

Presence of neutrophils in the lungs prior to infection with respiratory virus alters disease severity in mice

A thesis submitted for the degree of Doctor of
Philosophy

to

Imperial College London

Amber Owen

Section of Respiratory Infections

National Heart and Lung Institute

Imperial College London

Statement of Originality

I certify, to the best of my knowledge, that the experimental and written content presented in this thesis is a product of my own work. Any resources used or assistance provided have been appropriately referenced and acknowledged. This thesis has not been submitted for another degree or other purposes. - Amber Owen 2024

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Abstract

Respiratory viruses such as respiratory syncytial virus (RSV) and influenza A viruses (IAV) are amongst the leading causes of global death by communicable disease. Certain individuals, including infants and the elderly are at increased risk of severe disease following respiratory viral infection. However, the immunologic factors underlying increased risk are not fully understood. Using human challenge studies, increased neutrophil activation in the nasal mucosa before experimental RSV infection was found to best correlate with developing symptomatic disease. Similarly, in mouse models of RSV infection, intranasal CXCL1 to recruit neutrophils shortly before RSV infection drove exacerbated disease. In this thesis, mouse models were used to further explore how neutrophils in the respiratory tract before respiratory viral infection can alter the immune response and subsequent disease. Firstly, intranasal exposure to bacteria or their PAMPs before RSV infection was shown to increase early weight loss. This was not driven solely by neutrophils in the lungs before infection but by elevated expression of *Il1a* and *Tnfa*. Secondly, by increasing the time between neutrophil recruitment and RSV infection, this was shown to result in opposing effects on disease, protecting mice from weight loss. This was associated with the presence of apoptotic neutrophils and potentially through them altering the alveolar macrophage response to the virus. Finally, it was shown that neutrophils recruited by intranasal CXCL1 at different timepoints could exacerbate or protect mice from weight loss following IAV infection. By comparing the RSV and IAV infection models, it was revealed that the timepoint of neutrophil recruitment relative to recognition of the virus, rather than the timepoint of infection, likely dictates disease outcomes. Together, these findings increase our understanding of the environmental and immunologic factors that increase risk of severe disease following respiratory viral infection and might help in the development of future broad-spectrum interventions.

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Abbreviations

<i>A. baumannii</i>	<i>Acinetobacter baumannii</i>
ACE2	Angiotensin converting enzyme 2
ACK buffer	Ammonium chloride potassium buffer
AECs	Airway epithelial cells
ALR	Absent in melanoma-2- like receptors
AM	Alveolar macrophage
ANOVA	Analysis of variance test
ARDS	Acute respiratory distress syndrome
AWERB	Animal Welfare and Ethical Review Board
BAI1	Brain-specific angiogenesis inhibitor 1
BAL	Bronchoalveolar lavage
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumin
C/EBP	CCAAT-enhancer-binding protein
Ca ²⁺	Calcium ions
CARD	Caspase recruitment and activation domain
CCI12	C-C motif 12
CCL2	C-C motif ligand 2
CCL5	C-C motif ligand 5
CCL7	C-C motif 7
CCR2	C-C chemokine receptor 2
CDC	Centers for Disease Control and Prevention
cDC	Conventional dendritic cell
CDG	Chronic granulomatous disease
cDNA	Copy deoxyribonucleic acid
CFU	Colony forming units
CLR	C-type lectin receptor
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
cRNP	Copy viral ribonucleoprotein
CT	Cycle threshold
CTL	Cytotoxic T lymphocyte
CXCL1	C-X-C motif ligand 1

CXCL10	C-X-C motif ligand 10
CXCL2	C-X-C motif ligand 2
CXCL8	C-X-C motif ligand 8
CXCR2	C-X-C chemokine receptor 2
DAMPs	Damage association molecular patterns
DC	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DR5	Death receptor 5
dsRNA	Double stranded ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FasL	Fas cell surface receptor ligand
FCS	Foetal calf serum
FFU	Focus forming units
FMO	Fluorescence minus one control
FoxP3	Forkhead box P3
<i>Gapdh</i>	Glyceraldehyde 3-phosphate dehydrogenase gene
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte/myeloid progenitor cell
GzmB	Granzyme B
HA	Haemagglutinin
IAV	Influenza A virus
IBD	Inflammatory bowel disease
IBV	Influenza B virus
ICAM1	Intercellular adhesion molecule 1
IFN	Interferon
IFNAR	Interferon alpha receptor

Ig	Immunoglobulin
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-17	Interleukin-17
IL-1R	Interleukin-1 receptor
IL-1 α	Interleukin-1 alpha
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
in	Intranasal
ip	Intraperitoneal
IRF-3	Interferon regulatory factor 3
ISG	Interferon stimulated gene
IV	Influenza virus
LB	Lysogeny broth
LPS	Lipopolysaccharide
LRTI	Lower respiratory tract infection
LTA	Lipoteichoic acid
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MAb	Monoclonal antibody
Mac1	Macrophage-1 antigen
MAPK	Mitogen activated protein kinase
MAVS	Mitochondrial antiviral-signalling protein
mCRAMP	Mouse cathelicidin- related antimicrobial peptide
MDA5	Melanoma differentiation-associated protein 5
MerTK	Mer tyrosine kinase
mexAM	Mouse <i>ex vivo</i> alveolar macrophages
MHC-I	Major histocompatibility complex I
MHC-II	Major histocompatibility complex II
MLKL	Mixed lineage kinase domain-like pseudokinase
MMP-9	Matrix metalloprotease 9
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary-response protein 88

NA	Neuraminidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
Nf-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NLR	Nucleotide oligomerisation domain-like receptor
NLRP3	Nucleotide oligomerisation domain-like receptor family pyrin domain containing 3
NO	Nitric oxide
NOD	Nucleotide oligomerisation domain
NPY	Neuropeptide Y
O_2^-	Superoxide anions
PAD4	Peptidyl arginine deiminase 4
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell
PFU	Plaque forming units
PGE ₂	Prostaglandin E2
PKB	Protein kinase B
POMC	Proopiomelanocortin
PRR	Pattern recognition receptor
PSGL1	P-selectin glycoprotein ligand 1
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
Rag2	Recombination activating 2
RdRp	RNA-dependent ribonucleic acid polymerase
RIG-I	Retinoic acid-inducible gene-I
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RLR	Retinoic acid-inducible gene-I like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium

RSV	Respiratory syncytial virus
RT-qPCR	Real time quantitative polymerase chain reaction
SARS-CoV-2	Severe-acute-respiratory-syndrome coronavirus 2
SEM	Standard error of mean
SLE	Systemic lupus erythematosus
SP	Surfactant protein
ssRNA	Single-stranded ribonucleic acid
TAM	TYRO3, AXL and MERTK
T _{CM}	T central memory cells
TCR	T cell receptor
T _{EM}	T effector memory cells
T _{FH}	T follicular helper cell
TGF- β	Transforming growth factor beta
Th1	Type 1 helper T cell
Th17	Type 17 helper T cell
Th2	Type 2 helper T cell
TLR	Toll-like receptor
TNFR1	Tumour necrosis factor receptor 1
TNF- α	Tumour necrosis factor- alpha
TRAIL	TNF-related apoptosis inducing ligand
T _{Reg}	T regulatory cells
TRIF	TIR domain-containing adaptor protein inducing IFN- β
T _{RM}	Tissue resident memory cells
vRNP	Viral ribonucleoprotein
α -CCR2	Anti-CCR2 antibodies
α -IL-1 α	Anti-IL-1 α antibodies
α -IL-1 β	Anti-IL-1 β antibodies
α -Ly6G	Anti-Ly6G antibodies
α -rat kappa IgG	Anti- rat kappa chain IgG antibodies
α -TNF- α	Anti-TNF- α antibodies

1. Introduction

Through the development of modern medicine and vaccination, the global burden of infectious diseases has steadily declined. Despite this, lower respiratory tract infections (LRTIs) caused by viral pathogens remain in the top 5 causes of death globally (1). Moreover, respiratory viruses are considered a leading cause of mortality by a communicable disease and are the leading cause of mortality amongst infants under the age of 5 (2,3). The emergence of SARS-CoV-2 in 2019, shone a spotlight on the devastating consequences of respiratory viruses both on public health and global economies (4). However, there are many other respiratory viruses such as influenza viruses, adenovirus, rhinovirus and respiratory syncytial virus (RSV) which circulate each year, having a significant impact on the health of at-risk individuals (5).

Respiratory viruses are found ubiquitously throughout our environment (6). By the age of 2, most children will have been infected by a respiratory virus, developing symptoms typically described as “the common cold” (7). In the case of many respiratory viruses, infection does not lead to the development of full immunity, thus reinfection can occur throughout life (8). Infection begins when a viral particle is inhaled and comes into contact with the cells which line our respiratory mucosa (9). Here they bind to a range of specific receptors expressed on the surface of our airway cells, allowing them to fuse with the host cell and inject their viral genome (9). Once inside, the virus co-opts the host cell machinery, using them as factories to mass produce large quantities of progeny (10). This invasion does not go unnoticed by the host cells, which trigger a cascade of immune responses to clear the infection (11). Ultimately, it is these immune responses which lead to the development of symptoms (11). Classically, respiratory viral infection remains confined to the upper airways where they typically result in mild symptoms such as a runny or blocked nose, a cough or sore throat etc (12). However, in susceptible individuals, respiratory viruses can spread to the lower respiratory tract where they can cause more severe symptoms, such as pneumonia or bronchiolitis (12). Understanding the immune determinants which predispose an individual to severe disease following infection is therefore of great importance in the prevention and treatment of respiratory viruses. This thesis explores the roles neutrophils play in exacerbating disease following infection with different respiratory viruses in the context of mouse models.

1.1 Respiratory Syncytial Virus

First isolated in 1956, Respiratory Syncytial Virus (RSV) was quickly recognised as major cause of respiratory associated illness, hospitalisation and death amongst infants (2). The virus is a negative sense, single stranded RNA virus which belongs to the *Pneumoviridae* family (13). RSV is a human specific virus of which there are two circulating strains, A and B.

Whilst there are no other natural mammalian hosts to RSV, there are closely related viruses which cause infections in animals and can be used to model the disease, including bovine RSV in cows and pneumonia virus in mice. Mice can also be experimentally infected with human RSV at high viral titre, which has proved a useful model in investigating the immune response to the virus infection (14–17). When inhaled, RSV targets airway epithelial cells with a preference for ciliated cells (18). *In vitro*, RSV can drive the fusion of neighbouring cell membranes leading to the formation of multinucleate cellular plaques known as syncytia, a process through which the virus gets its namesake (18).

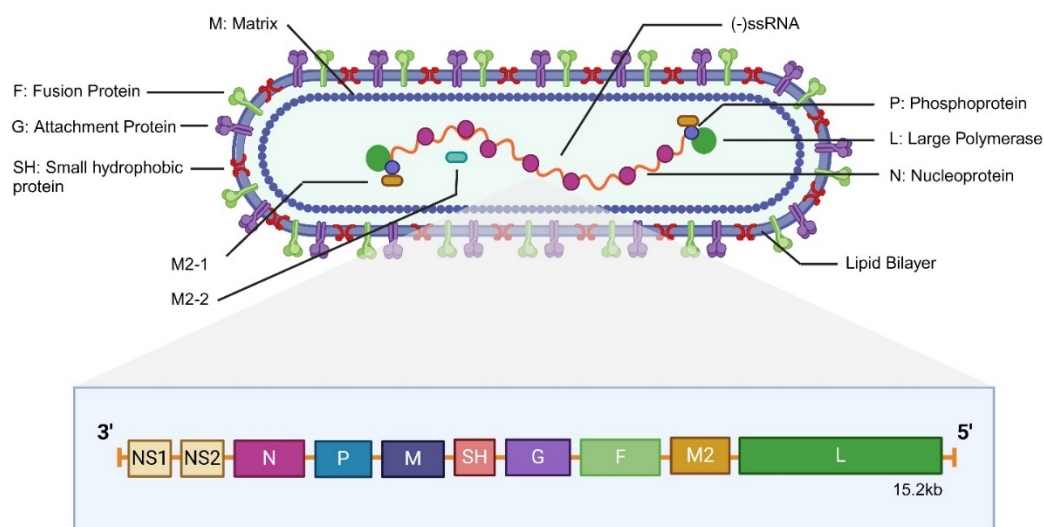


Figure 1.1 Schematic illustrating the structure of RSV

RSV is a filamentous, 15.2kb negative-sense single stranded RNA virus ((-)ssRNA). Its genome encodes for 9 structural proteins N, P, M, SH, G, F, M2-1, M2-2 and L, organised as indicated, and 2 non-structural proteins NS1 and NS2. Adapted from Cordova-Davalos et al (19). Created using BioRender.

1.1.1 RSV Virology

The 15.2kb RSV genome consists of 10 genes enveloped in a lipid membrane studded with structural proteins, forming long filamentous particles 60-200nm in diameter and up to 10µm in length (Fig. 1.1) (20). The 10 genes encode for 11 proteins: two non-structural proteins NS1 and NS2, and 9 structural proteins N, P, M, SH, G, F, M2-1, M2-2 and L. Three of these structural proteins, G, F and SH are transmembrane glycoproteins which insert into the lipid envelope. The G protein facilitates attachment of the virion to the host cell through interactions with glycosaminoglycans on the cell surface (21,22). Membrane fusion with the host cell is then initiated by the F proteins which form a trimer on the surface of the virion known as the pre-fusion complex (23). This complex will then undergo dramatic conformational changes generating enough energy to drive fusion of the two membranes (24). The F protein is also

important in driving fusion of an infected cell's membrane with that of neighbouring cells to form syncytia (25). The SH transmembrane protein is less well characterised than the G and F proteins. Whilst little is known on its significance during RSV infection, it has previously been shown to form pentameric pores which may act as cation channels. The formation of these pores has previously been suggested to reduce apoptosis of infected cells as well as inhibit TNF- α production and TNF receptor signalling (26).

Following entry of the viral genome into the cell, replication is enabled by the remaining 6 structural proteins (27). For replication to be initiated, the viral genome must be coated in a protective protein coat known as the nucleocapsid. Formation of the RSV nucleocapsid is driven by the N protein which can bind to both the genome and the antigenome creating helical structures (28). This in turn enables the formation of the nucleocapsid/polymerase complex formed by interaction of the P protein with the N protein of the nucleocapsid and subsequently the L protein which possesses the polymerase domain (29,30). Both the M2-1 and M2-2 proteins also play key roles in transcription of the antigenome and subsequent RNA replication. M2-1 also interacts with the P protein and the viral genome where it is believed to act as a transcription processivity factor, allowing the polymerase complex to carry out continuous synthesis of the antigenome (31). In contrast the M2-2 protein is thought to be important in switching polymerase activity from transcription to replication of new viral genomes (32). Studies on viral morphogenesis have indicated that the M protein may have several key roles. Whilst it does not appear to drive formation of viral filaments, it can be found associated with viral inclusion bodies in the cytoplasm, where viral replication takes place (27,33). Here it is thought to silence synthesis of viral RNA and transport the newly formed viral RNA to the plasma membrane for packaging into virions (33).

The two remaining proteins encoded for by the RSV genome are the non-structural proteins NS-1 and NS-2. Neither of these proteins play a role in viral genome replication, instead they appear to disrupt the host immune response to the virus. Particularly, NS-1 and NS-2 appear to disrupt type I interferon (IFN) receptor signalling by preventing translocation of IRF-3 to the nucleus (34). In addition, NS-1 and NS-2 have also previously been shown to promote survival of the host cell by inducing the early expression of anti-apoptotic factors such nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and protein kinase B (PKB) (35).

1.1.2 RSV Epidemiology and Clinical Manifestation

The epidemiology of RSV endemics is thought to be determined largely by climate. In the UK, the RSV season usually occurs between October and March, with cases tending to peak in December (36). In recent years, RSV seasons have been disrupted due to COVID-19 pandemic interventions, with low incidence occurring in the winter of 2020-21 but a large surge

in cases occurring in the summer of 2021, correlating with relaxation of social distancing measures (3). However, RSV seasonality now appears to have returned to normal according to RSV-NET data collected by the Centers for Disease Control and Prevention (CDC) (37). Before the age of 2, 90% of infants will have already been infected with RSV (38). Whilst primary infection does result in the generation of neutralising antibodies, these appear to be only partially effective at providing protection and wain rapidly. Therefore, natural reinfection is common throughout life (39). In healthy adults, RSV infection mostly results in a common cold, with the individual suffering from mild symptoms including a cough, sore throat and runny nose (12). However, infection with RSV can become much more insidious in susceptible populations, predominantly infants and the elderly. When the virus spreads from the upper airways to the lower airways, RSV can drive acute inflammation of the bronchioles or alveoli, termed as bronchiolitis or pneumonia respectively (2). RSV is the most common cause of bronchiolitis in infants, which can often require hospitalisation and ventilation (40). It is estimated that 3.2 million children under the age of 5 are hospitalised globally each year due to RSV infection, resulting in 59,600 in-hospital deaths, making RSV the leading cause of infant mortality due to communicable disease (5). Risk of severe lower respiratory tract infections in adults also increases with age. In the UK, RSV is believed to account for 5.9% of all respiratory associated deaths in those over the age of 50, resulting in over 8000 deaths per season (41,42). Other individuals considered at risk of severe lower respiratory tract infections following RSV infection, include those who suffer from cardiac disease, asthma, COPD or are immunocompromised (43).

1.1.3 RSV Treatments and Interventions

Despite first being isolated in 1956, vaccines against RSV have only recently been licensed for use in the elderly and pregnant women (44,45). The development of these vaccines is a huge breakthrough in the field of RSV following a long arduous road of historical complications (46). In the 1960s, the first formalin-inactivated RSV vaccine was trialled in infants. Infamously, this vaccine did not provide protection, rather enhanced disease resulting in more severe cases and sadly two fatalities (47). This failure was the result of poor understanding how the human immune system safely clears the virus, and spurred decades of research into the immune response to RSV (47). Despite this recent success in RSV vaccines development, they are still not widely available and data on their long-term efficacy are limited. In the elderly, two vaccines are now available, GSK Arexvy and Pfizer Abrysvo. Both vaccines are recombinant protein vaccines containing the RSV F protein antigen, stabilised in the pre-fusion conformation. Arexvy contains the F protein from RSV A virus, whilst Abrysvo is bivalent, containing F protein from both RSV A and B viruses (45,48). On-going phase III trials have

reported an efficacy of 85-94% against severe RSV infection after 2 weeks- 6 months (45,48). Abrysvo has also been approved for pregnant women. In phase III trials, this vaccine reported 82% efficacy against severe RSV infection in 3-month-old infants, which dropped to 69% efficacy in infants aged 6 months old (44). This vaccine did not prevent infection in infants, and was only 57% effective at reducing medically attended moderate cases of RSV in 3 month old infants (44). It is also not clear how long maternally acquired protection against RSV in infants will last, and no vaccines are yet available for older children. In addition to vaccines, an injectable monoclonal antibody therapy against RSV, Nirsevimab, has also recently become available. Nirsevimab was found to be 77% effective at reducing severe RSV infection in infants (49). The future of RSV prevention and treatment is therefore very promising. However, continued research into understanding what puts people at risk will further support this pioneering progress.

1.2 Influenza Virus

Throughout history, influenza viruses (IV) have been responsible for many severe and devastating pandemic outbreaks. Perhaps the most notable occurred in 1918, which resulted in an estimated 21-100 million deaths globally, the greatest mortality count caused by any disease outbreak in human history (50). IVs can be subdivided into four types; A, B, C and D, all of which are segmented, negative-strand RNA viruses belonging to the Orthomyxoviridae family (51). Influenza A viruses (IAV) can be further classified into subtypes based on their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). There are 16 subtypes of HA, of which 3, H1, H2 and H3, have been identified on strains which have resulted in widespread human disease (51). Similarly, there are 9 NA subtypes, of which only 2, N1 and N2, have been identified on pandemic strains. These subtypes are used in the nomenclature of IAV strains (51). For example, the 1918 pandemic was caused by an H1N1 strain of IAV, whilst the 1968 pandemic which originated in Hong Kong was caused by an H3N2 IAV (52,53). Whilst IAV are considered the bigger threat to human health, influenza B (IBV) and C viruses can also infect humans, however disease is usually milder. Influenza D viruses largely infect cattle and have not been known to transmit to humans (54).

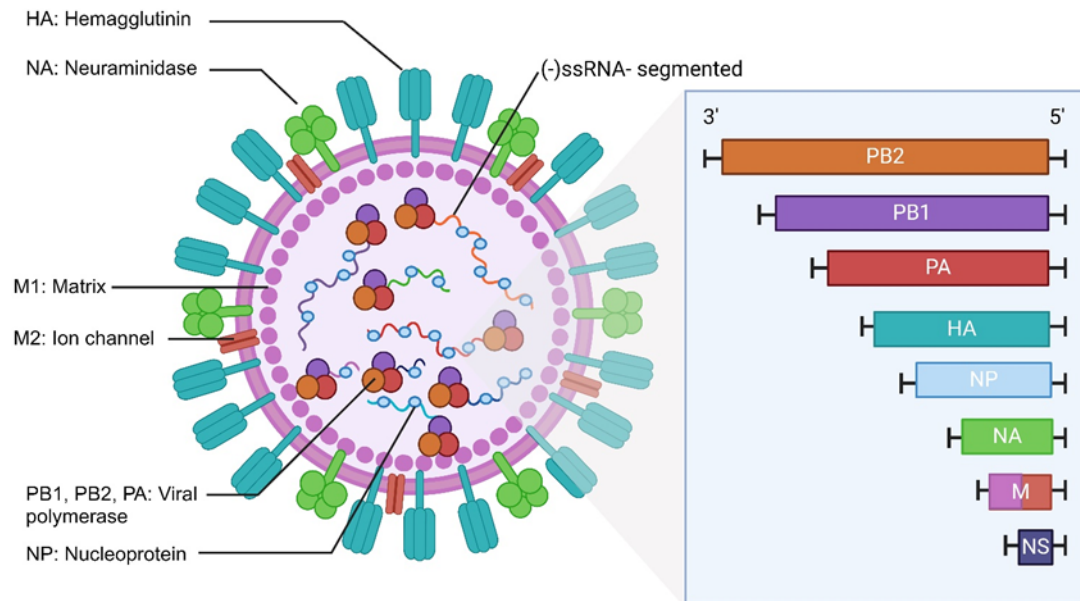


Figure 1.2 Schematic illustrating the structure of Influenza A virus

RSV is a segmented negative-sense single stranded RNA virus ((-)ssRNA). Its genome is comprised of 8 segments which encode for 9 proteins: PB2, PB1, PA, which together form the polymerase complex, HA, NP, NA, M1, M2 and NS. Adapted from AbuBakar et al (55). Created using BioRender.

1.2.1 Influenza Virus Virology

The negative-sense RNA of IAV and IBV is comprised of 8 segments encased within a lipid envelope, forming spherical or filamentous virions 100nm in diameter and up to 300nm in length (Fig. 1.2) (51). The genome segments are numbered 1-8 in order of decreasing length, and each encode for one or two proteins (Fig. 1.2). The glycoproteins which are found studded throughout the virion envelope, HA and NA are encoded for by segments 4 and 6 respectively (51). HA initiates viral entry by binding to sialic acid motifs present on host cells. Binding to sialic acid results in endocytosis of the virion and the resulting pH changes drive conformational changes in the HA exposing the fusion peptide (56). This results in the fusion of the virion with the host cell membrane allowing for the release of the viral ribonucleoprotein (vRNP) into the cytoplasm (57). Also studded in the virion envelope are the M2 proteins which are encoded for by segment 7. These proteins are believed to form a pore which prior to viral membrane fusion acts to pump protons into the virion thus driving acidification (58). This process is believed to aid in the release of the vRNP into the host cells by disrupting interactions with the M1 protein (58). Following entry, vRNPs are trafficked to the host cell nucleus by the host machinery, here the RNA-dependent RNA polymerase (RdRp) initiates replication. The RdRp is formed of three separate proteins, PB2, PB1 and PA encoded for by segments 1-3 of the influenza genome (59). First, transcription of the +RNA strand is initiated by binding of the PB1 subunit to the 3' end of the viral RNP (60). Once the +RNA copy strand

is transcribed, it is released and assembles with NP proteins encoded for by segment 5 of the viral genome, forming copy RNPs (cRNPs) (60). The RdRp can then initiate transcription of the cRNPs forming new vRNPs which can be packaged into new virions. Nuclear export of the virions is mediated by binding of the M1 protein. M1 associates with NEP, encoded for by segment 8, which exports the M1-virion complex out into the cytoplasm via pores in the nuclear membrane (51). M1 will accumulate at the membrane where it associates with newly synthesised HA, NA and M2 which has undergone posttranslational modification at the Golgi apparatus and subsequently trafficked and inserted into the host cell membrane (58). The accumulation of M1-vRNPs at the membrane is thought to initiate viral budding, but it is not until NA cleaves host cell sialic acids, thus releasing HA binding, that the new virion is released (58). The remaining non-structural protein, NS1, encoded for by segment 8 of the influenza genome, does not play a role in replication or trafficking of the viral genome. Instead it is known to disrupt host IFN receptor signalling (61).

1.2.2 Influenza Virus Epidemiology and Clinical Manifestations

IAV has a global distribution. In the northern and southern hemisphere, IAVs are responsible for annual seasonal outbreaks during the winter months, whilst in tropical locations, IAV causes infections throughout the year (62,63). It is estimated that seasonal IAV outbreaks are responsible for 290,000–650,000 mortalities globally each year (64). Seasonal Influenza disproportionately affects the elderly, with over 65s representing 75-80% of all Influenza associated deaths (65). However, like RSV, children under the age of 5 are also at significant risk. Each year, around 90 million children under 5 are infected with IAVs, of which 28,000-111,500 succumb to the disease (66). Like RSV, other comorbidities such as cardiopulmonary diseases and autoimmune disorders are also risk factors for severe disease following influenza virus infection. Infection with IAV presents with fever, cough, rhinitis, muscle aches and headaches. In severe cases, where the virus infects the lower respiratory tract, IAV can cause pneumonia, often requiring hospitalisation (67). Re-infection with IAV occurs throughout life, due to the many antigenic strains and their ability to undergo rapid mutation. IAV can undergo two forms of genetic mutation termed antigenic drift and antigenic shift (68,69). Antigenic drift is the process of rapid and continuous point mutations to the HA and NA of IAV (68). Antigenic shift occurs when two or more influenza A viruses infect the same cell within a host and combine, generating a new strain with different surface antigens (69). In addition to humans, influenza viruses can infect other animal hosts, including birds and pigs, creating reservoirs of the virus where antigenic mutations can occur. This creates a high risk of zoonosis, where mutated virus spreads from livestock or wildlife to humans, and a new pandemic strain emerges (69).

1.2.3 Influenza Virus Treatments and Vaccines

Vaccinations against seasonal IVs are widely available and are part of the current UK vaccination programme for adults over the age of 65 and children aged 2-4 (70). There are total 6 IV vaccines licensed in the UK, 4 of which are inactivated viral vaccines and 2 are live attenuated vaccines (71). These vaccines are all tetra- or quadrivalent, meaning they provide protection against two subtypes of IAV and one or two subtypes of IBV (71). Despite their widespread availability, the efficacy rates of seasonal IV vaccines are low, averaging 38% over the past 10 years and falling as low as 19% in 2014-15, according to the CDC (72). This variation in efficacy rates can be in part accounted antigenic drift and shift (68,69). This continuous mutation to the virus or threat of an antigenically distinct virus, means new IV vaccines need to be formulated each year based on predictions on what the predominant outbreak strain for that season might be. Therefore, continued research into IV viruses will benefit the development of new broad efficacy vaccines and treatments.

1.3 The Immune Response to Respiratory Viruses

The exact determinants which make an individual more susceptible to severe disease following respiratory viral infections are not yet fully understood. However, it is evident that severity is largely directed by a dysregulated immune response to the virus, and not the virus itself (8,73). Using animal models, human studies and *in vitro* experiments the protective immune response towards respiratory viruses has been well characterised (74,75).

When a respiratory virus is first inhaled by a host there are several host defence mechanisms it must overcome to cause productive infection. The first of these is the physical barriers provided by the mucociliary system (76). The epithelial cells which line the larger airways are differentiated into different subtypes, including ciliated and goblet cells (76). Goblet cells secrete mucins from their apical surface which form a thick gel like matrix called mucus which coats our airways. Viral particles can easily become trapped in this mucus before ever reaching an airway epithelial cell. The virus is then swept up and out of the airways by the beating of ciliated cells (77). The importance of the mucociliary system as a first line defence is evidence in individuals where this system has become disrupted, such as COPD, asthma and cystic fibrosis patients, who are more susceptible to respiratory infections (78). In the lower airways, type II alveolar cells produce surfactant, comprised mainly of phospholipids and four main proteins: surfactant protein (SP)-A, SP-B, SP-C, and SP-D. Surfactant coats the cells of the lower airways and provides a protective layer that can effectively prevent viral fusion (79). SP-A and SP-D have previously been shown to be able to bind to the HA and NA on IAV, as well as to RSV glycoproteins, preventing them from binding and fusing with the

host cell (80,81) In addition to the physical barrier provided by the mucociliary system, the lung microbiome has also been suggested to play a protective role against respiratory pathogens (82). Commensal lung pathogens are thought to occupy that niche, creating a homeostasis that is suboptimal for pathogenic pathogens to colonise or infect airway cells (82). Changes to the microbiota before, during and after respiratory viral infection have been linked to increased disease susceptibility in infants (83).

Once past the physical barriers, the virus will next need to overcome cells resident within the airways. Alveolar macrophages (AMs) patrol the lower airways and have previously been shown *ex vivo* to take up RSV without subsequent replication of the virus (84). The predominant cells which the virus will come into contact with are their target cells, the airway epithelial cells (85). Like most mammalian cells, airway epithelial cells express a range of pattern recognition receptors (PRRs), which can recognise foreign components of pathogens, known as pathogen associated molecular patterns (PAMPs) (86,87). PRR recognition of respiratory virus in epithelial cells is critical for driving the release of inflammatory cytokines and chemokines which initiate the immune response (86,88). In addition, viral recognition in AMs is also important and has been shown to be critical in driving the production of type I IFNs during RSV infection (15).

1.3.1 Pattern Recognition Receptors Sense Respiratory Virus

PRR is a broad term used to describe 5 classes of receptors expressed by host cells that can recognise PAMPs: Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide oligomerisation domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs) (86). These receptors recognise different components from bacterial, fungal, parasitic and viral pathogens (86). The PRRs which are predominantly responsible for recognising respiratory viral pathogens belong to the TLR and RLR families (89,90).

1.3.1.1 Toll-like receptors

TLRs are a family of glycoproteins which are expressed in a broad range of cells types including airway epithelial cells and innate immune cells such as AMs, dendritic cells (DCs) and neutrophils (88). In humans, 10 TLR proteins have so far been identified (TLR1-10), whilst mice have 13 TLRs (TLR1-9 are conserved in both species) (91). All TLRs are transmembrane proteins, possessing a single membrane spanning domain (92). They can be found inserted into the outer membrane of cells, where they can sense extracellular PAMPs or inserted into endosomal membranes, where they can sense PAMPs in the endosomes (92). Most TLRs form homodimers except TLR2 which forms heterodimers with either TLR1 or TLR6 (93). On

the outer side of the membrane, TLRs have a leucine rich domain which is responsible for interacting with microbes and sensing PAMPs. On the inner side of the membrane, their cytosolic tails are responsible for interacting with adaptor proteins which initiate the signalling cascade (92). All TLRs, excluding TLR3, signal via the myeloid differentiation primary-response protein 88 (MyD88) protein, resulting in the activation of the transcription factor NF- κ B and IRF7 (94). In contrast, TLR3 signals via TIR domain-containing adaptor protein inducing IFN- β (TRIF) to activate both IRF3 and NF- κ B transcription factors (95). TLR4 has also been shown to signal via TRIF as well as MyD88 (96). Activation of NF- κ B leads to the transcription and release of a range of proinflammatory cytokines and chemokines, whilst IRF3 and IRF7 are also important in inducing type I IFNs (97,98). During RSV infection or IAV infection, TLR2, TLR3, TLR4 and TLR7 have all been shown to be important in initiating the immune response (Fig. 1.3). TLR7 is expressed intracellularly on endosomal membranes and recognises ssRNA released into the endosome following viral membrane fusion (99,100). During RSV infection, TLR7 signalling has previously been shown to be important in balancing the IL-12/IL-13 axis to drive a predominantly Th1 response to the virus (101). In *Tlr7*^{-/-} mice, RSV infection led to increased mucus secretion associated with elevated levels of IL-13 and IL-17 (101). During IAV infection, TLR7 has been shown to be critical not only in initiating an innate immune response, but also in directing the adaptive immune response towards the virus and in generating long-term memory response (102). TLR7 agonists are thus often considered as important agonists when developing IAV vaccines (103). Like TLR7, TLR3 is also expressed in endosomes and can recognise double stranded RNA (dsRNA) intermediates produced during viral replication (104). TLR3 also promotes a Th1 response to respiratory virus, with *Tlr3*^{-/-} mice having increased mucus secretion, eosinophilia and elevated levels of IL-13 following RSV infection (105,106). As it signals via the TRIF pathway, activation of TLR3 is also important in driving the release of MyD88 independent cytokines such as CXCL10 and CCL5 (106). Furthermore, children with inherited TLR3 deficiency were found to be at higher risk of acute respiratory distress syndrome (ARDS) following IAV infection. This was suggested to be due to lack of type I IFN induction following infection (107). TLR2 and TLR4 are both expressed on the outer surface of cells where they can bind to components of the viral capsid (93,108). During RSV infection TLR4 can recognise the RSV F protein and has been shown to be critical in providing protection against severe disease (109). Studies in *Tlr4*^{-/-} mice, have demonstrated reduced viral clearance during RSV infection, whilst in humans, polymorphisms to *Tlr4* have been strongly linked with increased risk of severe disease and bronchiolitis in infants (90,110). The role of TLR4 during IAV infection is less understood, however there is some evidence that its activation may contribute to lung injury and pathology (111). TLR2 has also been shown to drive innate immune activation following RSV infection and IAV infection, potentially through recognition of the G and NP proteins,

respectively (112,113). Genetic polymorphisms in *Tlr2* have also been associated with increased risk of severe RSV bronchiolitis in infants (114).

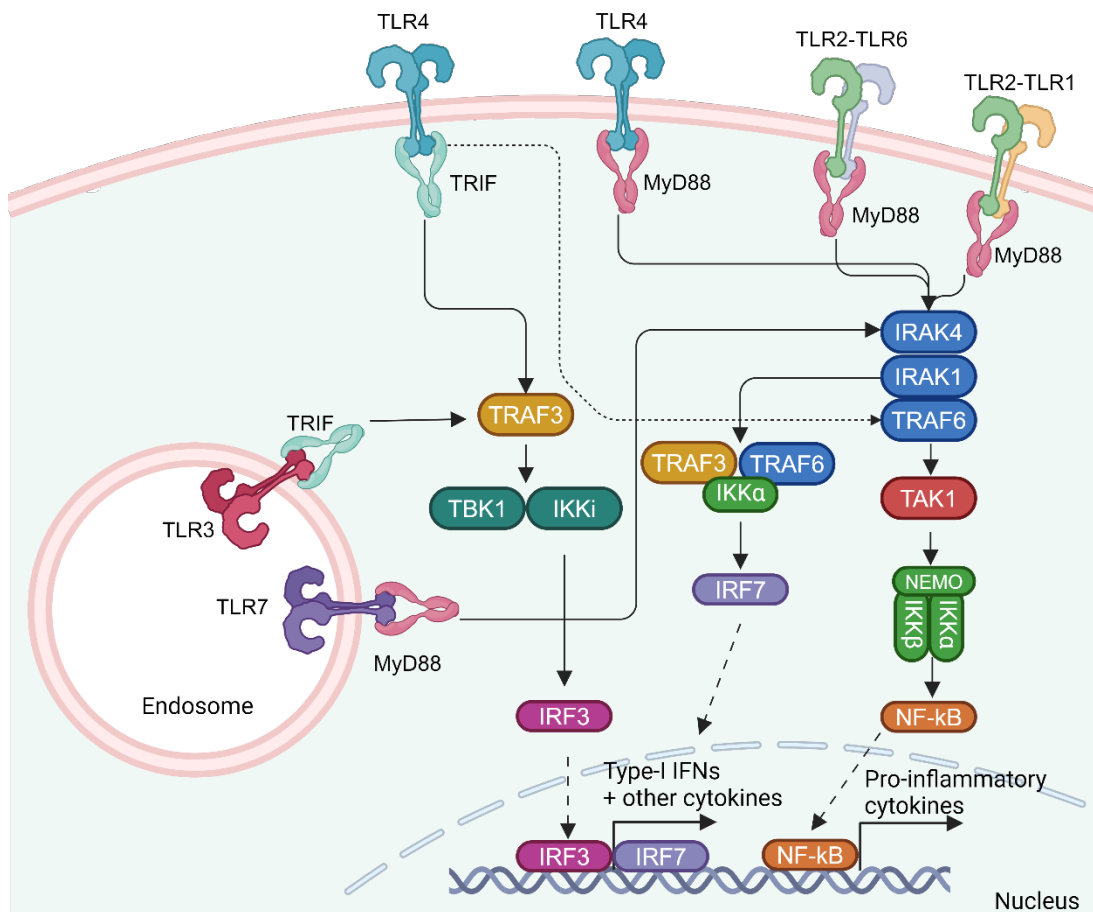


Figure 1.3 TLR signalling pathways

Diagram demonstrating the signalling pathways initiated by TLRs that sense respiratory viral infections. TLR2-TLR1 and TLR2-TLR6 heterodimers are localised on the cell surface membrane alongside TLR4. TLR3 and TLR7 are localised on endosomal membranes. TLR2, TLR4 and TLR7 signal via MyD88 to initiate a cascade culminating in translocation of NF-κB and IRF7 into the nucleus and transcription of pro-inflammatory cytokines. TLR3 and TLR4 signal via TRIF culminating in the translocation of NF-κB and IRF3 into the nucleus and transcription of type I IFNs (94,95,115). Created using BioRender.

1.3.1.2 RIG-I-like receptors

RLRs are intracellular sensors of viral RNA. There are two main RLRs, RIG-I and melanoma differentiation-associated protein 5 (MDA5) which are both found in the cytoplasm of most cell types in the body (88). The RIG-I and MDA5 proteins both possess a C-terminal helicase domain which can bind to both ssRNA and dsRNA, and an N-terminal caspase recruitment and activation domain (CARD) which is responsible for initiating the signalling cascade (116). RLRs signal via the adaptor protein mitochondrial antiviral-signalling (MAVS) which interacts with the CARD domain (Fig. 1.4) (117). Activation of MAVS triggers the signalling cascade

which ultimately leads to the activation of IRF3 and NF- κ B and the production of type I interferons and proinflammatory cytokines (Fig. 1.4) (117). RIG-I binds to the 5'-triphosphate end of viral RNA, including that of RSV and IAV, whilst MDA5 binds to long dsRNA with no end specificity (118,119). During RSV infection, activation of RLRs and downstream MAVS signalling has been shown to be crucial in activating the innate immune system but not in generating primary adaptive immune responses (84,120). In contrast, during IAV infection *RigI*^{-/-} mice have been shown to be important in priming functional T cell responses by promoting antigen presentation by dendritic cells (DCs) (121). RLR and MAVS signalling in AMs is also essential for the production of type I IFNs, discussed below (84).

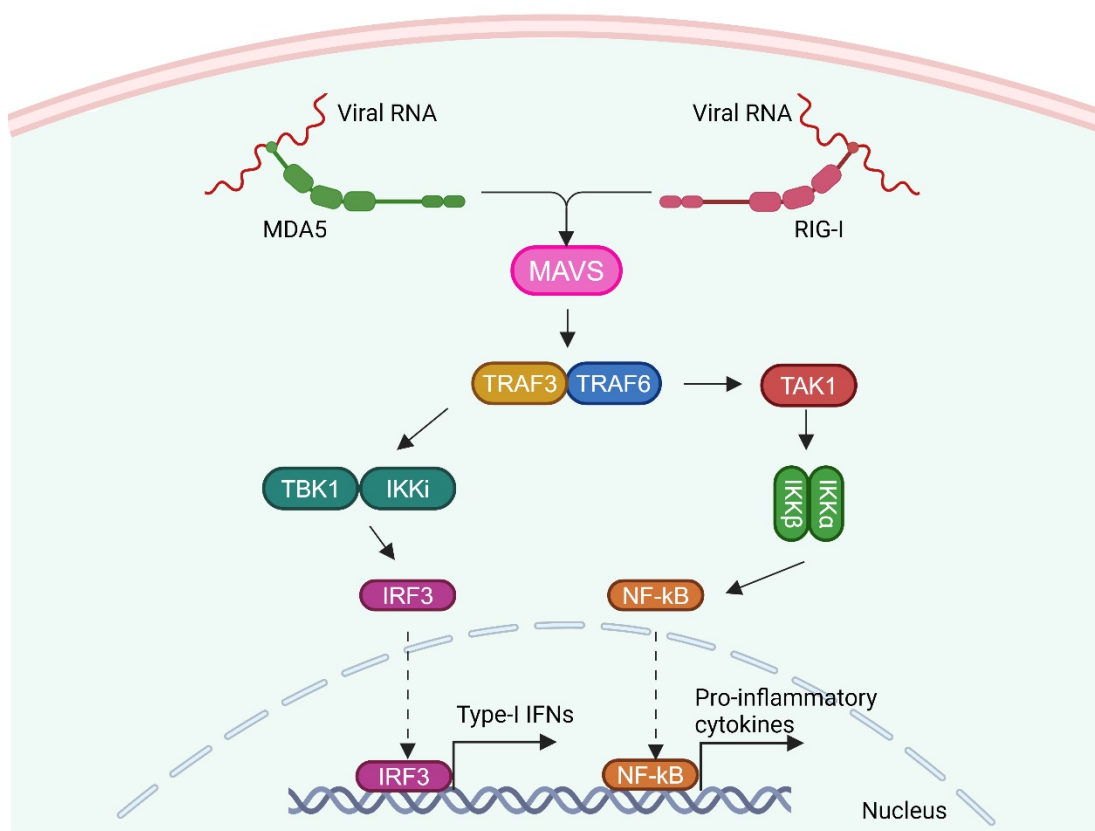


Figure 1.4 RLR signalling pathways

Diagram demonstrating the signalling pathways initiated by RLRs important in sensing respiratory virus. RIG-I and MDA5 are both found in the cytosol and can sense viral RNA. They both signal via MAVS which activates the TRAF3 signalling pathway to initiate IRF3 transcription of type I IFNs and TRAF6 to initiate NF- κ B transcription of pro-inflammatory cytokines (117). Created using BioRender.

1.3.2 Airway Resident Innate Immune Response

Shortly after recognition of respiratory viruses by the resident airway cells, the innate immune system is rapidly activated. The cells and cytokines of the innate immune system provide an

immediate response to limit viral replication and spread of the virus, but also is essential in directing the adaptive immune response.

1.3.2.1 Airway epithelial cells

More than just a physical barrier to prevent viral dissemination, the epithelial cells which line the respiratory tract will also initiate an inflammatory cytokine and chemokine response to respiratory virus (122). Airway epithelial cells (AECs) have been shown to express TLR4 on their apical surface. In addition, there is some data which suggests human AECs also express TLR3 and TLR7 on their surface, rather than in intracellular endosomes (123). RLRs are also constitutively expressed by AEC (124). AEC are thought to be the source of many pro-inflammatory cytokines early after respiratory viral infection and are important in initiating subsequent immune responses (122). Stimulation of normal human bronchoepithelial cells with RSV F and G proteins stimulates the release of chemokines like IL-8, CXCL10, CCL2 and CCL5 from their basolateral surface (125). These chemokines initiate rapid recruitment of innate inflammatory cells including neutrophils and monocytes (126,127). These chemokines are also released by bronchoepithelial cells following IAV infection (128). AECs are known to be major sources of IL-1 α following RSV infection, but can release many cytokines including IL-1 β , IL-6, and TNF- α during RSV and IAV infection (122,128–130). Following IAV infection, AECs are also an important source of type I IFNs (131). The role of these cytokines is discussed in more detail below.

1.3.2.2 Alveolar macrophages

AMs are highly specialised macrophages resident in the lower airways, where they patrol the alveoli (132). They are long-lived cells which have a broad range of functions in maintaining homeostasis and controlling infection (133). In a healthy, uninfected lung, AMs are responsible for the clearance of apoptotic cells and debris (134). When the epithelial cells of the airways begin to die, they express specific 'eat-me' signals which flag them for phagocytosis by AMs. The most well studied of these is phosphatidylserine which during apoptosis is flipped from the inner leaflet of the plasma membrane to be exposed on the outer surface of the cell (135). These 'eat-me' signals are recognised by a variety of engulfment receptors present on the surface of AMs, including BAI1, integrins α V β 3 or α V β 5 and TYRO3, AXL and MERTK (TAM) family receptors, which all signal via (Rac) to drive actin polymerisation, initiating engulfment (134). In addition to phagocytosis of apoptotic cells, AMs will also phagocytose and clear invading pathogens (136,137). The recognition of apoptotic cells versus the recognition of pathogens by AMs needs to initiate opposing responses (133,138). Whilst effective inflammatory responses are required following infection, such responses need to be inhibited following clearance of dead cells to prevent pathology and maintain homeostasis (134).

Similarly, AMs play a key role in resolution following infection and in the repair of damaged tissues (139). Because of these contrasting functions, AMs display incredible plasticity and have the ability to alter their phenotype along a spectrum from more anti-inflammatory to more pro-inflammatory. Historically, macrophages were phenotypically classified into two categories, classically activated (M1) and alternatively activated (M2) (140). M1 macrophages were considered to be more pro-inflammatory, associated with the production of proinflammatory cytokines such as IL-6, IL-1 α/β and TNF α , whilst M2 macrophages were considered to be more anti-inflammatory or pro-resolution, associated with increased production of IL-10 and TGF- β (141). However, it has become clear that this classification of AMs is much too narrow, and in fact AMs do not exist as one phenotype or the other, but along a broad spectrum (142).

AMs are amongst the first cells that respiratory viruses will come into contact with following infection. It is not clear if AMs actively phagocytose free virus, however it has been demonstrated that AMs can take up or be infected by RSV or IAV without productive viral replication (84,143). AMs express both RLRs and TLRs, which recognise the virus directing crucial early innate immune response. As previously discussed, activation of the RIG-I pathway in AMs during RSV and IAV infection is crucial for a robust type I IFN response (84,143). In addition, AMs will also upregulate their expression of multiple proinflammatory cytokines including IL-6, IL-1 β and TNF- α following RSV or IAV infection, as well as secrete multiple chemokines such as CXCL10 and CCL5 which drive the recruitment of innate and adaptive immune cells (84,143,144). These early innate immune responses are vital in preventing lung damage and rapid spread of the virus in the lower respiratory tract. Mice deficient in AMs show increased alveolar damage and lethality following IAV infection, as a result of increased infection of the alveolar epithelial cells (145). Depletion of AMs in mice using clodronate liposomes during RSV infection increased the peak of viral load associated with reduced recruitment of natural killer (NK) cells but had no effect on T cell responses (146). Similarly, it has been reported that activation and expansion of mouse AMs *in vivo* prior to RSV infection protected mice from weight loss, reduced viral titres and lung injury (147).

1.3.2.3 Type I Interferons

Type I IFNs are a group of proteins secreted by virus infected cells which exert their effects predominantly by signalling through the IFN- α receptor (IFNAR). There are several IFNs belonging to this group, including several IFN- α s and IFN- β , which are the best studied (148). IFNAR signalling during respiratory viral infection acts through multiple mechanisms to aid in protection. Firstly, type I IFNs can drive cell-intrinsic changes through the induction of interferon stimulated genes (ISGs), which restrict viral replication within cells (149). Extrinsic

to the cell, type I IFNs modulate both the innate and adaptive immune response, activating immune cells and promoting antigen presentation whilst also limiting over production of proinflammatory cytokines (150,151). Type I IFNs can be produced by many cells of the body, however during RSV or IAV infection, the major producers of IFN- α are AMs and plasmacytoid DCs, respectively (15,152). As discussed previously, induction of IFN- α production by AMs during RSV infection is dependent on RLR and MAVS signalling with levels peaking within the first day post infection (15). Using the *Mavs*^{-/-} mouse models have therefore enabled a better understanding of the role of IFN- α during RSV infection. Such studies have revealed that IFN- α production by AMs is essential in recruiting monocytes and activating neutrophils recruited into the lungs following RSV infection, as well as a general reduction in innate cytokines and chemokines (15,16,153). *Mavs*^{-/-} mice therefore show increased viral load and weight loss following RSV infection (120). However, they stills elicit robust primary CD4⁺ and CD8⁺ T cell responses comparable to WT mice (120,154). Despite a robust primary T cell response, type I IFN receptor signalling is required for the expansion of CD8⁺ Tissue resident memory T cells (T_{RM}) (154). Similarly, during IAV infection, *Mavs*^{-/-} and *Rigi*^{-/-} show delayed viral clearance, increased weight loss and poor survival compared to WT mice (121). Unlike RSV infection, IFNAR signalling appears to be more important in directing a functional T cell response toward IAV. *Rigi*^{-/-} mice infected with IAV show poor CD4⁺ and CD8⁺ T cell priming by dendritic cells (DCs), leading to lower frequencies of effector T cells compared to WT mice (121).

1.3.2.4 Pro-inflammatory cytokines

In addition to type I IFNs there are many pro-inflammatory cytokines released by AECs and AMs, as well as by recruited immune cells, following respiratory viral infection that are important in driving inflammation. Some of the more well characterised of these include IL-1 α , IL-1 β , IL-6 and TNF- α (84,122,128–130,143,144). IL-1 α is considered an alarmin released in an inactive pro-form. Cleavage of pro-IL-1 α by proteases such as elastase or granzyme B released by recruited immune cells, this enhances IL-1 α activity promoting its signalling via the IL-1 receptor (IL-1R) (58). IL-1R signals via MyD88 to activate NF- κ B and the release of further inflammatory cytokines and chemokines (59). Also, a member of the IL-1 family, IL-1 β shares the IL-1R with IL-1 α , thus can have similar effects on inflammation. IL-1 β is also produced in a pro-form which requires cleavage by caspase-1, which is activated by NLR family pyrin domain containing 3 (NLRP3) inflammasomes (155). Unlike IL-1 α , IL-1 β cleavage is essential for IL-1R signalling (155). During IAV and RSV infection, IL-1 β is processed and produced by AMs, but NLRP3 activation in AMs requires external signalling from recruited neutrophils (144). Despite signalling via the same receptor, IL-1 α and IL-1 β have previously

been described to not share redundancy during bacterial infections, suggesting they have distinct functions in inflammation (156).

IL-6 is considered a pleiotropic cytokine which exerts control over multiple inflammatory process during respiratory viral infection (157). For example, IL-6 can promote the expression of the M-CSF receptor on monocytes, promoting their differentiation into macrophages (158). In addition, IL-6 can drive the differentiation of CD8⁺ cytotoxic T cells, polarise CD4⁺ T cell responses and regulate DC differentiation (159–161). During RSV infection in mice, IL-6 was shown to be important in promoting the maturation of T regulatory cells (T_{Reg}), promoting resolution in the lungs (162). Binding of TNF- α to its receptor (TNFR1) drives inflammation through two separate mechanisms. Firstly, TNFR1 can signal via both mitogen activated protein kinase (MAPK) and NF- κ B pathways to directly induce the expression of pro-inflammatory mediators (163). Secondly, TNFR1, can induce both apoptotic and lytic cell death. This occurs through activation of receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which subsequently cleaves caspase 8 to initiate caspase 3 mediated apoptosis, or activates mixed lineage kinase domain-like pseudokinase (MLKL) to form pores in the cell leading to necroptosis (163). Necroptosis leads to the release of damage associated molecular patterns (DAMPs) which signal via PRRs to ultimately promote further inflammation (163). Whilst all of these cytokines are induced upon RSV or IAV infection, they have also been implicated as drivers of severe disease (164–167). This will be discussed further throughout the thesis.

1.3.3 Innate Immune Cell Responses

Soon after recognition of respiratory viruses by the resident airway cells and the induction of cytokines and chemokines, innate immune cells will begin migrating and infiltrating into the lungs and airways. The events during this early phase of infiltration have been extensively profiled using mouse models of RSV and IAV infection and through human studies (8,15,16,84,168–172). Whilst the magnitude and timings of the immune responses which occur in mouse models versus human infection do vary, the sequence of events is the same (Fig. 1.5)(8). Similarly, during IAV infection the sequence of cell infiltration is comparable to that of RSV but is likely subject to more variability dependent on the strain of the virus (75,173). In this section, the timings of immune response to RSV in mice will be described as it is the most relevant to the content explored in this thesis. The first circulating innate immune cells to migrate into the airways in large numbers are neutrophils, which respond rapidly to chemokines like CXCL1 and CXCL2, peaking in number around day 1 post infection (Fig. 1.5)

(15). Neutrophils and their role during respiratory viral infection will be discussed in more detail later.

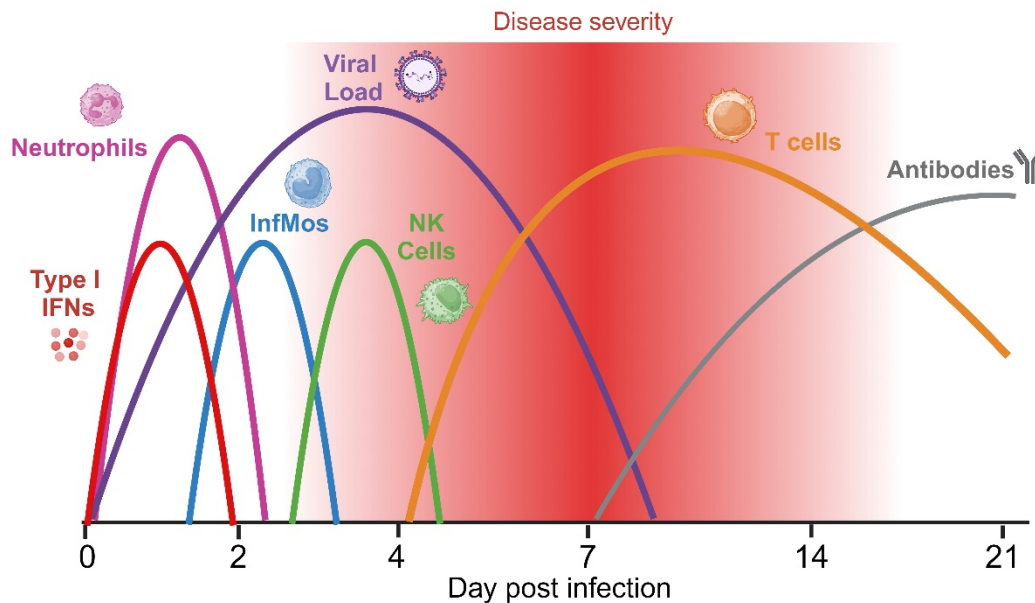


Figure 1.5 Timings of the immune response to RSV in mice

Diagram describing the timings in which immune cells are recruited into the lungs following infection with RSV in mouse models. Shortly after infection, type I IFNs are induced and neutrophils are rapidly recruited into the lungs, peaking around day 1 post infection. This is followed by recruitment of inflammatory monocytes and NK cells. During the first 4 days post infection, the virus exponentially replicates, with viral load peaking around day 4 post infection. After day 4 post infection, T cells begin infiltrating into the lungs which mirrors the decline in viral load. Adapted from Openshaw et al (8). Created using BioRender.

1.3.3.1 Inflammatory Monocytes

Following neutrophils, inflammatory monocytes are the next innate immune cells to infiltrate into the airways, peaking 2 days after infection (Fig.1.5) (8). Inflammatory monocytes are recruited by chemokines CCL2, CCL7 and CCL12 released predominantly by airway epithelial cells and stromal cells (125,174). Once in the lung, they respond to viral infection by releasing an array of proinflammatory cytokines including IL-1, IL-6, IL-8, IL-10 and TNF- α , as well as express TNF-related apoptosis-inducing ligand (TRAIL) (175–177). The production of inflammatory mediators by inflammatory monocytes contributes to protection against respiratory virus, however excessive recruitment of inflammatory monocytes can also be detrimental. Their role in protection has previously been evidenced using CCL2 KO mice infected with IAV, which have greatly reduced recruitment of inflammatory monocytes. These mice displayed increased weight loss, lung pathology and increased viral load (178). Whilst in mouse models of IAV which have increased production of CCL2, leading to increased recruitment of inflammatory monocytes, morbidity was significantly increased (179). Similarly, in an IAV and bacterial co-infection mouse model, the recruitment of TRAIL⁺ monocytes were

shown to drive epithelial damage, contributing to pathology and disease severity (177). During RSV infection, inflammatory monocytes have been shown to be important in early control of viral replication (15). *Mavs*^{-/-} mouse models of RSV infection, where inflammatory monocyte recruitment is lost, exhibit more weight loss compared to WT mice. This exacerbation in weight loss is ameliorated by intranasal delivery of recombinant CCL2 to restore monocyte recruitment, demonstrating their contribution to protection (15).

1.3.3.2 NK Cells

NK cells are the next innate immune cells to migrate into the lungs and airways, peaking at day 4 post infection (Fig. 1.5) (146). During RSV infection, the recruitment and activation of NK cells is dependent on AMs, demonstrated by the use of clodronate AM depletion in mice (146). NK cells play an important role in restricting viral replication by killing infected cells through a number of mechanisms. NK cells possess granules which contain cytotoxic mediators such as perforin and granzymes, which disrupt cell membranes, inhibit the cell cycle and drive DNA damage (180). In addition, NK cells can directly induce apoptosis of virally infected cells by expressing ligands, such as FasL, on their cell surface which can engage and activate death receptors (180). During both RSV and IAV infection, NK cells are also a major source of IFN- γ (181,182). IFN- γ has a multitude of functions, one of which is to limit viral infection by disrupting the viral replication cycle (183). This can be achieved through a number of mechanisms dependent on the virus, for example, during IAV infection of airway epithelial cell lines, IFN- γ treatment greatly reduced sialic acid expression on the cell surface, limiting viral adhesion and entry (184). IFN- γ is also important in bridging the gap between the innate and adaptive immune responses by promoting antigen presentation, influencing CD4⁺ T cell polarisation and generating cytotoxic CD8⁺ T cells (185).

1.3.4 The Adaptive Immune Response

During the first days of infection, whilst the innate immune response is rapidly responding, the virus is continuing to replicate and viral titres increase exponentially (16). In mice infected with RSV, viral load tends to peak at day 4 post infection, after which it rapidly declines (186). In humans, viral load peaks around day 6 post infection (171). Decline in viral load coincides with initiation of the adaptive immune response, specifically the recruitment of T cells (Fig. 1.5) (187). The recruitment and activation of the adaptive immune response is largely dependent on the cells and cytokines of the innate immune response, in particular antigen presenting cells.

1.3.4.1 Antigen presentation

The predominant cells responsible for antigen presentation during respiratory viral infection are DCs. DCs are sentinel innate immune cells which patrol most tissues in the body, taking up viral antigen either through phagocytosis of infected/dead cells or debris, or through direct viral infection (188). They process this antigen and then present it on their surface via major histocompatibility complex (MHC) I and II molecules. This process of antigen presentation is vital for the activation and maturation of both CD4⁺ and CD8⁺ T cells, generation of T cell memory, as well as activation of B cells (188). DCs can be subtyped and classified dependent on their localisation, function and expression of cell surface markers and transcription factors. Overall, there are two major groups of DCs, those which remain resident in the lymph organs or those which seed the non-lymphoid organs, like the lung, and migrate to lymph organs spontaneously or under inflammation (189). Resident in the lungs of mice, there are two classes of DCs important in responding to RSV or IAV infection, conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (190,191). cDCs can be further subtyped based on their surface expression of CD11b and CD103: CD11b⁻ CD103⁺ cDCs (cDC1s) and CD11b⁺ CD103⁻ cDCs (cDC2s) (190). During RSV infection of mice, there is an influx of cDC2s and pDCs into the lung, with numbers peaking at day 2-3 post infection (191). In contrast, cDC1s have been found to decrease in number in the lungs during the first 48h of RSV infection (191). The decrease in number of cDC1s may in part be explained by their migration to the lymph nodes. Both cDC1s and cDC2s have been found to travel to lymph organs where they present RSV antigen to naïve CD8⁺ and CD4⁺ T cells via MHC-I and -II, respectively (191). cDCs will also upregulate their expression of costimulatory molecules CD80 and CD86 during RSV infection, essential for T cell activation (191). In addition to presenting antigen, DCs also produce large amounts of cytokines and chemokines which help direct the adaptive immune response. During IAV infection, pDCs have been suggested to be a major source of type I IFNs (192). Whilst pDCs can produce type I IFNs when infected with RSV, the evidence for this is somewhat conflicting (193). cDC1s are also a major source of IL-12, which is required for effective Th1 polarisation of CD4⁺ T cells (194). During RSV infection, cDC2s have been shown to gain an inflammatory phenotype similar to that of cDC1s, producing IL-12 to promote Type I helper T cell (Th1) differentiation (195).

1.3.4.2 The Primary T cell Response

The generation of a successful T cell response begins in the thymus, where progenitor cells develop into single positive CD4⁺ or CD8⁺ naïve T cells. These naïve T cells then exit the thymus and migrate to the lymphoid tissue where they are activated through antigen presentation from DCs (196). CD8 will bind to MHC-I expressed on the surface of antigen

presenting cells, whilst CD4 binds to MHC-II (197,198). This engagement facilitates the recognition of the antigen by a compatible T cell receptor (TCR). TCRs through their association with CD3 at the cell surface, will initiate the signalling cascade which drives T cell expansion and activation (199).

Following activation CD4⁺ T cells can further differentiate into a multitude of subtypes, including the classical Th1 and Type 2 helper T cells (Th2) cells, as well as Type 17 helper T cells (Th17), T follicular helper (T_{FH}) cells and T_{Reg} cells (200). Differentiation is dependent largely on stimulations by cytokines present in the inflammatory environment. IL-12 is required for efficient development of Th1 CD4⁺ T cells (201). Th1 responses are considered proinflammatory and during infection they aid in the activation of other immune cells by providing costimulatory signals to B cells and cytolytic CD8⁺ T cells (CTLs) (200). In contrast, differentiation of Th2 cells requires IL-4 signalling (202). Unlike Th1 cells, Th2 cells are considered to have a more allergic phenotype, contributing to tissue repair and clearing worms and parasitic infections, but also contribute to the development of allergic pathology (200). Following both RSV or IAV infection in mice, Th1 cells comprise the greatest percentage of CD4⁺ T cells recruited to the lungs (203,204). This skewed Th1 response is considered protective during RSV infection, whilst Th2 weighted responses have been associated with more severe disease in infants (205). Mouse models of RSV vaccination have also revealed that the failure of the 1960s inactivated-formalin vaccine, which resulted in more severe disease following RSV infection in children, can be attributed to a predominant Th2 response and loss of an effective Th1 response (206). Similarly, in mouse models of IAV infection, polarisation toward a Th2 biased response through treatment with IL-4, resulted in delayed viral clearance (207). Furthermore, Th1 CD4⁺ T cells have been shown to be essential in generating and maintaining a functional population of memory CD8⁺ T cells following primary IAV infection (208).

The activation and expansion of CD8⁺ T cells is critical for protection against respiratory viral infection. In addition to recognising antigen presented on MHC-I, CD8⁺ T cell differentiation also requires IL-12 signalling (209). CD8⁺ T cells differentiate into cytotoxic T lymphocytes (CTLs) which can recognise infected cells and induce cell death through the release of enzymes such as granzyme B (GzmB) and through FasL engagement (210). CTLs are also an important source of TNF- α and IFN- γ (211). During RSV infection in both humans and mice, CD8⁺ T cells infiltrate the lungs where they play an important role in viral clearance. Depletion of CD8⁺ T cells in RSV infected mice leads to increased viral titres at day 7 post infection, whilst adoptive transfer of RSV primed CD8⁺ T cells into naïve mice significantly reduced viral loads (212,213). Prolonged viral shedding following RSV infection has also been noted in children with immunologic disorders resulting in CD8⁺ T cells deficiency (214). During IAV infection in mice, depletion of CD8⁺ T cells does not significantly impact viral clearance (215).

However, a robust CD8⁺ T cell response during primary infection is important for the development of long-term immunity to IAV (216). Despite the evidence that CD8⁺ T cells are important in providing protection toward respiratory viruses, they are cytotoxic and ultimately cause damage to the host tissue driving pathology. In mouse models of RSV, the peak of disease severity as measured by weight loss (day 6-7 post infection) coincides with the peak of T cell recruitment (8). This weight loss appears to be attributed to CD8⁺ T cells, as whilst depletion slows viral clearance it also ameliorates weight loss (212). Similar results can also be seen in mice depleted of CD8 T cells during IAV infection (217). Overall, CD8⁺ T cells are important in viral clearance and protection from respiratory virus, but they also appear to be major contributors to pathology and must be tightly regulated.

1.3.4.3 The Primary B cell and Antibody Response

When DCs carrying antigen arrive at the lymph organs, they not only present antigen to activate T cells but also to immature B cells. B cells reside within follicles in the lymph node. Following recognition of antigen, they migrate to the edge of the follicular tissue where they start to proliferate and begin producing antibodies (218). There are 5 main classes of antibodies which are classified based on their heavy chain (Fc) region, which is not responsible for antigen binding but instead directs the antibodies effector function. These classes include immunoglobulin (Ig) M, IgD, IgA, IgE and IgG (which can be further subtyped into IgG1, IgG2, IgG3 and IgG4) (219). At this earlier stage of proliferation, B cells will produce IgM specific toward the viral antigen (218). IgM is secreted by the B cells and enters the circulation, locating and binding to virally infected cells and driving activation of the complement classical pathway (219). B cells also present the viral antigen via MHC-II on their surface. This is engaged by the TCR of CD4⁺ Th1 cells which provide costimulatory signals to drive antibody isotype switching from IgM to IgG or IgA, as well as further proliferation and the formation of short-lived plasma blasts (218,220). IgG and IgA antibodies all have neutralising activity, meaning they bind to free virus and prevent it from infecting cells. In addition, IgG1, IgG3 and IgA can also opsonise an infected cell, flagging it for phagocytosis by macrophages (219). In addition to forming short-lived plasma blasts, some B cells will then migrate to the germinal centre of the follicle and undergo the process of somatic hypermutation and affinity maturation to generate high-affinity antibody-secreting cells (218). These cells produce large quantities of IgA and IgG, but soon after the virus is cleared, they decline in number (218). During primary RSV infection in children, IgM is detected within the first few days of infection, whilst IgG and IgA arise 10 days after infection, peaking 3 weeks later (221). Antibodies provide great protection against IAV and RSV. Using guinea pig models of IAV transmission, administration of virus specific IgG protected from weight loss, whilst administration of virus

specific IgA reduced transmission (222). In infants, treatment with monoclonal antibodies directed toward RSV has been seen to greatly reduce hospitalisations due to RSV infection (223). However, given that antibody titre's peak after resolution of symptoms, it is unlikely they contribute to protection during a primary RSV infection (221). Indeed, mice treated with anti-mu to deplete IgM antibodies early during RSV infection display no changes in weight loss nor viral clearance (212). Instead, it is much more likely that antibodies contribute more to protection during reinfection.

1.3.5 Adaptive Memory Responses

Respiratory viruses circulate seasonally, meaning we constantly come into contact with them throughout life (6,8). Therefore, the development of long-term memory responses during primary infection or through vaccination is important in providing protection against reinfection. During primary infection, a fraction of the activated T and B cells will develop into long-lived memory cells, which are rapidly activated upon reinfection (224). Both CD4⁺ and CD8⁺ memory T cells arise following T cell activation by antigen presentation but is not clear whether they commit to a memory or effector phenotype, or if effector T cells later differentiate into long lived memory cells (225). Recent studies analysing DNA methylation and chromatin accessibility patterns point toward the latter model, with memory T cells having similar patterns to effector T cells (226,227). There are three main forms of CD4⁺ and CD8⁺ memory T cells generated following respiratory viral infections; central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and T_{RM} (224). Both T_{CM} cells and T_{EM} cells enter the circulation and migrate between the blood and lymphoid organs (224). As their name suggests, T_{RM} cells reside within the affected peripheral tissues, which in the case of respiratory virus is the lung. Upon reinfection, they are amongst the first cells to come into contact with and be activated by viral antigen. Following RSV or IAV infection, both CD4⁺ and CD8⁺ T_{RM} cells have been detected in the lungs and airways of mice and humans (154,187,228). CD8⁺ T_{RM} cells, in particular have been shown to be important at providing protection against RSV, and can do so in the absence of antibody responses (187,229). In the context of IAV infection, CD8⁺ T_{RM} cells have also been shown to provide protection against heterosubtypic infection (230).

Following a primary infection or vaccination there are several proliferation events through which memory B cells are formed. Following activation at the lymph follicle edges, B cells which receive strong and durable signals from T_{FH} cells will migrate to the germinal centre and undergo somatic hypermutation (231,232). Prior to this, some B cells which may not receive strong enough signals from T_{FH} cells can differentiate into germinal centre independent memory B cells (231). At the germinal centre, somatic hypermutation and affinity maturation will select for a population of germinal centre dependent memory B cells whilst others enter

the circulation and secrete high quantities of antibody (218). Following an infection, some of these circulating B cells will migrate to the bone marrow or respiratory mucosa where they can survive as long-lived plasma cells or tissue resident memory B cells (218). B cell memory and antibody responses are effective in protecting against homosubtypic IAV infection. Levels of IgA both in the serum and nasal mucosa correlate well with protection following vaccination with live attenuated IAV vaccine (233). However, the majority of antibodies generated by memory B cells following IAV infection are against HA and NA, which are the most susceptible proteins to be altered by antigenic drift (234). Therefore, B cell memory and antibody responses do not provide good protection against heterosubtypic IAV infection (234). Unlike IAV, RSV does not undergo rapid mutation events like antigenic shift or drift, but despite this reinfection reoccurs throughout life. A study investigating antibody responses to RSV in healthy adults revealed that RSV infection induced almost no IgA RSV-specific memory B cells (235). In addition, IgG responses to RSV were transient and short lived (235). Weak memory B cell responses following RSV infection could in part explain why reinfection can occur.

1.4 Neutrophils

Neutrophils are innate immune cells which possess a multitude of effective antimicrobial functions. Following infection, neutrophils migrate in large numbers to the compromised tissue, often being the first innate immune cells to arrive and often outnumbering any other inflammatory cell (236). Once at the site of infection, they become activated and exert their antimicrobial functions (236,237). The importance of neutrophils in providing immediate protection against pathogens, especially bacterial and fungal, is evidenced in cases of congenital or autoimmune acquired cases of neutropenia, in which patients suffer with increased incidence of infection (238,239). In addition to eliminating pathogens, neutrophils can also provide important signals to link the innate and adaptive immune responses as well as promote resolution and wound healing (237,240,241). Despite the benefits of neutrophilic inflammation, their antimicrobial mechanisms are cytotoxic and ultimately also drive tissue damage (236). Neutrophils are therefore often considered as a double-edged sword during disease and thought to contribute greatly to pathology.

1.4.1 Development

Neutrophils are the most abundant immune cell type found circulating in the body (242). This vast population provides us with a rapid innate immune defence which is always ready to respond to infection or injury. However, despite this large circulating population, neutrophils have an incredibly short lifespan surviving on average only 2-3 days (243). Therefore, new

neutrophils need to be constantly produced in order to maintain the vast population. It is estimated that under homeostasis, mice produce 10^7 new neutrophils each day, whilst humans produce 10^{11} (240). Neutrophil development takes place in the bone marrow (244). During haematopoiesis, haematopoietic stem cells stimulated by granulocyte-colony stimulating factor (G-CSF) will commit to a common granulocyte/myeloid progenitor (GMP) cells which can further differentiate to give rise to either granulocyte or myeloid lineages (245). Single-cell transcriptomic studies have revealed the presence of multiple neutrophil precursor cells in the bone marrow of both humans and mice (240). These studies indicate the granulocyte/myeloid progenitor cells differentiate to become pre-neutrophils which no longer express markers associated with other leukocyte lineages (240). For differentiation into pre-neutrophils, GMPs require activation of the CCAAT-enhancer-binding protein (C/EBP) family of transcription factors (246). Under steady-state conditions, granulopoiesis is largely controlled by C/EBP- α , with *C/ebp- α ^{-/-}* selectively lacking granulocytes but not monocytes (247). Pre-neutrophils in turn differentiate into immature neutrophils which lack CXCR2 expression, that subsequently differentiate into CXCR2⁺ mature neutrophils (246). Expression of CXCR2 enables mature neutrophils to mobilise along chemokine gradients and exit the bone marrow, entering the circulation (248). During an infection, neutrophils are rapidly recruited, activated, and spent in large quantities, leading to a steep increase in demand on haematopoiesis to replenish the neutrophil population (249). Cytokines and chemokines released by infected resident and immune cells are responsible for driving this increased neutrophil production, termed “emergency haematopoiesis” (249). Increased production of both G-CSF, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-6 have all been shown to drive emergency haematopoiesis following certain infections (250,251).

1.4.2 Recruitment

To migrate from the circulation into infected tissues, neutrophils follow a cascade of steps beginning with tethering and rolling followed by adhesion, crawling and transmigration through the endothelium and epithelium (252). Following recognition of the infection, cytokines released by airway resident immune cells upregulate the expression of P-selectin and E-selectin on endothelial cells (253). Neutrophils then bind to these selectins via expression of glycosylated ligands such as P-selectin glycoprotein ligand 1 (PSGL1), on their surface (252). Rolling is mediated by the direction of blood flow, pushing neutrophils from selectin to selectin as they move through the blood vessel (254). To track to the lungs, neutrophils require a chemoattractant gradient generated by the release of chemokines by resident airways cells. The predominant chemokine involved in neutrophil recruitment in humans is IL-8 (CXCL8) which is analogous to murine CXCL1 and CXCL2 (252). Chemokines leave the tissue and

enter into the blood vessels where they bind to heparin sulphates expressed on endothelial cells creating a chemoattractant gradient (255). Rolling neutrophils which encounter the sequestered chemokines will bind to them via the CXCR2 receptor (256). This binding induces conformational changes to integrins expressed on the surface of the neutrophil which allow it to better adhere to ICAMs expressed on endothelial cells (257). Following adhesion to the endothelium, neutrophils do not necessarily undergo transmigration at that exact site. Instead, adhesion upregulates the motility of neutrophils which exhibit crawling behaviour (257). During this process, neutrophils appear to extend their plasma membrane out in different directions in order to probe the surrounding area, looking for the best location to transmigrate (258). Transmigration occurs at tight junctions, and once the neutrophil has located one it will crawl toward it in a process dependent on neutrophil macrophage-1 antigen (MAC1) binding to endothelial intercellular adhesion molecule 1 (ICAM1) (257). Subsequently, the neutrophil will begin the process of transmigration, passing through the endothelial tight junctions, the basement membrane and the lung epithelial tight junctions in order to extravasate into the airways (258).

1.4.3 Antimicrobial mechanisms

In the context of infection, neutrophils are the frontline foot soldiers that possess a selection of weaponry to fight invading bacteria, fungi and viruses (Fig. 1.6).

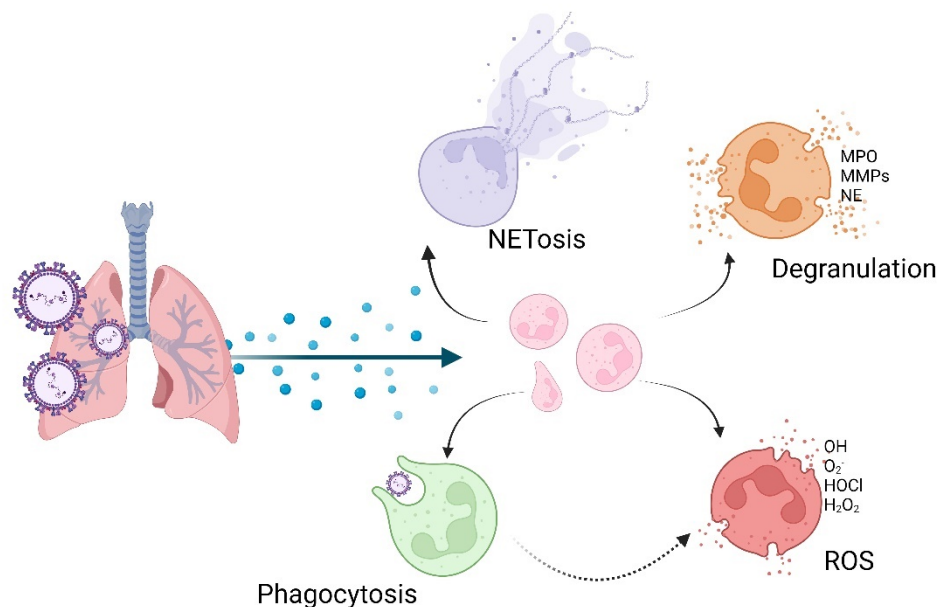


Figure 1.6 Neutrophil antimicrobial mechanisms

Following infection neutrophils employ a multitude of antimicrobial mechanisms including the ability to phagocytose pathogens, undergo programmed cell death to release NETs, produce large quantities of ROS and degranulate to release degradative proteases and antimicrobial peptides (259). Created using BioRender.

One of these weapons is their ability to phagocytose pathogens. Phagocytosis by neutrophils is dependent upon opsonisation of the pathogen by components of the complement system or immunoglobulins (237). The opsonised pathogens are then recognised by opsonic receptors expressed by the neutrophil and the pathogen is phagocytosed into a vacuole within the neutrophil (237,260). Phagocytosis in turn triggers an oxidative burst resulting in the generation of large quantities of reactive oxygen species (ROS) (260). The predominant enzyme responsible for ROS production, is nicotinamide adenine dinucleotide phosphate (NADPH), which pumps electrons across the phagosome membrane, creating superoxide anions (O_2^-) (261). Further ROS can be formed from O_2^- including H_2O_2 , HO and HOCl. ROS can inactivate pathogen membrane proteins as well as prevent DNA synthesis, driving cellular damage (261). The importance of ROS production by neutrophils is evident in patients with chronic granulomatous disease (CGD) who have a genetic defect in NADPH. These patients suffer from frequent severe bacterial and fungal infections, particularly *Aspergillosis* (262). ROS are not discriminate, and in addition to damaging pathogens will also damage cellular components of the lung epithelial and endothelial barrier (263). Increased levels of ROS are therefore thought to contribute greatly to pathology during severe ARDS (264).

Neutrophils can be defined as granular cells, meaning they contain membrane enclosed pockets of antimicrobial peptides, enzymes and inflammatory mediators known as granules. There are four different kinds of granules found in the cytoplasm of neutrophils: primary granules, secondary granules, tertiary granules and secretory granules (265). Primary granules, also known as azurophilic granules, contain the majority of the antimicrobial peptides and degradative enzymes including neutrophil elastase (NE), myeloperoxidase (MPO), cathepsins and defensins (265). Secondary and tertiary granules contain components which are important in digesting collagen, such as matrix metalloprotease 9 (MMP9), required for migration and extravasation into tissues (265). The process of degranulation requires an increase in intracellular Ca^{2+} which can be driven by chemoattractant binding to its cognate receptor (266). Ca^{2+} influx drives exocytosis of granules, whereby they translocate to and fuse with the plasma membrane or phagosome membrane, releasing their contents into the extracellular or vacuole space (266). The proteases stored in primary granules target and degrade components of pathogens which results in death of the pathogen. For example NE can cleave specific proteins on the outer membrane of *E.coli* which results in loss of membrane integrity and ultimately lysis of the bacterium (267). The release of these proteases into the phagosome has also been shown to be responsible for killing of pathogens such as *S. pneumonia* (268). Like ROS, the proteases and antimicrobial peptides releases through degranulation are also non-specific thus can also damage the airways (265,269,270). NE and MPO can cleave important components of extracellular matrix including fibronectin, elastin

and collagen (269). In addition, NE has also been shown to cleave e-cadherins *in vitro*, leading to loss of monolayer integrity of cultured airway epithelial cells (270).

1.4.4 Neutrophil cell death

Neutrophils have a high capacity to undergo multiple forms of cell death including apoptosis, necrosis, necroptosis, pyroptosis and NETosis, potentially explaining their short lifespan (243). The process of neutrophil cell death can play an important role in antimicrobial function, but also in directing the immune response toward inflammation or resolution (Fig.1.7) (243,271,272).

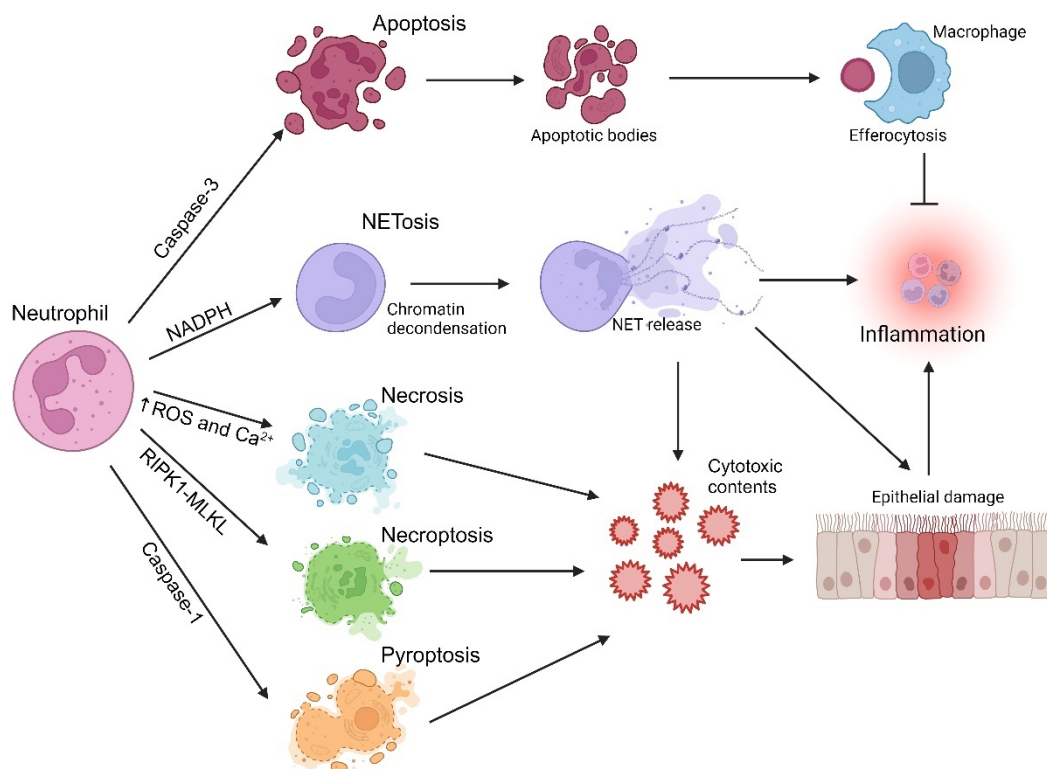


Figure 1.7 Neutrophil cell death pathways

Diagram depicting the different pathways through which neutrophils can die and how this can contribute to inflammation. Apoptosis is mediated by caspase-3 activation leading to the neutrophil being packaged into membrane bound apoptotic bodies that are cleared by macrophages (134,135,273). NETosis, driven by NADPH activity, begins with chromatin decondensation, followed by the rupture of the neutrophil membrane and release of NETs. NETs can drive epithelial damage, act as DAMPs and directly drive inflammation (274). Necrosis is uncontrolled cell death initiated by the accumulation of ROS and Ca^{2+} , which causes the neutrophil to swell and eventually rupture its membrane resulting in the release of its cytotoxic contents which can drive epithelial damage and inflammation (275). Necroptosis and pyroptosis, driven by RIPK1-MLKL and caspase-1 respectively, also lead to rupture of the neutrophil membrane and release of cytotoxic contents (243,276,277). Created using BioRender.

The majority of neutrophils in the blood under steady state, as well as those recruited to the tissues without substantial infection or inflammatory signals will undergo spontaneous

apoptosis (273). Apoptosis is a form of programmed cell death which can be initiated via two pathways, the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is initiated by mitochondrial stress, often driven by the accumulation of ROS (278). This results in the release of proapoptotic mediators that drive the activation of caspase-9 which ultimately activates caspase-3. The extrinsic pathway requires activation of death receptors by FasL or TNF- α , which activates caspase-8, which in turn activates caspase-3 (279). Activation of caspase-3 triggers the apoptotic programme (Fig. 1.7). Neutrophil chromatin will begin to condense before the nucleus fragments. Vesicles which contain nuclei fragments or other organelles surrounded by plasma membrane, known as apoptotic bodies, will then begin to bleb off the cell breaking it apart into smaller pieces (273). This process of dismantling the cell without rupturing the plasma membrane means that many neutrophilic cytotoxic and inflammatory mediators are not released. Thus, neutrophil apoptosis does not contribute to further inflammation (280,281). In fact, apoptosis of neutrophils is believed to be anti-inflammatory and contribute to resolution (280). This effect is mediated through the clearance of apoptotic neutrophils by macrophages through a process termed efferocytosis. As described previously, macrophages can recognise and bind to a range of “eat-me” signals which are exposed on the outer membrane of apoptotic neutrophils, such as phosphatidylserine (134,135). The apoptotic neutrophils are then engulfed by the macrophage. *In vitro* engulfment of apoptotic neutrophils by macrophages has previously shown an immunosuppressive effect, inhibiting the production of proinflammatory cytokines such as IL-1 β , GM-CSF and TNF- α following LPS stimulation (282). Whilst co-culture of apoptotic neutrophils with macrophages resulted in increased levels of anti-inflammatory mediators such as prostaglandin E2 (PGE₂) and TGF- β (282).

Unlike apoptosis, neutrophil necrosis is a form of uncontrolled, proinflammatory cell death (243). During necrosis, the cytoplasm and nucleus of the neutrophil swells until eventually the plasma membrane ruptures releasing the contents of the neutrophil. Necrosis is not programmed, and often occurs under extreme pro-inflammatory stress resulting in a lack of oxygen, nutrients or an accumulation of toxic components (243). Induction of necrosis requires the accumulation of ROS and increase in intracellular Ca²⁺ (Fig. 1.7) (275). In addition to necrosis, neutrophils can also undergo programmed forms of inflammatory cell death which also drive the rupture of the plasma membrane and release of the cellular contents (243). This includes necroptosis, which is initiated by activation of the RIPK3-MLKL, and pyroptosis which requires activation of caspase-1 (Fig. 1.7) (243). Both pathways result in the formation of pores in the plasma membrane (276,277). The release of the neutrophil contents through these methods of cell death further contributes to inflammation by acting as DAMPs or by contributing to epithelial damage (Fig. 1.7) (283).

NETosis is a form of cell death which is specific to neutrophils (243). During NETosis, neutrophils will rupture their outer membrane, sending out web-like structures composed mostly of chromatin studded in proteins, known as neutrophil extracellular traps (NETs) (274). These NETs, as their name suggests, trap pathogens and hold them in areas of high degradative enzyme concentration, thus contributing to pathogen killing (284). There is evidence that NETs trap and degrade a wide range of extracellular pathogens including both gram-negative and gram-positive bacteria, fungus, parasites and free viruses (284–287). The role of NETs during human infection is not fully understood, however there is some evidence to suggest neutrophils from neonates have an inability to form NETs and that this may contribute to increased risk of bacterial infections (288). In addition to their antimicrobial function, NETosis is also able to modulate the immune system to drive inflammation (274). Because NETs are studded in cytotoxic proteins, like histones, they also cause damage to the lung epithelia and endothelia (289). In addition to the release of DAMPs, NETs themselves can also act as DAMPs, driving PRR signalling (290). In the absence of pathogen, such as during mouse models of atherosclerosis, NETs have been shown to signal via TLR2 and TLR4 to drive the expression of IL-6 and IL-1 β from macrophages. The expression of these cytokines in turn increased the recruitment of myeloid cells and also drove differentiation of Th17 cells (291).

1.5 Neutrophils and Respiratory Viral Infection

1.5.1 Neutrophils During RSV Infection

Following infection with respiratory virus, neutrophils are rapidly recruited into the lungs of both mice and humans (8,16). During RSV infection in infants, increased airway neutrophilia is correlated with more severe disease, however in the past it has not been so clear on whether these neutrophils are beneficial in clearing the virus or are contributing more to pathology (292). Using mouse models of RSV, the dynamics of neutrophil recruitment and their role during infection has been revealed. As previously described, neutrophils require a chemokine signal to respond and migrate to sites of infection (252). In the lungs of mice infected with RSV, CXCL1 appears to be predominantly released by cells of the stromal compartment in a manner dependent upon TLR- MyD88 signalling (16). In contrast, once recruited to the lungs and airways, the neutrophils become activated by entering an inflammatory environment (16). One way is via the type I IFN receptor and as detailed above, the production of IFN- α during RSV infection is predominantly by AMs in an RLR-MAVS dependent manner (16). There is evidence that the antimicrobial mechanisms elicited by activated neutrophils are effective against RSV. For example, different proteins released during degranulation have shown to

restrict RSV infectivity. *In vitro* MMP9 treatment of airway epithelial cells infected with RSV, showed reduced viral titres (293). Similarly, the cathelicidin LL37 has been shown to bind to RSV virions and disrupt their viral envelope, resulting in reduced viral binding and entry into cells (294). Neutrophils have also been suggested to phagocytose RSV virions which have become opsonised by surfactant D protein, resulting in the production of ROS (295). In addition, there is evidence that NETs can trap RSV and prevent it from infecting cells *in vitro* (296). Despite the breadth of *in vitro* evidence of the efficacy of neutrophil defence mechanism against RSV, it is less clear whether they are functioning in protection or pathology *in vivo*. To investigate this, Kirsebom et al, infected mice with RSV and 12h later gave an intra-nasal dose of CXCL1 to drive further neutrophil recruitment into the lungs (17). The presence of increased number of neutrophils in the lungs and airways did not result in any changes to the lung viral load, suggesting neutrophils do not contribute significantly to viral clearance during RSV infection (17). Furthermore, increased neutrophils did not increase weight loss following RSV infection compared with RSV only controls, nor did they contribute to the levels of inflammatory mediators including IFN- α , IL-6, TNF- α and IL-1 β (17). In addition, depletion of neutrophils during RSV infection in mice had no effect on viral clearance, weight loss or the immune response towards the virus (17). Overall, this data suggests that during RSV infection neutrophils are not drivers of disease pathology nor do they play an important role in protection.

1.5.2 Neutrophils During IAV infection

Neutrophils are also recruited into the lungs following IAV infection, however their role in protection and pathology remains unclear (297,298). Unlike during RSV infection, neutrophils may play a role in controlling viral replication and clearance of IAV. In a study where neutrophils were depleted during IAV infection in mice, viral replication was increased in the respiratory tract (299). This protective role of neutrophils may be strain specific, with one study demonstrating neutrophil depletion resulted in higher viral titres in mice infected with more pathogenic strains of IAV compared to less pathogenic strains (300). In contrast, neutrophils have also been suggested to act as a vehicle for IAV and promote its dissemination as evidence of viral replication has been identified in human lung neutrophils from patients post mortem (301). However, IAV replication in neutrophils from the lungs of mice was not observed in prior studies (300). In addition to increasing viral load, depletion of neutrophils in mice infected with pathogenic strains of IAV resulted increase disease severity, characterised by increased weight loss (300). Such an effect has been reported in several other mouse studies of IAV infection (299,302). Furthermore, neutrophils recruited during IAV infection in mice have also been reported to be important in both the recruitment of CD8⁺ T cells and sustaining their

effector functions (303,304). Despite depletion studies demonstrating the importance of neutrophils during RSV infection, increased neutrophilia has been implicated in disease and the development of ARDS (297,305–307). In particular, increased levels of NETs have been found in the plasma of patients with severe IAV infection (307). Neutrophils isolated from these patients also demonstrated increased ability to form NETs *in vitro* (307). In mice, NETs have been found entangled in the alveoli in areas of high lung injury, suggesting they may drive tissue damage (305). Furthermore, mice deficient in MPO, a protease found in neutrophil granules and often found associated with NETs, show reduced damage in the lung (306). The evidence therefore points toward the dual functionality of neutrophils during IAV infection, suggesting they are protective, but dysregulation or excessive production of neutrophil mediators facilitates lung injury.

1.5.3 Neutrophils Before RSV infection

To better understand the immune determinants that drive susceptibility to severe disease following RSV infection, experimental human challenge studies of healthy adults have been successfully used. When a cohort of healthy adults are challenged with RSV, despite previous natural infection confirmed by the presence of serum neutralising antibodies, about 50-60% will become infected (170,235). Amongst the infected individuals, about 50% will display cold-like symptoms, whilst the other 50% remain asymptomatic (170). Utilising this, Habibi et al sequenced the nasal mucosa of healthy adults before infection with RSV and then at multiple timepoints after RSV infection (170). The expression profiles of the infected individuals who developed symptoms versus those that did not develop symptoms was then compared. Out of all the differentially expressed genes, increased neutrophil associated genes in the nasal mucosa before infection with RSV, correlated with the greatest likelihood of developing symptoms. To better understand how the presence of neutrophils in the airways before RSV infection might alter the immune response to the virus, these findings were translated into a mouse model. To recruit neutrophils, mice were given an intranasal dose of CXCL1 12h before RSV infection. Interestingly, despite neutrophils not impacting on disease during RSV in mouse models, neutrophil recruitment before RSV infection resulted in exacerbated weight loss (170). Therefore, it appears the role neutrophils play in exacerbating disease outcomes following RSV infection in both humans and mice, is specific to them being present in the airways before infection. In mouse models, this exacerbation was associated with an increase in early viral load at 18h infection but not increases in innate inflammatory cytokines (170). Exacerbation was also associated with an increase in the recruitment of CD8⁺ T cells at day 8 post infection. Using an anti-CD8 antibody depletion strategy, it was found that this increase in CD8⁺ T cells was responsible for driving the disease exacerbation (170). Taking this data

together it appears that neutrophils in the airways before infection may promote viral replication leading to an increased adaptive immune response resulting in exacerbated disease. The role of neutrophils in the lung before IAV infection has not previously been studied.

2. Hypotheses and Aims

The findings presented by Habibi et al. demonstrate neutrophils in the airways before RSV infection can increase risk of disease following RSV infection (170). However, this opened a number of questions which were not included in the scope of the study. Predominantly, there is no explanation as to why some of the participants may have had neutrophils present in their airways before infection. As a result, intranasal recombinant CXCL1 was used to drive neutrophil recruitment before infection in the mouse model, which does not represent a physiological mechanism. In addition, it is not clear when neutrophils need to be recruited into the airways before RSV infection. Neutrophils are known to be short-lived cells which might suggest they need to be recruited shortly before infection to have any effect on disease following RSV infection (272). Alternatively, there is the potential that neutrophils are able to induce longer lasting changes to the lung environment which subsequently exacerbate the immune response to RSV infection. The mouse model reported by Habibi et al recruited neutrophils 12h before infection and the dependency of disease exacerbation on these timings was not investigated (170). Finally, the study presented by Habibi et al was specific to RSV infection and did not investigate how neutrophils in the airways before infection with other respiratory viruses might affect disease outcomes (170). Individuals at higher risk of severe disease following RSV infection are also at risk of severe disease following infection with other respiratory viruses such as IAV and SARS-CoV-2 (5,308). Therefore, it is also possible neutrophils in the airways before infection with these viruses could exacerbate disease outcomes. However, whilst the immune response to these viruses are sequentially similar, they likely vary in magnitude and timings. Additionally, certain immune cell populations may play differential roles in protection and pathology during infection with different respiratory viruses.

These questions led to the formulation of three hypotheses detailed in Figure 2.1.

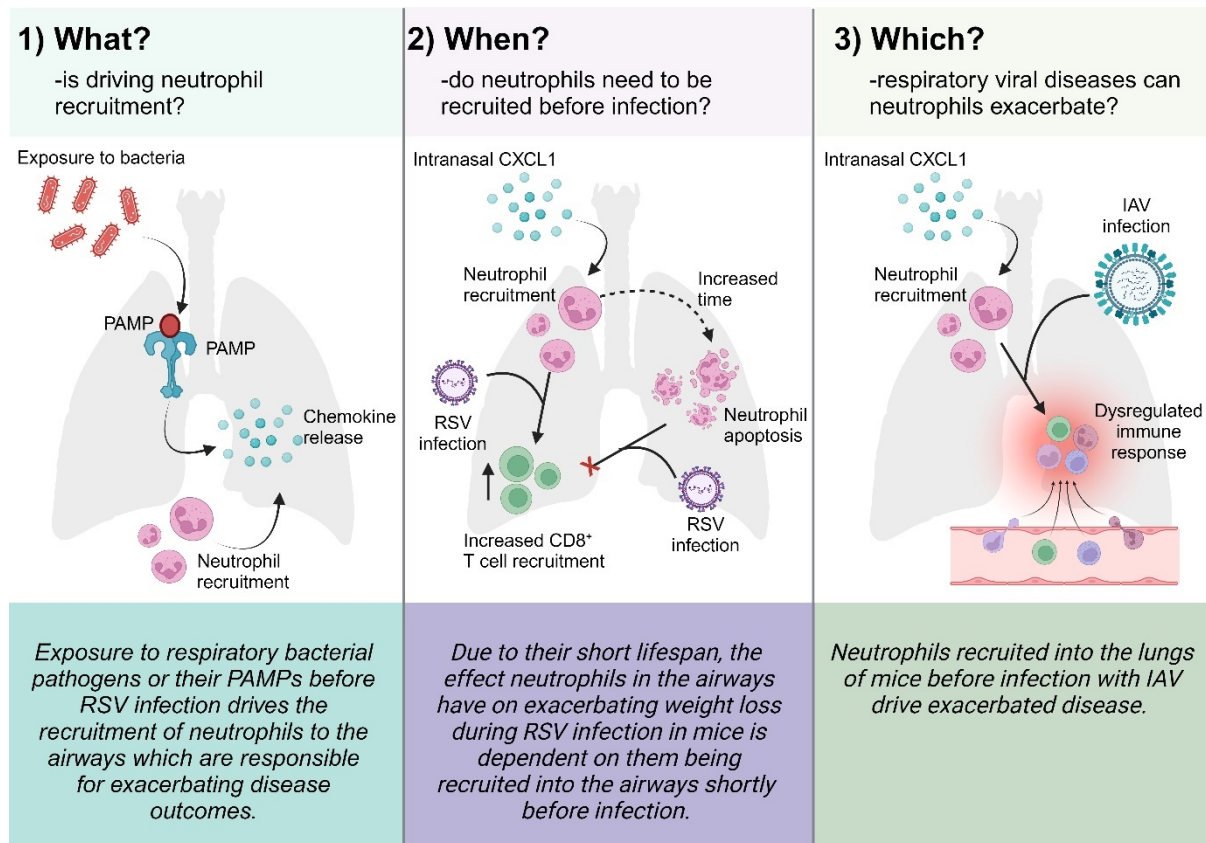


Figure 2.1 Hypotheses

Schematic detailing the formulation of three hypotheses from findings presented by Habibi et al (170), which form the basis of the work presented in this thesis

To begin answering these, a series of aims were outlined and subsequently explored:

- 1) *Exposure to respiratory bacterial pathogens or their PAMPs before RSV infection drives the recruitment of neutrophils to the airways which are responsible for exacerbating disease outcomes.*
 - a) To demonstrate if exposure to intranasal bacterial PAMPs can drive neutrophil recruitment to the airways of mice.
 - b) To investigate how exposure to bacteria or PAMPs before RSV infection alters weight loss.
 - c) To determine if neutrophils recruited by exposure to PAMPs are responsible for mediating any changes to weight loss.
- 2) *Due to their short lifespan, the effect neutrophils in the airways have on exacerbating weight loss during RSV infection in mice is dependent on them being recruited into the airways shortly before infection.*
 - a) To investigate how extending the time between intranasal CXCL1 and subsequent RSV infection affects weight loss.

- b) To identify if extending the time between CXCL1 and RSV infection changes the immune response to the virus.
- 3) *Neutrophils recruited into the lungs of mice before infection with IAV drive exacerbated disease.*
 - a) To determine if neutrophils recruited by intranasal CXCL1 can have the same effect on weight loss following IAV infection as it does following RSV infection.
 - b) To determine if increased neutrophil recruitment during IAV infection can affect disease outcomes or viral clearance.
 - c) To investigate how elevated neutrophils in the lung before or after IAV infection alter the immune response to the virus.

3. Reagents and Materials

Table 3.1 Media composition

Media	Components	Final Conc.	Supplier	Cat. no
Complete DMEM (cDMEM)	Dulbecco's Modified Eagle Medium (DMEM)	-	Sigma-Aldrich (UK)	D6429
	Foetal Calf Serum (FCS)	10%	Sigma-Aldrich	F9665
	Penicillin Streptomycin	1%	Sigma-Aldrich	P4333
	L-glutamine	1%	Gibco, Life Technologies (UK)	25030024
LB Broth	Miller's LB Broth	-	Sigma-Aldrich	L3522
LB Agar	Miller's LB Broth with Agar	-	Sigma-Aldrich	L3147
BAL media	PBS	1x	Sigma-Aldrich	D8537
	EDTA	0.5mM	Gibco, Life Technologies	15575020
	Distilled water	-		
ACK Lysis Buffer	NH ₄ Cl	0.15M	Sigma-Aldrich	A9434
	KHCO ₃	1mM	Sigma-Aldrich	P9144
	EDTA	0.1mM	Gibco, Life Technologies	15575020
	PBS	1x	Sigma-Aldrich	D8537
FACS buffer	Bovine Serum Albumin (BSA)	1%	Sigma-Aldrich	A3294
	EDTA	5mM	Gibco, Life Technologies	15575020
	Roswell Park Memorial Institute (RPMI) 1640	-	Sigma-Aldrich	R8758
Complete RPMI (cRPMI)	Foetal Calf Serum (FCS)	10%	Sigma-Aldrich	F9665
	Penicillin Streptomycin	1%	Sigma-Aldrich	P4333
	L-glutamine	1%	Gibco, Life Technologies	25030024
	Complete RPMI (cRPMI)	-	-	-
MexAM media	Murine GM-CSF	30ng/ml	Peprtech (UK)	315-03
	Recombinant human TGF- β	10ng/ml	Peprtech	100-21
	Rosiglitazone	1 μ M	Sigma-Aldrich	R2408

Table 3.2 Depletion antibodies

Target	Antibody	Clone	Dose	Supplier	Cat. no
Neutrophils	Anti-Ly6G	1A8	150 or 50µg	Assay Genie (IE)	IVMB0025
	Anti-Rat Kappa IgG	MAR18.5	50µg	Assay Genie	IVMB0245
	Rat Kappa IgG2 (isotype control)	2A3	150 or 50µg	Francis Crick Institute (UK)	-
Monocytes	Anti-CCR2	MC-21	20µg	Matthias Mack	-
	Rat IgG2b (isotype control)	1-2	20µg		IVMB0200
IL-1 α	Anti-IL-1 α	ALF-161	250µg	Assay Genie	IVMB0005
IL-1 β	Anti-IL-1 β	B122	250µg	Assay Genie	IVMB0085
TNF- β	Anti-TNF- α	TN3-19.12	250µg	Assay Genie	IVMB0007
	Armenian Hamster IgG (isotype control)	PIP	250 or 750µg	Assay Genie	IVMB0182

Table 3.3 Flow cytometry staining panels

Panel	Target	Fluorochrome	Clone	Final Conc.	Supplier	Cat. no
Innate	Live/Dead	Aqua	-	500	InvitroGen (UK)	L34966
	Siglec-F	BV786	E50-2440	0.5µg/ml	BD Bioscience (UK)	740956
	MHC-II	efluor450	M5/114-15.2	1µg/ml	eBioscience (UK)	48-5321-80
	CD26	BV605	H194-112	2µg/ml	BD Bioscience	745125
	Sirp1α	BV711	EBio4406	1µg/ml	BD Bioscience	740766
	CD45	AF488	30-F11	1.25µg/ml	BioLegend (UK)	103121
	XCR1	PerCP-Cy5.5	ZET	1µg/ml	BioLegend	148208
	CD11c	PE-CF594	HL3	1µg/ml	BD Bioscience	562454
	Ly6G	PE-Cy7	1A8	1µg/ml	BioLegend	127618
	CD11b	AF700	M1/70	0.5µg/ml	eBioscience	56-0112-82
	CD64	APC	X54-5/7.1	2µg/ml	BioLegend	139306
	CD3	eFluor780	145-2C11	2µg/ml	eBioscience	47-0031-82
	CD49b	BUV737	HMa2	0.5µg/ml	BD Bioscience	741752
Adaptive	Live/Dead	Aqua	-	500	Invitrogen	L34966
	Siglec-F	BV786	E50-2440	0.5µg/ml	BD Bioscience	740956
	CD11c	BV421	HL3	1µg/ml	BD Bioscience	562782
	PD-1	BV605	29F.1A12	2µg/ml	BioLegend	135220
	CD45	PerCP-Cy5.5	30-F11	0.5µg/ml	BioLegend	135220
	Ly6G	AF488	1A8	5µg/ml	BioLegend	127626
	CD4	PE	GK1.5	0.5µg/ml	eBioscience	12-0041-83
	CD3	AF700	17A2	2µg/ml	eBioscience	56-0032-82
	CD19	APC	1D3	2µg/ml	BioLegend	152410
	CD8a	efluor780	53-6.7	0.5µg/ml	eBioscience	47-0081-82
Combined	CD69	BUV737	H1.2F3	2µg/ml	BD Bioscience	612793
	L/D	Aqua	-	500	Invitrogen	L34966
	Siglec-F	BV786	E50-2440	0.5µg/ml	BD Bioscience	740956
	CD45	BV605	30-F11	0.25µg/ml	BioLegend	103140
	CD26	BV711	H194-112	2µg/ml	BD Bioscience	740678
	MHC-II	efluor450	M5/114-15.2	1µg/ml	eBioscience	48-5321-80
	Ly6G	AF488	1A8	5µg/ml	BioLegend	127626
	XCR1	PerCP-Cy5.5	ZET	1µg/ml	BioLegend	148208
	CD11b	PE-Cy7	M1/70	2µg/ml	BD Bioscience	552850
	CD11c	PE-CF594	HL3	1µg/ml	BD Bioscience	562454
	CD49b	PE	HMa1	2µg/ml	BioLegend	108908
	CD3	AF700	17A2	2µg/ml	eBioscience	56-0032-82
	CD8	efluor780	53-6.7	0.5µg/ml	eBioscience	47-0081-82
	CD64	APC	X54-5/7.1	2µg/ml	BioLegend	139306
	CD69	BUV737	H1.2F3	2µg/ml	BD Bioscience	612793
	CD4	BUV395	GK1.5	1µg/ml	BD Bioscience	563790

Table 3.3 Continued

Panel	Target	Fluorochrome	Clone	Final Conc.	Supplier	Cat. no
Neutrophil	Live/Dead	Aqua	-	500	Invitrogen	L34966
	Siglec-F	BV786	E50-2440	0.5µg/ml	BD	740956
					Bioscience	
	Sirp1α	BV711	EBio4406	1µg/ml	BD	740766
					Bioscience	
	Ly6C	efluor450	HK1.4	0.25µg/ml	eBioscience	48-5932-82
	Apotracker	AF488	-	0.8µM	BioLegend	427402
	CD45	PerCP-Cy5.5	30-F11	0.5µg/ml	BioLegend	135220
	Ly6G	PE-Cy7	1A8	0.5µg/ml	BioLegend	127618
	CD11c	PE-CF594	HL3	1µg/ml	BD	562454
					Bioscience	
	CD63	PE	NVG-2	1µg/ml	BioLegend	143903
	CD11b	AF700	M1/70	0.5µg/ml	eBioscience	56-0112-82
	CXCR2	APC-Cy7	SA044G2	1µg/ml	BioLegend	149313
Intracellular	CD64	APC	X54-5/7.1	1µg/ml	BioLegend	139306
	CXCR4	BUV737	2B11	1µg/ml	BD	741783
					Bioscience	
	Ly6G	APC	1A8	1µg/ml	BioLegend	127614
	Gzmb (anti-human)	PE	TWAJ		eBioscience	12-9966-42

Table 3.4 ELISA kits/reagents

ELISA	Component	Supplier	Cat. no
MPO	Mouse Myeloperoxidase DuoSet ELISA	R&D Systems (US)	DY3667
MMP-9	Mouse Total MMP-9 DuoSet ELISA	R&D Systems	DY6718
NE	Mouse Neutrophil Elastase/ELA2 DuoSet ELISA	R&D Systems	DY4517-05
IL-1 β	Mouse IL-1 beta/IL-1F2 DuoSet ELISA	R&D Systems	DY401
Gzmb	Mouse Granzyme B DuoSet ELISA	R&D Systems	DY1865
CXCL1	Mouse CXCL1/KC DuoSet ELISA	R&D Systems	DY453
IL-6	Purified Rat Anti-Mouse IL-6 (Capture Antibody)	BD Bioscience	554400
	Biotin Rat Anti-Mouse IL-6 (Detection Antibody)	BD Bioscience	554402
	Recombinant Mouse IL-6 (Standard)	BD Bioscience	554582
	HRP-Streptavidin	Thermo Scientific (UK)	SNN2004/S911
IFN- α	Monoclonal mouse IFN- α (Capture Antibody)	Hycult Biotechnology (NL)	HM1001
	Rabbit Poly-clonal Anti- IFN- α (Primary Detection Antibody)	PBL Biomedical Laboratories (US)	32100-1
	Biotin conjugated Mouse Anti-Rabbit IgG (Secondary Detection Antibody)	Jackson ImmunoResearch (UK)	211-065-109
	Recombinant IFN α	Hycult Biotechnology	HC1040b
Albumin	HRP-Streptavidin	Thermo Scientific	SNN2004/S911
	Affinity purified Goat anti-Mouse Albumin Coating Antibody	Bethyl Laboratories (US)	A90-134A
	HRP Conjugated Goat anti-mouse Albumin Antibody	Bethyl Laboratories	A90-134P
	Mouse Reference Serum	Bethyl Laboratories	RS10-101-5

Table 3.5 qPCR primers and probes

qPCR	Supplier	Cat. no/ Sequence
<i>Il6</i>	Thermo Scientific	Mm00446190_m1
<i>Il1b</i>	Thermo Scientific	Mm00434228_m1
<i>Il10</i>	Thermo Scientific	Mm00439614_m1
<i>Ifna5</i>	Thermo Scientific	Mm00833976_s1
<i>Ifng</i>	Thermo Scientific	Mm01168174_m1
<i>Cxcl1</i>	Thermo Scientific	Mm04207460_m1
<i>Cxcl2</i>	Thermo Scientific	Mm00436450_m1
<i>Ccl2</i>	Thermo Scientific	Mm00445235_m1
<i>Cxcl10</i>	Thermo Scientific	Mm00445235_m1
<i>Tgfb</i>	Thermo Scientific	Mm01178820_m1
<i>Tnfa</i>	Thermo Scientific	Forward: CATCTTCTCAAAATTCGAGTGACAA
	Thermo Scientific	Reverse: TGGGAGTAGACAAGGTACAACCC
	Eurofins Scientific (LU)	Probe: CACGTAGCAAAC
RSV L gene	Thermo Scientific	Forward: GAACTCAGTGTAGGTAGAATGTTTGCA
	Thermo Scientific	Reverse: TTCAGCTATCATTTTCTCTGCCAAT
	Eurofins Scientific	Probe: TTTGAACCTGTCTGAACATTCCCGGTT
X31 M gene	Thermo Scientific	Forward: AAGACCAATCCTGTCACCTC
	Thermo Scientific	Reverse: CAAAGCGTCTACGCTGCAG
	Eurofins Scientific	Probe: TTTGTGTTTACGCTCACCGT

Table 3.6 Miscellaneous Reagents

Use	Reagent	Final Conc.	Supplier	Cat. No
RSV immunoplaque assay	Methanol	≥99.8%	VWR (UK)	20846.326
	Hydrogen Peroxide 30%	2%	Sigma-Aldrich	216763
	Goat anti-RSV: biotin	8µg/ml	Bio-Rad	7950-0104
	Extravidin Peroxidase	20µM	Sigma-Aldrich	E2886
	Fast DAB substrate	--	Sigma-Aldrich	11718096001
Mouse models	Recombinant CXCL1	5µg	BioLegend	573708
	Lipopolysaccharide (<i>Escherichia coli</i>)	1µg	InvivoGen(UK)	L23352
	Lipoteichoic acid (<i>Staphylococcus aureus</i>)	50µg	InvivoGen	PLS-41-02
	Isoflurane gas (Iso-Vet)	1000mg/g	Chanelle Pharma (UK)	-
	Pentoject (Phenobarbital)	20%	Animalcare Ltd (UK)	-
Lung cell processing	Collagenase D	1mg/ml	Roche (UK)	11088882001
	DNase I from bovine pancreas	30µg/ml	Sigma-Aldrich	D5025
	gentleMAC C-tubes	-	Miltenyi Biotec (UK)	130096334
	100µm cell strainer	-	Greiner Bio One (UK)	542000
	Trypan blue	0.1%	Sigma-Aldrich	T8154
Flow cytometry	Purified Rat Anti-Mouse CD16/CD32 (FC Block)	2.5µg/ml	BD Bioscience	553141
	PMA	100ng/ml	Sigma-Aldrich	P8139
	Ionomycin	1000ng/ml	Abcam (UK)	ab120370
	GolgiPlug Protein Transport Inhibitor	1:1000	BD Bioscience	555029
	Fixation buffer (Paraformaldehyde)	2%	BioLegend	420801
	Permeabilisation (Perm)/Wash buffer	1x	BioLegend	412002
	CompBeads Anti-Rat and Anti-Hamster Ig κ /Negative Control Compensation Particles Set	-	BD Bioscience	552845
	CompBeads Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	-	BD Bioscience	552843
mexAM culture	Trypsin-EDTA	0.5%	Gibco, Life technologies	59418C

Table 3.6 continued

Use	Reagent	Conc.	Supplier	Cat. No
RNA extraction	TRIzol	-	InvitroGen	15596026
	Chloroform	-	VWR	22706.361
	β-mercaptoethanol	1%	Sigma-Aldrich	M6250
	RNeasy Mini Kit	-	Qiagen (UK)	74104
cDNA and qPCR	RNAse Free-DNase digest	-	Qiagen	79254
	Quantitect Probe PCR Master Mix	-	Qiagen	204345
	TMB solution	-	InvitroGen	15596026
ELISA	Tween 20	0.05%	Sigma-Aldrich	P1379
	Streptavidin- HRP	0.1 µg/ml	Agilent Technologies (UK)	CJ30H-1

Table 3.7 Instruments

Instruments	Supplier	Use
CX-2000 UV cross-linker	Analytik Jena AG (DE)	UV irradiation
gentleMACS Dissociator	Miltenyi Biotech	Lung cell processing
BD LSR Fortessa Cell Analyzer	BD Bioscience	Flow cytometry acquisition
FLUOstar Omega	BMG Labtech	Reading ELISA plates
TissueLyser LT	Qiagen	RNA extraction from lung
NanoDrop 1000 Spectrophotometer	Thermo Scientific	RNA quantification
2720 Thermal Cycler	Applied Biosystems (UK)	cDNA conversion
Applied Biosystems 7500 Fast Real-Time PCR System	Thermo Scientific	qPCR

Table 3.8 Software

Software	Supplier	Use
FACSDiVa	BD Bioscience	Flow cytometry acquisition
FlowJo v.10	Tree Star (US)	Flow data analysis
FlowJo UMAP plugin	Tree Star	UMAP
MARS data analysis software	BMG Labtech	ELISA data analysis
GraphPad PRISM v.10	GraphPad Software Inc (US)	Data visualisation and statistic
BioRender	BioRender, CA	Graphics and Illustrations

4. Methods

4.1 Pathogens

4.1.1 Growth of RSV

Plaque purified human A2 strain RSV (originally from ATCC) was grown in HEp2 cells (from ATCC). HEp2 cells were seeded in cDMEM at 10^7 cells in a T175 flask and were left to grow overnight at 37°C until they had reached 80% confluency. The cells were then washed with serum free DMEM, infected with RSV at an MOI of 0.1 in 4ml of serum free DMEM. Flasks were incubated at 37°C for 2h then topped up with 21ml of cDMEM and incubated for 24h. The percentage of FCS was then reduced from 10% to 2% by removing 20ml of media, centrifuging to pellet any material in the aspirate and resuspending this in serum free DMEM before adding this back to the flask. Flasks were incubated at 37°C for a further 24h or until 80% of cells had detached and were floating in the media. The remaining cells were detached using a cell scraper and the media was sonicated on ice in a water bath for 2 minutes to lyse cells. The samples were subsequently centrifuged at 480xg for 5 min at 4°C and the supernatant was aliquoted and stored in liquid nitrogen. For *in vivo* studies, virus was grown in antibiotic free media.

4.1.2 Immunoplaque assay for quantification of RSV titre

HEp2 cells were plated in cDMEM in 96 well-plates and left over night to form a monolayer overnight. Virus stock was then serially diluted 1:2 in serum free DMEM beginning with an initial dilution of 1:100. Dilutions were then added to the cell monolayers and incubated for 2h at 37°C. Wells were topped up with DMEM + 2% FCS and incubated for a further 20h at 37°C. Monolayers were then fixed with absolute methanol containing 2% hydrogen peroxide for 20min, then washed with 1% BSA. To stain infected cells, biotin conjugated goat anti-RSV antibody was added to the fixed cells at a concentration of 8µg/ml (1:500) and incubated for 2h at room temperature. Plates were then washed 3x with 1% BSA, then extravidin peroxidase was added to the cells for 1h at a concentration 20µM (1:500). After washing 5x with 1% BSA, Sigma Fast DAB substrate, prepared in distilled NANOpure H₂O according to the manufacturer's instructions, was added to each well. Cells were checked under a microscope for colour development then after a minimum of 5min plates were washed with dH₂O and left to dry. Infected cells were counted at appropriate dilutions, allowing calculation of focus forming units (FFU).

4.1.3 X31

Influenza virus strain X31 (H3N2) was grown in eggs and filtered (0.2mm) prior to titration and supplied by John Cauley, WHO Collaborating Centre for Reference and Research on Influenza, Francis Crick Institute.

4.1.4 Growth of *Acinetobacter baumannii*

Bacterial/RSV co-infection studies were carried out using gram-negative bacteria *Acinetobacter baumannii* clinical isolate strain ATCC 17978 (obtained from Sophie Higham and John Tregoning, Imperial College London). *A. baumannii* from a frozen glycerol stock was streaked out on to an LB agar plate 3 days prior to the infection timepoint and grown overnight at 37°C. The following day a sweep of cultures was taken from an area of high growth on the agar plate and used to inoculate 5ml of LB broth, which was grown at 37°C, 250rpm overnight. To generate the infection dose, 200µl of the overnight liquid culture was used to inoculate 20ml of LB broth in a 250ml Erlenmeyer flask and grown at 37°C, 250rpm for 3-3.5h until the culture had reached an OD of 0.7. At this point the culture was estimated to be 1×10^{10} colony forming units (CFU) /ml and was subsequently resuspended in PBS at an estimated 5×10^6 CFU/ml which was used for infecting mice as described below.

4.1.5 Colony forming unit assay for quantification of bacterial dose

To calculate the actual CFU mice received, the infection dose was serially diluted 1:10 in PBS. Six of the dilutions, 10^{-1} - 10^{-6} were dripped onto LB agar plates in parallel lines using a multichannel pipette, then incubated overnight at 37°C. Actual CFU could then be calculated (following infection) by counting the colonies.

4.2 *In vivo* procedures

4.2.1 Mice

Female C57BL6/J mice aged 6-10 weeks, purchased from Charles River UK were used as wildtype mice in all experiments. In some instances, age matched *Rag2^{-/-}* (provided by Marina Botto, Imperial College London) on C57BL6/J mice were used. All animal experiments were reviewed and approved by the Animal Welfare and Ethical Review Board (AWERB) within Imperial College London and approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 and the ARRIVE guideline.

4.2.2 Intranasal PAMP or rCXCL1 exposure

For intranasal delivery of rCXCL1, mice were gently anaesthetised using isoflurane gas 0.5, 2, 4 or 7 days before respiratory viral infection. Mice then received a 5µg dose of rCXCL1 diluted in 100µl PBS, which was dropped onto the nose for inhalation using a pipette. For

delivery of PAMPs, mice were anaesthetised using isoflurane gas 12h before RSV infection and received either 1,2 or 5µg LPS or 50µg LTA diluted in 100µl PBS. All control mice were given an intranasal dose of 100µl PBS.

4.2.3 Bacterial infection

In co-infection studies, naïve mice were infected with *A. baumannii* 12h prior to infection with RSV. Following anaesthesia using isoflurane gas, mice were intranasally infected with 100µl of 1×10^6 CFU/ml stock, thus receiving 1×10^5 CFU.

4.2.4 Respiratory viral infection

Mice were anaesthetised and infected intranasally with 100µl containing either 7×10^6 FFU RSV or 250 plaque forming units (PFU) X31 or PBS as a control. Mice were weighed across the course of the experiment as a measure of disease severity.

4.2.5 Neutrophil depletion

Two protocols to deplete neutrophils were used in this thesis. In the first of these, mice received an intraperitoneal (ip) injection of 150µg anti-Ly6G MAb (1A8; Rat IgG2a, κ) or isotype control (2A3; Rat IgG2a, κ) 1 day before intranasal LPS and then every other day after throughout the course of the experiment. The second protocol was adapted from work published by Boivin et al (309). Mice received 50µg anti-Ly6G Mab one day before intranasal LPS and then again, every day throughout the course of the experiment. Mice also received a 25µg dose of α-Ly6G intranasally at the time point of infection, diluted with the RSV, and a second intranasal dose of α-Ly6G on day 3 post infection. In addition to α-Ly6G, mice received a secondary depletion antibody, 50µg anti-rat kappa IgG i.p every other day throughout the course of infection.

4.2.6 Monocyte depletion

To deplete monocytes, mice received an i.p injection containing 20µg dose of anti-CCR2 MAb (MC-21; Rat IgG2b) (obtained from Matthias Mack, University Regensburg) or the relevant isotype control (1-2; Rat IgG2b) diluted in PBS, 6h before intranasal LPS and then again every day throughout the course of the experiment.

4.2.7 Cytokine blockage

To block IL-1α, IL-1β and TNF-α function, mice received an ip injection of either 250µg α-IL-1α Mab (ALF-161; Armenian Hamster IgG), α-IL-1β (B122; Armenian Hamster IgG), α-TNF-α (TN3-19.12; Armenian Hamster IgG) or all three in combination, 6h before intranasal LPS and then again once a day throughout the course of the experiment. Control mice for single

cytokine depletion were given a 250 μ g ip injection of isotype control (Armenian Hamster IgG), whilst control mice for the combination cytokine depletion received 750 μ g isotype control.

4.3 Processing of murine tissues

4.3.1 Schedule 1

Mice were humanely culled by ip overdose of pentobarbital (pentoject). Death was confirmed by cutting the femoral artery.

4.3.2 Bronchoalveolar lavage (BAL)

To obtain airway cells, the trachea was exposed post-mortem, and a small incision was made. The lungs were then flushed 3 times using 1ml of BAL media. Airway cells were pelleted, and supernatants were stored at -80°C for use in ELISA. Airway cells were then resuspended in 100 μ l ACK lysis buffer for 2min to remove any red blood cells. The remaining cells were subsequently resuspended in cDMEM for re-stimulations, DMEM for UV irradiation or in FACS buffer for flow cytometry. Total live airway cells were enumerated by counting live cells using 0.1% trypan blue under a microscope.

4.3.3 Lung tissue

The chest cavity of mice was exposed post-mortem, and lungs were perfused by intra-cardiac injection of 10ml PBS. The right upper lobe from each mouse was snap frozen in liquid nitrogen and stored at -80°C for later use in RNA extractions. The remaining four lobes were placed in cDMEM containing 1mg/ml collagenase D and 30 μ g/ml DNase I, in gentleMACS C-tubes. The lungs were then processed using a gentleMACS dissociator, following manufacturer's protocol lung_1.01. Following incubation for 1h at 37°C, shaking, the lungs were processed further using the gentleMACS dissociator, following manufacturer's protocol lung_2.01. The digested material was then pelleted and resuspend in ACK lysis buffer to remove red blood cells. Following ACK lysis, the samples were filtered through a 100 μ m cell strainer to generate a single cell solution. Total live lung cells could be calculated by counting cells in 0.1% trypan blue and multiplying the total counts by 1.2 to compensate for the lobe reserved for RNA extraction.

4.3.4 Blood

A minimum of 70 μ l blood was taken post-mortem from the femoral artery and suspended in 1mM EDTA diluted in PBS to prevent clotting. To lyse red blood cells, blood samples were then suspended in 10ml of ACK lysis buffer for 5min. Blood samples were washed in cDMEM then resuspended in FACS buffer for flow cytometry staining.

4.4 Flow Cytometry

4.4.1 Restimulations

For the analysis of T cell cytokines, 250,000 processed lung cells or all obtained BAL cells were restimulated with 100ng/ml PMA and 1000ng/ml Ionomycin and incubated for 1h at 37°C. BD GolgiPlug protein transport inhibitor (containing brefeldin A) was then added to samples at a final dilution of 1:1000 and samples were incubated at 37°C for a further 3h. After restimulations, cells were washed in FACS buffer and stained for flow cytometry as detailed below.

4.4.2 Extracellular staining

250,000 processed lung cells from each mouse or all processed BAL cells obtained were stained for flow cytometry. First, all cells were treated with 2.5µg/ml Fc block in FACS buffer for 20min at 4°C to prevent non-specific binding. Cells were then washed in PBS and stained with one of the antibody panels detailed in Table 3.3 for 30min at 4°C. Antibodies were diluted in PBS. In each experiment, extra lung cells were pooled from infected mice and an unstained control, live/dead control, general stain control and a fluorescence minus one (FMO) control for each antibody were stained. Following antibody staining, cells were washed in FACS buffer then fixed in Fixation Buffer (2%) PFA for 20min at 4°C.

4.4.3 Intracellular staining

To stain for intracellular markers, fixed, extracellular stained lung or BAL cells were permeabilised using 1x Perm/Wash buffer diluted in dH₂O for 15 min at 4°C. Cells were then stained with intracellular antibodies diluted in Perm/Wash buffer, including 2.5µg/ml FC block, for 1h at 4°C. Cells were then washed with Perm/Wash buffer then resuspended in FACS buffer and immediately acquired as described below.

4.4.4 Acquisition

Samples were acquired on a BD LSRFortessa Cell Analyzer using the FACSDiva software. Before acquisition, laser compensation was carried out using the unstained control, live/dead control and singly stained CompBeads Anti-Rat and Anti-Hamster Ig κ /Negative Control Compensation Particles Set or CompBeads Anti-Mouse Ig, κ/Negative Control Compensation Particles Set depending on the staining antibody isotype. Following compensation, the machine was set to acquire 250,000, live, CD45⁺ cells per sample.

4.4.5 Analysis

Flow cytometry data were analysed using FlowJo software using the gating strategies outlined in Figure 4.1-3. First single, live, CD45⁺ cells were gated out. Subsequently, each cell

population could be sequentially gated out using the appropriate markers. Once gated, the percentage CD45⁺ of live cells along with the percentage each cell population of CD45⁺ was exported to excel. Using the percentages, the total counts of each population was calculated using the total cell counts for each sample, obtained as described above by counting with trypan blue.

4.4.6 Gating Strategy

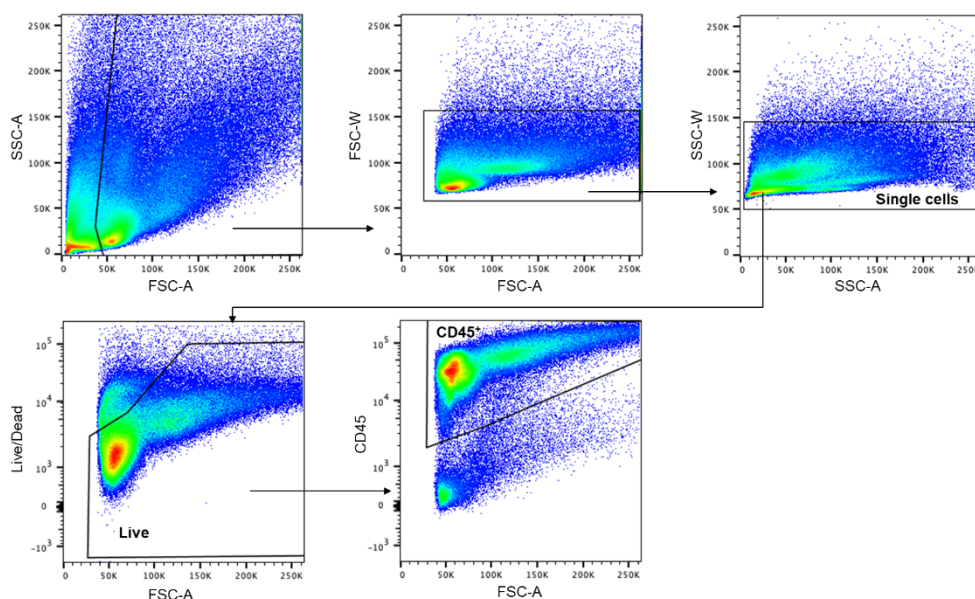


Figure 4.1 Gating of single, live, CD45⁺ cells

Example flow cytometry plots from the lungs of RSV infected mouse showing the gating strategy used to calculate live, CD45⁺ cells.

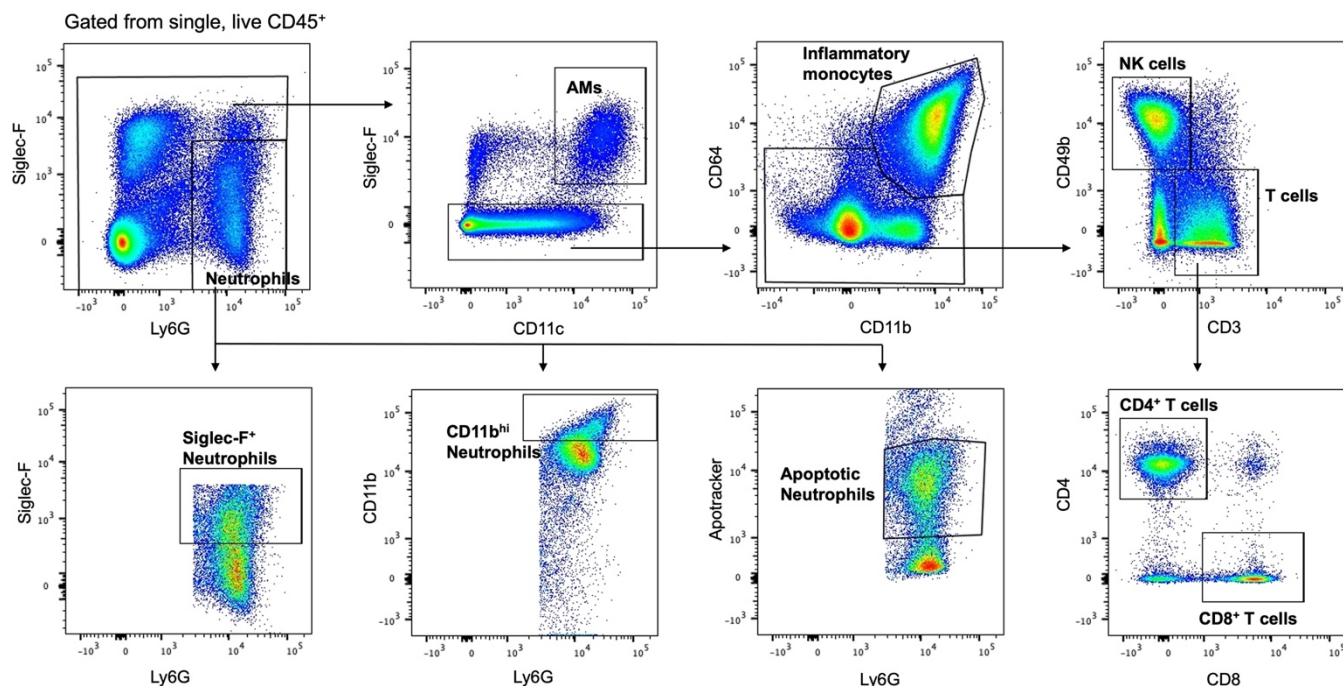


Figure 4.2 Innate/ Combined/ Neutrophil panel gating

Example flow cytometry plots from the lungs of RSV infected mouse showing the gating strategy used to calculate cell populations following staining with the innate, combined or neutrophil panel.

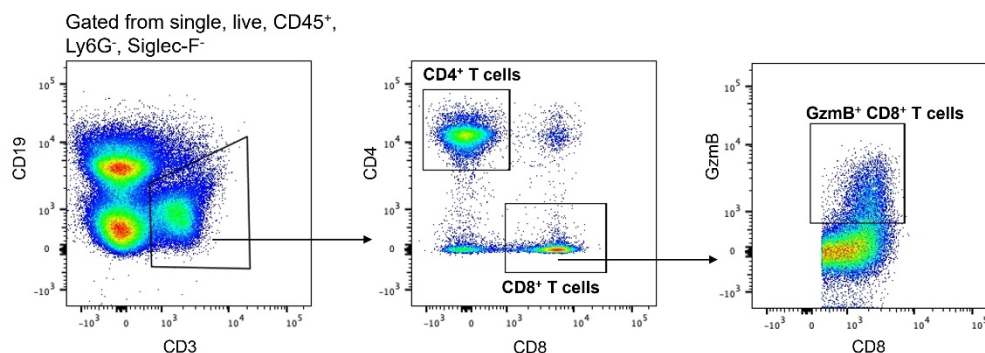


Figure 4.3 Adaptive panel gating

Example flow cytometry plots from the lungs of RSV infected mouse showing the gating strategy used to calculate cell populations following staining with the adaptive panel.

4.4.7 Uniform Manifold Approximation and Projection (UMAP)

To carry out dimensionality reduction of neutrophil flow cytometry data the UMAP plugin for FlowJo was used. FlowJo data was first concatenated, merging 10,000 events per sample. UMAP was then run using CD11b, Siglec-F and Apotracker Green fluorescence channels.

4.5 Apoptotic Neutrophils

4.5.1 Generation of Apoptotic Neutrophils

BAL cells were obtained, as described above, from mice treated with intranasal CXCL1 for 6 or 12h. Cells were then treated with UV radiation for 10min on low-adherence plates, then incubated at 37°C for 2h to induce apoptosis. Apoptotic cells were pooled and centrifuged at 480xg for 5 min. The supernatant was discarded, and the pellet was resuspended in DMEM for i.n.s or cRPMI for co-cultures.

4.5.2 Intranasal delivery of apoptotic neutrophils

12h before RSV infection, mice were anaesthetised using isoflurane gas and 5×10^6 apoptotic neutrophils resuspended in 100µl antibiotic free DMEM were delivered intranasally.

4.6 Mouse *ex vivo* Alveolar Macrophages (mexAMs)

4.6.1 Generation mexAMs

Naïve mice were culled using i.p. injection with phenobarbital and BAL was carried out as described above, then repeated with another 1ml of BAL media. Murine *ex vivo* (mex) AMs were generated based on an adapted protocol originally published by Gorki et al (310). The BAL was then centrifuged and the supernatant was discarded. The cell pellet was resuspended in mexAM media and counted using 0.1% trypan blue. Cells were diluted to a concentration of 2×10^2 cells/ml and plated onto a 6 well plate, 2ml per well. To remove any erythrocytes, cells were incubated at 37°C for 2-3h to allow the AMs to adhere. The media was then removed, cells were gently washed with PBS and fresh media was added to each well. The cultures were subsequently incubated at 37°C for 5-7 days until they had reached over 80% confluency. At this point cultures were split for expansion. Media was removed and each well was washed with PBS before being treated with 0.05% Trypsin- EDTA with phenol red for 5min at 37°C. cRPMI was then added to inactivate the trypsin and cell detachment was facilitated by pipetting up and down with a p1000 pipette. The media from all wells were collected and pooled then pelleted at 300g for 10 minutes. Cells were resuspended in mexAM media, counted and diluted to 2×10^5 cells/ml before being plated at 2ml per well in a 6-well plate. MexAM cultures were expanded until the desired cell count was obtained.

4.6.2 Neutrophil- mexAM *in vitro* co-cultures

MexAMs were seeded onto a 96-well plate at 100,000 cells per well in mexAM media and incubated at 37°C for 2h to allow them to adhere. Cells obtained from the BAL of mice treated with CXCL1 for 6h or 48h, or apoptotic neutrophils were resuspended in mexAM media, then added at a 1:1 ratio to mexAM cultures and incubated for 2h at 37°C. Supernatant was then

removed and cultures were then infected with RSV in mexAM media at an MOI of 4 and incubated at 37°C for 18h. Supernatants were harvested for ELISA and cells were lysed in TRIzol for RNA extraction.

4.7 Quantification of Protein and Gene expression

4.7.1 Enzyme-linked Immunosorbent Assay (ELISA)

Levels of cytokines and other effector proteins in BAL or cell culture supernatants were quantified using ELISA. The majority of mediators were measured using R&D DuoSet ELISA kits (Table 3.4) following the manufacturers protocol. A protocol alteration was made to the volume of supernatant added to each well from 100µl to 50µl. Quantification of IL-6 and IFN- α were carried out as previously described using the reagents detailed in Table 3.4. Briefly, 96-well plates were coated with capture antibody diluted in coating buffer overnight at 4°C. The next morning, plates were washed using PBS+ 0.05% Tween20 and blocked using 1% BSA for 2h at room temperature. Plates were washed and 50µl sample or standard were added to the wells for 2h. Detection antibody diluted in 1% BSA was added to each well for 1h at room temperature. For IFN- α a secondary detection antibody was subsequently added to each well. After the detection step(s) streptavidin- HRP diluted in 1% BSA was added to each well and incubated in the dark at room temperature for 30min. After washing, TMB solution was added and incubated until the colour had developed when the reaction was then stopped using 1M H₂SO₄. Albumin was quantified using the reagents detailed in Table 3.4. Capture antibody was diluted in 0.05M Carbonate-Bicarbonate to coat a 96-well plate and incubated at room temperature for 1h. Plates were washed and blocked for 30min at room temperature. Samples and standards were added to the plate and incubated for 1h. Detection antibody diluted in 1% BSA was added to each well after washing and incubated for a further hour. TMB solution was added to each well and the experiment was stopped using 0.2M H₂SO₄ when the colour had developed. All ELISA plates were read using FLUOstar Omega, with absorbance set to 450nm. Data were analysed using the Mars software.

4.7.2 RNA extraction

The right upper lobe was lysed in RLT buffer + 1% β - mercaptoethanol using mechanical homogenisation with a TissueLyser LT. The homogenate was subsequently centrifuged at 14,000g to remove any solid material. Supernatant was mixed 1:1 with 70% ethanol before being transferred to a Qiagen RNeasy Mini spin column. To extract RNA from BMDMs or mexAMs were lysed in TRIzol. Lysates from 3 replicate samples were pooled. Chloroform was then added and tubes were vigorously shaken for 15s. Samples were centrifuged at 12,000g for 10min at 4°C. The aqueous phase separated to the top above the gel was transferred to a

new Eppendorf and mixed 1:1 with 70% ethanol before being transferred to a Qiagen RNeasy Mini spin column. Following transfer of the samples to the RNeasy Mini spin columns, RNA was extracted using the Qiagen RNeasy Mini kit following the manufacturers protocol including an on-column DNase (Qiagen) digest. RNA was eluted in 40µl RNase-free water and quantified using a NanoDrop.

4.7.3 cDNA conversion

RNA was diluted to 2µg in RNase-free water and converted to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Once mixed with the kit buffer and enzyme, samples were run on a Thermocycler at 37°C for 60min, followed by 95°C for 5 min and then cooled to 4°C.

4.7.4 Real Time Quantitative PCR (qPCR)

To measure gene expression, cDNA samples were mixed with Quantitect Probe PCR Master Mix and the primers and probes listed in Table X. All qPCRs were run using an Applied Biosystems 7500 Fast Real-Time PCR System set to 45 cycles. To calculate gene expression, CT values were normalised to the housekeeper gene *Gapdh*, by subtracting the *Gapdh* CT values from the CT value of the gene of interest. The resulting values were then set to an inverse scale using the formula 2^{-CT} so that data could be more easily interpreted.

4.8 Pathogen Load

4.8.1 Viral load

To quantify RSV or X31 viral load, qPCR was used to detect L gene or M gene, respectively. Custom primers and probes detailed in Table.X were diluted in Quantitect Probe PCR Master Mix with cDNA and run on the Applied Biosystems 7500 Fast Real-Time PCR System as described above. For L gene, a standard curve of known copy number was used so that L gene copy number/µg RNA could be calculated. This value was normalised to *Gapdh*.

4.8.2 Bacterial Load

To quantify bacterial load in the lungs and airways of mice infected with *A. baumannii*, BAL was collected as previously described and whole lungs were suspended in antibiotic free DMEM then dissociated using sterile scissors and mashed through 100µm cell strainer. Lung and BAL suspension were serially diluted 1:10 in PBS. Six of the dilutions, neat-10⁻⁵ were dripped onto LB agar plates in parallel lines using a multichannel pipette, then incubated overnight at 37°C. Actual CFU could then be calculated (following infection) by counting the colonies.

4.9 Statistical Analysis

All data were plotted and statistically analysed using GraphPad PRISM 10 software. Normal distribution of data was checked using a Shapiro Wilk test and by Q-Q plots. Unpaired data determined to be normally distributed were analysed using One-way ANOVA with Tukey's post comparison test. Unpaired data which did not fit a normal distribution were analysed using Kruskal-Wallis test with Dunn's post comparison. For paired datasets, such as weight loss, data were analysed using a Two-way ANOVA with Šidák post comparison test. A p value ≤ 0.05 was considered as statistically significant.

5. Exposure to bacteria and/or their antigens exacerbates disease during the innate phase of RSV infection.

5.1 Introduction

Increased markers of neutrophilic activation detected in the airways before infection with RSV has previously been associated with the development of symptomatic disease in humans (170). In mouse models, neutrophils recruited by intranasal CXCL1 before RSV infection, were found to lead to exacerbated weight loss ultimately by driving increased CD8⁺ T cell recruitment later in the course of disease (170). Whilst this mouse model can provide insight into the mechanism through which neutrophils exacerbate subsequent RSV infection, it does not explain why some individuals would have neutrophils in their airways before infection. CXCL1 release and neutrophil recruitment to the lungs does not occur spontaneously and requires an inflammatory stimulus (252). In the environment and air we breathe, there are many stimuli which can drive airway neutrophilia, including aero-allergens (such as pollen or house dust mite) air pollutants, inhalation of cigarette smoke and use of e-cigarettes (311–314). However, one of the most common and well-studied environmental causes of airway inflammation is exposure to other pathogens such as bacteria, or their pathogen associated molecular patterns (PAMPs).

Bacterial co-infections are commonly associated with severe RSV infection (315–318). A previous study of infants hospitalised with severe RSV infection, found 20% of patients tested positive for bacterial co-infection, whilst a further 20% had detectable low grade bacterial growth in their lungs (317). Respiratory viral infections complicated by bacterial co-infection are therefore fairly well-studied. In many cases, patients with severe RSV infection will acquire nosocomial bacterial infection following hospitalization (316). This has led to the majority of mouse models of respiratory viral and bacterial co-infections to consider bacterial infection as secondary to the virus (319). However, bacteria and their PAMPs are ubiquitous in our environment and therefore have the potential to precede respiratory viral infection (320). Increased levels of bacterial lipopolysaccharide (LPS) in the home environment have previously been associated with increased incidence of hospitalization following RSV infection in children (321). Similarly, changes in the composition of the lung microbiota in infants which preceded RSV infection, have been associated with increased risk of severe disease (83). In particular, an increase in gram-negative gammaproteobacteria was detected in the lungs of infants who subsequently experienced severe RSV infection (83). This is an interesting observation, as the gammaproteobacteria *Acinetobacter baumannii* has previously been found to be one of the most prominent organisms present in household dust particles which

are known to aggravate lung inflammation (322). *A. baumannii* is an opportunistic pathogen, capable of colonising the respiratory tract and causing disease in immunocompromised individuals (323,324). In addition, *A. baumannii* is enriched in the microbiota of patients suffering from chronic inflammatory lung disease such as COPD and asthma (322). Taken together, this makes *A. baumannii* an interesting environmental bacterium to study in the context of lung inflammation and its subsequent effect on RSV infection.

Exposure to bacterial PAMPs can result in lung inflammation characterized by rapid neutrophil recruitment (325). This is mediated by toll-like receptors (TLRs) present on the surface of resident airway cells which detect PAMPs and initiate an inflammatory signalling cascade. For example, gram-negative LPS or gram-positive lipoteichoic acid (LTA) are recognized by TLR4 or TLR2, respectively (326,327). Both these receptors signal via the MyD88/TRIF pathway which activates NF- κ B transcription and the release of pro-inflammatory cytokines and chemokines including CXCL1 (326,327). Intranasal administration of both LPS or LTA in mice has previously been shown to drive neutrophil recruitment (328,329). Therefore, in this chapter the hypothesis that exposure to bacteria or bacterial PAMPs before RSV infection results in neutrophil recruitment which can exacerbate RSV disease outcomes was addressed.

5.2 Results

5.2.1 *A single intranasal dose of LPS drove neutrophil recruitment into the lungs and airways of mice*

Intranasal delivery of LPS in mice has previously been reported to drive neutrophil recruitment into the lungs and airways of mice (328). Additionally, as a general immune agonist, LPS can drive the recruitment of other innate inflammatory cells and the release of pro-inflammatory cytokines (326,328). Therefore, the dynamics of neutrophil recruitment and inflammation following intranasal LPS was compared to intranasal recombinant CXCL1 (referred to from here as CXCL1).

Mice were intranasally administered either 1µg LPS, 5µg CXCL1 or PBS and culled at 6h, 12h, 18h or 24h post exposure (Fig. 5.1a). These doses were selected based on titration experiments carried during my MRes project in the Johansson lab demonstrating neutrophil recruitment without inducing significant weight loss. Lungs and bronchoalveolar lavage (BAL) were collected to study the immune cell composition in the lungs and airways using flow cytometry. PBS control data is represented as a baseline average (grey dashed line) representing 10 mice culled at 6-24h after intranasal. In the lungs, neutrophil recruitment following CXCL1 (blue) peaked rapidly at 6h post intranasal, after which numbers steadily declined over time (Fig. 5.1b). In comparison, neutrophil recruitment peaked 12h after intranasal LPS (red), at comparable numbers to CXCL1 (Fig. 5.1b). CXCL1 induced significantly more neutrophil recruitment into the airways compared to intranasal LPS (Fig. 5.1b). In mice which received LPS, neutrophil recruitment followed the peak in expression of the neutrophil chemokine *Cxcl2* at 6h post intranasal. CXCL1 did not induce *Cxcl2* expression (Fig. 5.1c). LPS also resulted in recruitment of inflammatory monocytes into the lungs and airways, which was not observed in mice exposed to CXCL1 (Fig. 5.1d). This was matched by the expression of the monocyte chemokine *Ccl2* which peaked 6h after intranasal LPS but was not induced by CXCL1 (Fig. 5.1e). Neither intranasal LPS nor CXCL1, altered the population of AMs in the lungs or airways compared with PBS controls (Fig. 5.1f). Following CXCL1, at 12h post exposure, neutrophils were the major immune cell in the lungs and airways, accounting for 40% and 90% of CD45⁺ cells (Fig. 5.1g-h). Despite driving the recruitment of inflammatory monocytes, neutrophils also accounted for the greatest proportion of recruited cells in the lungs and airways, 12h after intranasal LPS at 34% and 68% of CD45⁺ cells (Fig. 5.1g-h).

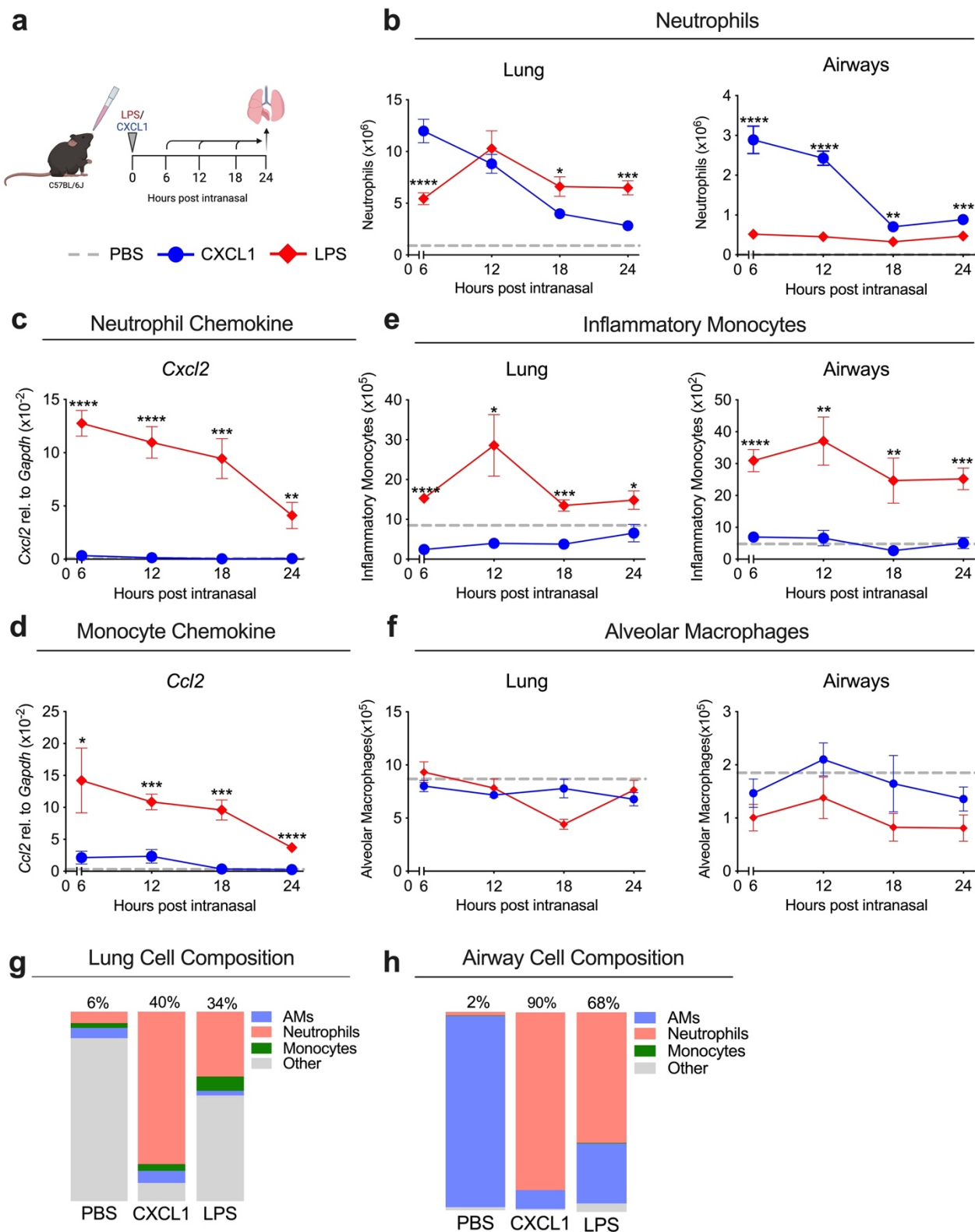


Figure 5.1 Intranasal LPS drove recruitment of neutrophils and inflammatory monocytes to the lungs and airways

a) Graphic describing the experimental setup. Mice were intranasally exposed to either 5 μ g CXCL1, 1 μ g LPS or PBS as a control. 6, 12, 18 or 24h later, airway (BAL) and lung cells were collected for analysis by flow cytometry, ELISA and qPCR. **b)** Total number of neutrophils in the lung and airways (BAL) following CXCL1 (blue circles) or LPS (red diamonds) compared to PBS baseline (grey dashed line). **c)** Expression of *Cxcl2* in the lung, measured

by qPCR and normalised to *Gapdh*. **d)** Total numbers of inflammatory monocytes in the lungs and airways following CXCL1 or LPS. **e)** Expression of *Ccl2* in the lung, measured by qPCR and normalised to *Gapdh*. **f)** Total numbers of alveolar macrophages in the lungs and airways following CXCL1 or LPS. Percentage contribution of innate immune cells to total CD45+ cells in the **g)** lung and **h)** airways, following PBS, CXCL1 or LPS at 12h after administration. The red part and the percentage at the top of each chart represents neutrophils, green represents inflammatory monocytes and blue represents AMs. Data are pooled from two separate experiments. CXCL1 n=6; LPS n=6; PBS baseline n=10. CXCL1 and LPS data are plotted as mean $\pm$ SEM. For line graphs, PBS values were averaged and plotted as a baseline (grey dashed line). Statistical analysis was carried out using Graphpad Prism software. At each timepoint an unpaired Student's t test was carried out to compare the LPS data with the CXCL1 data. Asterisks represent the p value; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.2.2 Recruitment of neutrophils was associated with increased neutrophil proteases and albumin leakage into the airways

During their recruitment and activation, neutrophils release granular proteases which cleave extracellular matrix and tight junctions. This aids in their migration through the tissue but can cause damage to epithelial barriers (269,270). Therefore, levels of neutrophil granular proteases in the airways were measured overtime following intranasal CXCL1 and LPS. Azurophilic granular proteases MPO and NE steadily increased in the airways overtime following intranasal CXCL1 and LPS (Fig. 5.2a-b). Levels of airway MPO peaked 18h after intranasal CXCL1 or LPS and levels were not significantly different between the two groups (Fig. 5.2a). NE peaked 12h after intranasal rCXC1 at higher levels compared to LPS, which peaked 18h after intranasal (Fig. 5.2b). In contrast, the tertiary granular protease MMP-9 peaked early at 6h post LPS and CXCL1 exposure at comparable levels, then declined across the 24h (Fig. 5.2c). As these proteases are known to disrupt epithelial barriers, leakage of serum albumin into the airways was detected by ELISA as a measure of barrier integrity. Albumin in the airways peaked early, 6h after intranasal CXCL1 or LPS (Fig. 5.2d). Airway albumin levels trended lower in mice which received intranasal LPS compared to CXCL1, but this was not significant. For both CXCL1 and LPS, albumin levels declined across the time course (Fig. 5.2d). The dynamics of albumin in the airways matched the dynamics of neutrophil recruitment into the lungs (Fig. 5.1b). This suggests recruitment of neutrophils by CXCL1 and LPS temporarily disrupts the integrity of the lung epithelial barrier.

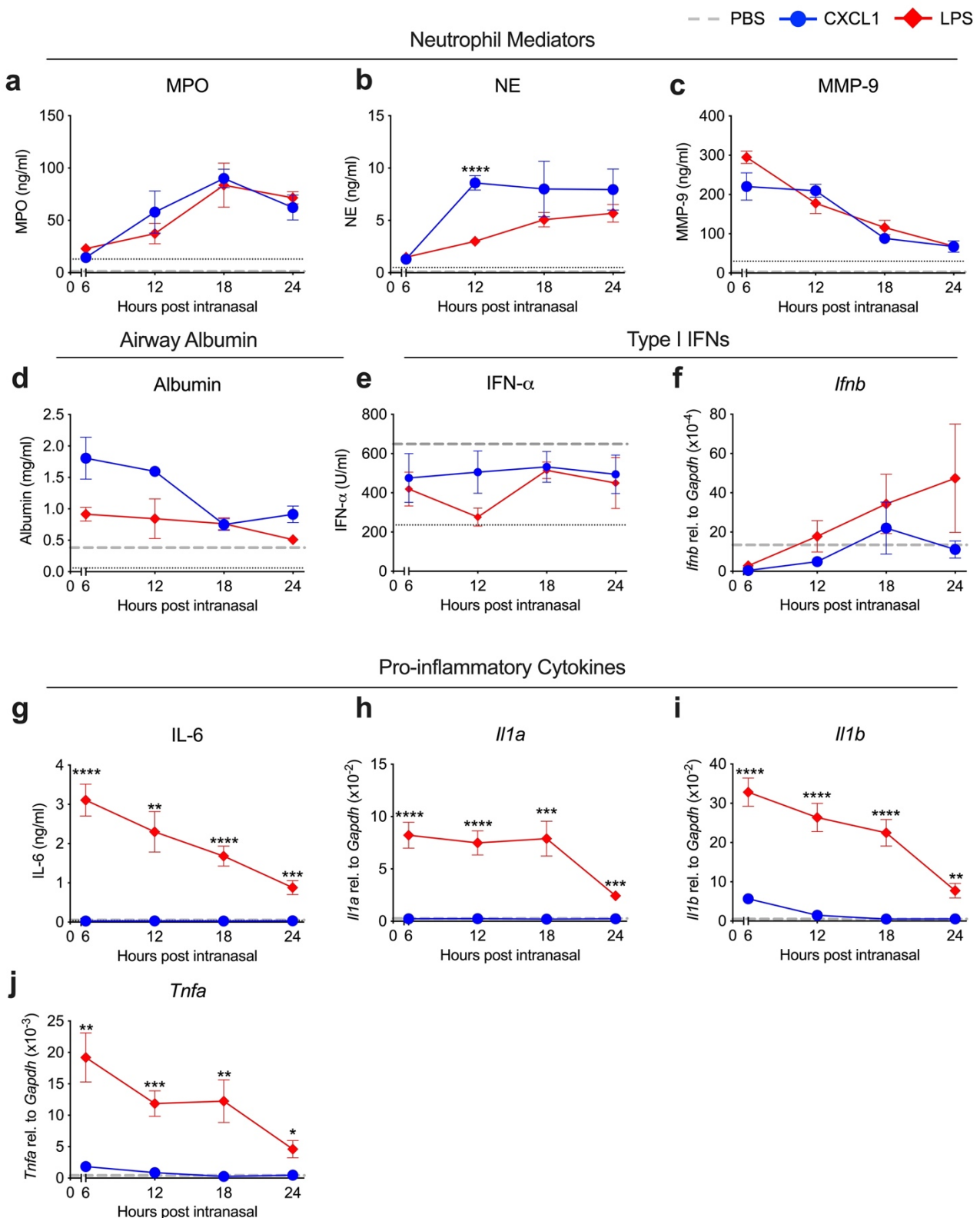


Figure 5.2 Intranasal LPS increased levels of neutrophil mediators and expression of proinflammatory cytokines

Levels of neutrophil granular proteins **a**) myeloperoxidase (MPO), **b**) neutrophil elastase (NE) and **c**) matrix metalloprotease (MMP-9), in BAL supernatant measured by ELISA. **d**) Levels of airway albumin in the BAL detected by ELISA. **e**) Levels of IFN- α in the BAL detected by ELISA. **f**) Whole lung gene expression of *Ifnb* relative to *Gapdh* measured by qPCR. **g**) Levels of IL-6 in the BAL detected by ELISA. Whole lung gene expression of **h**) *Il1a*, **i**) *Il1b* and **j**) *Tnfa* relative to *Gapdh* measured by qPCR. Data are pooled from two separate experiments. Grey dashed

lines represent PBS baseline. Black dotted lines represent lower limit of detection for ELISA data. CXCL1 n=6; LPS n=6; PBS baseline n=10. CXCL1 and LPS data are plotted as mean $\pm$ SEM. For line graphs, PBS values were averaged and plotted as a baseline (grey dashed line). Statistical analysis was carried out using Graphpad Prism software. At each timepoint an unpaired Student's t test was carried out to compare the LPS data with the CXCL1 data. Asterisks represent the p value; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.2.3 Intranasal LPS but not CXCL1 drove the expression of pro-inflammatory cytokines in the lungs

LPS signals via TLR4 to initiate inflammation (326). In addition, damage to the epithelia which can occur during neutrophil recruitment due to release of proteases, can generate damage associated molecular patterns (DAMPs) which can also signal via TLRs to induce an inflammation (330). Therefore, the expression of pro-inflammatory cytokines in the lung following intranasal LPS or CXCL1 were profiled, by ELISA and qPCR, and compared. Compared to PBS baseline, neither LPS nor CXCL1 increased levels of IFN- α in the airways (Fig. 5.2e). A small increase in expression of *Ifnb* was measured in the lungs of mice 24h after LPS exposure, but not CXCL1 (Fig. 5.2f). CXCL1 did not induce changes in the levels or expression of any pro-inflammatory cytokine including IL-6, *Il1a*, *Il1b* and *Tnfa* (Fig. 5.2g-j). In contrast, IL-6, *Il1a*, *Il1b* and *Tnfa* were significantly increased in the lungs of mice exposed to LPS, compared with those which were exposed to CXCL1 (Fig. 5.2g-j). Expression for all these cytokines peaked early at 6h after LPS and declined across the 24hs (Fig. 5.2g-j). Taken together, this data demonstrates neutrophil recruitment by CXCL1 is unable to induce further inflammation. In contrast, intranasal LPS induces a broad pro-inflammatory response in the lungs.

5.2.4 Intranasal LPS before RSV infection drove early weight loss in mice

To investigate how lung inflammation driven by LPS exposure could affect disease outcomes to RSV infection, mice were exposed LPS 12h before RSV infection (Fig. 5.3a). This time point was selected as it was the peak of neutrophil recruitment into the lungs (Fig. 5.1b). To determine the optimum dose of LPS and avoid lethality with subsequent RSV infection, mice were intranasally administered a single dose of either 5 μ g, 2 μ g or 1 μ g LPS, 12h prior to RSV infection (7×10^6 FFU). To measure disease severity, loss of original body weight was monitored up to day 8 post RSV infection (Fig. 5.3a). As previously published (15,17,170), RSV infection only (grey squares) resulted in minor weight loss of 5% original weight, at day 1 post infection which was not statistically significant compared to PBS controls and was followed by rapid recovery by day 2 post infection. Subsequently, these mice lost significantly more weight on day 5-7 post RSV infection compared to PBS controls (Fig. 5.3b). Intranasal exposure to 5 μ g

LPS, without subsequent infection, also drove a minor dip in weight of 5% original weight, peaking on day 2 post infection, which was also not statistically significant compared to PBS controls. 1µg LPS, without subsequent infection resulted in no weight loss compared to PBS controls throughout the experiment (Fig. 5.3c). Exposure to LPS at all concentrations, before RSV infection, resulted in early and rapid weight loss (Fig. 5.3d-f). Exposure to 1µg LPS before RSV infection result in significantly more weight loss compared to RSV only mice between days 1-3 post infection, peaking at 15% original weight on day 2 post infection. These mice then recovered by day 4 post infection and lost significantly less weight than RSV only mice on days 5-6 post infection (Fig. 5.3d). When intranasal LPS dose was increased to 2µg before RSV infection, a similar pattern in weight loss was observed, but mice lost a higher percentage (20%) of their original body weight on day 2 post infection (Fig. 5.3e). When exposed to 5µg LPS before RSV infection, mice rapidly lost weight and did not recover. Weight loss was significantly more than RSV only mice at all timepoints up to day 5 post infection, and by day 6 post infection all mice had reached human endpoints and were culled (Fig. 5.3f). Overall, these data demonstrate that LPS exposure before RSV infection exacerbates disease, driving rapid and early weight loss.

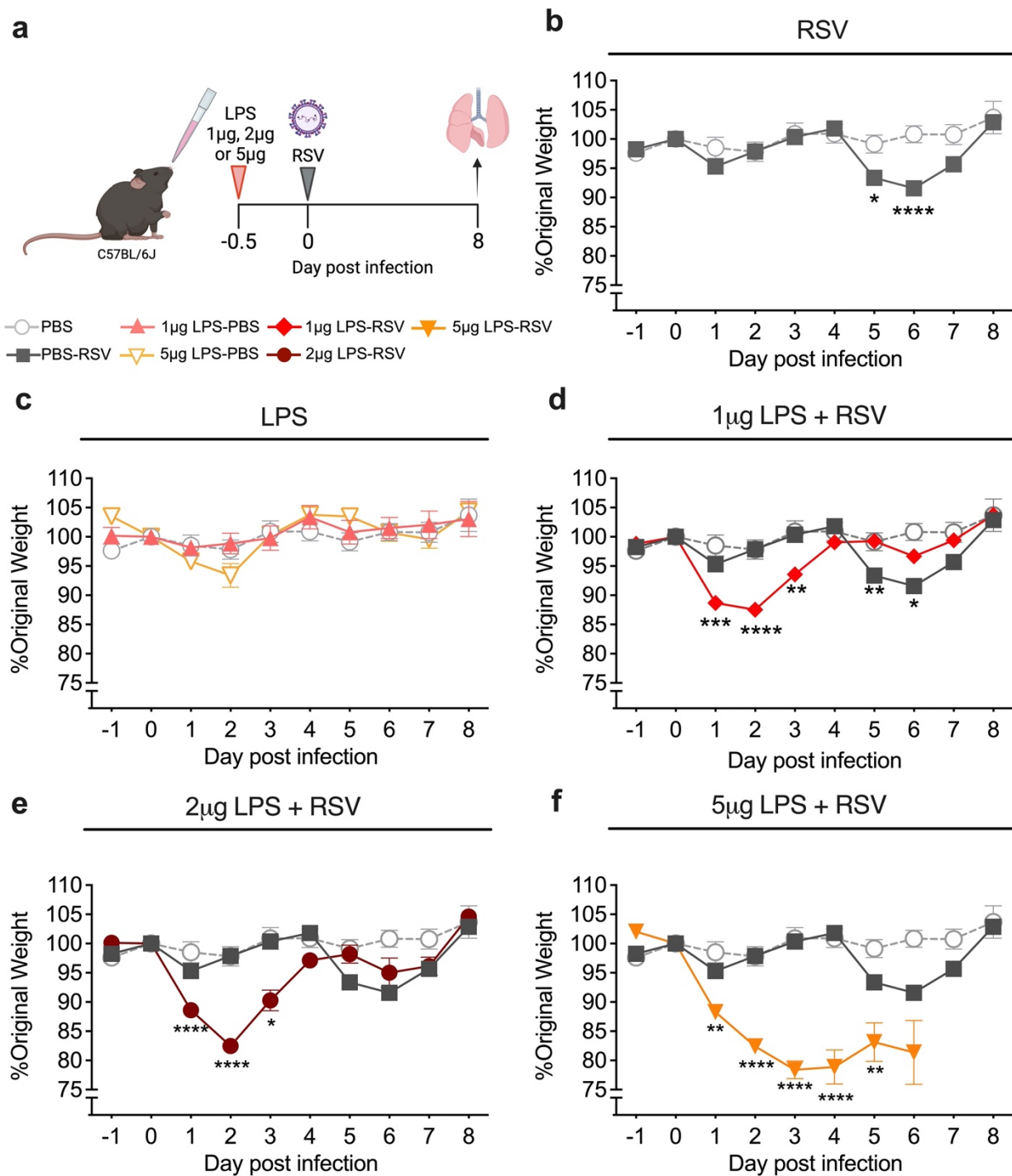


Figure 5.3 Intranasal exposure to LPS before RSV infection drove early weight loss

a) Graphic showing experimental design. Mice were intranasally exposed to either 1µg, 2µg or 5µg LPS, or PBS. 12h later mice were infected with RSV (7×10^6 FFU) or received intranasal PBS for uninfected controls. Mice were weighed daily up to day 8 post infection. **b)** Daily weight of RSV infected mice compared to PBS controls represented as percentage of original body mass on day 0. Statistics compare RSV mice to PBS controls. **c)** Daily weight of LPS exposed, uninfected mice compared to PBS controls, represented as percentage of original body mass on day 0. Daily weight of mice exposed to **d)** 1µg, **e)** 2µg or **f)** 5µg LPS and subsequently infected with RSV compared to RSV only mice, represented as percentage of original body mass on day 0. Statistics compare LPS-RSV mice with RSV controls. 5µg and 2µg LPS-RSV, 5µg LPS and 1µg LPS-PBS data were collected in one

experiment, n=4. PBS only, RSV only, 1 μ g LPS-RSV data are pooled from 2 separate experiments. PBS only n=5; RSV only and 1 μ g LPS-RSV n=9. Data are plotted as mean $\pm$ SEM. Statistical analysis was carried out using Graphpad Prism software. A two-way ANOVA with multiple comparisons test was carried out. Asterisks represent the *p* value comparing groups as stated; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.2.5 Early weight loss in mice exposed to LPS before RSV infection was associated with prolonged innate inflammation in the lungs

Having found intranasal LPS exposure before RSV infection to drive early and significant weight loss, the effect of LPS on viral replication and the immune response in the lungs was profiled. The 1 μ g dose of LPS was studied in all future experiments, as this dose resulted in a significant biological effect without substantial severity or lethality. Mice exposed to LPS before RSV infection were culled on day 1, 2, 4 and 8 post infection. Day 0 (12h after LPS) data is the same data presented for 12h post LPS presented in Fig. 5.1 & 5.2. Viral load in the lungs was quantified by measuring L gene copy number/ μ g RNA by qPCR and the recruitment of innate and adaptive immune cells were measured by flow cytometry (Fig. 5.4a). Viral load in the lungs of infected mice remained elevated between day 1-4 post infection, then declined between day 4-8 post infection. In mice exposed to LPS before infection, viral load trended lower at days 1-4 post infection, and was statistically significant on day 4 post infection. Viral clearance between days 4-8 post infection was not altered by LPS exposure before infection (Fig. 5.4b). This suggests that the early weight loss in mice exposed to LPS before RSV infection is not driven by viral replication and is likely a result to the immune response towards the virus. Neutrophils were recruited into the lungs and airways of mice following RSV infection, peaking on day 1 post infection. As shown in Fig. 5.1b, neutrophil numbers peaked 12h after intranasal LPS after which numbers declined (Fig. 5.4c&d). In mice exposed to LPS before RSV infection, neutrophil numbers did not increase further after day 0 post infection. However, in the lung's neutrophils persisted at higher numbers compared to LPS only mice at days 1 and 2 post infection. At these timepoints, neutrophil numbers in the lung were also significantly higher compared to RSV only mice (Fig. 5.4c). In the airways of LPS-RSV mice, neutrophil numbers were not altered compared to LPS only mice but were significantly higher compared to RSV only mice on day 1 and 2 post infection (Fig. 5.4d). RSV infection also drove the recruitment of inflammatory monocytes, peaking at day 1 post infection in the lungs and day 2 post infection in the airways. In LPS exposed mice, inflammatory monocytes in the lungs rapidly declined between day 0 (12h post LPS) and day 1 post infection. However, in LPS-RSV infection, inflammatory monocytes continued to increase during the first 2 days of infection. In these mice, inflammatory monocytes in the lung were significantly higher than LPS only mice at days 1-4 post infection, and significantly higher than RSV only mice at days

2-4 post infection (Fig. 5.4e). In the airways, inflammatory monocyte numbers did not significantly differ compared to RSV only mice (Fig. 5.4f). The population of resident AMs in the lung of RSV infected mice began to increase between day 4 and 8 post infection compared to PBS controls. In LPS exposed mice, the AM population increased between days 1-4 post infection, after which number declined to baseline by day 8 post infection. In LPS-RSV mice, the AM population did not increase as rapidly as in LPS only mice, but significantly more AMs were detected in the lungs at day 4 post infection compared to RSV only mice. By day 8, AM numbers trended lower in the lungs of LPS-RSV mice compared to RSV only mice, but this was not statistically significant (Fig. 5.4g). These data suggest the AM population expands more rapidly in the lungs of mice exposed to LPS before RSV infection compared to RSV only mice. In the airways, the AM population expanded in all three groups compared to PBS controls between day 1-2 post infection. Numbers remained elevated on day 4 post infection, after which the population declined to baseline by day 8 post infection. AM numbers in the airways did not significantly differ between RSV only, LPS only and LPS-RSV groups (Fig. 5.4h). Overall, the early weight loss in mice exposed to LPS before RSV infection was associated with prolonged elevation of innate immune cells in the lungs.

In addition to the innate immune response, the T cell response in the lungs at days 4 and 8 post infection was profiled. By day 4 post infection, CD4⁺ and CD8⁺ T cells were increased in the lungs of RSV infected mice compared to PBS controls (Fig. 5.4i-j). At day 8 post infection, CD8⁺ T cells but not CD4⁺ T cells continued to increase. LPS exposure before RSV infection did not significantly alter the population of CD4⁺ or CD8⁺ T cells at either time point compared to RSV only mice. However, numbers of both T cell subtypes trended lower at day 8 post infection. Therefore, the protection in weight loss observed at days 5-7 post infection in mice exposed to LPS before RSV infection could not be linked to any significant changes in the T cell response.

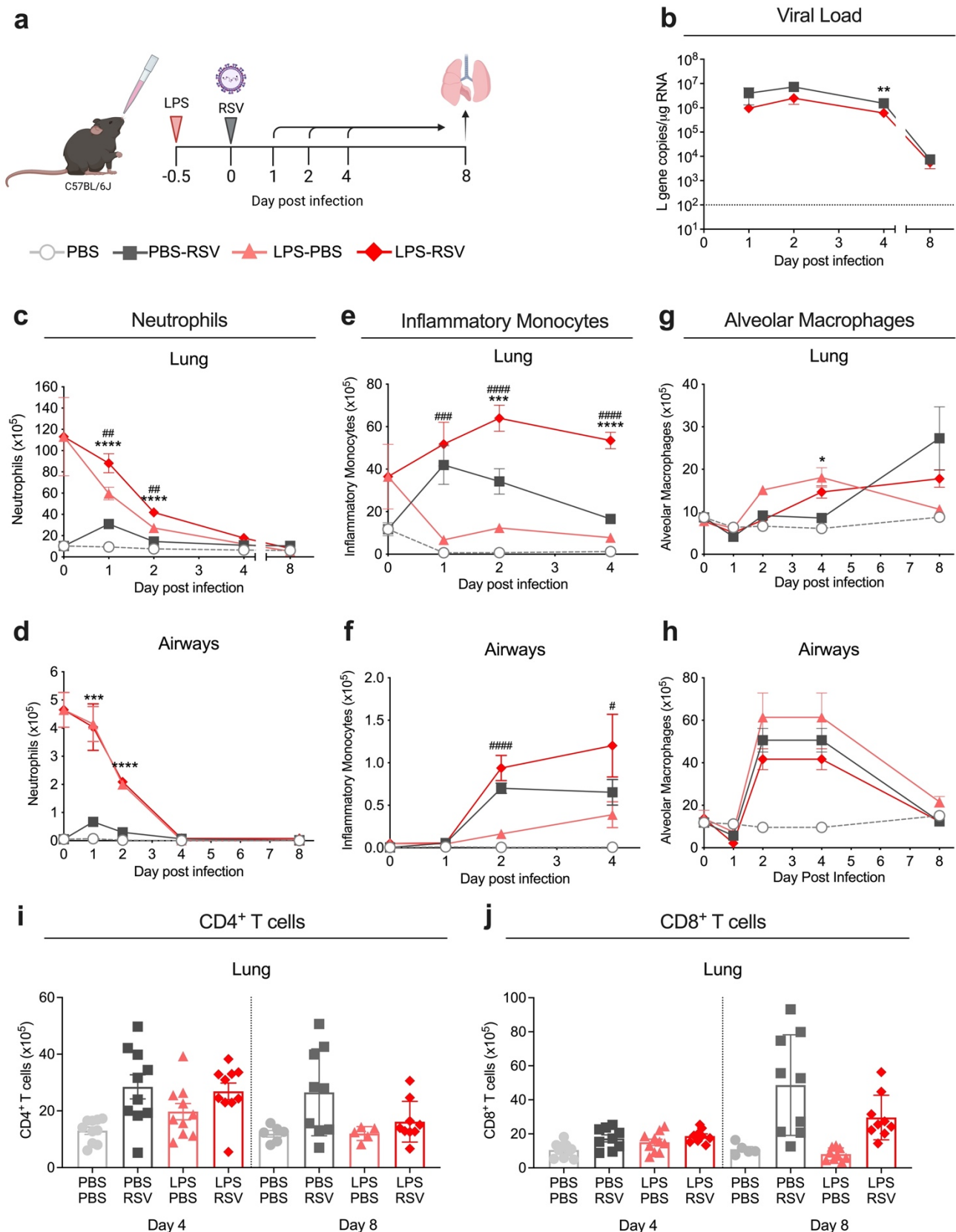


Figure 5.4 . Exposure to LPS before RSV infection prolonged elevated neutrophil and inflammatory monocytes in the lungs

a) Graphic showing experimental design. Mice were intranasally exposed to $1\mu\text{g}$ LPS or PBS 12h before RSV infection (7×10^6 FFU). Mice were culled and lungs and BAL harvested at day 1, 2, 4 and 8 post infection and

analysed using flow cytometry, ELISA and qPCR. **b)** Viral load represented as RSV L gene copy number/ μ g RNA measured by qPCR, normalised to *Gapdh*. Total number of neutrophils recruited into the **c)** lungs and **d)** airways. Total numbers of inflammatory monocytes recruited into the **e)** lungs and **f)** airways. Total numbers of alveolar macrophages in the **f)** lungs and **g)** airways. Total numbers of **i)** CD4⁺ T cells and **j)** CD8⁺ T cells in the lungs at day 4 and 8 post infection. At days 0-4 data are pooled from 2 experiments per time point. Day 0 data are the same as presented in Fig. 5.1 for 12h after exposure to LPS or PBS baseline. At day 1, 2 and 4 after infection, n=10. At day 8, PBS only and LPS only data are from 1 experiment n=5; RSV only and LPS-RSV, data are pooled from 2 experiments n=9. Time courses are plotted as mean $\pm$ SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. A one-way ANOVA with multiple comparisons test was carried out for each timepoint. Asterisks represent the *p* value for LPS-RSV data compared to RSV infected controls; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001. Hashtags represent the *p* value for LPS-RSV compared to LPS only controls; # $\leq$ 0.05, ## $\leq$ 0.01, ### $\leq$ 0.001, #### $\leq$ 0.0001.

5.3.6 Prolonged neutrophilia in the lungs of mice exposed to LPS before RSV infection was not associated with increased albumin leakage into the airways

It is possible that the prolonged neutrophilia in the lungs of mice exposed to LPS before RSV infection could lead to increased disruption of the lung epithelium. Therefore, the levels of neutrophil associated granular proteases and leakage of albumin into the airways was measured by ELISA. Both MPO and NE levels continued to increase 12h after LPS exposure peaking on day 1 (36h post LPS). RSV infection also drove an increase in these proteases, peaking on day 1 post infection and correlating with the peak of neutrophil recruitment into the lungs of these mice (Fig. 5.4c). In mice exposed to LPS before RSV infection, MPO and NE levels were significantly higher compared to both LPS only and RSV only mice at days 1 and 2 post infection (Fig. 5.5a-b). MMP-9 also increased in the lungs of RSV infected mice, peaking on day 1 post infection. In contrast, MMP-9 did not increase further in the lungs of LPS exposed mice after day 0 (12h post LPS). However, in mice exposed to LPS before RSV infection, MMP-9 levels were significantly higher at day 1 and 2 post infection compared to RSV only mice and day 1 post infection compared to LPS only mice (Fig. 5.5c). Taken together, the persistently increased neutrophil numbers in the lungs of mice exposed to LPS before RSV infection correlated with increased levels of neutrophil proteases in the airways. Despite the increase in these proteases on day 1 post infection, levels of airway albumin were not significantly increased in mice exposed to LPS before RSV infection compared with RSV only mice. This suggests that LPS exposure before RSV infection does not result in further loss of epithelial barrier integrity.

Neutrophil Granule Proteins

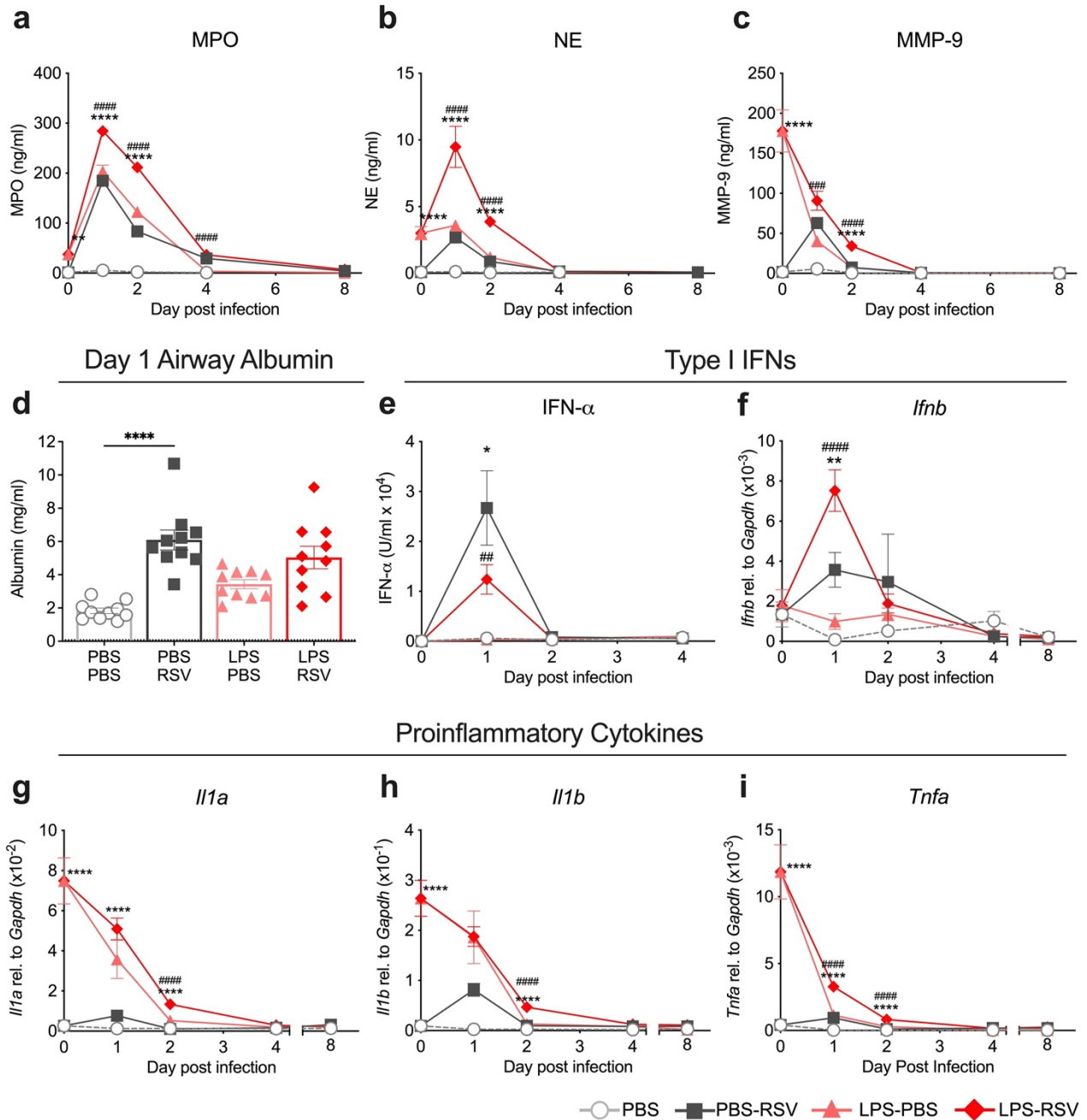


Figure 5.5 LPS exposure before RSV infection elevated neutrophil mediators in the airways and prolonged expression of proinflammatory cytokines

Levels of neutrophil granular proteins **a)** myeloperoxidase (MPO), **b)** neutrophil elastase (NE) and **c)** matrix metalloproteinase (MMP-9), in BAL supernatant measured by ELISA. **d)** Levels of airway albumin in the BAL on day 1 post infection detected by ELISA. **e)** Levels of IFN- α in the BAL detected by ELISA. **f)** Whole lung gene expression of *Ifnb* relative to *Gapdh* measured by qPCR. **g)** Levels of IL-6 in the BAL detected by ELISA. Whole lung gene expression of **h)** *Il1a*, **i)** *Il1b* and **j)** *Tnfa* relative to *Gapdh* measured by qPCR. At days 0-4 data are pooled from 2 experiments per time point. Day 0 data are the same as presented in Fig. 5.1 for 12h after exposure to LPS or PBS baseline. At day 1, 2 and 4 after infection, $n=10$. At day 8, PBS only and LPS only data are from 1 experiment $n=5$; RSV only and LPS-RSV, data are pooled from 2 experiments $n=9$. Time courses are plotted as mean $\pm$ SEM. For

bar graphs, error bars represent SEM. Dashed lines represent lower limit of detection. Statistical analysis was carried out using Graphpad Prism software. A one-way ANOVA with multiple comparisons test was carried out for each timepoint. Asterisks represent the p value for LPS-RSV data compared to RSV infected controls; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 . Hashtags represent the p value for LPS-RSV compared to LPS only controls; # ≤ 0.05 , ## ≤ 0.01 , ### ≤ 0.001 , #### ≤ 0.0001 .

5.2.7 Mice exposed to LPS before RSV infection had prolonged expression of proinflammatory cytokines

Type I IFNs play an important role in controlling respiratory viral replication and providing protection against disease (15). Therefore, the IFN- α and *Ifnb* response was profiled in mice exposed to LPS before RSV infection by ELISA and qPCR. RSV infection on its own, increased levels of IFN- α in the airways and expression of *Ifnb* in the lungs, peaking on day 1 post infection (Fig. 5.5e-f). In mice exposed to LPS before RSV infection, levels of IFN- α in the airways were significantly lower compared to RSV only mice (Fig. 5.5e). In contrast, expression of *Ifnb* was significantly increased in the lungs of mice exposed to LPS before RSV infection compared to RSV only mice (Fig. 5.5f). These data suggest LPS exposure results in an *Ifnb* bias type I IFN response towards RSV.

Unlike type I IFNs, intranasal LPS was previously shown to drive the expression of pro-inflammatory cytokines *Il1a*, *Il1b* and *Tnfa* (Fig. 5.2h-j). Therefore, the expression of these cytokines following subsequent RSV infection was profiled by qPCR. RSV infection only drove increased expression of all three cytokines compared to PBS mice, peaking at day 1 post infection (Fig. 5.5g-i). In mice exposed to LPS and not subsequently infected with RSV, expression of these cytokines declined between day 0 (12h post LPS) and day 2 post infection (60h post LPS). In mice exposed to LPS before RSV infection, expression of *Il1a*, *Il1b* and *Tnfa* were not further increased. However, expression of all three cytokines in the lungs of LPS-RSV mice at day 0-2 post infection were higher compared to RSV only mice and remained elevated for longer compared to LPS only mice (Fig. 5.5g-i). Expression of *Il1a* was significantly higher compared to RSV only mice at days 0-2 post infection, and significantly higher compared to LPS only mice at day 2 post infection (Fig. 5.5g). *Il1b* expression was significantly higher compared to RSV only mice at day 0 and 2 post infection and significantly higher compared to LPS only mice at day 2 post infection (Fig. 5.5h). *Tnfa* expression was also significantly higher compared to RSV only mice at days 0-2 post infection and was significantly higher compared to LPS only mice at days 1 and 2 post infection (Fig. 5.5i). Taken together, these data suggest LPS exposure before RSV infection results in prolonged expression of pro-inflammatory cytokines.

5.2.8 Intranasal exposure to gram-negative bacteria *Acinetobacter baumannii* drove inflammation comparable to LPS exposure

Exposure to the gram-negative PAMP LPS, drove early weight loss in mice subsequently infected with RSV. Next, it was investigated if these findings could be replicated by bacterial infection/exposure before RSV infection. The gammeproteobacteria *A. baumannii* was chosen due to its association with indoor dust particles and chronic inflammatory lung disease (322). Mice were intranasally infected with a low dose (5×10^5 CFU) antimicrobial susceptible strain of *A. baumannii* (ATCC 17978 provided by John Tregoning and Sophie Higham, Imperial College London). This strain and infection dose were selected based on previous data which demonstrated low dose intranasal infection with ATCC 17978 resulted in minimal weight loss and rapid clearance from the lungs and airways (331). Mice were culled at 12h, 18h and 36h post infection and bacterial load in the lungs and airways was quantified based on colony forming units (Fig. 5.6a). In the lung and airways, bacterial load quickly declined, suggesting intranasal exposure to *A. baumannii* resulted in short-lived, non-productive infection (Fig. 5.6b-c). Despite this rapid clearance, *A. baumannii* infection resulted in an influx of neutrophils into the lungs and airways (Fig. 5.6d). Neutrophil numbers in the lungs trended lower following *A. baumannii* infection compared to LPS exposure, however, this was not significant (Fig. 5.6d). *A. baumannii* infection also resulted in recruitment of inflammatory monocytes into the lung, but again at lower numbers compared to LPS exposure (Fig. 5.6e). Both neutrophils and inflammatory monocytes decreased between 12h and 18h post infection (Fig. 5.6d-e). At 12h post infection, *A. baumannii* also significantly increased the expression of *Il1a*, *Il1b* and *Tnfa* in the lungs compared to PBS controls (Fig. 5.6f-h). Overall, low dose *A. baumannii* infection led to the recruitment of innate immune cells and expression of pro-inflammatory cytokines comparable to LPS exposure.

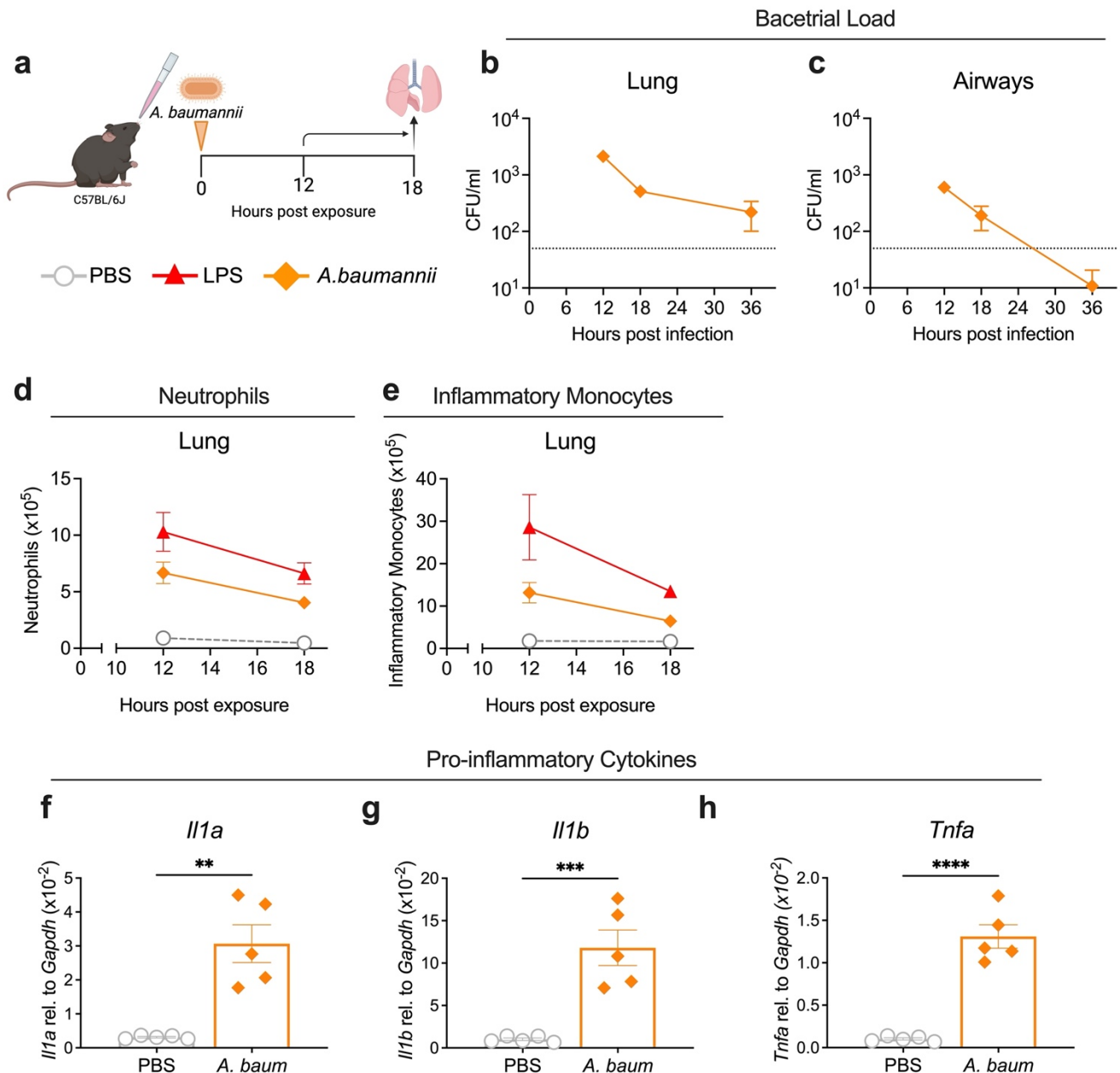


Figure 5.6 Intranasal *Acinetobacter baumannii* drove lung inflammation

a) Graphic showing experimental design. Mice were intranasally administered 5-8x10⁵ CFU *Acinetobacter baumannii*. At different time points, lungs and BAL were collected and processed either for CFU counts or for flow cytometry and qPCR. **b)** CFU/ml counted from lung homogenate plated onto LB agar at 12, 18 or 36h after *A. baumannii* infection. **c)** CFU/ml counted from BAL plated onto LB agar at 12, 18 or 36h. Dotted line represents the limit of detection determined from samples collected from PBS control mice. **d)** Total numbers of neutrophils in the lungs and airways following intranasal *A. baumannii* compared to LPS at 12 and 18h. **e)** Total numbers of inflammatory monocytes in the lungs following intranasal *A. baumannii* compared to LPS. Expression of **f)** *Il1a*, **g)** *Il1b* and **h)** *Tnfa* in the lung at 12h after *A. baumannii* infection, measured by qPCR and normalised to *Gapdh*. Data are from one experiment per time point, n=5. LPS data are the same data originally presented in Figure 5.1. Statistical analysis was carried out using GraphPad prism. An unpaired student's *t* test was carried out comparing *A. baumannii* infected mice to PBS controls. Asterisks represent the *p* value; *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001.

5.2.9 Infection with *Acinetobacter baumannii* 12h before RSV infection drove early weight loss associated with increased innate inflammation

To investigate if the inflammation driven by *A. baumannii* infection could also alter disease outcomes of subsequent RSV infection, mice were infected with *A. baumannii* 12h before RSV infection. This time interval was chosen as neutrophils and inflammatory monocyte numbers were seen to decline after 12h. Mice were weighed up to day 8 post infection and the infiltration of immune cells and viral load in the lungs was studied at day 1 and day 8 post infection using flow cytometry and qPCR (Fig. 5.7a). *A. baumannii* infection before RSV infection resulted in early weight loss which was significantly increased compared to RSV only mice on day 1 and 2 post RSV infection. By day 4 post RSV infection, these mice recovered to their original weight, before losing a smaller percentage (5%) at day 6 post RSV infection. The weight loss on day 6 post infection trended lower compared to RSV only mice but was not statistically significant (Fig. 5.7b). At day 1 post RSV infection neutrophils in the lung were significantly increased in the co-infected mice compared to RSV only mice (Fig. 5.7c). The AM population in the lung at day 1 post RSV infection was not significantly altered compared to either RSV, *A. baumannii* or co-infection (Fig. 5.7d). Inflammatory monocytes were not significantly increased in the lungs of co-infected mice compared to RSV only mice at day 1 post infection (Fig. 5.7e). In addition to immune cell recruitment, on day 1 post RSV infection the expression of *Il1a*, *Il1b* and *Tnfa* were all significantly increased in the lungs of mice infected with *A. baumannii* before RSV infection compared to RSV only mice (Fig. 5.7f-h). The increased inflammation on day 1 post RSV infection was not associated with any changes to viral load, which did not differ significantly between co-infected and RSV only mice (Fig. 5.7i). In addition to innate cell recruitment, the recruitment of T cells was profiled on day 8 post RSV infection. No significant differences were observed in the CD4⁺ or CD8⁺ T cell response between mice infected with *A. baumannii* before RSV infection and RSV only mice (Fig. 5.7j-k). Together, these data show a striking similarity in disease exacerbation and inflammation as mice exposed to LPS before RSV infection.

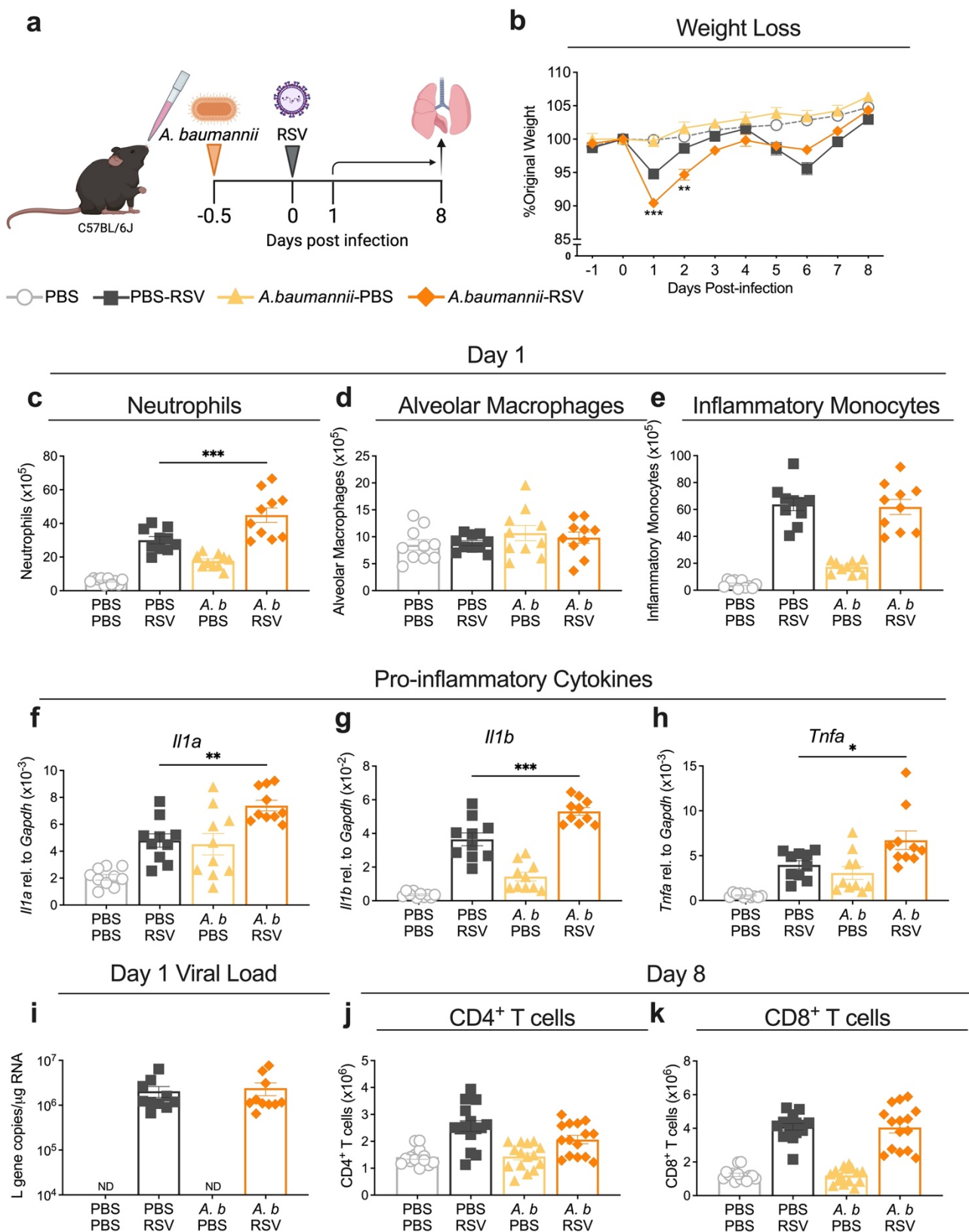


Figure 5.7 Exposure to *A. baumannii* before RSV infection induced early weight loss

a) Graphic showing experimental design. Mice were infected with 5×10^5 CFU *A. baumannii* (*A. b.*) 12h before RSV infection. Mice were weighed up to day 8 after infection. On day 1 after infection lungs and BAL were collected for analysis of the immune response by flow cytometry, ELISA and qPCR. **b)** Daily weight of mice represented as percentage of original body weight on day 0, in co-infected mice compared with RSV or *A. baumannii* infection only

mice. **c)** Total number of neutrophils, **d)** alveolar macrophages or **e)** inflammatory monocytes in the lungs at day 1 after RSV infection. Expression of **f)** *Il1a*, **g)** *Il1b* and **h)** *Tnfa* in the lung, measured by qPCR and normalised to *Gapdh*. **i)** Viral load represented as L gene copy number/ μ g RNA measured by qPCR and normalised to *Gapdh* on day 1 after RSV infection. ND= not detectable. Total numbers of **j)** CD4⁺ T cells and **k)** CD8⁺ T cells in the lungs at day 8 post infection. At day 1 after infection, data are pooled from 2 experiments: n=10. On day 8 after infection, data are pooled from 3 experiments: n=15. Weight loss is plotted as mean $\pm$ SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was carried out to compare the *A. baumannii*-RSV group with RSV only controls. For all other data, a one-way ANOVA with multiple comparisons test was carried out to compare the *A. baumannii*-RSV group with RSV only controls. Asterisks represent the *p* value compared to RSV only controls; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.2.10 Exposure to the lipoteichoic acid (LTA) before RSV infection drove early weight loss associated with increased innate inflammation

So far, exposure to the gram-negative PAMP, LPS and infection with gram-negative bacteria *A. baumannii* before RSV infection has been shown to drive early weight loss associated with elevated inflammation. Therefore, it was next investigated if this exacerbation is unique to gram-negative bacteria or could be replicated by exposure to gram-positive PAMPs. To do this, mice were intranasally exposed to 50 μ g LTA 12h prior to RSV infection and weighed across the course of 8 days (Fig. 5.8a). This dose was selected based on a neutrophil recruitment titration experiment. Mice were weighed up to day 8 post infection and the infiltration of immune cells and viral load in the lungs was studied at day 1 and day 8 post infection using flow cytometry and qPCR (Fig. 5.8a). Intranasal exposure to LTA before RSV infection also resulted in early weight loss compared to RSV only mice, peaking at ~10% on day 2 post infection (Fig. 5.8b). After day 2 the mice then recovered to their original weight by day 4 post infection before subsequently losing 5% their weight again at day 6 post infection. The weight loss at day 6 post infection was comparable to the RSV only mice (Fig. 5.8b). Mice exposed to LTA before infection had significantly higher numbers of neutrophils in the lungs compared to both RSV only and LTA only mice at day 1 post infection (Fig. 5.8c). RSV infection did not result in any alterations to the AM population in the lungs at day 1 post infection. Similar to LPS, an increase in AM numbers was recorded in the LTA only group, but this was not seen in the LTA-RSV group (Fig. 5.8d). At day 1 post infection, inflammatory monocytes were comparable between the RSV only and the LTA-RSV groups (Fig. 5.8e). The expression of *Il1a*, *Il1b* and *Tnfa* were all significantly increased in the mice exposed to LTA before RSV infection compared to RSV only mice (Fig. 5.8f-h). No significant differences in viral load were measured between the RSV only mice and mice exposed to LTA before RSV infection (Fig. 5.8i). At day 8 post infection the mice exposed to LTA before RSV infection had an increased trend in both CD4⁺ and CD8⁺ T cells compared to RSV only mice, but this was not statistically significant (Fig. 5.8j-k). Therefore, these data suggest exposure to gram-positive PAMPs

before RSV infection also results in early weight loss associated with increased innate inflammation.

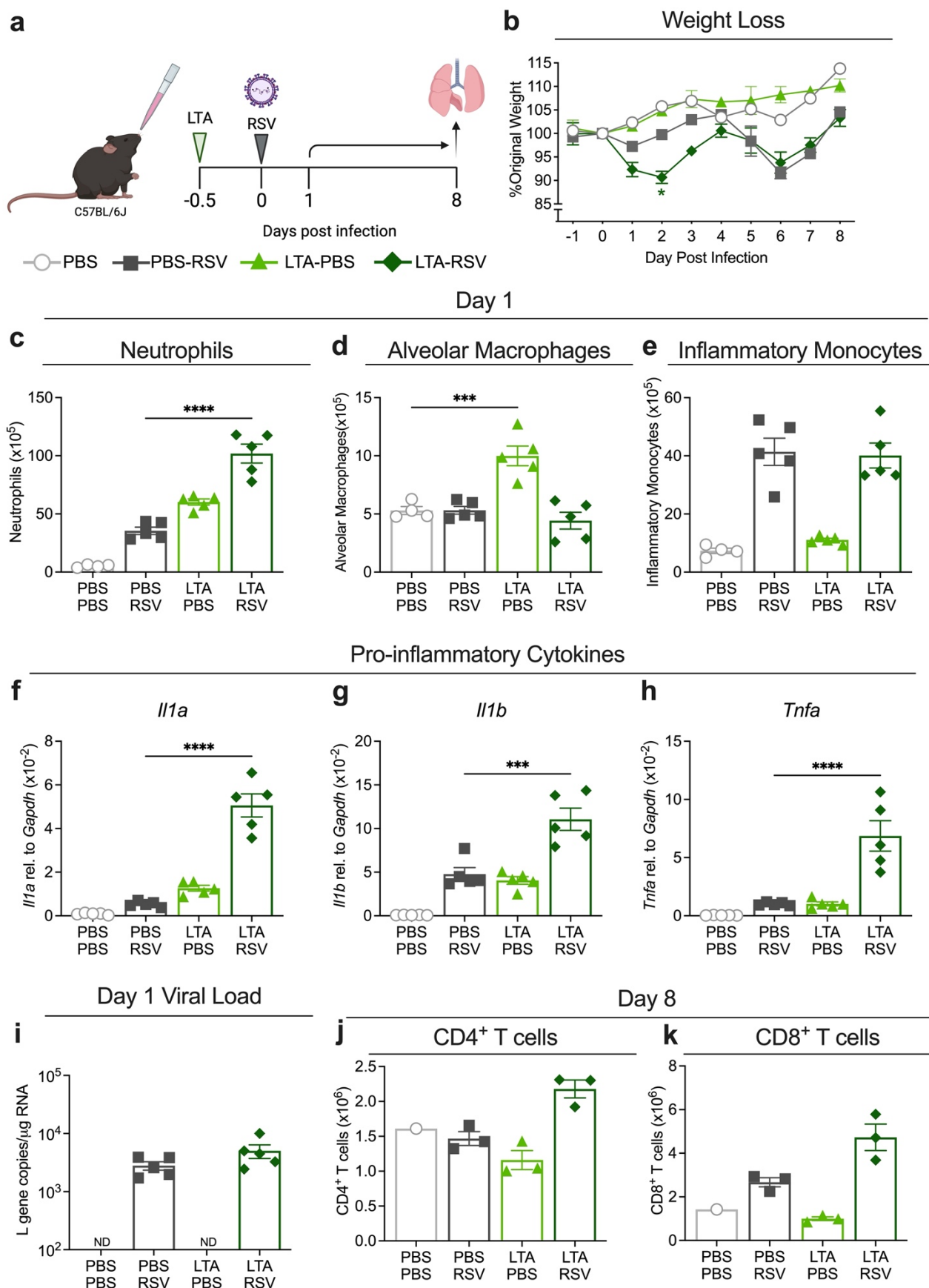


Figure 5.8 Exposure to LTA before RSV infection induced early weight loss

a) Graphic showing experimental design. Mice were exposed to 50µg LTA 12h before RSV infection and weighed up to day 8 after RSV infection. On day 1 after infection and lungs and BAL were collected for analysis by flow cytometry or qPCR. **b)** Weight loss represented as percentage of original body weight on day 0. Total number of **c)** neutrophils, **d)** alveolar macrophages or **e)** inflammatory monocytes in the lung at day 1 after RSV infection. Expression of **f)** *Il1a*, **g)** *Il1b* and **h)** *Tnfa* in the lung, measured by qPCR and normalised to *Gapdh*. **e)** Viral load represented as L gene copy number/µg RNA measured by qPCR and normalised to *Gapdh* on day 1 after RSV infection. ND= not detectable. Total numbers of **j)** CD4⁺ T cells and **k)** CD8⁺ T cells in the lungs at day 8 post infection. On day 1 after infection data are from one experiment, n=5. On day 8 post infection, data are from one experiment. PBS, n=1; RSV, LTA and LTA-RSV, n=3. Weight loss is plotted as the mean ± SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. For weight loss, as data were paired, a two-way ANOVA with multiple comparisons test was carried out to compare the LTA-RSV group with RSV only controls. For all other data, a one-way ANOVA with multiple comparisons test was carried out to compare the LTA-RSV group with RSV only controls. Asterisks represent the *p* value compared to RSV only controls; *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001

5.2.11 Early weight loss in mice exposed to LPS before RSV infection was not driven by T cells.

Having found exposure to bacteria or bacterial PAMPs before RSV infection to exacerbated disease in three separate mouse models, the next step was to identify the mechanism responsible for the accelerated weight loss. Weight loss at days 5-7 post RSV infection is known to be mediated by CD8⁺ T cells (332). However, weight loss in mice exposed to LPS before RSV infection occurred earlier after infection and was associated with exacerbated innate inflammation. Therefore, to conclusively show the adaptive immune response is not responsible for the early weight loss, *Rag2*^{-/-} mice were exposed to LPS 12h before RSV infection (Fig. 5.9a). *Rag2* encodes for proteins that form the RAG complex which is important in T development. Mice deficient in *Rag2* are therefore deficient in both mature CD4⁺ and CD8⁺ T cells (333). *Rag2*^{-/-} mice exposed to LPS before RSV infection lost comparable weight to the WT controls, suggesting disease exacerbation is driven not by T cells but the innate immune response (Fig. 5.9b). At day 4 post infection, mice were culled and the immune response in the lungs and airways was studied. T cell deficiency in *Rag2*^{-/-} mice was confirmed by flow cytometry, with *Rag2*^{-/-} mice having no detectable CD4⁺ or CD8⁺ T cells compared to WT (Fig. 5.9c-d). At day 4 post infection, viral load did not differ between WT and *Rag2*^{-/-} mice (Fig. 5.9e). The recruitment of neutrophils and inflammatory monocytes in mice exposed to LPS before RSV infection did not differ between *Rag2*^{-/-} mice and WT (Fig. 5.9f-g). Interestingly, in PBS mice, baseline neutrophils in the lung were significantly higher in *Rag2*^{-/-} mice compared to WT (Fig. 5.9f). The population of AMs in the lung at day 4 post infection was not significantly different between *Rag2*^{-/-} mice and WT mice exposed to LPS before RSV infection. These

data suggest early weight loss in mice exposed to LPS before RSV infection is driven by the innate immune response.

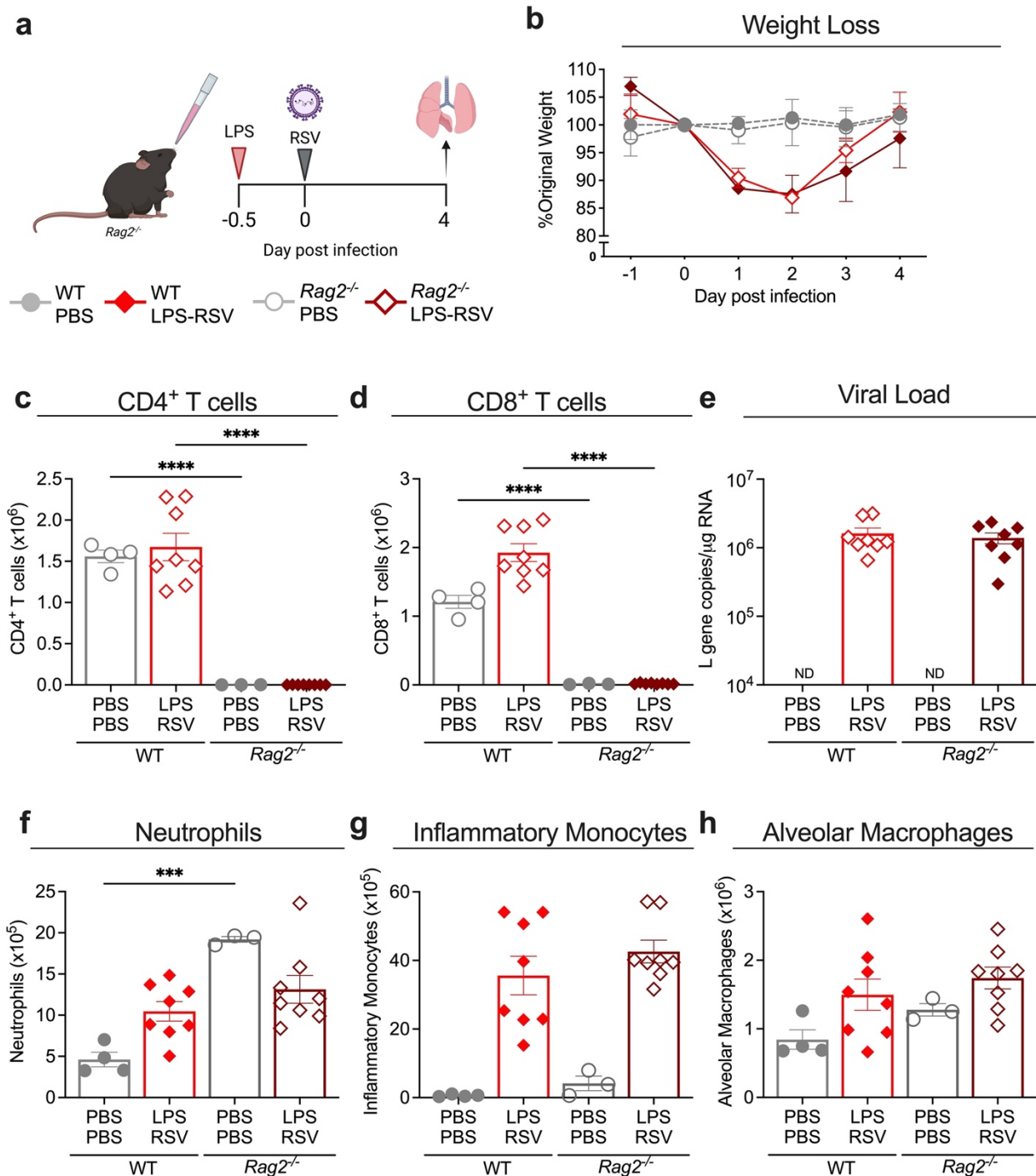


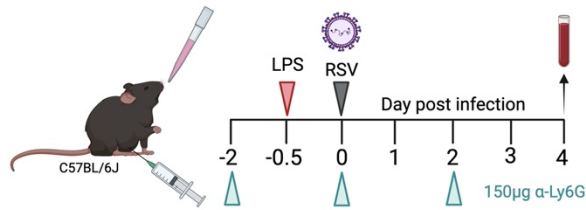
Figure 5.9 Exposure to LPS before RSV infection in *Rag2*^{-/-} mice does not differ to WT mice

a) Graphic showing experimental design. *Rag2*^{-/-} and C57BL/6J (WT) mice were intranasally exposed to LPS 12h before RSV infection. Mice were weighed up to day 4 at which point lungs and BAL were collected for flow cytometry analysis. **b**) Daily weight of mice represented as percentage of original body weight on day 0. **c**) Total number of CD4⁺ T cells or **d**) total number of CD8⁺ T cells in the lung at day 4 after infection in *Rag2*^{-/-} compared to WT mice. **e**) Viral load in the lungs measured as L gene copy number/μg RNA measured by qPCR and normalised to *Gapdh*.

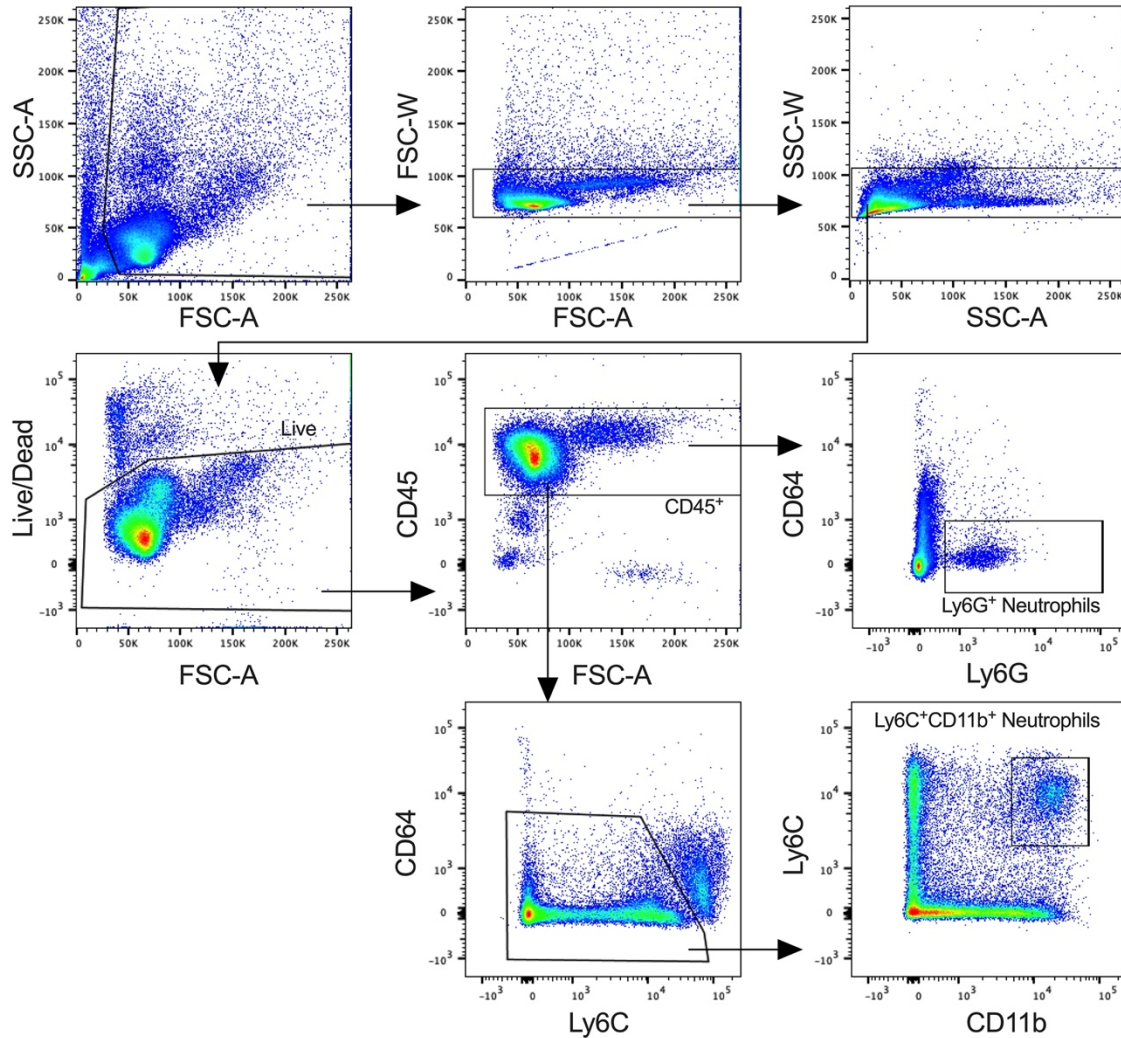
ND= not detectable. Total numbers of **f**) neutrophils, **g**) inflammatory monocytes or **h**) alveolar macrophages in the lung at day 4 post infection in *Rag2*^{-/-} mice compared to WT mice. Data are pooled from 2 experiments. WT PBS only n= 4; WT LTA- RSV n=8; *Rag2*^{-/-} PBS n=3; *Rag2*^{-/-}LPS-RSV n=8. Weight loss is plotted as the mean $\pm$ SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. For weight loss, as data were paired, a two-way ANOVA with multiple comparisons test was carried out to compare the *Rag2*^{-/-} LPS-RSV group with the WT LPS-RSV group. No statistical significance was found. For flow cytometry data, a one-way ANOVA with multiple comparisons test was carried out to compare *Rag2*^{-/-} mice with WT controls. Asterisks represent the *p* value; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.2.12 Neutrophil depletion using α -Ly6G antibodies was rapidly overcome in mice exposed to LPS before RSV infection.

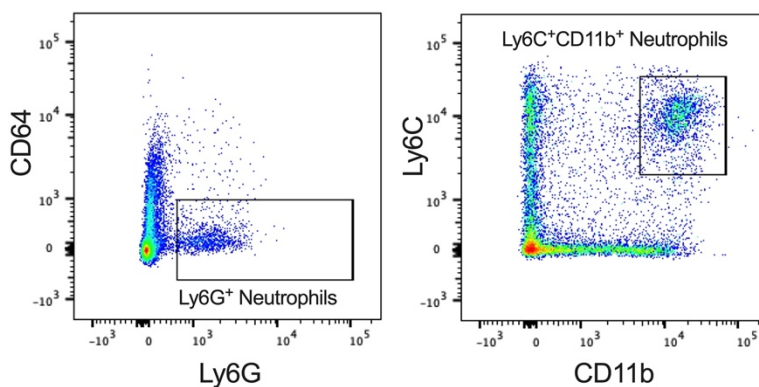
Based on the data presented by Habibi et al (170), It was initially hypothesised that intranasal exposure to bacteria or bacterial PAMPs would drive neutrophil recruitment into the lungs which would result in exacerbated disease upon RSV infection. To confirm the role of neutrophils, *in vivo* neutrophil depletion experiments were designed. Neutrophils can be identified and separated from other immune cell populations based on their cell surface expression of Ly6G (17). Therefore, antibodies directed toward Ly6G (α -Ly6G) have been used to deplete neutrophils with varying degrees of efficacy reported (17,309,334). In mouse models of RSV infection using C57BL/6J mice, intraperitoneal injection of 150 μ g α -Ly6G every other day has previously been shown to successfully deplete neutrophils (17). Following the same depletion protocol, mice received an i.p injection of α -Ly6G (or isotype control) on day - 2 post infection (1 day prior to intranasal LPS). Mice were then infected with RSV on D0 and received an i.p injection of α -Ly6G every other day until day 4 post infection (Fig. 5.10a). To confirm successful depletion of neutrophils, blood samples were collected post-mortem on D4 post infection and analysed using flow cytometry. When α -Ly6G depletion is carried out, neutrophils cannot be appropriately quantified using expression of Ly6G as all fluorescent antibodies share the same clone as the depletion antibody (1A8), thus epitopes can be masked by the α -Ly6G. Therefore, neutrophils were instead quantified as Ly6C⁺ CD11b⁺ cells, as depicted in the example gating strategy showing blood from an isotype mouse (Fig. 5.10b). Interestingly, populations of both Ly6G⁺ neutrophils and Ly6C⁺ CD11b⁺ neutrophils were identified in mice which received α -Ly6G depletion (Fig. 5.10c). This suggests that in this model, neutrophil depletion was not successful. Confirming this, no significant difference was seen in the percentage of Ly6C⁺ CD11b⁺ neutrophils in the blood in isotype vs α -Ly6G treated mice, (Fig. 5.10d).

a**b**

Blood Gating Strategy - Isotype

**c**

Blood Neutrophils - αLy6G

**d**

Blood Neutrophils %

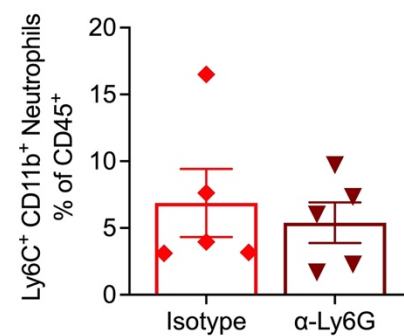


Figure 5.10 Neutrophils could not be depleted in mice exposed to LPS before RSV infection using only α -Ly6G antibodies

a) Graphic showing experimental design. Mice received 150 μ g isotype or α -Ly6G antibodies i.p. every other day starting 2 days prior to RSV infection. Mice were then given an intranasal dose of LPS 12h prior to RSV infection (7×10^6 FFU). At day 4 post infection mice were culled blood samples were collected for analysis by flow cytometry. **b)** Example flow cytometry plots outlining the gating strategy used to identify neutrophils in the blood. **c)** Example flow plots showing neutrophil populations in α -Ly6G treated mice. **d)** Percentage neutrophils (gated as Ly6C⁺CD11b⁺ cells) of CD45⁺ cells in the blood from α -Ly6G treated mice compared to isotype controls. Data are representative of 1 experiment, n=5. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. An unpaired student's *t* test was carried to compare α -Ly6G to isotype mice. No statistical differences were found.

5.2.13 Combination α -Ly6G and α -rat IgG Kappa treatment significantly reduced neutrophil recruitment following intranasal LPS and RSV.

It has previously been reported that C57BL/6 mice can rapidly overcome α -Ly6G depletion (309). α -Ly6G, which is a rat IgG2a drives Fc opsonisation resulting in neutrophil depletion through macrophage phagocytosis. Boivin et al (309) suggested, this is relatively slow depletion mechanism which can be overcome by neutrophil haematopoiesis. Given that LPS, rapidly induces emergency neutrophil granulopoiesis, it is likely that α -Ly6G depletion was easily overcome in this model. To obtain more complete and sustained neutrophil depletion, Boivin et al (309) reported the use of a secondary antibody directed toward rat IgG kappa chain (α -rat kappa IgG). This antibody is a mouse IgG2a, which instead of Fc opsonisation, drives complement-mediated cell death which is considered a faster and more effective depletion mechanism. Thus, using both α -Ly6G and α -rat kappa IgG results in isotype switching that allows for better more sustained neutrophil depletion (309). Therefore, based on the protocol described by Boivin et al (309), mice were given 50 μ g α -Ly6G by i.p injection every day beginning 2 days before RSV infection (1 day before LPS). 50 μ g α -rat kappa IgG was then injected i.p every other day beginning 1 day before RSV infection. In addition, mice also received 25 μ g α -Ly6G i.n. on day 0 post infection and 50 μ g α -Ly6G i.n. on day 3 post infection (Fig. 5.11a). Mice were exposed to intranasal LPS 12h before infection with RSV and weighed across the course of infection. On day 4 post infection, mice were culled, and neutrophil depletion was assessed by flow cytometry in the lungs, BAL and blood (Fig. 5.11a). Previously, neutrophil depletion could not be measured in the lungs due to difficulties in separating neutrophil populations from monocytes which also express CD64, CD11b and Ly6C. Therefore, lung, BAL and blood cells were permeabilised and stained for intracellular Ly6G (Int-Ly6G), using an antibody with a different fluorophore to the extracellular Ly6G (Ex-Ly6G) (309). To confirm the Int-Ly6G antibody was staining intracellular Ly6G only,

extracellular stained lung cells were stained with the Int-Ly6G antibody in the absence of permeabilization buffer (No Perm Control). These cells were predominantly Ex-Ly6G⁺, with lower expression of Int-Ly6G compared to permeabilised cells (Fig. 5.11b). Lung neutrophils from isotype mice were found to stain positive for both Ex-Ly6G and Int-Ly6G, whilst neutrophils from α -Ly6G mice were only positive for Int-Ly6G (Fig. 5.11b). Taken together, the example plots in Fig. 5.11b demonstrate the use of intracellular Ly6G staining as a method to identify neutrophils in mice treated with α -Ly6G antibody.

In the lung, the percentage of neutrophils at day 4 post infection was comparable between PBS mice and mice exposed to LPS before RSV infection which received the isotype control. In contrast, the percentage of neutrophils in lungs was significantly reduced in mice exposed to LPS before RSV infection which received α -Ly6G and α -rat kappa IgG compared to isotype controls (Fig. 5.11c). Similarly, in the BAL, neutrophil numbers were very low by day 4 post infection. Despite this a significant difference in the percentage of neutrophils was noted in the α -Ly6G/ α -rat kappa IgG mice compared to isotype mice (Fig. 5.11d). The effect of α -Ly6G/ α -rat kappa IgG treatment was most clearly seen in the blood. In PBS and isotype mice neutrophils accounted for 10-15% of CD45⁺ cells. This significantly fell to below 5% in the α -Ly6G/ α -rat kappa IgG treated mice (Fig. 5.11e). Overall, this data suggests a significant reduction in neutrophils in the circulation and subsequently less recruitment into the lungs and airways of α -Ly6G/ α -rat kappa IgG treated mice exposed to LPS before RSV infection.

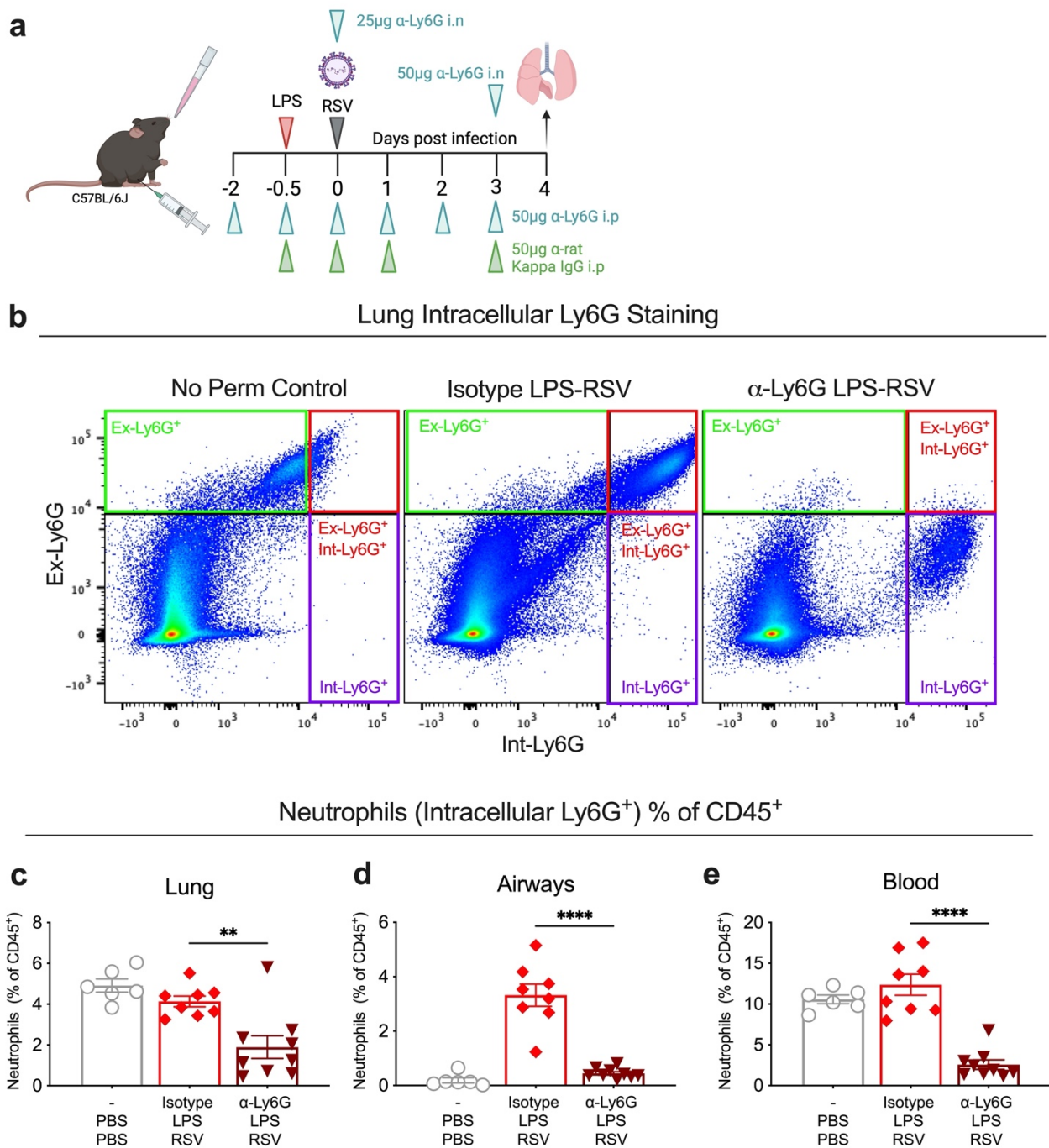


Figure 5.11 Neutrophils could be reduced in mice exposed to LPS before RSV infection using α -Ly6G and α -rat kappa IgG antibodies

a) Graphic demonstrating neutrophil depletion protocol which was adapted from that published by Boivin et al (2020). Mice received an intra-peritoneal (ip) injection of 50 μ g anti-Ly6G antibody (α -Ly6G) daily, ip injections of anti-rat kappa IgG (α -rat igG) on the indicated days, as well as intranasal α -Ly6G on the indicated days. Mice were intranasally exposed to LPS 12h before RSV infection and weighed up to day 4 after infection when lungs, BAL and blood were collected. **b)** Flow cytometry plots generated in FlowJo software showing expression of extracellular Ly6G (ex-Ly6G) staining versus intracellular Ly6G (int-Ly6G) on lung cells from isotype or α -Ly6G treated mice compared to non-permeabilised control lung cells from isotype mouse. Each plot represents one mouse/sample. Percentage of neutrophils (gated as intracellular Ly6G⁺ cells) from total CD45⁺ cells in the **c)** lungs, **d)** airways and **e)** blood of α -Ly6G treated mice compared to isotype controls. Data are pooled from 2 experiments. For neutrophil

depletion experiments: PBS n=6; Isotype LPS-RSV n=8; α -Ly6G LPS-RSV n=9. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. A one-way ANOVA with multiple comparisons test was carried out to compare α -Ly6G LPS-RSV group with isotype LPS-RSV controls. Asterisks represent the p value compared to isotype controls; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.2.14 Neutrophils recruited by LPS before RSV infection did not drive early weight loss

Having successfully reduced circulating neutrophils and recruitment into the lungs and airways following LPS exposure before RSV infection, the contribution of neutrophils to disease severity and inflammation were investigated. Reducing the number of neutrophils in the lungs and airways had no effect on weight loss during the first 4 days of RSV infection, compared to isotype controls (Fig. 5.12a). This suggests early weight loss in mice exposed to LPS before RSV infection is not driven by the presence of neutrophils in the lungs before or during infection. Interestingly, neutrophil depletion resulted in a significant decrease in viral load in the lungs on day 4 post infection compared to isotype controls (Fig. 5.12b). Neutrophils recruited by RSV have previously been shown not to contribute to viral clearance (17). Rather, inflammatory monocytes are thought to control viral replication during the innate phase of infection (15). Therefore, the effect of neutrophil depletion on other innate immune cell populations in the lung was assessed by flow cytometry. Neutrophil depletion had no significant impact on the total number of AMs in the lung of α -Ly6G/ α -rat kappa IgG treated mice compared to isotype controls (Fig. 5.12c). Inflammatory monocytes trended higher in α -Ly6G/ α -rat kappa IgG treated mice, but this was not statistically significant compared to isotype controls (Fig. 5.12d). Therefore, it remains unclear if the significant decrease in viral load directly or indirectly is due to the loss of neutrophils.

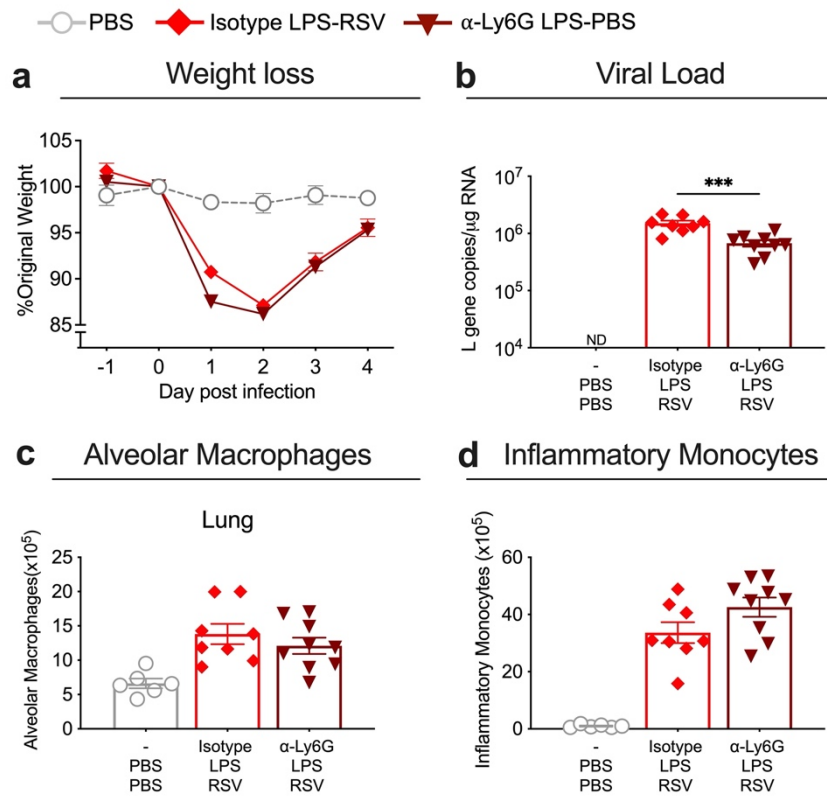


Figure 5.12 Neutrophil depletion in mice exposed to LPS before RSV infection did not alter weight loss

a) Daily weight of mice represented as percentage of original body weight on day 0 after infection in α-Ly6G treated mice exposed to LPS before RSV infection, compared to isotype treated mice. **b)** Viral load in the lungs represented as L gene copy number/μg RNA measured by qPCR and normalised to *Gapdh*. ND= not detectable. Total number of **c)** alveolar macrophages and **d)** inflammatory monocytes in the lungs of α-Ly6G treated mice compared to isotype controls. Data are pooled from 2 experiments. PBS n=6; Isotype LPS-RSV n=8; α-Ly6G LPS-RSV n=9. Weight loss is plotted as the mean ± SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was carried out to compare α-Ly6G LPS-RSV group with isotype LPS-RSV controls. For flow cytometry data, a one-way ANOVA with multiple comparisons test was carried out to compare α-Ly6G LPS-RSV group with isotype LPS-RSV controls. Asterisks represent the *p* value compared to isotype controls; *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001.

5.2.15 Early weight loss in mice exposed to LPS before RSV infection was partially driven by inflammatory monocytes

In addition to neutrophils, mice exposed to LPS before RSV infection also had increased recruitment of inflammatory monocytes into the lungs and airways. As neutrophils are not responsible for driving early weight loss, the contribution of monocytes was investigated. Monocytes can be successfully depleted in mouse models using antibodies directed towards the monocyte chemokine receptor CCR2 (α-CCR2) (335,336). Mice were treated with 20μg α-CCR2 antibodies (obtained from Matthias Mack, University Hospital Regensburg) or the

relevant isotype by ip injection, 6h before intranasal LPS exposure and then again on day 0 and 1 post infection. Mice were infected with RSV 12h after LPS exposure and weighed up to day 2 post infection (Fig. 5.13a). Monocyte depletion in the lungs of α -CCR2 compared to isotype controls was confirmed by flow cytometry (Fig. 5.13b). Mice treated with α -CCR2 and exposed to LPS before RSV infection lost comparable weight to isotype mice on day 1 post infection. However, on day 2 post infection weight loss in α -CCR2 treated mice was significantly less compared to isotype controls (Fig. 5.13c). This suggests α -CCR2 treated mice were recovering faster and that inflammatory monocyte recruitment partially contributes to early weight loss in mice exposed to LPS before RSV infection. Consistent with previous studies of monocyte depletion during RSV infection, viral load was significantly higher in α -CCR2 treated mice compared with isotype controls (Fig. 5.13d) (15). Depletion of monocytes did not affect the recruitment of neutrophils into the lung (Fig. 5.13e). The population of AM was significantly decreased by α -CCR2 treatment compared to isotype controls (Fig. 5.13f). However, this could be a result of reduced differentiation of monocytes into alveolar macrophages rather than depletion of AMs (337). Inflammatory monocytes are known to be sources of pro-inflammatory cytokines, in particular TNF- α (15). However, the expression of *Il1a*, *Il1b* and *Tnfa* were not significantly altered by monocyte depletion, suggesting these cytokines are expressed by another source in this model (Fig. 5.13f-h).

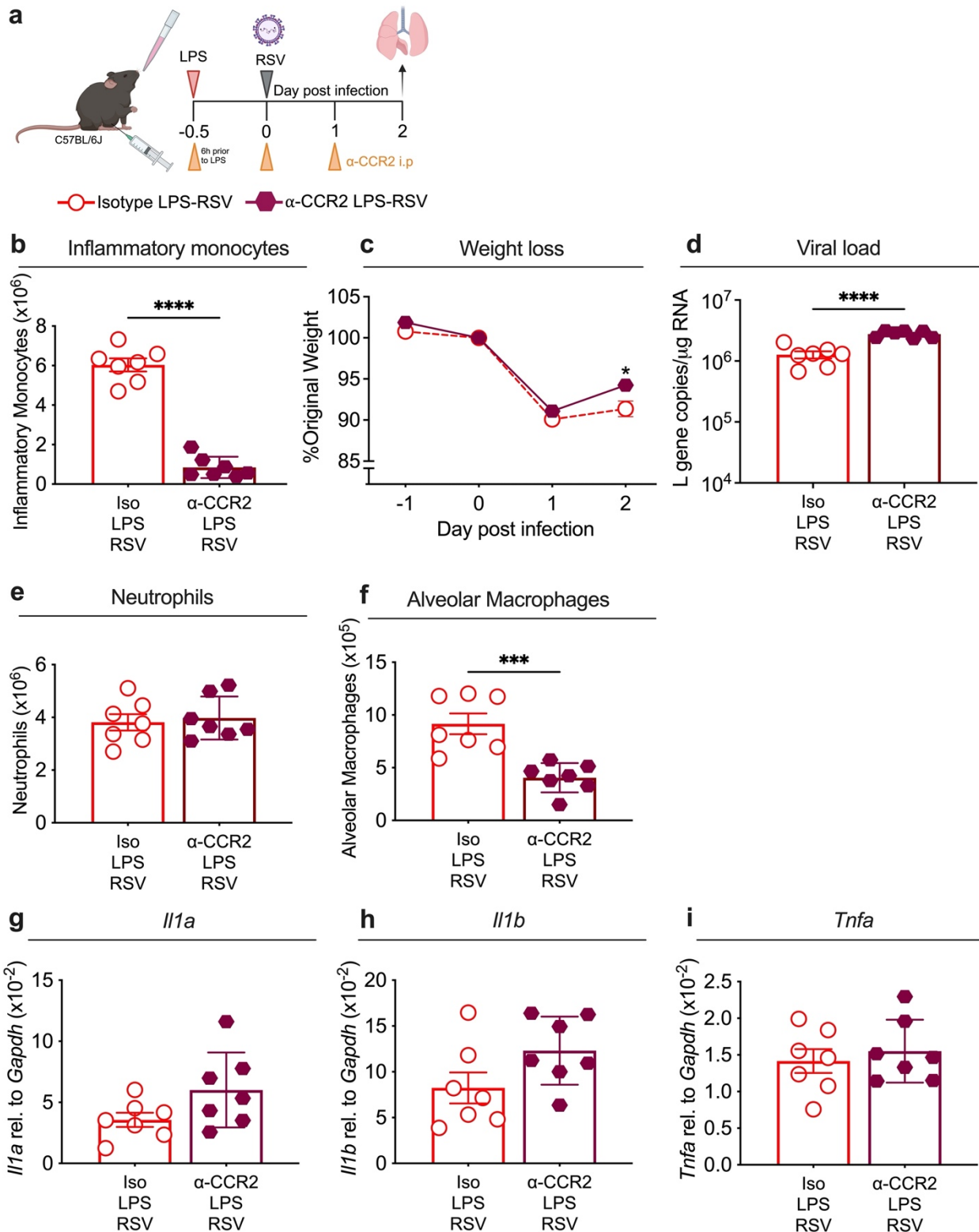


Figure 5.13 Monocyte depletion in mice exposed to LPS before RSV infection partially ameliorated weight loss

a) Graphic demonstrating monocyte depletion protocol. Mice received an ip injection of 20µg anti-CCR2 (α-CCR2) or relevant isotype 6h prior to intranasal LPS, then again daily. Mice were infected with RSV 12h after LPS and weighed up to day 2 post infection. **b)** Total number of inflammatory monocytes in the lungs at day 2 post infection

in α -CCR2 treated mice compared to isotype. **c)** Daily weight of mice represented as percentage of original body weight on day 0, in α -CCR2 treated mice compared to isotype controls. **d)** Viral load in the lungs measured as L gene copy number/ μ g RNA measured by qPCR and normalised to *Gapdh*. Total number of **e)** neutrophils and **f)** inflammatory monocytes in the lungs at day 2 post infection in α -CCR2 treated mice compared to isotype. Expression of **g)** *Il1a*, **h)** *Il1b* and **i)** *Tnfa* in the lung, measured by qPCR and normalised to *Gapdh*. Data are pooled from 2 experiments. α -CCR2 n=7; α -CCR2 isotype n=7. Weight loss is plotted as the mean $\pm$ SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was carried out to compare α -CCR2 LPS-RSV group with isotype LPS-RSV controls. For flow cytometry data, a one-way ANOVA with multiple comparisons test was carried out to compare α -CCR2 LPS-RSV group with isotype LPS-RSV controls. Asterisks represent the *p* value compared to isotype controls; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.2.16 *IL-1 α* and *TNF- α* were responsible for driving early weight loss in mice exposed to LPS before RSV infection

Neutrophils were found not to be responsible for driving early weight loss in mice exposed to LPS before RSV infection, and inflammatory monocytes were found to contribute only partially. Therefore, something else must be contributing this exacerbated disease. Expression of *Il1a*, *Il1b* and *Tnfa* was found to be significantly increased in the lungs in mice exposed to LPS, LTA or *A. baumannii* before RSV infection. To determine if these cytokines play a role in accelerating weight loss in mice exposed to LPS before RSV infection mice, all three were blocked in combination using antibodies. Mice received an ip injection containing anti-IL-1 β (α -IL-1 β), anti-IL-1 α (α -IL-1 α) and anti-TNF- α (α -TNF- α) in combination (α -combo) or the relevant isotype control, 6h before intranasal LPS and then again on day 1 and 2 post RSV infection (Fig. 5.14a). Mice which received the α -combo blocking antibodies were completely protected from weight loss induced by LPS and RSV, losing significantly less weight on days 1 and 2 post infection compared to isotype controls (Fig. 5.14b). This suggests that at least one of these antibodies is responsible for driving the early weight loss in mice exposed to LPS before RSV infection. To further assess the role of these antibodies in driving early weight loss, mice received ip injections of either only α -IL-1 β , α -IL-1 α or α -TNF- α , or the isotype control. Mice treated with α -IL-1 β lost comparable weight to isotype control mice. In contrast, mice treated with α -IL-1 α or α -TNF- α had partial amelioration in weight loss. α -IL-1 α treated mice had significantly less weight loss on day 1 and 2 post infection, and α -TNF- α treated mice lost significantly less weight on day 2 post infection compared to isotype controls (Fig. 5.14c). Taken together these data suggest blocking all three cytokines is required for full protection from weight loss, but in particular IL-1 α and TNF- α play a predominant role in driving weight loss. Viral load in the lungs at day 2 post infection was not significantly altered by blocking IL-1 α , IL-1 β , TNF- α or all three in combination (Fig. 5.14d). However, neutrophils and inflammatory monocytes in the lungs on day 2 post infection were significantly decreased in

mice treated with α -IL-1 α , α -TNF- α or α -combo, but not α -IL-1 β (Fig. 5.14e-f). The AM population in the lung was not affected by cytokine blockage (Fig. 5.14g). These data suggest that not only are these cytokines, predominantly α -IL-1 α and α -TNF- α , responsible for driving early weight loss, but also in dysregulating the recruitment of inflammatory cells into the lung.

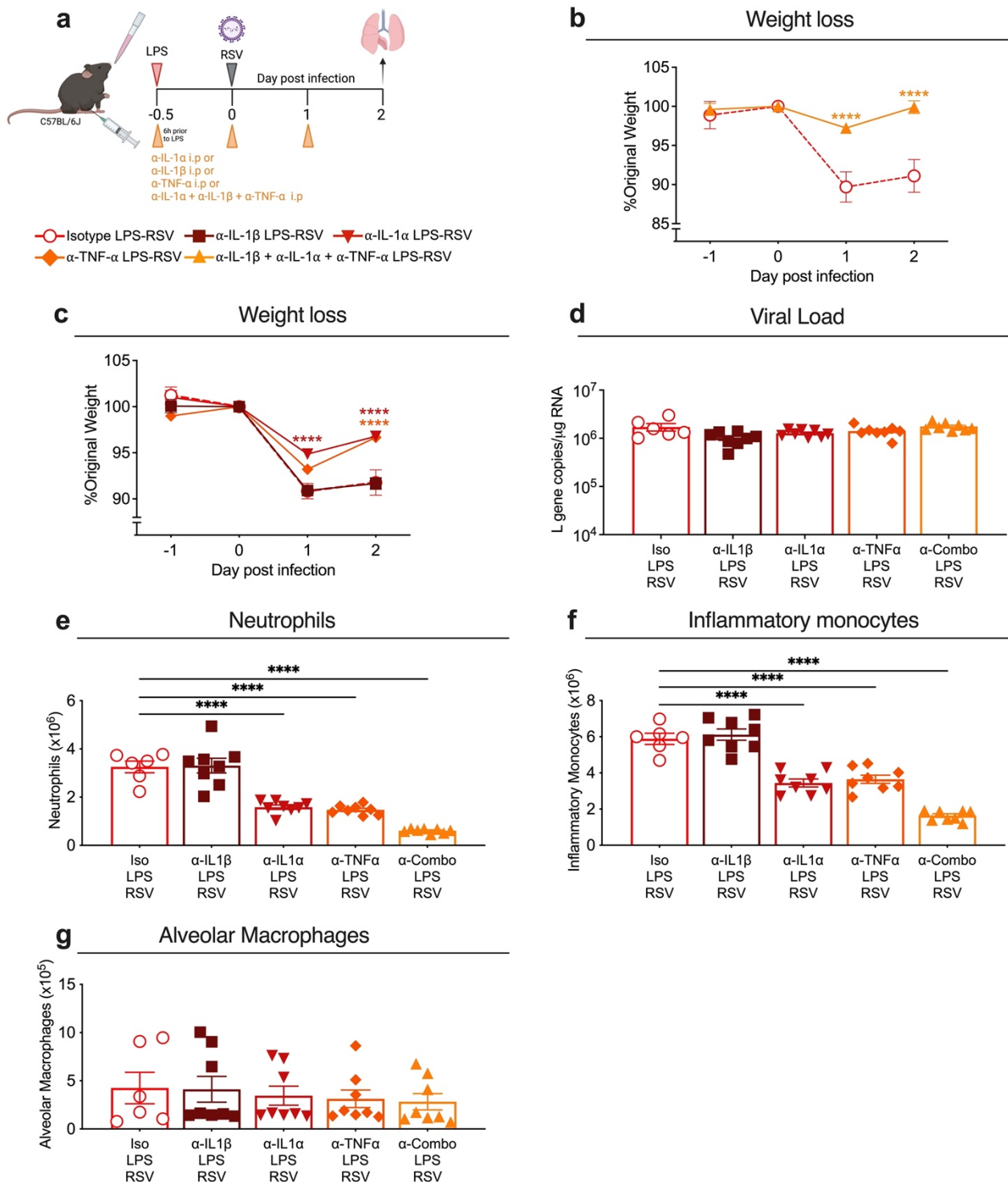


Figure 5.14 Depletion of IL-1 α and TNF- α ameliorated exacerbated weight loss

a) Graphic demonstrating the cytokine depletion protocol. Mice received an ip injection of 250 μ g anti-IL-1 α (α -IL-1 α), anti-IL-1 β (α -IL-1 β), anti-TNF- α (α -TNF- α), all three in combination (α -combo) or relevant isotype 6h prior to intranasal LPS, then again daily. Mice were infected with RSV 12h after LPS and weighed up to day 2 post infection.

b) Daily weight of mice represented as percentage of original body weight on day 0 after infection in α -IL-1 α + α -IL-1 β + α -TNF- α (α -Combo) treated mice exposed to LPS before RSV infection, compared to isotype (750 μ g) treated mice. **c)** Daily weight of mice represented as percentage of original body weight on day 0 after infection in α -IL-1 α , α -IL-1 β or α -TNF- α treated mice exposed to LPS before RSV infection, compared to isotype (250 μ g) treated mice. **d)** Viral load in the lungs of cytokine depleted mice compared to isotype controls, represented as L gene copy number/ μ g RNA measured by qPCR and normalised to *Gapdh*. Total number of **e)** neutrophils, **f)** inflammatory monocytes and **g)** alveolar macrophages in the lungs. Data are pooled from 2 experiments. α -cytokine isotype LPS-RSV n=6; α -Combo isotype LPS-RSV n=4; α -IL-1 α , α -IL-1 β , α -TNF- α and α -Combo LPS-RSV n=8. Weight loss is plotted as the mean $\pm$ SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was carried out to compare depletion groups with isotype LPS-RSV controls. For flow cytometry and qPCR data, a one-way ANOVA with multiple comparisons test was carried out to compare depletion with isotype LPS-RSV controls. Asterisks represent the *p* value compared to isotype controls; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

5.3 Discussion

The findings from this chapter demonstrate that intranasal exposure of mice to bacteria or bacterial PAMPS before RSV infection results in exacerbated disease outcomes. When exposed to LPS, LTA or *A. baumannii* 12h before RSV infection, mice experienced rapid early weight loss occurring between days 1-3 post infection. This was not driven solely by the inhaled bacteria or PAMP, which at the doses used in these models, did not drive weight loss. Following RSV infection, mice experience minor weight loss early after infection, but this is typically not significant compared to PBS controls. Therefore, the early weight loss likely occurred as a result of bacterial PAMPs altering the immune response towards RSV.

An increased neutrophil activation signature in the nasal mucosa of healthy adults before RSV infection, was found to correlated with increased risk of symptomatic illness following infection (170). Subsequently, it was demonstrated that neutrophils recruited into lungs and airways by intranasal CXCL1 12h before RSV infection exacerbated weight loss in mice (170). However, the presence of neutrophils in the airways of human participants could not be explained. In this chapter, the hypothesis that exposure to bacteria or bacterial PAMPs drives neutrophil recruitment to the lungs which subsequently exacerbate disease upon RSV infection was tested. Indeed, intranasal LPS, LTA or *A.baumannii* all resulted in significant neutrophil recruitment into the lungs and this was associated with significantly more weight loss compared with RSV only controls. However, the dynamics of this weight loss differed entirely from that of mice exposed to CXCL1 before RSV infection. Bacterial PAMP exposure before RSV infection drove early weight loss occurring during the innate phase of infection, whilst CXCL1 exacerbated weight loss during the adaptive phase of infection, between days 5-6 post infection (170). This indicated that the mechanism through which bacterial PAMP exposure exacerbates RSV infection is different from the mechanism through which CXCL1 exacerbates RSV infection. In confirmation with these observations, neutrophils did not appear to contribute to this early weight loss. Despite previous studies reporting successful neutrophil depletion during RSV infection in mice using α -Ly6G (17), robust neutrophil depletion proved difficult in mice exposed to LPS before RSV infection. This could potentially be explained by LPS ability to drive emergency granulopoiesis in the bone marrow of mice, which could rapidly overcome depletion (338). Even through use of secondary α -rat kappa IgG, which has previously been reported to improve neutrophil depletion in C57BL/6 mice, only a partial reduction in neutrophil recruitment into the lungs and airways was achieved (309). Therefore, this data does not conclusively prove their role, but does demonstrates they are not major contributors to early weight loss in mice exposed to LPS before RSV infection. These findings are in line with data which show increased neutrophilia during RSV infection does not exacerbate disease (17). However, there is no explanation why neutrophils recruited by LPS before RSV infection do

not exacerbate weight loss during the adaptive phase of infection. Instead, mice exposed to LPS before RSV infection were significantly protected from weight loss at days 5 and 6 post infection. This suggests the exacerbated innate immune response driven by LPS is preventing the mechanism through which neutrophils in the lungs before RSV infection leads to disease exacerbation.

CD8⁺ T cells are known to be responsible for driving weight loss at days 5-7 post RSV infection but are also essential for viral clearance (212,213,332). However, by using *Rag2*^{-/-} which lack a mature T and B cell response, it was conclusively demonstrated early weight loss in mice exposed to LPS before RSV infection is driven by the innate immune response (333). This was not surprising given weight loss occurred before day 4 post infection, when T cells are known to infiltrate into the lungs and airways (8,332). *Rag2*^{-/-} mice also had comparable viral load to isotype controls on day 4 post infection. This was an interesting observation given that viral load on day 4 post infection was significantly reduced in the lungs of mice exposed to LPS before RSV infection compared to RSV only mice. LPS exposure is therefore resulting in enhanced viral clearance. Due to the importance of T cells in RSV clearance, *Rag2*^{-/-} are unable to control viral replication and experience long term infection (339). Therefore, the enhanced viral clearance in LPS exposed mice is again likely due to the innate immune response rather than the adaptive response.

During the early stages of RSV infection, viral replication is known to be mediated by type I IFNs (15). This was demonstrated using *Mavs*^{-/-} mice which are unable signal via the RLR pathway and induce type I IFNs in response to RSV infection (15). These mice have significantly higher viral load early after RSV infection which was found to be due to a loss of inflammatory monocyte recruitment in these mice (15). Consistent with this, monocyte depletion using α -CCR2 was found to significantly increase viral load in mice exposed to LPS before RSV infection. This suggests the decreased RSV viral load in LPS exposed mice is likely a result of the increased inflammatory monocyte recruitment. Inflammatory monocyte recruitment during RSV infection is driven by chemokines released in response to IFNAR signalling (15). Despite the increased inflammatory monocyte recruitment, IFN- α was significantly reduced in the lungs of mice exposed to LPS before RSV infection. Interestingly, mice exposed to LPS before RSV infection had increased expression of *Ifnb* at day 1 post infection compared to RSV only mice. It is not clear why *Ifnb* may be significantly increased whilst IFN- α was decreased in mice exposed to LPS before RSV infection. However, macrophages have previously been shown to release IFN- β in response to bacterial infection (340). In addition, inflammatory monocytes have been shown to produce IFN- β during RSV infection, suggesting the increase in the recruitment of these cells could be responsible for the increased expression of *Ifnb* (341). Potentially, an early increase in *Ifnb* could result in negative

feedback on the release of IFN- α . In addition, IFN- α and IFN- β have previously been suggested to have differing biological functions during viral infection (342).

In addition to controlling viral replication during the innate phase of RSV infection, inflammatory monocytes were also found to partially contribute to the early weight loss observed in mice exposed to LPS before RSV infection. Inflammatory monocytes have previously been linked to early weight loss in mouse models of RSV infection. α -CCR2 depletion has been shown to ameliorate weight loss and promote survival in juvenile mice infected with IAV (343). Similarly, RSV infected *Mavs*^{-/-} mice, which lack inflammatory monocyte recruitment, do not experience any weight loss at day 1 post infection (15,154). Intranasal delivery of recombinant CCL2 restored this early weight loss (15). It therefore makes sense that increased inflammatory monocyte recruitment driven by prior LPS exposure is contributing to exacerbated early weight loss. However, inflammatory monocyte depletion did not alter weight loss on day 1 post RSV infection in mice exposed to LPS before infection. Instead, depletion accelerated their recovery. This suggests inflammatory monocytes are not driving the early weight loss, rather prolonging it. Interestingly, unlike mice exposed to LPS, mice infected with *A. baumannii* before RSV infection begin to recover on day 2 post infection. Therefore, it is possible inflammatory monocytes are not contributing to weight loss in this model. Co-infection did result in inflammatory monocyte recruitment, but this was not significantly different on day 1 post infection and numbers were not quantified on day 2 post infection.

How inflammatory monocytes prolong weight loss in this model is not clear. Monocytes have been shown to be major producers of TNF- α during RSV infection (15). Prolonged production of TNF- α by CD8⁺ T cells has been suggested to exacerbate disease in RSV infected mice (167). However, monocyte depletion did not affect the expression of *Tnfa* nor did it alter the expression of other cytokines found to be elevated in mice exposed to LPS before RSV infection. This suggests LPS exposure before RSV infection is primarily inducing TNF- α from a separate source. In addition to cytokine production, inflammatory monocytes have also been suggested to induce apoptosis of epithelial cells, contributing to pathology and sustained weight loss in mice (177). In mice coinfecting with IAV and *Streptococcus pneumoniae* increased recruitment of monocytes expressing TNF-related apoptosis inducing ligand (TRAIL) were identified as major inducers of lung pathology and lethality (177). TRAIL engages death receptor 5 (DR5) expressed on epithelial cells resulting in the induction of apoptosis of that cell (344). It would be interesting to detect expression of TRAIL on the inflammatory monocytes recruited in the LPS-RSV model and if anti-TRAIL treatment affects disease outcomes.

Early weight loss in mice exposed to LPS before RSV infection was found to be orchestrated by elevated expression of *Il1a*, *Il1b* and *Tnfa*. Blocking these antibodies in combination resulted in amelioration of early weight loss. This protection appeared to be mediated predominantly by blocking IL-1 α and TNF- α , as α -IL-1 β blockage had no effect on weight loss. RSV infection on its own induced the expression of all three of these cytokines. However, importantly when mice were exposed to LPS before infection, the expression of these cytokines was significantly elevated and prolonged. This indicated dysregulated control over their expression. Uncontrolled and elevated cytokine production, is associated with poor disease outcomes following respiratory viral infection, including RSV, IAV and SARS-CoV-2 infection (345–347). It is possible that exposure to bacterial PAMPs before RSV infection primes lung resident cells to express uncontrolled levels of pro-inflammatory cytokines. This was shown previously where intranasal delivery of LPS and SARS-CoV-2 S protein together led to dysregulated and increased expression of pro-inflammatory cytokines in the lungs of mice (348). The source of these cytokines in mice exposed to bacterial PAMPs before RSV infection is not clear, however as previously discussed it does not appear to be inflammatory monocytes. The rapid expression of these cytokines after intranasal LPS suggests a source resident in the lung such as AMs or epithelial cells. AMs have been shown to release IL-1 α , IL-1 β and TNF- α in response to LPS stimulation or RSV infection (15,144,349–352). Airway epithelial cells have also been shown to release pro-inflammatory cytokines in response to LPS or viral infection (353,354).

Regardless of their source, these cytokines appear to have a profound effect on directing inflammation and severity in LPS exposed, RSV infected mice. In addition to ameliorating weight loss, blocking IL-1 α , TNF- α or all three cytokines in combination also significantly reduced the recruitment of neutrophils and inflammatory monocytes. This suggests that IL-1 α and TNF- α are important in driving the elevated infiltration of innate immune cells observed in these mice. It is not clear how these cytokines signal this recruitment. Both IL-1 α and TNF- α have been shown to upregulate the expression of adhesion molecules on the surface of monocytes, ultimately promoting their recruitment and extravasation through tissues (355,356). In addition, IL-1 α has been shown to drive a pro-inflammatory response in fibroblasts in the lung which have previously been suggested to be major sources of CXCL1 during RSV infection (16,357). The excess recruitment of these innate immune cells driven by IL-1 α and TNF- α could be responsible for the early weight loss. Whilst depletion of neutrophils and inflammatory monocytes on their own did not fully ameliorate weight loss in the same way, potentially depletion of both populations together would provide protection. It would also be interesting to connect the loss of these innate immune cell populations and/or cytokines to changes in lung pathology using histology.

Pro-inflammatory cytokines can also drive weight loss independent of lung pathology in mouse models. This is because weight loss can occur as a result of appetite loss driven by cytokine signalling in the brain (358). Combination injection of both TNF- α and IL-1 β into the cerebral ventricles drives loss of appetite and subsequent weight loss in mice (359). Appetite is thought to be controlled by the interconnected Arc neurons with opposing effects: neuropeptide Y (NPY) neurons which stimulate appetite and proopiomelanocortin (POMC) neurons which suppress appetite (360). TNF- α has previously been shown to suppress the production of NPY from neurons in the hypothalamus, whilst IL-1 β has been shown to drive production of POMC. The excess expression of these cytokines in mice exposed to LPS, LTA or *A.baumannii* 12h before RSV infection, may work individually or in tandem to suppress appetite in mice for longer during the first days of infection. The ability of these cytokines expressed in the lungs to enter circulation and cross into the brain was not assessed in this model. TNF- α , IL-1 α and IL-1 β have all previously been shown to be able to cross the blood brain barrier (361–363). Specifically, IL-1 α levels have been found to increase in the brain following both RSV infection and intranasal delivery of IL-1 α (166). In this study, IL-1 α antibody blockade was also found to completely protect mice from RSV induced weight loss (166).

The findings that IL-1 α but not IL-1 β , functions to drive early weight loss in mice exposed to LPS before RSV infection, were surprising given both cytokines bind and act through the same receptor, IL-1R. It is important to note successful IL-1 β blockade could not be confirmed in this study, therefore the role of this cytokine cannot be concluded. However, despite their common receptor IL-1 α and IL-1 β have previously been shown to have differential effects in mouse models of infection. In mice infected sub-cutaneously with group A *Streptococcus* had spatially different effects, with IL-1 α inducing a unique transcriptomic profiling of the liver, whilst IL-1 β appearing to exert a greater effect on the transcriptome in the spleen (156). In the lungs, IL-1 α but not IL-1 β , was also previously shown to be important in driving neutrophil recruitment following infection with the bacterial pathogen *Legionella pneumophila* (364). These studies support the findings in this thesis, indicating IL-1 α and IL-1 β can exert differential functions during infection and that this is not a new phenomenon. The biological mechanism which results in this non-redundancy is not clear. One possible explanation may be the cellular source and localisation of these cytokines. As previously discussed, the source of these cytokines in this model has not yet been elucidated, however previous data suggests IL-1 α may predominantly be produced by epithelial cells whilst IL-1 β is more likely produced from AMs (144,357). Therefore, it is possible that when released by epithelial cells, IL-1 α more readily enters the circulation, eventually crossing the blood brain barrier and exerting control on appetite. In contrast, if predominantly expressed by AMs, IL-1 β may remain in the airways, exerting a more localised effect on inflammation. In support of this hypothesis, whilst IL-1 α

levels increase in the brain following RSV infection, IL-1 β levels in the brain were not increased (166). In the context of the LPS-RSV model, it would not only be interesting to identify the cellular source of these cytokines but also detect levels of these cytokines in the circulation and brain. In addition, in this chapter, IL-1 α and IL-1 β were only quantified by gene expression. This was due to limitations with detecting these cytokines in the BAL using standard ELISA kits. Both IL-1 α and IL-1 β are initially released in a pro-form. Pro-IL-1 β and requires NLRP3 activation of caspase 1 for its cleavage into the active form, only then can IL-1 β can signal via IL-1R (365). In contrast, pro-IL-1 α is cleaved by enzymes like calpain, GzmB and NE. However, pro-IL-1 α is active and can signal through IL-1R, cleavage only further promotes its activity (366). Therefore, in the mice exposed to LPS before RSV infection, IL-1 α may be having a faster effect on inflammation compared to IL-1 β which due to its processing requirements may act later in the course of infection.

In summary, exposure to bacterial PAMPs promotes the expression of pro-inflammatory cytokines, particularly *Il1a* and *Tnfa*, and drives the recruitment of innate immune cells. When mice are in then infected with RSV 12h later, this dysregulates the expression of *Il1a* and *Tnfa* leading to prolonged innate immune cell recruitment into the lungs. The elevated inflammatory response in the lung ultimately leads to rapid early weight loss following infection potentially by increasing IL-1 α and TNF- α in the brain (Fig. 5.15). Several studies of RSV and respiratory bacteria co-infection have been carried out in the past, but the majority focus on how RSV can drive susceptibility to bacterial infection (367–370). In these studies mice were first infected with RSV and subsequently infected with the bacterial pathogen. Infecting with RSV prior to bacterial exposure may be relevant in the context of hospital acquired bacterial pneumonia following admittance for RSV infection. However, it is not relevant when considering how environmental exposure to bacteria and their PAMPs or changes to the lung microbiota could direct severity toward RSV. Few studies have utilised mouse models which have been exposed to bacteria before RSV infection. Those which have, have not focused on the innate immune response or disease exacerbation (371,372). Therefore, in this chapter we show for the first time that bacterial PAMPs exacerbate disease outcomes in mice during the innate phase of RSV infection. Exposing mice to LPS, LTA or *A. baumannii* before RSV infection demonstrates this mechanism is likely not unique to LPS exposure and can be replicated by both gram-negative and gram-positive bacteria. Therefore, these findings could help us better understand requirements for the treatment, identify at risk individuals and develop broad spectrum interventions towards RSV infection in humans.

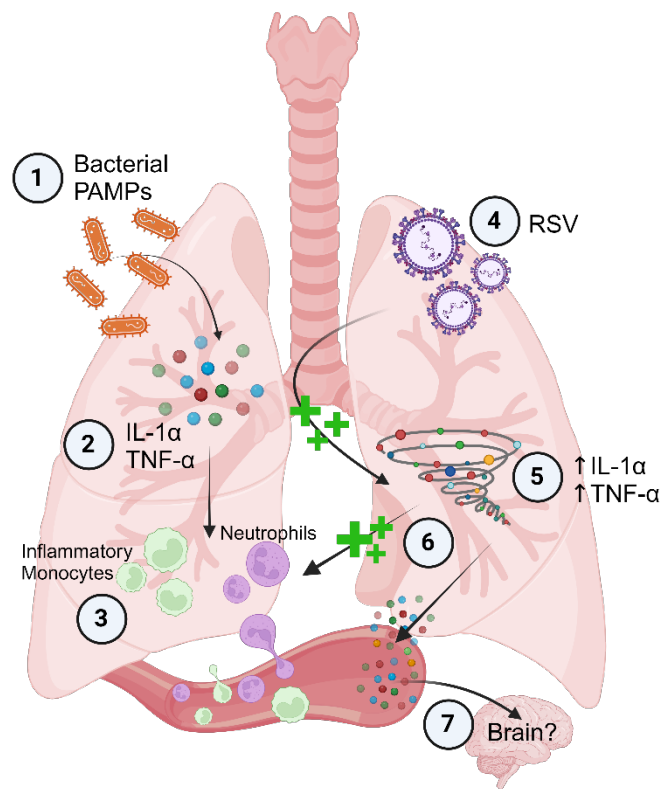


Figure 5.15 Graphical Summary

(1) Intranasal exposure to bacterial PAMPs increases the expression of cytokine and chemokines, in particular, (2) IL-1 α and TNF- α . (3) The expression of cytokines and chemokines drives the recruitment of innate immune cells including neutrophils and inflammatory monocytes into the lung. (4) When mice are infected 12h later with RSV this further (5) promotes the expression of IL-1 α and TNF- α in the lung. (6) These cytokines prolong the recruitment of neutrophils and inflammatory monocytes into the lung and (7) potentially enter the circulation and travel to the brain to exert control over appetite. Created using BioRender.

6. Neutrophils recruited into the lungs can dynamically and transiently alter the immune response to RSV

6.1 Introduction

Neutrophils have traditionally been considered as basic cells which function in frontline defence against microbes and are homogenous in their phenotype (373). However, more recently it is accepted neutrophils are dynamic in their phenotype and can have specialised functions in modulating the immune response (373). Despite their short life-span, neutrophils will undergo gradual and continuous alterations in their phenotype in a process termed neutrophil aging (243,374,375). Using mouse models, the aging process has been shown to be driven by the microbiota in a TLR and MyD88 dependent manner (375). The continuous exposure to the microbiota “primes” neutrophils causing them to upregulate genes associated with neutrophil activation, including TLR, NLR and NF- κ B signalling pathways (375). Aged neutrophils also have increased surface expression of adhesion and migration associated molecules including CD11b and CXCR4, suggesting they may better respond to chemotactic signals (375,376). The contribution of aged neutrophils to disease is not entirely clear, but they have been shown to have differential effects on inflammation and immune cell responses in contrast to their newly matured counterparts (375,377). For example, in response to LPS *in vitro*, aged neutrophils have been shown to produce more proinflammatory cytokines and ROS (375). Aged neutrophils have also been found to be particularly abundant in the skin of psoriasis patients (377). When co-cultured with T cells, these aged neutrophils promoted the release of IL-17 and IFN- γ from T cells, suggesting they can directly modulate the T cell response (377).

Neutrophils not only have the propensity to shape the immune responses during their life span, but also in their death (243). Neutrophils can undergo a number of different forms of cell death, the majority of which will contribute to tissue damage and promote inflammation (243). Under stress and inflammation, neutrophils can undergo proinflammatory forms of cell death, including NETosis, necrosis, necroptosis and pyroptosis (273). Through these mechanisms, the neutrophil membranes become compromised, and their toxic cytoplasmic contents are released into the extracellular environment (273). This includes a multitude of inflammatory mediators and DNA, which can further drive inflammation as well as degradative effector proteins which can damage neighbouring cells leading to the release of DAMPs (273). In contrast, under homeostatic conditions or the absence of stress/ inflammation, neutrophils will predominantly undergo apoptosis (378). Apoptosis is considered a non-inflammatory form of cell death as the neutrophil contents will be packaged up into membrane bound blebs, preventing the release of any inflammatory mediators (378). Neutrophil apoptosis has been

shown to be essential in promoting resolution and tissue repair, limiting further inflammation (379). In the lung, this is largely mediated by AMs which phagocytose apoptotic material, in a process termed efferocytosis (380). Efferocytosis induces gene expression changes in macrophages shifting them toward a more anti-inflammatory phenotype which has previously been characterised by increased expression of PGE₂ and TGFβ (381). This change in phenotype has been demonstrated to alter the macrophage response to inflammatory stimuli. For example, macrophages challenged with LPS following efferocytosis displayed lower production of TNF-α (382).

In mouse models of RSV infection, recruitment of neutrophils into the lungs by intranasal CXCL1 12h before infection, results in exacerbated weight loss (170). During RSV infection, the presence of more neutrophils in the lungs does not drive exacerbated weight loss (17). Therefore, exacerbated disease results from how these neutrophils alter the lung environment and subsequent immune response to RSV. It was shown that neutrophils in the lungs before RSV infection, resulted in increased recruitment of CD8⁺ T cells later in the course of disease. These CD8⁺ T cells were in turn responsible for driving the increased weight loss (170). However, following recruitment by intranasal CXCL1 and in the absence of any other stimuli, it is likely that neutrophils will rapidly age and initiate cell death. Given their ability to shape immune environments changes during their life-span, it is unclear if extending the time between neutrophil recruitment and RSV infection would have the same effects on disease. Therefore, in this chapter, the neutrophils were recruited at increasing time intervals before RSV infection in mice and the resulting effect on weight loss and inflammation was studied.

6.2 Results

6.2.2 *Intranasal CXCL1 2 days before RSV infection protected mice from weight loss*

Intranasal CXCL1 drives rapid neutrophil recruitment into the lungs, peaking 6h after intranasal followed by a decline in numbers (Fig. 5.1b). To study how changing the time interval between intranasal CXCL1 and RSV infection affects disease outcomes, were administered an intranasal dose of CXCL1, then subsequently with RSV either 0.5, 2, 4 or 7 days later (Fig. 6.1a). Weight loss was monitored up to day 8 post infection as a measure of disease severity. In line with previously published data (170), mice which received CXCL1 0.5d before RSV infection lost significantly more weight at day 5-6 post infection compared to RSV only controls (Fig. 6.1b). In contrast, mice which received CXCL1 2d before RSV infection were significantly protected from weight loss, displaying no weight loss comparable to PBS controls (Fig. 6.1c). Significant protection from RSV induced weight loss was also seen in mice exposed to CXCL1 4d before RSV infection (Fig. 6.1d). Mice which were infected with RSV 7d after intranasal CXCL1 did not differ significantly in weight loss compared with RSV only mice (Fig. 6.1e). Changes in weight loss were not associated with any changes in viral load measured as L gene copy number/ μ g RNA by qPCR, on day 8 post infection (Fig. 6.1f). This suggests neither exacerbation nor protection is associated with changes to the dynamics of viral clearance. Overall, this data demonstrates extending the time period between neutrophil recruitment and RSV infection from 0.5d to 2d results in opposing effects on disease. If neutrophils are recruited 7 days before RSV infection, they no longer alter disease, demonstrating their effect on RSV infection is transient and short-lived.

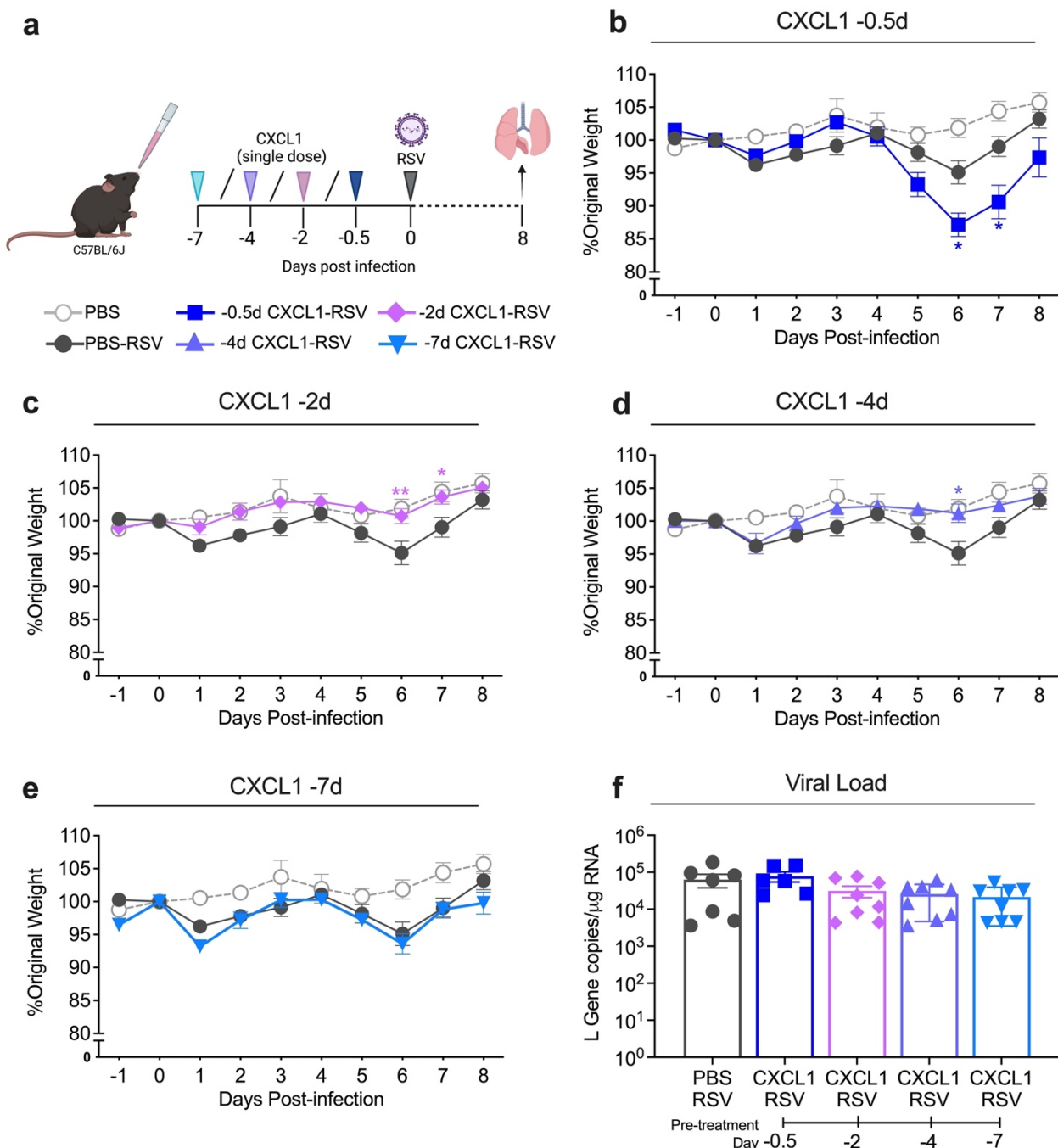


Figure 6.1 Neutrophil recruitment 2-4 days before RSV infection protected mice from weight loss

a) Graphic describing experimental design. Mice received a single intranasal dose of $5\mu\text{g}$ CXCL1. 0.5 (12h), 2, 4 or 7 days later mice were infected with RSV (7×10^6 FFU). Weight loss represented as percentage of original body weight on day 0 post infection in mice which received CXCL1 **b)** 0.5d, **c)** 2d, **d)** 4d or **e)** 7d before RSV infection, compared to RSV only controls. **f)** Viral load at day 8 post infection measured as L gene copy number/ μg RNA normalised to *Gapdh* quantified using qPCR. For -0.5d CXCL1-RSV and RSV only data are pooled from 3 separate experiments. -2d CXCL1-RSV, -4d CXCL1-RSV and -7d CXCL1 data are pooled from 2 separate experiments. PBS only is from one experiment and the same data is presented in b-e. -0.5d CXCL1-RSV n=11, -2d CXCL1-RSV, -4d CXCL1-RSV, -7d CXCL1-RSV n=8; RSV only n=7; PBS only n=3. Weight loss is plotted as mean $\pm$ SEM. For bar graphs, error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was carried out

to compare the CXCL1-RSV groups with RSV only controls. For viral load, a one-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-RSV groups with RSV only controls. Asterisks represent the p value compared to RSV only controls; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

6.2.2 CXCL1 given 2 days before RSV infection reduced the recruitment of Granzyme⁺ CD8⁺ T cells into the lungs and airways

Having found intranasal CXCL1 2d before RSV infection to result in protection from weight loss between days 5-7 post infection compared to CXCL1 0.5d before RSV infection which exacerbated weight loss, these two timepoints were selected for further comparison. CXCL1 0.5d before RSV infection was previously shown to drive weight loss by increasing the recruitment of CD8⁺ T cells into the lung. Therefore, it is possible protection from weight loss could also be associated with changes to the T cell response. To investigate this, mice were exposed to CXCL1 either 0.5 or 2d before RSV infection and lungs and BAL were collected for flow cytometry analysis on day 6 and 8 post infection (Fig. 6.2a). RSV infection increased CD8⁺ T cell recruitment into the lungs and airways on day 6 post infection which continued to increase on day 8 post infection. Confirming previous findings, CD8⁺ T cells were significantly increased in the lungs and airways of mice which received CXCL1 0.5d before RSV on day 8 post infection. On day 8 post infection, CD8⁺ T cells did not differ from RSV controls in the lungs and airways of mice which received CXCL1 2 days before RSV infection (Fig. 6.2b-c). However, on day 6 post infection CD8⁺ T cells trended lower in these mice compared to RSV controls (Fig. 6.2b). This was more clearly seen in the airways here CD8⁺ T cell recruitment was significantly lower compared to RSV controls (Fig. 6.2c). In addition to a trend in lower numbers on day 6 post infection, a lower percentage of CD8⁺ T cells recruited to the lungs of mice which received CXCL1 2 days before infection expressed granzyme B (Gzmb) compared to RSV controls (Fig. 6.2d). RSV infection also induced the recruitment of CD4⁺ T cells into the lungs and airways on day 6 post infection, which continued to increase on day 8 post infection compared to RSV controls (Fig. 6.2e-f). CD4⁺ T cells in the lung were not affected by CXCL1 0.5d or 2d before infection on day 6 or day 8 post infection (Fig. 6.2e). In the airways, CXCL1 0.5d before infection significantly increased CD4⁺ T cell recruitment on day 6 and 8 compared to RSV controls. CXCL1 2d before RSV had no effect on airway CD4⁺ T cells compared to RSV controls (Fig. 6.2f). These data suggest that whilst an increase in CD8⁺ T cell recruitment was associated with exacerbated disease, protection was associated with a trend to slower CD8⁺ T cell recruitment with reduced Gzmb expression.

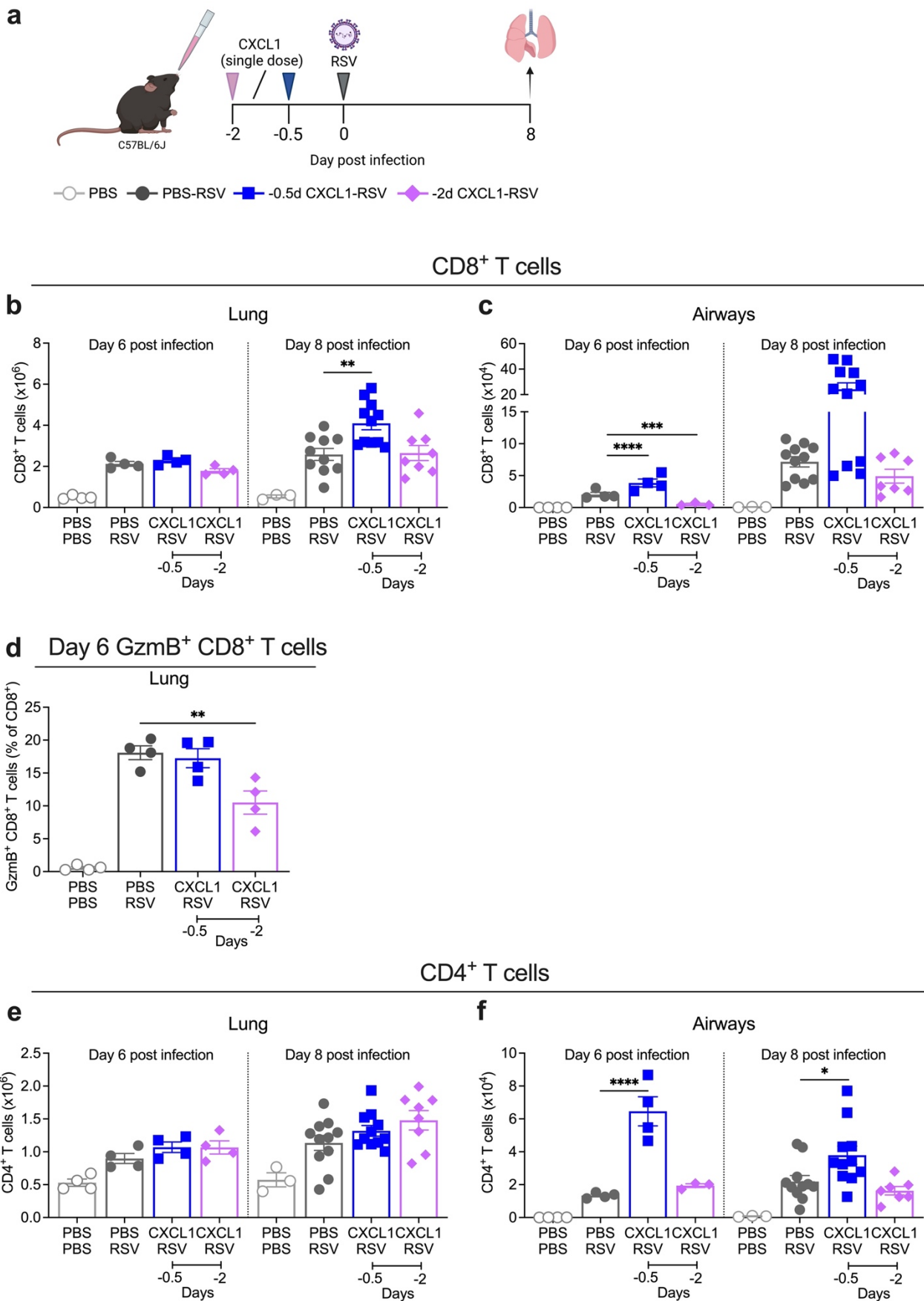


Figure 6.2 Lower expression of GzmB on CD8⁺ T cells in mice which received CXCL1 2d before RSV infection

a) Graphic describing the experimental design. Mice received intranasal CXCL1 either 0.5 or 2d before RSV infection. On day 6 or 8 post infection lungs and BAL were collected for flow cytometry analysis of the adaptive immune response. Total numbers of CD8⁺ T cells in the **b)** lung and **c)** airways (BAL) at day 6 and 8 post infection. **d)** Percentage of lung CD8⁺ T cells expressing GzmB (granzyme B) on day 6 post infection. Total numbers of CD4⁺ T cells in the **e)** lung and **f)** airways (BAL) at day 6 and 8 post infection. On day 6, data were collected in one experiment, n=4 (-2d CXCL1-RSV BAL, n=3). On day 8 post infection, -0.5d CXCL1-RSV and RSV only data are pooled from 3 separate experiments; -2d CXCL1-RSV data are pooled from 2 separate experiments; PBS only is from one experiment. -0.5d CXCL1-RSV n=11; -2d CXCL1-RSV n=8; RSV only n= 7; PBS only n=3. Error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. A one-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-RSV groups with RSV only controls. Asterisks represent the *p* value; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

6.2.3 Neutrophils and their mediators in the lungs of mice exposed to CXCL1 2 days before infection were not altered compared to RSV control mice

Next, it was assessed how intranasal CXCL1 would alter the neutrophil population in the lungs after RSV infection. Mice received CXCL1 either 0.5 or 2d before RSV infection and the neutrophil response in the lungs and airways were profiled at 18h post infection using flow cytometry and ELISA (Fig. 6.3a). In mice exposed to CXCL1 0.5d before RSV infection, the neutrophil population in the lungs and airways were significantly higher compared to RSV only controls (Fig. 6.3b-c). This increase in neutrophil counts was not noted in the lungs and airways of mice exposed to CXCL1 2d before RSV infection, where neutrophils were significantly lower than the 0.5d CXCL1 group but comparable to the RSV only group (Fig. 6.3b-c). Corresponding with the increased neutrophil populations, levels of neutrophil granular proteases NE, MPO and MMP-9 were all significantly increased in the airways of mice which received CXCL1 0.5d before RSV infection compared with RSV controls (Fig. 6.3e-g). In mice which received CXCL1 2d before RSV infection, levels of these proteases were comparable to RSV only mice (Fig. 6.3e-g). As recruitment of neutrophils and their proteases are known to disrupt epithelial barriers, leakage of serum albumin into the airways was measured by ELISA. Airway albumin was significantly increased in mice which received CXCL1 0.5d before RSV infection compared to RSV only controls, but not in mice which received CXCL1 2d before infection (Fig. 6.3d). These data suggest intranasal CXCL1 0.5d before RSV infection results in elevated neutrophilia in the lungs at 18h post RSV infection. This elevated neutrophilia is lost in mice which received CXCL1 2 days before infection.

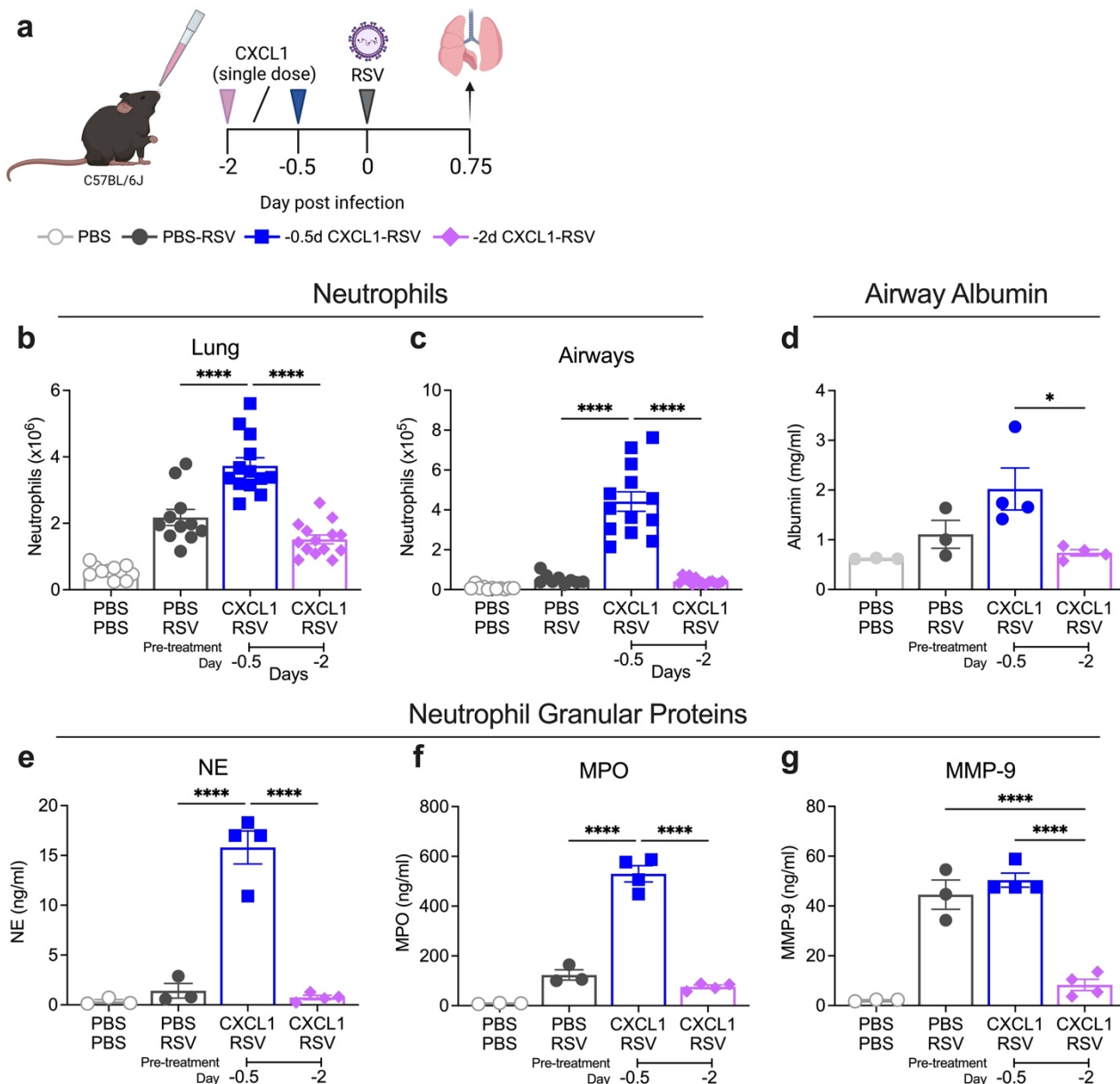


Figure 6.3 Neutrophilic inflammation at 18h post infection was not altered in mice which received CXCL1 2d before RSV infection compared with RSV only mice

a) Graphic describing experimental setup. Mice received a single intranasal dose of CXCL1 0.5 or 2d before RSV infection. At 18h post infection, lungs and BAL were collected for flow cytometry, ELISA and qPCR analysis of the innate immune response. Total number of neutrophils in the **b)** lung and **c)** airways at 18h post infection. **d)** Levels of albumin detected in the airways at 18h post infection measured by ELISA. Levels of neutrophil mediators including **e)** neutrophil elastase (NE), **f)** myeloperoxidase (MPO) and **g)** matrix metalloprotease-9 (MMP-9) in the airways measured by ELISA. For flow cytometry data, data are pooled from 3 separate experiments. -0.5d rCXCL-RSV n=13; -2d CXCL1-RSV n=14; RSV only and PBS only n=11. For ELISA data, data are from one experiment. -0.5d CXCL1-RSV and -2d CXCL1-RSV n=4; RSV only and PBS only, n=3. Error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. A one-way ANOVA with multiple comparisons test was carried out to compare between groups. Asterisks represent the *p* value; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

6.2.4 Protection from RSV induced weight loss was associated with increased alveolar macrophages in the lung.

The effect of intranasal CXCL1 before RSV infection on other innate immune cell populations in the lungs and airways was also assessed at 18h post infection using flow cytometry. The population of AM was not altered by RSV infection compared to PBS controls. In mice which received CXCL1 0.5d before RSV infection a trend to higher AM numbers was seen, but this was not significant compared to RSV controls. In mice which received CXCL1 2d before RSV infection the AM population in the lungs was significantly increased (Fig. 6.4a). In the airways, AMs were not significantly altered by RSV or CXCL1 before RSV infection but trended higher in mice which received CXCL1 2 days before infection (Fig. 6.4b). At 18h post RSV infection, inflammatory monocytes were increased in the lung compared to PBS controls. Intranasal CXCL1 either 0.5d or 2d before RSV infection had no effect on inflammatory monocyte recruitment at this time point (Fig. 6.4c). A similar trend could be noted in inflammatory monocytes in the airways, although numbers were overall low, RSV infection increased recruitment and CXCL1 had no significant effect on this (Fig. 6.4d). This data suggests that in addition to recruiting neutrophils, intranasal CXCL1 before RSV infection induces an increase in the AM population in the lung at 18h post infection. To identify if protection or an increased AM population correlated with any changes in early viral load, this was measured in the lungs at 18h post infection using qPCR to quantify L gene copy number/ μ g RNA. In agreement with previous data (170), viral load at 18h post infection was significantly higher in the mice which received CXCL1 0.5d before infection compared with RSV only controls (Fig. 6.4e). However, in mice which received CXCL1 2d before infection, viral load was comparable with RSV only mice (Fig. 6.4e). This suggests that CXCL1 2 days before infection does not alter early viral load, thus protection is more likely to be driven by the immune response to the virus.

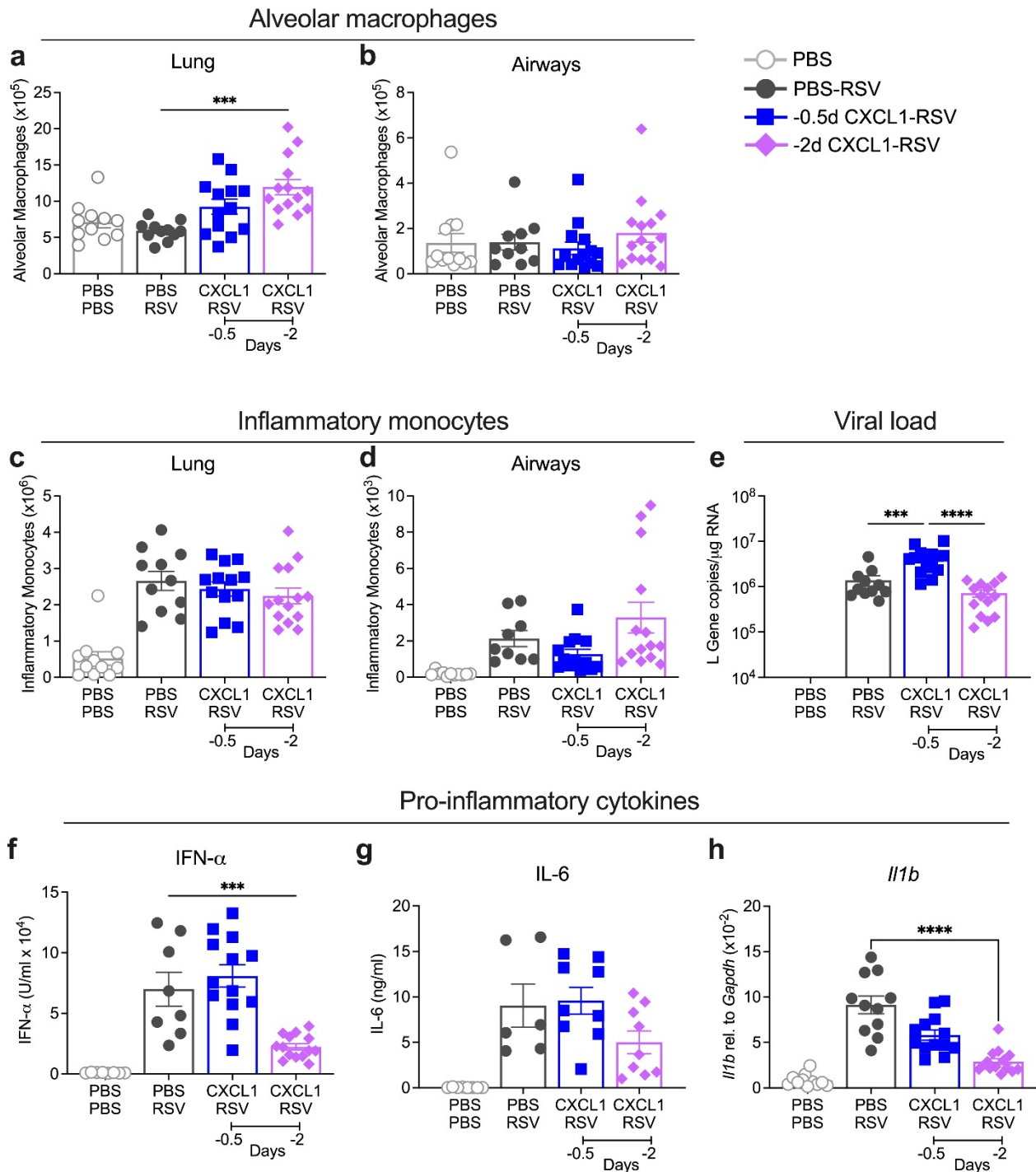


Figure 6.4 Intranasal CXCL1 2 days before RSV infection increased AM in the lung and dampened AM associated pro-inflammatory cytokines

Total number of AMs in the **a**) lung and **b**) airways at 18h post infection. Total number of inflammatory monocytes in the **c**) lung and **d**) airways at 18h post infection. **e**) Viral load in the lungs at 18h post infection measured as L gene copy number/ μ g RNA normalised to *Gapdh* quantified using qPCR. Levels of **f**) IFN- α and **g**) IL-6 in the BAL supernatant at 18h post infection measured by ELISA. **h**) Expression of *Il1b* relative to *Gapdh* in the lung at 18h post infection measured by qPCR. Data are pooled from 3 separate experiments. -0.5d rCXCL-RSV n=13; -2d CXCL1-RSV n=14; RSV only and PBS only n=11. For ELISA data, data are from one experiment. -0.5d CXCL1-RSV and -2d CXCL1-RSV n=4; RSV only and PBS only, n=3. Error bars represent SEM. Statistical analysis was

carried out using Graphpad Prism software. A one-way ANOVA with multiple comparisons test was carried out to compare between groups. Asterisks represent the *p* value; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

6.2.5 Intranasal CXCL1 2 days before RSV infection dampened pro-inflammatory cytokine expression in the lung

As AM are known to be important in releasing pro-inflammatory cytokines including type I IFNs, IL-6 and IL-1 β (15,144), levels/expression of these cytokines were assessed by ELISA and qPCR at 18h post RSV infection. Levels of IFN- α in the airways were increased at 18 post RSV infection compared to PBS controls. Intranasal CXCL1 0.5d before RSV infection did not affect IFN- α levels. In contrast, IFN- α was significantly reduced in mice which received CXCL1 2 days before RSV infection (Fig. 6.4f). Similarly, IL-6 was increased in the airways following RSV infection and this was not affected by CXCL1 0.5d before infection. In mice which received CXCL1 2 days before infection, IL-6 trended lower but this was not statistically significant (Fig. 6.4g). Expression of *Il1b* in the lungs was also increased following RSV infection compared to PBS controls. In mice exposed to CXCL1 0.5d before infection, *Il1b* expression was significantly lower compared to RSV only mice and was reduced further in mice which received CXCL1 2d before infection (Fig. 6.4h). This data suggests that CXCL1 2d before infection dampens the pro-inflammatory response to RSV.

6.2.6 Intranasal CXCL1 increased the AM population in the lung before RSV infection

In mice pre-exposed to CXCL1 2d before RSV infection, protection from weight loss was associated with a decline in neutrophil numbers coupled with an increase in AM numbers. To identify if these changes occur in the lung before RSV infection mice were given an intranasal dose of CXCL1 and lungs were collected 0.5d or 2d later, without subsequent RSV infection (Fig. 6.5a). The neutrophil and AM population was profiled using flow cytometry. Following CXCL1, neutrophil numbers were greatly increased in the lungs at 0.5d after CXCL1, peaking around $\sim 1 \times 10^7$ cells. 2 days after CXCL1, these numbers had declined almost to PBS baseline numbers (Fig. 6.5b). 0.5d after CXCL1, AMs were not significantly changed compared with PBS controls. However, a significant increase in AM numbers was quantified 2d after CXCL1 (Fig. 6.5c). Therefore, these data suggest the increase in AMs in the lungs of mice exposed to CXCL1 2 days before RSV infection occurs before RSV infection.

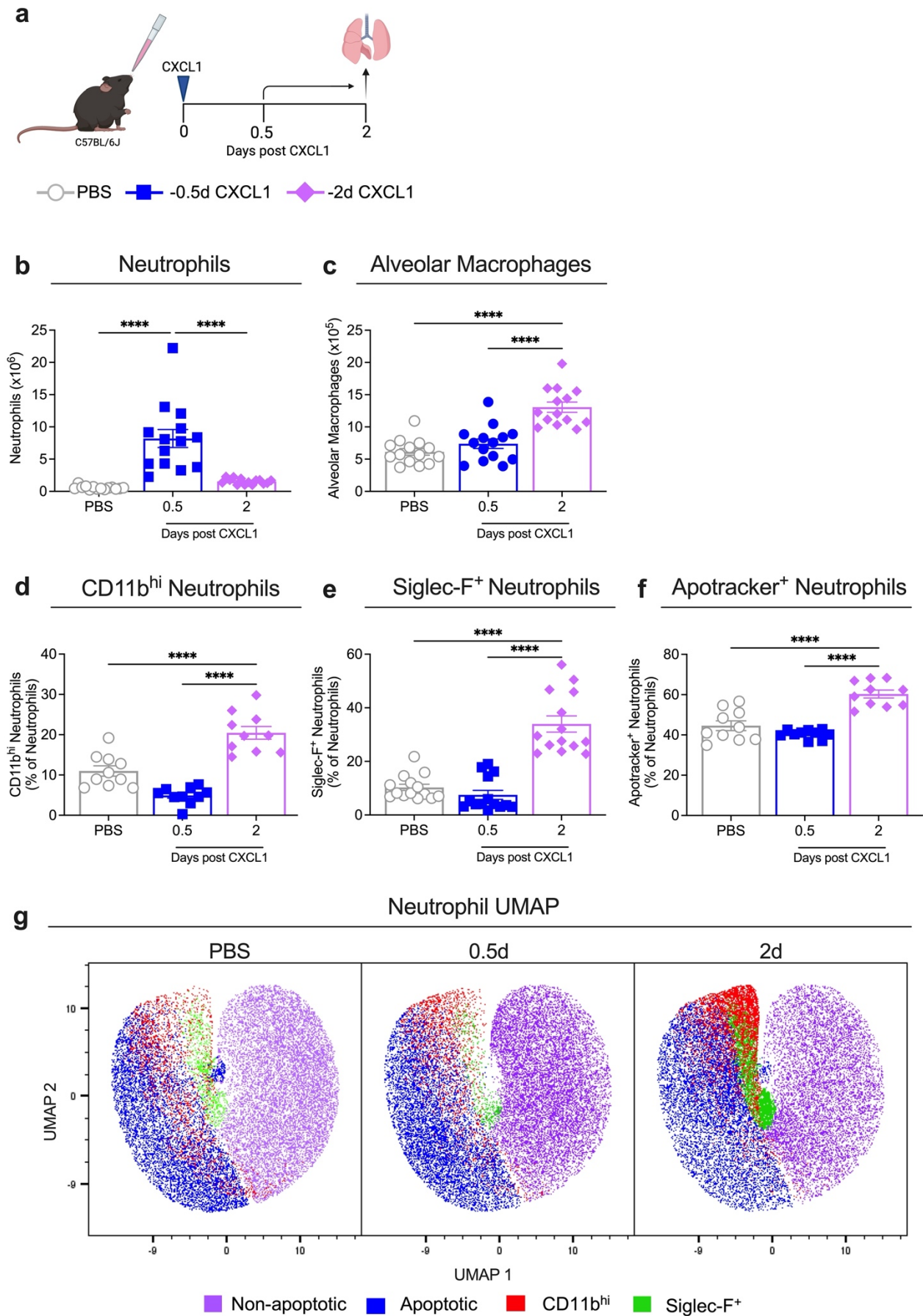


Figure 6.5 Neutrophils recruited to the lungs by CXCL1 rapidly altered their phenotype and underwent apoptosis

a) Graphic describing experimental design. Mice received an intranasal dose of CXCL1 and 0.5 or 2d later, lungs were collected for flow cytometry. Total number of **b)** neutrophils or **c)** AMs in the lung overtime following intranasal CXCL1. Percentage of neutrophil populations which stained positive for **d)** CD11b, **e)** Siglec-F or **f)** Apotracker Green, overtime following intranasal CXCL1. **g)** UMAP projection of neutrophil population in the lung based on CD11b, Siglec-F and Apotracker Green expression. For bar graphs, data are pooled from 2 separate experiments, n=10. For UMAP, data from 5 mice per group were pooled. Error bars represent SEM. Statistical analysis was carried out using Graphpad Prism software. A one-way ANOVA with multiple comparisons test was carried out to compare between groups. Asterisks represent the *p* value; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

6.2.7 Following recruitment into the lungs and airways by intranasal CXCL1, neutrophils altered their phenotype and rapidly underwent cell death

Previous studies have shown that neutrophils have the potential to influence the AM populations based on their phenotype and viability (138,144,383). Therefore, it was studied how the population of neutrophils recruited into the lungs by CXCL1 changed over time. Whilst 2d after CXCL1 neutrophil numbers were significantly decreased (Fig. 6.5b), the neutrophils which remained had specific changes in marker expression. Compared to 0.5d after CXCL1, neutrophils 2d after CXCL1 had significantly higher expression of CD11b (Fig. 6.5d). In addition, 2d after CXCL1 neutrophils had also upregulated their expression of the sialic acid binding receptor Siglec-F which was significantly lower on neutrophils 0.5d after CXCL1 (Fig. 6.5e). Corresponding with the sharp decline in neutrophil numbers between 0.5d and 2d after CXCL1, the neutrophils remaining at 2d stained greater for the apoptosis marker Apotracker Green (Fig. 6.5f). To better visualise the changes in these neutrophil populations, the flow cytometry data was projected into UMAPs based on their expression of the previously described markers. 0.5d after CXCL1, there was a dense population of non-apoptotic, CD11b^{lo}, Siglec-F⁻ neutrophils (purple). 2d after CXCL1, this population was reduced, and the neutrophil population shifted towards more apoptotic (blue), CD11b^{hi} (red), Siglec-F⁺ (green) neutrophils (Fig. 6.5g). The expression of these markers all clustered together, demonstrating that over time following CXCL1 neutrophils appear to be undergoing phenotypic alterations and increased apoptosis.

6.2.8 In vivo delivery of apoptotic neutrophils 0.5d before RSV infection protected mice from exacerbated weight loss

2d after CXCL1, neutrophil numbers had rapidly declined and those which remained were more apoptotic compared with PBS controls. Therefore, it was investigated if the presence of apoptotic neutrophils before RSV infection contributed to protection from weight loss. To generate apoptotic neutrophils, mice were given an intranasal dose of CXCL1 and 6h later BAL was collected (Fig. 6.6a). At this time point, neutrophils constituted over 90% of all BAL cells, therefore BAL cells will be referred to as BAL neutrophils from here forward (Fig. 5.1h). Apoptosis of BAL neutrophils was induced by exposure to UV radiation for 10 minutes followed by incubation at 37°C for 2h. Flow cytometry analysis revealed UV treated BAL cells stained positive for Apotracker green, confirming they were apoptotic (Fig. 6.6b). Five million apoptotic BAL neutrophils were then delivered intranasally to mice 12h before RSV infection and mice were weighed across the course of 8 days (Fig. 6.6a). Intranasal delivery of apoptotic neutrophils before RSV infection resulted in significant protection from weight loss at days 5, 6 and 7 post infection compared with RSV controls (Fig. 6.6c). The adaptive immune response in the lungs and airways of mice was studied at day 8 post infection. No significant differences were noted in the total number of CD8⁺ T cells in the lung or airways (Fig. 4.6d). However, numbers trended lower in the airways (Fig. 6.6e). Similar to CD8⁺ T cells, total number of CD4⁺ T cells in the lungs were also not significantly changed by UV BAL neutrophils compared to RSV only mice but trended lower in the airways (Fig. 6.6f-g). Compared to mice which received CXCL1 2 days before RSV infection, mice which received UV apoptotic BAL neutrophils 0.5d before RSV infection also show protection from RSV induced weight loss. This demonstrates the protective effect of apoptotic neutrophils in the lung before RSV infection.

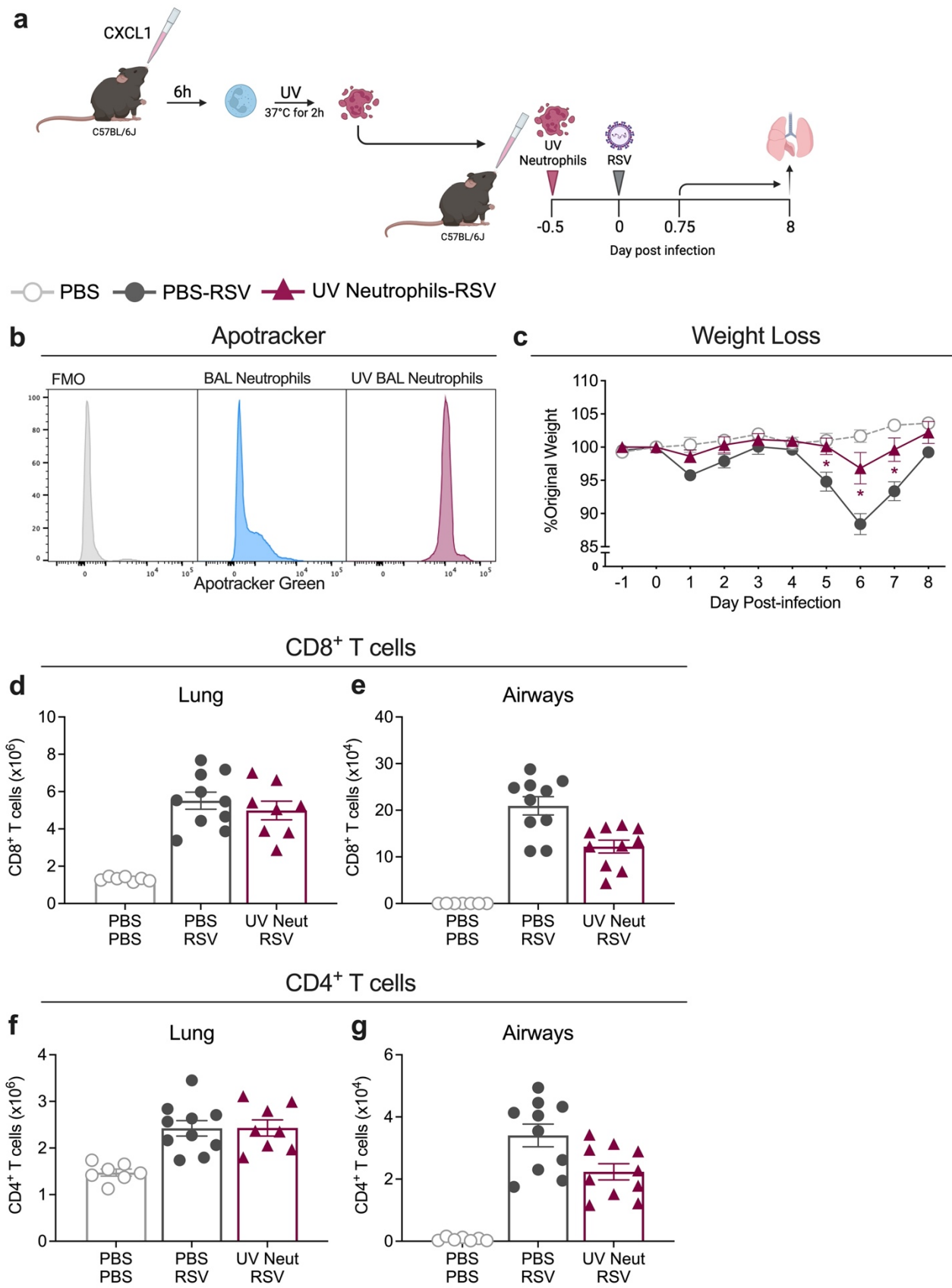


Figure 6.6 Apoptotic neutrophils in the airways before RSV infection protected mice from weight loss

a) Graphic describing experimental design. Mice received an intranasal dose of CXCL1 and 6h later mice were euthanised and BAL cells were collected. BAL cells (BAL neutrophils) were treated with UV radiation to induce apoptosis (UV neutrophils). UV neutrophils were then intranasally transferred to mice 12h before RSV infection. Mice were weighed daily and on day 8 lungs and BAL were collected for flow cytometry. **b)** Example flow cytometry histograms showing expression of Apotracker Green in viable BAL neutrophils and UV BAL neutrophils compared to FMO control. **c)** Weight loss represented as percentage of original body weight on day 0 post infection of mice which received UV neutrophils intranasally 12h before RSV infection compared to RSV only controls. Total number of CD8⁺ T cells in the **d)** lung and **e)** airways at day 8 post infection. Total number of CD4⁺ T cells in the **f)** lung and **g)** airways at day 8 post infection. Data are pooled from 2 separate experiments. UV neutrophils- RSV n=8; RSV only n=10; PBS only n=7. Weight loss is plotted as mean $\pm$ SEM. For bar graphs, error bars represent SEM. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was carried out to compare the UV neutrophil-RSV group with RSV only controls. For bar graphs, a one-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-RSV groups with RSV only controls. Asterisks represent the *p* value; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

6.2.9 Intranasal delivery of apoptotic neutrophils 0.5d before RSV infection increased the AM population in the lungs

Having found protection in mice which received apoptotic neutrophils before RSV infection to not be associated with changes to the adaptive immune response, the innate immune response at 18h post infection was studied. Total neutrophil numbers in the lung and airways were comparable between RSV infected mice and mice which received apoptotic neutrophils 0.5d before RSV infection (Fig. 6.7a-b). The mice which received apoptotic neutrophils 0.5d before RSV infection had significantly higher numbers of AMs in the lung compared with PBS and RSV only controls (Fig. 6.7c). AM numbers in the airways were not significantly different between all groups (Fig. 6.7d). Viral load in the lungs was comparable between RSV only mice and mice which received apoptotic neutrophils 0.5d before RSV infection (Fig. 6.7e). Overall, intranasal delivery of apoptotic neutrophils 0.5d before infection resulted in similar changes to innate immune cells populations in the lung at 18h post infection as seen in mice which received CXCL1 2d before infection.

As AM numbers were increased by intranasal delivery of apoptotic neutrophils before infection, the levels/expression of the AM associated pro-inflammatory cytokines was measured by ELISA and qPCR. IFN- α in the airways of mice which received apoptotic neutrophils 0.5d before RSV infection was significantly reduced compared to RSV only mice (Fig. 6.7f). In contrast, IL-6 levels in the airways and *Il1b* expression was not affected by apoptotic neutrophils before RSV infection. These data suggest that apoptotic neutrophils in the lungs

before infection can alter the pro-inflammatory cytokine response to RSV but not in the same way as CXCL1 2d before infection.

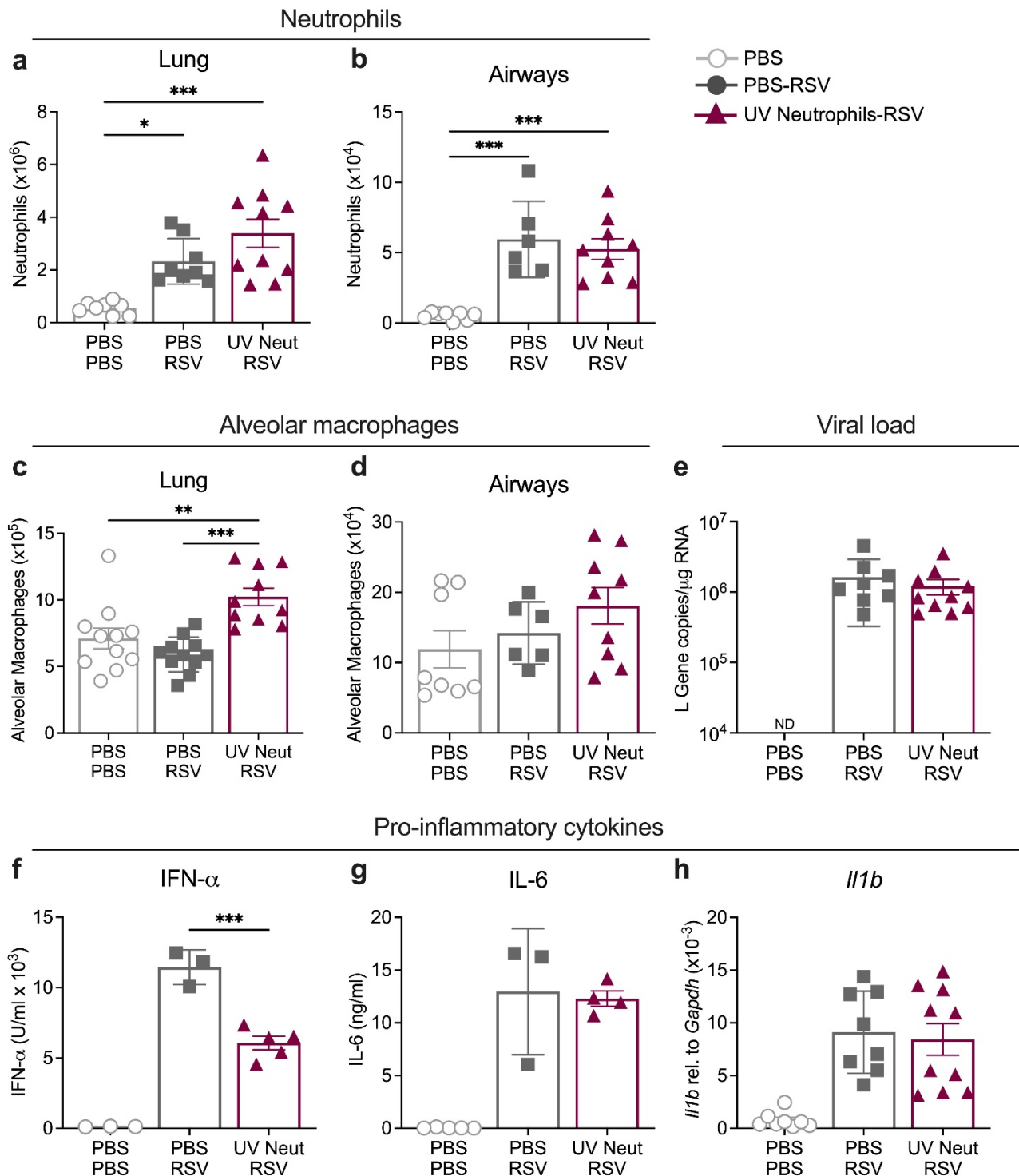


Figure 6.7 AM population at 18h post infection was increased in mice which received apoptotic neutrophils before infection

Total number of neutrophils in the **a)** lung and **b)** airways at 18h post infection. Total number of AMs in the **c)** lungs and **d)** airways at 18h post infection. **e)** Viral load in the lungs at 18h post infection measured as L gene copy

number/ μ g RNA normalised to *Gapdh* quantified using qPCR. Levels of **f**) IFN- α and **g**) IL-6 in the BAL supernatant at 18h post infection measured by ELISA. **h**) Expression of *Ii1b* relative to *Gapdh* in the lung at 18h post infection measured by qPCR. Flow cytometry and qPCR data are pooled from 2 experiments. UV neutrophils-RSV n=10; RSV only n=8; PBS only n=8. For ELISA data are from 1 experiment. UV neutrophils-RSV n=5; RSV only n=3; PBS only n=3. Error bars represent SEM. A one-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-RSV groups with RSV only controls. Asterisks represent the *p* value; * $\leq$ 0.05, ** $\leq$ 0.01, *** $\leq$ 0.001, **** $\leq$ 0.0001.

6.2.10 Apoptotic neutrophils altered the AM inflammatory response to RSV *in vitro*

Intranasal CXCL1 2 days before RSV infection results in protection from RSV induced weight loss, associated with increased apoptotic neutrophils, increased numbers of AMs in the lung and a decreased pro-inflammatory response. Similarly, intranasal delivery of apoptotic neutrophils 0.5d before RSV infection also results in protection from RSV induced weight loss associated with increased numbers of AMs in the lungs and an altered type I IFN response. Apoptotic neutrophils are cleared through the process of efferocytosis by AMs, which has previously been shown to alter their inflammatory phenotype (138,383). Therefore, it was hypothesised that following recruitment into the lungs by CXCL1 neutrophils begin to undergo apoptosis. These apoptotic neutrophils are in turn cleared by AMs, which alters their phenotype resulting in a dampened proinflammatory response to the virus. To investigate this, viable or apoptotic neutrophils were co-cultured with mouse *ex vivo* AMs (mexAMs) in a 1:1 ratio for 2, 4, 6 or 26h then infected with RSV at an MOI of 4. At 18h post infection, supernatants were collected for ELISA, and RNA extracted from lysed mexAMs for qPCR (Fig. 6.8a). Apoptotic neutrophils were generated by UV radiation as previously described. Co-culture ratios, timings and infectious dose were based on neutrophil-BMDM co-culture data previously presented by Imperial College MSc student, Sophie Guan, in her MSc dissertation. The levels/expression of pro-inflammatory cytokines associated with AMs were quantified. Supernatant levels of IFN- α were increased mexAM cultures without co-culture (media) following RSV infection compared to uninfected. When co-cultured with viable neutrophils for 2h, this did not affect IFN- α in the supernatant. In contrast, a time dependent decrease in IFN- α levels was observed in mexAMs co-cultured with apoptotic neutrophils for increasing periods of time before RSV infection. The reduction in IFN- α was statistically significant in mexAMs co-cultured with apoptotic neutrophils for 26h (Fig. 6.8b). Levels of IL-6 exhibited a similar pattern, with RSV infection increasing IL-6 in the supernatant of mexAM single cultures. Again, co-culture with viable neutrophils had not effect on IL-6 but in mexAMs co-cultured with apoptotic neutrophils, IL-6 was decreased in a time-dependent manner, with co-culture for 26h showing a significant decrease compared to mexAM infected controls (Fig. 6.8c). Expression

of *Il1b* was also induced in mexAMs following RSV infection compared to uninfected controls. Interestingly, expression was significantly potentiated in mexAMs co-cultured with viable neutrophils before infection. In contrast, co-culture with apoptotic neutrophils before infection significantly for 4h or longer, significantly reduced the expression of *Il1b* (Fig. 6.8d). Together, this data suggests increasing the time mexAMs are exposed to apoptotic neutrophils before RSV infection, significantly dampens their pro-inflammatory response to the virus.

To gain an idea if this could be due to mexAMs shifting towards a more anti-inflammatory phenotype, the expression of *Tgfb1* was measured. *Tgfb1* has previously been suggested to be a macrophage anti-inflammatory marker (384). RSV infection did not increase *Tgfb1* expression in mexAMs compared to uninfected controls. Co-culture with viable neutrophils also did not increase expression of *Tgfb1* in mexAMs. However, when co-cultured with apoptotic neutrophils for increasing time periods before RSV infection, *Tgfb1* expression increased in a time dependent manner (Fig. 6.8e). This increase was statistically significantly increased when cells were co-cultured for 26h before infection and also occurred in the absence of RSV infection (Fig. 6.8f). This suggest increasing the time mexAMs are exposed to apoptotic neutrophils shifts them towards a more anti-inflammatory phenotype.

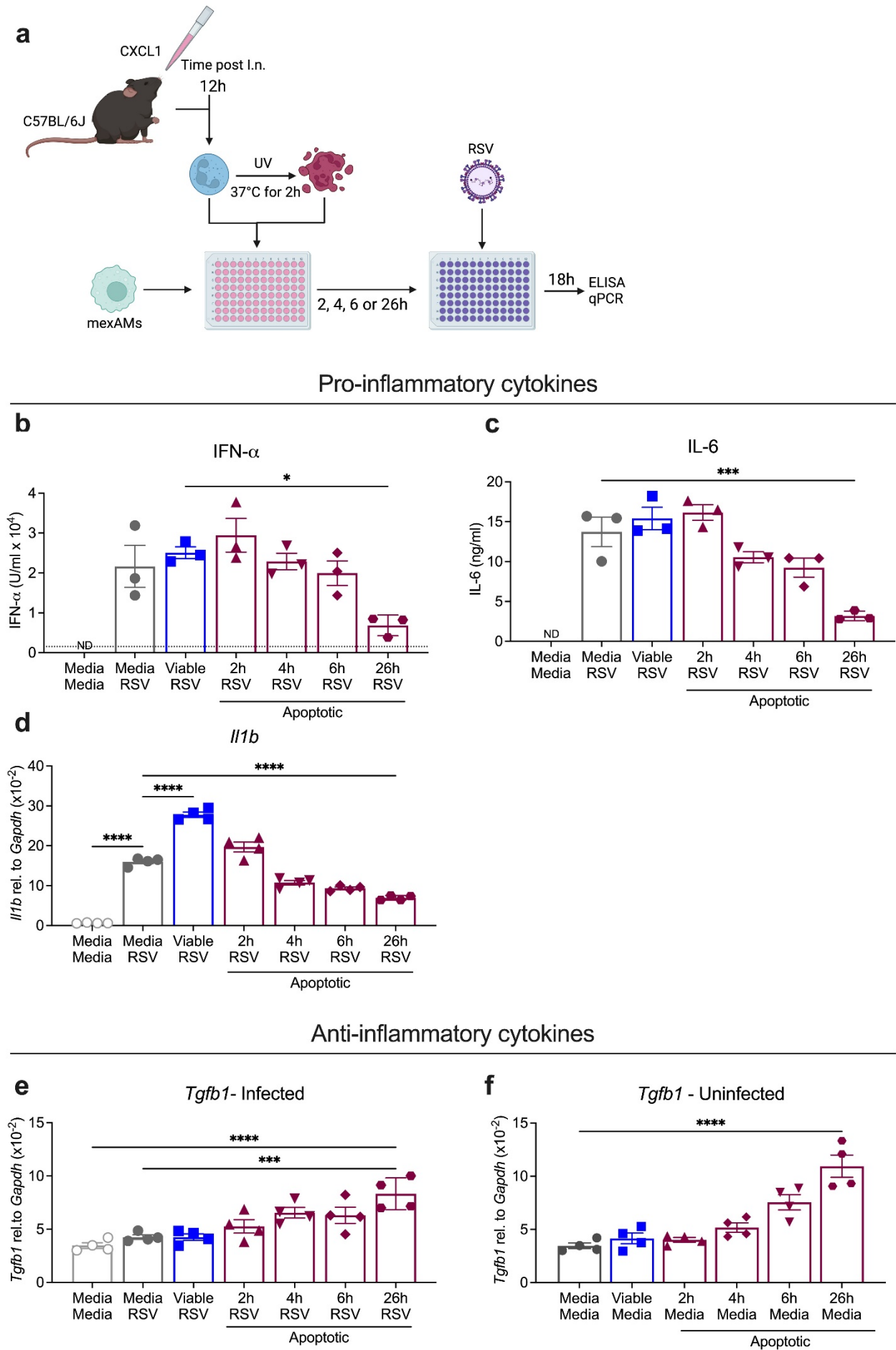


Figure 6.8 The pro-inflammatory response of mexAMs to RSV infection was altered by neutrophil co-culture

a) Graphic describing experimental design. Mice received an intranasal dose of CXCL1 and 12h later BAL cells (BAL neutrophils) were collected. Apoptotic neutrophils were generated by exposing viable BAL neutrophils to UV radiation. Viable apoptotic neutrophils were co-cultured with mexAMs for 2h, whilst apoptotic neutrophils were co-cultured with mexAMs for. 2, 4, 6 or 26h before mexAMs were infected with RSV MOI of 4. 18h after infection supernatants were collected and cells were lysed for qPCR. Supernatant levels of **b)** IFN- α or **c)** IL-6. **d)** MexAM expression of *Il1b* relative to *Gapdh*. Expression of *Tgfb1* relative to *Gapdh* in **e)** RSV infected mexAMs or **f)** uninfected mexAMS. Data are from 1 experiment. For qPCR data, RNA was extracted from 3 wells of mexAMs treated under the same condition pooled, n=4. For ELISA, each data point is supernatant from one co-culture, n=3. . Error bars represent SEM. A one-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-RSV groups with RSV only controls. Asterisks represent the *p* value; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

6.3 Discussion

Neutrophils recruited into the lungs of mice 12h before RSV infection drive exacerbated disease severity as measured by weight loss (170). In this chapter, the time between neutrophil recruitment and subsequent RSV infection was extended. Recruiting neutrophils just 36h earlier, at 2 days before RSV, had the opposite effect on disease, with mice losing significantly less weight than RSV only controls. This protective effect was observed when neutrophils were recruited up to 4 days before RSV infection. When neutrophil recruitment was extended to 7 days before RSV, any effect on weight loss was lost. These findings presented in this chapter demonstrate the transient and dynamic nature through which neutrophils can modify the inflammatory response to RSV in the lungs.

It is likely the opposing outcomes on weight loss are a result of how neutrophils change during their lifespan. Overtime following intranasal CXCL1, several changes were noted in the expression of the cell surface markers on the recruited neutrophils. This included increased expression of the β_2 integrin CD11b. CD11b is expressed on the surface of circulating neutrophils at low levels, but is stored intracellularly within secretory granules (385). Following *in vitro* exposure to LPS, CD11b is rapidly mobilised and expressed on the neutrophil surface within 5 minutes (386). *In vivo*, this expression of CD11b has been shown to function during transepithelial migration of neutrophils into the lungs (387). All neutrophils recruited into the airways will therefore express CD11b, even in the absence of RSV infection or other inflammatory signals (16). However, at day 2 post CXCL1, a secondary population of CD11b⁺ neutrophils with higher CD11b expression had greatly increased. Outside of its role in migration, CD11b expressed on neutrophils has also been suggested to function in phagocytosis (388). Increased expression of CD11b on neutrophils by exposure to TNF- α has previously been shown to contribute to increased phagocytosis of *Bordetella pertussis* (389). The contribution of neutrophil phagocytosis toward viral clearance during RSV infection is not known. However, it is likely to be negligible due to the intracellular nature of viruses (17). Supporting this, no difference was found in the viral load of mice treated with CXCL1 2 days prior to RSV infection compared with RSV only mice. It is therefore unlikely that increased CD11b expression on neutrophils is directly contributing to the protection from weight loss measured in these mice.

In addition to CD11b, neutrophils in the lungs 2 days after intranasal CXCL1 exposure had increased expression of Siglec-F on their surface. Siglec-F is a sialic acid binding immunoglobulin-like lectin more commonly associated with eosinophils and AMs (390). Studies reporting Siglec-F⁺ neutrophils have only emerged within the last few years (311,391–394). These studies have described Siglec-F⁺ neutrophils as a morphologically and

phenotypically distinct subset, derived from conventional neutrophils (311,391–394). In the airways of mice, Siglec-F⁺ neutrophils have been found to emerge following exposure to allergens including ovalbumin and diesel exhaust air pollutants (311,392). In this context, Siglec-F⁺ neutrophils were demonstrated to have a more activated phenotype and contributed to worsening of type 2 airway inflammation (311,392). Siglec-F⁺ neutrophils have also been identified in the nasal mucosa of mice following a re-infection model of *B. pertussis* (391). The accumulation of Siglec-F⁺ neutrophils was driven by IL-17. In addition to the absence of Siglec-F⁺ neutrophils, *Il17*^{-/-} mice showed reduced clearance of *B. pertussis* during re-infection (391). In this study, they demonstrated that Siglec-F⁺ neutrophils had an increased capacity to undergo NETosis and suggested they provide protection upon *B. pertussis* re-infection due to increased bactericidal activity (391). In this chapter, the ability of neutrophils to undergo NETosis was not studied. However, *in vitro* studies have previously shown that RSV can induce NET formation in ROS and peptidyl arginine deiminase 4 (PAD4) dependent pathway, which are capable of trapping RSV particles (395).

Increased expression of CD11b and Siglec-F have previously been associated with aged, long-lived neutrophils (375,393). Supporting this, the data presented in this chapter demonstrates the expression of both these markers is upregulated over time following CXCL1 in the absence of infection or any other inflammatory signals. Using UMAP projections, it was shown that expression of increased CD11b and Siglec-F co-localised to the same population of neutrophils. Aged neutrophils have previously been shown to have increased gene expression of pro-inflammatory mediators and Siglec-F⁺ neutrophils have also been suggested to have an over-active phenotype (311,375). Contrary to this, the data presented in this chapter points toward a dampened innate inflammatory response toward RSV associated with protection from weight loss later on during the course of infection. However, it is possible that aged neutrophils can influence the immune response of other cell types towards RSV. For example, aged neutrophils have previously been shown to drive expression of IFN- γ but not TNF- α from CD4⁺ T cells, both in mouse models of sepsis and in the blood of psoriasis patients (377,396).

In addition to neutrophil aging, both CD11b and Siglec-F have previously been associated with apoptosis. One study demonstrated that neutrophils isolated from *Cd11b*^{-/-} mice showed decreased apoptosis following infection with *Bordetella bronchiseptica* *in vitro*, compared with neutrophils from WT mice (397). Whilst apoptosis of Siglec-F⁺ neutrophils has not previously been reported, Siglec-F engagement has previously been shown to drive apoptosis of eosinophils (398). In this chapter, compared to 12h after intranasal CXCL1, neutrophil numbers 2 days after CXCL1 were significantly reduced, and a greater percentage of those neutrophils which remained were Apotracker Green⁺. Furthermore, the highest expression of

Apotracker Green co-localised with CD11b^{hi} Siglec-F⁺ neutrophils. Apoptotic neutrophils can shape inflammatory responses and have previously been associated with protection during respiratory infection. In a study of patients with nonresponding community acquired pneumonia, increased neutrophil apoptosis in the BAL was associated with lower levels of inflammatory cytokines and reduced duration of hospital stay (399). Given that apoptotic material is known to be cleared by macrophages, it was interesting that protection in mice which received CXCL1 2 days before RSV infection was associated with an increase in the AM population in the lungs. It has previously been shown *in vitro* and in mouse models of peritonitis and atherosclerosis, that efferocytosis of apoptotic neutrophils induces proliferation of macrophages (383). The effect of efferocytosis on AM proliferation *in vivo* has not previously been studied. However, one study identified murine AMs to exhibit higher proliferation rates compared to monocyte derived macrophages, following LPS induced lung injury and suggested this could be a result of increased efferocytosis capacity of recruited, dying neutrophils (400). In contrast, another study where BAL neutrophils recruited by CXCL1 were co-cultured with AMs *in vitro*, suggested the opposite, that neutrophil cell death inhibits AM proliferation (401). The data in this chapter supports the hypothesis of the former, as intranasal delivery of apoptotic neutrophils prior to RSV infection resulted in an increase in the AM population in the lungs. However, efferocytosis of apoptotic neutrophils by AMs was not conclusively demonstrated.

Efferocytosis of apoptotic neutrophils has previously been shown to dampen the macrophage inflammatory responses by promoting a shift towards a more anti-inflammatory phenotype (382). This shift in phenotype is important in promoting resolution following infection and to prevent chronic airway inflammation. Impaired efferocytosis of apoptotic cells is a common hallmark of a number of chronic airway disease including COPD, asthma, cystic fibrosis and idiopathic pulmonary fibrosis (402–405). In the case of each of these lung diseases, impaired efferocytosis is associated with uncontrolled inflammation and lack of resolution (406). In the context of respiratory viral infection, boosting efferocytosis in IAV infected mice, through intranasal delivery of the BCG vaccine, was suggested to enhance clearance of apoptotic cells in the lungs. The authors suggest this result in reduced inflammation and promoted survival (407). Impaired efferocytosis capacity has also been noted in lung monocytes and macrophages isolated from patients with severe COVID-19, which was suggested to contribute to excessive pro-inflammatory cytokine production and increased pathology (408).

Correlated with protection in mice which received CXCL1 2 days before RSV infection, an overall reduction in pro-inflammatory cytokines at 18h post RSV infection was measured in the lung. AMs have been shown to be major sources of several of these cytokines during RSV infection including IFN- α , IL-1 β , TNF- α and IL-6 (84,144,146). Therefore, it was hypothesised

that efferocytosis of apoptotic neutrophils by AMs shifts them to a more anti-inflammatory phenotype, thus dampening their inflammatory response to RSV. To begin testing this hypothesis, apoptotic neutrophils were co-cultured with mexAMs *in vitro* before being infected with RSV. Unlike BMDMs, MexAM cultures have been shown to be phenotypically and functionally similar to AMs in their response to inflammatory stimuli (310). Indeed, when mexAMs were exposed to RSV, expression of IFN- α , IL-1 β and IL-6 were all increased compared to uninfected controls. *In vivo* AMs in the lung will be exposed to neutrophils soon after intranasal CXCL1. However, the timepoint at which neutrophils begin to undergo apoptosis or when efferocytosis occurs is not known. Therefore, in co-culture models apoptotic neutrophils were co-cultured with mexAMs for increasing periods of time before RSV infection. Interestingly, increasing the time period between co-culture and RSV infection resulted in a decrease the expression of some pro-inflammatory cytokines and an increase in the expression of anti-inflammatory cytokines. This suggested that apoptotic neutrophils were able to alter the mexAM response to RSV.

In particular, supernatant concentrations of IFN- α , IL-6 and mexAM expression of *Il1b* were significantly reduced following RSV infection after 26h co-culture of mexAMs with neutrophils. Neutrophils are known to be essential in driving NLRP3 inflammasome activation and subsequent release of IL-1 β from AMs during IAV and RSV infection (144). Indeed, this was confirmed in mexAM co-cultures where viable neutrophils significantly potentiated *Il1b* expression. The reduction in *Il1b* expression when co-cultured with apoptotic neutrophils could be due to the loss of specific signalling requirements due to cell death. For example, mouse cathelicidin-related antimicrobial peptide (mCRAMP) signalling was shown to be a requirement for neutrophil mediated AM inflammasome activation (144). Therefore, it would be interesting to investigate how apoptosis alters mCRAMP expression. Elevated levels of IL-1 β have been identified in the airways of patients with severe RSV infection (164). However, blocking IL-1 β during RSV infection in mice was previously shown to exacerbate weight loss (166). Similarly, increasing levels of IL-6 have also been found in the airways of patients with severe RSV infection (165). Despite this, depletion of IL-6 during RSV infection in mice has been shown to increase the recruitment of CD8⁺ T cells leading to increased disease severity (162). Similarly, polymorphisms in the gene encoding IL-6 have been linked with increased susceptibility to RSV infection (409). Taken together, this data suggests that reduced levels/expression of IL-6 and *Il1b* from mexAMs and *in vivo* in mice which received CXCL1 2 days before RSV infection may not be mechanistically important in the resulting protection. Supporting this, neither of these cytokines were altered after RSV infection in mice which received apoptotic neutrophils before infection, despite these mice also being protected from weight loss. AMs have been shown to be the main source of Type I IFNs following RSV

infection in mice (15). In mice protected from RSV induced weight loss, either intranasal CXCL1 2d before infection or apoptotic neutrophils 0.5d before infection, IFN- α was significantly reduced. Co-culture with mexAMs with apoptotic neutrophils also reduced the release of IFN- α into the culture supernatant. Whilst this data strongly supports the hypothesis that AMs in these models have a reduced inflammatory response, it is difficult to link this with protection based on previous studies. IFN- α is known to be important in directing the immune response towards RSV (15). Loss of IFN- α production or receptor signalling in *Mavs*^{-/-} and *Ifnar*^{-/-} mice respectively, is associated with increased disease severity (14,15). It is therefore unclear how reduction in type I IFNs could result in protection from weight loss in mice.

Conversely, the expression of the anti-inflammatory macrophage marker *Tgfb1* was significantly increased overtime in mexAMs co-cultured with apoptotic neutrophils, independent of RSV infection. This has previously been seen following macrophage efferocytosis of apoptotic cells (410). TGF- β is not known to play a role during RSV infection and was not expressed by infected mexAMs which were not co-cultured with neutrophils. However, TGF- β is known to play an important role in immune regulation and resolution (411). Notably, TGF- β has been shown to limit CD8⁺ T cell differentiation and effector functions (412). Whilst increased *Tgfb1* expression may play a role in protection, no mechanistic link between AM phenotype and resulting protection was made.

Protection from weight loss in mice which received CXCL1 2 days before RSV infection occurred between days 5-7 post infection during the adaptive phase of the immune response, driven by CD8⁺ T cells (332). Therefore, it is likely changes to the innate immune response results in altered adaptive responses. Whilst CXCL1 12h before RSV infection increased the recruitment of CD8⁺ T cells, a trend to lower CD8⁺ T cell recruitment was observed in the lungs of mice which received CXCL1 2d before RSV on day 6 post infection. By day 8 post infection, CD8⁺ T cell numbers were comparable to RSV controls, suggesting a slight delay in their recruitment. This was more clearly seen in the airways, where CD8⁺ T cells were significantly reduced on day 6 post infection compared to RSV controls. In addition to slower CD8⁺ T cell recruitment, those that were recruited had lower expression of GzmB. GzmB is a serine protease released by cytotoxic T cells which acts to induce death of virally infected cells (413). Therefore, GzmB can cause significant host tissue damage (413). Increased levels of GzmB in the airways has previously been associated with severe RSV infection in children (414). Recently, reduced recruitment of GzmB⁺ CD8⁺ T cells has also been associated with protection from weight loss in RSV infected mice (166). It was suggested that GzmB cleaves pro-IL-1 α , releasing the active form which ultimately enters into the circulation and brain and exerts control on appetite to induce weight loss (166). GzmB is often used as a general marker of CD8⁺ T cell activation following RSV infection (166,414). No other markers of CD8⁺ T cell

activation such as IFN- γ and TNF- α , were presented in this chapter due to problems with antibody staining and ELISA detection limits. However, it would be interesting in future experiments to profile these as well. The role of these cytokines in driving weight loss at days 5-7 post RSV infection in mice is still debated. Adoptive transfer of IFN- γ KO CD8⁺ T cells has previously resulted in significant protection from weight loss following RSV infection (415). Similarly, in studies where TNF- α is neutralised during RSV infection, mice also lost less weight compared to infection only controls (167,416). However, other studies have reported that depletion of either IFN- γ or TNF- α during RSV has no effect on weight loss at days 5-7 post RSV infection (358,417). From the data on CD8⁺ T cells presented in this chapter their role in protection in mice which received CXCL1 2 days before RSV infection cannot be concluded. Subtle differences point towards a potential role but more in depth studying of their activation is required.

In addition to CD8⁺ T cells it would be interesting to better profile the CD4⁺ T cell response in mice exposed to CXCL1 before RSV infection. CD4⁺ T cell recruitment was not changed in RSV infected mice by intranasal CXCL1 2d before infection. However, RSV induces a mixed Th1 and Th2 response in mice (74). An imbalance in this response leading to increased Th2 cells, characterised by IL-4 and IL-13 expression, has been implicated in reduced viral clearance and increased pathology in mice (206,418). This imbalance is what is thought to have led to the failure of the 1960s formalin-inactivated RSV vaccine which enhanced disease in infants (206,418). On the flip side, Th1 responses, characterised by IFN- γ and TNF- α expression, are important in directing CD8⁺ T cell responses to clear RSV (419). But as increased CD8⁺ T cells are known to drive weight loss, an over active Th1 response can also be detrimental (332). In addition to exploring Th1/Th2 responses it would also be of interest to profile the FoxP3⁺ Treg response in mice exposed to CXCL1 2d before RSV infection. FoxP3⁺ T cells have been shown to elicit important control on cytotoxic T cell responses, limiting the accumulation of CD8⁺ T cells in the lung following RSV infection, thus limiting weight loss (420,421). Overall, Protection from weight loss between days 5-7 post infection is likely a result in unidentified changes in the T cell response. However, it is still unclear how the changes identified in the innate immune response, driven by intranasal CXCL1 2d before RSV infection could link to changes in the adaptive response.

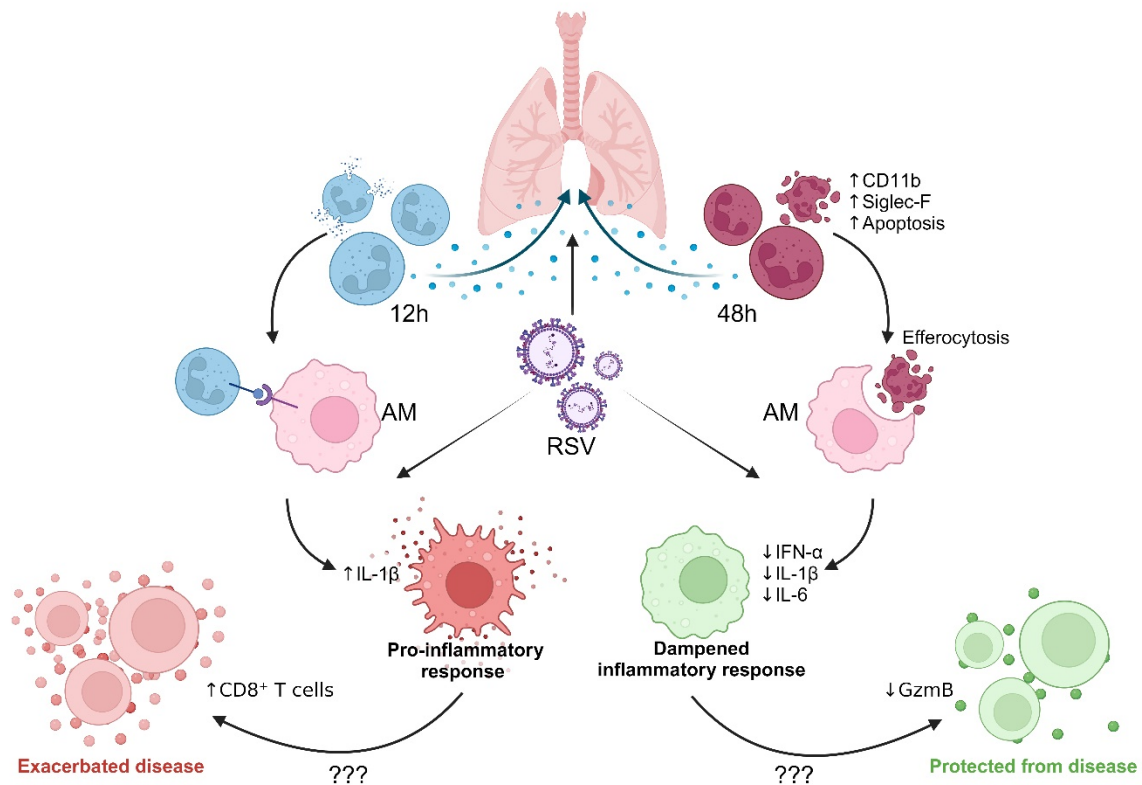


Figure 6.9 Graphical Summary

Apoptotic neutrophils in the airways before RSV infection are cleared by AMs through efferocytosis. This ultimately dampens the AM inflammatory response to RSV. These early changes to the innate immune response lead to changes in the CD8 $^{+}$ T cell response that are associated with protection from disease. This is characterised by reduced GzmB expression. Created using BioRender.

In summary, the presence of neutrophils in the lungs of mice before RSV infection has differential effects on disease outcomes depending on the length of time between neutrophil recruitment and infection and neutrophil viability. The presence of viable neutrophils in the lung at the time of RSV infection leads to exacerbated disease by increasing the recruitment of CD8 $^{+}$ T cells through unknown mechanisms. In these mice, AMs exhibit a pro-inflammatory response to the virus which could play a role in orchestrating the resulting inflammation. In contrast, the presence or clearance of apoptotic neutrophils in the lung before RSV infection, dampens the AM pro-inflammatory response to the virus. Through unknown mechanisms this likely results in changes to the CD8 $^{+}$ T cell response, characterised by lower GzmB production. Ultimately, these changes to the inflammatory response may protect mice from RSV induced weight loss (Fig. 6.10). The work presented in this chapter demonstrates the effect neutrophil recruitment to the lungs before RSV infection has on disease outcomes is dependent on their phenotype and life-stage. This exemplifies the dynamic nature of neutrophils and the different roles they can play during infection, making them incredibly hard to target in therapeutic strategies. The findings presented here demonstrate how they can have a protective role

during RSV infection. Further investigation into the mechanism in which this protection is achieved could help with the development of preventative therapies against severe disease induced by RSV.

7. Neutrophil recruitment before or after IAV infection have opposing outcomes on disease severity.

7.1 Introduction

Infection with seasonal influenza A virus (IAV) places a substantial strain on healthcare systems each year. Global studies of IAV burden have previously estimated that IAV is accountable for 290,000–650,000 deaths per annum (64,422). Like many respiratory viruses, IAV circulates seasonally each year (64). Most individuals will experience milder symptoms such as cough, headache and fever, often described as the flu (51). However, similar to RSV, IAV has a disproportionate burden amongst elderly adults over the age of 65 and children under the age of 5 (422). In these individuals, IAV infection has a higher risk of causing severe infection requiring hospitalisation and mechanical ventilation (64). In addition to circulating seasonally, IAV poses a significant threat as an emerging pandemic virus due to its ability to rapidly undergo antigenic drift and antigenic shift genetic mutation in animal reservoirs (69). These mutation events can result in the generation of a new IAV virus with unique antigens that cannot be neutralised by antibodies from previous heterotypic IAV infection or vaccination (69). Therefore, existing immunity towards the mutated IAV in the population is low resulting in increased risk of severe disease. What pre-disposes one individual to developing severe disease following IAV infection is unclear, but like RSV it is believed to be driven to the immune response towards the virus (423). Whilst an annual IAV vaccine is readily available, efficacy rates vary due to a number of factors including uptake rates, and predictions on the predominate seasonal IAV strain used to formulate the vaccine being incorrect (424). Therefore, understanding what drives severity following IAV infection may help with developing better treatments, care and preventions.

Having demonstrated and discussed the role neutrophils can play during RSV infection, this chapter investigates how neutrophils can influence disease outcomes during IAV infection. Neutrophils are recruited into the lungs during IAV infection, but their role in contributing to protection or pathology is still unclear (425). Neutrophils have previously been associated with increasing disease severity in both mice and humans, with studies suggesting they contribute greatly to lung damage and the development of ARDS (305–307). On the contrary, there is also evidence to suggest they play a beneficial role during IAV infection, with several studies reporting anti-Ly6G depletion of neutrophils results in exacerbated weight loss, higher viral titres and worsened lung pathology (300,302,426). However, the effect of increasing the magnitude of neutrophils recruited into the lung by intranasal CXCL1 the early phases of IAV infection using a mouse model of IAV infection has not been studied. It is also unclear if neutrophils present in the lung before IAV infection results in exacerbated disease outcomes.

7.2 Results

7.2.1 Increased neutrophil recruitment 12h following X31 infection resulted in exacerbated weight loss.

To study how neutrophils may contribute to disease during influenza virus infection, mice were infected with 250PFU X31 and 12h later given an intranasal dose of CXCL1 (Fig. 7.1a). Weight loss was monitored up to day 8 post infection as a measure of disease severity. Mice infected with X31 began to lose weight on day 2 post infection, and gradually continued to lose weight until day 6 post infection, when weight loss peaked between 10-15% of original body weight (Fig. 7.1b). Mice which received intranasal CXCL1 12h after X31 infection (X31-CXCL1) had a similar pattern of weight loss, beginning to lose weight on day 2 post infection and peaking at day 6 post infection (Fig. 7.1b). At day 6 post infection, peak weight loss did not significantly differ between X31 infected mice and X31-CXCL1 mice. However, the X31-CXCL1 mice did lose significantly more weight early after infection on day 2 and 3 post infection (Fig. 7.1b). On days 1,2,4,6 and 8 post infection, lungs and BAL were harvested and analysed using flow cytometry to assess immune cell populations and immune mediators in the lungs and airways. Following X31 infection, neutrophils began to infiltrate into the lung between day 1 and 2 post infection, with total numbers peaking and remaining elevated between day 2 and 6 post infection (Fig. 7.1c). In X31-CXCL1 mice, neutrophil numbers rapidly increased and peaked at day 1 post infection, after which numbers rapidly declined and did not significantly differ from X31 only mice at day 2 post infection (Fig. 7.1c). Total numbers of AMs also appeared to be increased at day 2, 6 and 8 but not day 4 post X31 infection. In the lungs of X31-CXCL1 mice, AMs were further increased compared to X31 mice at days 4 and 6 post infection (Fig. 7.1d). Recruitment of inflammatory monocytes into the lungs following X31 infection, also began between days 1 and 2 post infection and continued to increase at day 4 post infection (Fig. 7.1e). Total numbers of monocytes were significantly lower in the lungs of X31-CXCL1 mice at day 2 post infection (Fig. 7.1e). Recruitment of both CD4⁺ and CD8⁺ T cells into the lungs occurred following day 4 post infection (Fig. 7.1f-g). CD4⁺ T cell numbers peaked on day 6 post infection, whilst CD8⁺ T cells continued to increase on day 8 post infection (Fig. 7.1f-g). Neither CD4⁺ nor CD8⁺ T cell numbers were affected by intranasal CXCL1 12h following infection (Fig. 7.1f-g). Corresponding with the initiation of weight loss and the recruitment of innate immune cells into the airways viral load, measured by M gene copy number, peaked on day 2 post infection then rapidly declined. X31-CXCL1 mice had significantly higher viral load at day 2 post infection compared to X31 mice (Fig. 7.1h). Overall, the data suggests the presence of increased neutrophil numbers in the lungs 12h following infection associates with

exacerbated disease associated with significantly higher viral load and delayed monocyte recruitment.

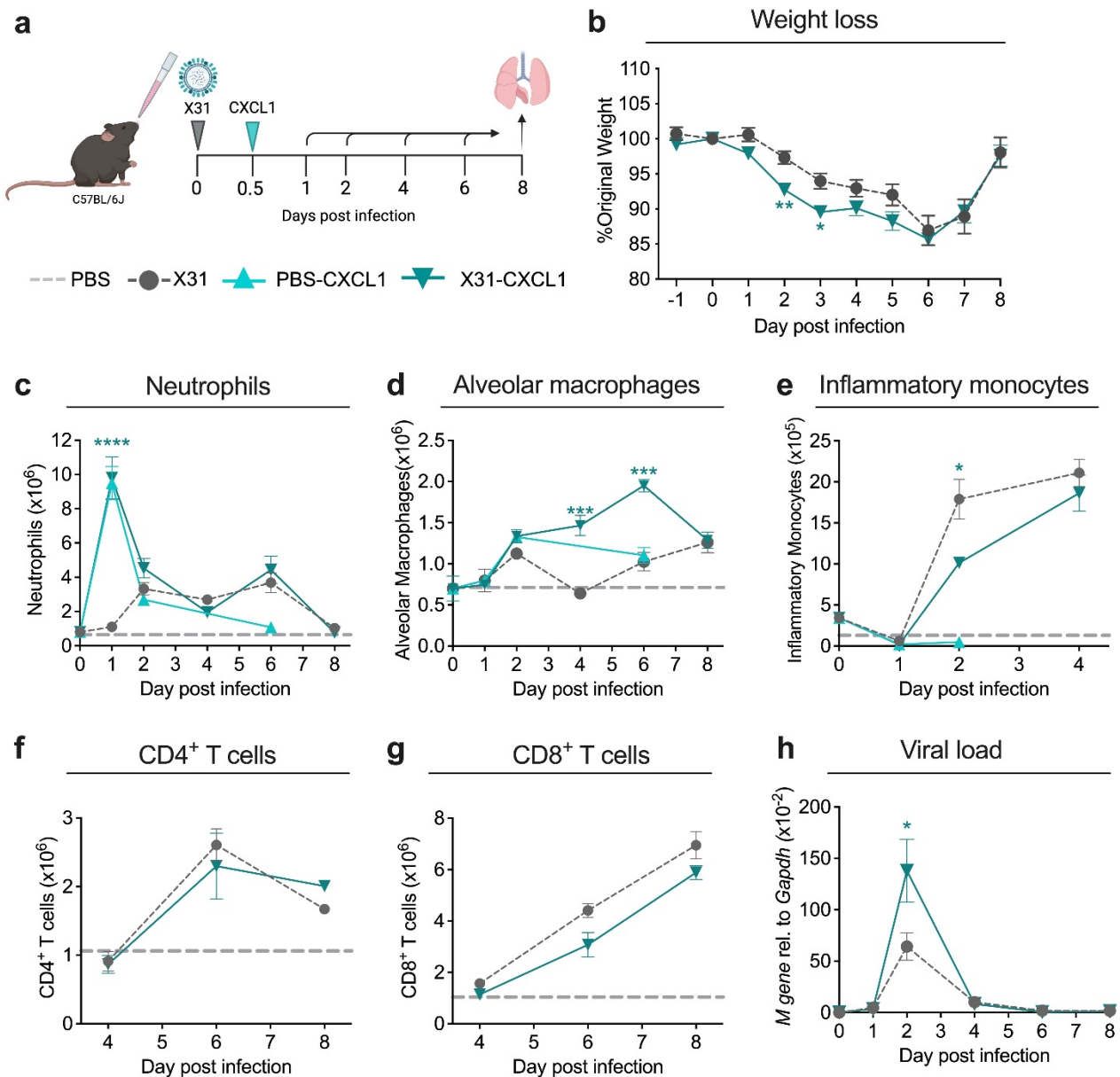


Figure 7.1 Intranasal CXCL1 12h after X31 infection exacerbated weight loss

a) Graphic describing the experimental design. Mice were infected with X31 (250PFU) and 12h later received an intranasal dose of CXCL1 (5µg). Mice were weighed across the course of 8 days and the innate and immune response were profiled in the lung at days 1, 2, 4, 6 and 8 post infection by flow cytometry. **b)** Weight loss in mice which received CXCL1 12h after X31 infection compared with X31 only mice. Represented as percentage of original body weight on day 0. Total numbers of **c)** neutrophils, **d)** alveolar macrophages and **e)** inflammatory monocytes in the lung at days 1-8 post infection. Total numbers of **f)** CD4⁺ T cells and **g)** CD8⁺ T cells on day 4-8 post infection. **h)** Viral load in the lung measured by *M* gene expression relative to *Gapdh* measured by qPCR, at days 1-8 post infection. Data were collected from 1 experiment per time point. For day 1 and 2 X31 n=4; PBS-CXCL1 and X31-CXCL1 n=3. For day 4 and 8, n=5. For day 6, n=3. Data are plotted as mean ± SEM. Grey dashed lines represent PBS baseline, n=12. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was

carried out to compare the X31-CXCL1 group with X31 only controls. For all other graphs a one-way ANOVA with multiple comparisons test was carried out to compare the X31-CXCL1 group with X31 only controls. Asterisks represent the p value compared to X31 only controls; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

7.2.2 Exacerbated weight loss in X31-CXCL1 mice was associated with increased *Il1b* and *Tnfa* expression and delayed IL-6 release.

Having found an association between increased neutrophilia in the lungs shortly after X31 infection and exacerbated disease, the effect of neutrophil recruitment on the cytokine response to X31 was profiled using qPCR and ELISA. Gene expression of *Il1a*, *Il1b*, *Tnfa* and *Ifnb* in the lung and levels of IL-6 and IFN α in the BAL, were all increased on day 2 post X31 infection (Fig. 7.2a-f). *Il1a* expression peaked on day 2 post infection then declined again by day 4 post infection. In the lungs of X31-CXCL1 mice, *Il1a* expression was not altered compared to X31 mice (Fig. 7.2a). *Il1b* expression also peaked on day 2 post infection and remained elevated on days 4 and 6 post infection. In contrast, X31-CXCL1 mice had significantly higher expression of *Il1b* on day 1 post infection compared to X31 only mice (Fig. 7.2b). Expression of *Tnfa*, declined following day 2 post infection and remained low on days 4 and 6 post infection, after which expression increased again on day 8 post infection (Fig. 7.2c). X31-CXCL1 mice had significantly higher expression of *Tnfa* on day 1 post infection compared to X31 mice, but significantly lower expression on day 2 post infection. On day 8, expression of *Tnfa* trended lower in X31-CXCL1 mice compared to X31 only, but this was not statistically significant (Fig. 7.2c). Levels of IL-6 in the BAL remained elevated between days 2-6 following X31 infection then declined by day 8. Interestingly, IL-6 levels in the BAL from X31-CXCL1 mice gradually increased overtime, remaining at significantly lower levels on days 2 and 4 post infection, but peaking at comparable levels to X31 mice on day 6 post infection (Fig. 7.2d). Expression of *Ifnb* also peaked on day 2 post infection and declined on day 4 and 6 post infection (Fig. 7.2e). Intranasal CXCL1 after X31 infection had no effect on *Ifnb* expression (Fig. 7.2e). Taken together, these data suggest increased neutrophil recruitment following X31 infection does not have a general effect on inflammatory mediators, but rather drive an early increase in *Il1b* and *Tnfa* expression whilst delaying IL-6 release.

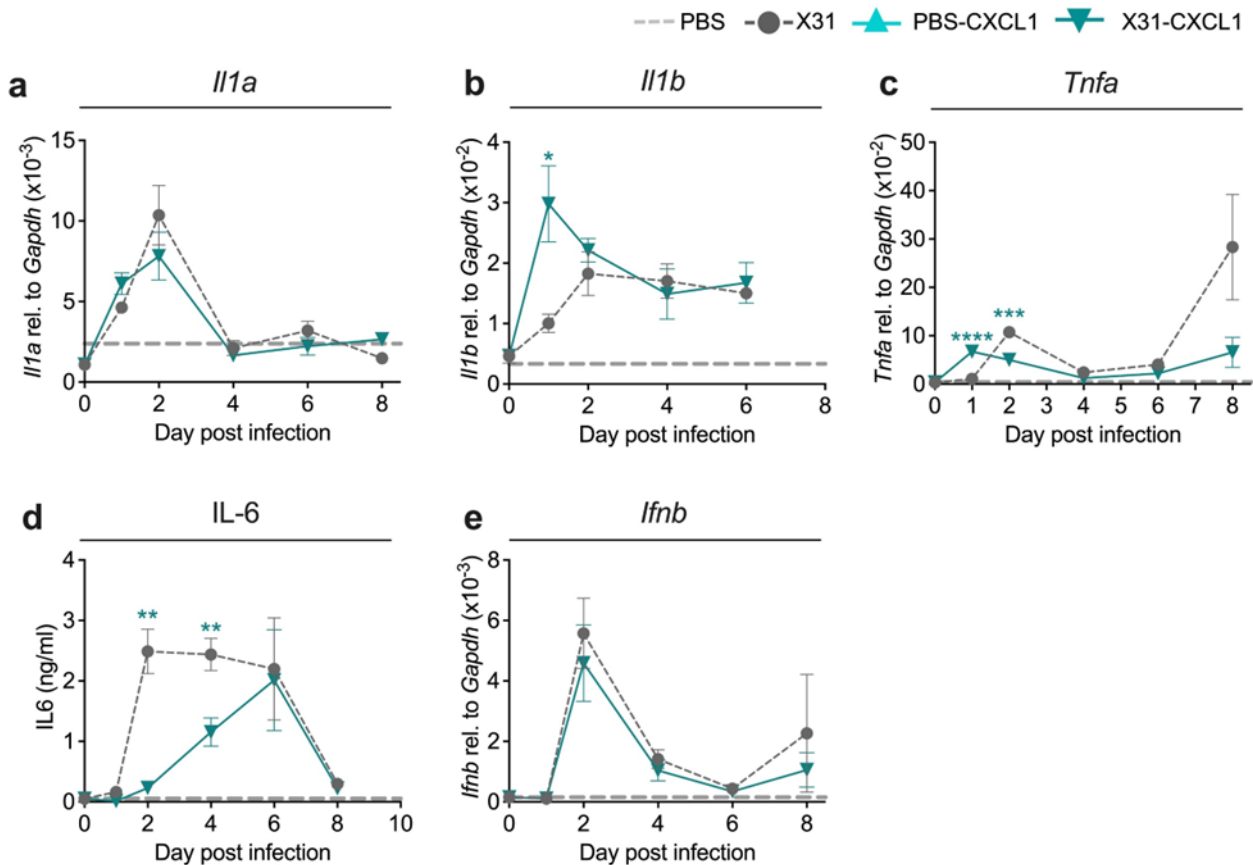


Figure 7.2 Intranasal CXCL1 after X31 infection increased expression of *Il1b* and delayed the IL-6 response

Expression of **a)** *Il1a*, **b)** *Il1b* and **c)** *Tnfa* in the lungs at days 1-8 post X31 infection relative to *Gapdh* measured by qPCR. **d)** Levels of IL-6 detected in the BAL by ELISA at days 1-8 post X31 infection. **e)** Expression of *Ifnb* in the lungs at days 1-8 post X31 infection relative to *Gapdh* measured by qPCR. Data were collected from 1 experiment per time point. For day 1 and 2 X31 n=4; PBS-CXCL1 and X31-CXCL1 n=3. For day 4 and 8, n=5. For day 6, n=3. Data are plotted as mean $\pm$ SEM. Grey dashed lines represent PBS baseline, n=12. A one-way ANOVA with multiple comparisons test was carried out to compare the X31-CXCL1 group with X31 only controls. Asterisks represent the *p* value compared to X31 only controls; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

7.2.3 Neutrophils recruited 12h before X31 infection resulted in protection from weight loss.

Unlike during RSV infection, increased neutrophil recruitment during X31 infection was found to drive exacerbations in weight loss early after infection during the innate phase of infection. However, like in mouse models of RSV infection, increased neutrophil recruitment 12h following X31 infection had no impact on weight loss later during the adaptive phase of infection. As demonstrated in previous studies and chapters of this thesis, neutrophil 12h recruitment before RSV infection does exacerbate weight loss during the adaptive phase of infection. Therefore, it was hypothesised the same could be true for X31 infection. To study this, mice were given an intranasal dose of CXCL1 and 12h later infected with 250PFU X31

(Fig. 7.3a). Weight loss was monitored up to day 8 post infection as a measure of disease severity. All the data presented for X31 control mice are the same data presented and described above in Fig. 7.1. The mice which received CXCL1 12h before X31 infection (CXCL1-X31), lost less weight than the X31 mice between day 2-7 post infection. This protection from weight loss was significant between days 5-7 post infection (Fig. 7.3b). Neutrophil numbers in the lungs peaked at 12h after intranasal CXCL1 at the point of X31 infection (day 0 post infection) (Fig. 7.3c). In the first day following X31 infection, neutrophil numbers rapidly declined to baseline levels. On day 2 post infection, neutrophil recruitment increased again following the same trend as X31 mice across the time course (Fig. 7.3c). Interestingly, at day 2 post infection, CXCL1-X31 mice had significantly increased AMs in the lungs compared to X31 only mice (Fig. 7.3d). Numbers decreased again by day 4 post infection but remained significantly elevated up to day 6 post infection compared to X31 mice (Fig. 7.3d). Inflammatory monocyte recruitment into the lungs of CXCL1-X31 mice followed the same trend as in X31 mice and was not altered by prior CXCL1 exposure (Fig. 7.3e). Neither CD4⁺ nor CD8⁺ T cell recruitment were affected by prior exposure to CXCL1 (Fig. 7.3f-g). Viral load, as measured by M gene expression relative to *Gapdh*, once again peaked on day 2 post infection and was not altered in the lungs of CXCL1-X31 mice compared to X31 mice (Fig. 7.3h).

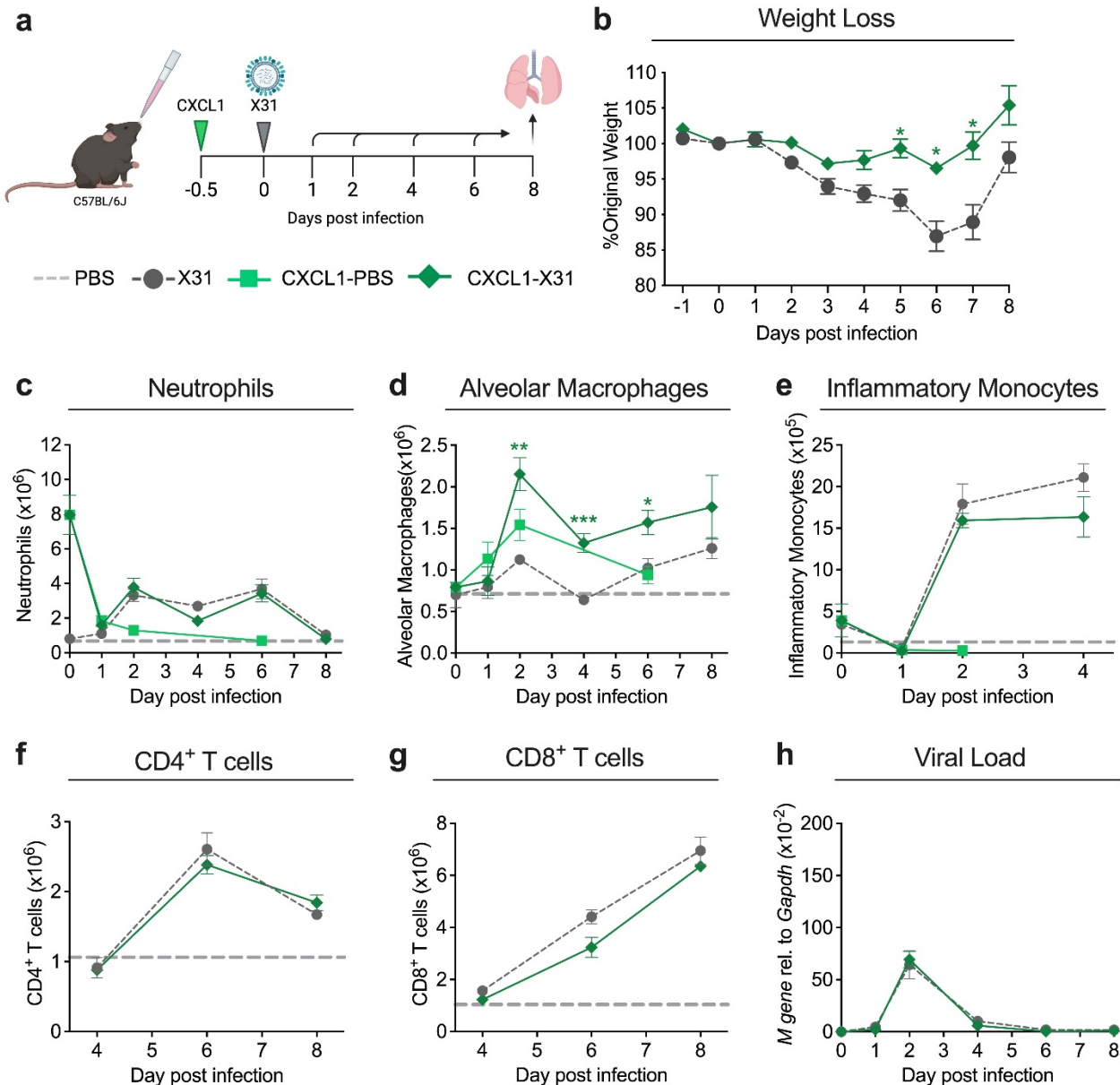


Figure 7.3 Intranasal CXCL1 12h before X31 protected mice from weight loss

a) Graphic describing the experimental design. Mice received an intranasal dose of CXCL1 (5µg) and 12h later were infected with X31 (250PFU). Mice were weighed across the course of 8 days and the innate and immune response were profiled in the lung at day 1, 2, 4, 6 and 8 post infection by flow cytometry. **b)** Weight loss in mice which received CXCL1 12h before X31 infection compared with X31 only mice. Represented as percentage of original body weight on day 0. Total numbers of **c)** neutrophils, **d)** alveolar macrophages and **e)** inflammatory monocytes in the lung at days 1-8 post infection. Total numbers of **f)** CD4⁺ T cells and **g)** CD8⁺ T cells on day 4-8 post infection. **h)** Viral load in the lung measured by *M* gene expression relative to *Gapdh* measured by qPCR, at days 1-8 post infection. Data were collected from 1 experiment per time point. X31 data is the same as presented in Fig. 7.1. For day 1 and 2 X31 n=4; CXCL1-PBS n=3; CXCL1-X31 n=4. For day 4 and 8, n=5. For day 6, X31 and CXCL1-PBS, n=3; CXCL1-X31 n=4. Data are plotted as mean ± SEM. Grey dashed lines represent PBS baseline, n=12. For weight loss, as data was paired, a two-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-X31 group with X31 only controls. For all other graphs a one-way ANOVA with multiple

comparisons test was carried out to compare the CXCL1-X31 group with X31 only controls. Asterisks represent the p value compared to X31 only controls; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

7.2.4 Protection from weight loss in CXCL1-X31 mice was associated with decreased expression of *Tnfa*.

Protection from weight loss in mice was associated with a significant increase in AMs in the lungs following the peak of neutrophil recruitment. To investigate if the alterations to this population could be associated with changes to the expression or release of inflammatory cytokines, the lungs and BAL were analysed using qPCR and ELISA. All the data presented for X31 control mice are the same data presented and described above in Fig. 7.2. Gene expression of *Il1a* in the lungs of CXCL1-X31 did not differ at any time point compared to X31 mice (Fig. 7.4a). *Il1b* expression in the lung of CXCL1-X31 mice showed a similar induction as X31 mice and did not significantly differ at any time point (Fig. 7.4b). *Tnfa* expression was found to be significantly lower in the lungs of CXCL1-X31 mice compare to X31 at day 2 post infection (Fig. 7.4c). Levels of IL-6 in the BAL of CXCL1-X31 mice were comparable to X31 mice and did not differ significantly at any time point (Fig. 7.4d). *Ifnb* expression was also comparable in the lungs of CXCL1-X31 mice and X31 mice at all timepoints (Fig. 7.4e).

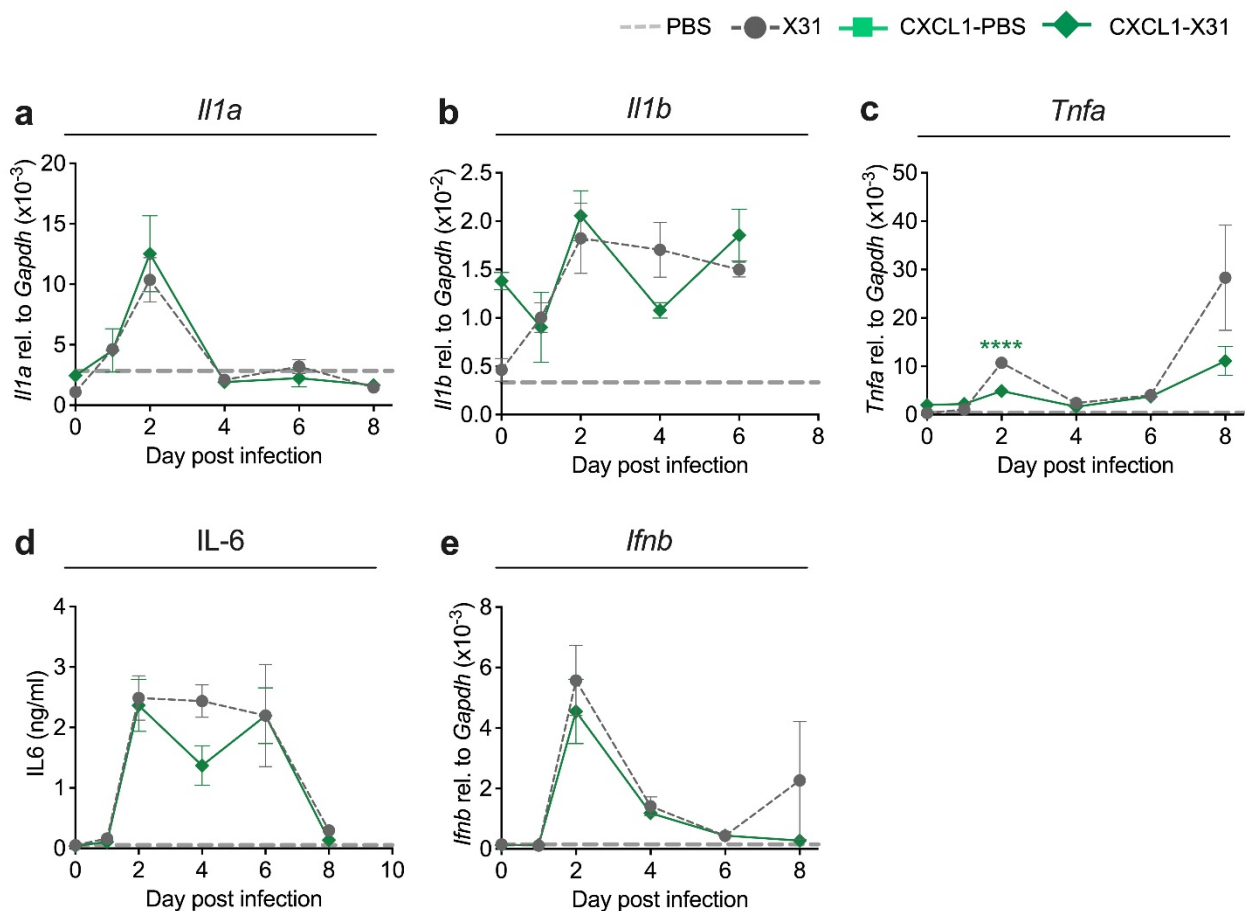


Figure 7.4 Intranasal CXCL1 12h before X31 infection decreased the expression of *Tnfa*

Expression of **a) *Il1a*, b) *Il1b* and c) *Tnfa*** in the lungs at days 1-8 post X31 infection relative to *Gapdh* measured by qPCR. **d)** Levels of IL-6 detected in the BAL by ELISA at days 1-8 post X31 infection. **e)** Expression of *Ifnb* in the lungs at days 1-8 post X31 infection relative to *Gapdh* measured by qPCR. Data were collected from 1 experiment per time point. X31 data is the same as presented in Fig. 7.2. For day 1 and 2 X31 n=4; CXCL1-PBS n=3; CXCL1-X31 n=4. For day 4 and 8, n=5. For day 6, X31 and CXCL1-PBS, n=3; CXCL1-X31 n=4. Data are plotted as mean $\pm$ SEM. Grey dashed lines represent PBS baseline, n=12. A one-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-X31 group with X31 only controls. Asterisks represent the *p* value compared to X31 only controls; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

7.2.5 Protection from X31 weight loss in CXCL1-X31 mice was associated with an increase in CD11b^{hi} Siglec-F⁺ neutrophils in the lung.

Intranasal exposure of mice to CXCL1 12h before X31 infection drove an early peak in neutrophil numbers at day 1 post infection. This ultimately resulted in significant protection from X31 induced weight loss. In contrast, CXCL1 12h after X31 infection further increased the peak of neutrophil numbers at day 2 post infection, ultimately leading to exacerbation in weight loss. To better understand why these differences in timings could be resulting in differing effects on weight loss we compared their expression of age associated markers on neutrophils (375,393). CD11b and Siglec-F expression were selected based on the data presented in Chapter 2 of this thesis. In X31 only mice, the percentage of Siglec-F⁺ neutrophils were not significantly increased at any time point post infection and remained at 10-20% of the neutrophil population (Fig. 7.5a). In contrast, Siglec-F⁺ neutrophils were significantly increased in CXCL1-X31 mice up to 50-60% of the total neutrophil population at day 1 post infection (Fig. 7.5a). This was independent of X31 infection as a similar increase was noted in the CXCL1-PBS mice (Fig. 7.5a). In X31-CXCL1 mice, percentage expression of Siglec-F on neutrophils also appeared to increase over time but peaked at only 20% day 2 post infection (Fig. 7.5b). Following X31 infection, the population of CD11b^{hi} neutrophils expanded from ~5% to ~15% of total neutrophils, between day 0 day 2 post infection (Fig. 7.5c). On day 1 post infection, the percentage of CD11b^{hi} neutrophils were significantly increased to ~25% total neutrophils in the lungs of CXCL1-X31 mice (Fig. 7.5c). By day 2 post infection, the percentage dropped back down to levels comparable to X31 only (Fig. 7.5c). In X31-CXCL1 mice, percentage CD11b^{hi} neutrophils were comparable to X31 only mice at all time points post infection (Fig. 7.5d). Overall, these data suggests that in addition to an early peak in neutrophil numbers in the lungs of CXCL1-X31 mice at day 1 post infection, the neutrophils have an altered phenotype.

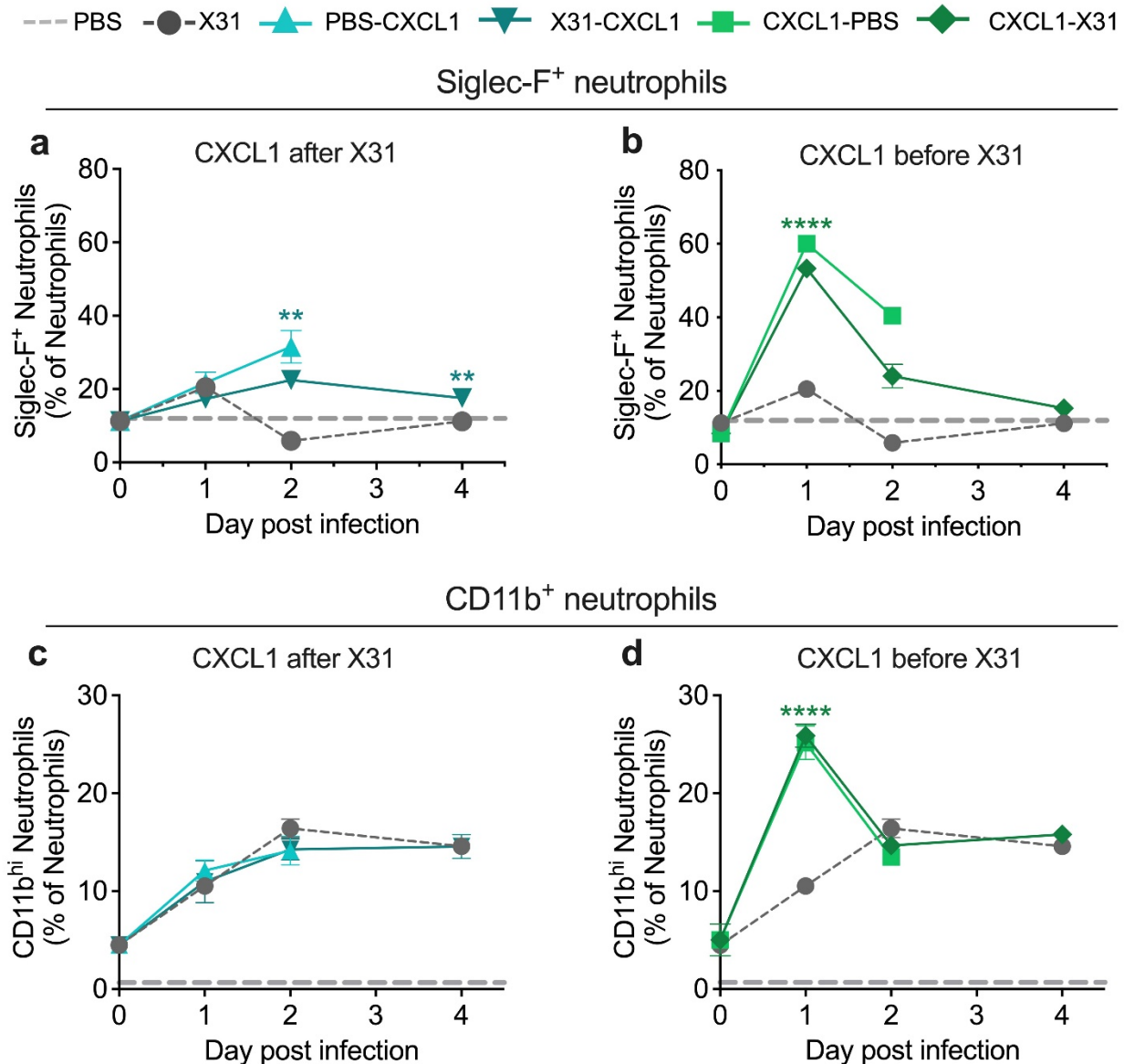


Figure 7.5 Neutrophils had higher expression of age associated markers in mice exposed to CXCL1 before X31 infection

Percentage of total neutrophils which express Siglec-F in the lungs of mice which received intranasal CXCL1 **a)** 12h after or **b)** 12h before X31 infection at days 1, 2 and 4 post infection. Percentage of total neutrophils with high CD11b expression in the lungs of mice which received intranasal CXCL1 **a)** 12h after or **b)** 12h before X31 infection at days 1, 2 and 4 post infection. Data were collected from 1 experiment per time point. X31 data is the same as presented in Fig. 7.2. For day 1 and 2 X31 n=4; PBS-CXCL1, PBS-CXCL1, CXCL1-PBS n=3; CXCL1-X31 n=4. For day 4 and 8, n=5. For day 6, X31 PBS-CXCL1, X31-CXCL1, PBS-CXCL1 n=3; CXCL1-X31 n=4. Data are plotted as mean $\pm$ SEM. Grey dashed lines represent PBS baseline, n=12. A one-way ANOVA with multiple comparisons test was carried out to compare the CXCL1-X31 group with X31 only controls. Asterisks represent the p value compared to X31 only controls; * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , **** ≤ 0.0001 .

7.2.6 Infection of mice with X31 results in a delayed innate inflammatory response compared to RSV infection.

When infected with X31, neutrophil recruitment 12h before infection results in protection from weight loss, whilst recruitment 12h after infection results in exacerbation in weight loss. These findings are in contrast to the previously reported findings of neutrophil recruitment before and during RSV infection in mice, where neutrophils recruited 12h after RSV infection have no impact on weight loss, whilst recruitment 12h before infection exacerbates weight loss (Fig. 6.1b-c) (17,170). In addition, the findings from chapter 2 demonstrate recruiting neutrophils 2 days before RSV infection results in protection. To understand why neutrophil recruitment may have opposing effects on this model of X31 infection compared to the RSV model, the dynamics of the immune response were compared. The X31 data presented in this figure have previously been presented and described in Fig. 7.1 and Fig. 7.3. The RSV data have previously been presented and described in chapter 1, Fig. 5.3-5. Mice were infected with either 250PFU X31 or 7×10^6 FFU RSV. Weight loss was monitored up to day 8 post infection as a measure of symptom severity. The dynamics of weight loss following X31 infection varied compared to RSV. Whilst RSV infected mice predominantly lost weight between days 5-7 post infection and recovered by day 8 post infection, X31 infected mice begin to gradually lose weight earlier beginning on day 2 post infection (Fig. 7.6a). Viral load in the lungs was quantified at each time point by measuring expression of M gene and L gene for X31 and RSV respectively. M gene expression in the lungs of X31 infected mice was low on day 1 infection, after which it increased, peaking on day 2 post infection before decreasing again on day 4 post infection (Fig. 7.6b). In contrast, on day 1 post RSV infection L gene copy number was high and remained elevated up to day 4 post infection (Fig. 7.6b). Corresponding with the delay in detectable virus following X31 infection compared to RSV, the initiation of innate immune cell recruitment was also delayed. Following RSV infection, neutrophils were rapidly recruited into the lungs, peaking at day 1 post infection before undergoing rapid decline (Fig. 7.6c). In contrast, in the lungs of X31 infected mice, neutrophils peaked on day 2 post infection (Fig. 7.6c). Unlike during RSV infection, neutrophil numbers remained high up to day 6 post X31 infection, only declining to baseline levels by day 8 post infection (Fig. 7.6c). A similar delay was also noted in the recruitment of inflammatory monocytes following X31 infection, which also peaked on day 2 post X31 but at day 1 post RSV (Fig. 7.6d). CD4⁺ T cells were increased on day 4 post infection in RSV infected mice, but only started to increase in X31

infected mice on day 6 post infection (Fig. 7.6g). The dynamics of CD8⁺ T cell recruitment were comparable in both infection models (Fig. 7.6f).

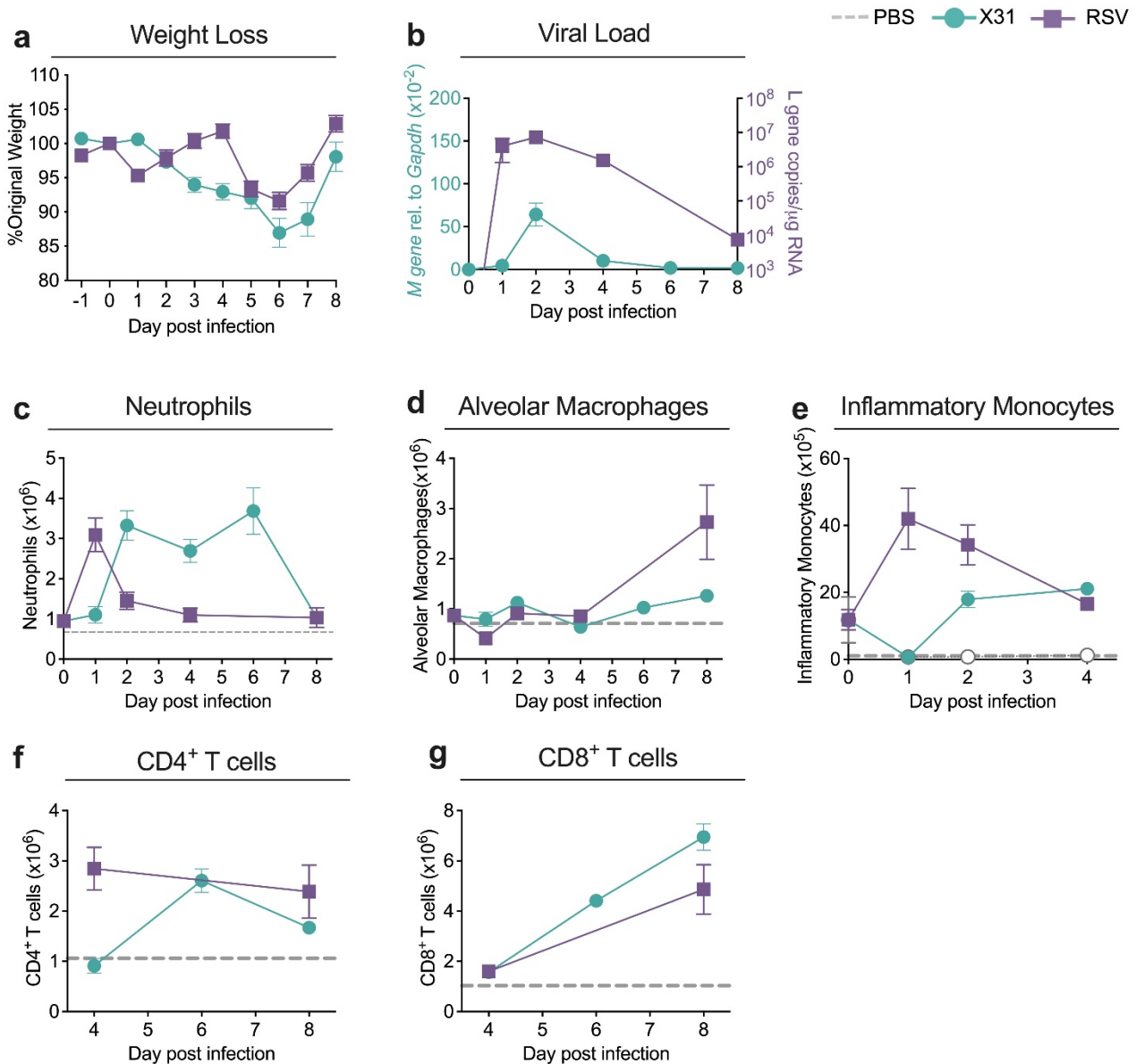


Figure 7.6 The immune response in the X31 mouse model was delayed compared to the RSV mouse model

To compare the immune response in the mouse model of X31 with the mouse models of RSV, the X31 data from Fig. 7.1 and 7.3 was plotted with the RSV collected and presented in Chapter 1. **a)** Weight loss represented as percentage of body weight at the time point of infection. **b)** Viral load in the lungs represented by *M gene* relative to *Gapdh* for X31 (left y axis) or *L gene* copy number/μg RNA normalised to *Gapdh* for RSV (right y axis) measured by qPCR. Time courses of the total number of **c)** neutrophils, **d)** alveolar macrophages, **e)** inflammatory monocytes, **f)** CD4⁺ T cells and **g)** CD8⁺ T cells in the lung measured by flow cytometry. Data are plotted as the mean ± SEM for each time point. Dashed line represents PBS baseline, n=12. X31 were collected from one experiment per timepoint. N=3-5. RSV data were collected from 2 independent experiments per time point n=10. Statistical analysis was deemed not appropriate to carry out.

In addition to the recruitment of innate immune cells, the dynamics of the inflammatory cytokine response in the mouse models of X31 and RSV infection were compared. Both X31 and RSV induce the expression of *Il1a*, *Il1b*, *Tnfa*, IL-6 and *Ifnb* (Fig. 7.7a-f). In the case of all these cytokines, expression after RSV infection peaked on day 1 post infection (Fig. 7.7a-f). In contrast, expression of these cytokines were low or undetectable on day 1 post X31 infection (Fig. 7.7a-f). Instead, all these cytokines peaked on day 2 post infection, corresponding with detection of the viral M gene by qPCR and infiltration of innate immune cells (Fig. 7.7a-f). Taken together, these data suggest a delay in the initiation of the immune response towards the virus in the X31 model compared to the RSV model.

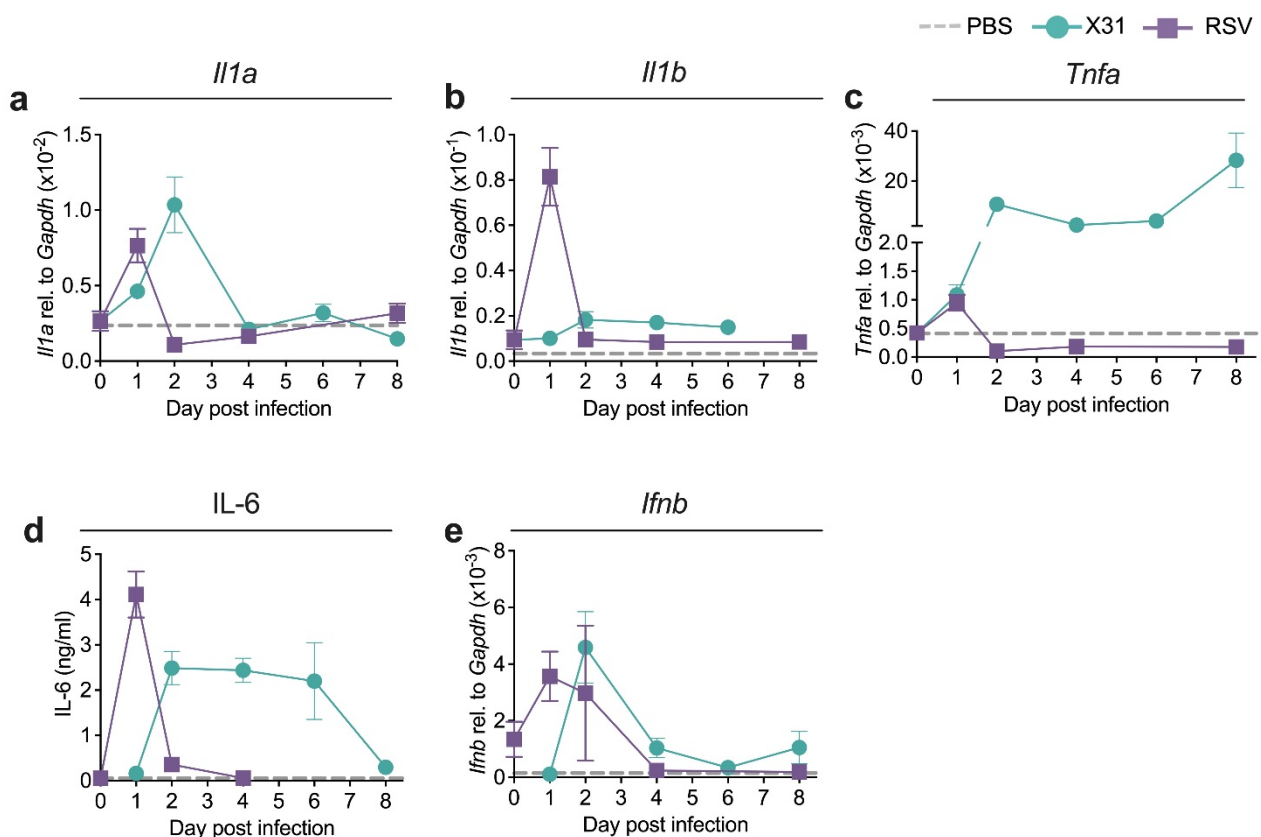


Figure 7.7 The expression of cytokines in the X31 mouse model had delayed onset in comparison to the RSV mouse model

To compare the immune response in the mouse model of X31 with the mouse models of RSV, the X31 data from Fig. 7.2 and 7.4 were plotted with the RSV collected and presented in Chapter 1. Expression of **a) *Il1a***, **b) *Il1b*** and **c) *Tnfa***, in the lung, normalised to Gapdh and measured by qPCR. **d)** Levels of IL-6 in the BAL supernatant measured by ELISA, **e)** Expression of *Ifnb* in the lung, normalised to Gapdh and measured by qPCR.

Data are plotted as the mean $\pm$ SEM for each time point. Dashed line represents PBS baseline, n=12. X31 were collected from one experiment per timepoint. N=3-5. RSV data were collected from 2 independent experiments per time point n=10. Statistical analysis was deemed not appropriate to carry out.

7.3 Discussion

The findings of this chapter demonstrate the dynamic effect neutrophils can have on the immune response and subsequent severity of IAV infection in mice. Similar to RSV mouse models of infection and the data presented in Chapter 2, neutrophil recruitment can either exacerbate or protect mice from IAV induced weight loss, dependent on the timepoint at which they are recruited. However, there were clear differences in effects of recruiting neutrophils between the two infections models.

Neutrophil recruitment using intranasal CXCL1 12h after IAV infection resulted in exacerbated weight loss, suggesting neutrophils may contribute to disease severity during IAV infection. This is in contrast to previous findings in the IAV mouse model, in which depletion of neutrophils resulted in significantly increased weight loss and mortality. This has led to the suggestion that neutrophils play a protective role during IAV infection (300,302,426). The contradictions in these findings do not mean that one of them is not true. Neutrophils are considered a “double-edged sword” in many respiratory diseases, and tight regulation of their recruitment is thought to be necessary to prevent chronic inflammation (427). It is possible that recruitment of neutrophils is necessary to limit disease severity by limiting viral replication and/or by directing subsequent immune responses. Indeed, neutrophil depletion in X31 infected mice resulted in increased viral load in the lungs between days 1-9 post infection (300,426). In these studies, they suggest that the higher viral titres are responsible for the increased weight loss, but the authors do not compliment this with any immunological outputs such as immune cell infiltration or cytokine responses (300,426). At the same time, it can also be possible that elevated neutrophil recruitment can enhance disease severity. This is due to the non-specificity of their actions, which whilst they may have effective anti-viral action, they can also damage resident cells within the lungs (259). An exemplifier of this, is the association between the production of NETs and increased disease severity during IAV infection in both mice and humans (305,307). NET production was found to be increased in the plasma from severe IAV patients, whilst in IAV infected mice NETs have been found entangled in the alveoli in areas of high tissue damage which was associated with increased progression to ARDS (305,307). In addition to NETs, other neutrophil components such as NE, MPO and ROS can also cause epithelial cell death and excessive tissue damage (269,270,306,428). This is a key reason why neutrophil recruitment following infection must be tightly regulated. Therefore, it is possible that increased neutrophil recruitment by intranasal CXCL1 12h following X31 infection drives excessive tissue damage in the lung which in turn drives increased weight loss. Lung pathology was not assessed in this model, and it remains unclear if lung damage directly correlates with weight loss in IAV mouse models.

Weight loss in mouse models of respiratory viral infections have previously been directly linked to components of the immune response, rather than directly resulting from damage or viral load. As discussed previously, weight loss during RSV infection is well known to be driven by CD8⁺ T cells (332). In mice exposed to CXCL1 12h prior to RSV infection, the resulting weight loss was due to increased recruitment of CD8⁺ T cells (170). The dynamics of CD8⁺ T cell recruitment during IAV infection in mice also track with the dynamics of weight loss (429). However, *Rag2*^{-/-} mice, which lack mature T cells, still lose weight following IAV infection, suggesting T cells are not responsible for weight loss in mouse models of IAV (430). Fitting with this, no significant alterations in the magnitude of the T cell responses were noted in the lungs of X31-CXCL1 mice. In addition, weight loss was exacerbated on days 2 and 3 post infection, during the innate phase of infection, prior to T cell recruitment. The enhanced weight loss in these mice was therefore likely not mediated by the adaptive immune response and the mechanism likely lies in the innate immune response. Considering the theory that increased neutrophils in the lung may enhance tissue damage, this could lead to more weight loss by promoting inflammation. Damaged airway cells can release damage associated molecular patterns (DAMPs) which can engage PRRs to activate other immune cells and/or drive the release of pro-inflammatory cytokines (330). However, enhanced airway inflammation was not noted in the lungs of X31-CXCL1 mice. Instead, increased weight loss was associated with a significant decrease in monocyte recruitment and IL-6 at days 3-4 post infection. This suggests the increased weight loss may not be driven by the neutrophils directly, rather in how they change the immune response to the virus.

The mechanisms through which intranasal CXCL1 12h after IAV infection resulted in reduced levels of IL-6 in the airways is unclear, however it may be playing a key role in driving exacerbated weight loss. IL-6 is well documented to play a multitude of important roles during IAV infection, including the generation of CD4⁺ memory T cells, and limiting virus-mediated neutrophil cell death (431,432). Furthermore, IAV infected *Il6*^{-/-} mice have been seen to lose more weight and have increased mortality compared to wild type mice (433). The enhanced disease in these *Il6*^{-/-} mice was attributed to increased apoptosis of infected epithelial cells and increased proliferation, migration and survival of fibroblast, both of which leading to dysregulated lung repair (433). Interestingly, in this study *Il6*^{-/-} mice also had reduced monocyte recruitment into the lungs following IAV infection. It is possible the reduction in IL-6 levels noted in the X31-CXCL1 mice result in the reduced recruitment of monocytes. It would be interesting to confirm this by treating these mice with intranasal IL-6 and observing if monocyte recruitment is restored. Monocytes have also been implicated in protection during IAV infection in mice. A study of IAV infection in monocyte chemoattractant protein 1 (MCP-1) deficient mice demonstrated these mice had impaired viral clearance and lost more weight

compared to WT mice on day 2 and 8 post infection. However, weight was not monitored on the days 3-7 post infection in this study, limiting the reliability of the data. In a study of IAV co-infection with dengue virus in *Ifnar1^{-/-}* mice, neutrophil recruitment to the lungs was significantly increased compared to IAV only mice (434). The co-infected mice subsequently lost more weight and had decreased survival compared to IAV only or dengue only mice (434). Interestingly, when monocytes from WT mice were transferred to the co-infected mice, survival was significantly improved (434). This study may suggest that monocytes are important in providing protection against IAV particularly in the context of increased neutrophil recruitment following a co-infection. However, it is important to consider *Ifnar1^{-/-}* were used as WT mice are not susceptible to dengue infection, which adds another layer of complexity when interpreting these findings as these mice have altered immune responses to respiratory virus (15,434). It would be interesting to confirm the role of monocytes in this model, either through monocyte transfer or monocyte depletion studies.

When neutrophils were recruited 12h prior to X31 infection, mice were significantly protected from weight loss. Given that the presence of more neutrophils in the lungs during X31 infection resulted in exacerbated weight loss, the neutrophils recruited prior to X31 are likely altering the immune response towards the virus to result in protection. In mouse models of RSV infection, neutrophils recruited 2 days prior to infection resulted in protection from weight loss associated with an increase in neutrophil apoptosis and an increase in AMs in the lung. Interestingly, in the CXCL1-X31 mice, protection was also associated with a sharp increase in AM numbers between days 1 and 2 post infection. Neutrophil apoptosis was not measured in the CXCL1-X31 model, however the increase in AMs was preceded by a peak in Siglec-F⁺ and CD11b^{hi} neutrophils, which were previously shown to cluster with apoptotic neutrophils. In mouse models, AMs are essential for protection against IAV infection. *Csf2^{-/-}* which do not develop AMs, lose significantly more weight and have increased mortality following IAV infection compared to WT mice (435). Transfer of CD45⁺ cells from WT mice restored the AM population and ameliorated weight loss and mortality (435). Similarly, depletion of AMs using clodronate liposomes prior to IAV infection also resulted in increased weight loss and mortality (435). Neutrophil apoptosis has also been implicated in protection against IAV. Mice deficient in the proto-oncogene B cell lymphoma 6 (*Bcl6*), have improved survival and lose less weight than WT mice following IAV infection (436). It was revealed that this protection was mediated specifically by *Bcl6^{-/-}* neutrophils, which had higher rates of apoptosis, higher expression of apoptotic gene pathways and led to attenuated inflammation in the lung following IAV infection (436). As discussed in chapter 2, increased efferocytosis of apoptotic neutrophils by AMs may result in an increase in their proliferation as well as alter their inflammatory response to respiratory virus (383,407). BCG vaccination in mice has previously been suggested to provide

protection against IAV infection by boosting efferocytosis (407). This was associated with a decrease in the levels of the pro-inflammatory cytokine TNF- α and an increase in levels of IL-10 in the airways at day 2 post infection (407). Overall, in the IAV mouse model, unlike in the RSV mouse model protection was not associated with a general decrease in inflammatory cytokine expression. However, levels of TNF- α were significantly reduced at day 2 post infection in CXCL1-X31 mice compared with X31 only mice. AMs are known sources of TNF- α during IAV infection (143). Furthermore, TNF- α has been implicated as a driver of disease severity during IAV infection in mouse models (437). Inhibition of TNF- α using etanercept treatment has previously shown improvement in weight loss, survival and lung pathology in IAV infected mice (437). Therefore, it is possible protection is a result of the reduction in this cytokine. The role through which inhibition of TNF- α results in protection from severe disease in IAV infected mice is unclear and may be due to its effects on modulating the immune response or by its effects on the appetite and the sickness response in the brain as discussed in chapter 1 (363,437).

The findings in this chapter contrast with the previously published findings and the data presented in chapter 2, on the role of neutrophils prior to and during RSV infection (17,170). Exacerbation in weight loss is elicited when neutrophils are recruited 12h after IAV infection, whilst recruitment of neutrophils after RSV infection has no effect on weight loss (17). Weight loss was exacerbated in mice which were exposed to CXCL1 12h prior to RSV infection, but in the corresponding IAV model, neutrophils recruited 12h prior to infection resulted in protection from weight loss (170). Protection from weight loss in the mouse model of RSV infection was observed when neutrophils were recruited 2 days prior to infection. Whilst the observed effects on the timing of neutrophil recruitment and its subsequent effect on weight loss differed between the two models, some parallels could be drawn. In particular, in both mouse infection models, protection was associated with a phenotypic change in neutrophils and an increase in AMs in the lung. To understand why the effect of neutrophil recruitment on weight loss is different between the two models, we compared the dynamics of the immune response to RSV and X31. Interestingly, in general the immune responses to X31 infection were delayed compared to RSV infection. The recruitment of inflammatory cells and production of inflammatory cytokines was initiated on day 2 post infection, whilst in the RSV model, this occurs earlier on day 1 post infection. The published literature and data on immune responses to RSV and IAV in humans and mice have been reviewed and compared previously (172). Unsurprisingly, this review reports the significant differences in recognition of the viruses by lung cells, the mechanisms each virus employs to evade the immune system and the cross-talk between the innate immune response and subsequent adaptive immune response (172). RSV and IAV are genomically distinct myxoviruses. Whilst both are negative sense, single

stranded RNA viruses, RSV is a Paramyxoviridae, meaning its genome is non-segmented, whilst IAV is an Orthomyxoviridae meaning its genome is segmented (27,438,439). These viruses will therefore enter host cells and replicate through distinct mechanisms (27,60). This will subsequently lead to differences in their recognition by the host cells. A number of TLRs including TLR3, TLR7, TLR8 and TLR9, as well as RLRs are important in the recognition of respiratory viruses (172). However, studies have demonstrated differences in the requirements of these receptors in IAV infected mice compared to RSV infected mice. For example, *Tlr3*^{-/-} mice show increased survival following severe IAV infection, but in contrast have increased IL-13 and mucus production following RSV infection (106,440). The activation of immune cells and production of cytokines has also been shown to be viral specific. For example, following IAVs, the predominant source of type I IFNs has been shown to be pDCs, but depleting these cells in a mouse model of RSV infection has no effect on type I IFNs (441). In contrast, AMs were found to be the major sources of IFN- α following RSV infection (15). Taken together, it is not surprising that the mouse models of RSV and IAV infection studied in this thesis demonstrate different dynamics in the immune response. Differences between the two viruses aside, there are other explanations for the delay in the immune response that must be considered. Firstly, RSV A2 is a human specific virus, to cause disease in mice they must be inoculated at high titre (26). In contrast, the influenza virus used in this model is a mouse adapted strain, X31, which causes more severe disease in mice thus they are inoculated at a much lower titre (27). The viral burden in the lungs of the RSV infected mice will therefore be significantly higher earlier immediately following infection, potentially reducing the time it takes for recognition of the virus. In addition, there are many strains of influenza virus, some of which are more adapted to mice than X31 such as PR8 and many which are human specific (27). Indeed, when studying the literature on the immune response to influenza virus in mouse models, there is lots of variation in the strain used, the inoculum titre, the subsequent timings of weight loss and the dynamics of the immune response (28–31). In a study comparing H7N9 and H9N2 infection in mice, the induction of cytokine expression differed between the viruses, and infiltration of immune cells peaked a day earlier following H9N2 infection compared to H7N9 (31). This demonstrates the importance in considering the strain of virus used in mouse models and how this could impact on the findings of your study.

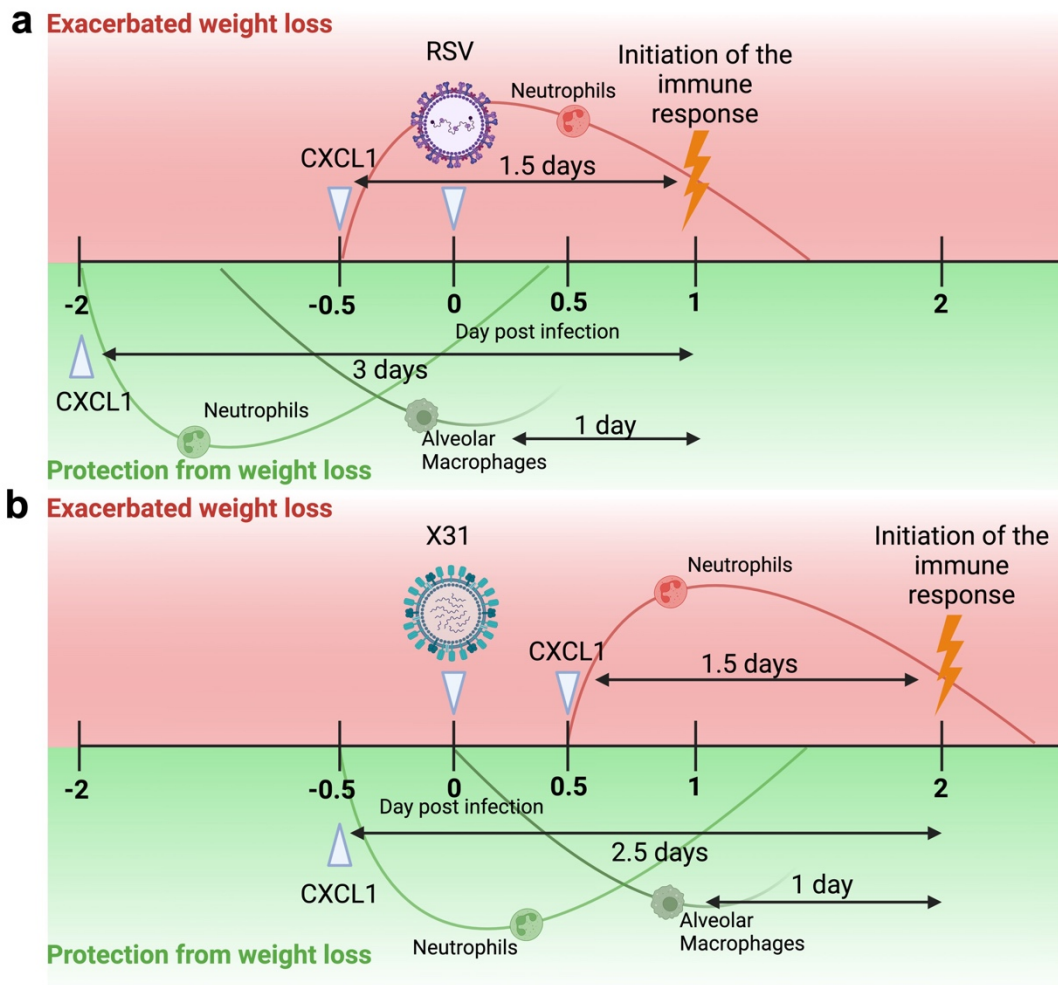


Figure 7.8 Graphical Summary

- a)** Figure detailing the effect of neutrophils on weight loss, recruited at different timepoints in respect to the initiation of the immune response in the mouse model of RSV.
- b)** Figure detailing the effect of neutrophils on weight loss, recruited at different timepoints in respect to the initiation of the immune response in the mouse model of X31.

Considering the delay in the immune response to IAV compared with the RSV mouse model, on further investigation the role neutrophils play in exacerbating or protecting mice from weight loss may actually be more comparable than initially thought. As depicted in Figure. 7.8, if we were to consider the time point of innate immune initiation rather than the time point of infection, the timings of neutrophil recruitment and subsequent effect on weight loss become the same. When neutrophils are recruited 1.5 days prior to initiation of the immune response, mice experienced exacerbated weight loss (Fig. 7.8). When neutrophils were recruited 2.5-3 days prior to initiation of the immune response, mice experienced protection from weight associated with an increase in AMs in the lung 1 day prior (Fig. 7.8). These findings demonstrate how neutrophils can exert influence over respiratory viral infection, resulting in either exacerbation or protection dependent on the time point in which they were recruited

relative to the immune response towards the virus. Understanding the mechanism through which neutrophils can provide protection could benefit the development of clinical preventions or interventions that may be broadly applicable across different respiratory viral infections. This study reveals the importance of considering the immune response to the virus as opposed to the time point of infection. In addition, the findings from this chapter demonstrate the importance of considering the mouse model, viral strain and infectious dose used to study severity following respiratory viral infection and when interpreting experimental findings.

8. Discussion

The work presented in this thesis addresses three hypotheses outlined in Figure 2.1:

- 4) *Exposure to respiratory bacterial pathogens or their PAMPs before RSV infection drives the recruitment of neutrophils to the airways which are responsible for exacerbating disease outcomes.*
- 5) *Due to their short lifespan, the effect neutrophils in the airways have on exacerbating weight loss during RSV infection in mice is dependent on them being recruited into the airways shortly before infection.*
- 6) *Neutrophils recruited into the lungs of mice before infection with IAV drive exacerbated disease.*

These hypotheses were based on published data that demonstrated the presence of neutrophils in the lungs of mice before RSV infection exacerbates weight loss (170). In Chapter 1, the first hypothesis was addressed, and mice were exposed to LPS, LTA or *A. baumannii* before RSV infection. Overall, the findings for chapter 1 lead us to reject this hypothesis. Whilst exposure to bacterial PAMPs 12h before RSV infection led to exacerbated weight loss, neutrophils were found not to be responsible for driving the exacerbation. The exacerbation driven by LPS was ultimately found to result from increased production of the inflammatory cytokines IL-1 α and TNF- α , which dysregulated the innate immune response to RSV (Fig. 8.1)

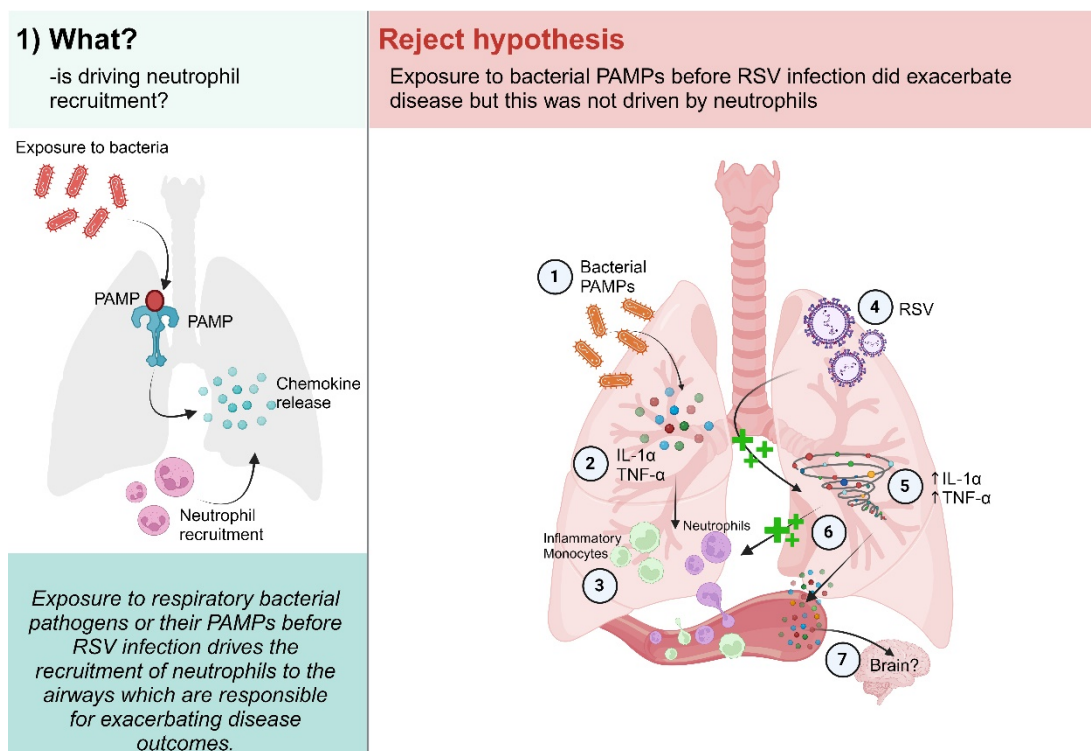


Figure 8.1 Chapter 1 hypothesis versus experimental findings

Schematic comparing the initial hypothesis addressed in chapter 1 with the actual findings which led to rejection of the hypothesis. Elements of Fig. 2.1 and Fig. 5.15 were combined to make this figure. Created using BioRender.

The dynamics of weight loss in mice exposed to LPS before RSV infection differed compared to mice exposed to CXCL1 before RSV, with mice losing more weight earlier after RSV infection (170). Whilst this was not dependent on neutrophils, LPS exposure did drive their recruitment into the lungs. It remains unclear why these neutrophils recruited by LPS did not replicate the exacerbation in weight loss at days 5-7 post RSV infection found after neutrophils were recruited using CXCL1 before infection, (170). In addition to not experiencing exacerbated weight loss at days 5-7 post RSV infection, mice exposed to LPS before infection were significantly protected from weight loss at these timepoints. CXCL1 12h before RSV infection results in exacerbated weight loss by increasing the recruitment of CD8⁺ T cells (170). In comparison, LPS before RSV infection does not lead to increased CD8⁺ T cell recruitment. As these cells are known to be responsible for driving weight loss at days 5-7 post RSV infection, this may explain why these mice do not experience weight loss exacerbation at these timepoints (332). The mechanism through which CXCL1 leads to increased CD8⁺ T cells has not yet been identified making it difficult to understand how LPS neutrophil recruitment does not have the same outcome. However, differences between the two models were observed. Importantly, downstream of neutrophils recruited by CXCL1 before RSV infection, these mice were found to have significantly higher viral load at 18h post infection (170). A greater viral load could mean more available antigen, which has previously been hypothesised to drive better T cell activation (442,443). Several studies have demonstrated the immune response towards different viruses can be shaped by viral load. A study monitoring participants before and after natural SARS-CoV-2 infection found higher initial nasal viral load correlated with better IgG responses (444). Experimental challenge with the attenuated yellow fever vaccine virus, has previously shown more rapid T cell responses in those with higher viral load up to a saturation limit, above which T cell responses did not increase further (442,443). Similarly, increased viral load has previously been linked with increased disease severity in humans and mice infected with RSV. Higher viral load in infants detected shortly after symptom onset has been associated with increased duration of hospitalisation (445,446). Experimental challenge of healthy volunteers with increasing viral titre also correlated with increased disease severity (447). Furthermore, titration experiments in mice have demonstrated increasing the initial RSV infectious dose leads to increased CD8⁺ T cell recruitment and increased weight loss (146). Exposure of LPS before RSV infection resulted in lower viral load in the lungs of mice compared to RSV only mice. Correlating with this, the total number of CD8⁺ T cells was not increased at day 8 post infection. Total numbers trended lower in the lungs of these mice at

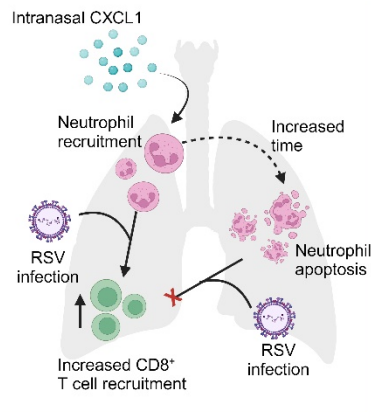
day 8 post infection, but this was not significant. Therefore, if early viral load does direct the magnitude of the T cell response during RSV infection, this might explain why these the LPS exposed mice did not experience weight loss exacerbation at days 5-7 post RSV infection. However, the effect of viral load and antigen availability on subsequent T cell responses in mouse models of RSV infection is not clear.

The restriction of viral replication and subsequent lower viral load in the lungs of mice exposed to LPS before RSV infection is likely due to the recruitment of inflammatory monocytes. When these cells were depleted in LPS exposed, RSV infected mice, viral load at day 2 post infection was significantly increased. Inflammatory monocytes have previously been shown to restrict RSV replication, however the mechanism through which they do this remains unknown (15). Whilst depletion of monocytes reduced weight loss exacerbation at day 2 post infection in mice exposed to LPS before RSV infection, it is possible that the impact on viral load could alter weight loss later on in the course of infection. It would therefore be interesting to study how monocyte depletion affects the reduction in weight loss observed between days 5-7 in mice exposed to LPS before RSV infection.

Protection from weight loss at days 5-7 post RSV infection was also observed in the mouse model presented in Results Chapter 2. In this chapter, the second hypothesis was addressed, and neutrophils were recruited by intranasal CXCL1 at longer time intervals before RSV infection. The findings lead us to accept this hypothesis, as it was found that when recruited a week before infection, neutrophils no longer exerted any effect on weight loss. However, surprisingly neutrophils recruited 2 days before RSV infection had the opposite effect on weight loss and mice were significantly protected.

2) When?

-do neutrophils need to be recruited before infection?



Due to their short lifespan, the effect neutrophils in the airways have on exacerbating weight loss during RSV infection in mice is dependent on them being recruited into the airways shortly before infection.

Accept hypothesis

-neutrophils recruited a week before RSV infection had no effect on disease. However, recruitment 2 days before infection:

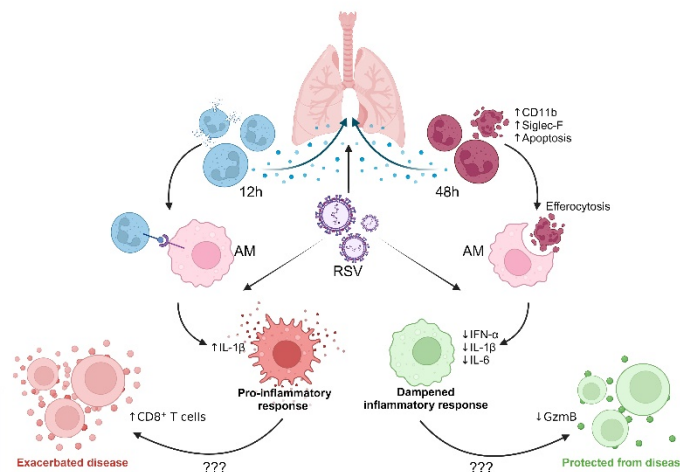


Figure 8.2 Chapter 2 hypothesis versus experimental findings

Schematic comparing the initial hypothesis addressed in chapter 2 with the actual findings which led the hypothesis to be accepted but also indicated a novel mechanism through which neutrophils may protect mice from RSV infection. Elements of Fig. 2.1 and Fig. 6.10 were combined to make this figure. Made in BioRender.

Given both CXCL1 2 days before infection and LPS 12h before infection led to protection from weight loss between days 5-7 post infection, it should be considered if this could be occurring through similar mechanisms. Unlike the LPS-RSV model, intranasal CXCL1 2 days before RSV infection did not result in significant changes to the recruitment of inflammatory monocytes. Similarly, protection was also not correlated with any changes to viral load. Instead, protection was found to be associated with apoptotic neutrophils and their potential clearance by AMs, which can alter their inflammatory response to RSV. Like CXCL1, intranasal LPS did induce a significant increase in the number of AMs in the lungs 2.5 days after exposure. However, at the time point of infection, the AM population was not significantly increased in LPS exposed mice and neutrophil numbers had not declined. This suggests the protection at days 5-7 in the LPS model is not mediated by efferocytosis of apoptotic neutrophils and is more likely to result from the increased recruitment of inflammatory monocytes. Supporting this, LPS has also previously been shown to delay apoptosis of neutrophils (448,449). Whilst this was not measured in the work presented in Results Chapter 1, a slower decline in neutrophil numbers was observed in the lungs following intranasal LPS compared to intranasal CXCL1. Furthermore, unlike CXCL1, LPS can directly activate AMs

and has been shown to induce macrophage polarisation towards a more pro-inflammatory phenotype (450). Therefore, instead of a shift towards a more anti-inflammatory phenotype noted in mexAMs co-cultured with apoptotic neutrophils, LPS may enhance the AM pro-inflammatory response to RSV.

The findings from the LPS-RSV and CXCL1-RSV mouse models demonstrate early weight loss is driven by the innate immune response, whilst weight loss later in the course of infection is driven by the adaptive immune response. Despite this, changes to weight loss at day 5-7 post infection correlate with significant changes to the innate immune response or viral load occurring early after infection. These early changes have not been mechanistically linked to the later immune response, but it is likely DCs play an important role in 'connecting the dots'. During RSV infection, cDC2s have been shown to increase in the lungs, whilst cDC1s leave the lungs and travel to the lymph nodes where they efficiently cross-present RSV antigen to CD8⁺ T cells (191). DCs therefore play an important role in initiating and directing the adaptive immune response to RSV. In mice exposed to increasing infectious dose titres of RSV, as well as CD8⁺ T cell recruitment and weight loss, the recruitment of cDC2s also increases with viral load (146). Therefore, increased early viral load in mice which received CXCL1 12h before RSV infection, may lead to increased antigen presentation by DCs which subsequently increase CD8⁺ T cell priming resulting in more T cell recruitment to the lungs and exacerbation of weight loss. Similarly, the reduced early viral load in LPS-RSV mice may lead to reduced antigen presentation and potentially less RSV specific T cell responses. Changes to viral load were not connected with protection from weight loss in mice which received CXCL1 2 days before RSV infection. However, AMs have been shown to regulate DC responses by limiting their antigen presentation functions (451). Therefore, changes to the AM inflammatory response to RSV driven by apoptotic neutrophils could limit DC functions. For example, exposure to monocyte derived TGF- β has been shown to promote the expression of MHC-II over MHC-I and reduce the expression of cognate receptors CD80 and CD86 on Langerhans cells, a subset of DCs found in the skin (452). This subsequently limited their maturation following LPS stimulation and has been suggested to potentially limit harmful inflammatory responses (452). Airway DC maturation and antigen presentation has also been shown to be limited by nitric oxide (NO) production by AMs (451). NO production or nitric oxide synthase expression or activity from AMs were not measured in this study. Whilst NO is typically thought to be released from more pro-inflammatory macrophages following an inflammatory stimulus such as LPS, it has also been shown to be increased following efferocytosis in AMs (453,454). In addition to DCs, NO from AMs can also directly regulate proliferation of T cells. This is thought to be mediated by disruption of IL-2 receptor (IL-2R) signalling via the JAK3/STAT5 pathway (455). Given their important role in initiation of the adaptive immune response, it

would be interesting to further characterise how DC populations are altered in the CXCL1-RSV and LPS-RSV mouse models.

In Results Chapter 3, the third hypothesis was addressed, and mice received intranasal CXCL1 either 12h before or after X31 infection. If the time point of infection is considered then, this hypothesis would be rejected as CXCL1 recruitment of neutrophils 12h before X31 resulted in protection from weight loss. However, as discussed previously, when the timepoint of immune activation was considered, recruitment of neutrophils had similar effects on weight loss following X31 infections as that in RSV infected mice. In this case, the hypothesis would be accepted as neutrophils recruited 12h before initiation of the immune response to X31 exacerbated disease.

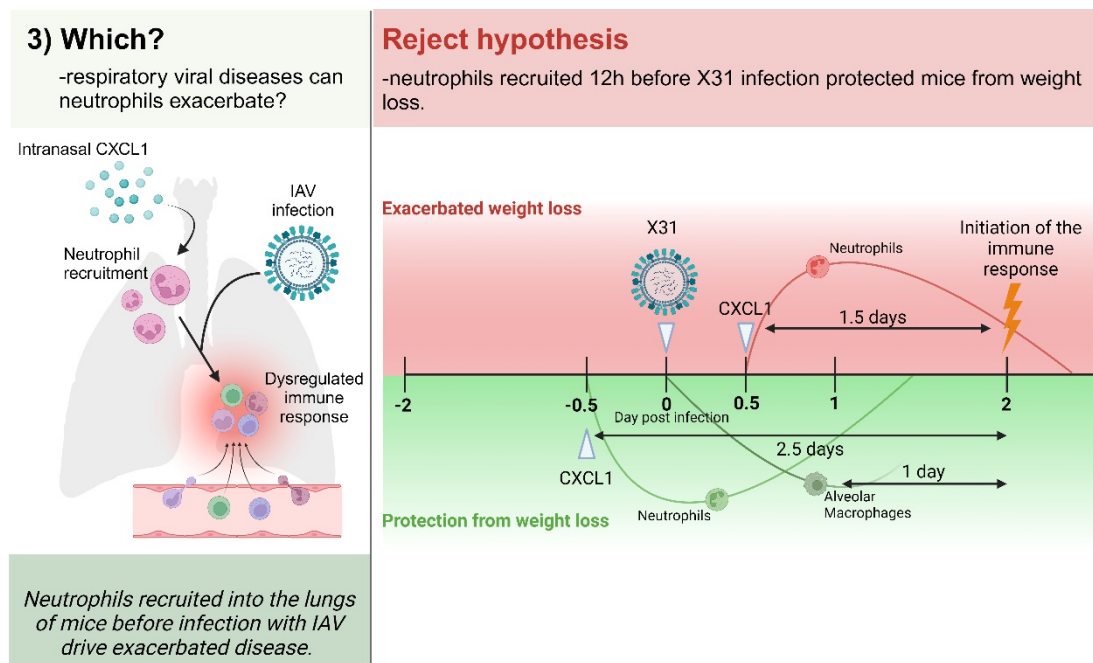


Figure 8.3 Chapter 3 hypothesis versus experimental findings

Schematic comparing the initial hypothesis addressed in chapter 3 with the actual findings which led the hypothesis to be rejected. Elements of Fig. 2.1 and Fig. 7.8 were combined to make this figure. Created using BioRender.

Like the CXCL1-RSV mouse model, reduction in weight loss in X31 mice also appeared to be mediated by an increase in AMs in the lung and phenotypic changes to the neutrophil population at the time point of immune activation. However, further experimental work is required to confirm if the mechanism of protection is the same in the X31 model as the RSV model. For example, can co-culturing neutrophils with mexAMs also alter their inflammatory response to X31.

Neutrophil recruitment driven by intranasal CXCL1 12h after X31 infection resulted in exacerbation of weight loss. This contrasted with RSV, where CXCL1 after infection has been shown to have no effect on weight loss (17). However, in both mouse models of RSV and X31 infection exacerbated weight loss occurs when neutrophils peak 1-1.5 days before activation of the immune response to the virus. Despite this similarity, it is likely that the mechanism which drives this weight loss exacerbation during X31 infection is different from the mechanism during RSV infection. As previously discussed, during RSV infection this is due to CD8⁺ T cells, but CD8⁺ T cells are known not to be responsible for driving weight loss in IAV infected mice (430). In addition, exacerbated weight loss occurred early at day 2-3 post X31 infection. The timings of this exacerbation better match the timings of exacerbated weight loss seen in mice exposed to LPS before RSV infection, further suggesting exacerbation is driven by the innate immune response. However, few other comparisons could be drawn between the LPS-RSV model and the X31-CXCL1 model. Overall, unlike in LPS-RSV mice, excluding neutrophil recruitment, the innate immune response was not exacerbated in X31-CXCL1 mice. Importantly, expression of the inflammatory cytokines *Il1a* and *Tnfa*, which were shown to drive a dysregulated immune response and exacerbated weight loss in mice exposed LPS before RSV infection, were not prolonged. Instead, certain aspects of the innate immune response were dampened in mice exposed to CXCL1 after X31 infection such as delayed expression of IL-6 and decreased inflammatory monocyte recruitment. *Tnfa* expression was also significantly reduced on day 2 post infection in X31-CXCL1 mice compared to X31 only mice. TNF- α has previously been suggested to have anti-viral properties against IAV, restricting its replication *in vitro* (456). As previously discussed, inflammatory monocyte recruitment can be dependent on TNF- α and vice versa, as TNF- α can drive monocyte recruitment and monocytes can express TNF- α (15,355). Therefore, like in RSV mouse models, inflammatory monocytes may be important in controlling viral replication early after X31 infection. Indeed, viral load was significantly elevated in the lungs of X31-CXCL1 mice on day 1 post infection compared to X31 controls. Uncontrolled viral replication has also been linked to increased disease severity following IAV infection, however unlike in RSV infected mice this appears to be independent of CD8⁺ T cell recruitment. *Rag2*^{-/-} mice infected with IAV, lose comparable weight to WT mice during the first 6 days of infection. Whilst the WT mice subsequently recover, *Rag2*^{-/-} mice rapidly lose more weight and succumb to infection (430). This pattern in weight loss correlated with viral load, which was comparable to WT mice up to day 6, but whilst WT mice then cleared the infection, viral load remained elevated in *Rag2*^{-/-} mice (430). Therefore, the increased viral load in the lungs early after infection in X31-CXCL1 may play a role in the exacerbated weight during the innate phase of infection in this model.

The work presented in this thesis expands upon what is already known about how neutrophils can influence disease outcomes during respiratory viral infections. Not only can neutrophils exacerbate disease in RSV infected mice, but due to their rapid life cycle and changing phenotype, neutrophils can also provide protection against RSV. Furthermore, these findings could be extended to a mouse model of IAV infection. It is possible neutrophils could also dynamically influence disease outcomes following infection with other respiratory viruses such as SARS-CoV-2. Increased neutrophil recruitment into the airways has been associated with increased disease severity in COVID-19 patients (457,458). Similarly, neutrophils and in particular NETs, have been implicated as drivers of organ failure in mouse models of SARS-CoV-2 infection (459). However, the effect of neutrophils in the airways before SARS-CoV-2 infection on disease has not yet been studied.

The emerging similarities in the pathways which appear to contribute to protection in the mouse models of RSV and IAV infection presented in this thesis, could provide insights on the development of future broad-spectrum treatments and interventions against respiratory viruses. For example, it is possible that treatments to shift macrophages towards an anti-inflammatory, repair orientated phenotype could subsequently dampen dysregulated immune responses towards respiratory viruses. Harnessing macrophage phenotype as a form of intervention is by no means a new concept and has been considered as a therapeutic strategy across many different disease states including rheumatoid arthritis (RA), systemic lupus erythematosus, inflammatory bowel disease (IBD), multiple sclerosis and type I diabetes (460). Many treatments for such autoimmune diseases suppress inflammatory macrophage activity and their associated cytokines which could potentially be effective in respiratory virus infected patients (460). For example, the monoclonal antibody mavrilimumab which blocks GM-CSF, has shown safety and efficacy in RA patients in phase II trials and has been associated with improved clinical outcomes in patients with severe COVID-19 pneumonia (461). However, translating drugs which inhibit macrophage activation and polarisation from use in chronic inflammatory diseases to treat acute respiratory viral infection is not without risk. Macrophages play a key role in providing protection against respiratory virus, often directing important early anti-viral and protective inflammatory responses, such as the production of type I IFNs (15). Therefore, completely inhibiting their function could be detrimental. Considering the findings presented in this thesis, a “softer” approach could be to enhance the efferocytotic capacity of AMs, promoting the clearance of neutrophils and promoting resolution. The antibiotic azithromycin has previously been shown to enhance the clearance of apoptotic cells by AMs from patients with COPD (462). Interestingly, early azithromycin treatment given as soon as symptoms of respiratory tract infection were detected in preschool children with a history of severe LRTI, was found to reduce the likelihood of

progression to severe LRTI (463). Taken together, this could provide evidence for the efficacy of pro-efferocytotic treatments during respiratory viral infections. However, the use of antibiotics for such therapies could be unethical given the growing threat of antibiotic resistance. A clinical trial investigating treatments which activate apoptotic receptors on AMs to enhance efferocytosis in COPD patients has recently been completed (ClinicalTrials.gov ID NCT04775394). However, details on these treatments and their efficacy are yet to be reported.

The reason why neutrophils were present in the airways leading to exacerbated disease outcomes in participants of the human RSV challenge study is still unknown (170). However, the findings presented in Results Chapter 1 suggest that this is likely not due to previous exposure to or infection with bacterial pathogens. This is supported by microbiota profiling of these participants, which did not show any obvious changes to bacterial populations in the airways (170). Despite this, the mouse model of bacterial PAMP exposure before RSV infection can still provide us with insights that could be clinically valuable. The majority of research on RSV and bacterial co-infections has considered the bacterial infection to be secondary to RSV (464). Whilst this is clinically important, and nosocomial bacterial infection often results as a complication of hospitalisation due to RSV infection, a significant proportion of patients present at hospital with an already established co-infection and there is no way of telling which infection preceded the other (316). In addition, studies have revealed how changes to the microbiota before RSV infection can correlate with exacerbated disease outcomes (83). Providing an insight into the mechanism through which bacteria can exacerbate RSV infection is therefore of clinical relevance. For patients who present with RSV and bacterial co-infection, antibiotics are usually the primary treatment (315). The mouse models presented in Results Chapter 1 demonstrate active bacterial infection is not required to exacerbate disease during RSV infection, and that exposure to bacterial PAMPs before infection is sufficient to drive rapid exacerbation. If the same mechanisms occur in patients, antibiotic intervention at the point of hospitalisation would therefore not be appropriate. IL-1 α and TNF- α were identified as key drivers of the immune dysfunction which led to exacerbated disease outcomes and therefore have the potential to be better therapeutic targets. There are several TNF- α inhibitors already available and licensed for the treatment of inflammatory diseases like RA and IBD, including the protein inhibitor etanercept and the monoclonal antibodies (mAbs) infliximab, adalimumab, golimumab, and certolizumab (465). Similarly, IL-1 family receptor inhibitors are also clinically available including anakinra and rilonacept (466). These treatments have so far not been trialled in RSV patients, however TNF- α blockade has proven success in reducing disease outcomes in mouse models of RSV (467,468).

Outside of the context of co-infections, increased IL-1 α and TNF- α could also explain the increased susceptibility to RSV infection in patients with different risk factors. For example, it is well documented that as we age, baseline inflammatory markers in the blood and tissues increase in a process termed inflammageing (469). Increased TNF- α has been found in the BAL of both elderly mice and humans compared to their younger counterparts (470). In addition, IL-1 α has previously been identified as a marker of cellular senescence (471). A baseline increase in these cytokines has also been observed in obese patients, which has previously been reported as a risk factor for severe RSV infection (472–474). In addition, age and obesity are both risk factors associated with increased disease severity following infection with IAV and SARS-CoV-2 (475–478). Therefore, targeting these cytokines could be beneficial when treating severe respiratory viral infections across a broad range of patients with varying risk factors.

Overall, placing the work in this thesis into a wider clinical context, the findings shed a new light on mechanism which could exacerbate or provide protection from disease following RSV infection. By further studying these mechanisms and comparing them with immune responses which occur in humans, there is the possibility to develop broad spectrum interventions or treatments which prevent the development of severe disease following respiratory viral infections.

9. Future Work

9.1 Investigate the significantly reduced weight loss at days 5-7 post infection in mice exposed to LPS before RSV infection.

Despite driving early weight loss at days 1-3 post RSV infection, prior LPS exposure also resulted in a significant reduction in weight loss at days 5-7 post RSV infection. This could not be explained by the data collected on the adaptive immune response in Results Chapter 1. Both CD8⁺ and CD4⁺ T cell recruitment was measured at days 4 and 8 post infection and no significant changes were observed. However, the adaptive immune response was not profiled in more depth. On day 8 post infection, there was a trend to lower numbers of CD8⁺ T cells in the lungs but due to variations in the data set this was not significant. Because CD8⁺ T cells are known to drive the weight loss at days 5-7 post RSV infection, it would be worth investigating their role in the LPS-RSV model in more detail (332). In particular, the reduction in weight loss at days 5-7 post infection, might not be due to changes in CD8⁺ T cell recruitment, rather to changes in their functionality. Cytokines and effector proteins associated with activated CD8⁺ T cells following RSV infection include IFN- γ , TNF- α and GzmB (479). Whilst these can all be measured by ELISA or qPCR, they can be produced by other cell types. Therefore, to use these cytokines as a measure of CD8⁺ T cell functionality, it is best to use flow cytometry and shown for GzmB production in Chapter 2. T cells can be re-stimulated using virus peptides or PMA and ionomycin. This allows for the subsequent intracellular staining of cytokines (154,479). TNF- α producing CD8⁺ T cells have previously been suggested to be responsible for driving weight loss during RSV infection (167). Similarly, GzmB producing T cells have been implicated as drivers of lung pathology in COPD and severe asthma (480,481). It is possible LPS could modulate changes to the production of these cytokines by CD8⁺ T cells which results in the protection from weight loss.

Whilst the CD8⁺ T cell response is more commonly associated with weight loss during RSV infection in mice, it would also be interesting to further dissect the CD4⁺ T cell response. When CD4⁺ T cells are depleted during RSV infection, a partial amelioration is seen, suggesting they play a role in driving disease (332). RSV infection predominantly drives a Th1 CD4⁺ T cell response. Th1 cells, characterised by IFN- γ and TNF- α production, are important in directing the CD8⁺ cytotoxic T cell responses. Therefore, the magnitude of the Th1 response could influence the magnitude and

functionality of the CD8⁺ T cell response and impact on weight loss following RSV infection. In addition to Th1 responses, other CD4⁺ T cell subsets have been implicated in disease following RSV infection. Some studies have suggested a skew towards Th2 biased responses can be correlated with increased disease severity in infants. (482,483). Th2 responses following RSV infection have also been linked to the development of asthma in infants (484). In contrast, Tregs play an important role in protection against RSV infection. In mice, selective depletion of Tregs greatly increases weight loss following RSV infection (485,486). It would be interesting to study if LPS exposure before RSV infection could affect these T cell populations and if this could be linked with changes to weight loss at days 5-7 post infection.

Although it is likely that changes to the T cell response at days 5-7 post infection are responsible for the protection from weight loss observed in LPS exposed, RSV infected mice, the exacerbated innate immune response likely directs the changes to the adaptive response. It would be interesting to study this further to identify a mechanism. For example, how does depletion or blockage of innate immune cell populations or proinflammatory cytokines affect the weight loss at days 5-7 post RSV infection. To this, the protocols used in Results Chapter 1 for neutrophil, inflammatory monocyte, IL-1 α , IL-1 β and TNF- α depletion/blockage could be repeated but extended to allow weight loss to be monitored up to day 8 post infection.

9.2 Determine if exposure to other pathogens or long term PAMP exposure before RSV infection also exacerbate disease through similar mechanisms.

In Results Chapter 1 we found that exposure to gram-negative LPS, *A. baumannii* infection, and gram-positive LTA before RSV infection all had comparable effects on weight loss and innate inflammation. This suggests that the mechanism is not specific and could be driven by exposure to any bacteria. It would be interesting to confirm this by infecting mice with a range of clinically relevant respiratory bacteria with differing pathogenicity. For example, how does infection with pathogenic gram-positive bacteria, such as *Streptococcus pneumoniae* which cause high cases of community acquired pneumonia in infants, affect disease outcomes with subsequent RSV infection? (487). A mouse model of intranasal *S. pneumoniae* infection before IAV infection has previously been described (488). However, in this model over 50% of mice succumbed to co-infection within the first 4 days of IAV infection. Therefore, to

fully profile the immune response the dose and strain of *S. pneumoniae* would require titration.

In addition to exposure to bacteria, it would be interesting to investigate how exposure to other pathogens before RSV infection can alter disease severity. For example, increased exposure to environmental fungus has previously been associated with higher incidence of lower respiratory tract infections in infants (489). Mouse models of respiratory fungal infection, such as *Aspergillus fumigatus* or *Cryptococcus neoformans* have been well described (490,491). Intranasal infection models with fungal pathogens have previously been shown to rapidly result in dissemination to other organs, such as the brain. Therefore, care would need to be taken in titrating the optimum exposure dose prior to RSV infection to prevent unnecessary severity in the model (491).

In addition, it was also only studied how a single exposure to bacterial PAMPs before RSV infection affected disease outcomes. However, physiologically, exposure to increased levels of environmental PAMPs is likely to occur repeatedly over a long period of time (321). Similarly, changes to the microbiota result from long-term growth and colonisation of the airways by a new strain of bacteria. Therefore, it would be interesting to set up a mouse models that would better replicate such scenarios. Repeated intranasal exposure models have been well described and studied in the context of allergen exposure and the development of allergic airway disease (492–494). In these models, mice are intranasally exposed to house dust mite multiple times a week, over the course of 3–4 weeks, which has been seen to result in inflammatory changes to the lungs (492–494). The effect of long-term intranasal exposure to LPS on inflammation in the lungs is less well studied. Repeated intratracheal exposure to LPS in mice over the course of 1 to 8 weeks, has demonstrated persistent cellular inflammation and a sustained increase in TNF- α expression in the lungs (495). Similarly, daily exposure to 4 μ g LPS over the course of 3 days resulted in sustained neutrophilia in the airways (496). It would be interesting to build upon these prior studies, to develop a protocol for long-term intranasal exposure to LPS, study how this alters the expression of inflammatory cytokines in the lung and ultimately how this affects RSV disease outcomes.

9.3 Investigate different ways to drive neutrophil recruitment into the lungs before RSV infection.

Neutrophils recruited by exposure to bacterial PAMPs before RSV infection, did not result in exacerbated disease following subsequent RSV infection. Therefore, bacterial exposure may not explain why neutrophils, which were associated with the development of symptomatic illness, were present in the airways before RSV infection in healthy adult volunteers (170). It would be interesting to study other mechanisms which drive airway neutrophil recruitment, and how they might influence RSV disease outcomes, to better identify at risk groups. Cigarette smoke is well known to drive neutrophil recruitment and is associated with the development of COPD. In addition, second-hand exposure has been associated with increased risk of severe LRTI following RSV infection in children (497). Therefore, it would be interesting to interrogate the role in neutrophil recruitment driven by cigarette smoke inhalation in driving disease severity to RSV infection. Mice can be exposed to cigarette smoke as previously described using a smoking apparatus chamber (498). In addition to cigarette smoke, exposure to air pollutants, particularly in city environments, has become increasingly linked with the development of airway disease in RSV bronchiolitis (499–501). Mice can be exposed to air pollutants both intranasally, and by aerosolisation of fine particulate matter, both of which have been shown to drive neutrophil recruitment (502). Also ever present in the air we breathe are allergens such as pollen and house dust mite which have also been shown to drive neutrophil recruitment in mice (312,492). The majority of studies looking at allergens and RSV focus on how RSV infection can sensitise children to allergens, leading to development of asthma. However, it would also be of interest to study how allergen levels in the environment may predispose individuals to more severe disease following RSV infection. Mice could be exposed to each of these three stimuli and infected with RSV either after an acute or chronic exposure. The effect of these stimuli on weight loss and inflammation could then be profiled, and the role of neutrophil recruitment determined by antibody depletion experiments described in chapter 1.

9.4 Further examine the role of efferocytosis of neutrophils by AMs in providing protection against respiratory viral induced disease.

In chapter 2, it was not conclusively shown that protection from weight loss in mice exposed to CXCL1 2 days before RSV infection was due to efferocytosis. There are

several ways which efferocytosis of apoptotic neutrophils could be quantified in mexAM cultures and *in vivo*. For example, green fluorescence protein expressing (GFP⁺) neutrophils could be co-cultured with mexAMs, which don't express GFP. Efferocytosis could then be quantified by measuring GFP⁺ expression associated with mexAMs (503). Similarly, apoptotic neutrophils can be labelled with a pH sensitive dye called pHrodo, which will only fluoresce when engulfed by macrophages (504). Both of these techniques could also be extended to *in vivo* models, where fluorescently labelled, apoptotic neutrophils are delivered intranasally before RSV infection (505). In mouse models where neutrophils are recruited from circulation rather than delivered intranasally, it is more difficult to selectively stain them with pHrodo. Therefore, another potential way to measure efferocytosis would be to permeabilise and stain for intracellular Ly6G expression in AMs (506). As AMs do not typically express Ly6G, the expression of intracellular Ly6G would be indicative that they have efferocytosed neutrophils. However, as seen in chapter 2, over time neutrophils begin to upregulate the expression of extracellular Siglec-F which is used to identify AMs. Similarly, AMs have high autofluorescence and can appear Ly6G⁺, therefore potentially leading to false positive data.

In addition to proving efferocytosis of neutrophils by AMs is taking place, it is important to demonstrate that this mechanism is a requirement for protection from RSV induced weight loss. To this, efferocytosis could be inhibited by targeting receptors which initiate and facilitate the process. There are many receptors involved in efferocytosis but one of the best described is MerTK (507). *Mertk*^{-/-} mice have been shown to have reduced clearance of apoptotic thymocytes (507). These mice could potentially be used to investigate the requirement of efferocytosis in providing protection towards RSV both *in vivo* and *ex vivo* by generating mexAM cultures from their BAL. In addition, an inhibitor towards MerTK has been developed, BMS794833, which has been shown to reduce efferocytosis *in vitro* and in mouse models (508).

9.5 Study how neutrophil recruitment before RSV infection might alter the development of the T cell memory response.

Generation of T_{RM} cell memory is important in providing protection against RSV re-infection (154). Neutrophils recruited at different time intervals before RSV primary infection can alter the recruitment and functionality of T cells (170). In addition, in mice

exposed to CXCL1 2 days before RSV infection, IFN- α was significantly reduced. Type I IFNs during primary infection have previously been shown to be essential in the generation of CD8⁺ T_{RM} cells (154). Therefore, it would be interesting to determine how neutrophils recruited by intranasal CXCL1, may alter T_{RM} populations upon re-infection. To do this, mice would be exposed to CXCL1 either 2 days or 12h before RSV infection. As previously described, mice would then be re-infected with RSV 21 days after primary infection and culled on day 4 post-secondary infection to study the T_{RM} populations (154). CD8⁺ T_{RM} cells are classically identified as CD62L⁻ CD44⁺ CD8⁺ CD103⁺ CD69⁺ cells, whilst CD4⁺ T_{RM} in the lung are identified as CD62L⁻ CD44⁺ CD4⁺ CD69⁺ cells (154).

9.6 Investigate how neutrophil recruitment before and after SARS-CoV-2 infection alters disease outcomes.

Having found neutrophils to influence disease outcomes in both mouse models of RSV and IAV infection, it would be interesting to study their role during SARS-CoV-2 infection. Neutrophils have been associated with increased disease severity in COVID-19 patients, but it is not conclusively known if they contribute to pathology or protection. Likewise, it is not known if neutrophils in the nose/airways before SARS-CoV-2 could be a risk factor for severe disease. Mice cannot be naturally infected by SARS-CoV-2 as they lack the ACE2 receptor required for viral entry. However, several mouse models of successful SARS-CoV-2 infection have already been developed. One of these requires transfecting mice with adenovirus vectors which express the gene for human ACE2 (509). These mice will therefore express hACE2 and can be infected with SARS-CoV-2 and have a measurable immune response (509). However, these mice do not lose significant weight throughout the course of infection, so whilst they can be used to study disease exacerbation, they may not be a suitable model for identifying protection (509). Transgenic mice which express hACE2 under the control of the K18 promoter have also been developed for the study of SARS-CoV (510). These mice do lose significant weight when infected with SARS-CoV-2, making them a good model to study protection (510). However, this model is associated with high lethality due to rapid viral dissemination to the brain, meaning it may not be the best model to study disease exacerbation (510). To overcome this lethality, humanised hACE2 knock-in mice have been developed, where the *hACE2* gene is inserted precisely downstream

of the mouse *Ace2* start codon and upstream of the *mAce2* stop codon. These mice therefore express hACE2 instead of mACE2 and have improved survival upon SARS-CoV-2 infection compared to K18-hACE2 mice (511). These mice could be exposed to CXCL1 before and after infection to investigate how neutrophils alter disease.

10. Concluding Remarks

To finish, this thesis builds upon work which demonstrates how neutrophils can and cannot exacerbate disease during RSV infection (17,170). It was demonstrated that exacerbation as a result of neutrophils present in the airways before infection is likely dependent upon the physiological mechanism which drives their recruitment. Neutrophils recruited by bacterial PAMP exposure before RSV infection were found not to be responsible for driving disease at day 6-7 after the infection. However, PAMP exposure did drive increased disease severity dependent on pro-longed pro-inflammatory cytokine expression early after infection. In addition, it was demonstrated that exacerbation following RSV infection was dependent on when neutrophils were recruited before RSV infection. Instead of exacerbating disease, neutrophils recruited to the airway at a longer time point before RSV infection can change the inflammatory response to the virus and subsequently protect mice from weight loss. Finally, this was extended to a mouse model of X31 infection where neutrophils could also exacerbate and protect mice, dependent on the time point they were recruited relative to infection. While this work begins to answer the hypotheses set out at the beginning of this thesis, it also opens new avenues of research that could better expand our knowledge on risk factors that determine severity to respiratory viral infections.

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