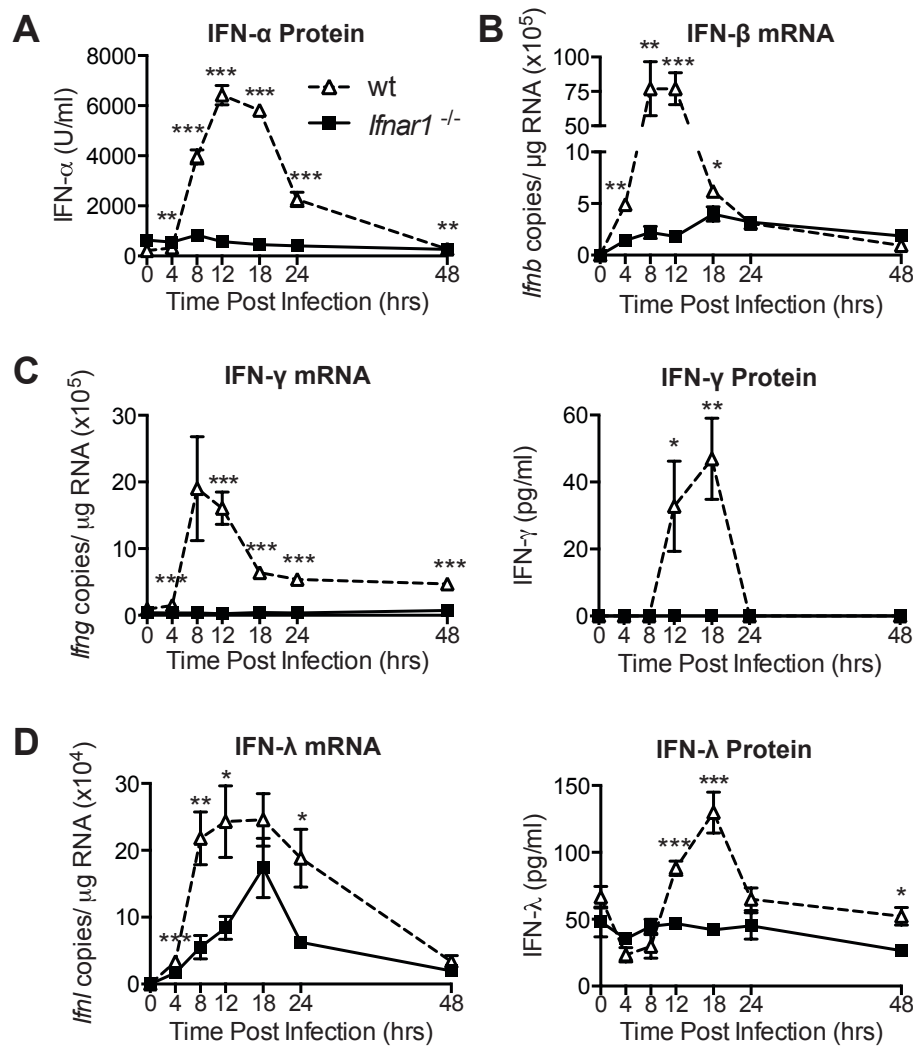


Figure 5-2 Interferon responses in *Ifnar1*^{-/-} and wt mice after RSV infection. C57BL/6 wt and *Ifnar1*^{-/-} mice were infected intranasally with 2×10^6 FFU of RSV. A) IFN- α protein levels were determined in bronchoalveolar lavage (BAL) fluid by ELISA. B) RNA was isolated from lung tissue and *Ifnb* gene expression was quantified by qPCR. C) IFN- γ , and D) IFN- λ protein expression in BAL fluid and mRNA expression in lung tissue were quantified. Copy numbers were determined using a plasmid standard. Data shown are representative of at least two individual experiments with 4 to 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$.

In order to assess if the reduced induction of IFN expression detected in *Ifnar1*^{-/-} mice was sufficient to effect the induction of anti-viral immune responses, the expression of interferon inducible genes (ISGs) was determined. CXCL10 expression could not be detected on mRNA level in *Ifnar1*^{-/-} mice, but it was highly induced in wt mice (Figure 5-3 A). An increase in mRNA levels above baseline was measured for MX-1, PKR, RIG-I and Viperin in both *Ifnar1*^{-/-} and wt mice (4-8 hrs p.i.), but wt mice showed a 3-6-fold increase

in mRNA expression up to 12 hrs p.i. (Figure 5-3 B-F). These data indicated that the loss of type I IFN signalling resulted in a global reduction of ISG expression early after RSV infection.

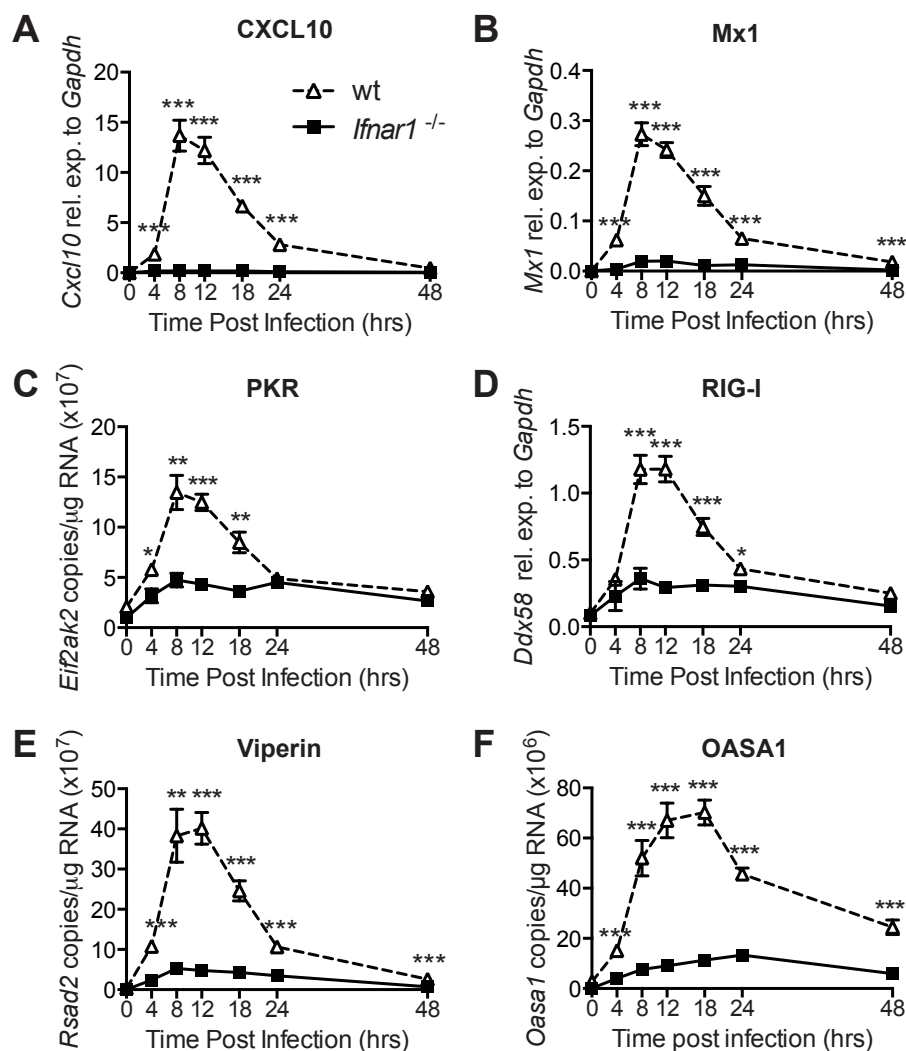


Figure 5-3 Interferon stimulated gene (ISG) induction after RSV infection. C57BL/6 wt and *Ifnar1*^{-/-} mice were infected intranasally with 2×10^6 FFU of RSV. Total RNA was isolated from lung tissue and mRNA levels of A) *CXCL10*, B) *Mx1*, C) *Eif2ak2* (PKR), D) *Ddx58* (RIG-I), E) *Rasad2* (Viperin) and F) *Oasa1* were determined by qPCR. Copy numbers for *Eif2ak2*, *Rasad2* and *Oasa1* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Mx1*, *Cxcl10* and *Ddx58*. Data shown are representative of at least two individual experiments with 4 to 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$.

5.1.2. Reduced proinflammatory cytokine responses in IFNAR1-deficient mice

A deficit in IFNAR signalling was associated with increased weight loss during RSV infection. It is well known, that proinflammatory cytokines released under inflammatory conditions can cause discomfort and relate to weight loss in this model (Borchers et al., 2013; Kelley et al., 2003). Thus, the expression of IL-6, IL-1 β and TNF- α in the lung of infected mice was measured by qPCR. All three cytokines were easily detectable in the lung of wt mice already at 4 hrs p.i. with peak of expression at 8 hrs p.i. (Figure 5-4). In contrast, IFNAR1 deficient mice displayed only a slight induction 4 hrs p.i and failed to amplify the signal at 8 hrs or later unlike wt mice (Figure 5-4).

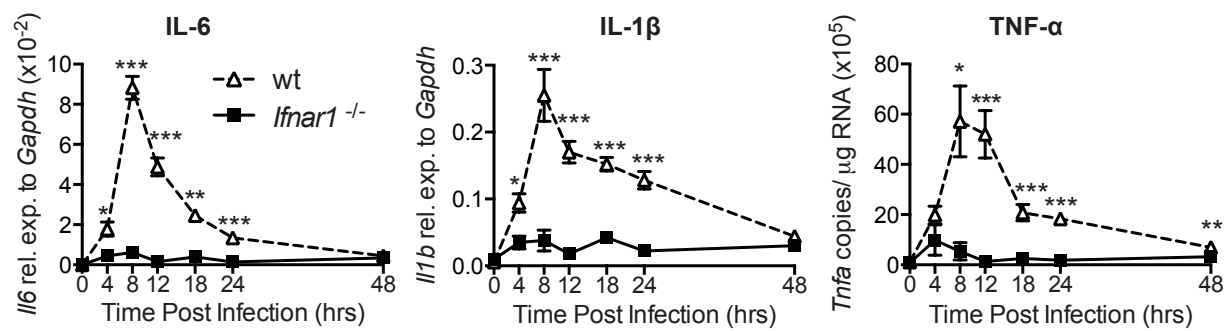


Figure 5-4 Reduced proinflammatory cytokine gene expression in *Ifnar1*^{-/-} mice after RSV infection.

C57BL/6 wt and *Ifnar1*^{-/-} mice were infected intranasally with 2x10⁶ FFU of RSV. Total RNA was isolated from lung tissue and mRNA levels of A) *Il6*, B) *Il1b* and C) *Tnfa* were determined by qPCR. Copy numbers for *Tnfa* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Il6* and *Il1b*. Data shown are representative of at least two individual experiments with 4 to 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. ***, p $\leq$ 0.001; **, p $\leq$ 0.01; *, p $\leq$ 0.05.

To gain a more detailed picture of the overall early cytokine responses to RSV, a multiplex Luminex cytokine and chemokine analysis was conducted to determine the protein abundance in BAL fluid of infected mice. For IL-6, IL-1 β , TNF- α and IL-12p40 an initial production was detectable in BAL fluid of *Ifnar1*^{-/-} mice, but not comparable to wt mice where a 5-10 fold higher production was measured (Figure 5-5 A, B). Other cytokines as IL-1 α , IL-5, CXCL9, CCL2, CCL3 and GM-CSF were completely absent in *Ifnar1*^{-/-} mice at all time points, whereas wt mice had a strong production peaking at 18 hrs p.i. (Figure 5-5 B). IL-2, IL-4, IL-10, IL-13 and IL-17A were not detectable in BAL fluid of either wt or *Ifnar1*^{-/-} mice (data not shown). Thus, the remarkably reduced proinflammatory cytokine and chemokine

responses observed in *Ifnar1*^{-/-} mice after RSV infection indicates a role for type I IFN signalling in the induction of these cytokines and chemokines.

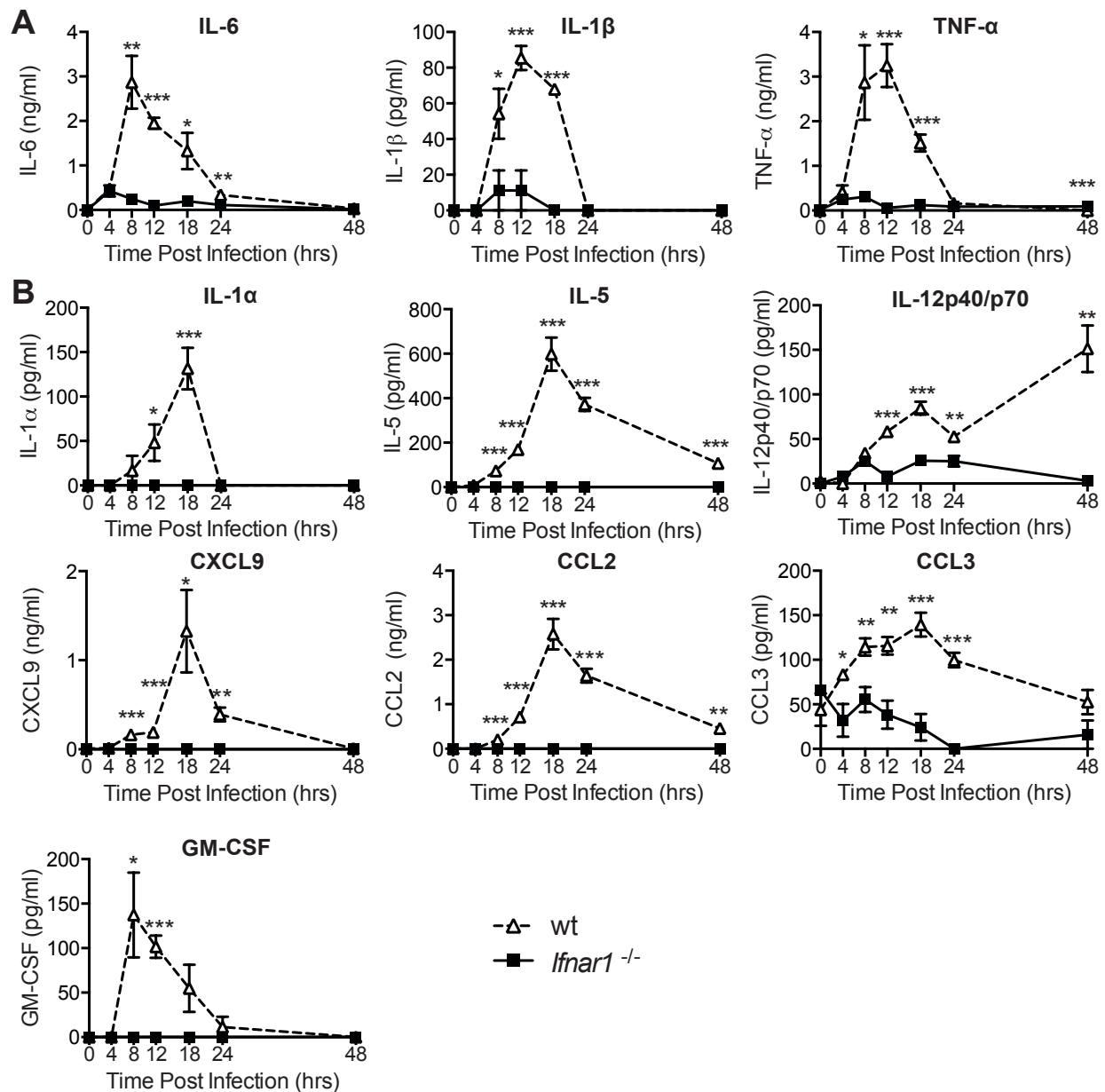


Figure 5-5 Abrogated proinflammatory cytokine protein expression in the BAL fluid of *Ifnar1*^{-/-} mice compared to wt mice during RSV infection. C57BL/6 wt and *Ifnar1*^{-/-} mice were infected intranasally with 2×10^6 FFU of RSV. Protein expression levels in BAL for A) IL-6, IL-1 β , TNF- α , B) IL-1 α , IL-5, IL-12p40, CCL2, CCL3, CXCL9 and GM-CSF were determined by Luminex. Data shown are representative of at least two individual experiments with 4 to 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Another hallmark of inflammatory responses is the recruitment of immune cells into inflamed tissues. In order to gain an insight into the cell recruitment, BAL was collected and differential cell count on H&E stained cytopsin slides was performed to determine the percentage of neutrophils and lymphocytes/monocytes during the course of infection. Notably, lymphocytes and monocytes are grouped together because of their morphological similarities, which made them difficult to distinguish. In wt BAL lymphocyte/monocytes recruitment was detectable by day 2 p.i. (most likely monocytes) and peaked at day 7 p.i., the time virus specific T cells are recruited to the site of infection (Openshaw and Tregoning, 2005)(Figure 5-6 A). In comparison, *Ifnar1*^{-/-} mice showed a slightly delay in recruitment of lymphocytes/monocytes into the lung, but by day 7 p.i. the frequency of lymphocytes/monocytes was similar to wt mice (Figure 5-6 A). This was consistent with other studies comparing T cell responses in *Ifnar1*^{-/-} mice with wt mice (Johnson et al., 2005). Neutrophil infiltration into wt lung was detected already at 4 hrs p.i., constituting 40% of BAL cells. Neutrophils persisted in the lung until day 1 p.i., after which they started to decline and reached baseline levels by day 7 p.i. (Figure 5-6 B). Interestingly, the early infiltration of neutrophils in *Ifnar1*^{-/-} mice did not differ from wt mice. Expression analysis of CXCL1, a chemoattractant important for neutrophil recruitment, revealed a similar gene and protein expression in knockout and wt mice early upon infection (4 hrs -12 hrs). Later during RSV infection, CXCL1 expression in wt mice was significantly increased compared to *Ifnar1*^{-/-} mice (Figure 5-6 C). Overall, the lack of type I IFN signalling led to a remarkable reduction of proinflammatory cytokine responses upon RSV infection, but had minimal effect on the recruitment of neutrophils and lymphocytes.

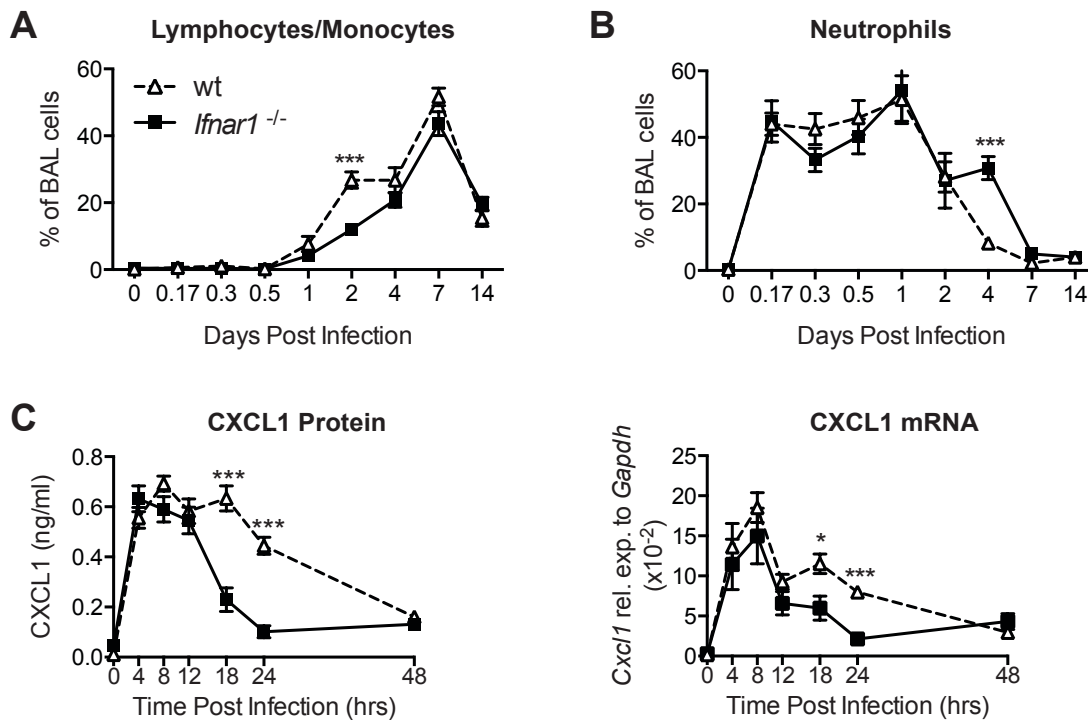


Figure 5-6 Normal infiltration of lymphocytes/monocytes and neutrophils into the lung of *Ifnar1*^{-/-} mice upon RSV infection. C57BL/6 wt and *Ifnar1*^{-/-} mice were infected intranasally with 2x10⁶ FFU of RSV. Percentage of A) lymphocytes/monocytes and B) neutrophils in BAL was determined by differential cell count. Data shown are pooled data from 3 individual experiments with 3 to 5 mice per group in each experiment. C) Protein expression level of CXCL1 in BAL fluid was determined by ELISA. Gene expression levels of *Cxcl1* in the lung tissue were determined by qPCR. Gene expression relative to *Gapdh* was calculated for *Cxcl1*. Data shown are pooled data from two individual experiments with 3 to 5 mice per group in each experiment. Results are presented as mean ± SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. ***, p<0.001; *, p<0.05.

5.1.3. Pulmonary challenge of IFNAR1 deficient mice with TLR agonists abrogates proinflammatory cytokine responses

To test whether the observed reduction of proinflammatory cytokine responses in *Ifnar1*^{-/-} mice upon RSV infection also applies to other stimuli of the airways, different non-infectious agonists of PRRs were intranasally administered into wt and *Ifnar1*^{-/-} mice. Samples were collected 24 hrs after intranasal instillation with CpG, PolyI:C or LPS (Table 4-2). Protein production of IFN-α in the BAL fluid was significantly increased after PolyI:C in wt mice while no production was detectable in *Ifnar1*^{-/-} mice (Figure 5-7 A). Upon treatment with the TLR9 agonist CpG, abrogated gene expression levels for IFN-λ were measured in *Ifnar1*^{-/-} mice (Figure 5-7 B). Gene expression levels of IFN-γ and CXCL10 were

reduced in *Ifnar1*^{-/-} mice independent of the respective TLR agonist tested (Figure 5-7 B, C). When assessing proinflammatory responses in the lung after TLR agonist challenge a reduced induction of IL-6 protein in the BAL fluid was observed for all agonists (Figure 5-7 D). Also reduced gene expression of IL-1 β and TNF- α in response to the TLR3 agonist PolyI:C in *Ifnar1*^{-/-} mice compared to wt mice (Figure 5-7 E) were detected. Challenging mice with the TLR4 agonist LPS showed a significant induction of IL-1 β mRNA in wt lung in contrast to *Ifnar1*^{-/-} mice (Figure 5-7 D and E).

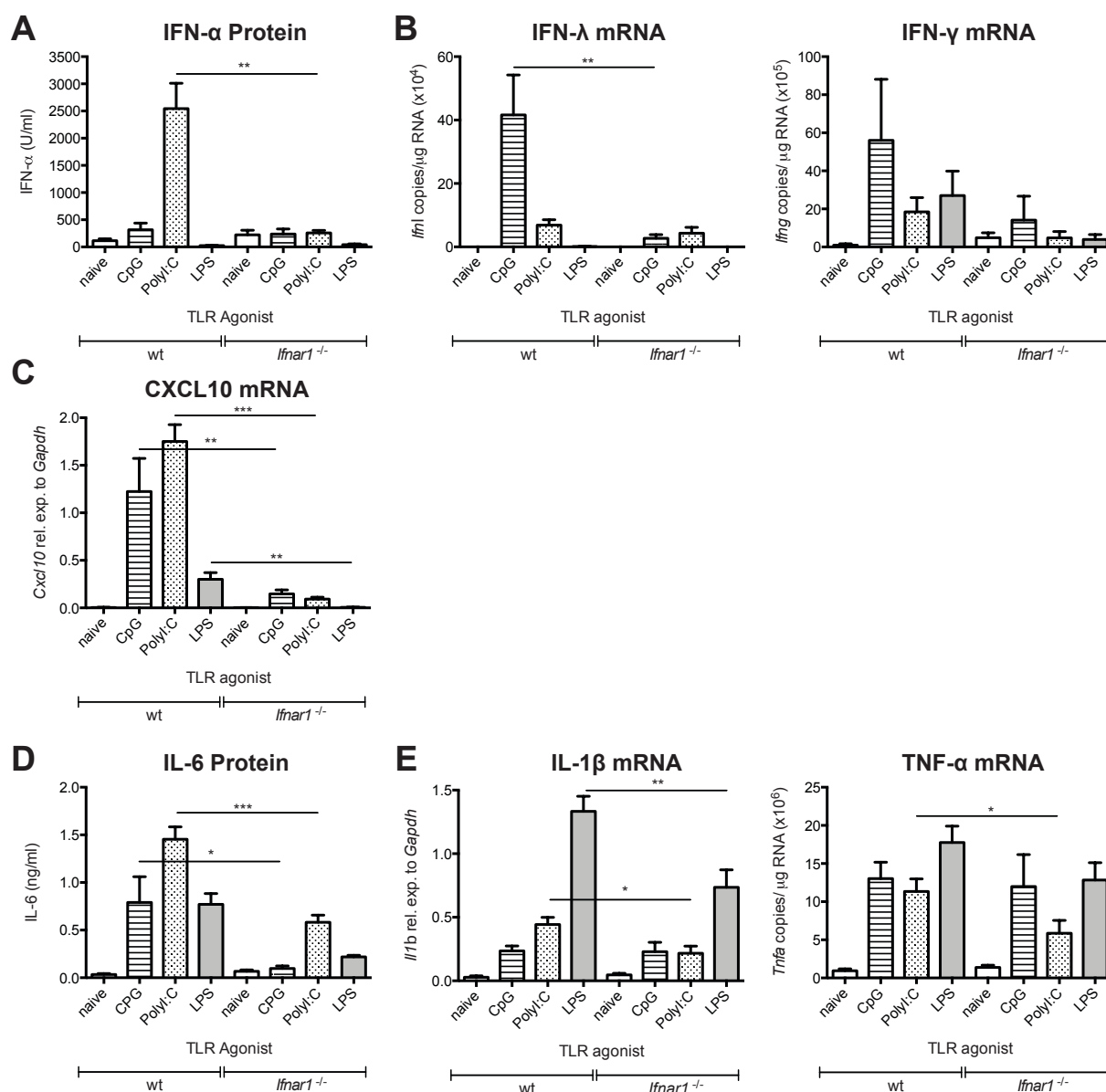


Figure 5-7 Cytokine responses after instillation of TLR agonists in lungs of *Ifnar1*^{-/-} and wt mice. C57BL/6 wt and *Ifnar1*^{-/-} mice were intranasally challenged with TLR agonists (CpG, 1.25 μ g/g body weight; PolyI:C, 3.5 μ g/g body weight; LPS, 500 ng/g body weight) and sacrificed 24 hrs later. Protein expression in BAL fluid was determined by ELISA and RNA expression in lung tissue was determined by qPCR. A) IFN- α

protein level. B) *Ifng* and *Ifnl* mRNA level. C) *Cxcl10* mRNA level. D) IL-6 protein level. E) *Il1b* and *Tnfa* mRNA level. Copy numbers for *Ifng*, *Ifnl* and *Tnfa* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Cxcl10* and *Il1b*. Data shown are pooled data from two individual experiments with 4 to 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significance of differences between treatment groups was determined by two-tailed, unpaired Student's t-test. ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$.

5.1.4. IFN- α amplifies proinflammatory cytokine production in the lung

The data obtained from the challenge and infection experiments suggest a global effect of type I IFN signalling in the induction of proinflammatory cytokine responses in the lung. To assess whether type I IFNs alone are sufficient to induce proinflammatory cytokines in the lung, IFN- α 11 (Miltenyi Biotech) a representative of IFN- α 's was intranasally administered into wt and *Ifnar1*^{-/-} mice. Subsequent gene expression analysis 12h post stimulation for IFN- γ , TNF- α , IL-6 and IL-1 β showed a significant induction ($p=0.001$) in the lung of wt mice upon treatment with recombinant IFN- α 11 (rIFN- α) compared to the PBS sham control (Figure 5-8). This rIFN- α dependent stimulation was not observed in *Ifnar1*^{-/-} mice, which demonstrates that the expression of this set of proinflammatory cytokines is fully dependent on functional type I IFN receptor signalling.

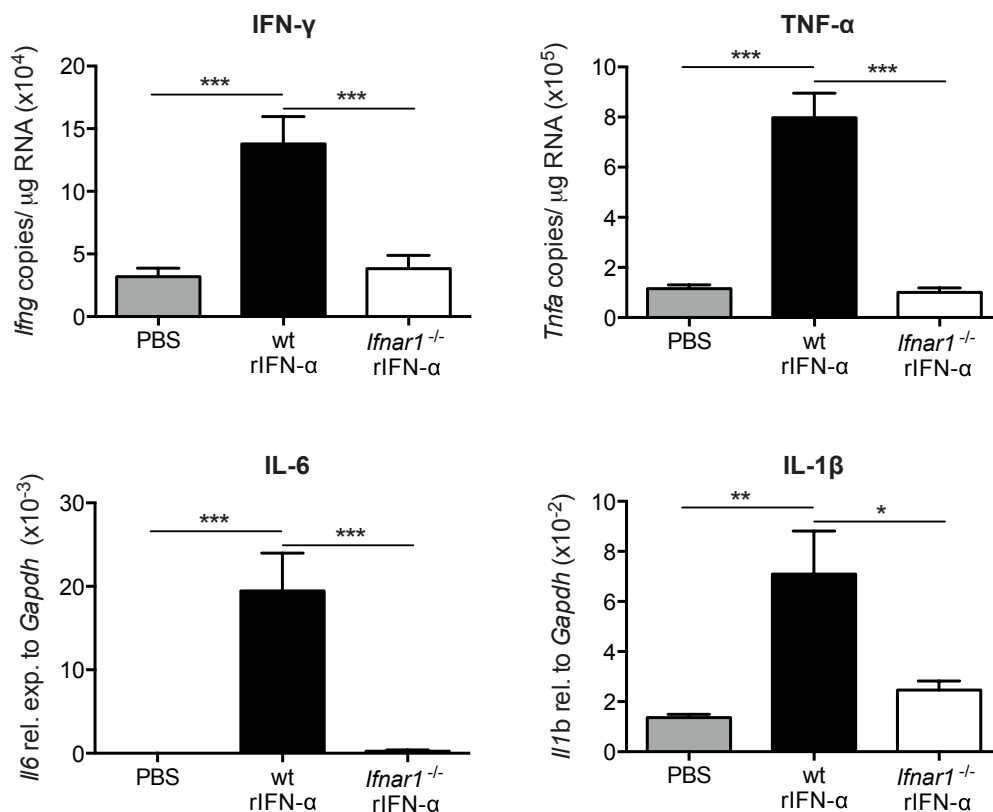


Figure 5-8 Administration of rIFN- α is sufficient for the induction of proinflammatory cytokines in the lung. C57BL/6 wt and *Ifnar1*^{-/-} mice were intranasally challenged with 500 ng recombinant IFN- α 11 or mock infected (PBS) and sacrificed 12 hrs post treatment. RNA expression of *Ifng*, *Tnfa*, *Il6* and *Il1b* in lung tissue was determined by qPCR. Copy numbers for *Ifng* and *Tnfa* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated *Il6* and *Il1b*. Data shown are pooled data from two individual experiments with 4 to 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by One-way ANOVA with Tukey's post hoc test. ***, $p \leq 0.001$; *, $p \leq 0.05$.

To prove that type I IFNs potentiate proinflammatory cytokines, wt mice were challenged with rIFN- α alone or together with an innate stimulus. LPS was chosen as innate stimuli, because it does not elicit high type I IFNs production on its own. As anticipated, mice that received both, LPS and rIFN- α , increased gene expression and protein expression for TNF- α , IL-6 and IL-1 β , respectively (Figure 5-9). In summary, the results highlight that signalling through the IFNAR enhances proinflammatory cytokine responses in the lung.

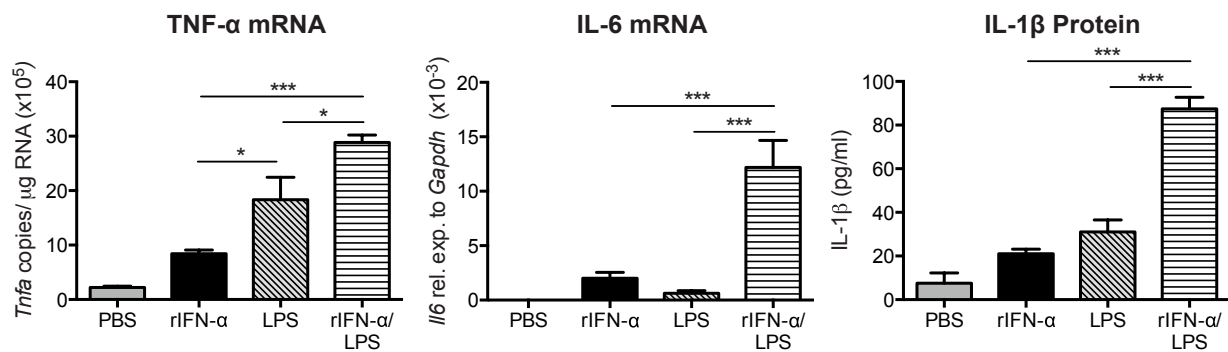


Figure 5-9 Recombinant IFN- α amplifies proinflammatory cytokine production in the lung. C57BL/6 wt were intranasally challenged with a suboptimal dose of LPS (50 ng/g body weight) with or without recombinant IFN- α 11 (500 ng/mouse) or mock infected (PBS) and sacrificed 24 hrs post treatment. RNA expression of *Tnfa* and *Il6* in lung tissue was determined by qPCR. Copy numbers for *Tnfa* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Il6*. Protein levels of IL-1 β in BAL fluid were determined by ELISA. Data shown are representative of two individual experiments with 4 to 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by One-way ANOVA with Tukey's post hoc test. **, $p \leq 0.01$; *, $p \leq 0.05$.

5.1.5. Discussion

Type I IFNs are the imminent response of the innate immune system after sensing viral infections. Signalling through their receptor, IFNAR, induces the expression of hundreds of different target genes, which regulate and modulate innate and adaptive immune responses to protect the host from virus invasion. Previous studies have addressed the role of type I IFNs in RSV infection with the main focus on the adaptive immune responses (Johnson et al., 2005). Nevertheless, the consequences of type I IFN signalling in initiating immune responses early during RSV infection are understudied. In this project a critical role for IFNAR signalling in controlling RSV infection by regulating the inflammatory response was provided. In addition a general role for type I IFNs in amplifying proinflammatory cytokine production was discovered.

The functional loss of type I IFN signalling was shown to increase the susceptibility of *Ifnar1*^{-/-} mice to RSV infection (Figure 5-1). This finding differs from those of other groups, claiming mice deficient in IFNAR expression are not more permissive to RSV infection. It is difficult to compare our study with others as they used mice on a different background

(Johnson et al., 2005) or a different inoculum (Mordstein et al., 2008), and both studies focused on only one time point to measure RSV titres while this study undertook analyses at multiple time points.

The autocrine positive feedback loop - mediated by IFNAR - is important in amplifying the initial type I IFN production during viral infection. Since no induction of type I IFNs was detectable in *Ifnar1*^{-/-} mice, it was thought that other type of IFNs (IFN- γ and/or IFN- λ) would compensate for the loss of type I IFN signalling to protect the host from viral spread and disease severity, as previously clearly demonstrated during influenza virus infection (Crotta et al., 2013; Jewell et al., 2010). All IFNs, type I, II and III, signal via distinct receptors and therefore independently respond to viral infections. However, it was surprising that neither IFN- γ nor IFN- λ protein expression were detectable in *Ifnar1*^{-/-} mice (Figure 5-2). The IFN- λ receptor is primarily expressed on epithelial cells, as opposed to leukocytes (Donnelly and Kotenko, 2010). In humans, IFN- λ 1 expression is regulated by PRR-activation of IRF3 and IRF7 (the latter constitutively expressed in pDCs) signalling pathways, similar to the early type I IFNs, IFN- β and IFN- α 4, whereas IFN- λ 2/3 gene expression depends solely on IRF7, which is induced downstream of IFN- λ receptor signalling (Honda and Taniguchi, 2006; Osterlund et al., 2007). Although the murine IFN- λ 1 gene is inactive in mice (Lasfar et al., 2006), the ability of type I IFNs to upregulate IFN- λ during RSV in wt mice might be due to their regulation of IRF7 expression downstream of IFNAR, and the absence of type I IFN signalling will abolish IFN- λ as seen in *Ifnar1*^{-/-} mice. However, influenza virus infected *Ifnar1*^{-/-} mice were shown to express IFN- λ 2/3 protein (Jewell et al., 2010) independent of type I IFN signalling. This is most likely mediated by pDCs, which constitutively express the transcription factor IRF7. Surprisingly, during RSV infection no such compensatory mechanism was detected in *Ifnar1*^{-/-} mice, suggesting a redundancy for type III IFNs in antiviral responses during RSV infection. Type I IFN signalling activates several STAT molecules to elicit a wide spectrum of gene expression to enhance the antiviral response; this includes STAT4, a transcription factor described to be important for IL-12-independent IFN- γ expression in NK cells (Mack et al., 2011; Nguyen et al., 2002). The extensive lack in expression of all IFN subtypes observed in *Ifnar1*^{-/-} mice resulted in insufficient upregulation of ISGs (Figure 5-3). The finding that a proportion of ISGs could still be induced above baseline in *Ifnar1*^{-/-} mice indicates induction of ISG expression independent of type I IFN signalling. Type I and III IFNs can induce similar ISG signature in lung epithelium (Crotta et al., 2013). This redundancy in IFN signalling

pathways will guarantee the initiation of antiviral host responses even when one pathway is inhibited or absent. Small amounts of autocrine IFNs might bind immediately to their respective receptor to induce ISG expression and are therefore not measured in assays such as ELISA. Additionally, studies suggest that ISGs can be activated by IRF proteins independent of type I IFNs, including IRF1 (Schoggins et al., 2011), IRF3 (Paladino et al., 2006), and IRF1/IRF3 (Dixit et al., 2010). Even if other signalling pathways are involved in ISG upregulation their contribution is minimal during RSV infection and confirms a key role for IFNAR in the induction of antiviral responses.

The expression of proinflammatory cytokines and chemokines was also significantly reduced in RSV infected *Ifnar1*^{-/-} mice (Figure 5-4 and Figure 5-5). This finding was strengthened by similar observations made after administration of different PRR agonist into the lungs of *Ifnar1*^{-/-} mice (Figure 5-7). Interestingly, IFN- α alone was sufficient to stimulate the expression of proinflammatory cytokines and even augmented the response after LPS challenge (Figure 5-8 and Figure 5-9). Almost all of the cytokine and chemokine expression measured was previously described to depend on the transcription factor NF- κ B (Blasius and Beutler, 2010; Pahl, 1999). NF- κ B is a key regulator of the immune and stress response in cells and is activated by a variety of pathogens and other stimuli. Active NF- κ B signalling regulates the expression of more than 150 genes, including cytokines and chemokines, immune receptors, proteins involved in antigen presentation, adhesion molecules, stress response genes, transcription factors and many others, to elicit an adequate host immune response (Pahl, 1999). Type I IFNs are described as stimulators of multiple pathways involved in NF- κ B activation. The formation of STAT3/PI3K complexes together with the IFNAR1 chain were shown to be involved in I κ B degradation and NF- κ B activation (Pfeffer, 2011). Additionally, protein kinase B (PKB, also known as AKT) is also involved in IFN-mediated classical NF- κ B activation (Pfeffer, 2011). IFNs stimulate also non-classical NF- κ B pathways involving NF-kappa-B induced kinase (NIK) and TRAF2 (Pfeffer, 2011). Together, these IFN-driven pathways are essential for cell survival (PI3K/Akt pathway) or apoptosis (NIK/TRAF2 pathway) (Pfeffer, 2011). Nevertheless, all cellular components involved in the mutual influence of NF- κ B and type I IFN pathways are not fully investigated and might depend on the cellular context. It is therefore a likely scenario that type I IFNs play a role in NF- κ B-induced expression of proinflammatory cytokines as presented in this study. Indeed, bone marrow derived DCs (BMDCs) from *Ifnar1*^{-/-} mice show reduced biological active IL-12p70 synthesis after RSV (Rudd et al.,

2007) or TLR challenge (Gautier et al., 2005) highlighting a synergising effect of NF- κ B and type I IFN pathways in inducing the optimal secretion of IL-12. However, NF- κ B translocation into the nucleus can be detected as early as 0.5 hrs post RSV inoculation without the necessity of viral replication (Haeberle et al., 2002). This might provide an explanation for the early induction of some of the measured cytokines such as IFN- β , IL-6, TNF- α , or IL-12 in the lungs of *Ifnar1*^{-/-} mice, but perhaps sustained NF- κ B activation may be in part IFN dependent.

In contrast to *Ifnar1*^{-/-} mice wt mice have the ability to further enhance this ‘first signal’ through the IFNAR-driven autocrine and paracrine amplification loop to maximise antiviral responses. Of particular interest within the feedback loop is that signalling molecules like TLR3, RIG-I, MDA5, MAVS, MyD88, IRF3 and IRF7 are themselves ISGs and therefore amplify the IFN response (Haller et al., 2006; Jaitin and Schreiber, 2007). Without a functioning IFNAR loop the detection of accumulating viral RNA and the further downstream processing of the signal is compromised in infected cells as they lack the feedback mediated boosted expression of the viral RNA sensors. This imbalance will eventually promote viral replication and spread early during infection. Moreover, the lack of responsiveness to RSV in *Ifnar1*^{-/-} mice could also suppress the recruitment of additional cells to the site of infection, which could otherwise contribute to local cytokine secretion. These usually specifically recruited cells are most likely of non-polymorph nuclear origin, since neutrophil recruitment was comparable in wt and *Ifnar1*^{-/-} mice (Figure 5-6). In sum, type I IFNs directly drive inflammation in the lung by amplifying primary signals initiated by other stimuli. Indeed, continuous IFNAR signalling was shown to induce a feed-forward signalling mechanism leading to IFN-mediated immunopathology in induced sterile inflammation (Bhattacharya et al., 2014). Similar data were found in acute influenza virus infections when excessive type I IFN secretion mediated inflammation and increases mortality (Davidson et al., 2014). In contrast, type I IFNs have a positive effect during RSV infection by decreasing the susceptibility to infection and reducing disease severity.

The abrogated antiviral responses in *Ifnar1*^{-/-} mice led not only to increased viral burden, but also heightened the morbidity as measured by weight loss. Disease severity was shown to coincide with T cell infiltration and can be abrogated by T cell depletion (Cannon et al., 1988). Nevertheless, the percentage of BAL lymphocytes (Figure 5-6) during RSV infection was unchanged in *Ifnar1*^{-/-} mice compared to wt mice. Previous studies described reduced CD8⁺ T cell numbers in the airways of *Ifnar1*^{-/-} mice with increased IFN- γ

secretion upon stimulation (Johnson et al., 2005). Heightened effector T cell function might therefore drive weight loss. One explanation for the elevated IFN- γ production observed in *Ifnar1*^{-/-} mice is the higher viral load in the lung at the time of T cell recruitment. The second possibility taken into account is the imbalance in signalling molecules present in genetically ablated *Ifnar1*^{-/-} cells. Low-level expression of molecules required for the induction of various signalling pathways in homeostasis is maintained by constitutive expression of IFN- β to assure for rapid responses upon exogenous stimuli. *Ifnar1*^{-/-} cells have decreased basal expression levels of STAT1, STAT2 and STAT3 shifting the ratio of STAT molecules in steady state (Gough et al., 2012). Reduced STAT1 might favour STAT4 activation, which is a key regulator of IFN- γ expression in T cells and NK cells (Mack et al., 2011; Nguyen et al., 2002). In other viral infections, type I IFN signalling resulted in the direct stimulation of virus-specific CD8⁺ T cell by influencing T cell expansion and survival (Aichele et al., 2006), and regulation of their cytolytic activity (Arimori et al., 2014). How RSV-specific T cells respond to the lack of type I IFN signalling needs further investigation. However, type I IFN signalling is not required for priming of CD8⁺ T effector cells in RSV infection but their immune-modulatory function certainly influences the quality of the T cell responses either directly or indirectly through other cells.

5.2. Alveolar macrophages orchestrate pulmonary antiviral immune responses through type I IFN induced recruitment of CD64^{hi} inflammatory cells

Type I IFNs are rapidly induced cytokines crucial to elicit antiviral immune responses. Signalling through IFNAR was identified to play a key role in RSV-induced lung inflammation and susceptibility (see previous section). The individual contribution of different pulmonary cell populations in the production of type I IFNs *in vivo* and which signalling pathways are involved in the detection of RSV infection remains elusive.

Several cell types in the lung were suggested as source of type I IFNs after exposure to RSV *in vitro* (Bhoj et al., 2008; Demoor et al., 2012; Guerrero-Plata et al., 2006; Jewell et al., 2007; Rudd et al., 2007; Schijf et al., 2013; Smit et al., 2006; Wang et al., 2006), but coherent *in vivo* observations are missing. To delineate the source of type I IFNs in the lung during RSV infections a reporter mouse model for the *Ifna6* gene was used. The further use of mice deficient in the RLR adapter MAVS and the TLR adaptors MyD88 and TRIF will help to identify the pathway required for RSV sensing and induction of type I IFN production.

5.2.1. Identification of GFP⁺ pulmonary innate leucocyte subsets

In order to identify type I IFN producing cells during RSV infection a transgenic mouse model, in which GFP is expressed under the control of the *Ifna6* promoter (Kumagai et al., 2007), was utilised to screen for RSV induced *Ifna6* expression in the lung. Different innate immune cell populations from the whole lung were characterized by flow cytometry at various time points post infection (p.i.). The initial gating of pulmonary leucocytes was performed on CD45⁺ cells and neutrophils were selected by high Ly6G expression (Figure 5-10). All remaining Ly6G^{lo} cells were further subdivided into Siglec-F^{hi} and Siglec-F^{lo} cells. By measuring the expression level of CD11c Siglec-F^{hi} cells were divided in two populations: Siglec-F^{hi}CD11c^{hi} cells, typical for alveolar macrophages (AMs), and Siglec-F^{hi}CD11c^{lo} cells representing eosinophils. CD11c expressing Siglec-F^{lo} cells consist of a mixed population of different subsets of pulmonary dendritic cells (DCs): CD103⁺ DCs (CD11c^{hi}CD11b^{lo}CD103^{hi}), CD11b⁺ DCs (CD11c^{hi}CD11b^{hi}Ly6C^{lo}) and plasmacytoid DCs (pDCs; CD11c^{int}CD11b^{lo}B220^{hi}Siglec-H^{hi}Ly6C^{hi}). Alternative gating on total Siglec-F^{lo} cells

allowed for distinguishing $CD64^{hi}CD11b^{hi}$ inflammatory monocytes, which are recruited into tissues upon an inflammatory response (Guilliams et al., 2013a). Different expression levels of CD11c further subdivided this population into monocyte-derived DCs (moDCs; $CD11c^{hi}CD11b^{hi}CD64^{hi}$) and inflammatory monocytes (infMo; $CD11c^{lo}CD11b^{hi}CD64^{hi}$); see (Figure 5-10) for a detailed visualisation of the gating strategy.

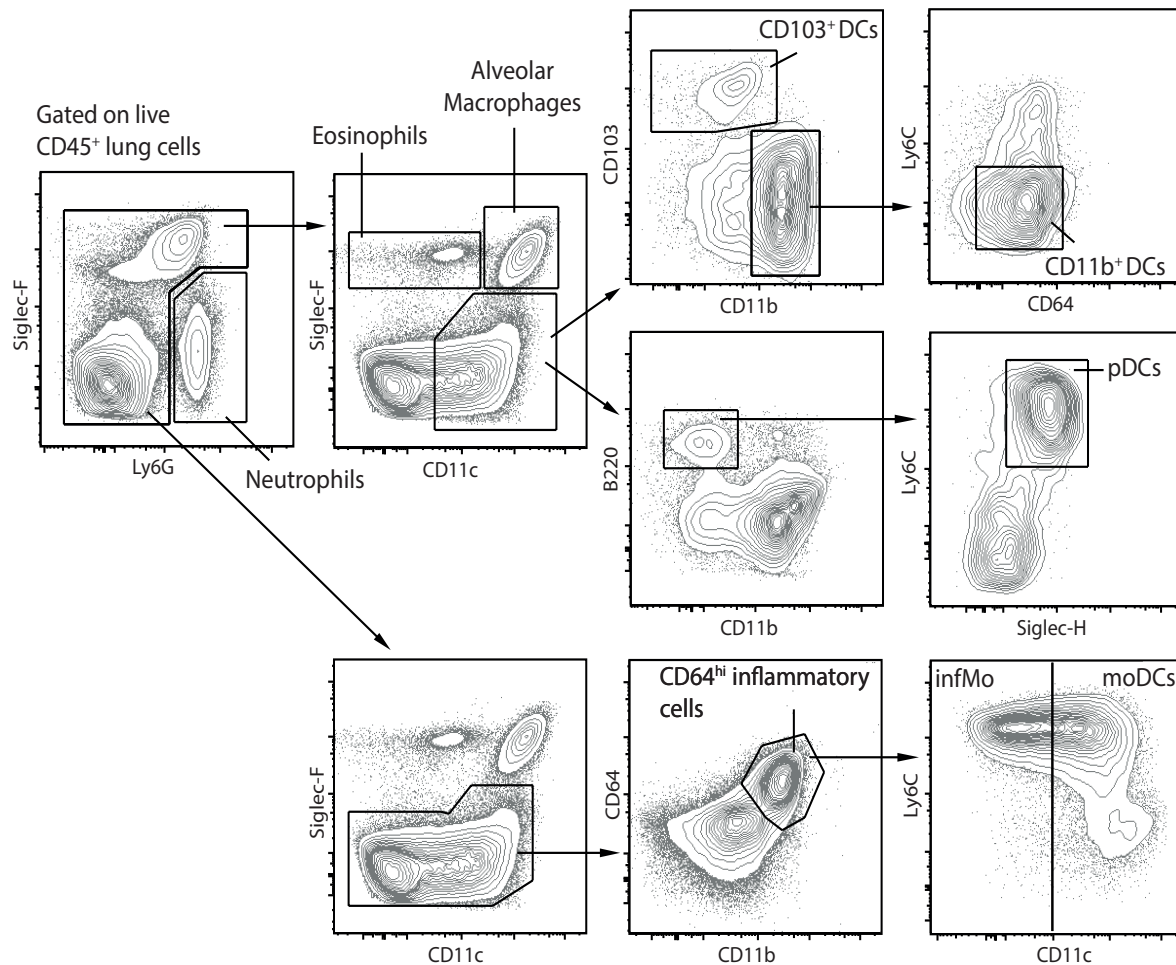


Figure 5-10 Gating strategy to characterise lung cell population by flow cytometry. Mice were infected with 2×10^6 focus forming units (FFU) of RSV. Lung cells were obtained by collagenase digestion of lung tissue and stained for indicated cell surface molecules. Representative plots for the gating are shown. Alveolar macrophages (AMs), dendritic cells (DCs), plasmacytoid DCs (pDCs), inflammatory monocytes (infMo), monocyte-derived DCs (moDCs).

Based on the gating strategy described in Figure 5-10 and by using the type I IFN reporter mouse model, the early enhanced GFP (eGFP)-reporter expression in pulmonary cell subsets of *Ifna6^{afp/+}* mice, infected intranasally with RSV from 8 hrs to day 4 post infection

was determined. Amongst all CD45 expressing pulmonary leukocytes, the GFP-reporter signal was detectable as early as 8 hrs p.i. with increasing expression peaking at 18 hrs p.i.. From 18 hrs onwards the signal declined and became almost undetectable by day 4 p.i. (Figure 5-11 A). Noteworthy, the actual GFP-reporter signal did not fully resemble active *Ifna6* expression throughout the course of infection, since mRNA levels of *Ifna6* peaked already at 8 hrs in the lung (Figure 5-11 B). Although pDCs and epithelial cells are reported to be the main type I IFN producers during RSV infection (Jewell et al., 2007; Schijf et al., 2013; Smit et al., 2006) no aberrant GFP signal was detected in either cell type using this particular reporter model (GFP detection in epithelial cells, unpublished observation, F. Kausar). The same lack of reporter expression was observed for all the other populations identified by the described gating strategy (Figure 5-11 C). Only within the highly autofluorescent Siglec-F^{hi}CD11c^{hi} AMs population, 5-10 % of the cells displayed a clearly detectable GFP signal (Figure 5-11 D, E). Interestingly, during the course of infection AMs upregulated the surface marker CD11b (Figure 5-12 A) and by day 3 all AMs were positive for CD11b expression (Figure 5-12 B and C).

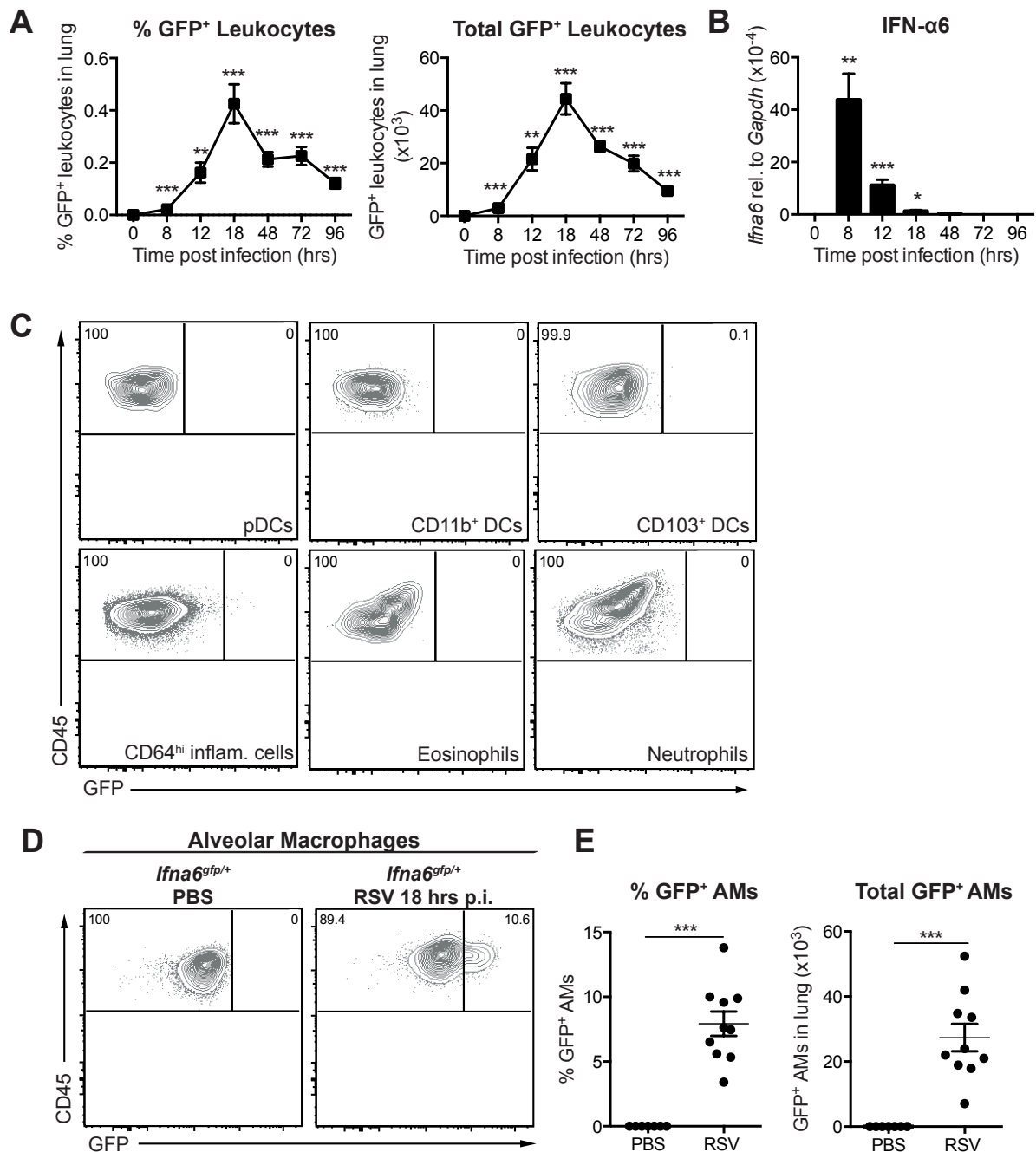


Figure 5-11 Alveolar macrophages are the only pulmonary cell type expressing IFN-α6/GFP in response to RSV infection. *Ifna6*^{gfp/+} mice were intranasally infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Percentage and numbers of GFP-reporter expressing pulmonary leukocytes in infected *Ifna6*^{gfp/+} mice. Data are representative of 3 independent experiments with 5 mice per group. B) Gene expression of *Ifna6* in lung tissue. Gene expression was calculated relative to *Gapdh*. C) Assessment of GFP-reporter expression in plasmacytoid dendritic cells (pDC; CD11c^{int}CD11b^{lo}B220^{hi}Siglec-H^{hi}, Ly6C^{hi}), CD11b⁺ DCs (CD11b⁺ DCs; CD11c^{hi}CD11b^{hi}CD64^{lo}Ly6C^{lo}), CD103⁺ DCs (CD11c^{hi}CD11b^{lo}CD103^{hi}), CD64⁺ inflammatory cells (CD11b^{hi}CD64^{hi}), eosinophils (CD11c^{lo}Siglec-F^{hi}) and neutrophils (CD11b^{hi}Ly6G^{hi}) from *Ifna6*^{gfp/+} mice at 18 hrs post infection. D) GFP-reporter expression in Siglec-F^{hi}CD11c^{hi} AMs. E) Percentage of GFP⁺ AMs and total numbers of GFP⁺ AMs in the lungs 18 hrs post infection. Data are pooled

from two independent experiments with 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

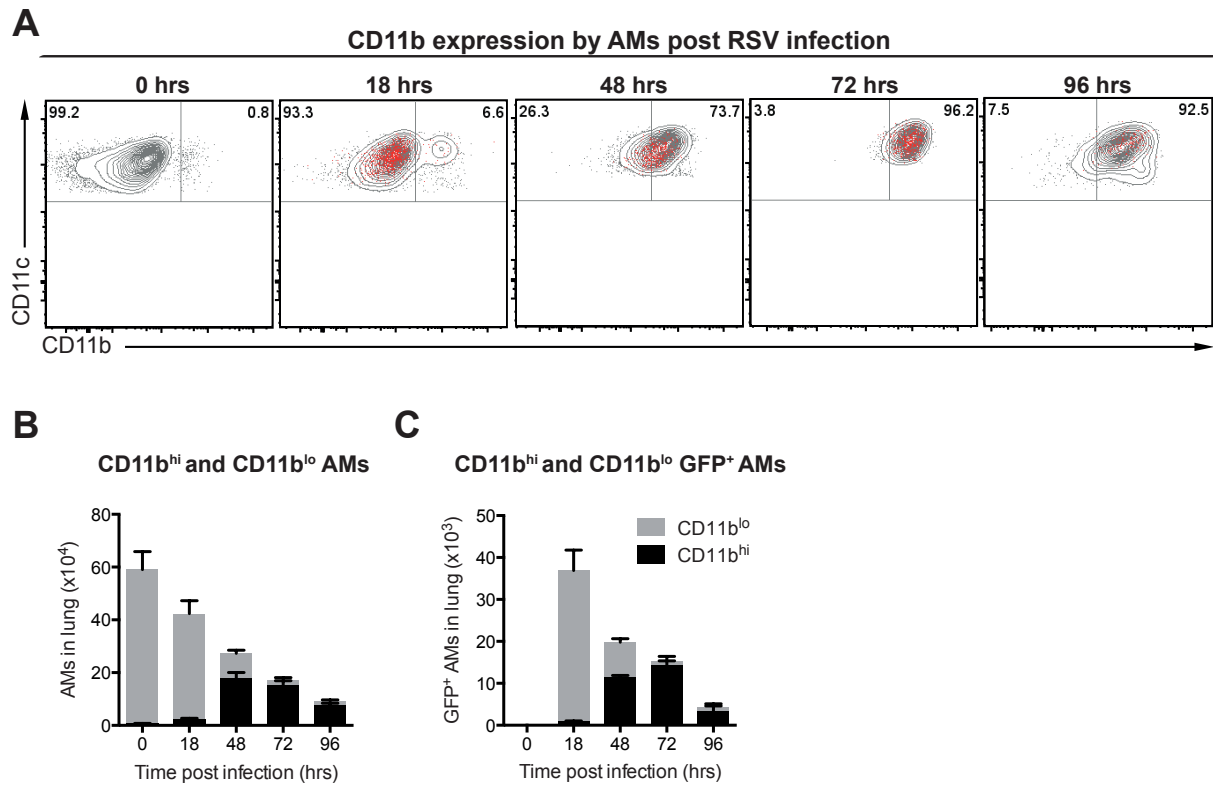


Figure 5-12 Upregulation of CD11b on alveolar macrophages during RSV infection. *Ifna6gfp/+* mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, $t=0$). A) Representative plots of CD11b expression on Siglec-F^{hi} AMs during the course of RSV infection. Total AM population in grey. Red dots represent GFP expressing AMs. B) Number of CD11b^{hi} and CD11b^{lo} AMs at indicated time points post infection. C) Number of GFP⁺ AMs positive or negative for CD11b expression. Data are representative of at least three independent experiments with 4 to 5 mice per group.

To assess if AMs are also the major source of other type I IFNs, different pulmonary innate cell subsets were FACS sorted (see gating strategy in Figure 5-10). Gene expression analysis for IFN- $\alpha 5$ and IFN- β revealed high levels of transcripts in GFP⁺ (*Ifna6*⁺) AMs supporting the notion that AMs are the major source of type I IFNs in RSV infection (Figure 5-13). In addition, mRNA transcripts for IFN- $\alpha 5$ and IFN- β were also detectable in GFP⁻ AMs and pDCs but 5 times less compared to the levels found in GFP⁺ AMs (Figure 5-13). In conclusion, AMs are the main source of type I IFNs during RSV infection.

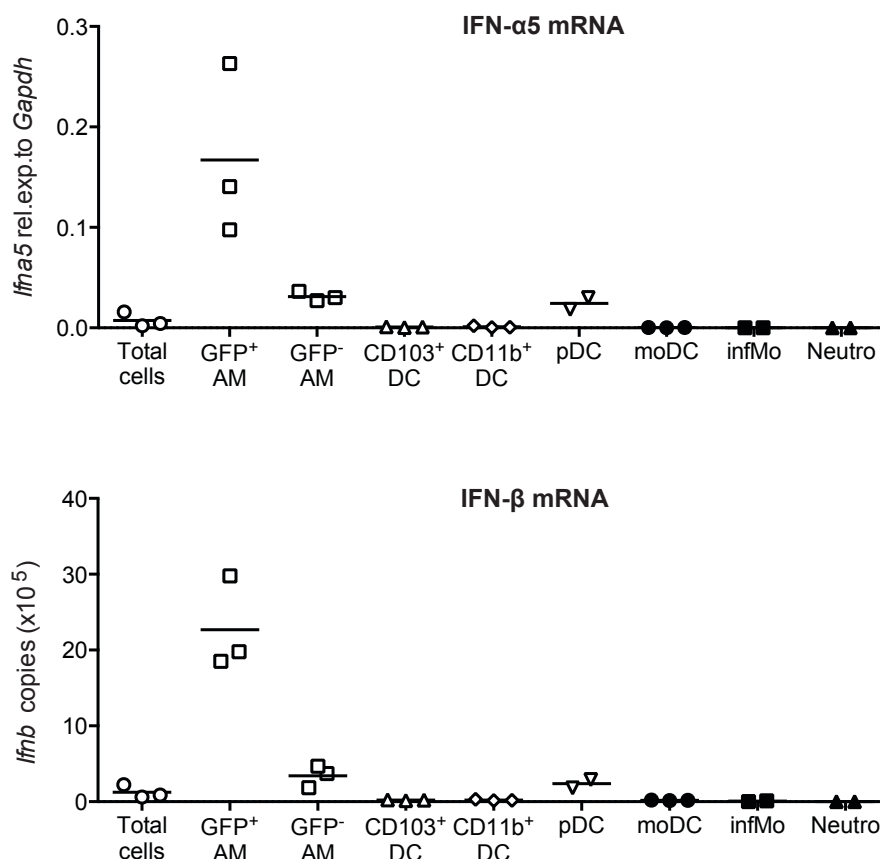


Figure 5-13 AMs are the major producers of type I IFNs during RSV infection. *Ifna6^{gfp/+}* mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV. Lung cell populations were FACS sorted 18 hrs p.i.. Gene expression for *Ifna5* and *Ifnb* for total cells and FACS sorted GFP⁺ and GFP⁻ AMs (Siglec-F^{hi}CD11c^{hi}), CD103⁺ DCs (CD11c^{hi}CD11b^{lo}CD103^{hi}), CD11b⁺ DC (CD11b⁺ DC; CD11c^{hi}CD11b^{hi}CD64^{lo}Ly6C^{lo}), pDC (CD11c^{int}CD11b^{lo}B220^{hi}Siglec-H^{hi}), monocyte-derived DC (moDC; CD11b^{hi}CD64^{hi}CD11c^{hi}), inflammatory monocytes (infMo; CD11b^{hi}CD64^{hi}CD11c^{lo}) and neutrophils (Neutro: CD11b^{hi}Ly6G^{hi}). Each individual point represents one individual experiment with 20-37 mice. Copy numbers for *Ifnb* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Ifna5*. Results present the mean.

5.2.2. IFN-α expression by AMs relies on RLR-MAVS pathways

To address which pattern recognition pathway is crucial for RSV recognition and the subsequent induction and production of IFN-α in AMs, *Ifna6^{gfp/+}* mice deficient in the RLR adaptor MAVS or TLR adaptors MyD88/TRIF were intranasally infected with RSV and IFN-α6/GFP-reporter expression in lung leukocytes was assessed 18 hrs p.i.. The induction of GFP-reporter expression in AMs required live virus infection, because no GFP signal could be detected after inoculation of ultraviolet (UV)-inactivated RSV (Figure 5-14 A). After RSV infection, GFP expression in AMs from MyD88/TRIF deficient mice (*Myd88/Trif*^{-/-}) was

clearly detectable (Figure 5-14 A), although the numbers of GFP expressing AMs in *Myd88/Trif*^{-/-} mice were reduced compared to wt AMs. This suggests a partial dependency on TLR signalling for a full IFN- α induction (Figure 5-14 B). In MAVS deficient mice (*Mavs*^{-/-}), the GFP signal in AMs was completely absent, concluding that the induction of IFN- α relies on RLR-MAVS pathways (Figure 5-14 B). Direct recognition of the virus by AMs was an additional requirement for the induction of GFP expression (S. Makris, unpublished observation).

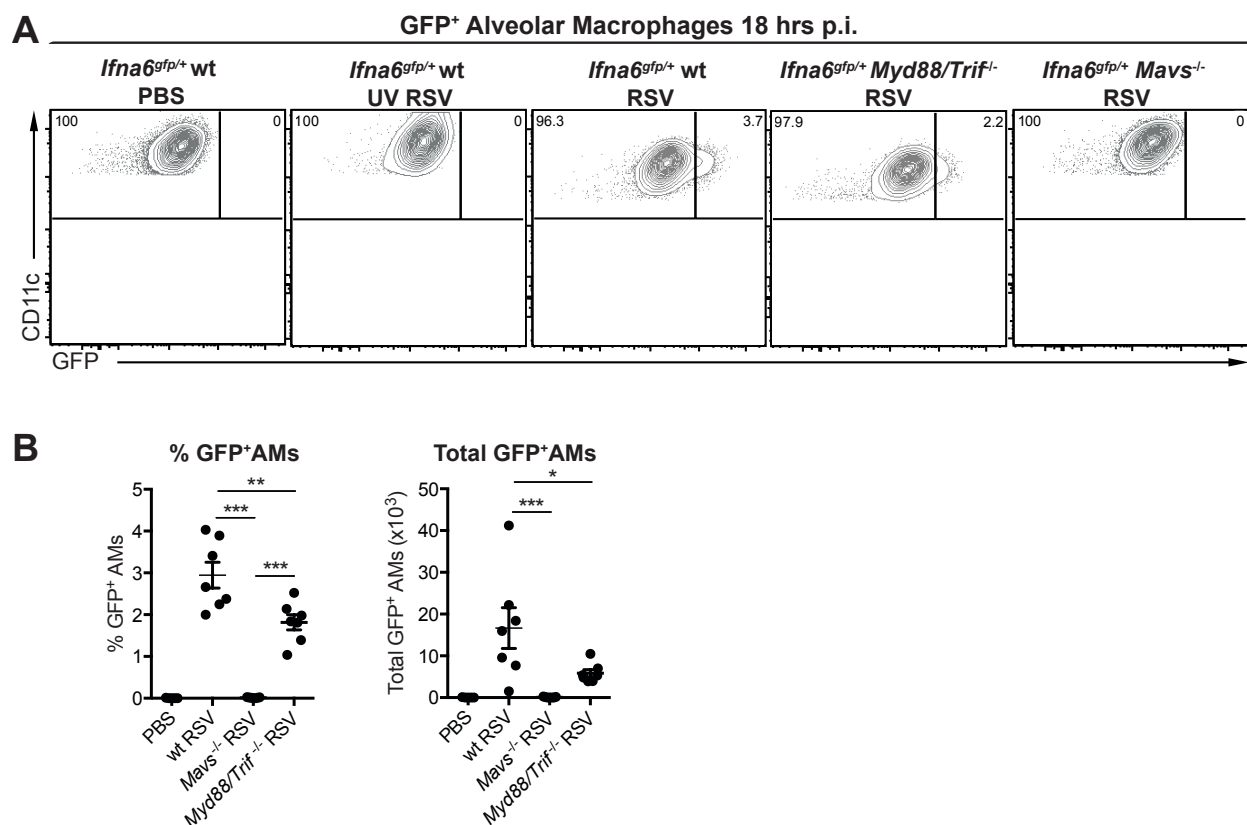


Figure 5-14 Type I IFN production by AMs relies on MAVS signalling. *Ifna6gfp/+* wt, *Ifna6gfp/+* *Mavs*^{-/-} and *Myd88/Trif*^{-/-} mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or UV-RSV or mock infected (PBS, t=0). A) GFP-reporter expression from AMs in *Ifna6gfp/+* wt, *Ifna6gfp/+* *Myd88/Trif*^{-/-} and *Ifna6gfp/+* *Mavs*^{-/-} mice 18 hrs post RSV infection or UV-RSV exposure. B) Percentage and number of GFP⁺ AMs 18 hrs p.i. Data are pooled from at least two independent experiments with 3 to 4 mice per group. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by One-way ANOVA with Tukey's post hoc test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

To assess the role of different PRRs in the early immune response to RSV infection, viral load and cytokine expression levels were determined in wt, *Mavs*^{-/-}, and *Myd88/Trif*^{-/-} mice 18 hrs p.i. . The viral burden was measured by detecting RSV L gene copy numbers in the lung using qPCR. As anticipated by the results from *Ifna6* reporter mice, infected *Mavs*^{-/-} mice had a significant higher viral burden compared to wt or *Myd88/Trif*^{-/-} mice (Figure 5-15 A). Similar to the flow cytometry data no mRNA induction for other members of the IFN family (IFN- β , IFN- λ and IFN- γ) was detectable in whole lung samples of *Mavs*^{-/-} mice (Figure 5-15 B). In contrast to the *Mavs*^{-/-} mice, *Myd88/Trif*^{-/-} mice showed 2-3 times reduced induction of IFN- β and IFN- λ gene expression compared to wt mice and no increase of IFN- γ gene transcripts compared to naïve mice (Figure 5-15 B). The lack of IFNs in *Mavs*^{-/-} mice was consistent with an absence of interferon inducible gene (ISG) transcripts (CXCL10, RIG-I; Figure 5-15 C). The production of IFNs was insufficient for the induction of CXCL10 in *Myd88/Trif*^{-/-} mice, but RIG-I transcripts were observed to reach levels comparable to wt. In terms of proinflammatory cytokine responses to RSV infection, IL-1 β and TNF- α transcripts were not induced in both *Mavs*^{-/-} and *Myd88/Trif*^{-/-} mice compared to wt mice (Figure 5-15 D), suggesting an interplay of RLR and TLR pathways in the induction of these two cytokines. RNA and protein levels of the proinflammatory cytokine IL-6 were reduced in *Myd88/Trif*^{-/-} mice and undetectable in *Mavs*^{-/-} mice (Figure 5-15 E). Taken together, RLR-MAVS pathways are crucial for the induction of an appropriate cytokine response during RSV infection. Seemingly, the recognition of RSV through TLRs plays a minor role in early cytokine responses. Unfortunately, we experienced severe problems breeding the *Myd88/Trif*^{-/-} mice, preventing further in depth studies on the effect of this pathway on RSV-induced inflammation. Due to this limitation, the following parts of this thesis will focus on the role of RLR pathways in the induction of antiviral immune responses during RSV infection, utilising the *Ifna6^{gfp/+} Mavs*^{-/-} reporter mice. From now on all results are obtained from *Ifna6^{gfp/+} wt* (wt) and *Ifna6^{gfp/+} Mavs*^{-/-} mice (*Mavs*^{-/-}).

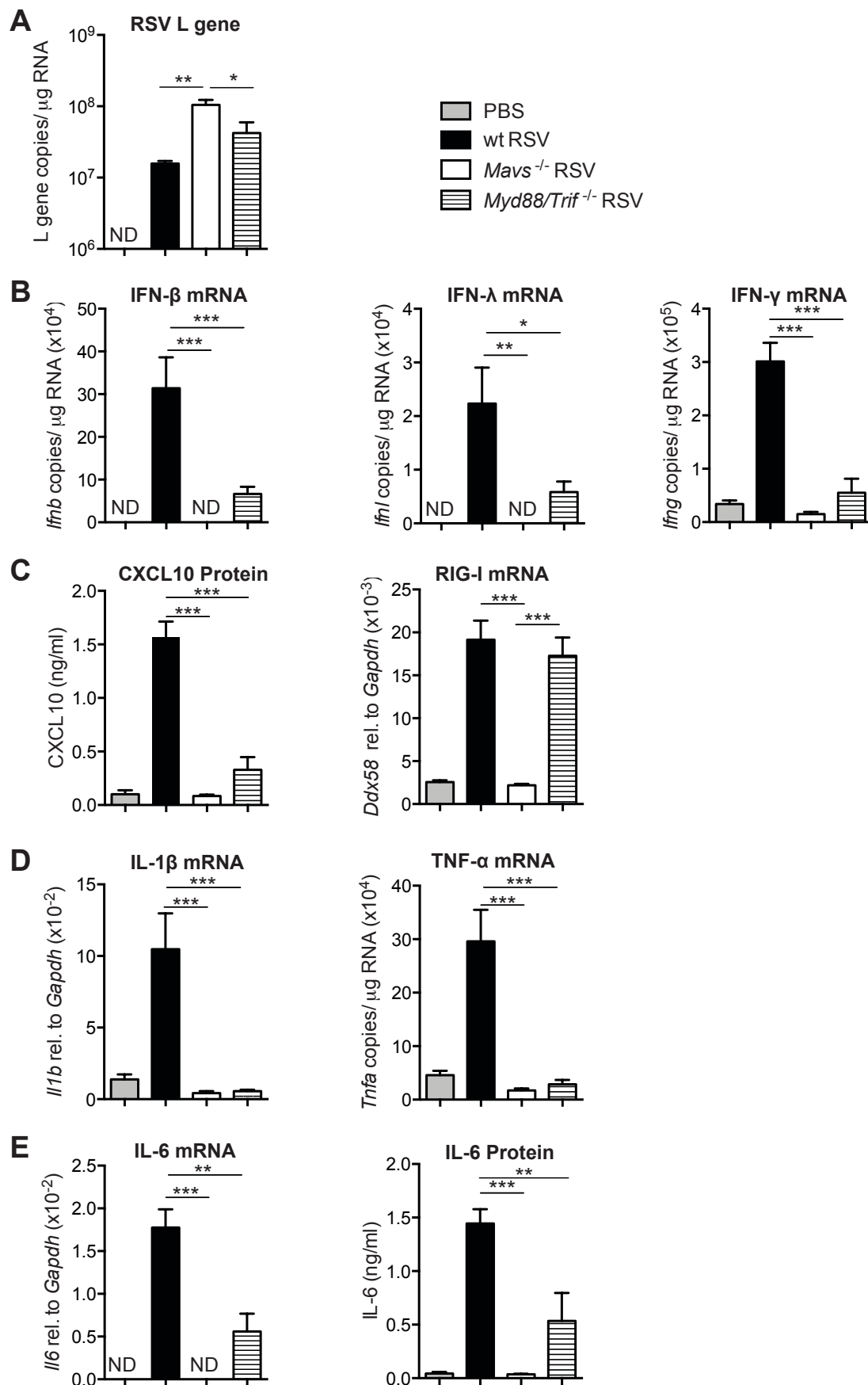


Figure 5-15 Early cytokine production in PRR adaptor deficient mice during RSV infection. *Ifna6^{gfp/+}* wt, *Ifna6^{gfp/+}* *Mavs*^{-/-} and *Ifna6^{gfp/+}* *Myd88/Trif*^{-/-} mice were intranasally infected with 2×10^6 focus forming units

(FFU) of RSV or mock infected (PBS, t=0), and sacrificed 18 hrs p.i. A) Viral load was determined by quantification of viral L gene copies in lungs of RSV-infected mice. B) RNA expression of *Ifnb*, *Ifnl* and *Ifng* was determined by qPCR. C) CXCL10 protein expression in lung homogenate was measured by ELISA and *Ddx58* RNA levels in lung tissue were determined by qPCR. D) *Il1b* and *Tnfa* transcript levels were determined by qPCR. E) *Il6* RNA levels in lung tissue were determined by qPCR, and IL-6 protein levels in lung homogenate were measured by ELISA. Copy numbers for RSV L gene, *Ifnb*, *Ifnl*, *Ifng*, and *Tnfa* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Il1b*, *Il6* and *Ddx58*. Data are representative of at least two independent experiments with 3 to 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by One-way ANOVA with Tukey's post hoc test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, ND; not detectable.

To further dissect the kinetics of type I IFN responses in AMs during RSV infection in *Ifna6gfp/+* wt compared to *Ifna6gfp/+ Mavs^{-/-}* mice time course experiments focusing on early immune responses (8hrs p.i. up to day 4) were performed. In wt mice, GFP-reporter expression was detectable in AMs at 8 hrs p.i., increasing constantly until it peaked at 18 hrs p.i., and was almost undetectable by day 4 p.i.. The kinetics and the total numbers of GFP-reporter⁺ AMs accounted fully for the observed GFP⁺ events in pulmonary CD45⁺ cells (see 5.2.1), emphasising the important role of AMs as the major source of type I IFNs in RSV infection. On the contrary, no GFP-reporter⁺ AMs were detectable at any time p.i. in the lungs of *Mavs^{-/-}* mice (Figure 5-16 A). Previous studies using Newcastle disease virus showed abrogated IFN- α responses in AMs from *Mavs^{-/-}* mice, which led to compensatory IFN- α secretion from pDCs (Kumagai et al., 2007). In our study, no GFP-reporter expression was observed in any lung cell in *Mavs^{-/-}* mice at any of the time points studied during RSV infection (Figure 5-16 B; data for day 5- day 9 p.i. not shown). The lack of eGFP reporter activation in AMs of *Mavs^{-/-}* mice reflects a global deficiency in antiviral IFN responses in the lung after RSV infection. No protein induction of other type I IFNs, such as IFN- α 3 and IFN- β , was detectable in *Mavs^{-/-}* mice compared to infected wt, which showed detectable expression of IFN- α 3 and IFN- β at 18 hrs p.i. (Figure 5-16 C). Also, no gene expression of other members of the IFN family (IFN- λ , IFN- γ) was observed in RSV challenged *Mavs^{-/-}* mice, which could otherwise compensate for the loss of type I IFN production (Figure 5-16 D). In agreement with lack of interferon expression, the induction of gene transcripts for interferon inducible genes (ISGs; Viperin, OAS1a, PKR, MX-1) was abrogated in *Mavs^{-/-}* mice compared to wt (Figure 5-16 E).

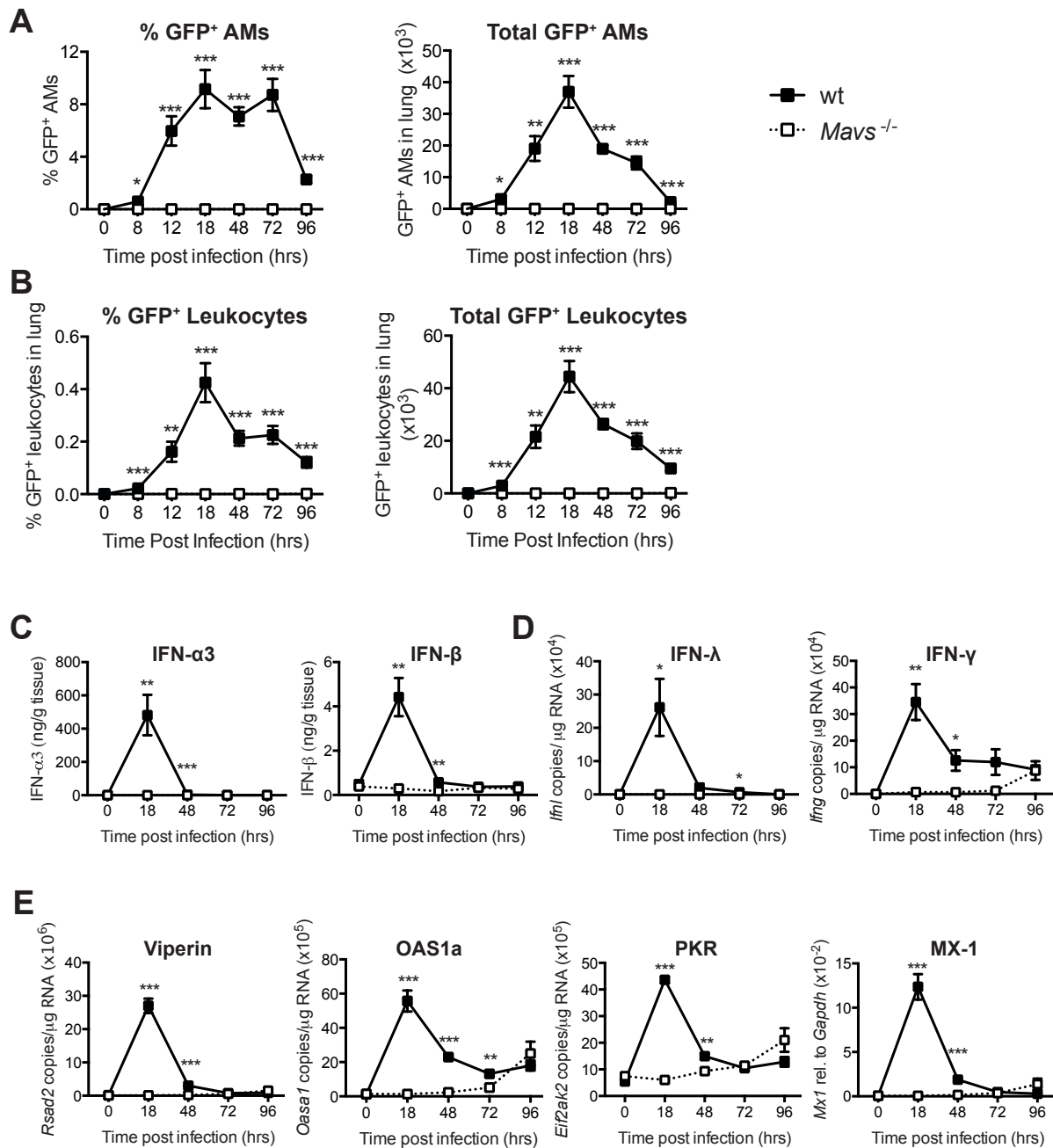


Figure 5-16 Type I IFN production is dependent on MAVS. *Ifna6^{gfp/+}* wt and *Ifna6^{gfp/+} Mavs^{-/-}* mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Percentage and number of GFP⁺ AMs in *Ifna6^{gfp/+}* wt and *Ifna6^{gfp/+} Mavs^{-/-}* mice during the time course of infection. B) Percentage and numbers of GFP-reporter expressing pulmonary leukocytes in infected *Ifna6^{gfp/+}* mice wt and *Ifna6^{gfp/+} Mavs^{-/-}* mice. C) IFN-α3 and IFN-β protein expression in lung homogenate from wt and *Mavs^{-/-}* mice were determined by Luminex analysis. RNA expression of D) *Ifnl*, *Ifng* E) *Rsad2*, *Oasa1*, *Eif2ak2* and *Mx1* was determined by qPCR. Copy numbers for *Ifnl* and *Ifng* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Rsad2*, *Oasa1*, *Eif2ak2* and *Mx1*. Data are representative of at least two independent experiments with 5 mice per group. Results are presented as mean ± SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

5.2.3. MAVS controls early cytokine responses in RSV

To examine whether the abrogated type I IFN responses in *Mavs*^{-/-} mice had an effect on the viral clearance and disease severity, the differences in viral load and viral induced pathology in infected *Ifna6^{gfp/+}* wt (further referred to as wt) and *Ifna6^{gfp/+}* *Mavs*^{-/-} mice (further referred to as *Mavs*^{-/-}) were compared. The lungs of *Mavs*^{-/-} mice were more permissive to RSV infection and replication than wt lungs. At almost all indicated time points, the viral load was significantly higher in *Mavs*^{-/-} mice compared to wt mice (Figure 5-17 A). In terms of pathology, RSV infection induced 10-13 % weight loss in *Mavs*^{-/-} mice at day 6 and 7 p.i. compared with only 4 % observed in wt mice (Figure 5-17 B).

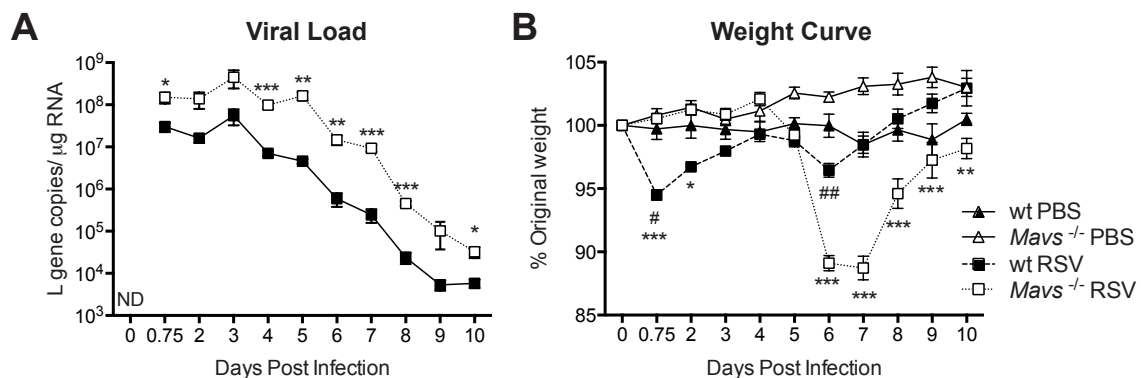


Figure 5-17 *Mavs*^{-/-} mice are more permissive to RSV replication and display increased weight loss after RSV infection compared to wt mice. Wt and *Mavs*^{-/-} mice were infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Viral load was determined by quantification of viral L gene copies in lungs of RSV-infected mice. B) Weight was monitored daily and plotted as a percentage of original weight. Statistically significant differences comparing uninfected mice with infected wt mice are represented by '#'; comparing wt mice and *Mavs*^{-/-} mice represented by '*'. Data are pooled from two independent experiments with 5 mice per group. Results are presented as mean ± SEM. Statistical significance of differences between groups was determined by A) two-tailed, unpaired Student's t-test, and B) 2-Way ANOVA with Bonferroni's post hoc test. *, P≤0.05; **, P≤0.01; ***, P≤0.001; ##, P≤0.01; #, P≤0.01; ND, not detectable.

To test whether the increased weight loss in *Mavs*^{-/-} mice was due to increased proinflammatory cytokine production the gene expression levels of TNF-α, IL-6 and IL-1β were determined. The mRNA levels of these proinflammatory cytokines were highly induced 18 hrs p.i. in wt mice and their expression levels declined later during infection.

However, no proinflammatory cytokine gene expression was detectable in *Mavs*^{-/-} mice at any time point early during RSV infection (Figure 5-18).

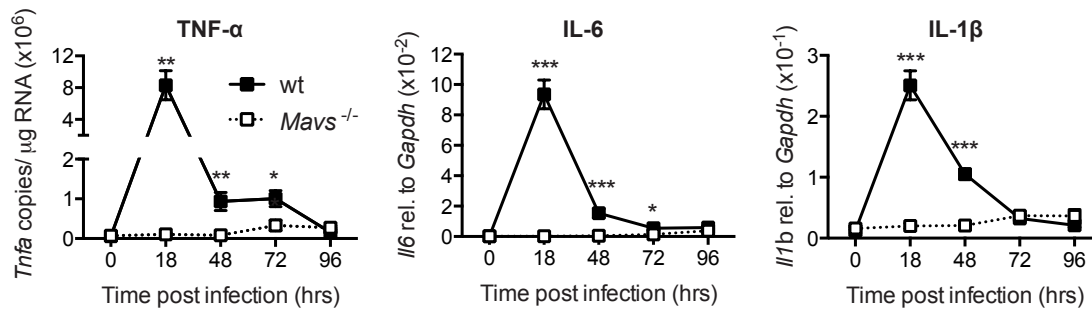


Figure 5-18 Proinflammatory cytokine induction in the lung of RSV infected mice is MAVS dependent.

Wt and *Mavs*^{-/-} mice were infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Gene expression for *Tnfa*, *Il6* and *Il1b* was determined by qPCR. Copy numbers for *Tnfa* was determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Il6* and *Il1b*. Data are representative of at least two independent experiments with 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, $P \leq 0.05$; **, $P \leq 0.01$.

In order to provide a more detailed picture of cytokine and chemokine responses induced during RSV infection in *Mavs*^{-/-} and wt mice, multiplex assay analysis were performed. Induction of the chemoattractants: CCL2, CXCL1, CXCL9 and CXCL10 in lung homogenate were observed in wt mice with a peak of production at 18 hrs p.i. except for CXCL10 peaking already at 12 hrs p.i. (Figure 5-19 A). A similar protein expression pattern in the lung of wt mice was present for the interleukins: IL-1 α , IL-5, IL-6 and IL12p40/p70 (Figure 5-19 B). None of these chemokines or interleukins were detectable at any time points in *Mavs*^{-/-} mice (Figure 5-19). Protein levels of GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-10, IL-13 and TNF- α were undetectable in any of the processed lung samples (data not shown).

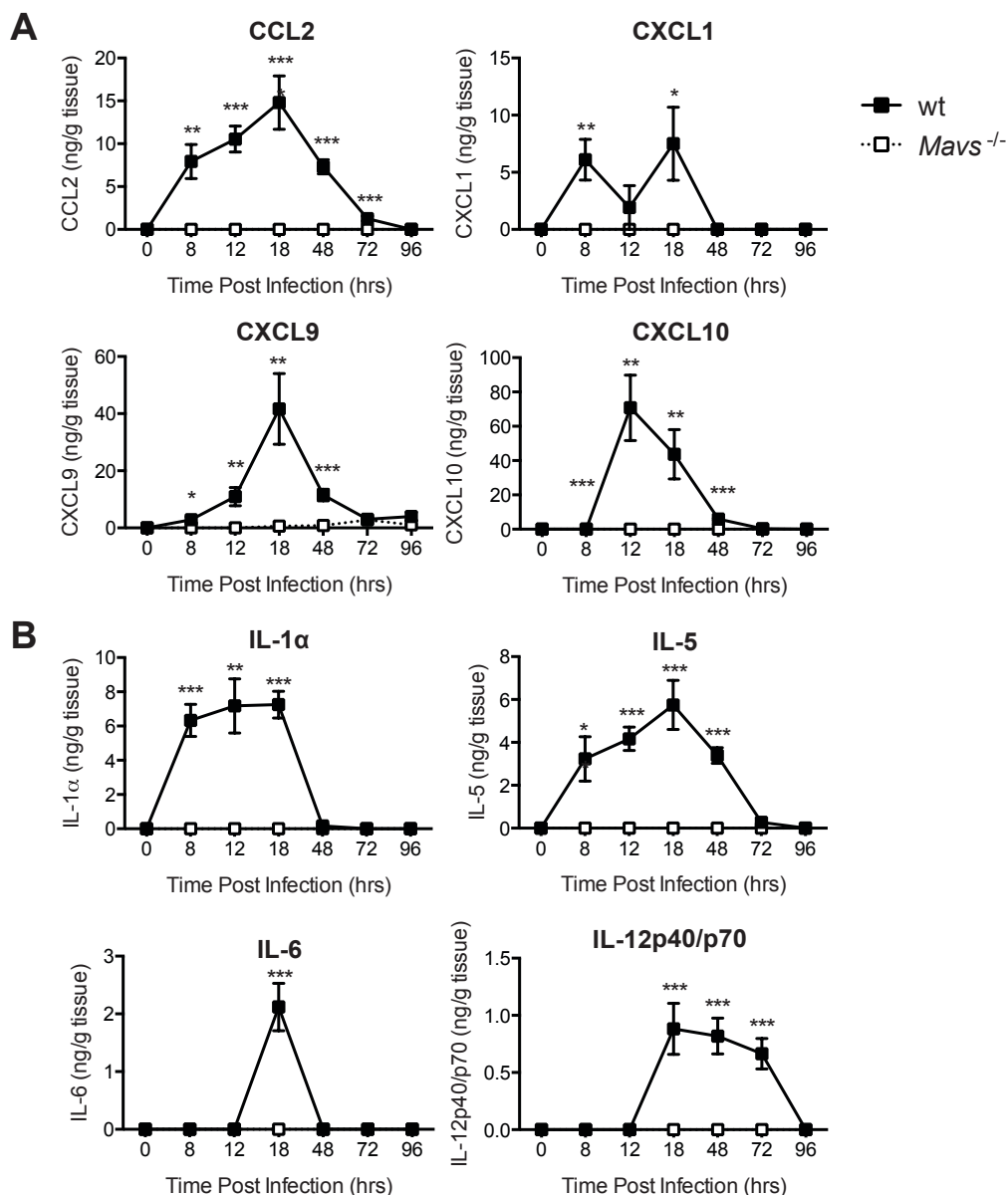


Figure 5-19 Abrogated proinflammatory cytokine protein expression in the lung of *Mavs*^{-/-} mice during RSV infection. Wt and *Mavs*^{-/-} mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Protein expression levels in lung homogenate for A) CCL2, CXCL1, CXCL9, and CXCL10, and B) IL-1 α , IL-5, IL-6, and IL-12p40/p70 were determined by Luminex. Data shown are pooled from two individual experiments with 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

5.2.4. Inflammatory CD64^{hi} cells are recruited to the lungs during RSV infection

In parallel to viral load and cytokine release, the effect of RSV infection on the cellular composition and the immune cell recruitment into the infected lungs was assessed, using the gating strategy shown in Figure 5-10. Upon RSV infection increased recruitment of leukocytes was detected in wt mice, whereas *Mavs*^{-/-} mice displayed no considerable changes in leukocyte numbers (Figure 5-20 A). Within the airways there is a highly developed network of different resident DC subsets (Lambrecht and Hammad, 2012). In response to RSV infection, CD103⁺ DCs were recruited to the lung of wt mice peaking at 18 hrs p.i. (Figure 5-20 B). Their numbers dramatically decline by 48 hrs p.i., presumably fulfilling their function of priming T cells by leaving the lungs and migrating to the lung-draining lymph nodes (LN). The CD103⁺ DC population in *Mavs*^{-/-} mice was unchanged during the course of infection (Figure 5-20 B). Uninfected *Mavs*^{-/-} mice showed significantly higher numbers of residing CD11b⁺ DCs in the lung than uninfected wt mice (Figure 5-20 C). In the course of infection an increase in CD11b⁺ DC numbers was observed in *Mavs*^{-/-} mice by 18 hrs p.i. and then slowly coming back to baseline levels. For wt mice the numbers of CD11b⁺ DCs stayed on baseline levels (Figure 5-20 C). The recruitment of pDCs was comparable in both strains of mice and peaked at 48 hrs p.i. (Figure 5-20 D). The number of AMs significantly decreased during RSV infection in wt mice compared to *Mavs*^{-/-} mice where numbers remained unchanged (Figure 5-20 E). Eosinophil numbers in wt mice did not change considerably during the course of infection, whereas an increase in eosinophil numbers was seen in *Mavs*^{-/-} mice (Figure 5-20 F). A reduced recruitment of neutrophils at 8 and 12 hrs p.i. was observed in *Mavs*^{-/-} mice compared to wt mice. Yet, this was not comparable to the rapid recruitment seen in wt mice peaking much earlier at 8 hrs p.i. and declining thereafter (Figure 5-20 G).

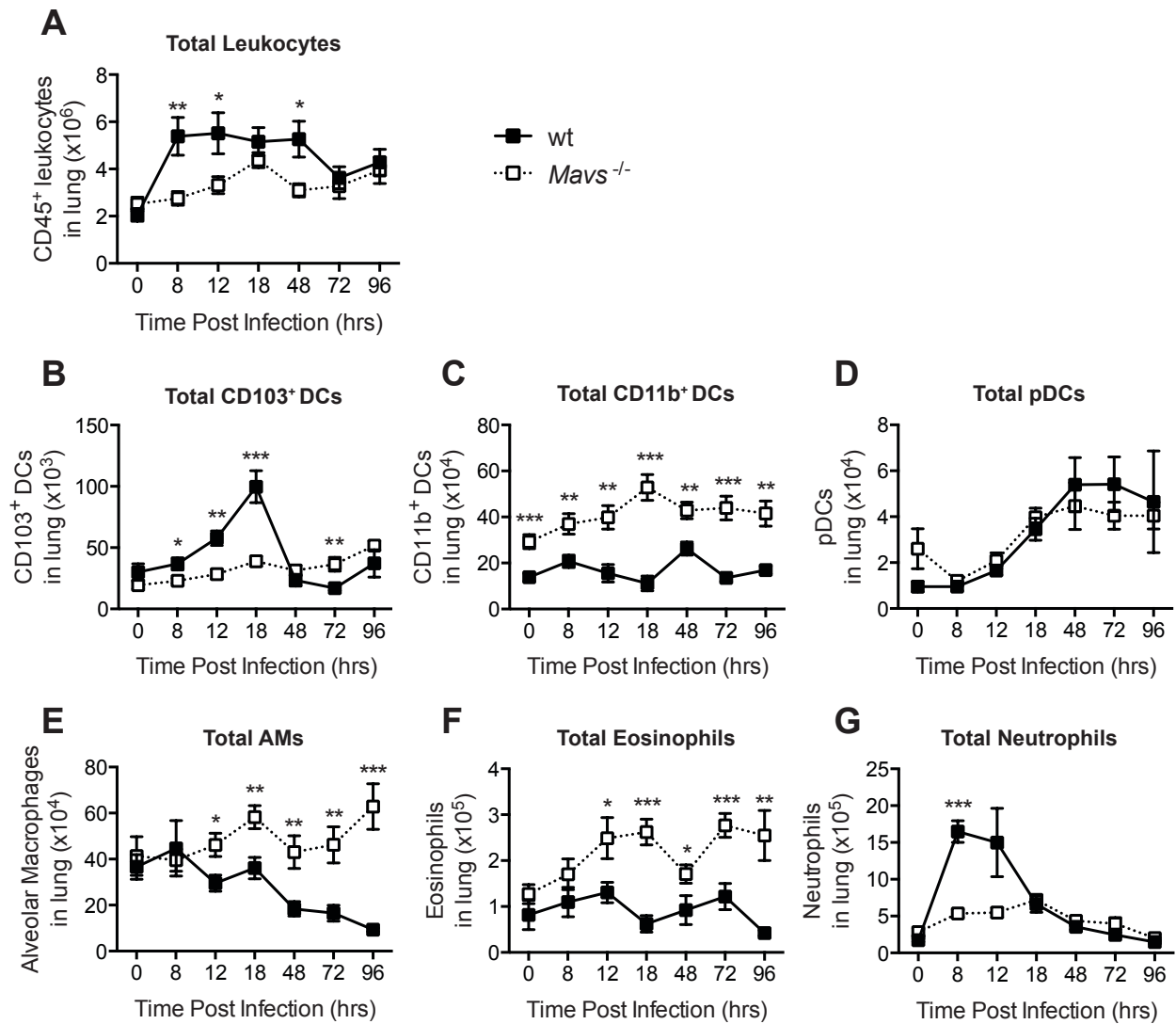


Figure 5-20 Dynamics of innate lung cell populations in wt and *Mavs*^{-/-} mice early during RSV infection. Wt and *Mavs*^{-/-} mice were infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). Lung cells were obtained by collagenase digestion of lung tissue and stained for surface expression of B220, CD11b, CD11c, CD45, Cd64, Ly6C, Ly6G, Siglec-F, and Siglec-H. Total numbers of A) CD45⁺ leukocytes, B) CD103⁺ DCs, C) CD11b⁺ DCs, D) pDCs, E) AMs, F) eosinophils, and G) neutrophils in the lung of wt and *Mavs*^{-/-} mice at indicated time points. Cell populations in the lung were analysed by flow cytometry. Data are representative of at least two independent experiments with 5 mice per group. Results are presented as mean ± SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, P≤0.05; **, P≤0.01; ***, P≤0.001.

A large proportion of lung cells stained positive for the surface marker CD64 and CD11b in wt mice resembling inflammatory monocytes (Figure 5-21 A) (Hammad et al., 2010; Plantinga et al., 2013). A failure in recruitment of these CD64^{hi} inflammatory cells was detected in the lungs of RSV-infected *Mavs*^{-/-} mice, although blood monocyte numbers were equivalent between the strains (Figure 5-21 A and B). The inflammatory monocytes recruited from the blood accumulated in the lung during infection and represented ~40 % of the total leukocyte population at day 2 p.i. in a wt lung (Figure 5-21 C). The recruitment of leukocytes, such as monocytes, is the consequence of the secretion of chemokines important for cell attraction. The chemoattractant CCL2 critical for monocyte egress from the bone marrow and together with CCL7 and CCL12 are mediating the recruitment into inflamed tissue (Struyf et al., 1998; Tsou et al., 2007). All three chemokines were induced after infection in wt mice, but a severe impairment in the induction of CCL2 protein and CCL7 and CCL12 mRNA was observed in *Mavs*^{-/-} mice, explaining the complete lack of CD64^{hi} inflammatory monocytes (Figure 5-21 D and E).

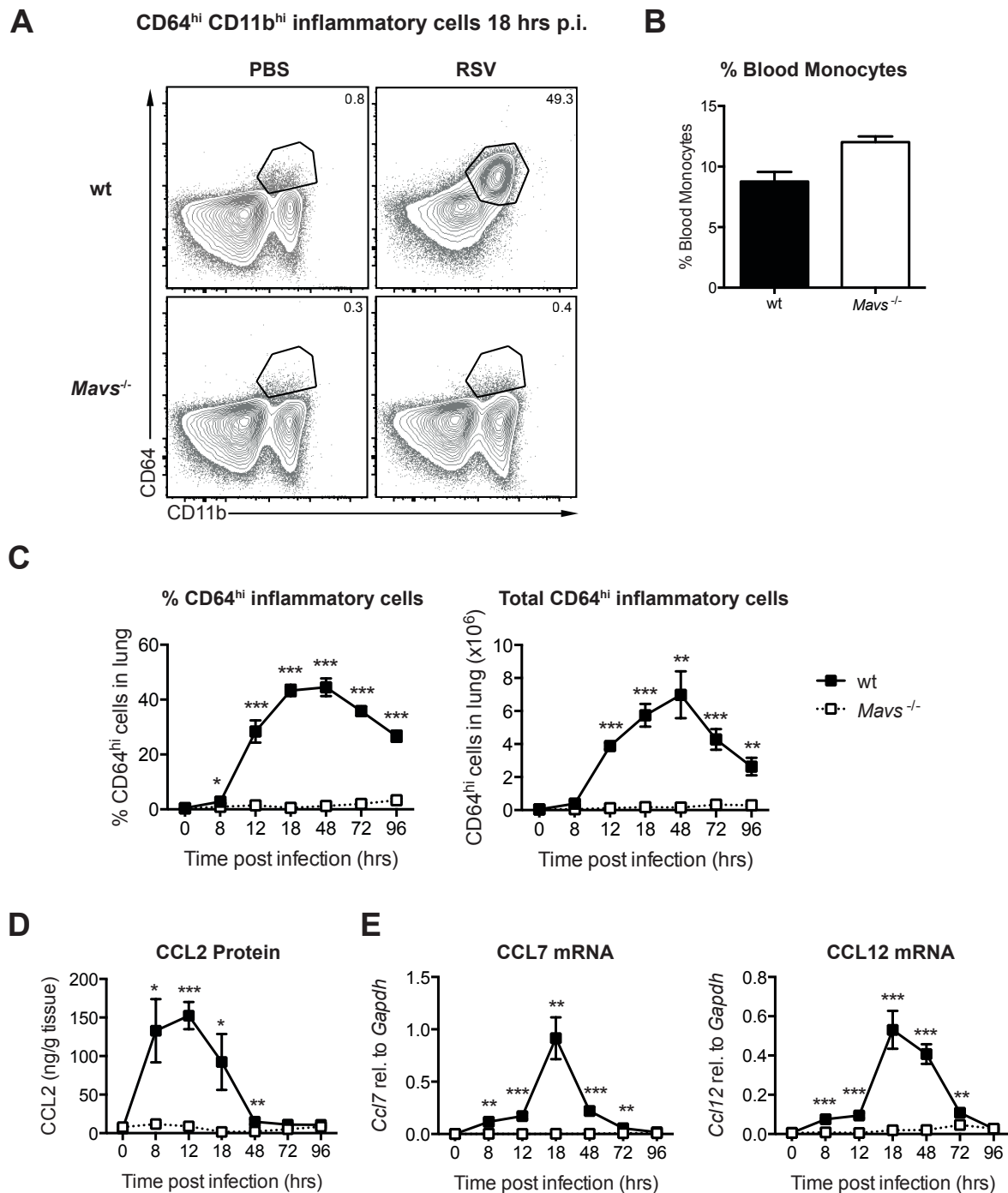


Figure 5-21 Diminished recruitment of CD64^{hi} inflammatory into the lung of *Mavs*^{-/-} mice during RSV infection. Wt and *Mavs*^{-/-} mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Representative plots of CD64^{hi}CD11b^{hi} inflammatory cells 18 hrs p.i. in wt and *Mavs*^{-/-} mice infected with RSV or mock infected with PBS. B) Percentage of Ly6C^{hi}CD11b^{hi} monocytes in the blood. C) Percentage and number of CD64^{hi} cells in the lung. D) CCL2 protein expression in lung homogenate was determined by ELISA. E) Gene expression levels of *Ccl7* and *Ccl12* in lung tissue was measured by qPCR and calculated relative to *Gapdh*. Data are representative of at least two independent experiments with 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

CD64^{hi} inflammatory cells express high levels of Ly6C, a marker often used to classify cells of monocytic origin in tissue ((Egawa et al., 2013; Grainger et al., 2013; Zigmond et al., 2012)) (Figure 5-22 A). Consistent with previous results in a model of house dust mite induced airway inflammation (Plantinga et al., 2013), Ly6C expression was progressively downregulated on CD64^{hi} inflammatory cells during RSV infection (Figure 5-22 B). Consequently, this leads to difficulties in discriminating monocyte-derived cells from tissue resident Ly6C^{lo} CD11b^{hi} DCs. Therefore, CD64 was used as the main marker to distinguish recruited inflammatory monocytes from Ly6C^{lo} CD11b^{hi} DC subsets in the lung (Plantinga et al., 2013). The differential expression of CD11c further subdivided CD64^{hi} inflammatory cells into previously described monocyte-derived DCs (moDCs; CD64^{hi}CD11b^{hi}CD11c^{hi}; (Segura and Amigorena, 2013)) and inflammatory monocytes (infMo; CD64^{hi}CD11b^{hi}CD11c^{lo}) (Dunay et al., 2008), (Figure 5-22 A and C). In the early event of infection most CD64^{hi} inflammatory cells expressed low levels of CD11c and were defined as infMo. By day 2 p.i., the majority of CD64^{hi} inflammatory cells expressed CD11c and could be classified as moDCs (Figure 5-22 C).

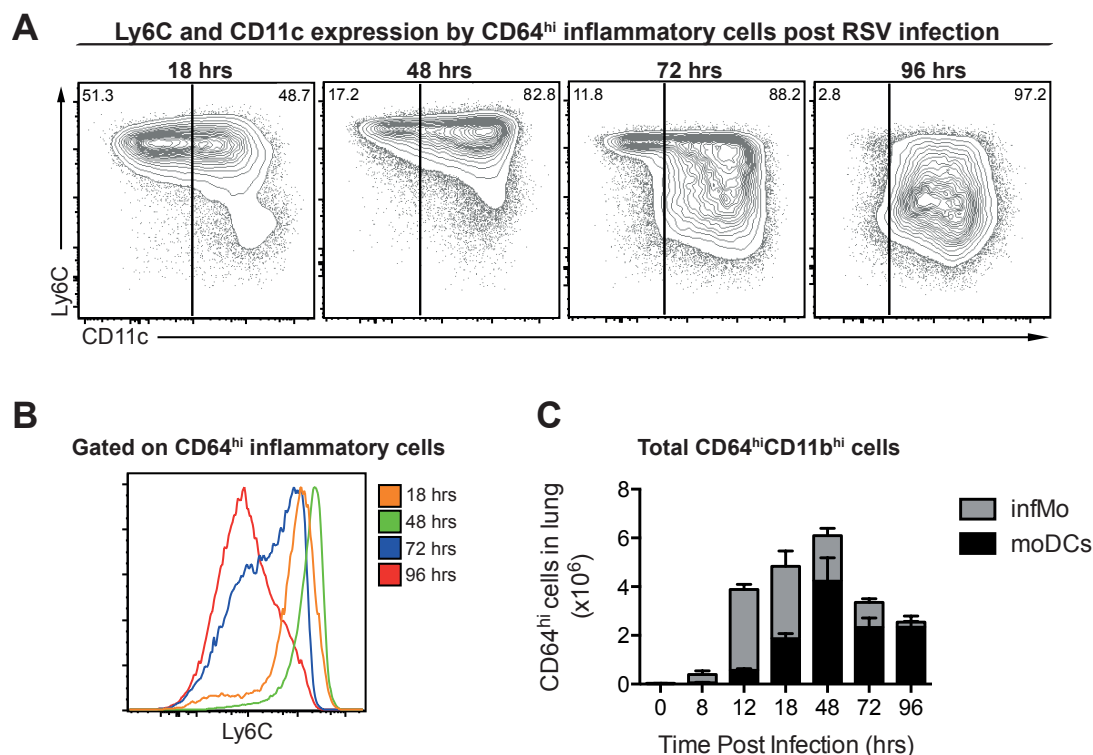


Figure 5-22 CD64^{hi} inflammatory cells consist of monocyte-derived DCs and inflammatory monocytes. Wt mice were intranasally infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Representative plots of Ly6C and CD11c expression by CD64^{hi}CD11b^{hi} inflammatory cells at indicated time points. B) Representative histograms of Ly6C expression by CD64^{hi}CD11b^{hi} inflammatory cells at indicated

time points. C) Number of monocyte-derived DCs (CD11c^{hi}) and inflammatory monocytes (CD11c^{lo}) that constitute CD64^{hi}CD11b^{hi} inflammatory cells. Data are representative of at least two independent experiments with 5 mice per group.

5.2.5. Type I IFN production by AMs leads to CCL2 dependent recruitment of CD64^{hi} inflammatory cells

Type I IFNs have been implicated to drive the secretion of the monocyte attractant CCL2 (Conrady et al., 2013; Majer et al., 2012). To examine whether the absence of CD64^{hi} inflammatory cells in *Mavs*^{-/-} mice was due to the failure of AMs to produce IFN- α , 4x10⁵ wt AMs or *Mavs*^{-/-} AMs were transferred i.n. into *Mavs*^{-/-} mice 24 hrs prior to RSV infection. A small but not significant induction of IFN- α was detected in *Mavs*^{-/-} mice receiving wt AMs on day 18 hrs p.i. (2 out of 5 mice had detectable levels of IFN- α ; Figure 5-23 A). Nevertheless, mRNA levels for CCL2 were elevated 48 hrs p.i. (Figure 5-23 B) indicating a partial rescue of local CCL2 levels. Although the CCL2 expression did not reach the levels observed in wt mice it was enough to partially restore the recruitment of CD64^{hi} inflammatory cells into the lungs of *Mavs*^{-/-} mice transferred with wt AMs; *Mavs*^{-/-} mice transferred with *Mavs*^{-/-} AMs remained incompetent to recruit monocytes (day 2 p.i.) (Figure 5-23 C and D). A hallmark of the CD64^{hi}CD11c^{hi} moDC subtype activity is the secretion of TNF- α (Serbina et al., 2003). An augmented transcription of TNF- α mRNA was detected in *Mavs*^{-/-} mice transferred with wt AMs compared to RSV-infected *Mavs*^{-/-} mice receiving *Mavs*^{-/-} AMs. In addition, the local production of IFN- γ was restored back to wt levels in *Mavs*^{-/-} mice that received wt AMs (Figure 5-23 E).

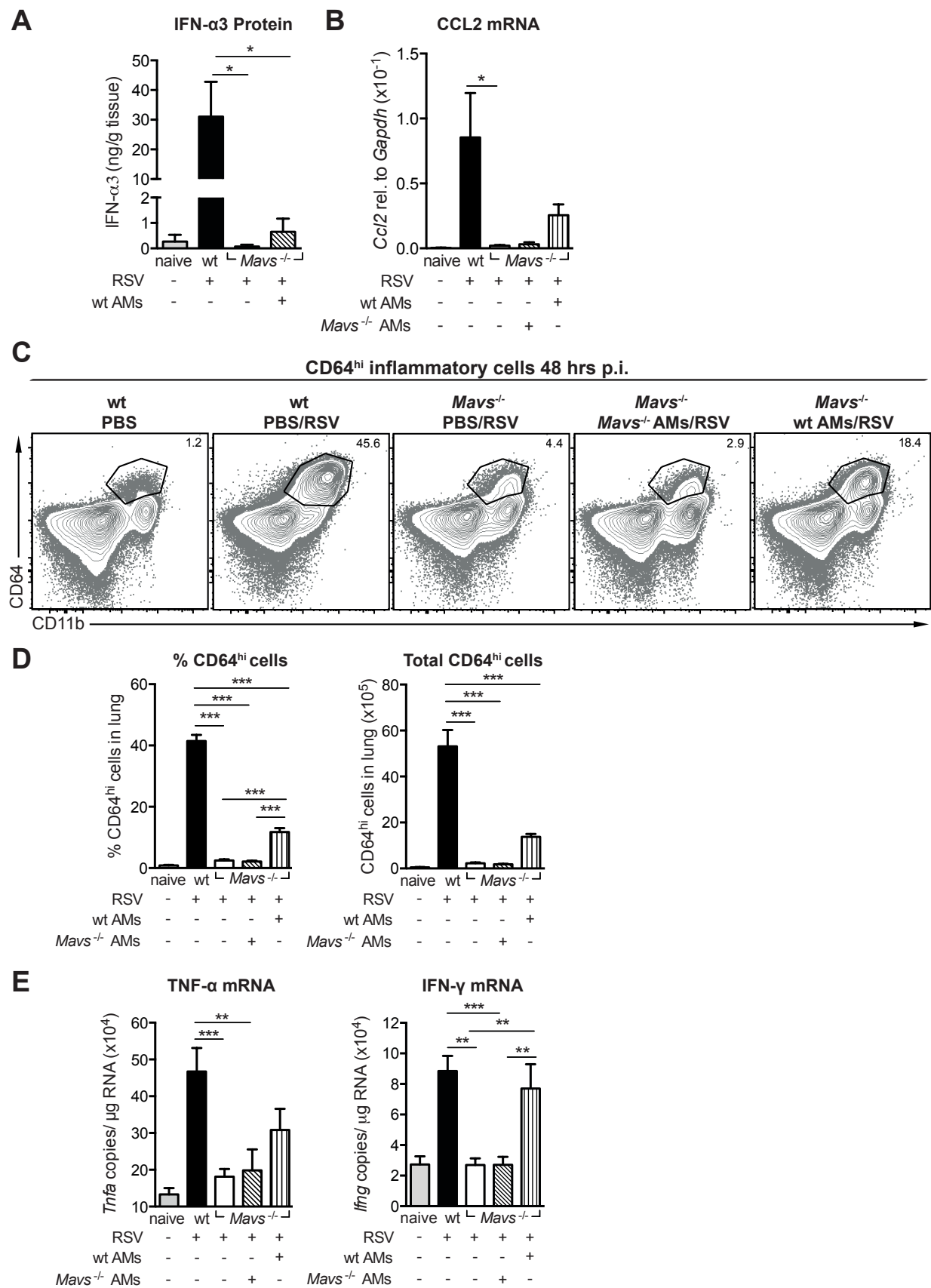


Figure 5-23 Partially restored recruitment of CD64^{hi} inflammatory cells into the lung of *Mavs*^{-/-} mice adoptively transferred with wt AMs prior to RSV infection. *Mavs*^{-/-} mice were intranasally adoptively

transferred with 4×10^5 AMs obtained from either wt or *Mavs*^{-/-} mice. 24 hrs post transfer mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0, stated as naïve). A) Protein levels of IFN- α 18 hrs p.i. in lung homogenate were measured by Luminex. Data show one experiment with 5 mice per group. B) Gene expression levels of *Ccl2* in the lung on day 2 p.i. were determined by qPCR and calculated relative to *Gapdh*. C) Representative plots of CD64^{hi}CD11b^{hi} inflammatory cells on day 2 p.i. in AM transferred mice. D) Percentage and number of recruited CD64^{hi} inflammatory cells on day 2 p.i. in AM transferred mice. E) Gene transcript levels in lung for *Tnfa* and *Ifng* were determined by qPCR on day 2 p.i.. Copy numbers were determined using a plasmid standard. Data represent pooled data from two independent experiments with 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by One-way ANOVA with Tukey's post hoc test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Reconstitution assays were performed in order to test whether the recruitment of CD64^{hi} inflammatory cells was a result of IFN- α production by wt AMs in *Mavs*^{-/-} mice. To substitute for the lack of type I IFN production *Mavs*^{-/-} mice received intranasal administrations of 500 ng recombinant IFN- α (rIFN- α) 6hrs post RSV infection. On day 2 p.i. an increase in CCL2 mRNA levels was measured in infected *Mavs*^{-/-} mice treated with rIFN- α . This confirmed the dependency of type I IFN signalling for CCL2 expression (Figure 5-24 A). This also coincided with the detection of CD64^{hi} inflammatory cells in the lungs of rIFN- α inoculated *Mavs*^{-/-} mice (Figure 5-24 B and C). Together with the partially restored recruitment of CD64^{hi} inflammatory cells, the gene expression levels of TNF- α and IFN- γ rose in infected, rIFN- α treated *Mavs*^{-/-} mice compared to infected control mice. Confirming the results obtained from the AM transfer and implicating a failure in AM type I IFN production as cause for the lack of monocyte recruitment during RSV infection of *Mavs*^{-/-} mice.

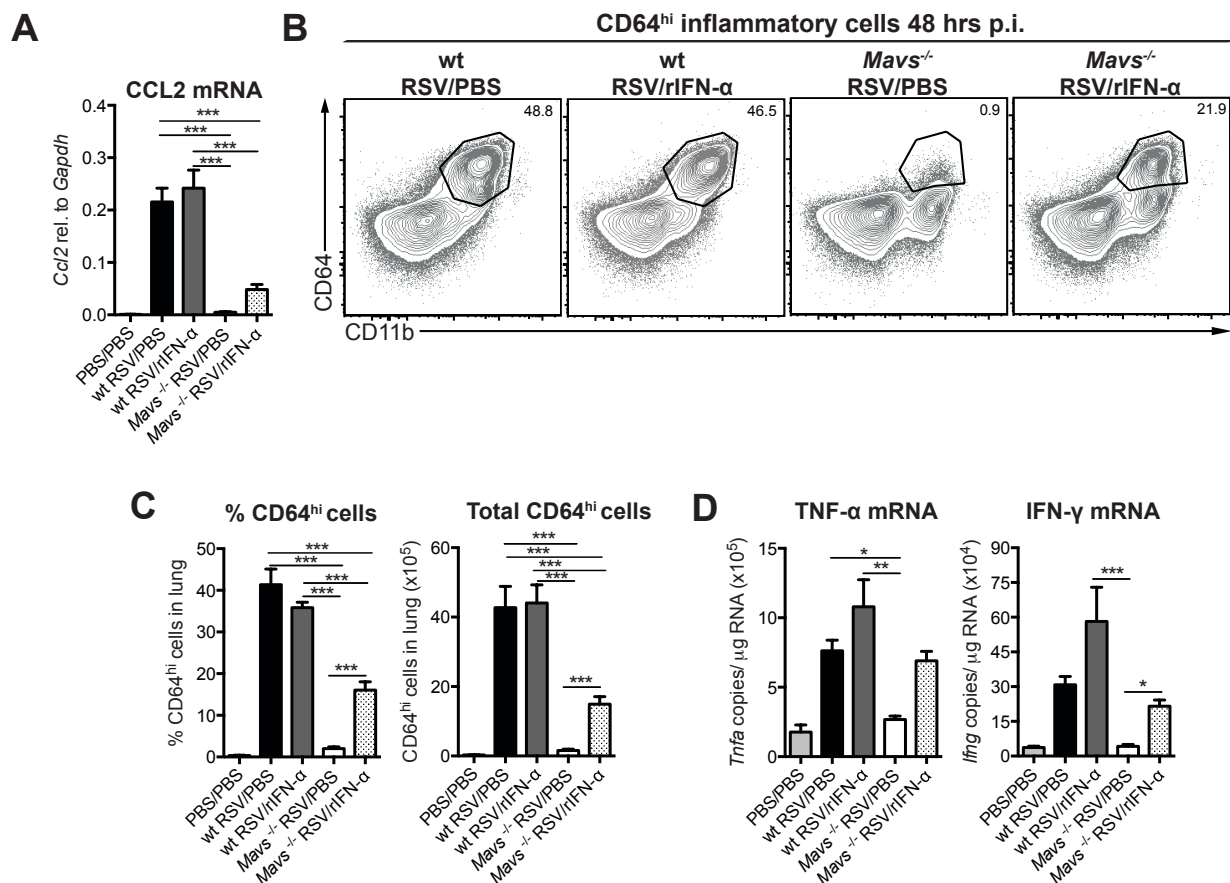


Figure 5-24 Recombinant IFN-α is sufficient to restore the recruitment of CD64^{hi} inflammatory cells into the lungs of *Mavs*^{-/-} mice after RSV infection. Wt and *Mavs*^{-/-} mice were infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). Six hours p.i. mice were intranasally inoculated with recombinant IFN-α11 (500ng/mouse). Mice were sacrificed 48 hrs p.i. A) Gene expression of *Ccl2* in lung tissue was determined by qPCR and calculated relative to *Gapdh*. B) Representative plots of CD64^{hi}CD11b^{hi} inflammatory cells recruited to the lung. C) Percentage and number of recruited CD64^{hi} inflammatory cells in the lung. D) Gene transcript levels in lung for *Tnfa* and *Ifng* were determined by qPCR. Copy numbers were assessed using a plasmid standard. Data are representative of at least two independent experiments with 5 mice per group. Results are presented as mean ± SEM. Statistical significances of differences between groups were determined by One-way ANOVA with Tukey's post hoc test. *, P≤0.05; **, P≤0.01; ***, P≤0.001.

Type I IFNs produced by transferred AMs or the administration of rIFN-α into *Mavs*^{-/-} mice will have a direct antiviral effect and will complicate clear conclusions about the role of CD64^{hi} inflammatory cells in RSV infection. To bypass type I IFN responses, *Mavs*^{-/-} mice were intranasally administered 25 μg of the monocyte chemoattractant CCL2 (recombinant CCL2, rCCL2), 6 hrs p.i. On day 2 p.i. 25 % of the pulmonary leucocyte population consisted of CD64^{hi} inflammatory cells in *Mavs*^{-/-} mice after rCCL2 administration (Figure 5-25 A and B). Interestingly, in this model with only monocytes being the responsive cell type the gene

expression for TNF- α and IFN- γ was fully restored in RSV-infected, CCL2 inoculated *Mavs*^{-/-} mice (Figure 5-25 C).

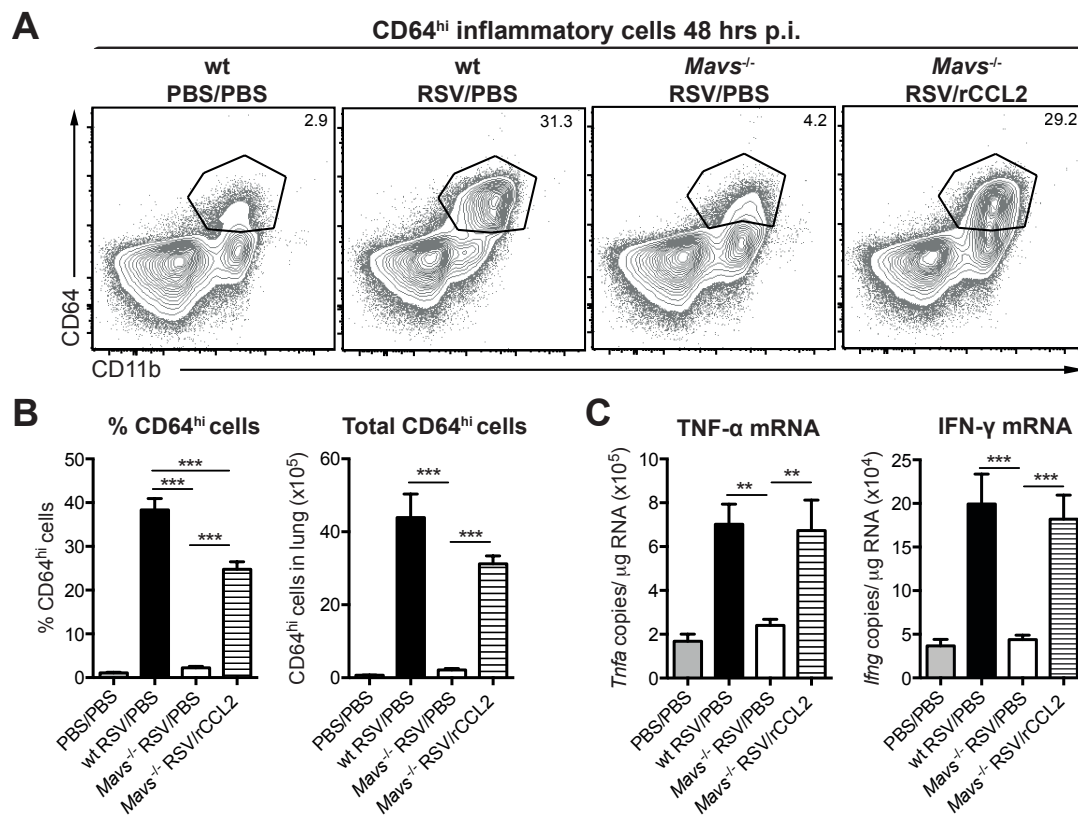


Figure 5-25 Recombinant CCL2 restores the recruitment of CD64^{hi} inflammatory cells into the lung of *Mavs*^{-/-} mice during RSV infection. Wt and *Mavs*^{-/-} mice were infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). Six hours p.i. mice were intranasally challenged with recombinant CCL2 (25μg/mouse). Mice were sacrificed 48 hrs p.i. A) Representative plots of CD64^{hi}CD11b^{hi} inflammatory cells recruited to the lung. B) Percentage and number of recruited CD64^{hi} inflammatory cells in the lung. C) Gene transcript levels in lung for *Tnfa* and *Ifng* were determined by qPCR. Copy numbers were determined using a plasmid standard. Data represent pooled data from two independent experiments with 5 mice per group. Results are presented as mean ± SEM. Statistical significances of differences between groups were determined by One-way ANOVA with Tukey's post hoc test. ***, P≤0.001; **, P≤0.01.

The mobilisation of monocytes from the bone marrow into the blood and from the blood into inflamed tissue is CCR2-dependent (Tsou et al., 2007). To prove the importance of inflammatory monocytes in the antiviral response, blood monocytes in wt mice were depleted with an antibody against CCR2 (Mack et al., 2001). The antibody was administered intraperitoneally 8 hrs prior to RSV infection and on day 1 p.i. to prevent recruitment of CD64^{hi} inflammatory cells into the lung; the depletion of Ly6C^{hi}CD11b^{hi} blood monocytes was confirmed by flow cytometry (Figure 5-26 A and B). Analysis of lung

tissue revealed a complete absence of inflammatory monocytes in mice treated with anti-CCR2 antibody compared to isotype controls were $\sim 35\%$ of pulmonary leukocytes consisted of CD64^{hi} inflammatory cells (Figure 5-26 C and D). In support of the notion that monocytes have a direct or indirect role in TNF- α induction during RSV infection, a more than 2 fold reduction of TNF- α gene expression in monocyte-depleted mice was observed. However, no differences were detectable for IFN- γ (Figure 5-26 E).

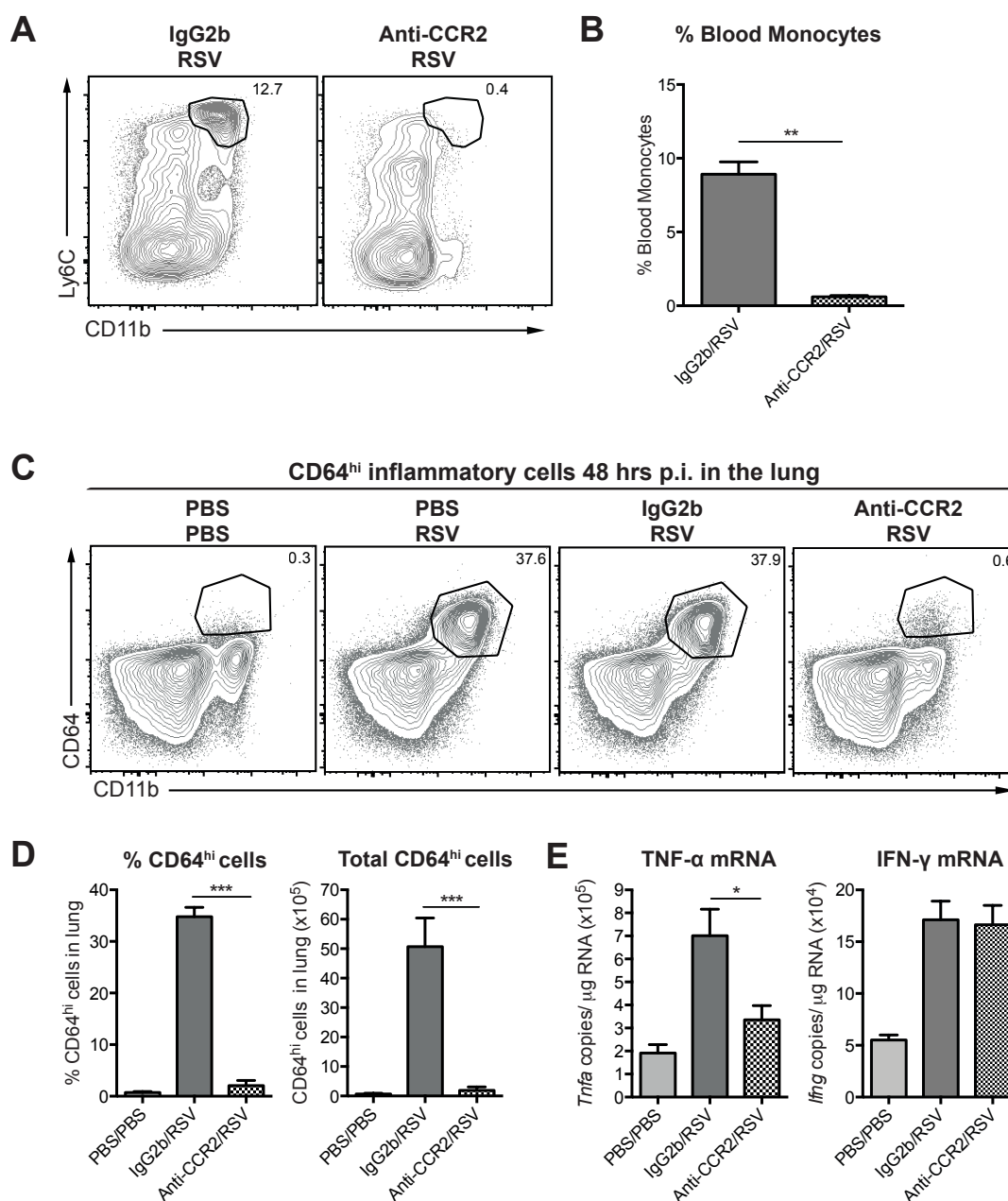


Figure 5-26 Depletion of blood monocytes abrogates recruitment of CD64^{hi} inflammatory cells into the lung of wt mice during RSV infection. C57BL/6 mice were treated with PBS or 20 μ g anti-CCR2 (clone MC21) or isotype antibody (IgG2b, clone A95-1) intraperitoneal at 8 hrs prior to RSV infection and 24 hrs p.i..

Wt mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0), and sacrificed 48 hrs p.i. A) Representative plots and quantification of Ly6C^{hi}CD11b^{hi} monocytes in the blood 24 hrs after last injection of anti-CCR2 or isotype antibody. B) Quantification of blood monocytes in anti-CCR2 and isotype control treated mice. C) Representative plots of CD64^{hi}CD11b^{hi} inflammatory cells recruited to the lung. D) Percentage and number of recruited CD64^{hi} inflammatory cells in the lung. E) Gene transcript levels in lung for *Tnfa* and *Ifng* were determined by qPCR. Copy numbers were determined using a plasmid standard. Data represent pooled data from two independent experiments with 4 to 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by B) two-tailed, unpaired Student's t-test, and D) and E) One-way ANOVA with Tukey's post hoc test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

5.2.6. CD64^{hi} inflammatory cells limit weight loss and control viral infection

As previously described, the disease severity in *Mavs*^{-/-} mice was increased during RSV infection compared to wt mice (see Figure 5-17 B). To address the role of recruited CD64^{hi} inflammatory cells in disease severity, the weight of RSV infected *Mavs*^{-/-} mice transferred with wt or *Mavs*^{-/-} AMs was monitored for the duration of 10 days. RSV induced weight loss of around 13 % in *Mavs*^{-/-} mice transferred with *Mavs*^{-/-} AMs on day 6 p.i. was similar to non-transferred infected *Mavs*^{-/-} mice (Figure 5-27 A). Surprisingly, *Mavs*^{-/-} mice that received wt AMs only lost 3 % of weight, very similar to RSV-infected wt mice (Figure 5-27 A). The decrease in weight loss in *Mavs*^{-/-} mice could be due to monocytes restricting RSV replication as the CD11c expressing subset of CD64^{hi} inflammatory cells was recently described to restrict HSV-1 replication (Conrady et al., 2013). To test whether this is also true during RSV infection, the viral load in the lung was determined on day 2 p.i. as CD64^{hi} inflammatory cell recruitment peaks on this day (see Figure 5-21 C). Indeed, reduced gene transcript levels for the viral L and N genes were detected in *Mavs*^{-/-} mice transferred with wt AMs compared to *Mavs*^{-/-} AM transferred *Mavs*^{-/-} mice (Figure 5-27 B) indicating a restrictive function of AMs/monocytes in RSV replication.

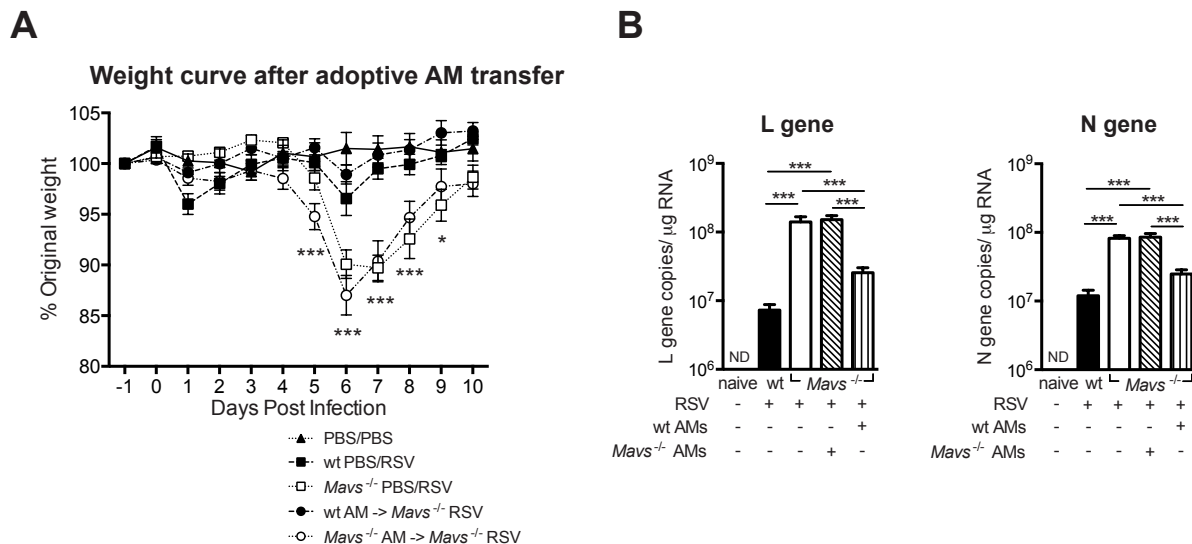


Figure 5-27 Transfer of wt AMs into *Mavs*^{-/-} mice prevents weight loss and controls RSV infection.

Mavs^{-/-} mice were adoptively transferred with 4×10^5 AMs obtained from either wt or *Mavs*^{-/-} mice. 24 hrs post transfer, mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Naive resembles PBS treated mice. A) Weight was monitored daily and plotted as a percentage of original weight. Statistically significant differences comparing infected wt-AM transferred *Mavs*^{-/-} mice with infected *Mavs*^{-/-}-AM transferred *Mavs*^{-/-} mice are shown. B) Viral load was determined by quantification of viral L and N gene copies in lungs of RSV-infected mice. Copy numbers were determined using a plasmid standard. Data are pooled from two independent experiments with 5 mice per group. Results are presented as mean $\pm$ SEM. ND=not detectable. Statistical significances of differences between groups were determined by A) 2-Way ANOVA with Bonferroni's post hoc test, and B) One-way ANOVA with Tukey's post hoc test. *, $P \leq 0.05$; ***, $P \leq 0.001$.

The impact on weight loss and viral load could be caused by a pleiotropic effect of the type I IFNs produced by the transferred wt AMs, even though no significant increase in IFN- α protein production was observed at 18 hrs p.i. (see Figure 5-23 A). To corroborate this possibility, rCCL2 was administered to RSV infected *Mavs*^{-/-} mice to examine the influence of recruited monocytes on disease severity and viral replication. An initial approach using a single dose of CCL2 6 hrs p.i., as previously described, led to no significant rescue of RSV infection mediated weight loss (data not shown). A likely cause of this could be due to the simple fact that a single ectopic application of rCCL2 cannot completely replicate the longer lasting endogenous response. To mimic a physiological production of CCL2, compared to the initial single dose experiment, CCL2 was administered at 6 hrs, 18 hrs, and 48 hrs p.i., thereby replicating the main kinetics of CCL2 production during RSV infection (see Figure 5-21 D). Administration of rCCL2 caused remarkable protection from weight loss in

infected *Mavs*^{-/-} mice, with a weight loss of about 4 % on day 6 p.i. similar to wt infected mice, compared to approximately 10 % weight loss in infected *Mavs*^{-/-} mice (Figure 5-28 A). In terms of viral load, treatment with rCCL2 led to a reduction in viral L and N gene copies already on day 2 p.i., which is the peak of CD64^{hi} inflammatory cell influx (Figure 5-28 B). Plaque assays for the detection of infectious virus in the lung were negative on day 2 p.i. in any of the mice (data not shown). Concomitant, with a study were infectious virus in BAL fluid was shown to be undetectable by day 2 using plaque assays showed a progressive increase in measurable plaques by day 3-5, indicating active viral replication at a late time point (Jafri et al., 2004). Respecting this observation, viral load plaque assays were performed on day 4 p.i. to allow for a better detection of live virus. Significant reduced levels of viral L gene and N gene were detected on day 4 p.i. in the lung of infected, rCCL2 inoculated *Mavs*^{-/-} mice compared to untreated infected controls (Figure 5-28 C) confirming the viral RNA transcript data from day 2 and 4 p.i.. But perhaps more convincing for a role of CD64^{hi} inflammatory cells in controlling viral load, indirect or direct, was the reduction of infectious virus in the lung by a factor of 10 compared to non-inoculated RSV infected *Mavs*^{-/-} mice (Figure 5-28 D).

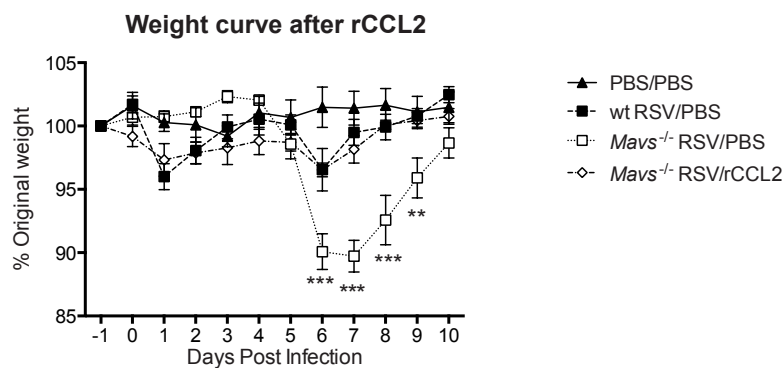
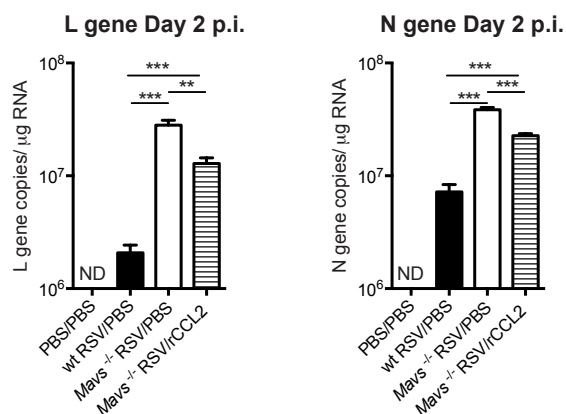
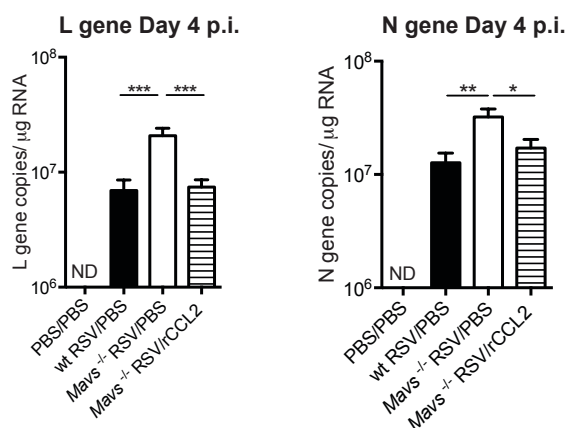
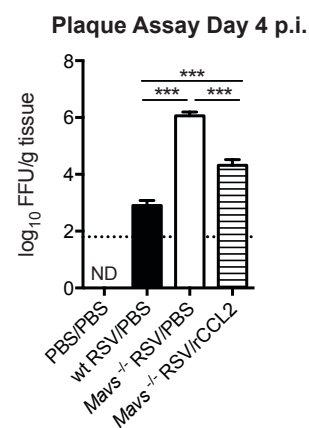
A**B****C****D**

Figure 5-28 CCL2 recruited CD64^{hi} inflammatory monocytes prevent weight loss and reduce viral load in *Mavs*^{-/-} mice. Wt and *Mavs*^{-/-} mice were intranasally infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). Intranasal administrations of PBS or recombinant CCL2 (25μg/mouse) were performed 6 hrs p.i. for mice sacrificed on day 2, and 6, 18 and 48 hrs p.i. for mice sacrificed on day 4. A) Weight was monitored daily and plotted as a percentage of original weight. Statistically significant differences between infected *Mavs*^{-/-} mice and infected *Mavs*^{-/-} mice that received rCCL2 are shown. Viral load was determined by quantification of viral L and N gene copies in lungs of RSV-infected mice on B) day 2 p.i., and C) day 4 p.i.. D) Infectious virus in lung homogenate was determined by Immunoplaque Assay. Dotted line represents the detection limit. Data are pooled from two independent experiments with 5 mice per group. Results are presented as mean ± SEM. Statistical significances of differences between groups were

determined by A) 2-Way ANOVA with Bonferroni's post hoc test, and B-D) One-way ANOVA with Tukey's post hoc test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Next, the role of recruited CD64^{hi} inflammatory cells in viral control and disease pathology was assessed in immunocompetent wt mice using the anti-CCR2 antibody to deplete monocytes in the blood. Anti-CCR2 antibody was injected 8 hrs prior to infection and from then on once a day for a period of 4 days, covering the kinetics of CD64^{hi} inflammatory cell recruitment into the lung. Depletion of monocytes during the first 4 days of infection had no obvious influence on RSV-induced weight loss (Figure 5-29 A). Anti-CCR2 treated mice lost weight similarly to isotype control treated mice on day 6 p.i. and weight recovery was observed by day 7 p.i. (Figure 5-29 A). Although an impact on weight loss could not be observed, the viral load determined by viral L and N gene transcripts was significantly increased in monocyte depleted mice on day 2 p.i. compared to controls (Figure 5-29 B). This initial increase in viral load vanished by day 4 p.i. as no differences in viral L gene copies or infectious particles could be detected (Figure 5-29 C). It is therefore likely that other compensatory mechanisms partially limit the effect of monocyte depletion on RSV disease severity in wt mice.

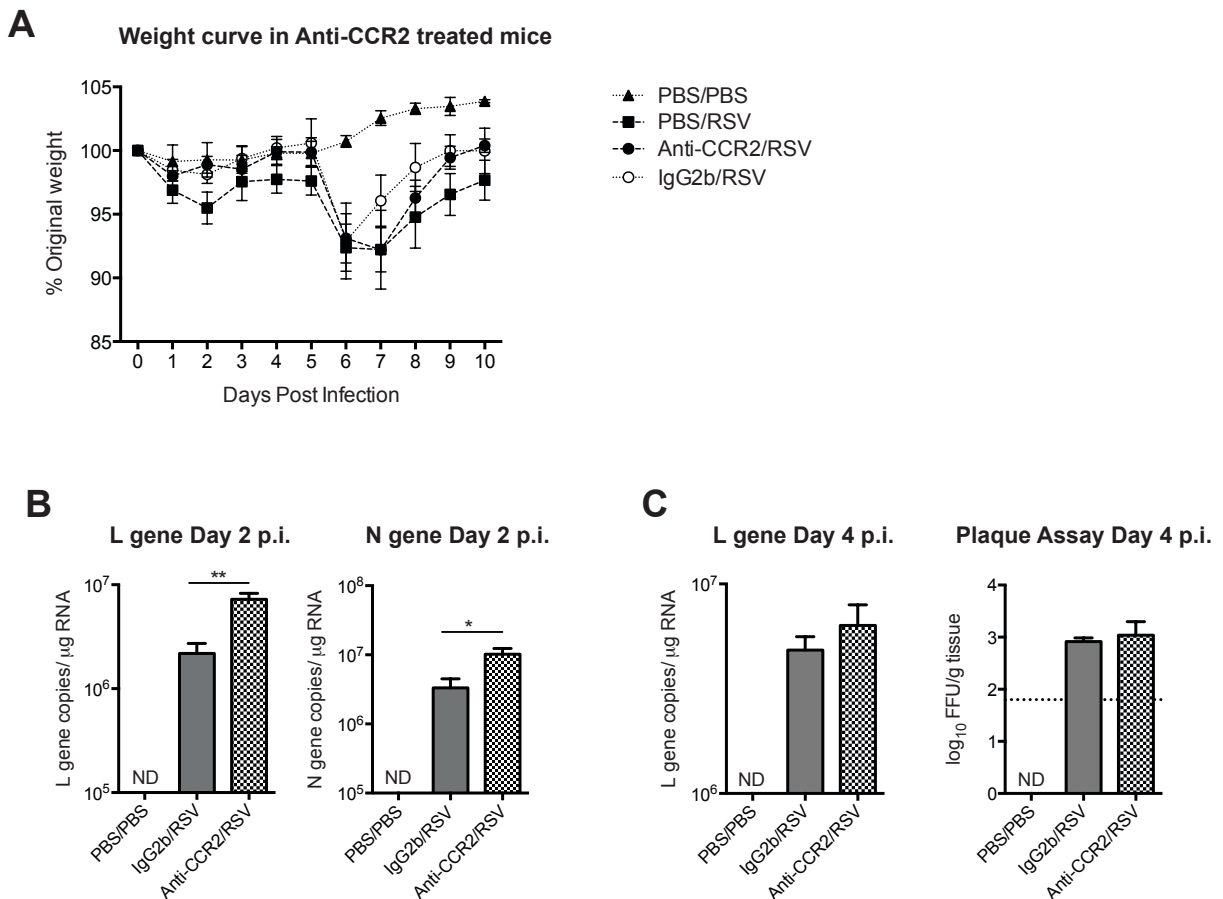


Figure 5-29 Loss of viral control in monocyte depleted wt mice but no impact on RSV induced pathology. C57BL/6 mice in A) and B) and *Ifna6gfp/+* wt mice in C) were treated with PBS or 20 μ g anti-CCR2 (clone MC21) or isotype antibody (IgG2b, clone A95-1) intraperitoneal at 8 hrs prior to RSV infection and daily up to day 4 after RSV infection. Mice were infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Weight was monitored daily and plotted as a percentage of original weight. Data represent one experiment with 5 mice per group. B) Mice were sacrificed 48 hrs p.i. Viral load was determined by quantification of viral L gene and N gene copies in lungs of RSV-infected mice. Data are pooled from two independent experiments with 4 to 5 mice per group. C) Infectious virus in lung homogenate was determined by Immunoplaque Assay. Dotted line represents the detection limit. Data represent one experiment with 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by A) 2-Way ANOVA with Bonferroni's post hoc test, and B) and C) One-way ANOVA with Tukey's post hoc test. *, $P \leq 0.05$; **, $P \leq 0.01$.

5.2.7. Discussion

Type I IFNs are potent immune modulators crucial for the activation of antiviral defence mechanism of the innate and adaptive immune system. In response to RSV infection type I IFN production and signalling was shown to be involved in the induction of proinflammatory cytokines in the lung and decreasing RSV-induced disease. The cellular source of type I IFNs and how the IFN-mediated inflammation influences the outcome of RSV infection remains unclear. The here presented study provides for the first time that alveolar macrophages (AMs) are the main producers of type I IFNs in the lung after RSV infection utilising the RLR-MAVS pathways for detection of the virus. Furthermore, the innate cytokine responses early upon virus inoculation are absent in the context of MAVS deficiency with no compensatory MyD88 or TRIF-mediated upregulation of type I IFNs or other cytokines. *In vivo* analyses of the *Ifna6^{gfp/+}* mouse model revealed AMs as the major source of type I IFNs in response to RSV infection (Figure 5-11). Previous data from other *in vitro* studies showed that most lung cells, like epithelial cells, fibroblast, blood monocyte-derived DCs, pDCs, and macrophages have the potential to secrete type I IFNs upon RSV infection (Bhoj et al., 2008; Demoor et al., 2012; Guerrero-Plata et al., 2006; Rudd et al., 2007; Wang et al., 2006), but this broader expression of type I IFNs was not observed *in vivo*. Epithelial cells are the primary target of RSV infection, and viral replication does not lead to type I IFN production in AII epithelial cells (F. Kausar, unpublished observation), probably due to the capability of RSV-genome encoded NS proteins decreasing the levels of the signalling molecule STAT2 and thereby inhibiting the type I IFN response (Ramaswamy et al., 2006; Swedan et al., 2009). In contrast to epithelial cells, STAT-signalling in RSV exposed AMs remains functional (Ravi et al., 2013), likely due to abortive replication of RSV in AMs and perhaps abrogated NS protein expression (Ravi et al., 2013). Quantitative PCR analysis for type I IFN transcripts (*Ifna5* and *Ifnb*) on FACS sorted lung cell populations further substantiated that AMs are the main source of type I IFNs during RSV infection. Nevertheless, low-level transcripts in sorted pDCs could be detected, yet AMs had five to ten times more *Ifna* or *Ifnb* expression (Figure 5-13).

Infection with live viral particles were a clear prerequisite to induce responses by AMs in the lung as UV inactivated RSV was not sufficient for type I IFN induction (Figure 5-14). This clearly indicated that initial events in RSV replication must occur in order to trigger AMs to start type I IFN production.

The detection of the virus and subsequent IFN secretion solely relied on cytosolic RLR-MAVS pathways, which is consistent with studies suggesting RIG-I or MDA5 as sensing receptors for RSV-derived RNA (Bhoj et al., 2008; Demoor et al., 2012; Liu et al., 2007; Loo et al., 2007). Moreover, no compensatory MyD88-mediated induction of type I IFNs could be detected in *Mavs*^{-/-} mice during RSV infection (Figure 5-15), although it has been suggested in a model of pulmonary NDV infection. In this NDV model, MyD88 activity in pDCs led to type I IFN production in a *Mavs*^{-/-} background (Kumagai et al., 2007). The redundant role for pDCs in type I IFN production during RSV infection is unclear, as one study suggests pDC depletion did not compromise type I IFN production (Jewell et al., 2007), and another report suggesting pDCs indispensable for type I IFN secretion (Smit et al., 2006). Our study suggests that AMs are the main producers supporting the findings of Jewell et al, but one has to be cautious as we observed a reduced overall level of type I IFN production in *Myd88/Trif*^{-/-} RSV infected mice by quantitative PCR. This reduced IFN production was not observed in an independent study measuring IFN protein levels in MyD88 deficient RSV infections (Bhoj et al., 2008). Summarising the results and the literature, it is feasible to attribute AMs as the main producers of type I IFN during RSV infections.

The expression of the majority of antiviral cytokines and chemokines was depended on MAVS-mediated signalling during RSV infection (Figure 5-15). The production of IFN- γ , IL-1 β and TNF- α was additionally abolished in MyD88/TRIF-deficient mice, which implied the complex regulation and interplay of different signalling pathways for these cytokines and their production. As a consequence of the lack of antiviral type I IFNs and other cytokines and chemokines (Figure 5-16, Figure 5-18 and Figure 5-19), MAVS-deficient mice had an advantageous environment for viral replication in the lung leading to significantly higher viral loads throughout the course of infection compared to wt mice (Figure 5-17). This increased viral load in *Mavs*^{-/-} mice was accompanied by a significantly increased weight loss during infection (Figure 5-17). Increased weight loss is a clear sign for enhanced disease, because RSV-induced weight loss correlates with airway hyperresponsiveness, airway resistance and inflammation (Pribul et al., 2008).

Cytokine and chemokine production after RSV sensing lead to profound changes in the composition of pulmonary immune cells in infected mice. In general, infiltration of monocytes and neutrophils in response to chemoattractants are a hallmark of inflammation. A profound recruitment of inflammatory monocytes identified as

CD64^{hi}CD11b^{hi} cells was detected in wt mice, dominating with up to 40 % of the total leukocyte population in the lung at day 2 p.i. (Figure 5-21). Recruited monocytes sequentially downregulated Ly6C in the lungs of wt mice during RSV infection (Figure 5-22), recapitulating the so called mo-waterfall which was first described in the gut (Tamoutounour et al., 2012) and later in other tissues such as the skin and lung (Plantinga et al., 2013; Tamoutounour et al., 2013). The down-regulation of Ly6C expression resembles the differentiation of inflammatory monocytes into either monocyte derived DCs (moDCs), monocyte derived macrophages or Ly6C low and CD11c low monocytes (infMo). To avoid confusion with resident Ly6C^{lo}CD11b^{hi} conventional DCs, CD64 is a reliable marker for discrimination of cells of monocytic origin, and was previously suggested as a useful marker during lung inflammation (Plantinga et al., 2013). Some of the infiltrating monocytes further gradually upregulated CD11c in the lung acquiring phenotypic features of monocyte-derived DCs (moDCs) also known as inflammatory DCs or Tip-DCs (Figure 5-22)(Segura and Amigorena, 2013). These CD64^{hi}CD11b^{hi} inflammatory monocytes were completely absent in *Mavs*^{-/-} mice consistent with the lack of the monocyte chemoattractants CCL2, CCL7 and CCL12 (Figure 5-21). Various cell types are suggested as a source of CCL2 secretion during viral infections such as structural cells (Conrady et al., 2013), monocytes (Seo et al., 2011), AMs (Demoor et al., 2012; Seo et al., 2011) or primary epithelial cells (Demoor et al., 2012). Importantly, the production of CCL2 was reported to be driven by IFNAR signalling (see chapter 5.1 and (Conrady et al., 2013; Majer et al., 2012)).

In this study, AM transfer experiments or rIFN- α treatments of *Mavs*^{-/-} mice proved the dependency of monocyte recruitment on MAVS-mediated type I IFN secretion from AMs and subsequent CCL2-driven inflammatory monocyte attraction into the RSV-infected lung (Figure 5-23, Figure 5-24 and Figure 5-25). Inflammatory monocytes can stimulate immune responses in various ways by releasing mediators or modulating cellular responses. Functional properties of inflammatory monocytes range from antigen-uptake, processing and presentation, secretion of immunomodulatory molecules, re-stimulating of effector T cells to the induction of memory T cells (Aldridge et al., 2009; Grainger et al., 2013; Hohl et al., 2009; León et al., 2007; Serbina et al., 2003; Wakim et al., 2008). Interestingly, upon induced recruitment (via intranasal inoculation of recombinant CCL2) of inflammatory monocytes into the lungs of RSV-infected *Mavs*^{-/-} mice a restriction in viral replication and a marked reduction in RSV-induced disease was observed, indicating a

contribution of CD64^{hi} inflammatory cells in the overall antiviral responses (Figure 5-28). This was consistent with reports in models of HSV-1 infections in the cornea (Conrady et al., 2013), HSV-2 in the vaginal tract (Iijima et al., 2010) or models of MCMV and vaccinia virus (Barbalat et al., 2009), where monocytes were shown to contain viral replication.

The impact of loss of recruited CD64^{hi} inflammatory monocytes on RSV replication in anti-CCR2 treated wt mice was less pronounced (Figure 5-29). In the initial and early control of viral replication (day 2 p.i.), monocytes indeed dampen RSV replication in the lung. However, the loss of viral control in monocyte-ablated mice was restored by day 4 after RSV inoculation. As a likely explanation, one needs to consider that wt mice induce appropriate type I IFN responses that will modulate cell intrinsic mechanisms to restrict viral replication in infected cells. Monocyte depleted wt mice can still stimulate other immune cells, like NK cells, which play an important role in RSV clearance and are recruited by day 3-4 in respiratory tract infections with RSV (Hussell and Openshaw, 1998).

5.3. Late immune responses to RSV are independent of MAVS signalling

Pulmonary RSV detection is mediated by RIG-I-like receptors, and signalling through MAVS elicited the secretion of type I IFNs and other cytokines and chemokines in the lung. Loss of MAVS expression severely impaired antiviral responses, leading to increased viral burden and disease severity. However, *Mavs*^{-/-} mice eventually recover and effectively clear the virus (Figure 5-17).

It is widely believed that early antiviral cytokines, like type I IFNs, are essential for the induction of protective adaptive immunity (Swiecki and Colonna, 2011). Surprisingly, several reports detected normal T cell numbers in the lungs of *Mavs*^{-/-} mice during RSV infections (Bhoj et al., 2008; Demoor et al., 2012), in the absence of innate immune responses. The late immune responses to RSV were examined, in order to gain further insights into the effect of antiviral immune responses and RSV-induced disease and to identify antiviral responses independent of MAVS.

5.3.1. Biphasic production of inflammatory cytokines and chemokines in the lung of RSV infected mice

To monitor antiviral immune responses at later time points than studied before - during the peak of RSV induced disease severity - the transcript levels of cytokines and chemokines were measured by qPCR in infected mice from day 4-7 p.i.. Interferon responses are thought to be induced quickly and transiently after initial infection, yet it was surprising that mRNA levels for IFN- β and IFN- λ were increased over background levels in wt mice, on all assessed time points and on day 5 p.i., respectively. Notably, *Mavs*^{-/-} mice had higher IFN- β transcripts and elevated IFN- λ mRNA levels than wt mice on day 5 p.i. (Figure 5-30 A). After the initial peak of IFN- γ gene expression observed in wt mice, (18 hrs p.i., see Figure 5-16 C) a second induction on day 5 after RSV infection was detectable (Figure 5-30 A). *Mavs*^{-/-} mice displayed continuous increased IFN- γ levels from day 5 to day 7 p.i. (Figure 5-30 A). Accompanied with the detectable IFN responses was the induction of ISGs; OAS1a, PKR, Viperin, and MX-1 in both strains with a significantly higher production seen in *Mavs*^{-/-} mice (Figure 5-30 B). To effectively clear RSV additional immune cells need to be recruited to the site of infection employing the production of chemokines.

The monocyte and T cell chemoattractants CCL2 and CXCL10 were similarly increased in both strains on day 5 p.i. (Figure 5-30 C). CXCL1, which is not induced during the late phase of infection in wt mice, was produced in *Mavs*^{-/-} mice on day 4 and 5 p.i. (Figure 5-30 C). The proinflammatory cytokines IL-6 and IL-1 β were highly induced in both mice strains on day 4 and 5 p.i. and declined afterwards. Only *Mavs*^{-/-} mice had a second significant increase in IL-6 gene transcripts on day 7 p.i. (Figure 5-30 D). Copy numbers of TNF- α were increased in both mice strains on all assessed time points with significant higher induction in wt mice on day 5 and 7 p.i. (Figure 5-30 D). Taken together, for most of the examined cytokines and chemokines, wt mice displayed a biphasic induction during the course of RSV infection; first at 18 hrs p.i. (see 5.2.2 and 5.2.3) and second on day 5 after the initial RSV infection. In *Mavs*^{-/-} mice the early cytokine response was completely abrogated (Figure 5-16, Figure 5-18, Figure 5-19), but a marked induction of cytokines and chemokines was detected in *Mavs*^{-/-} mice during the late induction indicated intact signalling pathways independent of MAVS in late immune responses after RSV infection (Figure 5-30).

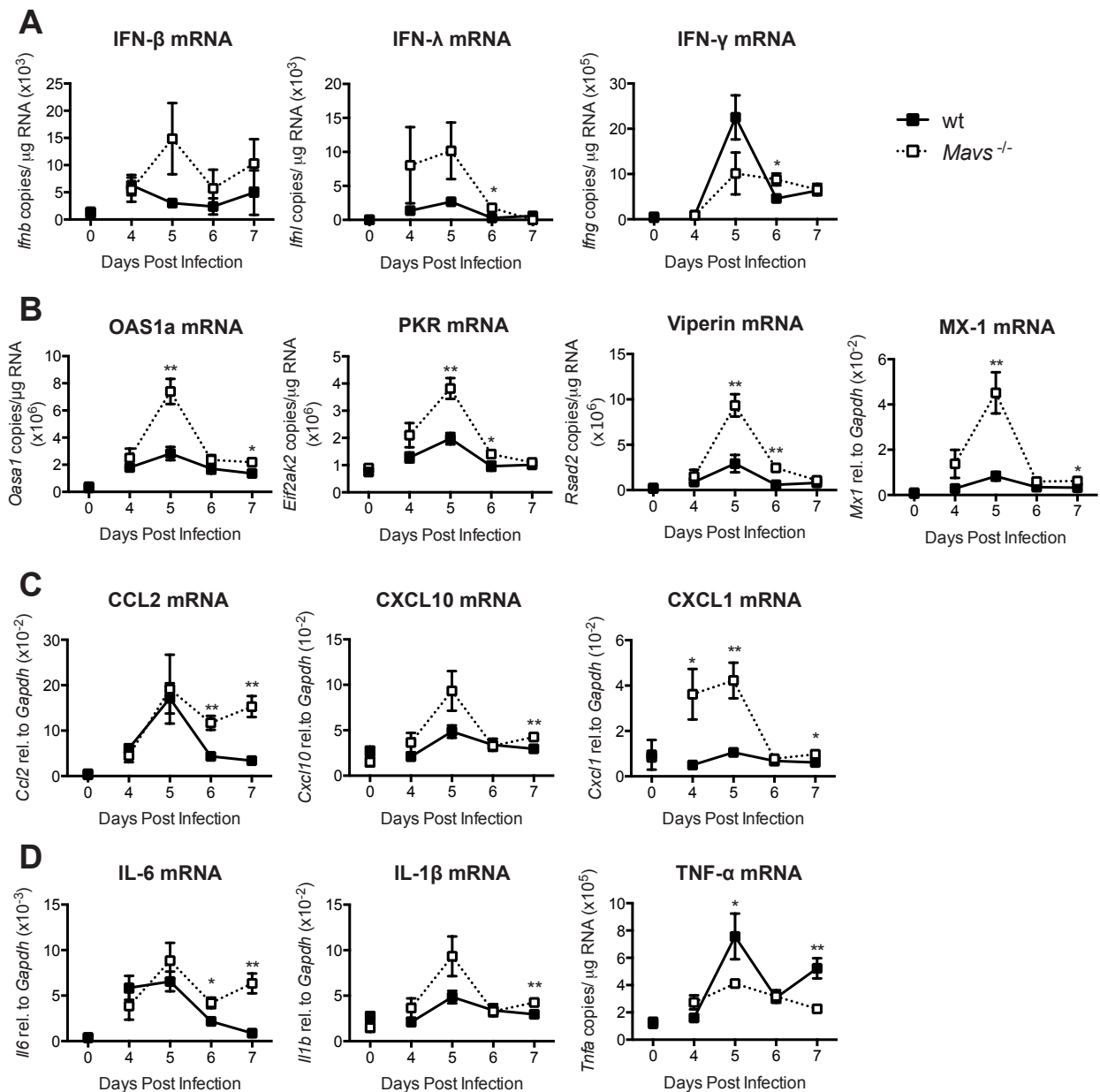


Figure 5-30 Late cytokine and chemokine responses induced by RSV are MAVS independent. Wt and *Mavs*^{-/-} mice were infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Gene expression levels for A) *Ifnb*, *Ifnl*, *Ifng*, B) and the ISGs; *Oasa1*, *Eif2ak2*, *Rsad2*, and *Mx1* was determined by qPCR. Copy numbers for *Ifnb*, *Ifnl*, *Ifng*, *Oasa1*, *Eif2ak2*, and *Rsad2* were determined using a plasmid standard. Gene expression relative to *Gapdh* was calculated for *Mx1*. C) Gene expression for the chemokines *Ccl2*, *Cxcl10* and *Cxcl1* relative to *Gapdh*. D) Proinflammatory cytokine expression for *Il6*, *Il1b* and *Tnfa* was determined by qPCR. Gene expression relative to *Gapdh* was calculated for *Il6* and *Il1b*. Copy numbers for *Tnfa* were determined using a plasmid standard. Data are representative of at least two independent experiments with 4-5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, $P \leq 0.05$; **, $P \leq 0.01$.

The differential expression of the various cyto- and chemokines raised the question about the dynamics of pulmonary innate cells during the late phase of RSV infection. Therefore, wt and *Mavs*^{-/-} mice were sacrificed on day 4-9 p.i. and lung cells were assessed by flow cytometry. Equivalent to the cytokine responses described (see Figure 5-30), leukocyte recruitment occurred a second time during the late phase of responses in wt mice, this time with comparable numbers detected in *Mavs*^{-/-} mice (Figure 5-31 A). As previously described, wt CD103⁺ DCs infiltrated the lung at 18 hrs p.i. and declined back to baseline levels by day 4 p.i. (see Figure 5-20 A). On day 5 p.i. CD103⁺ DC numbers increased by 3 fold and stayed up until day 9 p.i., the last time point of the time course (Figure 5-31 B). CD103⁺ DC numbers in *Mavs*^{-/-} mice were unchanged during the early responses, but were recruited to the lung on day 5 p.i., in similar numbers as in wt mice. The peak of CD103⁺ DC recruitment in *Mavs*^{-/-} mice was reached at day 8 p.i. and was significantly higher than in wt mice (Figure 5-31 B). CD11b⁺ DCs displayed minor changes in wt mice and no changes in *Mavs*^{-/-} mice during the early antiviral responses (4 hrs – 96 hrs; Figure 5-31 C). Whereas through the late phase responses a recruitment of CD11b⁺ DCs was detectable in *Mavs*^{-/-} mice on day 4, 8 and 9 p.i., but not in wt mice (Figure 5-31 C). Total pDC numbers showed biphasic recruitment into the lungs of infected wt and *Mavs*^{-/-} mice. After the initial recruitment of pDCs early during RSV infection (Figure 5-20 D), a second peak was observed on day 6 p.i. declining back to baseline levels on day 9 p.i. (Figure 5-31 D). This was detected in both wt and *Mavs*^{-/-} mice but was higher in *Mavs*^{-/-} mice on day 7-9 p.i.. AM numbers recovered back to baseline levels in wt mice after the acute reduction seen earlier in infection (see Figure 5-20 E). In *Mavs*^{-/-} mice a 3 fold increase of AM numbers was detectable on day 5, 6 and 7 p.i., and slowly coming back to baseline levels thereafter (Figure 5-31 E). The number of eosinophils was stable in wt mice, but in *Mavs*^{-/-} mice significantly increased numbers on day 5, 7, 8, and 9 p.i. were detectable (Figure 5-31 F). During the late response neutrophil recruitment was different in wt and *Mavs*^{-/-} mice, with transient recruitment in wt mice (day 8 p.i.) and two peaks of neutrophil recruitment in *Mavs*^{-/-} mice on day 5 p.i. and day 7-9 p.i. (Figure 5-31 G). This is consistent with the previously described levels of the neutrophil attractant CXCL1 detected in the lungs of *Mavs*^{-/-} mice but not wt mice (see Figure 5-30 C).

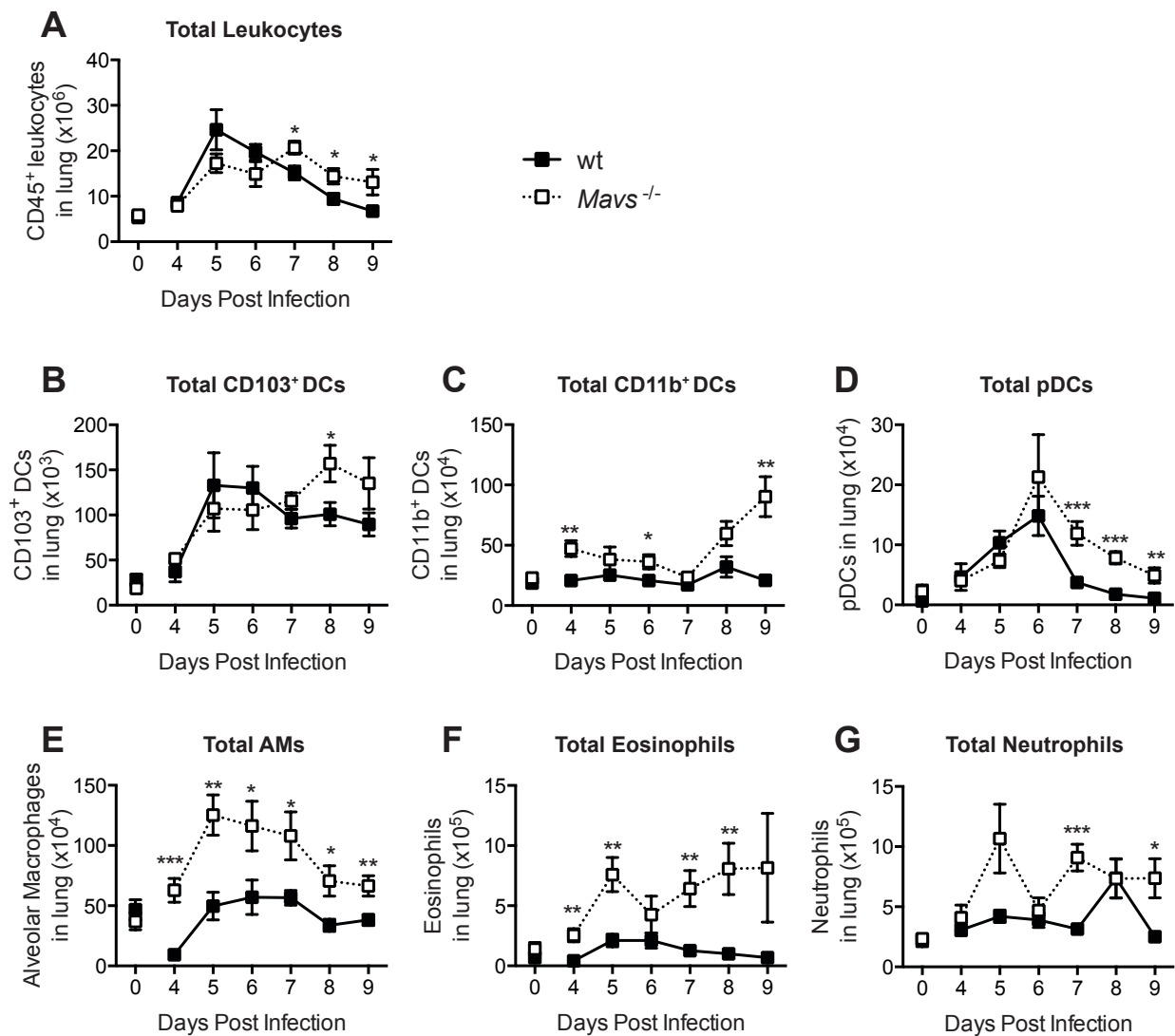


Figure 5-31 Dynamics of innate lung cell populations in wt and *Mavs*^{-/-} mice during peak of RSV pathology. Wt and *Mavs*^{-/-} mice were infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Lung cells were obtained by collagenase digestion of lung tissue and stained for surface expression of B220, CD11b, CD11c, CD45, CD64, Ly6C, Ly6G, Siglec-F, and Siglec-H. A) Total leukocytes, B) CD103⁺ DCs, C) CD11b⁺ DCs, D) pDCs, E) AMs, F) eosinophils, and G) neutrophils in the lung of wt and *Mavs*^{-/-} mice and indicated time points. Cell populations in the lung were analysed by flow cytometry and were gated as shown in **Figure 5-10**. Data are pooled from two independent experiments with 4-5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

While assessing the different cell populations in the lung a recurrent recruitment of CD64^{hi} inflammatory cells was seen in wt and *Mavs*^{-/-} mice. Similar numbers of CD64^{hi} inflammatory cells were detected in *Mavs*^{-/-} mice as in wt mice (Figure 5-32 A) indicating that this recruitment is independent of MAVS signalling. Yet, *Mavs*^{-/-} mice showed a minor

delay in peak recruitment (day 7 p.i.) compared to wt mice (day 6 p.i.) (Figure 5-32 B). Noteworthy, a fifth of these newly recruited CD64^{hi} inflammatory cells in *Mavs*^{-/-} mice resembled CD11c^{lo} infMo, which was not observed in wt controls (Figure 5-32 C). The majority of CD64^{hi} cells, distinguishable by their CD11c expression, were still characterised as moDCs in wt and *Mavs*^{-/-} mice. As indicated above, all CD64^{hi} inflammatory cells were conclusively moDCs in wt mice (Figure 5-32 C). The necessary chemoattractants for monocyte recruitment; CCL2, CCL7, and CCL12, were induced in both strains, with CCL2 protein expression and CCL7 and CCL12 mRNA levels peaking on day 5 p.i. in the lung of wt and *Mavs*^{-/-} mice (Figure 5-32 D).

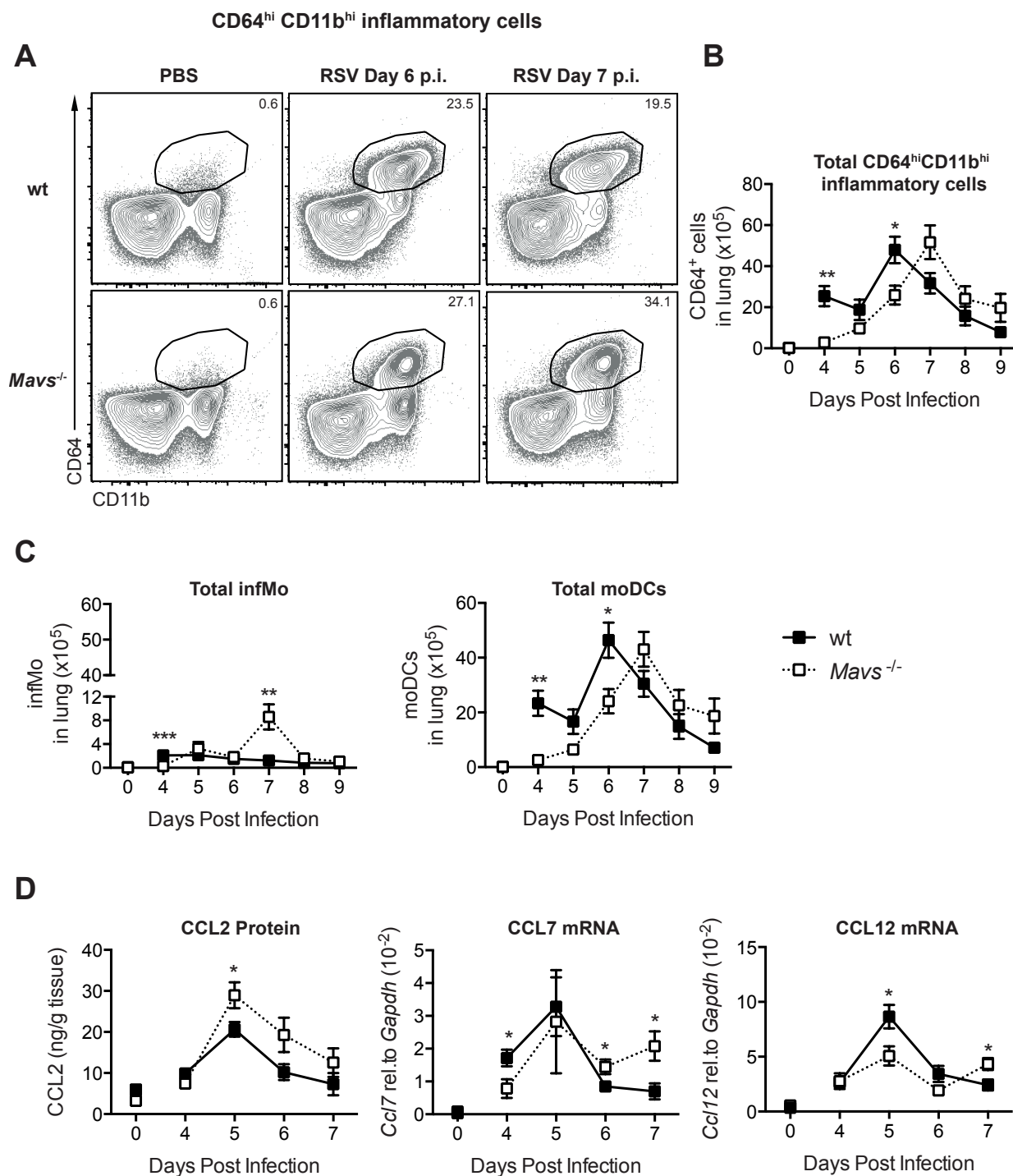


Figure 5-32 Recruitment of CD64^{hi} inflammatory cells during the late response to RSV infection is independent of MAVS signalling. Wt and *Mavs*^{-/-} mice were intranasally infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0), and sacrificed at indicated time points. A) Representative plots of CD64^{hi}CD11b^{hi} inflammatory cells recruited to the lung in mock (PBS) infected or RSV infected mice on day 6 and 7 p.i. B) Number of recruited CD64^{hi} inflammatory cells, C) CD64^{hi}CD11b^{hi}CD11c^{lo} infMo, and CD64^{hi}CD11b^{hi}CD11c^{hi} moDCs in the lung. D) Protein expression for CCL2 in lung homogenate was assessed by ELISA. Gene expression for *Ccl7* and *Ccl12* in lung tissue relative to *Gapdh* was determined by qPCR. Data are pooled from two independent experiments with 4-5 mice per group. Results are presented as mean ±

SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

To examine the contribution of CD64^{hi} inflammatory cells on disease pathology in *Mavs*^{-/-} mice, recruitment of blood monocytes was prevented by the administration of anti-CCR2 antibodies. A daily treatment of isotype control antibody (IgG2b) or anti-CCR2 on day 4-8 p.i. covered the previously described kinetics of CD64^{hi} inflammatory cell recruitment (see Figure 5-32 B). RSV-induced pathology was monitored by daily recording the weights. Notably, the ablation of monocytes had no effect on weight loss in monocyte-ablated animals compared to isotype controls (Figure 5-33).

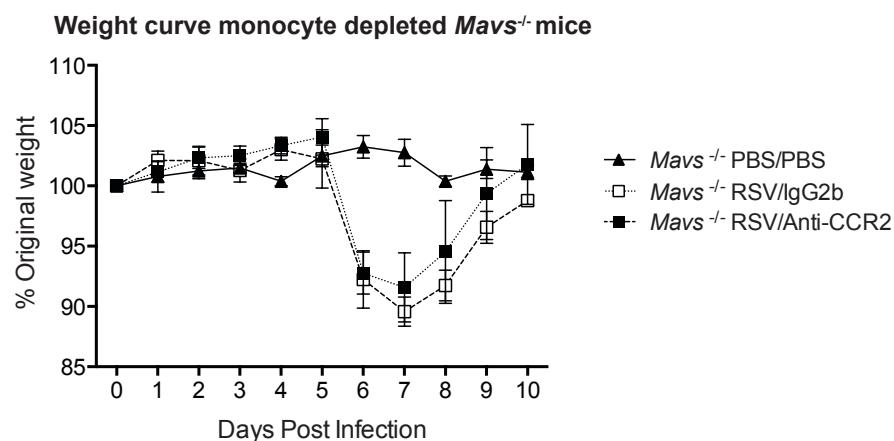


Figure 5-33 Preventing the late recruitment of CD64^{hi} inflammatory cells into the lungs of RSV infected *Mavs*^{-/-} mice had no influence on disease pathology. *Mavs*^{-/-} mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Mice were treated with PBS or 20 μ g anti-CCR2 (MC21) or isotype (IgG2b) control intraperitoneal daily from day 4 to 8 after RSV infection. Weight was monitored daily and plotted as a percentage of original weight. Data represent one experiment with 5 mice per group. There was not significant difference between the groups as assessed by 2-Way ANOVA with Bonferroni's post hoc test.

5.3.2. Normal priming and recruitment of T cells in *Mavs*^{-/-} mice

RSV infection leads to the induction of virus specific T cells, which play an essential role in the late and final viral clearance, regulating RSV induced inflammation, and mediating disease severity (Openshaw, 2005). In order to assess the role of MAVS signalling in the

induction of T cell responses, wt and *Mavs*^{-/-} mice were sacrificed on day 8 p.i. and antigen specific T cells were quantified by flow cytometry. No differences between wt and *Mavs*^{-/-} mice were detectable in terms of total CD4⁺ T cells and CD8⁺ T cells (Figure 5-34 A and B). Next, the specificity of the recruited CD8⁺ T cells was assessed using tetramers specific to the immunodominant RSV M peptide. Again, no differences could be detected with 35-40 % of infiltrating CD8⁺ T cells being specific for the M peptide in both strains (Figure 5-34 C). These data were coherent with other studies showing that T cell priming is independent of MAVS signalling (Bhoj et al., 2008). Elevated levels of the T cell-derived IFN- γ in the BAL of *Mavs*^{-/-} mice indicated for potential differences in T effector cell function, compared to wt mice (Figure 5-34 D).

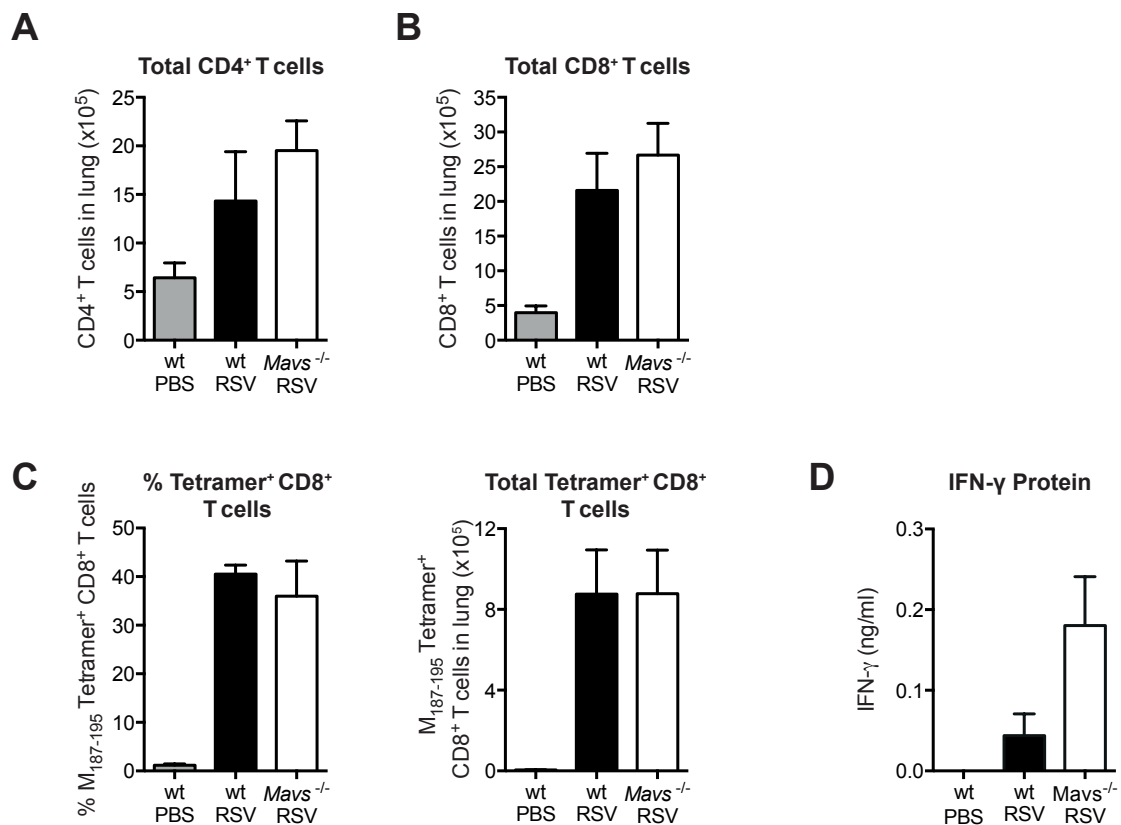


Figure 5-34 T cell recruitment into the lung of wt and *Mavs*^{-/-} mice is equivalent. Wt and *Mavs*^{-/-} mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). On day 8 p.i. lung cells were stained for CD3, CD4 and CD8. A) Total CD4⁺ T cells in lung. B) Total CD8⁺ T cells in the lung. C) RSV-specific CD8⁺ T cells were detected using M₁₈₇₋₁₉₅ tetramers staining on CD19⁻, CD3⁺ and CD8⁺ lung cells. Both frequency and total cell numbers are shown. Data represent two independent experiments with five mice per group. D) Protein levels for IFN- γ in the BAL were determined by ELISA. Data are pooled from 2 individual experiments. Results are presented as mean $\pm$ SEM. There was not significant difference between the groups as assessed by One-way ANOVA with Tukey's post hoc test.

5.3.3. Discussion

After the first peak of cytokine production observed in RSV infected wt mice (12-18 hrs p.i.) a second peak of cytokine and chemokine production was detected at the peak of disease manifestation in wt mice and this late time production was also detected in *Mavs*^{-/-} mice (Figure 5-30). The bi-phasic production of cytokines and chemokines in wt mice has previously been observed during RSV infection (Chavez-Bueno et al., 2005; Jafri et al., 2004; Miller et al., 2004). This induction resembles observations made in nasal lavage samples of experimental RSV challenged human adults, with a transient induction of CXCL8 post inoculation followed by an increase during viral shedding and illness (Noah and Becker, 2000). Additionally, CCL2, CCL3, and CCL5 were upregulated during symptomatic illness (Noah and Becker, 2000). The biphasic pattern of chemokine expression was not limited to CCL2, CXCL1, CXCL10, but also the proinflammatory cytokines IL-6, IL-1 β , and TNF- α were highly induced in both strains. Interestingly, IFN- β upregulation was detectable in *Mavs*^{-/-} mice but absent in wt mice.

This second induction suggested gene upregulation independently of RLR-MAVS-mediated signalling. Intracellular viral RNA sensing is not only mediated by RLRs in the cytosol. Activation of pathways downstream of the endosomal TLR3 and TLR7 will be increasingly important in RSV sensing upon phagocytosis of infected apoptotic cells in the late stage of infection (Welliver et al., 2008). Given this aspect, the increase in absolute numbers of TLR3 expressing CD103⁺ DCs and TLR7 expressing pDCs (Mildner and Jung, 2014) in both strains during RSV-induced illness substantiates a role for endosomal TLRs in the recognition of RSV at later stages (Figure 5-31).

Additionally, the increase in cytokine and chemokine induction at the peak of illness markedly changed the composition of immune cells in the lung of infected mice. Inflammatory monocytes that dominated the early infiltration in RSV infected wt mice but not in *Mavs*^{-/-} mice, were the predominant cell type during peak of pathology at day 6-7 p.i. in both strains, but were redundant for disease outcome (Figure 5-32). Neutrophils were highly abundant in the respiratory tract of *Mavs*^{-/-} mice (Figure 5-31) compared to wt mice, which is consistent with observations made in children with RSV LRTI. Clinical studies in infants with severe RSV-induced LRTI reported a marked increase in neutrophil numbers in the airways at peak of disease severity (Everard et al., 1994; Heidema et al., 2007;

Lukens et al., 2010; McNamara et al., 2003). Moreover, abundant neutrophil influx was found in autopsy material from infants who died from RSV LRTI (Welliver et al., 2008), and also in a re-assessment of the autopsy report and lung tissue from FI-RSV vaccinated children (Prince et al., 2001). Increased levels of the neutrophil chemoattractants CXCL1 in mice (Figure 5-31) and IL-8 in children with severe RSV LRTI (Abu-Harb et al., 1999; McNamara et al., 2005) and in experimental RSV infected adults (Noah and Becker, 2000) were the drivers of neutrophil trafficking. These findings suggest a major role of neutrophils in RSV pathology, which are increased in *Mavs*^{-/-} mice in the later stage of infection. A speculative but highly attractive connection is that a deficiency in MAVS signalling and/or deficiency in type I IFNs in humans promote susceptibility to LRTI.

A surprising finding was that, despite the absence of MAVS, the induction of RSV-specific T cell responses was unaffected during infection (Figure 5-34). It is commonly believed that the early activation of innate cytokine responses, especially type I IFNs, is required for the activation of appropriate adaptive immune responses (Swiecki and Colonna, 2011). A likely explanation for this is that other MAVS independent signalling pathways must play a role in the activation of adaptive immune responses. Bhoj et al described a migratory DC subset in RSV-infected *Mavs*^{-/-} mice, which was suggested to be involved in cross-priming of CD8⁺ T cells in the draining LNs (Bhoj et al., 2008). A specific characterisation of this DC subset and the specific molecular signals that lead to its activation remain unclear. Both, MyD88, essential for protective immunity to influenza virus (Koyama et al., 2007), or TLR3, and TRIF dependent mechanisms could trigger DC maturation and activation during RSV. However, a bystander activation of DCs by soluble mediators, such as IL-1 β (Pang et al., 2013) that can bind immediately to their respective receptors and would therefore be undetectable by currently available assays, could additionally play an essential role in activating adaptive immune responses to RSV.

The elevated cytokine levels observed in *Mavs*^{-/-} mice possibly drive tissue damage and pathology. Especially exacerbated T cell responses are reported to contribute markedly to illness and disease severity (Alwan et al., 1992; Cannon et al., 1988; Graham et al., 1991). IFN- γ is predominantly produced by CD4⁺, CD8⁺ T cells and NK cells during RSV infection (Hussell and Openshaw, 1998; Hussell et al., 1997). Indeed, elevated protein levels of IFN- γ in BAL fluid (Figure 5-34) and increasing numbers of activated IFN- γ ⁺ CD4⁺ and CD8⁺ T were reported in *Mavs*^{-/-} mice after RSV infection (Demoor et al., 2012).

The increased neutrophil migration and the excessive production of cytokines derived from T cells and other immune cells can together augment inflammation and pathology in the lung and affect the overall disease severity. Indeed, previous studies found correlations between RSV-induced weight loss and airway hyperresponsiveness, airway resistance and inflammation (Pribul et al., 2008).

5.4. Potential antiviral mechanism elicited by recruited CD64^{hi} inflammatory cells

During RSV infection alveolar macrophages are the major source of type I IFNs. Beside their potent activation of cell intrinsic antiviral mechanisms type I IFNs were shown to be essential for the recruitment of CD64^{hi} inflammatory monocytes during RSV infection. These recruited CD64^{hi} monocytes were demonstrated to have an important role in restricting viral replication and RSV-induced disease in *Mavs*^{-/-} mice, since the lack of recruitment of these cells resulted in 100 fold higher viral load during RSV infection (Goritzka et al., accepted for publication in *J Exp Med* 2015).

Inflammatory monocytes can modulate the immune response by the release of mediators or cell-contact dependent mechanisms to promote pathogen clearance. An attempt to delineate how CD64^{hi} monocytes mediate their antiviral properties to contain RSV replication was assessed in the following section.

5.4.1. Proinflammatory mediators secreted by CD64^{hi} inflammatory cells during RSV infection

Preventing weight loss and reducing viral load during RSV infection by inducing the recruitment of CD64^{hi} inflammatory cells into *Mavs*^{-/-} mice indicates the protective role of these cells during RSV infection. In HSV-1 or *L.m.* infections a reduction in pathogen burden was mediated by TNF- α and iNOS producing moDCs (Conrady et al., 2013; Serbina et al., 2003). The complete lack of TNF- α gene expression observed in *Mavs*^{-/-} mice could be connected to abrogated monocyte recruitment (see Figure 5-21 A, C and Figure 5-18). Flow cytometric analysis of RSV infected wt mice (18 and 48 hrs p.i.), detected around 1.5-2.5% of the CD64^{hi}CD11b^{hi} cells as TNF- α producers. Amongst these cells both moDCs and infMo contributed to TNF- α production (18 hrs p.i.), while moDCs represented the majority of TNF- α producers on day 2 p.i. (Figure 5-35 A). In addition, interleukin-1 β (IL-1 β) production was another characteristic of the CD64^{hi}CD11b^{hi} population at 18 hrs p.i., with equal contribution of moDCs and infMo. By day 2, IL-1 β ⁺ infMo declined in frequency and IL-1 β ⁺ moDC were unchanged (Figure 5-35 A and B). Noteworthy, around 0.4 % of CD64^{hi} inflammatory cells co-expressed TNF- α and IL-1 β (data not shown). CD64^{hi} inflammatory

cells were not the only source of TNF- α in wt mice as around 8 % of the AM population stained positive for TNF- α at 18 hrs p.i. but halved in frequency by day 2 p.i. (Figure 5-35 C). AMs from *Mavs*^{-/-} mice showed no detectable TNF- α signal (Figure 5-35 C). Cytokine staining for IL-10, IL-6, IFN- γ and iNOS was negative in both CD64^{hi} inflammatory cells and AMs.

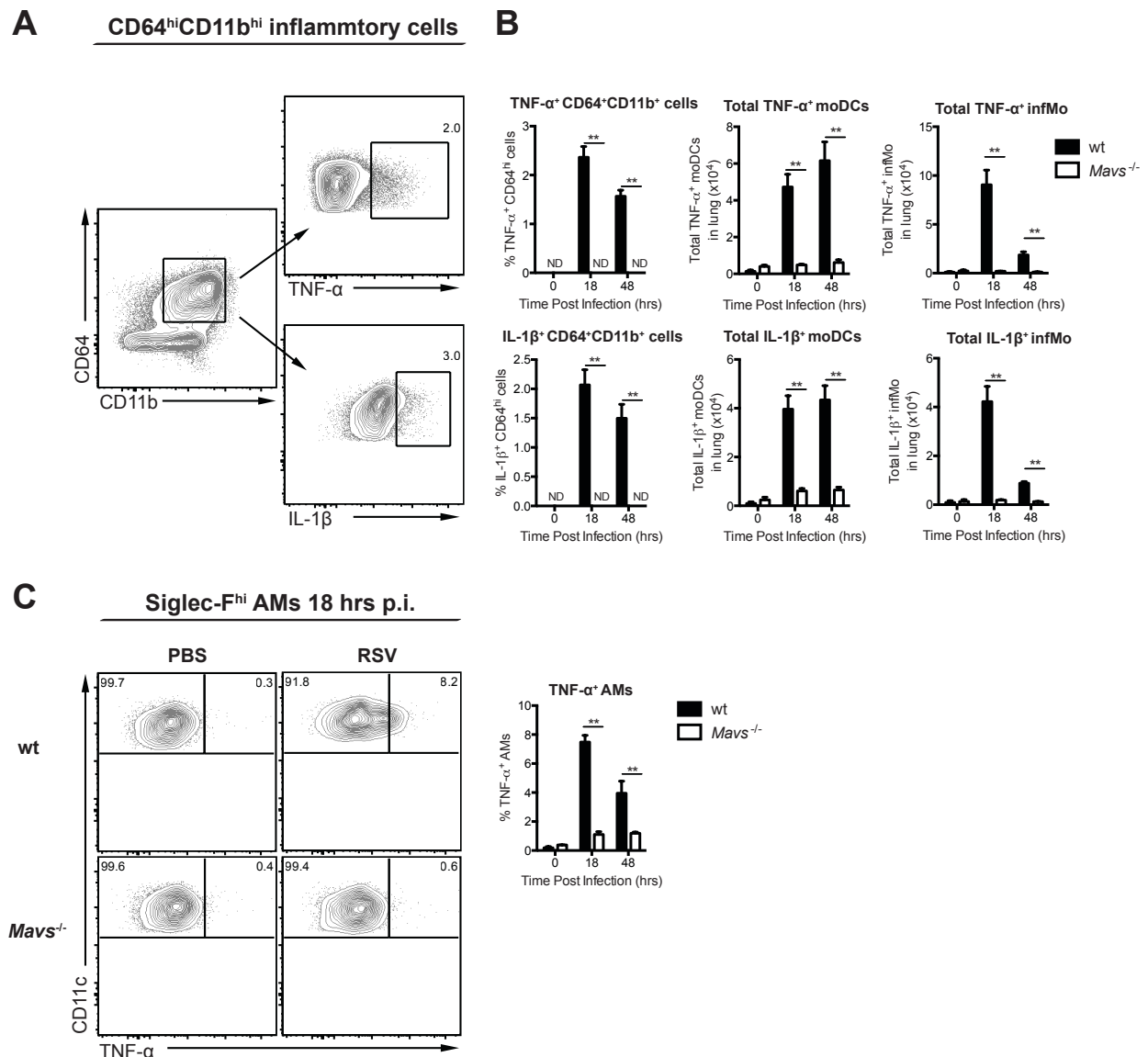


Figure 5-35 Intracellular cytokine expression in CD64^{hi} inflammatory cells and AMs from wt and *Mavs*^{-/-} mice after RSV infection. Wt and *Mavs*^{-/-} mice were intranasally infected with 2×10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). Lung cells were obtained by collagenase digestion of lung tissue and stained for surface expression of CD3, CD19, CD11b, CD11c, CD45, CD64, Ly6C, Ly6G, and Siglec-F and intracellular for TNF- α and IL-1 β . A) Representative plots of TNF- α and IL-1 β staining in CD64^{hi}CD11b^{hi} inflammatory cells from wt mice, 18 hrs p.i.. B) Percentage of TNF- α or IL-1 β positive CD64^{hi}CD11b^{hi} inflammatory cells and total number of TNF- α or IL-1 β positive moDCs and infMo, 18 and 48 hrs p.i.. C) Representative plot of TNF- α expression in AMs of RSV infected or PBS treated wt and *Mavs*^{-/-} mice 18 hrs p.i.,

and percentage of TNF- α positive AMs in the lung. Data represent two independent experiments with five mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. **, $P \leq 0.01$; ND, not detectable.

It is possible that the early production of TNF- α by CD64^{hi} inflammatory cells is crucial to limit viral replication and disease severity. To test this possibility, anti-TNF- α antibodies (25 μ g) were administered intranasally to wt mice at 4, 18 and 48 hrs after RSV infection. These time points will guarantee the depletion of TNF- α derived from innate cells, with limited affect on T cell derived TNF- α later during RSV infection. The intranasal depletion of TNF- α had no impact on weight loss as observed on day 6 p.i. when compared to infected controls (Figure 5-36 A). Additionally, no differences in viral load (measured by RSV L gene) was detected in the lung between TNF- α depleted and non-depleted groups (day 4 p.i.; Figure 5-36 B). To conclude, monocyte-derived TNF- α plays a minor role in early RSV-mediated pathology or viral control.

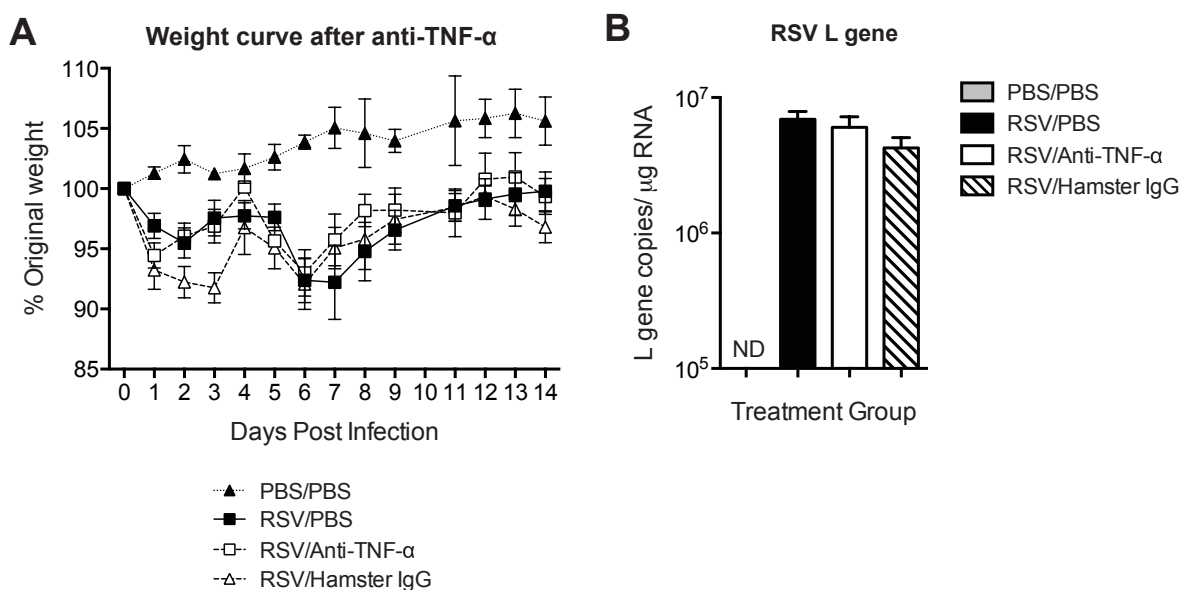


Figure 5-36 TNF- α is dispensable for RSV induced disease severity. Wt mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Mice were intranasally administered with PBS or 25 μ g of anti-TNF- α (clone TN3-19.12) or isotype control (hamster IgG) 4, 18 and 48 hrs p.i.. A) Weight was monitored daily and plotted as a percentage of original weight. B) Viral load was determined by quantification of viral L gene copies in lungs of RSV-infected mice. Data represent one experiment with 5 mice per group. Results are presented as mean $\pm$ SEM. There was not significant difference between the RSV-infected groups as assessed by A) 2-Way ANOVA with Bonferroni's post hoc test, and B) One-way ANOVA with Tukey's post hoc test.

5.4.2. Tissue growth factors are not impaired in *Mavs*^{-/-} mice during RSV infection

Maintenance of the airway epithelial integrity during respiratory viral infections is crucial to guarantee gas exchange. The defective immune surveillance in *Mavs*^{-/-} mice led to uncontrolled viral replication that could potentially increase lung damage. Increased lung damage has been reported to induce weight loss and increases the expression of growth factors (Jamieson et al., 2013; Monticelli et al., 2011). Amphiregulin, an epidermal growth factor, was similarly induced in the lung of wt and *Mavs*^{-/-} mice after RSV infection with mRNA levels peaking 72 hrs p.i. (Figure 5-37 A). Also the fibroblast growth factors (FGF) and vascular endothelial growth factor (VEGF) displayed high baseline protein levels in wt mice, but both initially declined during infection (8 hrs p.i.). Similar observations were made for *Mavs*^{-/-} mice with high baseline levels of VEGF protein that was almost undetectable at 12 hrs p.i. (Figure 5-37 B). From 18 hrs onwards into infection, a slow and continuous increase in FGF and VEGF was observed in both strains with VEGF levels significantly higher in *Mavs*^{-/-} mice at 18 and 72 hrs p.i. (Figure 5-37 B). To assess the severity of pathology, albumin concentration in the BAL fluid was measured as an indicator for epithelial permeability. Both strains had increased albumin levels after RSV infection indicating damage or increased permeability in the epithelial cell barrier, however, levels in wt mice were significantly higher compared to PBS treated mice (Figure 5-37 C). Taken together, inflammation and virus inflicted tissue injury induced tissue repair mechanisms comparable in wt and *Mavs*^{-/-} mice.

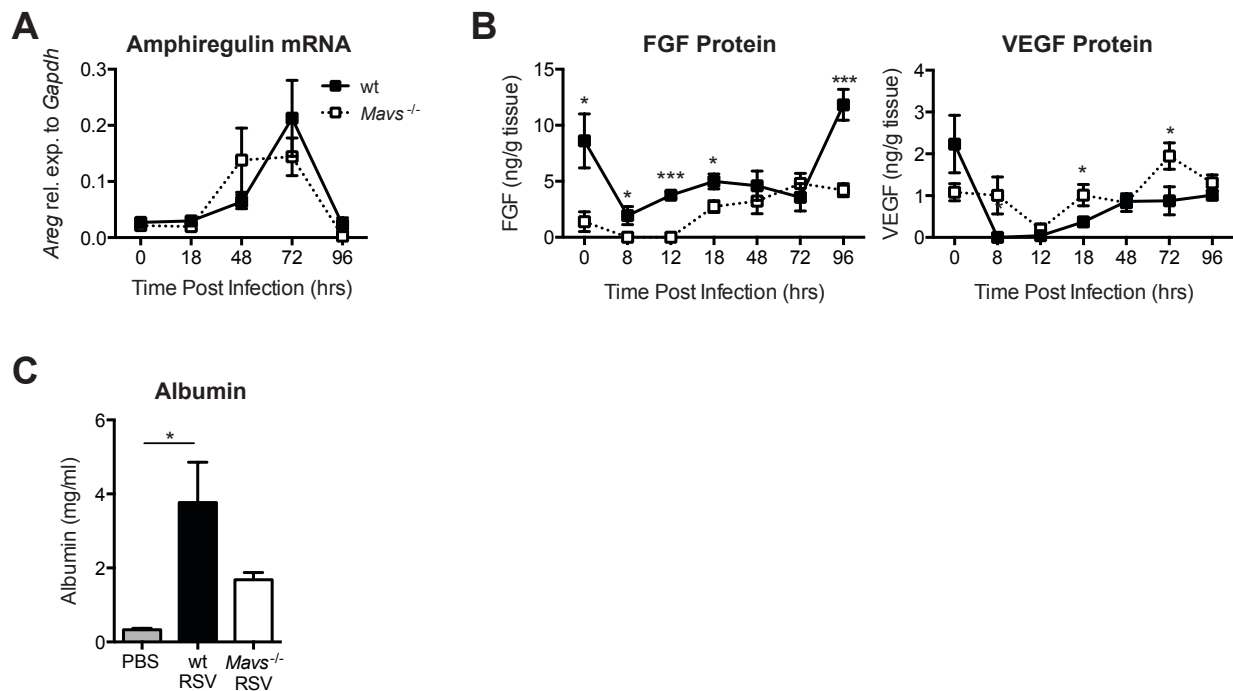


Figure 5-37 Growth factor induction and barrier function is not impaired in *Mavs*^{-/-} mice. Wt and *Mavs*^{-/-} mice were intranasally challenged with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Gene expression analysis of *Areg* (Amphyregulin) relative to *Gapdh* was determined by qPCR at indicated time points p.i.. Data represent one experiment with 5 mice per group. B) Protein expression of FGF and VEGF in lung homogenate was determined by Luminex. Data shown are pooled from two individual experiments with 5 mice per group in each experiment. C) Albumin levels in BAL fluid on day 4 p.i. were determined by ELISA. Data represent one experiment with 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by A) and B) two-tailed, unpaired Student's t-test, and C) One-way ANOVA with Tukey's post hoc test. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

5.4.3. CD64^{hi} inflammatory monocytes are a source of the immune modulator PGE2

The immune modulatory lipid prostaglandin E2 (PGE2), exerts anti-inflammatory responses during inflammation (Morici, 2013) and has recently been described to derive from inflammatory monocytes (Grainger et al., 2013). Cyclooxygenase 2 (COX-2), important for the enzymatically production of PGE2, was increased on transcription level in wt mice 18 and 48 hrs after RSV infection, coinciding with the peak of inflammatory monocyte recruitment. An increase in COX-2 mRNA was also detectable in *Mavs*^{-/-} mice but to a much lower degree compared to wt mice (Figure 5-38 A). Differences in PGE2 protein

expression were undetectable in lung homogenate, but protein in BAL fluid was significantly increased in wt mice compared to infected *Mavs*^{-/-} mice and uninfected controls (Figure 5-38 B). To link this expression with the recruitment of CD64^{hi} inflammatory cells, the macrophage transfer model was utilised. Although, *Mavs*^{-/-} mice transferred with wt AMs had increased PGE2 levels in BAL fluid compared to non-transferred mice (day 2 p.i.), no significant differences were detectable in comparison to mice transferred with *Mavs*^{-/-} AMs (Figure 5-38 D). In monocyte depleted wt mice less PGE2 was detectable compared to non-depleted mice, but this was not statistical significance (Figure 5-38 E). That monocytes are a source of PGE2 was demonstrated in the supernatant of overnight cultured FACS-sorted moDCs from infected wt mice at 18 hrs p.i. (Figure 5-38 F). Thus, the immune modulator PGE2 is induced during RSV infection and partially derives from inflammatory monocytes.

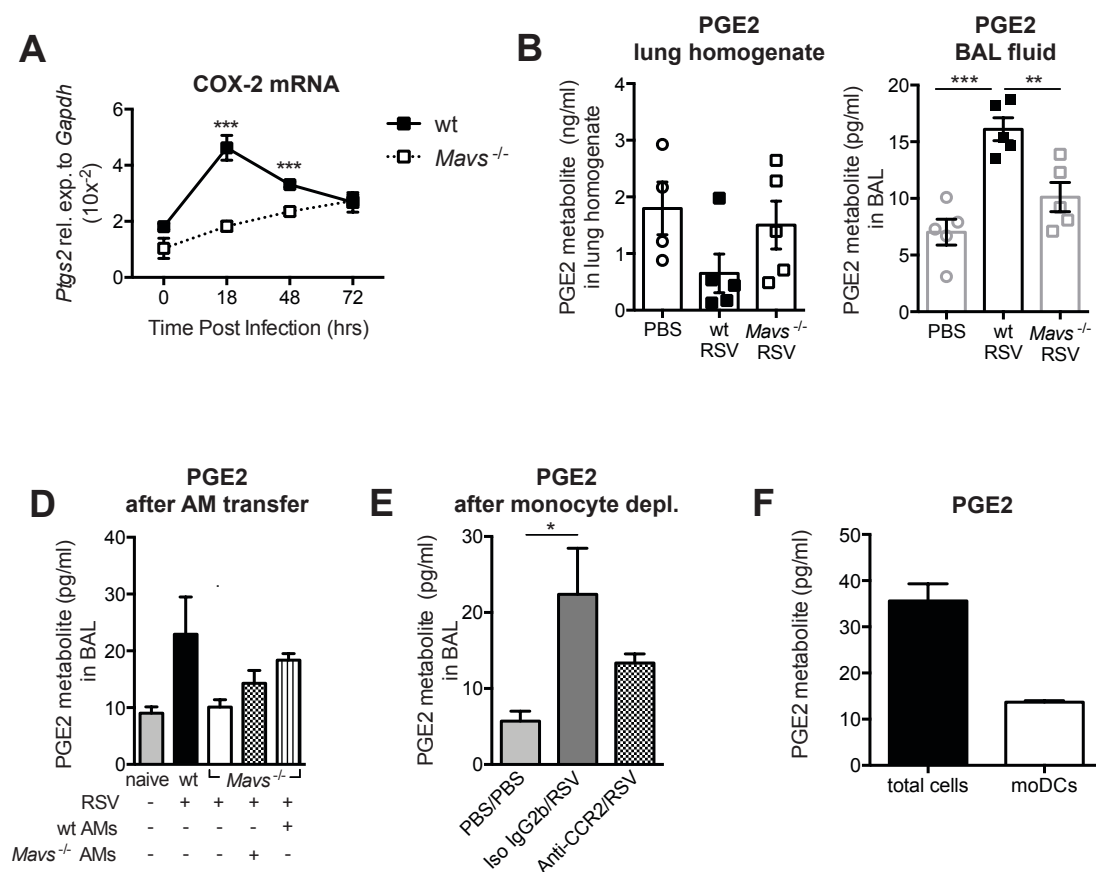


Figure 5-38 Prostaglandin E2 is induced during RSV infection. Wt and *Mavs*^{-/-} mice were intranasally infected with 2x10⁶ focus forming units (FFU) of RSV or mock infected (PBS, t=0). Naive resembles PBS treated mice. A) Gene expression analysis of *Ptgs2* (COX-2) relative to *Gapdh* was determined by qPCR at indicated time points p.i.. B) PGE2 (Prostaglandin E2) expression in lung homogenate and BAL fluid on day 2 p.i.. D) PGE2 in BAL fluid on day 2 p.i. from wt mice and *Mavs*^{-/-} mice transferred with wt AMs or *Mavs*^{-/-} AMs

on day 2 p.i. E) PGE2 in BAL fluid from in monocyte depleted C57BL/6 mice on day 2 p.i.. Data represent one experiment with 5 mice per group. F) Total lung cells and FACS-sorted CD64^{hi}CD11c^{hi} moDCs from 18 hrs p.i. were incubated overnight in medium. After 18 hrs incubation supernatants were assessed for PGE2. Data show one experiments with 37 mice. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by A) two-tailed, unpaired Student's t-test, and B-E) One-way ANOVA with Tukey's post hoc test. **, $P \leq 0.01$; *, $P \leq 0.05$.

5.4.4. TRAIL expression is not detectable on the surface of CD64^{hi} inflammatory monocytes

Tumor necrosis factor-related apoptosis-induced ligand (TRAIL) can be upregulated on various cell types (Davidson et al., 2014; Herold et al., 2008; Stacey et al., 2014), and elicits its antiviral properties by inducing apoptosis in infected cells. Type I IFN signalling dependent upregulation of TRAIL was recently linked to monocyte mediated epithelial cell death during influenza virus infection (Davidson et al., 2014; Herold et al., 2008). During the early response to RSV infection (18 hrs p.i.), wt mice had significantly elevated TRAIL transcript levels compared to *Mavs*^{-/-} mice (Figure 5-39 A). An increase in TRAIL expression was also identified on non-autofluorescent non-polymorph-nuclear cells in wt lung 18 and 48 hrs p.i. (Figure 5-39 B). But the diffuse expression of the surface markers CD11c, CD11b and CD64 made an assignment to a specific cell type impossible. In summary, TRAIL expression is induced during RSV infection but was not attributable to a specific leukocyte population.

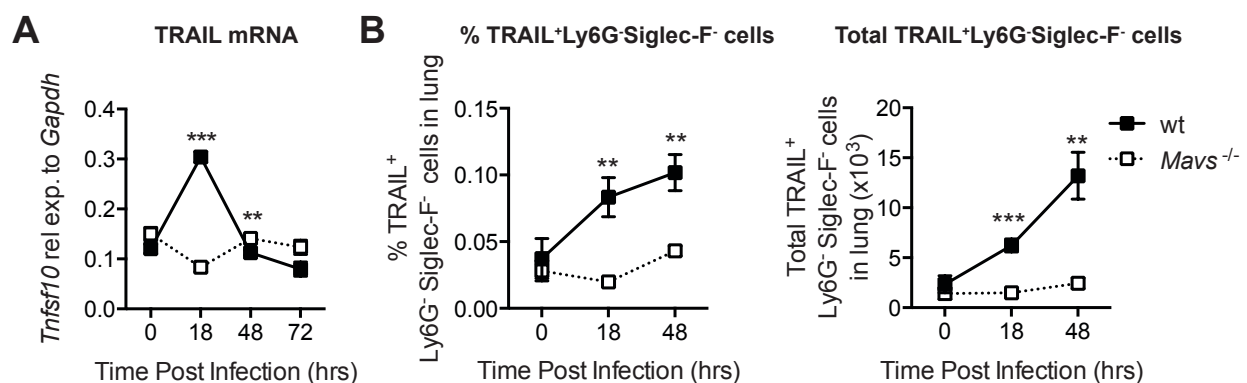


Figure 5-39 Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is not induced in *Mavs*^{-/-} mice during RSV infection. Wt and *Mavs*^{-/-} mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). A) Gene expression analysis of *Tnfsf10* (TRAIL) relative to *Gapdh*

was determined by qPCR at indicated time points p.i.. B) Percentage and number of TRAIL expressing cells negative for Siglec-F and Ly6G expression. Data represent one experiment with 5 mice per group. Results are presented as mean $\pm$ SEM. Statistical significance of differences between groups was determined by two-tailed, unpaired Student's t-test. ***, $P \leq 0.001$; **, $P \leq 0.01$.

5.4.5. CD64^{hi} inflammatory monocytes are involved in NK cell activation

NK cells are an important innate cell type that limit viral burden by killing virus infected cells. Interleukin 15 (IL-15), important for NK cell proliferation (Huntington, 2014), was transiently induced in wt mice shortly after infection (18 hrs p.i.), whereas *Mavs*^{-/-} mice displayed no induction of IL-15 expression early in RSV infection (Figure 5-40 A). By day 4 p.i., when NK cell numbers peak during RSV infection (Hussell and Openshaw, 1998), wt mice displayed a significant increase in NK cell numbers in the lung while this response was abrogated in *Mavs*^{-/-} mice (Figure 5-40 B). In addition NK cells show a more activated phenotype (CD69^{hi}) in wt mice than in *Mavs*^{-/-} mice (Figure 5-40 B). NK cell cytotoxicity can be mediated by TRAIL (Sarhan et al., 2013), but during RSV infection TRAIL expression was not significantly induced on the surface of NK cells (Figure 5-40 C). To evaluate a potential cross talk between CD64^{hi} inflammatory cells and NK cells, *Mavs*^{-/-} mice were administered with rCCL2 to recruit monocytes as previously described, and NK cell recruitment and activation was determined by flow cytometry (day 4 p.i.). Similar frequencies of NK cells were present in the lung of *Mavs*^{-/-} mice with or without administration of rCCL2 indicating no additional recruitment or *in situ* proliferation of NK cells; yet, resident NK cells in rCCL2/*Mavs*^{-/-} mice expressed high levels of the activation marker CD69 in comparison to untreated *Mavs*^{-/-} mice (Figure 5-40 D). The increased activation of NK cells observed correlated with the recruitment of CD64^{hi} inflammatory cells (Figure 5-40 E). The opposite approach, by depleting monocytes in wt mice, indicated that monocytes play a role in NK cell recruitment and/or proliferation but seemed to be redundant for NK cell activation (Figure 5-40 F). Taken together, in the absence of MAVS NK cells recruitment/proliferation and activation was diminished, but the latter could be restored by rCCL2-mediated CD64^{hi} cell recruitment.

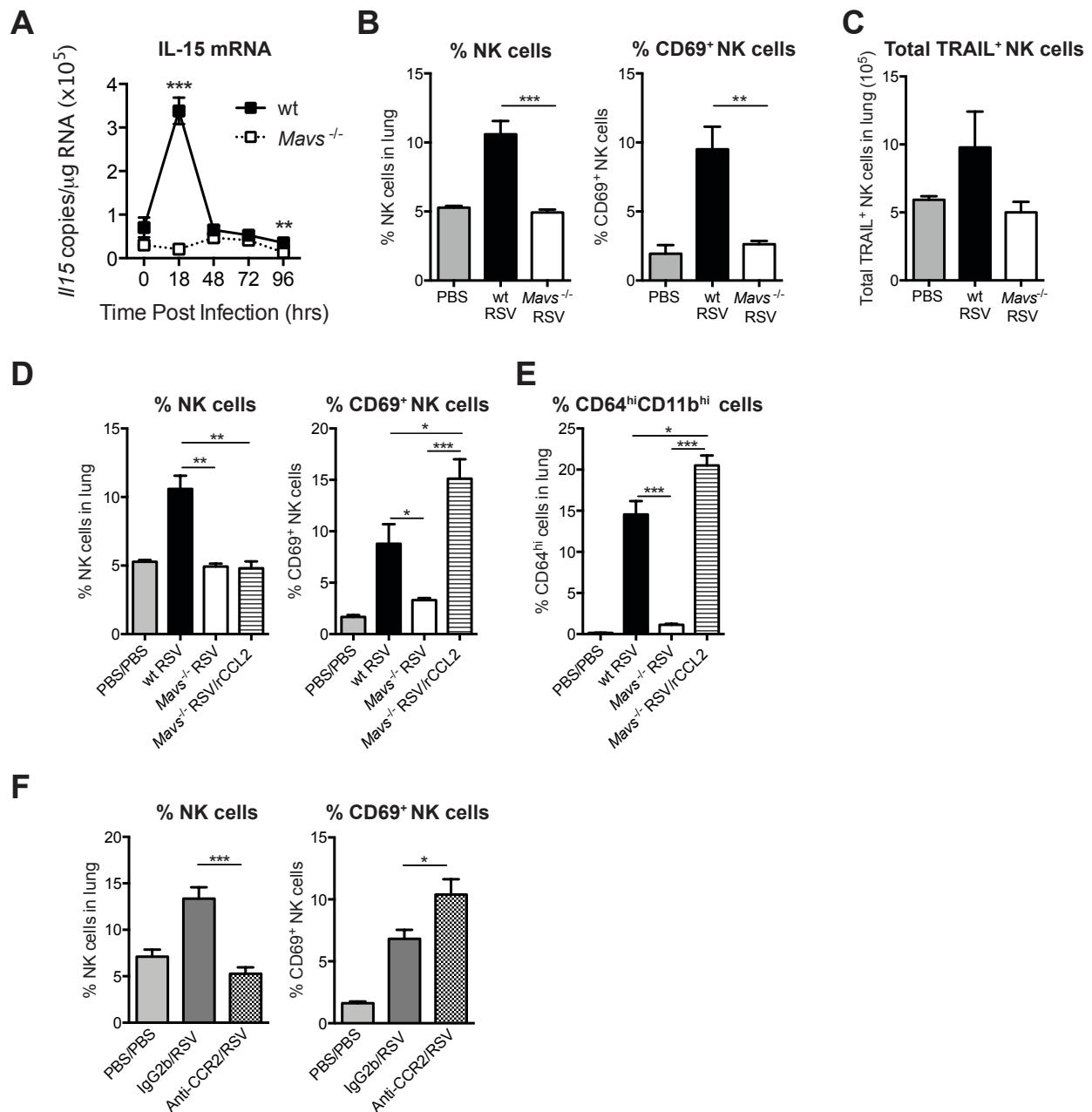


Figure 5-40 CD64^{hi} inflammatory cells influence NK cell activation in RSV infected *Mavs*^{-/-} mice. Wt and *Mavs*^{-/-} mice were intranasally infected with 2×10^6 focus forming units (FFU) of RSV or mock infected (PBS, t=0). Mice were sacrificed on various times p.i. for RNA analysis. A) Quantification of *Il15* copies in lungs of RSV-infected wt or *Mavs*^{-/-} mice. B) On day 4 lung cells were stained for CD3, CD4, CD8, CD19, CD11b, CD11c, CD45, CD64, Ly6G, CD69, Nkp46, and TRAIL. Percentage of total NK cells and CD69⁺ NK cells in the lung. C) TRAIL expression on lung NK cells. D) Percentage of total NK cells and CD69⁺ NK cells in the lung of *Mavs*^{-/-} mice and rCCL2 treated *Mavs*^{-/-} mice on day 4 p.i.. E) Percentage of CD64^{hi}CD11b^{hi} inflammatory cells in the lung of *Mavs*^{-/-} mice and rCCL2 treated *Mavs*^{-/-} mice on day 4 p.i.. F) Percentage of total NK cells and CD69⁺ NK cells in monocyte depleted wt mice. Data for A, C, E and F represent one experiment with 4-5 mice per group. Data for B and D represent 2 individual experiments with 5 mice per group in each experiment. Results are presented as mean $\pm$ SEM. Statistical significances of differences between groups were determined by A) two-

tailed, unpaired Student's t-test, and B-F) One-way ANOVA with Tukey's post hoc test. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

5.4.6. Discussion

In the present study a role for recruited CD64^{hi} inflammatory cells in reducing the viral burden and RSV-induced pathology was demonstrated in rCCL2 treated *Mavs*^{-/-} mice. A direct antiviral effect of inflammatory monocytes has been described during different viral infections, such as influenza virus, MCMV and vaccinia virus (Barbalat et al., 2009; Davidson et al., 2014; Herold et al., 2008). Prevention of viral replication can be mediated through extrinsic activation of caspase-8 by death-receptors to induce apoptosis in infected cells (McIlwain et al., 2013). TRAIL, a member of the tumor necrosis factor (TNF) superfamily, is a potent inducer of apoptosis upon binding to death receptors expressed on the cell surface (Manzo et al., 2009). In influenza virus infection the interaction of TRAIL on inflammatory monocytes with the DR5 receptor on airway epithelial cells leads to apoptosis of the latter (Davidson et al., 2014; Herold et al., 2008). This was also dependent on type I IFN signalling. It was shown that RNA levels of TRAIL were induced in wt mice during RSV infections compared to type I IFN devoid *Mavs*^{-/-} mice (Figure 5-39), but detection of TRAIL on the cell surface proved to be difficult due to a cleavage site on the extracellular stalk of TRAIL which allows release of TRAIL as soluble mediator (Manzo et al., 2009). This release of TRAIL might explain the difficulties in identifying a particular cell type responsible for TRAIL expression by flow cytometry in the present study. However, this inability to identify the specific source of TRAIL secretion does not exclude a role for TRAIL-mediated cell death in antiviral responses to RSV.

Increased cell death and vascular permeability are linked to loss of epithelial barrier function (Miller, 1923) and this is reflected in elevated plasma protein (e.g. albumin) levels in the airways of RSV-infected wt mice (Figure 5-37). Inflammatory cytokines such as TNF- α , IFN- γ and IL-1 β were shown to compromise the epithelial cell barrier (Ivanov et al., 2010) and were induced in wt mice after RSV encounter (Figure 5-16, Figure 5-18). Notably, vascular permeability induced by neutrophils was shown to deliver antimicrobial proteins, such as IgM and the complements factor C1q into the alveolar space of mice (Kantrow et al., 2009). Similar contribution to the immune defence in wt mice could be mediated by infiltrating neutrophils and monocytes during RSV infection. In contrast to wt mice, *Mavs*^{-/-} mice had less vascular leakage at the early time points after RSV infection (Figure 5-37), in line with diminished proinflammatory cytokine responses and cellular recruitment, this comes at the cost of higher morbidity later in RSV infection.

Inflammatory monocytes can shape the inflammatory environment through their secretion of different cytokines and molecules including TNF- α , IL-1 α , IL-1 β , IL-6, iNOS, IL-10 and PGE2 (Grainger et al., 2013). Their capacity to release high amounts of TNF- α and iNOS to mount antibacterial responses termed them Tip-DCs (Serbina et al., 2003). In the present study CD64^{hi} monocytes were a source of IL-1 β and TNF- α in response to RSV infection (Figure 5-35) and depletion of these cells markedly reduced transcripts of the latter (Figure 5-26). To evaluate the role of CD64^{hi} monocytes-derived TNF- α in RSV-induced disease, anti-TNF- α was administered intranasally (4, 18, 48 hrs p.i.) into wt mice, but no difference in illness was detectable in TNF- α depleted mice suggesting compensatory mechanisms (Figure 5-36). This was in contrast to a study from Rutigliano et al. (2004) where anti-TNF- α treatment attenuated RSV-induced illness (Rutigliano and Graham, 2004). Possible explanations for this discrepancy are different routes of administration and TNF- α depletion throughout the whole study, which additionally affected T cell-derived TNF- α . In conclusion from the present study, TNF- α secreted by CD64^{hi} inflammatory cells plays a minor role in immunocompetent mice during RSV infection.

The immune modulatory lipid PGE2 is an important mediator in inflammatory responses at mucosal surfaces and is known for its wide inhibitory effects on innate immune cells (Morici, 2013). The expression of PGE2 is highly regulated and promotes immunosuppressive mechanisms to prevent tissue damage and maintain tissue integrity (Grainger et al., 2013). However, pathogens like influenza virus take advantage of PGE2 by enhancing its expression to counteract protective immune responses (Coulombe et al., 2014). The enzyme COX-2 which is responsible for PGE2 synthesis was upregulated during RSV infection and PGE2-metabolites were detected in BAL fluid of infected mice at the peak of monocyte influx (day 2p.i., Figure 5-38), but absent in RSV-infected *Mavs*^{-/-} mice. Macrophages and epithelial cells were reported to be the primary source of PGE2 in neonatal lamb RSV infections (Radi et al., 2010). The reduced PGE2 expression in the lungs of monocyte-depleted mice identified CD64^{hi} inflammatory cells as a source of PGE2 during RSV infection, but its exact role in RSV immunity is not clear.

Recruited CD64^{hi} monocytes could also indirectly enhance antiviral responses by modulating the activity and cytotoxicity of immune cells, such as NK cells in the lung both in a cell contact dependent manner and by the release of soluble mediators. One possible mediator produced by infiltrating monocytes that could influence NK cells is the cytokine

IL-15, a potent activator of NK cell proliferation and differentiation (Huntington, 2014). During RSV infection NK cell recruitment into the lung occurs between day 3 and 4. They are an abundant, early source of IFN- γ , play an important role in RSV clearance, and influence subsequent CD8 T cell responses through their cytokine secretion (Harker et al., 2010; Hsussell and Openshaw, 1998). Although different cell types can express IL-15 mRNA only, macrophages, DCs, and monocytes produce the bioactive IL-15 protein (Waldmann, 2014). IL-15 expression is highly dependent on type I IFN signalling (Durbin et al., 2000), which led to an absence of IL-15 in *Mavs*^{-/-} mice (Figure 5-40). This possibly resulted in the observed lack of NK cells expressing the activation marker CD69 in *Mavs*^{-/-} mice (Figure 5-40). Interestingly, with the presence of CD64^{hi} monocytes in CCL2 treated *Mavs*^{-/-} mice increasing activation of resident NK cells was detected but no *de novo* recruitment. Trans presentation of bioactive IL-15 by CD64^{hi} monocytes to promote IFN- γ and granzyme B production in NK cells (Soderquest et al., 2011; Soudja et al., 2012) might be an unlikely scenario due to the type I IFN dependency for IL-15 expression (Durbin et al., 2000). Other cytokines important for NK cell activation are IL-18 and TNF- α (Serti et al., 2014; Soudja et al., 2012; Vogel et al., 2014), both reported as monocyte mediators during viral infection and the latter was detected in RSV infection (Figure 5-35). Another possibility for NK cell activation are cell-contact dependent interactions between monocytes expressing ligands that bind NK cell receptors, as described for macrophage-NK cell interactions *in vitro* (Michel et al., 2012). In contrast to rCCL2 treated *Mavs*^{-/-} mice, monocyte depleted wt mice displayed normal activation (CD69^{hi}) of NK cells, since type IFNs, important for NK cell activation during viral infection, were not abrogated in wt mice (Andoniou et al., 2005). But surprisingly monocyte depleted mice had diminished peripheral NK cell influx and/or proliferation in the lung (Figure 5-40). These preliminary data might suggest that NK cells crosstalk with monocytes to enter the inflamed lung and/or proliferate. How monocytes interact with NK cells during RSV infection needs further investigation, but monocytes were sufficient to promote resident NK cell activation, to support antiviral responses and RSV resolution.

In summary, the nature of the antiviral mechanism elicited by inflammatory monocytes remains unclear but it is speculated that a combination of several mechanisms is utilised by CD64^{hi} monocytes to restrict RSV replication in the lung.

6. Conclusions

Human respiratory syncytial virus (RSV) can cause mild upper respiratory tract infections, known as common cold, but RSV is also the prevalent cause of lower respiratory tract infections (LRTI) in children, the elderly, and immunocompromised individuals. The common forms of childhood LRTI are bronchiolitis and pneumonia with numerous risk factors being associated with severe RSV-induced LRTI. Currently, there is a lack of effective immunotherapies for RSV-associated LRTI and LRTI treatment only involves supportive care (Borchers et al., 2013; Collins and Melero, 2011).

Pathogen sensing is mediated by germ-line encoded receptors of the innate immune system to trigger appropriate immune responses against invading pathogens. Several of these PRRs have been implicated to be involved during RSV infection, including members of the TLR family, such as TLR2, TLR3, TLR4 and TLR7 (Kurt-Jones et al., 2000; Lukacs et al., 2010; Murawski et al., 2009; Rudd et al., 2006). Other studies suggested a crucial role for cytosolic RLRs in RSV sensing and for the induction of innate immune responses (Bhoj et al., 2008; Demoor et al., 2012).

Upon viral infections the secretion of type I IFNs comprises the imminent response triggered in infected cells. These cytokines induce robust antiviral and immunomodulatory responses to contain viral replication and spread (McNab et al., 2015). Sensing of viral PAMPs by PRRs leads to the transcriptional activation of IRF3 and IRF7 (the latter only in pDCs), and the secretion of type I IFNs by infected cells (Gürtler and Bowie, 2013). Secreted type I IFNs mediate signalling through their transmembrane receptor IFNAR, and stimulate the expression of hundreds of genes, that directly interfere with viral replication and viral spread (Platanias, 2005; Uddin and Platanias, 2004). In addition, type I IFNs modulate the immune response by promoting antigen presentation, cytokines production, DC and NK cell activity and macrophage function, and they stimulate protective adaptive immunity by enhancing T cell effector function and antibody production (González-Navajas et al., 2012; Swiecki and Colonna, 2011).

RSV can stimulate the production of type I IFNs *in vitro* and *in vivo*. Several studies confirmed an antagonising effect of RSV-NS proteins on type I IFN production and signalling (Bossert et al., 2003; Boyapalle et al., 2012; Ling et al., 2009; Ramaswamy et

al., 2006; Spann et al., 2004; Swedan et al., 2009; Thornburg et al., 2012). This mechanism of immune evasion in RSV infection was shown *in vitro* in epithelial cell lines and primary epithelial cells, representing the major target of RSV replication *in vivo*. Therefore, other pulmonary innate cell populations are of increasing importance for type I IFN secretion and initiating the primary immune response to RSV. How type I IFNs modulate early innate immune responses beyond local viral control during RSV infection is understudied.

To characterise the overall role for type I IFNs in RSV-mediated disease the consequences of IFNAR deficiency for innate immune response was studied in RSV infected *Ifnar1^{-/-}* mice. Loss of type I IFN signalling was characterised by increased viral burden and disease pathology. It was also shown that type I IFNs signalling was crucial in regulating the early inflammatory response in the lung (Goritzka et al., 2014). This suggested that type I IFNs elicit their antiviral potential beyond their cell-intrinsic viral control. To further delineate the type I IFN responses induced during RSV infection the cellular source of type I IFNs and how the IFN-mediated inflammation influences the outcome of RSV infection was studied.

Alveolar macrophages (AMs) are directly exposed to invading viral pathogens as they are positioned in the airways at the interface between the environment and lung tissue. This study provided for the first time evidence that AMs are the primary source of type I IFNs during RSV infection *in vivo*. A recent study showed that RSV infections of AMs *ex vivo* led to abortive production of infectious virus progeny (Ravi et al., 2013). This natural resistance of AMs will certainly prevent virus propagation in the lower airways and renders AMs less susceptible for RSV-induced cytopathic effects, in support of their antiviral responses. Furthermore, the present study showed that RSV recognition by AMs is mediated by RLRs depending on MAVS signalling. In addition, proinflammatory cytokine and chemokine secretion solely relied on RLR-MAVS pathways and most likely was dependent on AMs. This is supported by data from AM depletion models where proinflammatory cytokine secretion was abolished during RSV infection (Kolli et al., 2014; Pribul et al., 2008). Other studies showed that the activity of NF- κ B, the key transcription factor for proinflammatory cytokines, was entirely dependent on the presence of AMs in the lungs of RSV infected mice (Haeberle et al., 2002). Additionally, no alternative MAVS independent pathways for the induction of type I IFNs could be detected in *Mavs^{-/-}* mice. This places AMs in the centre of coordinating the immune

responses to RSV, by the secretion of type I IFNs, and possibly regulating other pro-inflammatory cytokines, that drives the inflammatory response during RSV infection and protect from pathology.

Type I IFNs are crucial to elicit intrinsic antiviral responses in infected cells to prevent viral replication. This study showed that AM-derived type I IFNs induced the production of CCL2 and other monocytes chemoattractants crucial for monocyte extravasation into the lung during RSV infection. These inflammatory monocytes can differentiate into inflammatory DCs or Tip-DCs in inflamed tissue and regulate immunity to viruses, bacteria, fungi and parasites (Segura and Amigorena, 2013). During RSV infection inflammatory monocytes become the dominant leukocyte subset detected in the lung by day 2 p.i. and were identified by their expression of CD64 to distinguish them from resident cDCs. With progressing disease CD64^{hi} inflammatory cells undergo a developmental transition from extravasated Ly6C^{hi}CD11c^{lo} monocytes to Ly6C^{lo}CD11c^{hi} inflammatory DCs or Tip-DCs. In *Mavs*^{-/-} mice this early accumulation of CD64^{hi} monocytes was completely absent, and IFNAR signalling was shown to play a role in early monocyte migration during RSV infection, as supported by studies using other infection models (Conrady et al., 2013; Majer et al., 2012). Interestingly, the induced recruitment of monocytes into RSV-infected *Mavs*^{-/-} mice through adoptive transfer experiments (AM transfer) or chemokine reconstitution experiments (rCCL2) led to 100-fold decreased viral load and decreased pathology in these mice, indicating the beneficial effect of CD64^{hi} inflammatory monocytes during RSV infection.

The exact architecture of the antiviral mechanism implemented by CD64^{hi} inflammatory cells could not be demonstrated, even though several functional properties attributed to these cells were assessed. It was shown that monocytes display an inflammatory signature by producing TNF- α and IL-1 β during RSV infection, a mechanism previously reported for the control of bacterial infections (Grainger et al., 2013; Mayer-Barber et al., 2011; Serbina et al., 2003). The control of viral replication and TNF- α levels measured in the lung directly correlated with monocyte infiltration (rCCL2 treatment) or depletion (anti-CCR2 treatment) in RSV infection. Monocyte-derived TNF- α was shown to activate NK cells during HSV-1 infection (Vogel et al., 2014), and the inflammasome/caspase-1 regulated IL-18, was shown to stimulate IFN- γ production from NK cells to limit HCV replication (Serti et al., 2014). Although only IL-1 β was measured, in this work it is likely to speculate that IL-18 is similarly upregulated as

they are both inflammasome/caspase-1 dependent. Similar mechanisms could explain the increase in NK cell activation and subsequent restrained viral replication after recruitment of monocyte into the lung of *Mavs*^{-/-} mice during RSV infections.

Type I IFN production induced by MAVS and subsequent IFNAR-signalling are the central mediators for the induction of early antiviral proinflammatory responses and monocyte migration during RSV infection, but it was also shown that during the later response to RSV infection MAVS independent pathways are regulating immune responses. Hence, other detection and signalling pathways likely gained increasing importance to continue antiviral responses during the course of infection and clearance. Uptake of accumulating infected apoptotic and necrotic cell debris by macrophages and DCs can trigger endosomal TLRs and stimulate cytokine and chemokine secretion dependent on MyD88 and TRIF, but not MAVS (Albert et al., 1998; Hashimoto et al., 2007; Hedlund et al., 2010). Additionally, inflicted injury of lung tissue exerted by the cytolytic activity of RSV itself and inflammatory mediators will generate danger signals and will in turn attract neutrophils and other cells to the site of inflammation (McDonald et al., 2010).

The induction of adaptive immune responses independent of functional type I IFN production and IFNAR-signalling suggests a potent activation and maturation of DCs involving other pathways independent of MAVS and IFNAR. TLR3 and TRIF signalling is most properly the key pathway involved in CD8⁺ T cell cross-priming, as suggested by Bhoj *et al.* (2008). In the absence of MAVS and MyD88 a not further characterised pulmonary DC subset was attributed to prime CD8⁺ T cell (Bhoj et al., 2008). It cannot be excluded that CD64^{hi} monocytes with phenotypical characteristics of DCs are involved in antigen uptake and T cell priming in wt mice during RSV infection. Several reports demonstrated that CD64^{hi} inflammatory monocytes can indeed present antigens to T cells (León et al., 2007; Plantinga et al., 2013; Zigmond et al., 2012) and dependent on the type of inflammation modulate the type of T cell response (Hammad et al., 2010; Nakano et al., 2009; Plantinga et al., 2013; Schreiber et al., 2013). Secretion of IL-12 stimulates Th1 cell polarisation and IFN- γ secretion (Nakano et al., 2009; Schreiber et al., 2013). Whereas in allergic inflammation monocytes can induce Th2 cell responses (Hammad et al., 2010; Plantinga et al., 2013). However, CD64^{hi} inflammatory monocytes were certainly dispensable in MAVS- or IFNAR-deficiency, where T cell priming and recruitment was intact. This is in contrast to protozoa infections or respiratory fungal

infections where inflammatory monocytes are essential for antigen uptake and the induction of T cell responses (Hohl et al., 2009; León et al., 2007). Furthermore, CD64^{hi} inflammatory monocytes could play a role in re-stimulation antigen-specific CD8⁺ T cells in peripheral tissue. In mucosal infections with HSV-2 and influenza virus stimulation of effector T cell responses are compromised in the absence of inflammatory monocytes (Aldridge et al., 2009; Iijima et al., 2010), suggesting a role for inflammatory monocytes in the magnitude of effector T cell responses during RSV infection.

Children with high RSV load at admission are more likely to develop severe RSV-induced LRTI (DeVincenzo et al., 2005; Fodha et al., 2007; Saleeby et al., 2011; Scagnolari et al., 2012), suggesting a strong correlation between the viral load and disease in RSV infection. Loss of viral control by CD64^{hi} inflammatory monocytes could lead to heightened effector T cell responses due to increased viral burden and thereby associated worsened disease. A study has demonstrated that the depletion of effector CD8⁺ T cell can improve weight loss in RSV-infected mice (Graham et al., 1991), in contrast administration of RSV-specific CD8⁺ T cells can augment pathology (Cannon et al., 1988). So it is possible that the increases detected in weight loss in *Mavs*^{-/-} and *Ifnar1*^{-/-} mice is due to exacerbated T cell responses. This is supported by a study from Demoor *et al.* (2012) who demonstrated elevated protein levels of IFN- γ in BAL fluid and increasing numbers of activated IFN- γ ⁺ CD4⁺ and CD8⁺ T cells in the absence of MAVS (Demoor et al., 2012), and Johnson *et al.* (2005) who detected increased IFN- γ levels in BAL fluid of *Ifnar1*^{-/-} mice (Johnson et al., 2005).

The present study demonstrated a protective role for type I IFNs in RSV infections, by regulating the early inflammatory responses and cell migration into the lung. A severe reduction in proinflammatory cytokines and in particular CD64^{hi} inflammatory monocyte trafficking was associated with increased illness and viral burden. The immunopathology observed in a type I IFN devoid host is most likely the result of RSV-mediated tissue damage, exacerbated T cell responses and/or neutrophil recruitment at peak of illness.

Recruitment of CD64^{hi} inflammatory monocytes improved the disease outcome in *Mavs*^{-/-} mice, suggesting that these cells are beneficial for the host and promote RSV clearance and immunity. This study coupled for the first time the dependency of CD64^{hi} inflammatory monocytes on AM-derived type I IFN production in respiratory tract

infections with RSV. The CD64^{hi} inflammatory monocytes represent a cell extrinsic type I IFN-mediated antiviral mechanism to further extend viral restriction after the transient expression of type I IFNs itself and before additional immune cells, like NK cells and T cells, will elicit their antiviral functions (Figure 6-1; Goritzka et al., accepted for publication *J Exp Med* 2015)).

The timing of LRTI cases being admitted to hospital aggravates in depth studies of early antiviral immune responses to RSV in children, since most of these transient responses will have vanished. Therefore, data on the very early events are virtually absent, but later in established RSV infection type I IFNs were either not detected or detected at low level in nasopharyngeal samples (Isaacs, 1989; Scagnolari et al., 2009; Taylor et al., 1989). One might speculate that inadequate innate responses in some children with RSV will hamper the viral control and the increased viral burden will provoke strong inflammatory response later during the course of infection. In consequence infiltrating inflammatory cells such as neutrophils and T cells will augment lung pathology and increase morbidity (Everard et al., 1994; Heidema et al., 2007; Johnson et al., 2006; Lukens et al., 2010; McNamara et al., 2003; Wang et al., 1998). To prevent exacerbated host immune responses to RSV in this scenario the administration of antiviral agents might improve the disease outcome. In contrast, the potential of type I IFNs to drive and amplify proinflammatory responses (Davidson et al., 2014; Goritzka et al., 2014) could, if type I IFNs are produced excessively, possibly cause the excessive immune responses and increased morbidity and mortality to RSV in some children, independent of the viral burden. Here administration of anti-inflammatory drugs could benefit the course of disease. Accordingly, reliable clinical markers need to be established to identify the cause of the inflammatory response from case to case, in order to decide on an appropriate therapy other than supportive care.

Utilising the antiviral properties of type I IFNs in regulating the inflammatory response and monocyte migration as detected in RSV infection could be another therapeutical approach. Trials with intramuscular administrations of IFN- α 2 to children with RSV-induced illness could not provide any conclusive data. With one group detecting clinical improvements in treated children (Sung et al., 1993) and another study dismissing any effect on the course of disease (Chipps et al., 1993). However, administration through aerosols might be more suitable to impact the local mucosa of

the airways, but need to be carefully assessed to avoid side effects by the administered type I IFNs.

To harness the antiviral effect identified within the recruited monocyte population a selectively recruitment independent of type I IFNs might be beneficial in dampening RSV replication to prevent excessive inflammatory responses later in the course of RSV infection.

In summary, type I IFN-mediated antiviral and inflammatory effects need careful regulation to contain RSV replication along with minimising damage of excessive inflammation to the host. One might speculate that genetically or environmentally changes affecting the type I IFN system predispose to severe LRTI with RSV, as seen in humans.

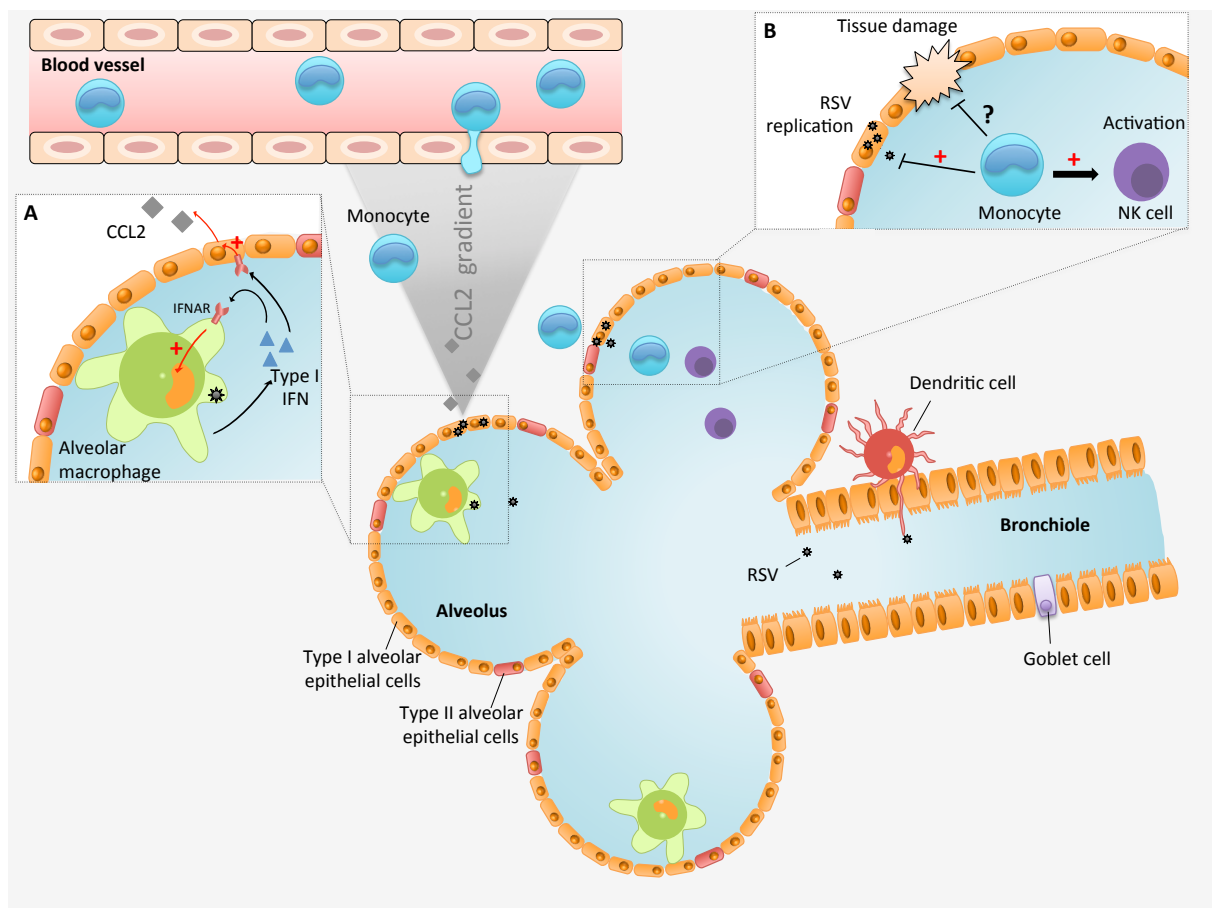


Figure 6-1 Early immune responses in the RSV infected lung. Upon RSV infection cytosolic sensing of virus particles by the RNA sensors RIG-I and MDA5 in alveolar macrophages activates MAVS pathways and leads to the upregulation of inflammatory cytokines and chemokines. A) Release of the antiviral type I IFNs stimulates the type I IFN receptor mediated upregulation and secretion of the monocyte chemoattractant CCL2. B) Following trafficking of blood monocytes along the CCL2 gradient into the inflamed lung, these monocytes restrict viral replication and spread and promote NK cell activation.

Future directions

Regulation of inflammatory responses by type I IFNs

In this study a regulatory role for type I IFNs in proinflammatory cytokine expression in the lungs was identified during RSV infection. The signalling pathways and proteins involved in the cross talk between IFNAR-signalling and NF- κ B-signalling are not clear and need further investigation.

The source of CCL2

The cellular source of CCL2 during RSV infection was not identified during the present study. FACS sorting of the individual pulmonary lung populations, including structural cells at different time points during RSV infection, could address this question. The purified cells can be further analysed for mRNA expression levels of *Ccl2*.

Mechanism of antiviral effect by monocytes

Although the recruitment of CD64^{hi} monocytes into *Mavs*^{-/-} mice was shown to have a clear impact on viral replication and pathology the exact mechanism by which inflammatory monocytes exert their antiviral function is yet unresolved. The observation that recruited CD64^{hi} inflammatory monocytes activate NK cells in the lungs of *Mavs*^{-/-} mice is very interesting. It remains to be analysed whether an indirect mechanism through the release of mediators or a direct cell-cell-contact dependent mechanism play a role in this activation. These questions could be addressed in a relatively simple co-culture system where sorted NK cells and monocytes from the lung can interact either through direct cellular contacts or are separate by a membrane to only allow for soluble factors to circulate between the cell types. Additionally, studying the effector function of monocyte-induced NK cells, by means of intracellular staining for IFN- γ and granzyme B, will provide deeper insight into monocyte-mediated NK cell activation and function.

To assess a potential direct antiviral effect of monocytes, co-culture systems of RSV infected cell lines and sorted mouse monocytes could be applied.

Furthermore, the involvement of monocytes in tissue damage or repair should be assessed at later time points during RSV infection. Since day 4 p.i. did not provide conclusive data regarding vascular permeability (albumin concentration in BAL was assessed). The differences in epithelial permeability in wt mice and *Mavs*^{-/-} mice compared to rCCL2 treated *Mavs*^{-/-} mice and monocyte depleted wt mice could be further assessed by measuring albumin levels in the airways or using immunofluorescence staining for tight junction proteins.

Influence of type I IFN on adaptive immune response

It was surprising that RSV-specific T cells were generated in mice lacking the early immune responses to RSV. Whether the quality of the T cell responses is affected and if T cells are driving RSV-induced disease need further investigations. Firstly, intracellular staining for T cell derived cytokines (IFN- γ , TNF- α) and the apoptosis mediating granzyme B will give indications about the qualitative effector responses of T cells in the lung and airways. Secondly, depletion of T cells will further characterise their contribution to the observed weight loss. Furthermore, the implication of the lack of innate responses on the quality and quantity of memory responses to successive RSV reinfections should be studied in-depth.

The DC subset identified in another study (Bhoj et al., 2008) possible responsible for the priming of CD8⁺ T cells in *Mavs*^{-/-} mice is not well characterised. Using a fluorescent labelled RSV-strain together with sophisticated surface antigen staining, will help to characterise the DC subtype harbouring RSV-antigen. Additionally, assessing the activation status of different DC subsets in the lung and LNs will further help to characterise detailed DC responses.

Signalling pathways involved in the second cytokine induction and cell recruitment during RSV

The second response of cytokine expression and cell recruitment during RSV infection was independent of MAVS signalling, therefore other pathways need to be taken into account for the observations made. One possibility is the phagocytic uptake of necrotic infected cells by immune cells, which triggers endosomal TLRs to signal through MyD88

or TRIF. Using knockout mice in MyD88 and TRIF will give further insights into the role of TLRs during late responses to RSV infection.

The role of monocytes in the induction of RSV illness

CD64^{hi} monocytes recruited later during RSV infection might in part drive exacerbated T cell responses, by re-stimulating antigen specific T cells in the lung and enhancing their antiviral responses. Depletion of monocytes from the blood will prevent the late recruitment and give the opportunity to study the T cell responses in the presence or absence of monocytes. If significant differences are detectable, the mechanism of interaction will be addressed in co-culture experiments of sorted lung monocytes and T cells.

Characterisation of responses in RSV infected human patients

RSV challenges in adults could be used to characterise the IFN responses and cell recruitment into the respiratory tract under controlled conditions. Upon infection samples throughout the course of infection will be obtained from the upper and lower respiratory tract to assess for antiviral cytokines (type I, II and III IFNs), proinflammatory cytokines (IL-6, IL-1 β , TNF- α , etc.), and chemokines (CCL2, IL-8, CXCL10). Cell recruitment into the airways can be assessed by flow cytometric analysis of bronchoscopy samples (lower airways) and nasal scrapes (upper airways). It would be of particular interest if cell recruitment resembles the observations made in mice in this study.

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