Phenotype of Pulmonary Macrophages and Role of Recruited Monocytes in Respiratory Syncytial Virus (RSV) infection

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ABSTRACT

Respiratory syncytial virus (RSV) is a major cause hospitalization of children with respiratory illness worldwide. RSV infection can lead to severe bronchiolitis in some infants. This is characterised by excessive inflammation leading to airway occlusion. Therefore, understanding and managing inflammation in RSV disease is of great importance.

Viral infection leads to activation and recruitment of innate cells into the airway and lungs which mediate the release of an array of cytokines and chemokines. Studies in murine model of influenza infection in the lung showed that recruited inflammatory monocytes promote pathology, but our knowledge of their role in RSV infection is limited. We hypothesized that inflammatory monocytes recruited from the bloodstream into the lung (rather than resident alveolar macrophages) contribute to inflammation during the early stages of RSV infection.

Infecting mice with human A2 strain RSV, F4/80^{pos} CD11b^{pos} CD11c^{int} Ly6C^{pos} inflammatory monocytes rather than resident F4/80^{pos} CD11b^{neg} CD11c^{pos} Ly6C^{pos} alveolar macrophages predominated in the airway and lung on day 2 of RSV infection. The frequency of these cells decreased both in the airway and lungs at day 4 suggesting changing roles for different cell types during the evolving response to RSV infection.

To determine the role of inflammatory monocytes, blood monocytes were depleted via intravenous injection of clodronate liposomes during RSV infection. This reduced numbers of F4/80^{pos} CD11b^{pos} CD11c^{int} Ly6C^{pos} cells both in the airway and lung at day 4 and 7 compared to simple RSV infection. This depletion caused attenuated weight loss, suggesting reduction in lung pathology. Although there was an increase in NK cell number in the airway at day 4 and 7 post infection with monocyte depletion, CD4^{pos} and CD8^{pos} T-cell percentage and number were effected.

We conclude that inflammatory monocytes profoundly affect the inflammatory responses to RSV infection and depleting them may be protective against RSV disease.

(Word count: 300)

Statement of Contribution

All of the laboratory work in this thesis is my own.

Siti Sarah Affendi 2014

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ABBREVIATIONS

A	
AM	Alveolar Macrophages
APC	Antigen presenting cell or Allophycocyanin
ARDS	Acute respiratory distress syndrome
B	
BAL	Broncho Alveolar Lavage
BM	Bone Marrow
BSA	Bovine Serum Albumin
C	
CCL2	CC-chemokine ligand 2
CCR2	CC-chemokine receptor 2
CD	Cluster of Differentiation
cDMEM	Complete Dulbecco's Modified Eagle Medium
cDNA	Complementary DeoxyriboNucleic Acid
CFSE	Carboxyfluorescein succinimidyl ester
CL	Clodronate Liposome
CR	Chemokine Receptor
CSF	Colony Stimulating Factor
D	
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
E	
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
F	
F	Fusion Protein
FACS	Fluorescence assisted cell sorting
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FI-RSV	Formalin Inactivated RSV vaccine

FITC	Fluorescein isothiocyanate
G	
G	Attachment proteins
GAGs	Glycosaminoglycans
H	
HKL	Heat-killed Listeria
I	
i.n	Intranasal
i.p	Intraperitoneal
i.v	Intravenous
ICOS	Inducible T-cell co-stimulator
IFA	Immunofluorescence Assay
IFN	Interferon
IL	Interleukin
IM	Interstitial Macrophages
K	
KC	Chemokine (C-X-C Motif) Ligand 1 or CXCL1
KO	Knock out
L	
LRTI	Lower Respiratory Tract Infection
M	
M	Matrix proteins
MFI	Medium Fluorescence Index
MHC	Major Histocompatibility Complex
mIL-4	Murine Interleukin 4
MPS	Mononuclear phagocyte system
N	
NFκB	Nuclear factor kappa-light chain-enhancer of activated β cells
NK Cells	Natural Killer Cells
NO	Nitric Oxide
NS	Non-structural protein

P	
PBS	Phosphate buffered saline
PCL	Phagocytic Cell Linker
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
R	
R10F	Complete Roswell Park Memorial Institute medium
RNA	RiboNucleic Acid
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute medium
RSV	Respiratory Syncytial Virus
RSV-WT	Respiratory Syncytial Virus-Wild Type
RT	Reverse Transcription
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S	
SH	Small Hydrophobic protein
T	
TCR	T cell receptor
TGF	Transforming Growth Factor
Th	T-helper
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor α

1.0 INTRODUCTION

1.1 PARAMYXOVIRUS

1.1.1 Introduction

The nomenclature Paramyxovirus is derived from three Greek words; para- beyond, myxo- mucus and virus- poison. Paramyxoviruses belong to the Paramyxoviridae family under the order of mononegavirales (Lamb, 2007). Mononegavirales is defined by the Greek word mono- single, which refers to the single stranded genomes of these viruses; the Latin word negare- negative, referring to the negative polarity of the viral genome; and virales is a suffix in taxonomy which signifies viruses (Kuhn, 2010). Hence, they are all negative sense, single-stranded, RNA viruses. They cause a number of important diseases in humans and animals alike with a vast host range; both land-based and aquatic. They can be further divided into three sub-families; Paramyxovirinae, Pneumovirinae and Morbilivirinae. Amongst the major human Paramyxovirinae viruses are the mumps virus (MuV) and parainfluenza viruses whilst respiratory syncytial virus (RSV) is classified as a member of Pneumovirinae and measles virus (MeV) is a member of the Morbilivirinae. This was based on the organization of the viral genome, protein sequences encoded within the genome, biological activity of the proteins and morphological characteristics of the viruses (Lamb, 2007).

1.1.2 Epidemiology

Human viruses of the Paramyxoviridae family are important pathogens. All of the viruses in this sub-family causes respiratory disease, particularly in children and they are highly contagious. Parainfluenza and RSV are the main contributors to cases of bronchiolitis croup and pneumonia in infants. In the past, measles and mumps have been major threats worldwide. Today, the success of immunization has decreased their occurrences in developed

countries. Measles however remains to be the major cause of death amongst infants that are undernourished in the Third World countries with over a million deaths recorded annually (Aaby et al, 1986).

1.2 RESPIRATORY SYNCYTIAL VIRUS

1.2.1 Epidemiology and Clinical Burden

One of the first studies of pneumonia was published by Goodpasture in the year 1939. Then in 1941 a comprehensive report on a RSV outbreak was made by Adams where a nosocomial infection in a neonatal nursery affected 32 children, killing nine (Adams, 1941). The first isolation of this virus was in 1956 from a chimpanzee with coryza illness by Morris and associates. From cell culture, it was found that this virus is capable of infecting other chimpanzees thus named "chimpanzee coryza agent" (Morris, 1956). In the following year, Chanock and his colleagues isolated the same virus from a child with croup in Baltimore (Chanock and Finberg, 1957). The virus was renamed respiratory syncytial virus due the syncytium formation from cell fusion in cell culture (Chanock, 1957; O' Donnell 2008).

The distribution of RSV disease was originally documented in parts of the world with temperate climate. Today, RSV is known as a global disease in all climates. RSV is extremely infectious with outbreaks occurring annually and seasonally. In temperate climate most cases were reported from November until March, whilst in the tropics, it is more common during rainy season (Falsey and Walsh, 2000; McNamara and Smyth, 2002; Simoes, 1999; Sorce, 2009).

Viral bronchiolitis is the most common cause of infantile hospitalisation in the western world. Approximately 70% out of the cases are caused by RSV infection making RSV one the most important causes of acute lower respiratory tract infection with bronchiolitis and pneumonia during infancy and early childhood. What makes RSV infection burdensome to the clinical world is the added weight it imposes towards the paediatric cases, particularly infants and children under the age of three. In the first 18 months of age, approximately 87% of these children have RSV antibodies and by the age of three almost all have been infected (Simoes, 1999). Furthermore, 2-3% out of these required hospitalisation and mechanical ventilation. Re-infection with the same strain of virus may occur regularly throughout life. The almost non-existence of immunity against RSV is still a big puzzle towards managing the disease. Development of asthma and difficulties in breathing in adulthood are believed to be due to infection during infancy (Samet et al., 1983). RSV bronchiolitis in infancy is also known to be a significant risk factor for recurrent wheezing, asthma and airway obstruction in children later in life (Kimpen and Simoes, 2001; Sigurs et al., 1995). However, recurrent bronchiolitis in infant is fairly uncommon (Braciale, 2005; Glezen, 2009; McNamara and Smyth, 2002). In recent years, RSV infection has increasingly caused significant disease and mortality in the elderly and immunocompromised patients (Falsey et al., 2005).

1.2.2 Structure and Genome

RSV is an enveloped RNA virus with a negative sense, single-stranded, non-segmented genome consisting of 10 genes encoding 11 proteins (Fig 1). RSV is one of the more complex members of the Paramyxoviridae family. At 15.2 kb length, RSV genome has a similar size to the other members but with additional genes and proteins; NS1, NS2, SH, M, and M2 (Collins and Melero, 2011; Easton et al., 2004).

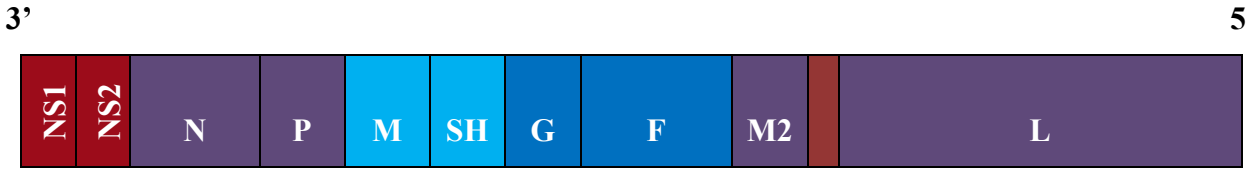


Fig 1: Genome organization of Human Respiratory Syncytial Virus

RSV virions are heterogeneous in size and shape with pleomorphic spherical or filamentous forms. They consist of a nucleocapsid core of N, P and L proteins with a lipid bilayer derived from host cell membrane that surrounds the genome. The viral envelope is covered with projecting spikes made up of three surface glycoproteins, G, F and SH that measures 11-20nm long separated by 6-10nm (Easton et al., 2004; Falsey and Walsh, 2000; McNamara and Smyth, 2002).

The G (attachment) glycoprotein mediates attachment of the virus to the cell surface but may not be the only protein that is responsible for viral attachment. Recombinant RSVs that lack the G protein still have the ability to infect some cell types as effectively as their wild type counterparts (Easton et al., 2004; Hacking and Hull, 2002; McNamara and Smyth, 2002). This raises the possibility that the F (fusion) protein, a highly conserved protein, has additional functions in viral attachment to the host's co-receptor. The F protein is involved in penetration of the virus into cells and syncytium formation between infected adjacent cells. Amongst the RSV glycoproteins, the G and F proteins are the most studied. These two proteins induce protective antibodies, making them major antigenic determinants of the virus (Easton et al., 2004; Hacking and Hull, 2002; Ogra, 2004).

Presence of the other surface proteins G and SH is also important for efficient syncytium formation (Easton et al., 2004; McNamara and Smyth, 2002). The small hydrophobic, SH

protein may also have an important role in syncytium formation where cell death via apoptosis may be blocked and inhibit TNF-α signalling (Fuentes, 2007). The M and M2 proteins are the non-glycosylated matrix proteins contribute to transcription of viral genome as well as viral assembly (Ogra, 2004; Simoes, 1999). The synthesis of RSV RNA is carried out by the large polymerase protein; L protein which is found in the nucleocapsid of the virus. The two remaining proteins are the non-structural proteins; NS1 and NS2 are only found in infected cells and not in virions (Ruuskanen and Ogra, 1993). These proteins play an important role in host defence via the inhibition of type-1 interferon signalling and production (Collins and Graham, 2008).

Strains of RSV have minimal antigenic heterogeneity. Studies using monoclonal antibody typing identified two subtypes of RSV; A and B. These two subtypes are commonly found co-circulating during a single outbreak and, simultaneous infection within one individual is possible (Bueno et al., 2008). The multiplicity of the viral strain genotypes were identified based on the amino acid sequence of their G and F glycoproteins (Johnson et al., 1987). In a more recent study, the major antigenic differences between the two strains were found in the G, F, N and P proteins. The G protein is most variable with only 53% homology in the amino acid sequence in the protein between the two strains (Ruuskanen and Ogra, 1993).

1.2.3 Life Cycle

Respiratory epithelial cells form the first line of defence against pathogens that enters the respiratory tract and the lungs. RSV preferentially infects airway epithelial cells that line the nasal cavity as well as the large and small airways (Easton et al., 2004; McNamara and Smyth, 2002). Viral entry is the most crucial step in the infection process. Upon attachment to the cell by the G protein onto the cell surface, the virus entered the cell via membrane

fusion by the fusion protein or the F protein (Hacking and Hull, 2002; Oshansky et al., 2009). Cell fusion then releases the viral nucleocapsid into the cell cytoplasm. Genomic replication is then followed by viral assembly and release of mature virions via cell budding at the apical surface or by fusing to adjacent cells forming syncytia. (Hacking and Hull, 2002).

1.2.4 Pathogenesis of RSV Infection

RSV typically infects the upper respiratory mucosa of the nose or the eyes via direct contact or aerosol. Infection is often limited to the upper respiratory tract (URT), but in severe RSV disease, infection of the lower airways may result which leads to epithelial necrosis, sub mucosal oedema, ciliary detachment, and production of cellular debris which can cause mucous plugging, small airway obstruction and atelectasis (Fig 2) (Falsey and Walsh, 2000; Hall, 2001; McNamara and Smyth, 2002).

Incubation and replication processes takes about 2-8 days before viral shedding. Clinical manifestations of RSV disease start showing 4-5 days after viral inoculation. Signs and symptoms in the lower respiratory tract (LRT) shows 1-3 days after the onset of rhinorrhea (Hall, 2001). However, the underlying mechanism by which the virus spreads from the upper respiratory tract (URT) to the LRT is not fully understood.

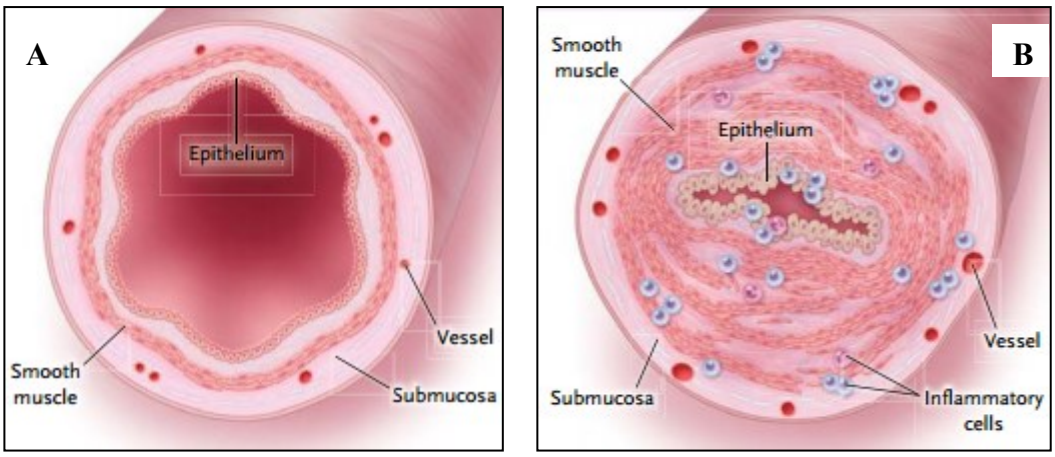


Fig 2 : Cross section of normal bronchiole (A) and affected by bronchiolitis (B). (Adapted from Hall *et al*, N Engl J Med 2001; 344:1917-1928) Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

Symptoms of RSV disease vary between individuals, ranging from mild cold to severe bronchiolitis with severe respiratory complications. Most cases of RSV-associated illnesses are reported in infants, children under the age of 3, the elderly and immunocompromised adults. This disease spreads rapidly amongst susceptible populations such as children in childcare centres and elderly people in long term care facilities. It is also common to have the disease spreading amongst family members. Babies born during autumn and winter have higher risk of severe RSV-related illness compared to those born in spring and summer (Hacking and Hull, 2002; Ogra, 2004).

1.2.5 Immunological Responses and Clinical Features

The severity of RSV-related bronchiolitis in infants may be due to the immunological mechanisms involved during infection. Upon infection with RSV, an array of cytokines and chemokines were released in the airway that contributes to bronchial hyper responsiveness, mucous secretion, inflammation and sneezing (Glezen, 2009; Sorce, 2009). In contrast, a

study by Bennett and colleagues showed the increased levels of pro-inflammatory cytokines/chemokine in RSV-infected children leads to a lesser need for extended supplemental oxygen therapy. This analysis suggests that pro-inflammatory immune response plays a vital role in protection against viral infection, particularly in RSV (Bennett et al., 2007). Nevertheless, there is a delicate balance between the protective and disease-enhancing effect of the host's immune response towards RSV.

RSV infection modifies the immunological environment in the airway. Upon entry into the alveoli, the epithelial barrier is broken via attachment of the viruses. Epithelial cells in the airway are targets for RSV infection which then becomes the site of activation for the innate immune response. This then leads to induction of IFN-α and IFN-β via activation of NFκB and TLR3. Simultaneously, engagement of RSV to TLR4 via the binding of the F protein of the viral envelope induces secretion of an array of cytokines and chemokine which in turn attracts inflammatory innate cells into the airway. Inflammation induced by viral inflammation further mediates release of more chemoattractants leading to more cellular infiltration into the airway (Fig 3) (Bueno et al., 2008).

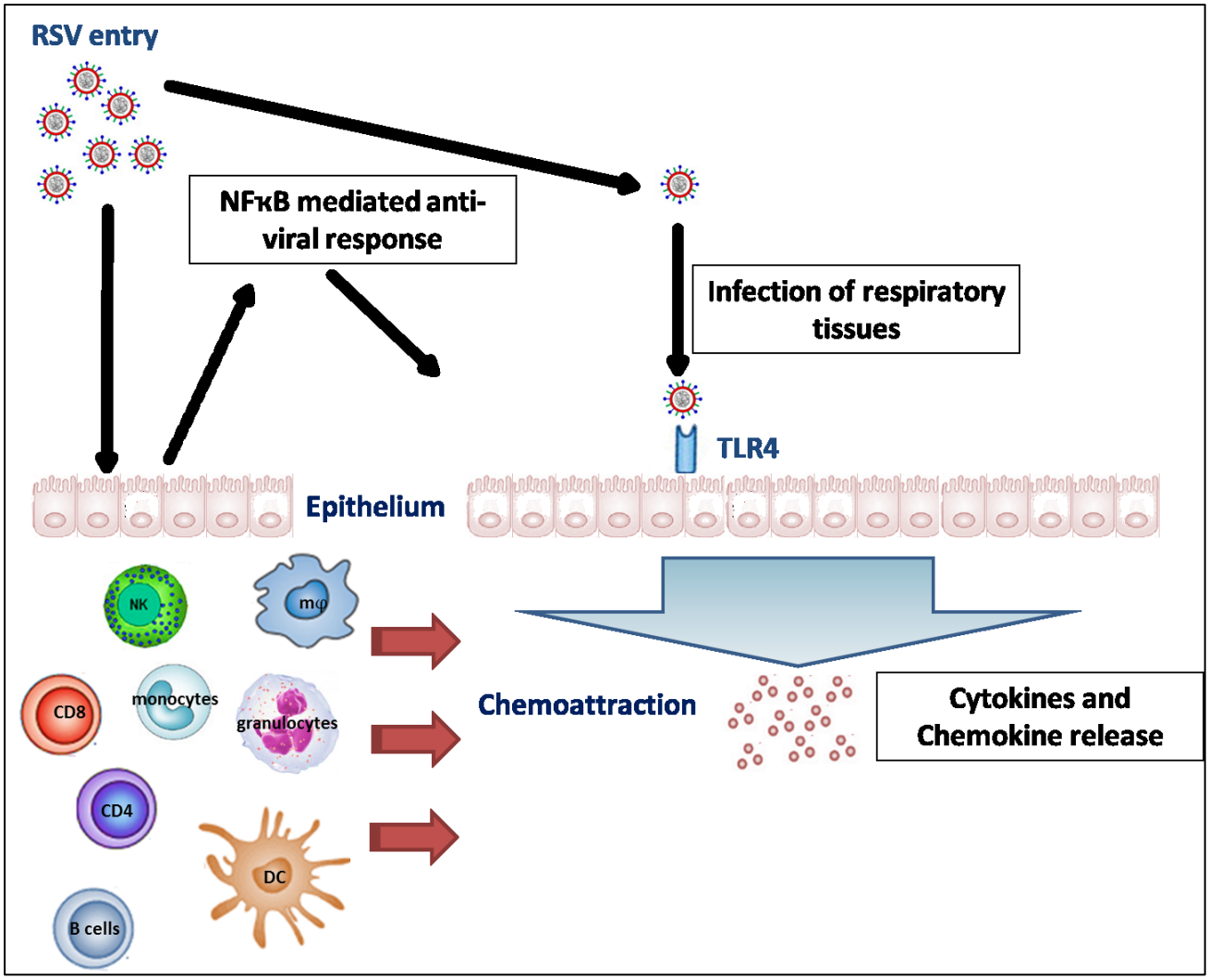


Fig 3 : Immune response and inflammation in RSV infection. (Adapted from Bueno *et al*, Int Immunopharmacol 2008;1320-1329).

1.2.6 RSV and Bronchiolitis

RSV is the leading cause of viral bronchiolitis worldwide (Bennett et al., 2007). Bronchiolitis is an acute respiratory illness affecting infants and young children that can be fatal. Infection and inflammation of the lower airways results in bronchiolar infiltration, airway oedema, mucous production by goblet cells, necrosis of the airway epithelium and cellular infiltration into the airway (Fig 4) ((Falsey and Walsh, 2000; Hall, 2001; Habibi and Openshaw, 2012; McNamara and Smyth, 2002). There is an association between RSV bronchiolitis and subsequent wheezing in childhood which is linked to prolonged respiratory sequalae.

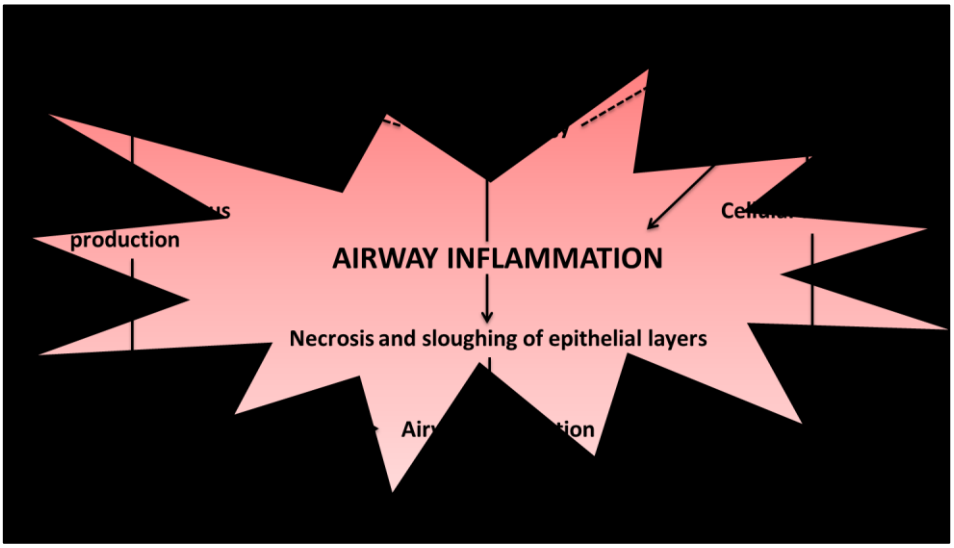


Fig 4 : Pathogenesis of RSV bronchiolitis. (Adapted from Habibi and Openshaw, Curr Opin Infect Dis 2012; 25:687-694).

1.2.7 Diagnosis and Treatment

RSV infections in adults can be diagnosed using several methods such as viral culture, antigen detection by Immunofluorescene Assay (IFA) or Enzyme Immunoassay (EIA), Direct Fluorescene Assay (DFA), Optical Immunoassay (OIA), RNA detection using reverse transcriptase PCR (RT-PCR), and serologically by detecting RSV-specific IgM antibodies in

the acute phase of infection or by detecting RSV-specific IgG antibodies between the acute phase and convalescent phase from serum samples (Aldous et al., 2005; Falsey et al., 2002; Shafik et al., 2011). Although direct immunofluorescence of nasopharyngeal aspirates and virus culture are still in practice, RT-PCR is becoming widely available in clinical settings.

Once diagnosis of the disease is confirmed, supportive treatments are given to ensure enough oxygenation and hydration. Nonetheless, positive pressure ventilation is required for severe cases where continuous positive airway pressure is available and bi-level positive airway pressure may reduce the need for intubation and ventilation (Domachowske and Rosenberg, 1999; Kimpen, 2001). The use of a synthetic nucleoside, Ribavirin has shown to have high efficacy of antiviral activity in vitro. However, it has been shown to have only moderate efficacy clinically which limits administration of the drugs to small groups of high risk individuals or those with severe cases of RSV disease (Bawage et al., 2013; Domachowske and Rosenberg, 1999).

1.2.8 Protection, Vaccine and Infection Control

The success of vaccines often relies on mimicking the protective immune response of natural infection without inducing the pathological effects. However, natural infection with RSV does not induce complete protection. Host responses are normally partially protective. Therefore, an appropriate protective induction of immune response is needed to have a successful vaccine against RSV. Most importantly, this induction must be free of detrimental immunopathology (Hall et al., 2013).

Currently, there are no safe and effective vaccines or drugs against these RSV-related illnesses. The failure of formalin-inactivated RSV (FI-RSV) vaccine trial in the early 1960s

was a major setback towards RSV vaccine development. Like any other formalin treatment to whole virus to create vaccines, the FI-RSV vaccine has the viral properties to aid immune recognition without the ability to replicate. Initially, the results from the vaccine trial were promising. However, administration of formalin-inactivated whole virus vaccine leads to development of a more severe disease in up to 80% of the vaccinees upon acquiring RSV infection naturally with two deaths reported. Investigation using serum antibodies against surface proteins G and F from the sera of the vaccinees were found to be low in vaccine efficacy with poor neutralising activity. (Kim et al., 1969).

In most current research, studies are focused on testing live attenuated vaccines that are safe for infants. Strains used do not stimulate a serum neutralizing antibody response because infants less than 6 months of age do not produce a consistent immune response towards natural RSV infection. Alternatively, passive immunization could be used. Studies have shown, infants that obtained high levels of neutralizing antibodies from their mothers are protected from severe RSV disease during their neonatal period and first months of life (Glezen et al., 1986).

Therapy with a monoclonal antibody against the RSV F protein (Palivizumab by Synagis) can reduce the rate of hospitalisation in high risk children significantly. Although this therapy is a promising option to treat RSV disease, the use of this monoclonal antibody is limited to preterm infants with cardio-pulmonary disorders, immunodeficiency or immunosuppression and individuals that are considered at high risk of severe RSV infection and hospitalisation. Also, the cost-effectiveness of this treatment is a major concern (Clark et al., 2000; Wang et al., 2008).

There are various infection control strategies recommended in controlling the disease. Hand washing has been employed to prevent the spread of RSV-related nosocomial infection in hospitals and clinical settings. Since infection control is not only preventing spread of disease between patients or from healthcare personnel to patients, it is also applicable to prevent disease transmission from a diseased patient to healthy individuals such as healthcare personnel or visiting family and friends. Therefore, the use of gowns, gloves and masks could reduce the chance of the disease being transmitted. Cohorting and isolation of infected patients is also recommended (Falsey and Walsh, 2000).

1.3 THE LUNG

1.3.1 Structure and Function

The lungs consist of two large, sponge-like, air-filled organs located in the thoracic cavity. The lungs are covered by a thin balloon-like structure called the pleura. The lubricated layers allow the lungs to move smoothly as they expand and contract.

Due to their physiological structure, the lungs have an enormous surface area. At the end of the trachea (windpipe), two tubes called the bronchi branch out and distribute air to the right and left side of the lungs.

Within the lungs, the bronchi branch into smaller bronchi and to even smaller tubular structures called bronchioles. As they spread to the ends of the lungs, at the end of each bronchiole there are tiny air sacs called the alveoli where the actual gaseous exchanges takes place. The interstitium is a tissue network between the air sacs which separates the endothelium and epithelium, and surround and supports the alveoli. This web-like network

contains fibroblasts, elastin and strong collagen fibres that give structural integrity and elasticity preventing the air sacs from being over stretched during the process of gaseous exchange.

1.3.2 Mechanisms of Defence

The main function of the lungs is to facilitate the respiratory process. The inhaled air is essential to fulfil the oxygen needs of the body. However, this exposes the respiratory tract to substances from the surrounding environment. During gaseous exchange, the lungs are potentially exposed to the diverse gases, air-borne particles and most importantly, harmful pathogens.

The epithelial surface of the URT and the LRT are largely exposed to the outside environment. Approximately 90% of inhaled particles are trapped in the mucous layer on the ciliated epithelium. The airway mucous consists mostly of mucin that is secreted from the epithelial surface and from the sub-mucousal glands. Tiny hairs or cilia which move in swaying motion line the bronchi. Mucous is secreted at an average of 0.089 litres onto the lining of these bronchi daily. Removal of large particles from the URT is another means of protection via the mucociliary clearance (Nicod, 1999).

Mucous is carried on the top of the cilia and brought upwards in the respiratory tract towards the mouth. This mechanism is the first line of defence for infection removing pathogens like bacteria and viruses from the lungs. Mucous also has bactericidal properties. Phagocytic cells such as alveolar macrophages and pulmonary DCs also play important role in pathogenic clearance.

1.4 MACROPHAGES

1.4.1 Introduction

Cells of the immune system originate from pluripotent stem cells which are divided into two major progenitors; the lymphoid progenitors and the myeloid progenitors. Cells that derived from the lymphoid progenitors include T-cells, natural killer (NK) cells and B-cells. Myeloid progenitors give rise to different cell lineages; erythroid, megakaryotic, granulocytic and monocytic lineages. From each different lineage, cells mature into erythrocyte (red blood cell), platelets, basophils, eosinophils, neutrophils, mast cells, dendritic cells and monoctyes.

Monocytes can further differentiate into macrophages and dendritic cells depending on the signals they receive. Macrophages are the most differentiated cells in the mononuclear phagocyte system (MPS) (Fig. 5). The MPS comprises of the family of cells which derives from the bone marrow progenitors, which then differentiates into blood monocytes and macrophages. Cells in the MPS have endocytic enzymes such as esterase, lysosomal hydrolase and ecto-enzymes. Their phagocytic capacities can be either non-specific or specific depending on the particles that are ingested for example, specific receptors are required for complement-coated particles. However, these features do not define macrophages entirely nor distinguish them from other mesenchymal cells (Hume, 2006).

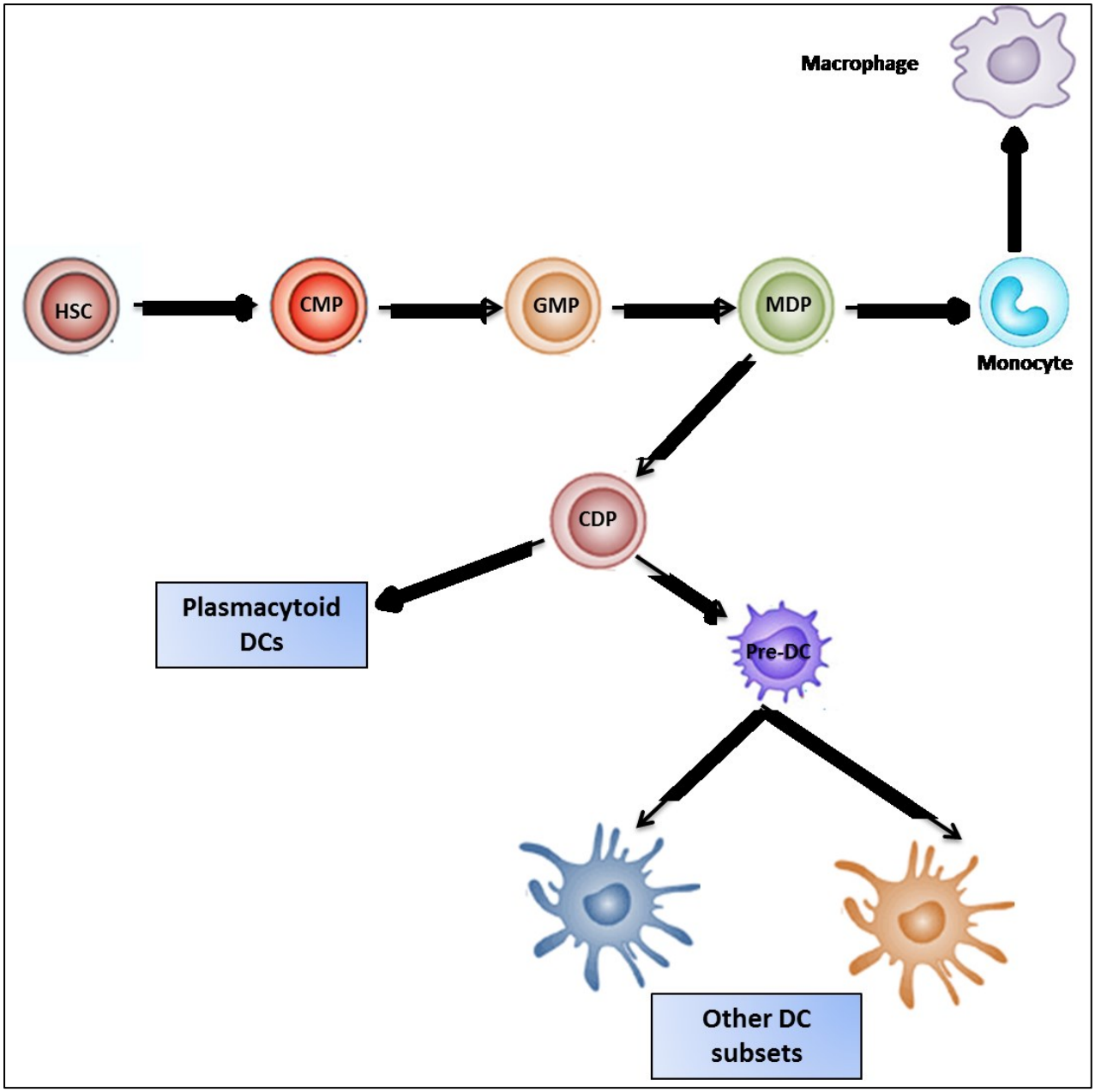


Fig 5 : Mononuclear Phagocyte System (MPS). (Adapted from Hume, Curr Opin Immunol. 2006; 18:49-53)

Macrophages are widely distributed throughout the body and localise in specific organs; namely the liver, lungs, lymphoid organs, the gastrointestinal tract (GIT), central nervous system, synovium, bone and skin. Macrophages are known for their diverse functions in a wide range of physiological and pathological processes (Geissmann et al., 2010; Hume et al.,

2002; Murray and Wynn, 2011). These large mononuclear cells were first identified more than a century ago by Elie Metchnikoff in Messina (Karnovsky, 1981).

1.4.2 Macrophage Activation and Polarization

Macrophages change their physiology in response to endogenous stimuli which are rapidly generated upon injury and infection giving rise to different populations of cells with distinct functions. These early stimuli are typically produced by innate immune cells. Macrophages also respond to signals produced by antigen-specific immune cells. They are activated and polarise into different phenotype depending on the immunological environment. Cytokines produced by immune cells give rise to macrophages with distinct physiological and immunological functions upon activation (Mosser, 2003; Murray et al., 2014; Rees, 2010). Macrophages can be distinctively polarized into classical activation and alternative activation.

In Th1 immunity, macrophages undergo classical activation which is primed by IFN-γ in combination with bacterial products like LPS, and other pro-inflammatory cytokines like TNF-α giving rise to macrophages of M1 phenotype. These M1 or classically activated macrophages (CAM) are microbicidal, pro-inflammatory and histotoxic (Rees, 2010). Generally, the M1 macrophages are identified with IL-12^{hi}, Il-23^{hi} and IL-10^{lo} phenotype. They are efficient producers of pro-inflammatory cytokines like IL-1, IL-6 and IL-23 (Fig 7) and effector molecules such as reactive oxygen species (ROS) and nitrogen intermediates. Cytokines produced by M1 macrophages are vital components for host defence against intracellular pathogens. These mediators are also associated with the development and

expansion of Th-17 cells that produce IL-17. However, it is pivotal to control activation of M1 macrophage because production of mediators following activation often leads to host-tissue damage (Langrish et al., 2005; Mosser and Edwards, 2008; Veldhoen et al., 2006).

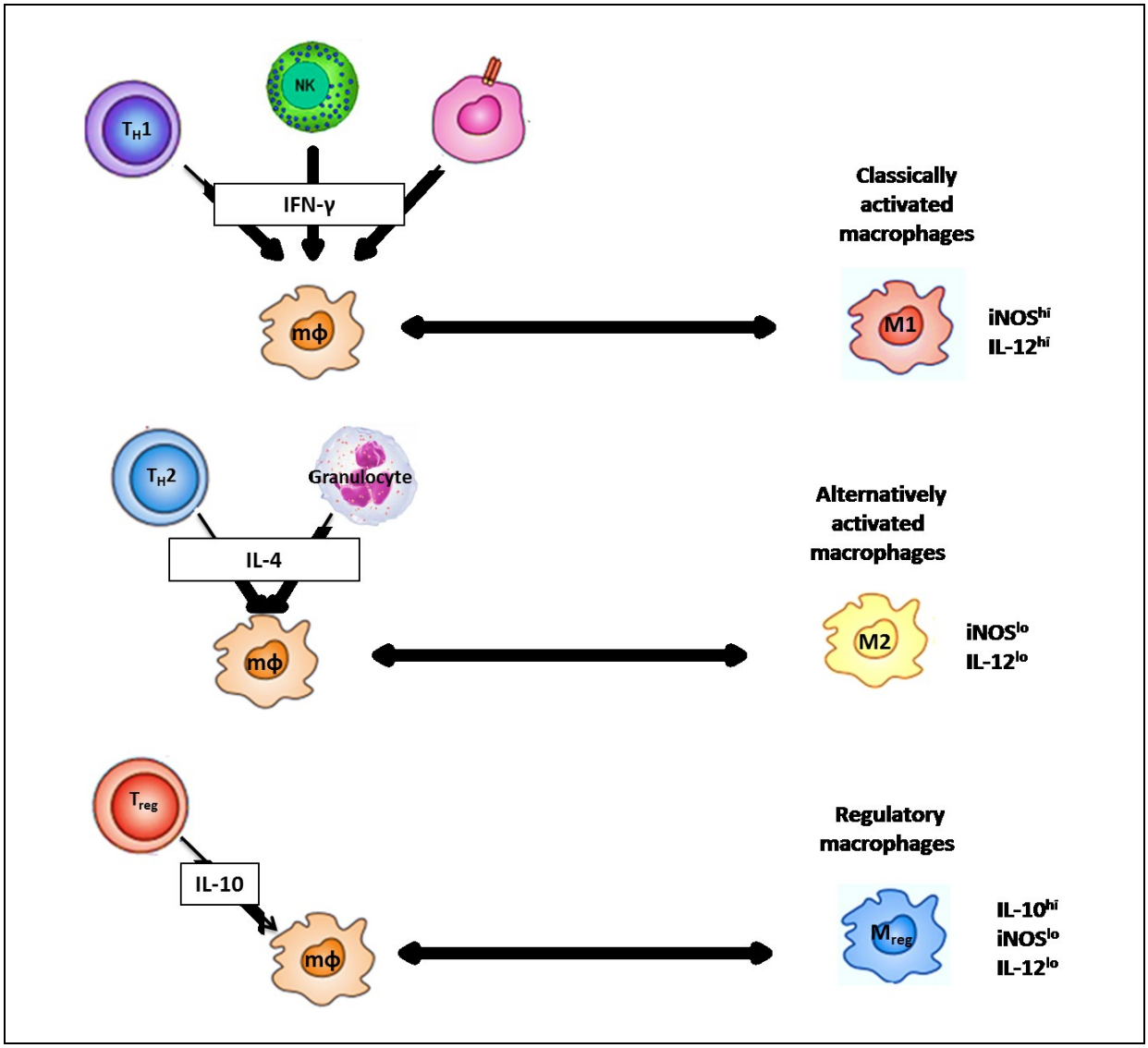


Fig 6: Macrophage activation and polarisation. (Adapted from Exploring the full spectrum of macrophage activation Mosser and Edward *et al*. Nat Rev Immunol.; 8(12):958-969)

Alternative activation of macrophages via induction by Th2 cytokines; IL-4 and IL-13 has gained interest amongst macrophage researchers in the last decade. These alternatively activated macrophages (AAM) are also known as the M2 macrophages that share the IL-12^{lo}, Il-23^{lo} and $iNOS^{lo}$ phenotype. They express high levels of the scavenger receptor, mannose receptor and the galactose-like receptor. M2 macrophages have been implied to have important roles in homeostasis, inflammation, tissue repair, malignancy and metabolic functions. Hence, they are also referred to as 'wound-healing' macrophages (Gordon and Martinez, 2010; Mosser and Edwards, 2008)

M1 and M2 macrophages differ in many physiological and immunological aspects as summarized in Table 2.

Table 1: Macrophage activation; classical activation versus alternative activation

	CLASSICALLY ACTIVATED (M1)	ALTERNATIVELY ACTIVATED (M2)
Immunological phenotype	Th1-like	Th2-like
Inflammation	Promote/Chronic	Resolve/ Wound healing
Extracellular matrix (ECM)	Destruct	Construct
Cell growth	Apoptosis	Proliferation
Activation	Primed by IFN-γ, activated by stimulus (i.e.: LPS, etc)	IL-4, IL-13; do not require priming
Chemokines released	IL-8 (CXCL8), IP-10 (CXCL10), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5)	MDC (CCL22), PARC (CCL18), TARC (CCL17)
Further reaction (inflammation)	Pro-inflammatory CKs: IL-1F2 (IL-1β), IL-6, TNFα (TNFSF1A)	Counteraction towards inflammation: IL-1Ra (IL-1F3), Ym1, Ym2, RELMα (FIZZ), IL-10, TGFβ

There is another type of activation that gave rise to an 'M2-like' phenotype, which are also known as the regulatory macrophages. Similarly to the M1 and M2 macrophages, these regulatory macrophages or Mreg can be activated following innate or adaptive immune responses (Sternberg, 2006), as well as in the later stage of adaptive immune response, responsible in dampening the immune response and limiting inflammation (Mosser, 2003). These Mregs produce IL-10 which has the ability to inhibit production of and activity of

various pro-inflammatory cytokines making them potent inhibitors and regulators of inflammation (Edwards et al., 2006).

Macrophages develop and operate within a changing microenvironment. In viral-induced inflammation, macrophages can significantly influence the severity, persistence and the magnitude of most inflammatory reactions and innate immune response. Generally, they play an important role in keeping the balance between the pro- and anti-inflammatory reactions by controlling the immune response (Geissmann et al., 2010; Gordon, 2003).

1.4.3 Resident Pulmonary Macrophages

Macrophages originate from precursor cells of the haemopoietic organs. They are transported via the bloodstream into the lungs and respiratory tract. Most studies of macrophages in the lung are limited to alveolar macrophages. There are two major populations of resident macrophages in the lung; the alveolar macrophages (AM) and the interstitial or tissue macrophages (IM). In inflammation, there is a recruitment of monocytes from the peripheral blood into the airway. These populations may have different functions in the regulation of inflammation (Lohmann-Matthes et al., 1994). Nevertheless, the immunological function and correlation with their activation state during infection/inflammation are understudied.

Alveolar macrophages (AM) are the most abundant cells in the naïve airways. They are placed at the interphase between air and the lung tissue. Due to their unique localisation, they are the only macrophage population in the body that are exposed to air and represent the

immune defences against inhaled constituents (Lohmann-Matthes et al., 1994) which requires adaptation to this environment.

AMs are different from other macrophages including IM in certain functions and in their phenotype. They are known to play an important role in regulating inflammation and protecting the lung from pathogens. In steady state, AM are quiescent, do not produce inflammatory cytokines, are less phagocytic than other macrophage populations and the expression of the phagocytic receptor CD11b is very low or almost non-existent (Garn et al., 2006; Holt, 1978; Weinberg and Unanue, 1981). They are long-lived cells with a very low turnover and in homeostasis, the macrophage population in the alveolar compartment did not require recruitment from the bone marrow to replenish (Jenkins et al., 2011; Murphy et al., 2008).

AMs generally have an efficient phagocytic capacity, and produce large amounts of reactive oxygen radicals (ROS) and nitric oxide in a Th1 immunological environment. Although AM are less susceptible to RSV infection than epithelial cells, exposure to RSV seems to have to activate their immunoregulatory functions and stimulates secretion of TNF-α (Franke-Ullmann et al., 1996; Franke-Ullmann et al., 1995; Garn et al., 2006). In contrast, MHC-class-II molecules and co-stimulatory molecules such as CD80 and CD86 are low in expression and secretion of pro-inflammatory cytokines; IL-1β and IL-6 is low (Franke-Ullmann et al., 1996; Garn et al., 2006).

The other population of resident macrophage in the lungs is the tissue or interstitial macrophages (IM). They can be found in the lung connective tissue and appear to have intermediate functional properties in terms of secondary defence and as APCs (Lohmann-

Matthes et al., 1994). Compared to their more popular counterpart; the AMs, they are less favoured in research.

Tissue resident macrophages are tissue specific heterogenous cells that fulfil niche-specific functions; ranging from clearance of cellular debris in homeostasis to responding to infection and a possible role in resolution of inflammation. In the steady state, they reside in and function in their respective tissues (Hashimoto et al., 2013; Murray and Wynn, 2011). The long existent immunological dogma is that these IMs derive from monocytes infiltrating the tissues in steady state and during inflammation, lacking the capacity to proliferate (Davies et al., 2013a; Davies et al., 2013b) in accordance with the MPS theory in the 1960s (van Furth and Cohn, 1968). In a study combining selective cell ablation and adoptive transfer, Landsman and Jung demonstrated that blood monocytes develop into lung parenchymal macrophages as an intermediary population which then develop further into AMs (Landsman and Jung, 2007).

The contribution of monocytes towards replenishment of tissue macrophages is still debatable. Recent studies highlighted various origins of tissue macrophages that were either bone-marrow derived or self-renewing from the existing local population. A recent finding demonstrated that local proliferation of tissue macrophages in the pleural cavity is induced by IL-4 in response to parasite infection and is independent of monocyte infiltration (Jenkins et al., 2011). Studies have also shown that bone marrow derived monocytes and resident tissue macrophage are distinctly different in terms of lineage and function. Their expansion in the peritoneum occurs at different stages of inflammation (Davies et al., 2013b; Gundra et al., 2014).

The differences between resident macrophage populations in the lung are summarized in Table 2.

Table 2: Comparison between two resident pulmonary macrophages; alveolar macrophages and interstitial macrophages

	ALVEOLAR MACROPHAGES (AM)	INTERSTITIAL MACROPHAGES (IM)
Localisation	At the interphase between air and lung tissue	In the lung connective tissue
Size	Large	Smaller, more uniformed size
Function	Inflammatory; Highly phagocytic and microbicidal	Proliferative capacity Immunoregulatory

1.4.4 Monocytes

Monocytes are circulating mononuclear leukocytes that develop in the bone marrow from dividing monoblasts of the long-term haematopoietic stem cells (LT-HSC). LT-HSC gives rise to progenitors that develop into common lymphoid precursors and common myeloid precursors. Once released into the bloodstream, the monocytes may enter different tissues and further differentiate into a range of tissue macrophages and DCs. They are irregular in shape with oval- or kidney-shaped nucleus; differ in size, trafficking and receptors expressed on their surfaces. Collectively, they are defined by the expression of the Csf-1 receptor; MCSF-R or CD115 (Auffray et al., 2009; Geissmann et al., 2003).

Peripheral blood monocytes are divided into two subsets in mice based on CCR2, CX_3CR1 and Ly6C expressions. The most prevalent subset is the $CCR2^{hi}$ CX_3CR1^{lo} $Ly6C^{hi}$

monocytes. They are known as inflammatory monocytes and contributes to 2- 3% of the circulating leukocyte population in homeostasis (Geissmann et al., 2003). During inflammation, they are rapidly recruited to inflamed tissues (Serbina et al., 2009) and constitute 60% of monocytes in mice (Rees, 2010). The second subset is the $CCR2^{lo}$ CX_3CR1^{hi} $Ly6C^{lo}$ monocytes. They are normally found adhered to the endothelial cells and migrate along the luminal surface. Thus, they are called the patrolling monocytes (Auffray et al., 2009).

In humans, monocytes are divided into three subsets based on their CD14 and CD16 expressions. The $CD14^{++}$ $CD16^{neg}$ subset; the classical monocytes are most prevalent in human blood. They are similar to the $Ly6C^{hi}$ population in mice. The remaining two are the $CD14^{pos}$ $CD16^{++}$ and $CD14^{++}$ $CD16^{+}$, known as the non-classical and intermediate monocytes respectively (Ziegler-Heitbrock et al., 2010). The non-classical monocyte population, also known as the patrolling monocytes in human is similar to the mouse $Ly6C^{lo}$ monocytes (Cros et al., 2010).

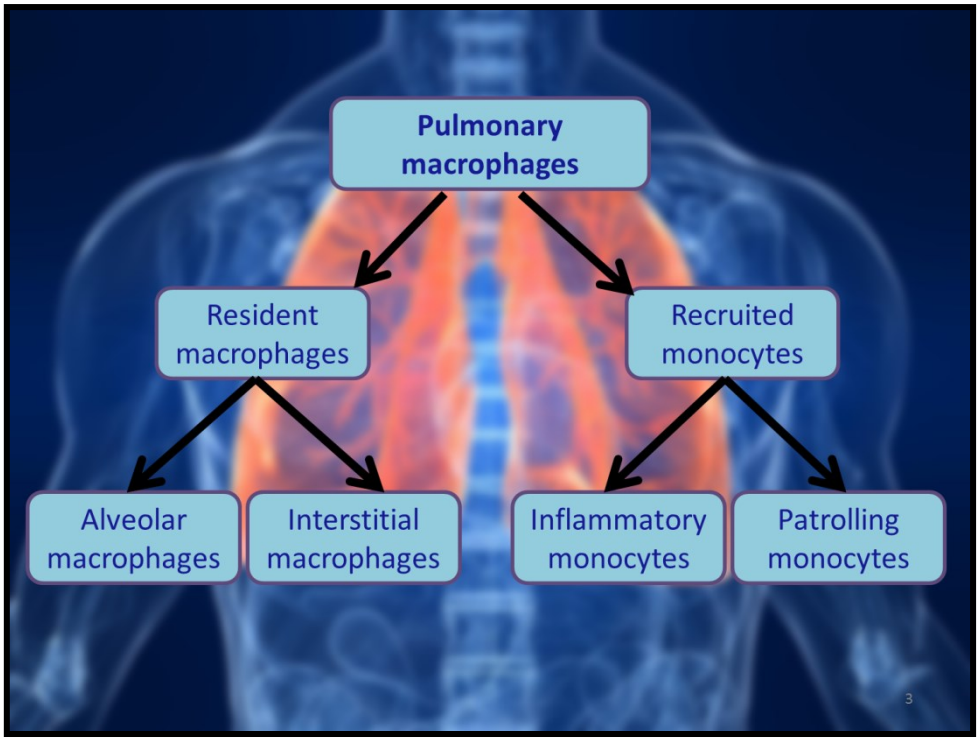


Fig 7: Resident and recruited pulmonary macrophages. (Adapted from http://www.natureworldnews.com/articles/5149/20131202/lung-cells-grown-first-time-human-stem.htm, Photo : iStockPhoto via University of Minnesota)

1.4.5 Characterisation of Pulmonary Macrophages and Monocytes

Macrophages can be characterised based on their localisation (Section 1.4.2 and Section 1.4.3), surface markers and how there are activated. F4/80 is a monoclonal antibody that is commonly used as a macrophage marker in the mouse. Found in 1981, F4/80 became a unique marker for mouse macrophages. It is expressed on macrophages from different sites such as the peritoneal cavity, lung, spleen and thymus, and also on blood monocytes (Austyn and Gordon, 1981). F4/80 is highly expressed on tissue macrophages and expressed at a lower level on monocytes. However, there are probably some cells from the subsets of DCs and eosinophils that also express F4/80 on their surfaces (Murray and Wynn, 2011).

Based on their surface markers, macrophage subsets in the lung have been characterised in several ways. Pribul and colleagues referred to the CD11c^{pos} MHCII^{lo} cells as lung macrophages, which they also refer to as alveolar macrophages (Pribul et al., 2008). In some other studies, alveolar macrophages are referred to as F4/80^{pos} CD11c^{pos} (Bedoret et al., 2009; Kirby et al., 2006), MHCII^{int} CD11c^{pos} CD11b^{lo} (Tighe et al., 2011), F4/80^{pos} CD204^{pos} CD11c^{hi} CD11b^{lo} (Rigaux et al., 2012) and CD64^{pos} CD11c^{pos} Siglec F^{pos} (Misharin et al., 2013). In most studies, tissue macrophage or IMs are commonly referred to as F4/80^{pos} CD11c^{neg} CD68^{pos} MHCII^{pos} (Bedoret et al., 2009), CD64^{pos} CD24c^{neg} CD11b^{hi} MHCII^{pos} (Misharin et al., 2013).

The third population of F4/80^{pos} cells are the monocytes recruited from the blood stream, which consist of two subsets: the inflammatory and the patrolling monocytes. Inflammatory monocytes are identified as CCR2^{hi} CX$_3$CR1^{lo} Ly6C^{hi} (Geissmann et al., 2003), F4/80^{pos} CD11b^{pos} CD115^{pos} Ly6C^{pos} Gr1^{pos} MHCII^{neg} (Strauss-Ayali et al., 2007), CD64^{pos} Ly6C^{pos} (Misharin et al., 2013), F4/80^{lo} CD11b^{pos} Ly6C^{pos} CD64^{pos} MHCII^{pos} (Jakubzick et al., 2013) and CD115^{pos} CD11b^{pos} MHC^{neg} (Breslin et al., 2013). The other monocytes subset are referred to as F4/80^{pos} CD11b^{pos} CD115^{pos} Ly6C^{pos} Gr1^{pos} MHCII^{neg} (Strauss-Ayali et al., 2007) and CD64^{neg} Ly6C^{neg} (Misharin et al., 2013).

1.4.6 Functions of Different Lung Macrophage Populations in Inflammation and Infection

Alveolar macrophages are important as a component of host defence against infection. In a functional comparison between the AMs and the IMs by Lohmann-Matthes et al in 1994, they found that AMs are more effective in producing cytokines involved in anti-microbial defence, whilst the IMs secrete more IL-6 and IL-1, and showed higher expression of MHC

class II. They concluded that AMs are better equipped for antimicrobial tasks, whilst the IMs are more immune-regulatory. Therefore, the alveolar macrophages may have an important role in the initiation and the resolution of inflammation (Lohmann-Matthes et al., 1994).

In 2009, Bedoret et al reported that lung IMs prevent a Th2 response in mice challenged with OVA and LPS. The IMs and not AMs produced a significantly high amount of IL-10 and were believed to have inhibited maturation and migration of LPS-induced dendritic cells (DCs). Furthermore, they have shown that IMs play a pivotal role in preventing the development of immune response against harmless antigens. They believe that IMs and AMs have complementary immunosuppressive roles against inhaled antigens with IMs being able to prevent sensitization to harmless antigens whilst AMs are capable of diminishing the effect of the immune response (Bedoret et al., 2009).

1.4.7 Monocytes/Macrophages in Respiratory Infection

Macrophages increase in numbers locally either by recruitment or proliferation of local populations. However, whether newly recruited inflammatory macrophages leave the lung after inflammation or replace resident ones is not known. Some studies claimed that resident tissue macrophages undergo rapid *in situ* proliferation in order to increase population density without recruitment of potentially tissue-destructive inflammatory cells. Moreover macrophage population dynamics in the lung in viral infection is not fully understood.

1.4.7.1 Influenza and Macrophages

Influenza virus infections induce various cytokines and chemokines in the host that regulates the immune response. During infection, monocytes are recruited to the site of inflammation.

In a mouse model of influenza infection, $CCR2^{pos}$ monocyte-derived cells are predominantly the cause of immune pathology during infection (Lin et al., 2008). Although H5N1 virus replicates productively in alveolar macrophages, higher levels of cytokine expression were observed from peripheral blood monocyte-derived macrophages than alveolar macrophages (Yu et al., 2011). In 2011, Lin and colleagues demonstrated that $CCR2^{pos}$ monocyte-derived cells play a major role in influenza-induced inflammation and pathology. Recruitment of monocyte-derived inflammatory cells was greatly reduced when they infect CCR2 KO mice with influenza. This observation is supported by very low expression of co-stimulatory molecules, NOS2 producing cells, weight loss and mortality (Lin et al., 2011).

1.4.7.2 Macrophages in RSV infection

The most important cellular components of the innate immune response in RSV infection include phagocytic cells like the macrophages (McNamara and Smyth, 2002). They are one of the first populations of cells alongside the epithelial cells to encounter RSV in the airway. But little is known about how RSV infections influence these cells, and vice versa (Kimpen, 2001). Pribul et al in 2008 demonstrated that pulmonary macrophage depletion with i.n. administration of clodronate liposome prior to RSV infection inhibited the early release of inflammatory cytokines into the airway and dampened the innate response. These findings suggest that lung macrophages play a central role in the early response to viral infection but significantly showed minor effect on the adaptive response at the peak of disease severity (Pribul et al., 2008).

In a study by Shirey et al, RSV infection was shown to induce T-and B-cell-independent differentiation of alternatively-activated macrophages (M2) *in vivo* and *in vitro*. In IL-4Rα-/-

mice, enhanced lung inflammation was observed indicating that the M2 differentiation was IL-4Rα/STAT6-dependent. Overall, their data demonstrated that RSV induces M2 macrophages to differentiate in response to IL-4 and IL-13, and mediate disease resolution via IL-4Rα/STAT6-, TLR4- and IFN-β-dependent signalling pathways (Shirey et al., 2010).

The different roles of AMs and IMs in terms of viral clearance, induction and resolution of inflammation, and the innate and adaptive response in respiratory infection, particularly in RSV are still ambiguous. Recent studies have shown the dynamics of different pathogen-macrophage interaction demonstrating the immunomodulation of these macrophages upon activation, suggesting their interchanging phenotype.

1.5 Project Hypothesis

Different pulmonary macrophage and monocytes populations have functionally distinct roles in regulating inflammation and pathology in RSV infection

1.6 Project Aims and Objectives

1. To characterise different population of pulmonary macrophages after RSV infection
2. To determine different roles of pulmonary monocytes/ macrophages in terms of viral clearance, induction and resolution of inflammation in respiratory viral infection

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 BUFFERS AND REAGENTS

Table 3: Buffers and reagents (in alphabetical order)

Buffers/ Reagent	Composition	Application	Supplier
7-Aminoactinomycin D (7-AAD)	7-Aminoactinomycin D (7-AAD); 1 mg/mL solution in DMSO	Flow cytometry	Invitrogen
ACK Lysis Buffer	8.29 g NH_4Cl (0.15 M), 1 g $KHCO_3$ (1 mM), 37.2 g Na_2EDTA (0.1 mM); H_2O added to a total volume of 1 L; pH adjusted to 7.2 – 7.4	Lysis of red blood cells (haemolysis)	Sigma-Aldrich
Anti-RSV antibody	Generated from the chimeric bovine/human (b/h) parainfluenza virus type 3 (PIV3) expressing the human PIV3 (hPIV3) fusion (F) and hemagglutinin-neuraminidase (HN) proteins from an otherwise bovine PIV3 (bPIV3) genome	Plaque assay	Biogenesis
Avidin-Horse Radish Peroxidase (AV-RP)	Peroxidase conjugate	RSV ELISA	BD Biosciences
CFSE (Carboxyfluorescein succinimidyl ester)	5-(and-6)-Carboxy fluorescein Diacetate, Succinimidyl Ester (5(6)-CFDA, SE; CFSE) -	Cell labelling *in vivo*	Invitrogen

	Mixed Isomers, 25mg		
Collagenase	Collagenase from *Clostidrium histolyticum*.	Tissue digestion	Sigma-Aldrich
Cytofix™	Fixation Buffer 4 % formaldehyde in PBS	Fixation of cells	BD Biosciences
DMSO (Dimethylsulfoxide), HYBRIMAX™	Hybri-Max™, sterile-filtered, BioReagent, ≥99.7%	Cryo-preservation, Tissue culture	Sigma-Aldrich
dNTP mix	10 mM each of dATP, dCTP, dGTP and dTTP in water	Polymerase chain reaction (PCR)	Promega
Dulbecco's PBS	(1x) without Ca & Mg	Tissue culture	PAA Laboratories GmbH
ELISA Conjugate	Streptavidin-HRP	ELISA	R&D Systems
ELISA Diluent	For IFN-γ: 1 % BSA, Tween® 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl) For others: 1 % BSA in PBS (w/v)	ELISA	Sigma-Aldrich
ELISA Stop Solution	2 NH_2SO_4	ELISA	Sigma-Aldrich
ELISA Substrate	TMB (3,3', 5,5;-tetramethylbenzidine) Chromogen Solution	ELISA	Invitrogen
ELISA Wash Buffer	0.05 % Tween® 20 in PBS	ELISA	Sigma-Aldrich
Fast DAB Substrate	3,3'-Diaminobenzidine	RSV ELISA	Sigma-Aldrich
Fc Block	CD16/CD32 (FcϒIII/II R) Purified Rat IgGb anti-mouse	Flow cytometry	BD Biosciences

Golgi Stop	Protein Transport Inhibitor containing monensin	Tissue culture	BD Biosciences
LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit	Amine reactive dye; for 405 nm excitation	Cell viability	Invitrogen
LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit	Amine reactive dye; for 633 or 635 nm excitation	Cell viability	Invitrogen
PBS	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4	ELISA	Sigma-Aldrich
Perm/Wash Buffer	FBS and saponin	Flow cytometry	BD Biosciences
PKH26 PCL	Phagocytic cell label fluorescent dye	Cell labelling *in vivo*	Sigma-Aldrich
Reastain Quick-Diff Kit	Methanol – Fixative Eosin – Solution 1 Haematoxylin – Solution 2	Cytospin (Differential count)	Gamidor Technical Services Ltd
RSV ELISA Stop Solution	2M Sulphuric Acid (v/v)	RSV ELISA	Sigma-Aldrich
Staining buffer (FACS buffer)	PBS/ 1 % BSA/ 0.1% Sodium azide	Flow cytometry	Sigma-Aldrich
Trypan blue	Trypan blue solution (0.4 %)	Viable cell counting	Invitrogen
TaqMan® Universal PCR Master Mix	DNA Polymerase, Uracil-DNA Glycosylase, dNTPs with dUTP	Real-time PCR	Applied Biosystem
Trypsin/EDTA (10x)	0.5 % Trypsin with EDTA	Tissue culture	Invitrogen

2.1.2 MEDIA AND MEDIA SUPPLEMENTS

Table 4: Media and media supplements (in alphabetical order).

Name	Composition	Application	Supplier
BAL Media	3.5 % Lidocaine in PBS used per mouse	Bronchoalveolar lavage	PAA Laboratories GmbH
Complete Dulbecco's Modified Eagle's Medium (cDMEM)	500 ml DMEM, 50ml FCS, 5 ml Pen-Strept, 5 ml L-Glutamine	Tissue culture	PAA Laboratories GmbH
Complete Roswell Park Memorial Institute 1640 medium (R10F)	500 ml RPMI 1640, 50ml FCS, 5 ml Pen-Strept, 5 ml L-Glutamine	Tissue culture	PAA Laboratories GmbH
Dulbecco's Modified Eagle's Medium (DMEM)	See media formulations given by manufacturer	Tissue culture	PAA Laboratories GmbH
Freeze Media	90 % FCS and 10% DMSO	Freezing cell aliquots	Invitrogen; Sigma-Aldrich
Foetal Calf Serum (FCS)	Foetal bovine serum; heat inactivated	Tissue culture	Invitrogen
L-Glutamine	L-Glutamine 200 nM (100x)	Tissue culture	Gibco
Penicillin-Streptomycin (Pen/Strept)	10,000 U/ml penicillin G sodium and 10, 000 µg/ml Streptomycin sulphate in 0.85 % saline. Working concentration = 100 u/ml Penicillin, 100 µg/ml Streptomycin	Tissue culture	Gibco

Roswell Park Memorial Institute 1640 medium (RPMI 1640)	See media formulations given by manufacturer	Tissue culture	PAA Laboratories GmbH, Austria

2.1.3 ANAESTHETICS

Table 5: Anaesthetics (in alphabetical order).

Name	Composition	Application	Supplier
Isofluorane (ISOFlo)	1-choro-2,2,2-trifluoroethyldifluoromethyl ether	Intranasal anaesthesia	Abbot Laboratories Ltd
Pentobarbitone	Pentobarbitone sodium 200 mg/ml	Intraperitoneal injection for culling	Animalcare Ltd

2.1.4 INSTRUMENTS

Table 6: Instruments (in alphabetical order).

Name	Application	Supplier
ABI Prism 7500 Fast Real-Time PCR System- Taqman	qPCR sequencing	Applied Biosystems
Canto II HTS	Flow cytometry, cell counting	BD Biosciences
Countess® Automated Cell Counter	Cell counting	Invitrogen / Life Technologies
Cytospin 3 centrifuge	Cytospins	Thermo Shandon

Eppendorf 5810R centrifuge	Centrifugation	Eppendorf, Hamburg
GeneAmp PCR System 9600	PCR – cDNA conversion	Applied Biosystems
GentleMACS™ Dissociator	Dissociation of tissues into single-cell suspensions	Miltenyi Biotec Ltd
Heraeus Megafuge 40R centrifuge	Centrifugation	Thermo Scientific
Herasafe™ Biological Safety Cabinet	Microbiological Safety Cabinet	Thermo Scientific
LEICE DME Inverted microscope	Differential cell count	Leica Microsystems
LSR Fortessa	Flow cytometry	BD Biosciences
LSR II	Flow cytometry, cell counting	BD Biosciences
MACSmix™ Tube Rotator	Enzymatic steps during tissue dissociation	Miltenyi Biotec Ltd
NanoDrop 1000	RNA specification	Thermo Scientific
NU 5500E air-jacketed automatic CO_2 incubator	Cell culture	NuAire, Inc.
QIAcube System (110 V)	RNA extraction	QIAGEN
SCANLAF MARS Bio Safety Cabinet	Microbiological Safety Cabinet	Labotal Scientific Equipment (1997) LTD
Sonicator	Sonication of cells	ULTRAWAVW Ltd.
SpectraMax® Plus384	Absorbance Microplate Reader (for ELISA)	Molecular Devices
TissueLyser LT	Tissue disruption / homogenisation	QIAGEN

2.1.5 SOFTWARE

Table 7: Software used in this project (in alphabetical order).

Name	Application	Supplier
7500 Fast System SDS Software	qPCR sequence detection analysis	Applied Biosystems® / Life Technologies
FlowJo	Flow cytometry data analysis	Tree Star Inc.
GraphPad Prism 4	Statistical analysis	GraphPad Software, Inc.
ND-1000 V3.7.1	RNA specification analysis	Thermo Scientific
SoftMax® Pro Software	ELISA analysis	Molecular Devices Corp

2.1.6 VIRUS STOCK

Respiratory Syncytial Virus

Respiratory syncytial virus A2 strain was originally donated by E.J. Stott, Institute of Research on Animal Diseases, Newbury, Berkshire, UK.

2.1.7 CELL LINES

HEp2 cells were obtained from ATCC® and used in the propagation and titration of virus stocks. The properties of the cell line is summarised in table below.

Table 8: Cell Lines

Name	Organism	Tissue	Age	Morphology	Growth properties	Source
HEp2	Human	HeLa containment	Adult	Epithelial	Adherent	ATCC

2.1.8 ANTIBODIES

2.1.8.1 Flow cytometry

Table 9: Antibodies used for flow cytometry

Antibody	Conjugate	Clone	Supplier
CD115 (CSF1R)	APC	AFS98	BioLegend
CD11b	PE-Cy7	M1/7	BD Biosciences
CD11c	V450	HL3	BD Horizon
CD206	PE	MR5D3	Abd Serotec
CD3	PerCP-Cy5.5	145-2C11	BioLegend
CD4	eFluor 605	GK1.5	eBioscience
CD45	Brillilant Violet 570	30-F11	Cambridge Biosciences
CD8a	Pac Blue	53-6.7	BD Biosciences
F4/80	APC	BM8	BioLegend
F4/80	PerCP-Cy5.5	BM8	BioLegend
Granzyme B	APC	FGB12	Invitrogen
IFN-Ɣ	PE-Cy7	XMG1.2	eBioscience
IL-10	FITC	JES5-16E3	BD Biosciences
IL-13	PE	eBio13a	eBioscience
IL-4	APC	11B11	eBioscience
Ly6C	AF 700	AL-21	BD Biosciences
Ly6G	PerCP-Cy5.5	1A8	BD Biosciences
MHC II	FITC	2G9	BD Biosciences
NKp46	AF700	29A1.4	BD Biosciences

2.1.9 ELISA KITS

Table 10: Cytokine and chemokine duo set kits used for ELISA

Cytokine / Chemokine	Supplier
CCL2 (MCP-1)	R&D Systems
IFN-γ	R&D Systems
IL-10	R&D Systems
IL-15	R&D Systems
IL-4	R&D Systems
IP-10	R&D Systems
KC (CXCL1)	R&D Systems
TNF-α	R&D Systems

2.2 METHODS

2.2.1 TISSUE CULTURE

2.2.1.1 Thawing cell aliquots

Cells were thawed in a water bath at 37°C. The cell suspension was transferred into a 15 ml tube and washed twice with cDMEM. The cells were then re-suspended in cDMEM to seed 10^7 HEp2 cells in a total volume of 25 ml into a 175 cm^2 flask.

2.2.1.2 Growth and maintenance of cells

Hep2 cells were cultured in cDMEM medium. 10^7 Hep2 cells were seeded in a total volume of 25ml into a 175 cm^2 vent capped flask and left to grow overnight at 37^0C, 5% CO_2 in cDMEM. Cells were passaged when 70-75 % confluent.

2.2.1.3 Cell passage

Confluent cells were washed gently with 10 ml D-PBS then 5 ml of 1x trypsin/EDTA was added and left at 37˚C for 15 minutes for cell disassociation. The trypsin was neutralised by addition of complete medium. Cells were washed and re-suspended in complete medium then split into a ratio of 1:5.

2.2.1.4 Viable cell counts

Trypan blue solution (0.1 %) was used at 10:1 dilution with cells suspended in PBS to determine cell viability. 10 µl of the cell/trypan blue solution was added to one chamber of a Neubauer haemocytometer and the number of viable cells were determined.

2.2.1.5 Freezing and storing cell aliquots

Cells were spun to a pellet then re-suspended in freeze media. Cells were transferred into cryovials and then into a cryogenic freezing container to reduce the rate of cooling, and frozen at -80°C. The frozen vials were transferred into a liquid nitrogen tank 3 days later for storage.

2.2.2 VIROLOGY

2.2.2.1 Growing Virus.

Human RSV A2 strain was used for all infections. HEp2 cells were grown as per section 2.2.1.2. The HEp2 cell monolayer was washed with serum free DMEM and infected with RSV A2 strain at 0.1 pfu per cell in serum free medium. In the Hep2 control flask, serum free medium was added. Virus was harvested when the cytopathic effect was more than 50%. After 2 minutes sonication, cells were centrifuged at 1500 rpm for 5 minutes before storage in liquid nitrogen until required.

2.2.2.2 RSV Plaque Assays (Immunostaining)

Polyclonal goat anti-RSV biotin conjugated antibody was used for anti-RSV plaque assay. Antibody was purchased from Biogenesis.

In a V-bottomed plate, the virus stock or samples to be tested were titrated using doubling dilutions starting at 1:10 – 1:100. At least 8 dilutions were prepared.

Medium from the plates containing confluent Hep2 cells were removed and 50 µl of the virus dilution prepared above were added into the wells in triplicates or quadruplicates. Plates were incubated for 90 to 120 minutes at 37 ºC. 100 µl of DMEM with 2% of FCS was added to each well and plates were incubated for a further 40 – 48 hours at 37 ºC.

After incubation, medium from the plates were removed and each well was washed once in 100 µl of PBS. Cells were fixed with 100 µl absolute methanol containing 2 % hydrogen

peroxide for 20 minutes. Each well was washed once in 200 µl of PBS/ 1% BSA. Cells were washed gently to avoid disruption of the cell monolayer. Plates were blotted dry after washing.

Biotinylated anti-RSV was prepared in 1:500 dilution in PBS/ 1% BSA. 100 µl of the diluted anti-RSV antibody was added to each well and plates were incubated for 2 hours at room temperature. After incubation, the plates were washed 3 times with 200 µl of PBS/ 1% BSA. Plates were blotted dry after washing.

100 µl of a dilution of 1:250 – 1:500 of extravidin peroxidase (predetermined with each batch) were added and plates were left in the dark to incubate for at least 1 hour at room temperature. Secondary antibody was added to each well at 1:500 dilution in PBS/ 1%BSA.

Plates were washed 5 times with PBS/1%BSA and blotted dry. Sigma Fast DAB substrate was prepared according to the manufacturer's recommendation at 1 set of tablets in 5 ml of distilled water. 50 µl of Sigma Fast DAB substrate preparation were added to each well. Once the plaques have acquired the desired colouration, the reactions were stopped by washing the cells with PBS.

Plaques were counted in each well. Dilutions of viruses that gave 80 – 150 plaques per well were chosen for plaque count. The mean of all replicates were determined. Plaques forming units per ml (pfu/ml) were calculated as:

$$\frac{\boldsymbol{plaques} \times \boldsymbol{fold\ dilution}}{\boldsymbol{volume\ of\ inoculum\ (ml)}}$$

2.2.2.3 RSV stock titration

Virus stock was tested in a plaque assay and titre of 1.77 x 10^7 pfu/ml was obtained. 100µl (8.85 x 10^5 pfu/ml) of virus was given intra-nasally per mouse at 1:2 dilution with PBS to give the standard weight loss curve of RSV disease in *BALB/c* murine model.

2.2.3 ANIMAL WORK

All animal work in the animal facility was approved and licensed appropriately by the U.K. Home Office and the animal ethics committee of Imperial College London.

2.2.3.1 Mice.

7 – 8 weeks old female *BALB/c* mice (Table 11) were purchased from Harlan, UK. All mice were maintained under specific pathogen-free conditions in accordance to institutional and United Kingdom Home Office guidelines.

Table 11: Mice used in this project

Common Name	Strain nomenclature	Origin	Coat colour	MHC haplotype
BALB/c	BALB/cAnNCrl	Harlan, UK	White	H-2^d

2.2.3.2 Intranasal infection of mice with RSV

After light general anaesthesia with isofluorane (Table 2.1.3), 8-week old BALB/c (Table 11) mice were infected intranasally with desired concentration of RSV in 100 µl total volume per mouse. Following the inhalation of the virus, each mouse was returned to its cage and observed until fully recovered. The weight of mice was monitored daily after challenge. At selected time points, mice were sacrificed by overdose intraperitoneal injection sodium pentobarbitone.

2.2.4 CELL COLLECTION AND PREPARATION

Work was carried out under aseptic conditions. Euthanised mice were pinned down on a board with the belly facing up. The fur was sprayed with 70 % alcohol. The fur was cut along the ventral midline from the belly to the chin and pulled back on the sides to reveal the thyroid gland. The thyroid gland and fat tissues were then pulled apart to expose the trachea. The abdominal wall was cut just below the ribcage and the diaphragm was detached away from the ribcage. Then the ribcage was cut along the sides to expose the lungs and heart.

2.2.4.1 Blood

Blood samples were taken from the left ventricle of the heart by a thoracotomy. Blood collected using syringe containing 0.5 M EDTA via cardiac puncture and transferred into Eppendorf tube containing 0.5 M EDTA. Blood was added directly into ACK lysis buffer, mixed by inverting the tube and let stand for several minutes to allow lysis of RBCs. After two washings, cells were re-suspended in FACS staining buffer and added into wells on a 96-well round-bottomed polypropylene plate.

2.2.4.2 Bronchoalveolar lavage fluid (BALF)

2.2.4.2.1 BALF collection

The membrane of the trachea was cut and removed. A small incision was made using a spring-bow scissors to make a hole at the trachea. Prior to lung perfusion and collection of lung tissues, BALF was collected into Eppendorf tubes by inflating the lungs 3 times with BAL media using a needle with tubing attached to a 2 ml syringe. BALF was kept on ice for transportation to the laboratory. BALF was centrifuged and the supernatant stored at -80°C prior to analysis.

2.2.4.2.2 BAL cells preparations

Cell pellets were re-suspended in 200 µl ACK lysis buffer and topped up with 800µl cDMEM. The mixture was centrifuged at 6000 rpm for 5 minutes and the supernatant was discarded. Then, cell pellets were re-suspended in 1000µl cDMEM and kept on ice until use.

2.2.4.2.3 Cytospin preparations

50 µl of each sample and 50 µl of RPMI were added to appropriate cytospins. Samples were centrifuged at 400 rpm for 5 minutes. Cells on the slides were differentially stained and observed under the microscope for differential cell count.

2.2.4.3 Lung cells

2.2.4.3.1 Lung cells collection

Lungs were perfused via the pulmonary artery with PBS-EDTA and collected in tubes or vials for different purposes.

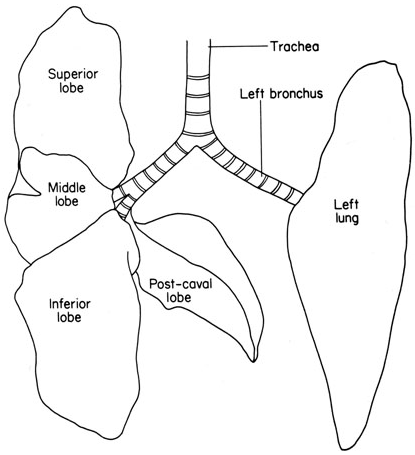


Figure 8: Anatomy of the lung: tranchea and lobes.

(Adapted from
http://www.informatics.jax.org/cookbook/figures/figure53.shtml)

The superior lobe (Fig 8) of the lungs was collected in cryovials and snap-frozen in liquid nitrogen for RNA extraction. The middle lobe (Fig 8) was collected in cryovials and snap-frozen in liquid nitrogen and stored in -80˚C freezer until used for lung ELISA if needed. The remaining lung lobes were collected in tubes containing R10F media and were kept on ice for transportation to the laboratory.

2.2.4.3.2 Lung cell preparation

Collagenase was added to lungs at 1:1 (collagenase: R10F media) ratio. After dissociation of tissues into single-cell suspensions using a gentleMACS Dissociator, cells were kept at 37°C for 1 hour on MACSmix for collagenase digestion.

Digested samples were then filtered and washed with R10F media. Cells were then treated with ACK lysis buffer and washed with R10F media. Cells were re-suspended in R10F media and counted in trypan blue using a cell counter, COUNTESS (Invitrogen).

2.2.5 PKH26 PCL: Fluorescent Cell Linker Kit for Phagocytic Cell Labelling

2.2.5.1 Optimization of PKH26 PCL Dye

PKH26 PCL 1X Dye Working Solution was diluted at 1:500 in Diluent C (provided with kit). The dye was used at concentrations of 1:5, 1:10 and 1:20 by diluting the Working Solution in Diluent C. 100 µl of PKH26 PCL was administered intravenously per mouse.

2.2.5.2 PKH26 PCL longevity studies

For this study, PKH26 PCL was used at a concentration of 1:10. PKH26 PCL was administered intravenously 100 µl per mouse and lungs were collected at day 7, 14, 21 and 42 post administration.

2.2.6 Carboxyfluorescein succinimidyl ester (CFSE) Staining

The lyophilized CFSE was reconstituted in DMSO. After diluting with serum free DMEM at 1:3 dilution. CFSE was given at a volume of 50 µl per mouse intranasally under anaesthesia.

2.2.7 Clodronate liposomes (Dichloromethylene-diphosphonate)

Clodronate encapsulated liposomes purchased from Clodronate Liposomes© Dr. Nico Van Rooijen, Belgium at 0.01 g/mL.

For the monocyte depletion experiment, mice were treated with clodronate liposomes 24 hours prior to infection and subsequent doses were administered every 48 hours. Clodronate liposomes were administered at 200 µl per mouse at concentration of 0.01 g/mL via intravenous injection.

2.2.8 Flow Cytometry

2.2.8.1 LIVE/DEAD Reactive Dye

Cells were washed once with PBS and re-suspended in PBS. The density of cells was adjusted with PBS to ~1 × 10^6 cells in a 1 ml volume. The reconstituted fluorescent amine reactive dye (Aqua or Near-IR) was added to 1 ml of the cell suspension.
Cells were incubated at room temperature or on ice for 30 minutes, protected from light. Cells were washed twice with 1 ml of FACS Staining Buffer, and re-suspended in PBS.

2.2.8.2 Surface Staining

Cell suspensions (~1.0 × 10^6 cells per well) were added to 96-well polypropylene U-bottomed plate(s). Cells were washed in PBS and then stained with LIVE/DEAD Fixable Amine Reactive Dye at 1:1000 dilution as per section 2.2.8.1. After washing with FACS staining buffer and blocking the CD16/CD32 receptor with Fc Block at 1:200 dilution, cells were stained with antibodies in FACS staining buffer. Staining antibodies used were as follows:

I. Characterisation of pulmonary macrophages experiment

Antibody	Conjugate
CD11b	PE-Cy7
CD11c	V450
CD206	PE
F480	APC
Ly6G	PerCP-Cy5.5
MHC II	FITC

II. Blood monocyte depletion experiment

(a) Blood cell staining

Antibody	Conjugate
CD115	APC
CD11b	PE-Cy7
CD11c	V450
CD45	Brillilant Violet 570
Ly6C	Alexa Fluor 700
Ly6G	PerCP-Cy5.5

(b) BAL and lung cell staining

Antibody	Conjugate
Ly6G	PerCP-Cy5.5
CD45	Brillilant Violet 570
CD11b	PE-Cy7
CD11c	V450
Ly6C	Alexa Fluor 700
F4/80	APC

After incubation with staining antibodies, cells were washed with FACS staining buffer, and fixed with Cytofix and incubated in the dark at 4°C. Cells were washed once in FACS staining buffer before analysing with the flow cytometer using Diva Software on the flow cytometer. Data obtained from the flow cytometer were analysed using Flow Jo.

2.2.8.3 Intracellular Staining

Cell suspensions (~1.0 × 107 cells per well) were added to 96-well polypropylene U-bottomed plate(s) and stimulated with 1 mg/ml PMA and 1 mg/ml ionomycin for 1 hour. After adding 20 µl/ml of Golgi Stop, then cells were incubated for further 3 hours at 37°C.

Cells were washed in PBS and then stained with LIVE/DEAD Fixable Amine Reactive Dye at 1:1000 dilution as 2.2.8.1. After washing with FACS staining buffer and blocking the CD16/CD32 receptor with Fc Block at 1:200 dilution, cells were surface stained with antibodies in FACS staining buffer. Staining antibodies used were as follows:

Antibody	Conjugate
CD3	PerCP-Cy5.5
CD4	eFluor 605
CD8a	Pac Blue
NKp46	AF700

Cells were washed with FACS Staining Buffer and then fixed for 30 minutes at 4ºC with Cytofix. FACS buffer were added on for a quick wash and cells were kept in FACS Buffer overnight prior to intracellular staining.

Cells were permeabilised with Perm/Wash for 5 minutes at 4°C. Fc Block (anti-CD16/CD32) was diluted at 1:200 with the staining buffer and 50 µl of the diluted Fc Block was added to each well. Then, the plate(s) were incubated in the dark at 4ºC for 15 minutes. Cells were stained with antibodies with FACS staining buffer. Staining antibodies used were as follows:

Antibody	Conjugate
IFN-Ɣ	PE-Cy7
IL-4	APC
IL-10	FITC
IL-13	PE
Granzyme B	APC

Then, the cells were washed in FACS staining buffer before analysing with the flow cytometer using Diva software. Data obtained from the flow cytometer were analysed using Flow Jo.

2.2.8.4 Granzyme B Intracellular Staining

Cell suspensions (~1.0 × 107 cells per well) were added to 96-well polypropylene U-bottomed plate(s) and stained with LIVE/DEAD Fixable Amine Reactive Dye at 1:1000 dilution as 2.2.8.1. After washing with FACS staining buffer and blocking the CD16/CD32 receptor with Fc Block at 1:200 dilution, cells were surface stained with antibodies in FACS staining buffer. Staining antibodies used were as follows:

Antibody	Conjugate
CD3	PerCP-Cy5.5
CD4	eFluor 605
CD8a	Pac Blue
NKp46	AF700

Cells were washed with FACS Staining Buffer and then fixed for 30 minutes at 4ºC with Cytofix. FACS buffer were added on for a quick wash and cells were kept in FACS Buffer overnight prior to intracellular staining.

Cells were permeabilised with Perm/Wash for 5 minutes at 4°C. Fc Block (anti-CD16/CD32) was diluted at 1:200 with the staining buffer and 50 µl of the diluted Fc Block was added to each well. Then, the plate(s) were incubated in the dark at 4ºC for 15 minutes. Cells were stained with antibodies with FACS staining buffer as follows:

Antibody	Conjugate
Granzyme B	APC

Then, the cells were washed in FACS staining buffer before analysing with the flow cytometer using Diva software. Data obtained from the flow cytometer were analysed using Flow Jo.

2.2.9 Enzyme Linked ImmunoSorbent Assay (ELISA)

96-wells immunosorb plates were coated with capture (1˚) antibody diluted in PBS according to manufacturer's recommendation. Plates were kept sealed at room temperature overnight. After washing each well 3 times with ELISA wash buffer, plates were blocked with ELISA blocking solution and left for a 2 hour incubation at room temperature. The plates were washed 3 times with ELISA wash buffer.

ELISA protein standards were prepared in ELISA reagent diluent in 8 doubling dilutions and added onto the plates in triplicates. Samples were also added onto plates in designated wells.

The plates were sealed and incubated at 4°C for 2 hours. After washing 3 times with ELISA wash buffer, detection (2˚) antibody preparation was added 100 µl per well. The plates were sealed and incubated at room temperature for 2 hours.

The plates were washed 3 times with ELISA wash buffer and 100 µl working dilution of Strepavidin-HRP were added to each well. The plates were sealed and incubated at room temperature for 20 minutes away from sunlight. Substrate solution was added 100 µl per well after washing the plates 3 times with ELISA wash buffer and the plates were sealed and incubated at room temperature for 20 minutes in the dark. Once the standard curve were sufficiently developed, stop solution was added 50 µl per well to stop the reaction.

Optical density of each plate was read at 490nm on SpectraMax® Plus384 plate reader. Data obtained from the reader were analysed using SOFTmax Pro. Version 3.1.2. All reagent and buffer preparation were prepared in accordance to manufacturer's recommendation.

2.2.10 Molecular Biology

2.2.10.1 Lung homogenisation

Lung tissues were homogenised using TissueLyser LT in RLT Buffer containing ß-mercaptoethanol to inactivate RNAses in the cell lysate. Supernatant from pelleted cell lysates were collected for RNA extraction.

2.2.10.2 RNA Extraction (QIACUBE)

RNA extraction was conducted using an automated instrument for DNA, RNA, or proteins purification. Instrument was used with QIAGEN spin-column kits. All reagents, buffers and samples were prepared in accordance to manufacturer's recommendation.

2.2.10.3 RNA Specification

Sample volume to get 2 µg of RNA (per sample) was determined using the NanoDrop 1000 Spectrophotometer and quantified using the ND-1000 V3.7.1 Software.

2.2.10.4 cDNA Synthesis

cDNA was synthesized via reverse transcribed from RNA using the QIAGEN Omniscript Kit. A master mix of 10x RT Buffer, dNTP (0.5 mM in each reaction), 1 µM random hexamers, 2.5 units of RNAse inhibitor and 4 units of Reverse Transcriptase were prepared. 2 µg of RNA was added to the mix and diluted in RNAse free water to make a total reaction volume of 20 µl.

2.2.10.5 Quantification of RSV L-gene

RSV L-gene copy number was determined using quantitative real-time PCR. A reaction mix containing RSV L-gene specific forward and reverse primers, conjugated probes and PCR master mix. The reaction mix was added into a FAST 96 well reaction plate at 11.5 µl per well. After adding 1 µl per well of DNA and standard, the reaction plate was sealed and

quantified on 96-well standard 7500 programme. Each cDNA sample was also quantified for the housekeeping gene, 18S to control for variability in cDNA levels

2.2.11 Statistical Analysis

The mean of each group is represented as ± standard error mean (SEM). The students T test or the Mann Whitney test was used to determine differences between two groups. For 3 or more groups, the differences were analysed by one-way ANOVA using Kruskal-Wallis test followed by Dunns post-test. A p value < 0.05 was considered statistically significant. All statistical analysis was performed with GraphPad Prism Software.

3.0 CHARACTERISATION OF PULMONARY MACROPHAGES

3.1 Introduction

Pathology of RSV disease is generally thought to be caused by excess inflammation following infection. Regulation of inflammation is essential in modulating the effects of RSV infection. Therefore, in the hope of having a better understanding of the pathogenesis of RSV disease it is pivotal to understand what drives and regulates inflammation.

T lymphocytes are thought to be a major cause of RSV disease in man. In the mouse model of RSV disease, weight loss and increases in airway hyper-responsiveness measurements correlate with T cell recruitment to the airway. Depletion of T cells in this model ablates both (Schwarze et al., 1999). Many studies in the last decade were focused on the role of T cells. In recent years, the role of innate cells in RSV disease has triggered interest amongst researchers; particularly macrophages and monocytes.

Macrophages play a role in regulating inflammation. Monocytes, the precursors of macrophages and precursors to some of the DC subsets are of importance in inflammation. These cells have important roles in innate immunity, as well as influencing adaptive immunity. Depending on the location and the activating stimuli, different subsets of macrophages and/or monocytes get recruited to the site of inflammation (Gordon, 2003; Lohmann-Matthes et al., 1994; Stein and Keshav, 1992).

Macrophages and monocytes can be and have been characterised in many ways based on their surface markers. Studies of different subpopulations of pulmonary macrophages suggested that these distinct populations have different functions in inflammatory diseases and lung injuries (Pluddemann et al., 2011). However, the immunological function of lung resident versus inflammatory macrophages and correlation with their activation state during infection and inflammation are understudied; particularly in RSV disease (Gordon, 2003; Pluddemann et al., 2011).

3.2 Experimental Aims and Objectives

The cells of interest in this study are the pulmonary macrophages and monocytes. The aim of the work in this chapter is to optimize a flow cytometry panel to characterize macrophages and monocytes in naïve and infected airway and lung based on their surface markers.

3.3 Near-IR amine reactive dye: An effective live/dead discriminator in managing auto-fluorescence of alveolar macrophages compared to Aqua amine reactive dye

The aim of this study was to optimize a staining protocol for defining different populations of pulmonary macrophages and monocytes. Amongst the populations that are predominant in the airway are the alveolar macrophages. Alveolar macrophages are inherently fluorescent, staining these cells for flow cytometry is challenging. Therefore, is it important to choose the right tool to discriminate live from dead cells for multi-coloured flow cytometry to begin with. Also, using a less distinguishing discriminator could interfere cell gating down the line because of the autofluorescent nature of dead cells.

Initially, we used LIVE/DEAD Aqua Amine Reactive Dye for live/dead discrimination. Cells were stained with LIVE/DEAD Aqua reactive dye (Fig 9B) or LIVE/DEAD Near-IR reactive dye (Fig 9D). In the 405 nm laser with band filter 510/50 (AmCyan channel), most the cells that are higher on forward and side scatter display auto-fluorescence in unstained lungs (Fig 9A). This is an important observation as the alveolar macrophages have high forward and side scatter on the flow cytometry plot.

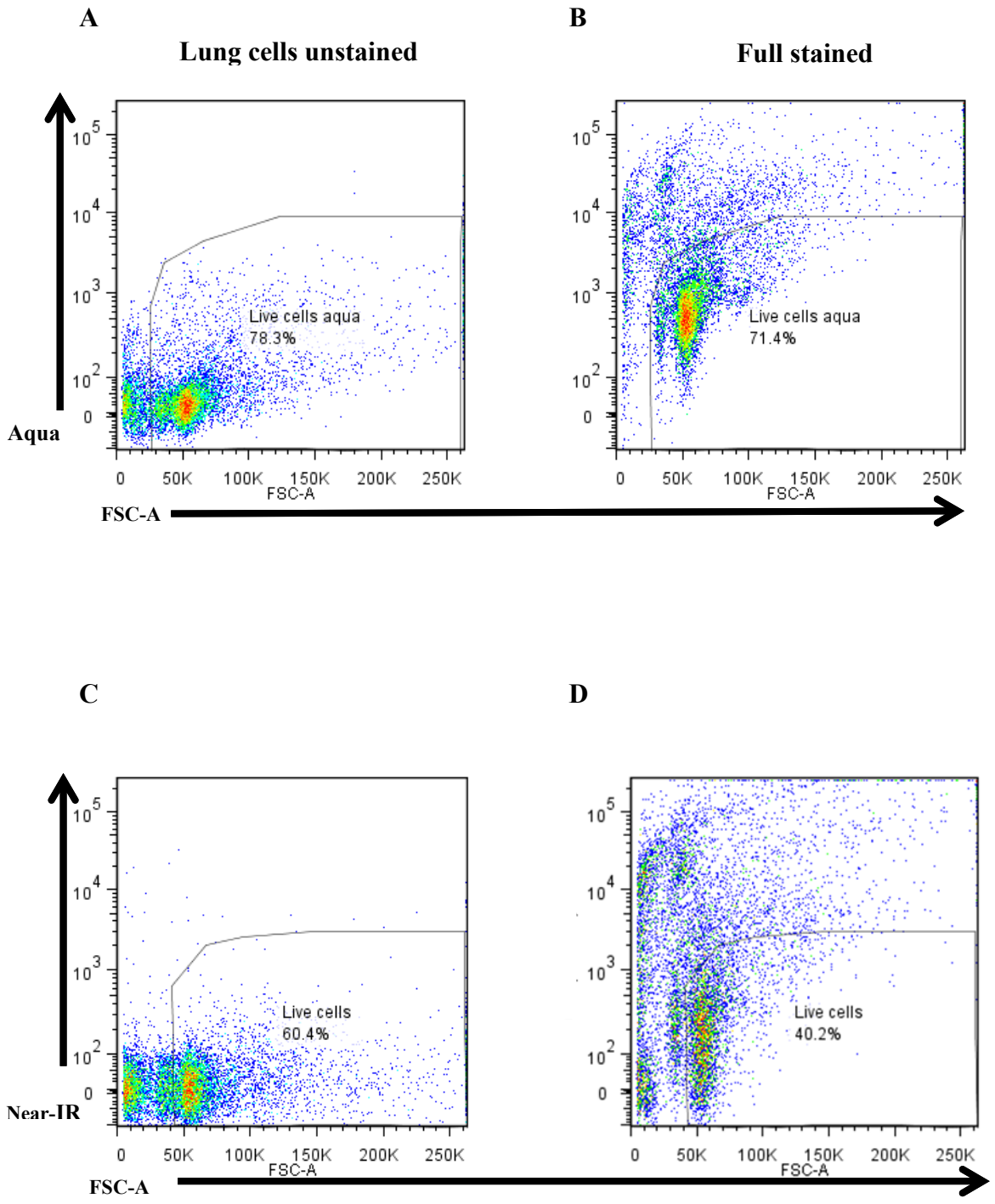


Fig 9 Alveolar macrophages (AMs) do not auto-fluoresce in the Near-IR channel compared to Aqua Reactive Dye Lung cells suspensioere was stained with LIVE/DEAD Aqua Reactive Dye (B) and LIVE/DEAD Near-IR Reactive Dye (D) at 1:1000 dilution. Some cells were left unstained for both dyes (A, C). Autofluorescense of pulmonary cells in the AmCyan (Aqua Reactive Dye) and APC-Cy7 (Near-IR) channels were compared using the flow cytometer.

Staining cells with dyes or conjugated antibodies cause cells to shift in the flow cytometry plot. In some of the lung samples, heat-killed cells were added as positive control for the live/dead stain (data not shown) to ensure that the live cells were gated accurately. In a full stained sample, cells displayed no clear distinction between live and dead cells when cells were stained with LIVE/DEAD Aqua reactive dye (Fig 9B). From these observations, LIVE/DEAD Aqua reactive dye was substituted with LIVE/DEAD Near-IR reactive dye and a comparison was made between the two dyes. Unstained pulmonary cells that have higher forward and side scatter in the APC-Cy7 channel (Fig 9C) did not auto fluoresce as they would in the AmCyan channel (Fig 9A) making the Near-IR amine reactive dye a better live/dead stain for macrophage studies.

3.4 F4/80-APC conjugated antibody gives better resolution in staining compared to F4/80-PerCP Cy5.5 conjugated antibody

F4/80 is a surface marker that is associated with macrophages and monocytes. Hence, cells that are F4/80^{pos} were selected. This macrophage "pan-marker" may also be expressed on dendritic cells. However, some studies show that F4/80 is only expressed on precursors of DCs. Nevertheless, F4/80 is the best tool available to identify macrophages and monocytes, making F4/80 an important marker in the macrophage staining panel.

In an airway allergy study by Bedoret and associates, alveolar macrophages (AMs), interstitial macrophages (IMs) and DCs were differentiated using F4/80 and CD11c as surface markers using flow cytometry. In their studies, they have shown that AMs are positive for both F4/80 and CD11c, IMs to be F4/80^{pos} CD11c^{neg}, whereas DCs are F4/80^{neg} but CD11c^{pos} (Bedoret et al., 2009).

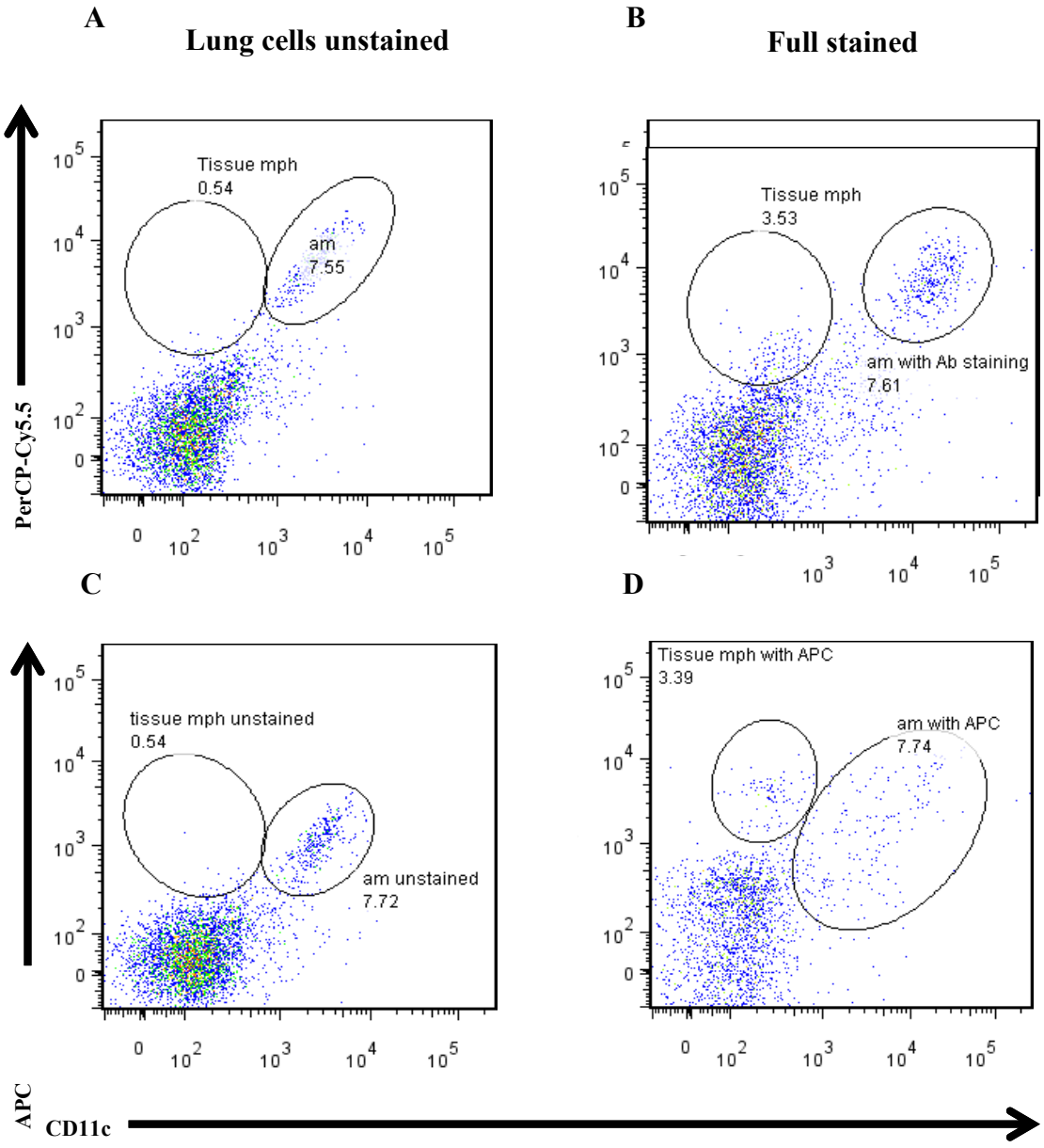


Fig 10 F4/80-APC conjugated antibody gives better resolution in staining on flow cytometry compared to F4/80-PerCP Cy5.5 conjugated antibody Lung cells were either unstained (A and C) for negative control or stained with CD11c on V450 and F4/80 on PerCp-Cy5.5 (B) and F4/80 on APC (D).

In this experiment, live lung cells were stained with F4/80 and CD11c to mimic what has been shown in Bedoret et al's study. The antibody optimisation started with PerCP-Cy5.5 conjugated F4/80 antibody and CD11c on V450. The three populations mentioned in Bedoret et al's studies were identified but F4/80 on PerCP-Cy5.5 did not give a clear resolution between the macrophage populations, especially the F4/80^{pos} CD11c^{neg} (IMs) cells (Fig 10B). Staining with APC conjugated F4/80 antibody gave a clear resolution between the macrophage populations as well as non-macrophage (i.e.: DCs, lymphocytes) cells (Fig 10D).

3.5 The two major populations of resident lung macrophages are the tissue macrophages (CD11c^{neg} CD11b^{pos}) and the alveolar macrophages (CD11c^{pos} CD11b^{neg})

Studies on AM and IM started in the late 1980s and suggested that interstitial macrophages are an intermediary stage of alveolar macrophages (Laskin et al., 2001). In a recent study on Cigarette Smoke-Induced emphysema whereby Xiong and associates investigated the role of CX3CR1 in recruitment of monocytes, inflammatory cytokine responses and tissue destruction that showed CX3CR1^{pos} tissue resident mononuclear phagocytes are responsible in initiation of innate immune response via TNF-α and IL-6. They also showed that IMs express CD11b but not CD11c, whereas the AMs were the exact opposite (Xiong et al., 2011).

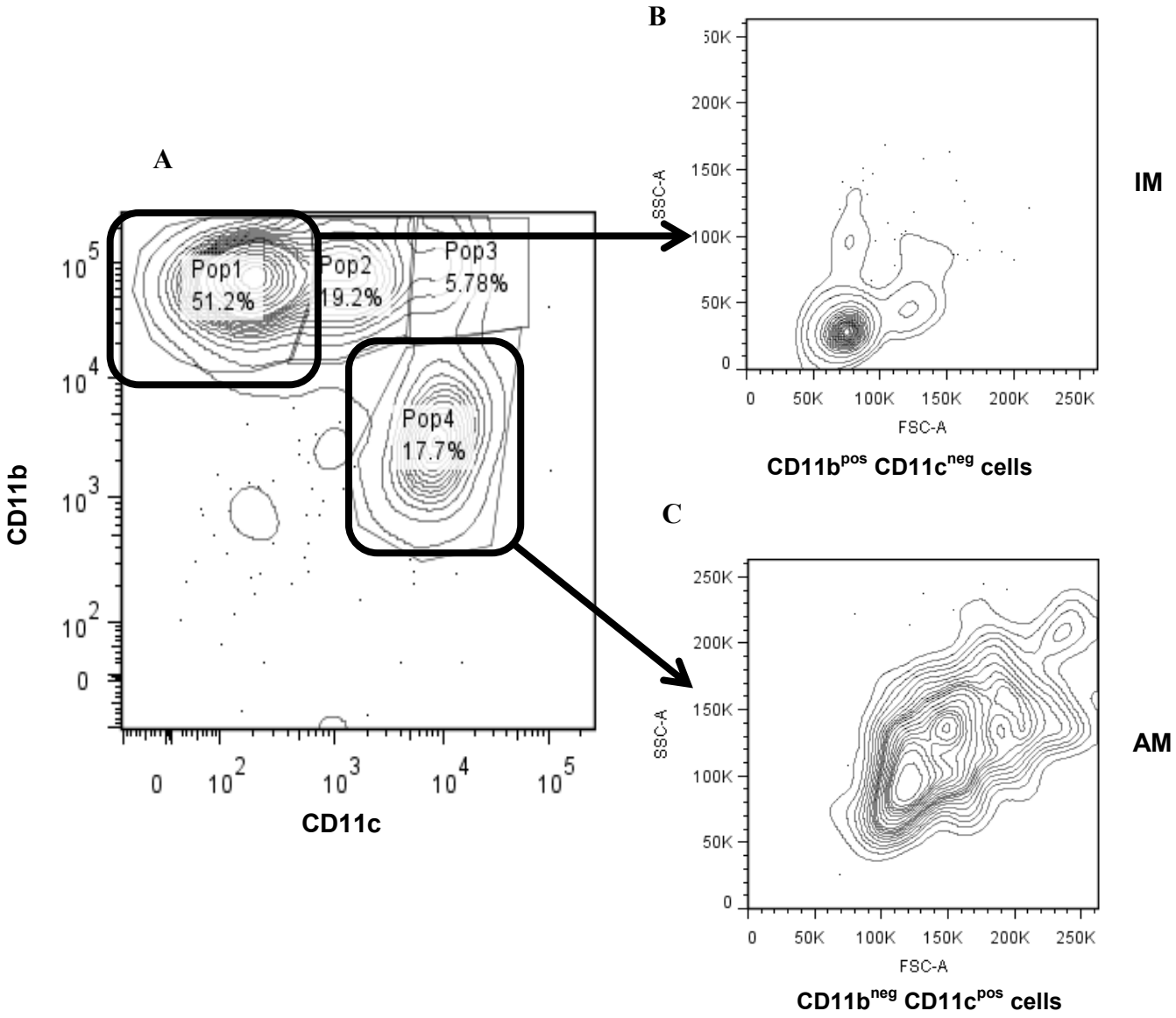


Fig 11 The two major pop populations of F4/80^{pos} cells (macrophages) in the lungs are the tissue and alveolar macrophages. Live F4/80^{pos} lung cells were gated on CD11c on V450 and CD11b on PE-Cy7 (A). The two populations ; tissue macrophages (B) and alveolar macrophages (C) were plotted on forward and side scatter by flow cytometery.

The aim of the this experiment is to established an optimized flow cytometry panel to characterise lung macrophages based on CD11b and CD11c expression on their surface. Naïve lung cells were stained with F4/80, CD11b and CD11c; and were gated on CD11b and CD11c to observe phenotypes.

This experiment confirms that the two major populations in the lung tissue are the $CD11b^{pos}$ $CD11c^{neg}$ macrophages or the interstitial or tissue macrophages and the alveolar macrophages or the $CD11b^{neg}$ $CD11c^{pos}$ cells. (Fig 11A). These two macrophage populations are not only different in their cell surface marker; they also differ in their size and granularity. These populations were plotted on forward (FSC) and side (SSC) scatter using flow cytometry analysis. Tissue macrophages or the $CD11b^{pos}$ $CD11c^{neg}$ cells are smaller in size and less granular (Fig 11B) compared to the alveolar macrophages or the $CD11b^{-}$ $CD11c^{+}$ cells, which are bigger and more granular (Fig 11C)

A study by Fukushima and colleagues documented that eosinophils express F4/80 and CD11b on their surface. Therefore in the next experiment, Ly6G antibody was added to the staining panel to exclude granulocytes (Fukushima et al., 2010).

3.6 Gating strategy to identify pulmonary macrophages and monocytes

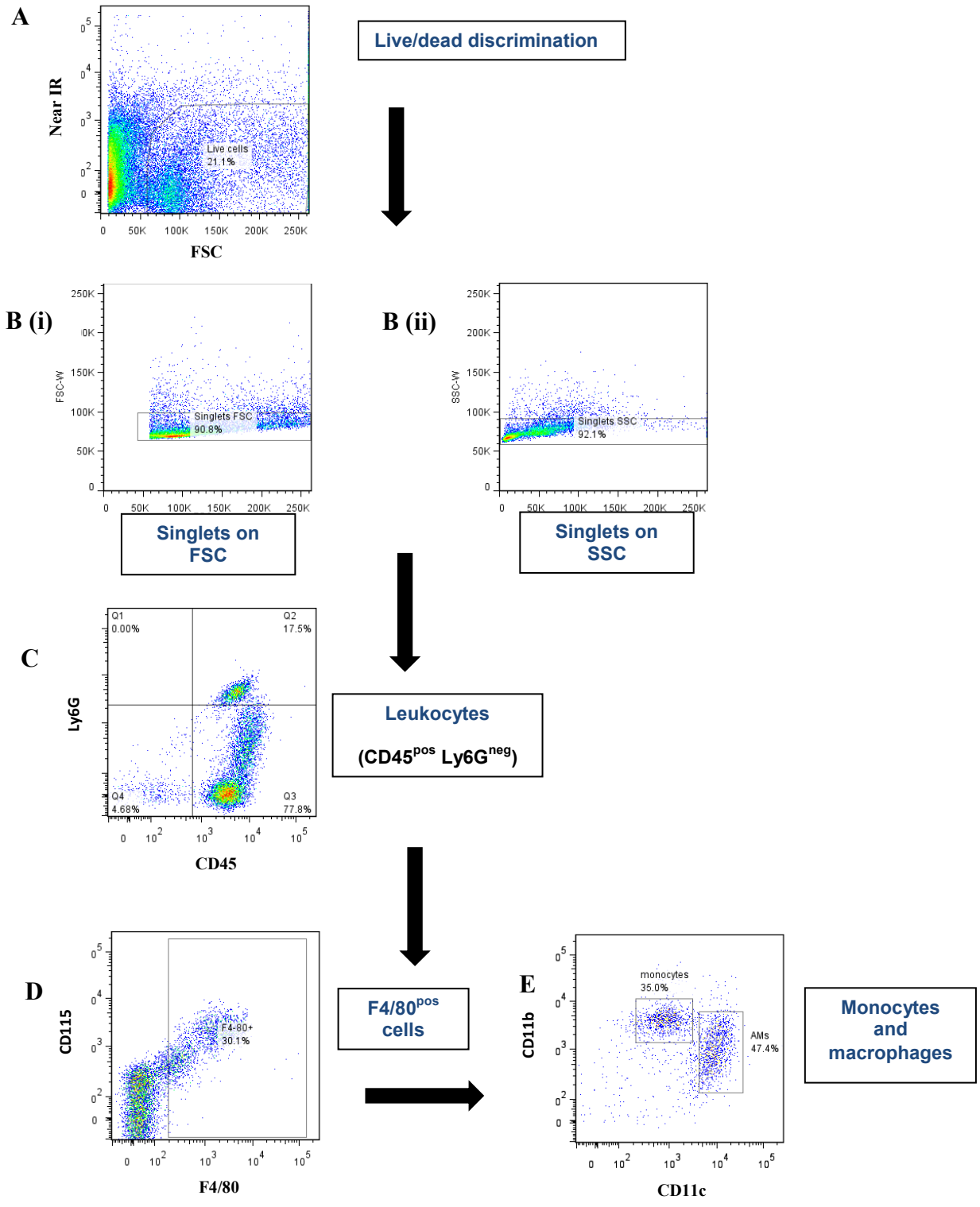


Fig 12 Representative plots confirming pulmonary macrophages and recruited monocytes BAL and lung cells were stained for flow cytometry analysis. A standardised gating strategy was established for this project to gate monocytes and pulmonary macrophages.

To confirm the origin and phenotype of the inflammatory macrophage/monocyte, it was necessary to establish a panel and a gating strategy to identify these cells (Fig 12). BAL and lung cells were stained with a live/dead fixable stain to eliminate dead cells (Fig 12A). Dead cells auto fluoresce during flow cytometry analysis. Due to the nature of the cells of interest in this study, particularly alveolar macrophages which are inherently auto-fluorescent, the live/dead discrimination was a pivotal step to avoid interferences in the accuracy of data analysis.

Live cells went through single cell selection twice based on their size (Fig 12B i) and granularity (Fig 12B ii) before identification based on cell surface markers. In a study on allergic conjunctivitis, Fukushima and associates found that the increased number of $CD11b^{pos}$ $F4/80^{pos}$ cells in allergic conjunctivitis is due to eosinophil infiltration rather than macrophages (Fukushima et al., 2010). Therefore, in the next step of gating leukocytes were selected as $CD45^{pos}$ $Ly6G^{neg}$ cells to exclude granulocytes that are $Ly6G^{pos}$.

3.7 Identification of recruited inflammatory monocytes in the airway during RSV infection

The main aim of this work is to identify recruited monocytes and to find a tool or markers to distinguish them from resident macrophages. During RSV infection, there is an influx of inflammatory cells into the pulmonary cavity and amongst the cells that get recruited into the airway and lung are the monocytes. Monocytes are a subset of leukocytes circulating the bloodstream. These cells derived from precursors in the bone marrow which can further differentiate into macrophages and dendritic cells (Auffray et al., 2009; Geissmann et al., 2003).

Mouse and human monocytes are similar in terms of their differentiation during infection and/or inflammation. Although their roles in immune defence are similar, their identification and expression of surface markers do not overlap (Geissmann et al., 2003; Gordon and Read, 2002).

In mice, monocytes are divided into different subsets based on their surface markers and chemokine receptor expression. The most prevalent subset is the $Ly6C^{hi}$ $CD11b^{pos}$ or the inflammatory monocytes. In an uninfected mouse these cells represent approximately 3% of the blood leukocyte population. During infection, they are rapidly recruited to the inflammatory sites via CCR2. The other subset expresses CCR2 and Ly6C at low levels but expresses high levels of CX3CR1. These monocytes adhere to the blood vessel lumen, migrating along the surface of the endothelial cells; an activity referred to as patrolling monocytes (Geissmann et al., 2003; Sunderkotter et al., 2004a; Sunderkotter et al., 2004b; Woollard and Geissmann, 2010).

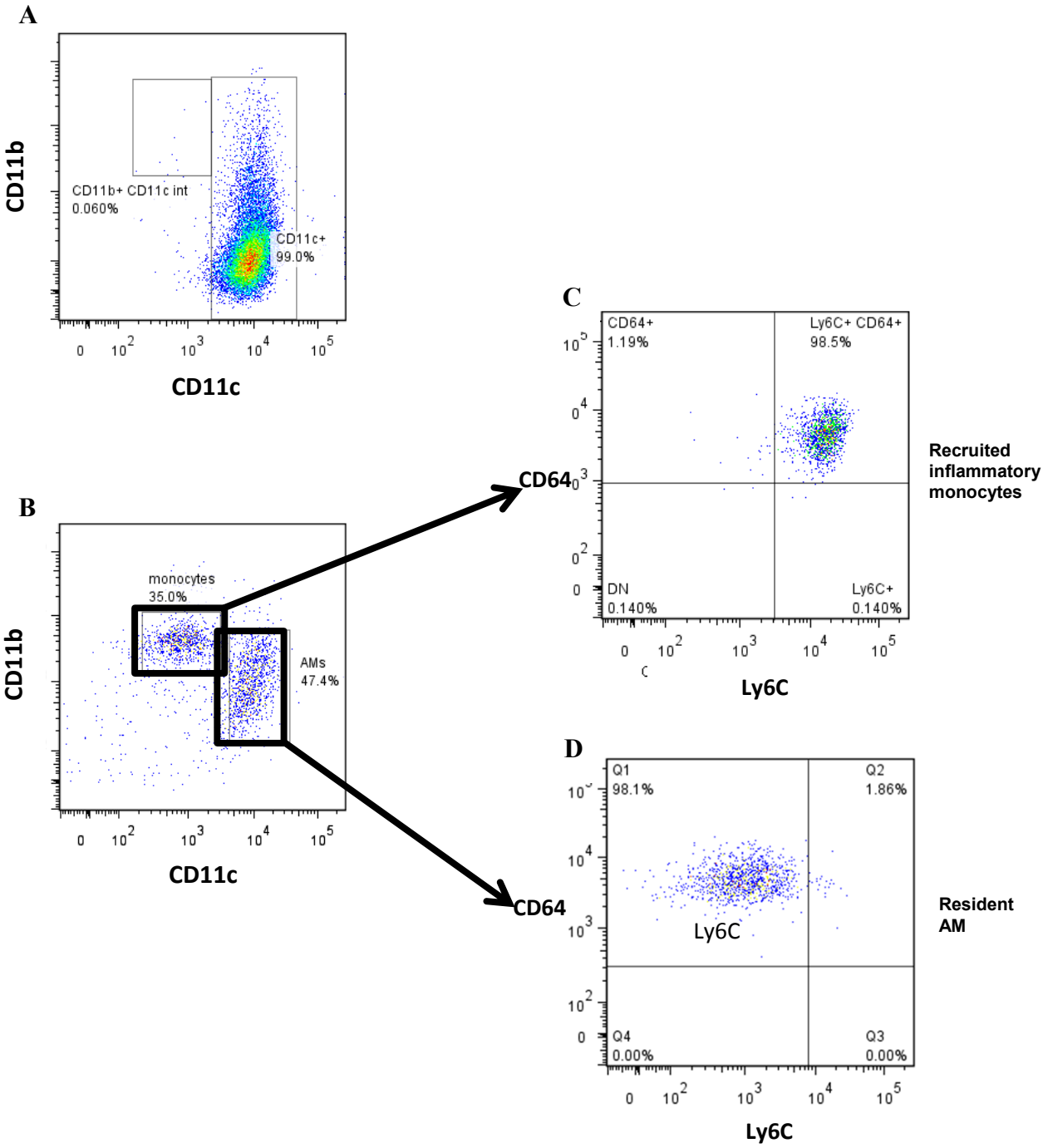


Fig 13: Identifying inflammatory monoctyes in the airway using Ly6C. Resident alveolar macrophages are the predominant F4/80^{+} cells found in the naïve airway (A). At day 2 following infection, monocytes (CD11b^{+} CD11c^{int}) are recruited into the airway (B). These recruited monocytes expressed Ly6C (C) which was not present on alveolar macrophages (D).

In this experiment, resident lung macrophages were characterised based on CD11b and CD11c expression on their surface. In the naïve airway, the predominant F4/80^{pos} cells are the alveolar macrophages (CD11b^{pos} CD11c^{pos}) (Fig 13A). An influx of CD11b^{pos} CD11c^{int} cells (monocytes) into the airway were seen at day 2 after RSV infection (Fig 13B). Ly6C expression on CD11b^{pos} CD11c^{int} cells after RSV infection suggests the presence of recruited inflammatory monocytes (Fig 13C). These markers were not present on resident alveolar macrophages (Fig 13D).

3.8 Distinguishing inflammatory monocytes from resident alveolar macrophages using PKH26 PCL Cell Linker

The aim of this experiment is to distinguish recruited monocytes and resident macrophages with the cell linker dye PKH26 PCL. PKH26PCL is a phagocytic cell marker, which is a novel technique that discriminates bronchoalveolar recruited monocytes from resident alveolar macrophages (Maus et al., 2001). According to the manufacturer's recommendation, the dye is said to be stable up to 21 days in phagocytic cells.

3.8.1 PKH26PCL longevity study

For this study, stability of the dye in the target cells is crucial. Therefore, the stability of this dye was tested in these macrophage subpopulations. In this study, the dye was used at 1:10 dilution and administered intravenously. Lungs were collected at day 7, 14, 21 and 42 after administration of the dye

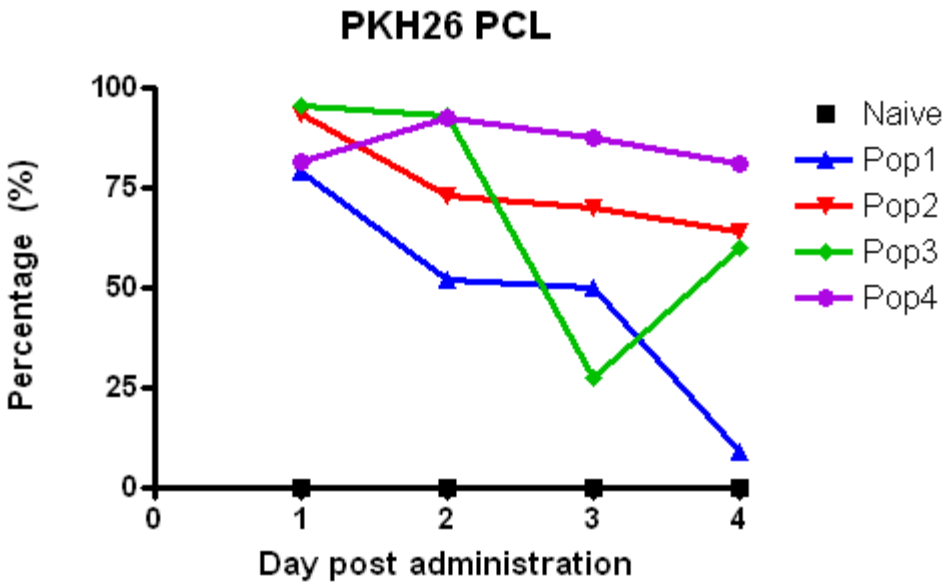


Fig 14 The fluorescent phagocytic cell linker PKH26 PCL dye remained stable in cells for at least 21 days and up to day 42 for Populations 2, 3 and 4. For this study, the dye was used at concentration 1:10. PKH26 PCL was administered intravenously per mouse at day 0 and nothing was given to the control group. Lungs were harvested at day 7, 14, 21 and 42.

Note:

Population 1: $CD11b^{pos}$ $CD11c^{neg}$

Population 2: $CD11b^{pos}$ $CD11c^{int}$

Population 3: $CD11b^{pos}$ $CD11c^{pos}$

Population 4: $CD11b^{neg}$ $CD11c^{pos}$

From this experiment, PKH26 PCL dye remained stable in the cells for at least 21 days as suggested by the manufacturer and up to day 42 for Populations 2, 3 and 4 (Fig 14). Between the two major populations of pulmonary macrophages, PKH26 PCL was more stable in Population 4 (i.e. AM) than in Population 1 (i.e. IM). This is probably due to the *in situ* proliferation capacity of the tissue macrophages as suggested in a study in a helminth model claiming that tissue macrophages undergo rapid *in situ* proliferation in order to increase population density (Jenkins et al., 2011). Although this was true in inflammation, in this study it shown that Population 1 (interstitial/tissue macrophages) has a higher turnover in the lung than Population 4 (alveolar macrophages) in naïve lung. Nevertheless, it is still ambiguous in RSV infection.

3.9 Distinguishing inflammatory monocytes from resident alveolar macrophages using CFSE staining

This study is also aiming to distinguish resident macrophages from recruited monocytes. In this study, the staining tool used was CFSE. Carboxyfluorescein diacetate, succinimidyl ester (CFSE) was originally used to label lymphocytes and to track their migration *in vivo* for months. Optimal labelling of cells allows approximately 7 – 8 cell divisions and last up to 8 weeks before the intensity of the dye dies down. The limitation to this dye is that if used in high concentration, it can be toxic to cells (Carson and Wilson, 2013; Parish et al., 2009).

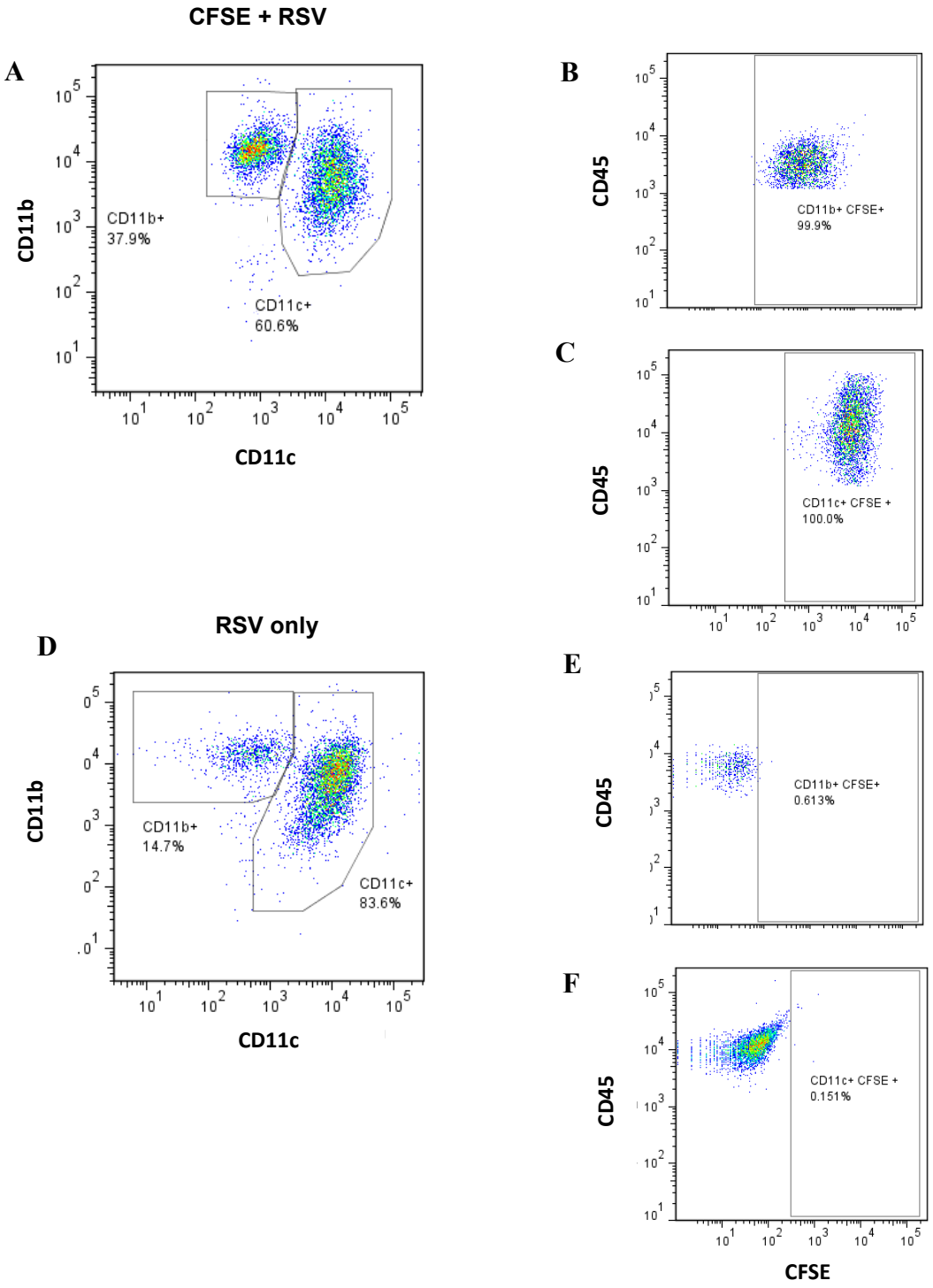


Fig 15: Distinguishing recruited inflammatory monocytes and resident alveolar macrophages using with carboxyfluorescein diacetate succinimidyl ester (CFSE). Single live airway cells that were $CD45^{pos}$ $F4/80^{pos}$ were labelled with CFSE intranasally 24 hours prior to RSV infection (A) to distinguish between the recruited monocytes (B) and the resident macrophages (C). Another group were only infected with RSV (D) and used as negative controls for recruited monocytes (E) and resident alveolar macrophages (F).

In this experiment, CFSE is used to differentiate resident alveolar macrophages from the recruited inflammatory monocytes two days after RSV infection. The hypothesis is that CFSE would stain the resident alveolar macrophages but not the recruited monocytes with local administration into the lung. CFSE was given 50 μl per mouse intranasally one day before RSV infection (Fig 15A) and a group was given RSV infection only as a negative staining control for CFSE (Fig 15D). BALF was collected at day 2 post RSV infection for flow cytometry analysis.

Intranasal administration of CFSE before RSV infection stains resident alveolar macrophages brightly (Fig 15C) with no evidence of recruitment of new $CFSE^{neg}$ macrophages from the periphery. The recruited inflammatory monocytes (Fig 15B) however show a dimmer staining with CFSE. These $CFSE^{dim}$ recruited monocytes may have migrated from the lung tissue in the airway during RSV infection due to local proliferation of tissue macrophages (interstitial macrophages).

3.8 Comparison between PKH26 PCL and CFSE staining for macrophages and monocytes

Defining recruited monocytes and to be able to distinguish them from resident macrophages is pivotal to this study. Ly6C demonstrated a clear definition of the recruited monocytes from the resident alveolar macrophages. To confirm the best tool to use, a comparison of staining of the $F4/80^{pos}$ cells were made between PKH26 PCL and CFSE.

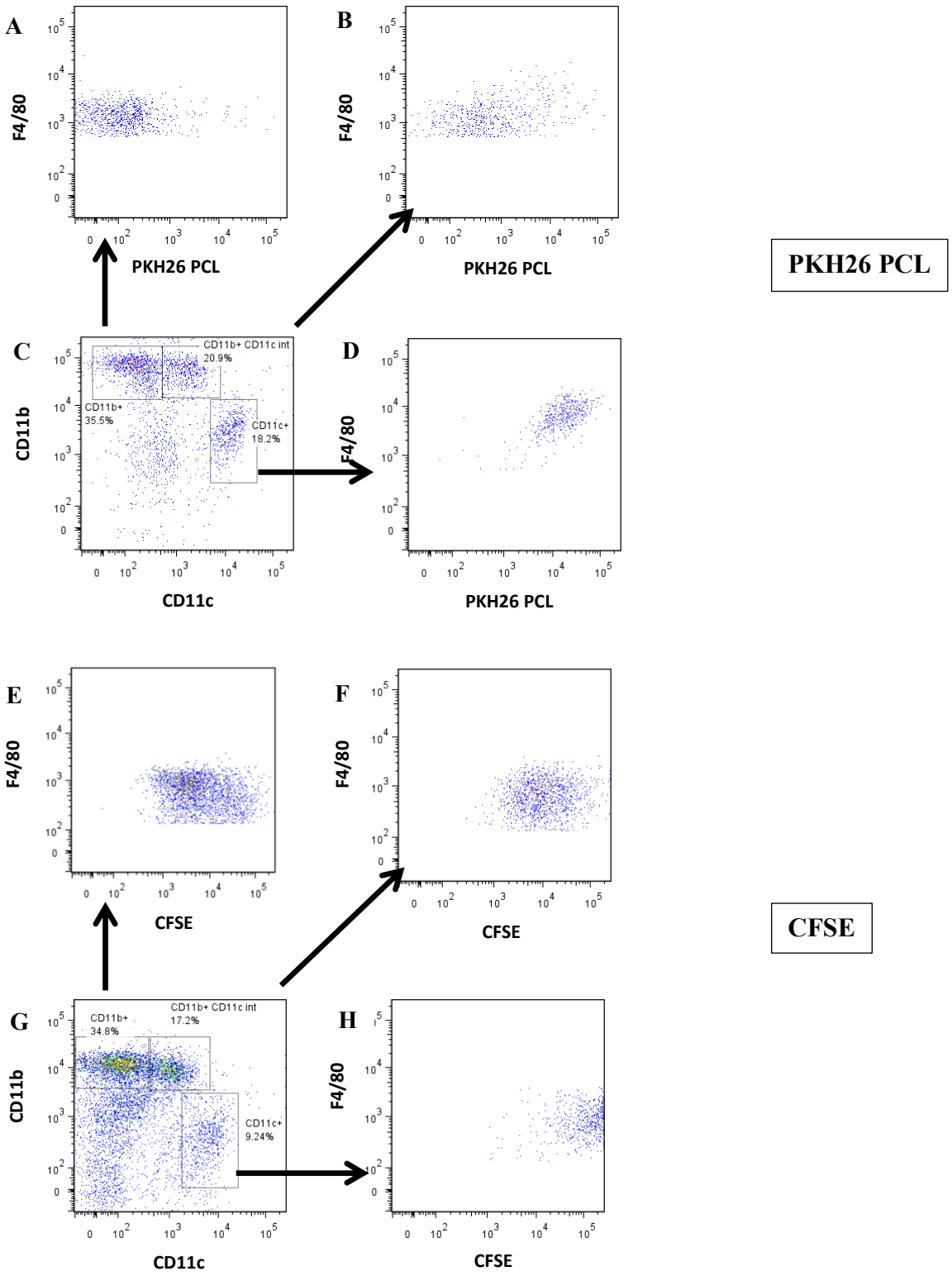


Fig 16: Comparison of PKH26 PCL and CFSE staining on F4/80^{pos} cells. Single live lung cells that were CD45^{pos} F4/80^{pos} were labelled with intravenous injection of PKH26PCL (C) and CFSE intranasally (E) 24 hours prior to RSV infection. Staining of F4/80^{pos} cell population were compared; CD11b^{pos} (A,E), CD11b^{pos} CD11c^{int} (B,F) and CD11c^{pos} (D,H).

In this experiment, lung cells were stained with either PKH26 PCL via i.v. injection or CFSE via intranasal administration 24 hours before RSV infection. PKH26 PCL dye distinctively labelled the $CD11^{pos}$ cells (Fig 16D) compared to the rest of the subsets. The $CD11b^{pos}$ cells (Fig 16A) and $CD11b^{pos}$ $CD11c^{int}$ cells (Fig 16B) were not affected by the dye.

However, labelling with CFSE was not as distinctive between the cell subpopulations. The $CD11c^{pos}$ cells were $CFSE^{hi}$ whilst the $CD11b^{pos}$ $CD11c^{int}$ cells and $CD11b^{pos}$ cells were partially stained. This is similar to what was seen in section 3.7 where there were no clear distinction in CFSE staining between the $CD11c^{pos}$ cells and the $CD11^{pos}$ $CD11c^{int}$ cells.

3.9 Summary of Chapter 3

In the beginning of this chapter, the obstacles and importance of establishing a staining panel for macrophages in naïve and infected lung were highlighted, from the choice of LIVE/DEAD discriminator to be used to discriminate dead cells from the live cells, to the detailed gating strategy of lung macrophage and monocytes populations. To determine the best way of distinguishing local macrophages in the lung from the recruited monocytes, several methods and staining tools were used. Although PKH26 PCL demonstrated a more distinct staining of the $F4/80^{pos}$ subsets compared to CFSE, Ly6C is the best marker to use in this study for identifying recruited monocytes and distinguish them from resident macrophages. $F4/80^{pos}$ subpopulations were characterized based on their surface markers as summarized in Table 12.

Table 12: Summary of pulmonary macrophage phenotype based on their surface markers in uninfected and infected airway and lung

Markers	Naïve		RSV infection		
	AM	IM	AM	IM	Recruited monocytes
CD45	+	+	+	+	+
Ly6G	-	-	-	-	-
F4/80	+	+	+	+	+
CD11b	-	+	+	+	+
CD11c	+	-	+	-	±
CD64	+	+	+	+	±
Ly6C	-	-	-	-	+
PKH26 PCL	+	±	+	-	-
CFSE	+	+	+	±	±

4.0 RECRUITMENT OF INFLAMMATORY MONOCYTES DURING THE TIME COURSE OF RSV INFECTION

4.1 Introduction

Monocytes are a subset of leukocytes circulating in the bloodstream. These cells are derived from precursors in the bone marrow which can further differentiate into macrophages and dendritic cells. In mice, monocytes are divided into different subsets based on their surface markers and chemokine receptor expression. The most prevalent subset in mouse lung is the $Ly6C^{hi}$ $CD11b^{+}$ or the inflammatory monocytes, which express high levels of the CC-chemokine receptor 2 (CCR2) and low levels of CX_3C-chemokine receptor 1 (CX_3CR1). They represent 2 -3 % of the blood leukocyte population in an uninfected mouse. During infection, these $Ly6C^{hi}$ monocytes are rapidly recruited to the inflammatory sites via CCR2, the receptor used by monocytes to migrate from the bloodstream into the lung tissue. In humans, they represent the $CD14^{++}$ $CD16^{neg}$ subsets also known as the classical monocytes (Geissmann et al., 2010; Geissmann et al., 2003; Sunderkotter et al., 2004).

In 2008, Lin and colleagues demonstrated that $CCR2^{pos}$ monocyte-derived cells play a major role in influenza-induced inflammation and pathology. Recruitment of monocyte-derived inflammatory cells was greatly reduced in $CCR2^{-/-}$ mice. This observation is supported by very low expression of co-stimulatory molecules, NOS2 producing cells, weight loss and mortality (Lin et al., 2008). In a study of *Klebsiella pneumoniae* infection in the lung, Herold and associates found that the increase in alveolar epithelial cell death in $CCR2^{-/-}$ compared to wild-type mice suggests the importance of recruitment of

monocytes/macrophages from the circulating pool as a protective response towards *K. pneumoniae* infection (Herold et al., 2011).

The other major subset of blood monocytes expresses CCR2 and Ly6C at low levels but expresses high levels of CX_3CR1. In humans, they are known as the intermediate monocytes with $CD14^{++}$ $CD16^{+}$ phenotype. These monocytes adhere to the blood vessel lumen, migrating along the surface of the endothelial cells; an activity referred to as patrolling. Although these $Ly6C^{low}$ monocytes are less prevalent in the blood stream, they are also recruited to the sites of inflammation with a possible wound healing function via differentiation into alternatively activated macrophages. (Geissmann et al., 2003; Sunderkotter et al., 2004)

This study will utilise the BALB/c murine model of RSV infection (Fig 17) to monitor monocyte recruitment into the airway and lung tissue in a time course of RSV infection. Mice were given intranasal infection of RSV (Section 2.2.3.2) and were sacrificed at 24 hours, 48 hours, day 4 and day 7 after RSV infection. The experiment will demonstrate whether the inflammatory macrophage/monocyte phenotype, i.e.: $Ly6C^{pos}$ seen during RSV infection is blood-derived or due to up-regulation of inflammatory marker by the resident cells.

4.2 Experimental Aims and Objectives

Identifying recruited inflammatory monocytes vs resident alveolar macrophages and resident tissue macrophages over a time course of RSV infection based on their surface markers:

(i) Recruited inflammatory monocytes: $CD11b^{pos}$ $Ly6C^{pos}$

(ii) Resident alveolar macrophages: $CD11c^{pos}$ $Ly6C^{neg}$

(iii) Resident tissue macrophages: $CD11b^{pos}$ $CD11C^{neg}$

Determining the peak of inflammatory monocyte recruitment over a time course of RSV infection

4.3 Experimental Model

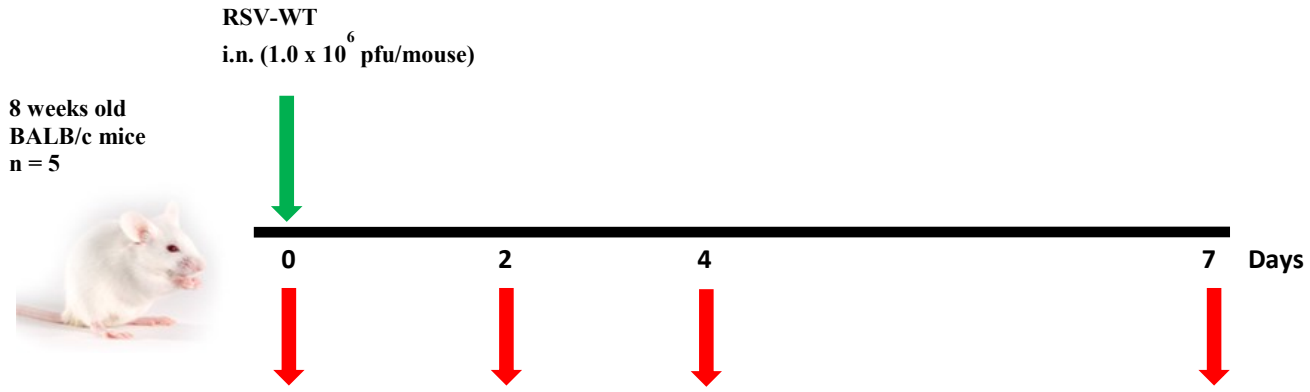


Fig 17 Murine model of RSV infection 8-weeks old BALB/c mice were treated with RSV i.n. At different time points, these mice were given terminal anaesthesia. BALF and lungs were collected for end point analyses. (n = 5 mice per group)

The general hypothesis is that recruitment of inflammatory cells in the acute stage of RSV infection is the main cause of severe inflammation in the disease. The main focus of this study is the recruitment of monocytes into the airway and lung tissue during the acute phase of RSV infection, which is believed to be the main contributor to excess inflammation in this disease.

4.4 F4/80^{pos} CD11b^{pos} CD11c^{int} cells recruited into the airway at day 2 post RSV challenge

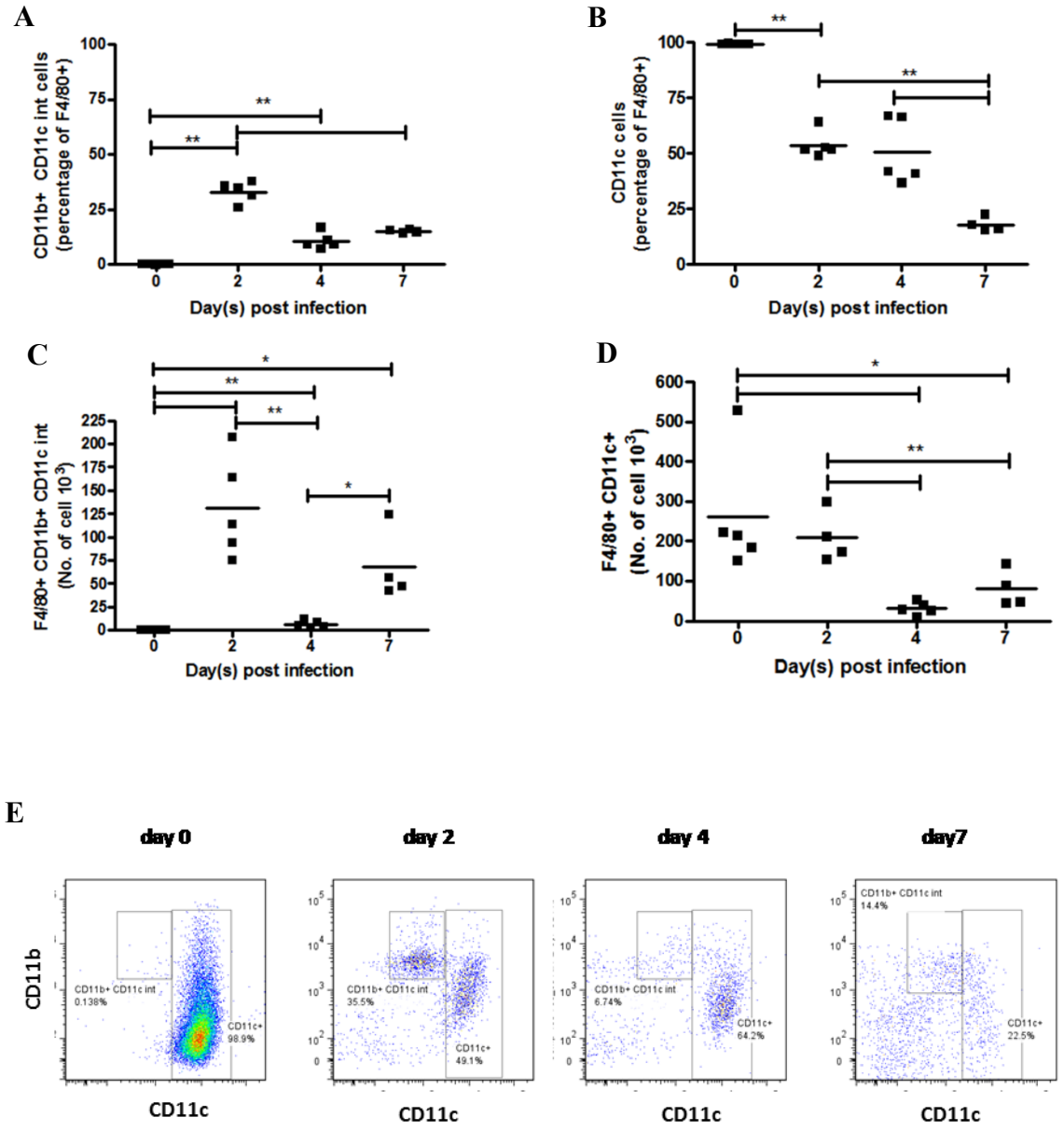


Fig 18 F4/80^{pos} CD11b^{pos} CD11c^{int} cells recruited into the airway at day 2 post RSV challenge Single live CD45^{pos} Ly6G^{neg} F4/80^{pos} BAL cells were gated to quantify the percentage (A and B) and number (C and D) of recruited monocytes and resident alveolar macrophages in the airway respectively. Flow cytometry plots represent the populations (E) throughout the time course of RSV infection. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$, ** = $p < 0.01$; data are otherwise not significant)

Recruited monocytes, as described in chapter 3 as CD11b^{pos} CD11c^{int} Ly6C^{pos} cells was seen infiltrating the airway at day 2 post RSV infection. The percentage (Fig 18A) was seen highest at day 2 and started to decrease at day 4 and remained low at day 7 post challenge. The number of CD11b^{pos} CD11c^{int} Ly6C^{pos} cells recruited into the airway after RSV infection showed similar trend (Fig 18C), with the highest number seen at day 2 post challenge and greatly decreased at day 4 post challenge. Interestingly, a slight but significant increase in cell number seen at day 7 post challenge may be contributed to the local proliferation of tissue macrophages. Several studies have shown that local tissue macrophages have the ability to self-replicate without or with minimal contribution from recruited monocytes (Hashimoto et al., 2013; Jenkins et al., 2011).

As previously mentioned in chapter 3, majority of F4/80^{pos} cells in the airway are the alveolar macrophages or the F4/80^{pos} CD11c^{pos} cells. The percentage of these cells (Fig 18B) seen highest prior to infection (day 0) and significantly declined to approximately 25% at day 7 post challenge. This is due to the massive infiltration of inflammatory cells during infection overwhelming the percentage of alveolar macrophages in the airway. In terms of cell number (Fig 18D), there was no significant difference between day 0 and 2 post challenge but there were significant decrease at day 4 and day 7 after RSV infection. In a naïve airway, the alveolar macrophages are typically of a F4/80^{pos} CD11c^{pos} phenotype. These cells were seen to up-regulate their CD11b expression at day 2 after RSV infection (Fig 18E). This population started to down-regulate CD11b expression at day 4 and are back to the original phenotype of F4/80^{pos} CD11c^{pos} by day 7 post challenge. In a pneumococcal challenge study in 2006, a similar finding of a novel population of AMs that express CD11b was reported (Kirby et al., 2006).

4.5 F4/80^{pos} CD11b^{pos} CD11c^{int} cells recruited into the lung at day 2 post RSV challenge

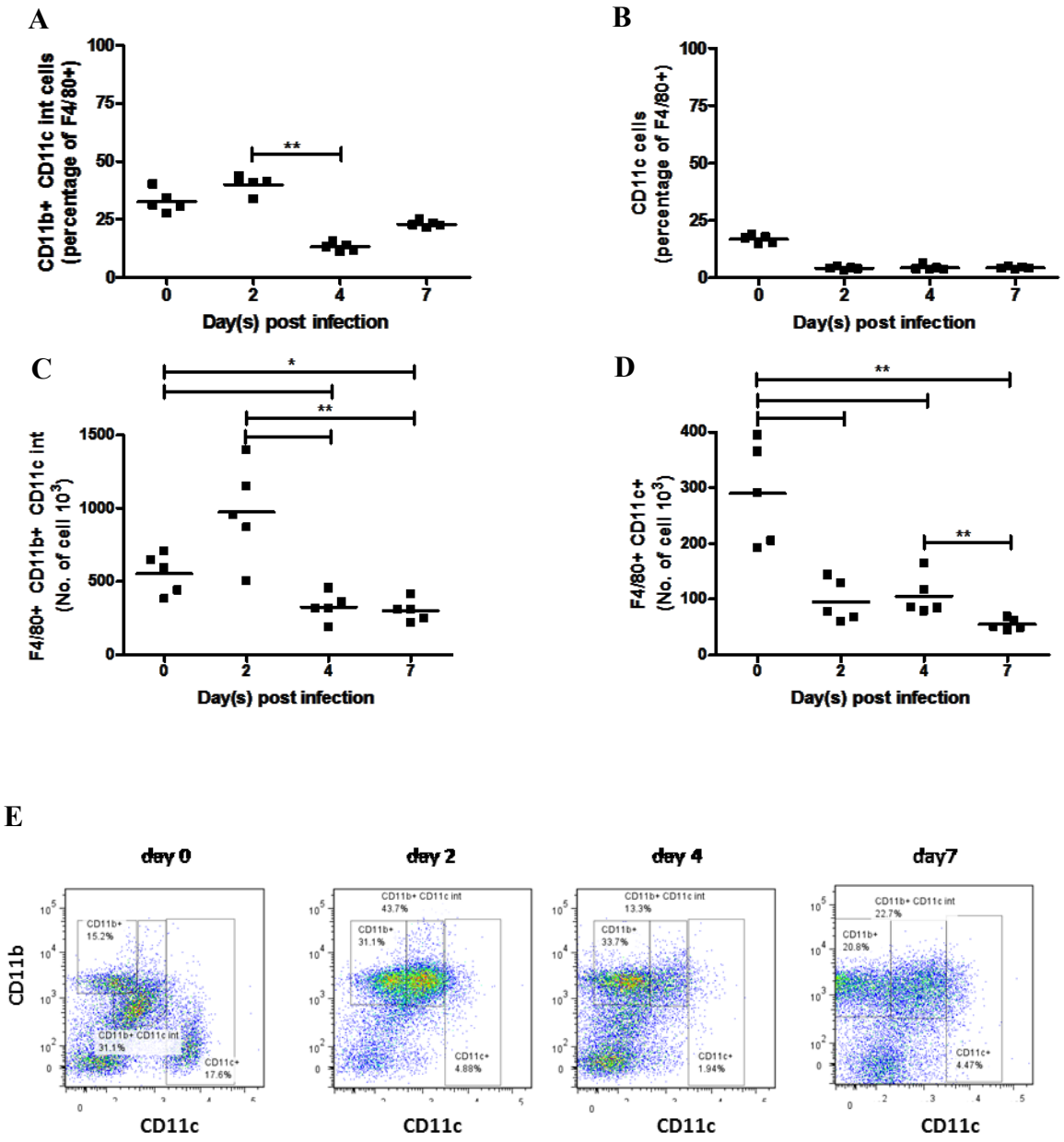


Fig 19 F4/80^{pos} CD11b^{pos} CD11c^{int} cells recruited into the lung at day 2 post RSV challenge Single live CD45^{pos} Ly6G^{neg} F4/80^{pos} lung cells were gated to quantify the percentage (A and B) and number (C and D) of recruited monocytes and resident alveolar macrophages in the airway respectively. Flow cytometry plots represent the populations (E) throughout the time course of RSV infection. (n=5 mice per group; values represent ± SEM, * = p <0.05, ** = p <0.01; data are otherwise not significant)

In the lung tissue, the kinetics of F4/80^{pos} CD11b^{pos} CD11c^{int} cells showed similar trend to the airway (Fig 19A). Although it was not significant, there was a slight increase in proportion seen at day 2 post infection. This cell population decreased significantly at day 4 and a slight increase but not significant seen at day 7 post infection. There was an increase in cell number (not significant) at day 2 and the number decreased significantly at day 4 and remained low at day 7 post infection (Fig 19C).

The percentage of alveolar macrophages in the lung tissues was low prior to infection, and it remained low throughout the time course of infection (Fig 19B). However, there was a significant drop in cell number at day 2 and the number remained fairly similar at day 4 before a decreasing significantly at day 7 post RSV challenge.

One of the aims of this experiment is to determine the phenotype of recruited monocytes after RSV challenge. After RSV infection, recruitment of F4/80^{pos} CD11b^{pos} CD11c^{int} was seen highest at day 2 post infection. The expression of CD64 and Ly6C were used to determine whether the recruited F4/80^{pos} CD11b^{pos} CD11c^{int} cells were of inflammatory phenotype. CD64 (most commonly known as Fc-gamma receptor 1, FcγRI) is a marker that is inherently found on macrophages and recently, alongside MAR-1 has been suggested to be the marker to distinguish monocyte-derived DCs from conventional DCs (Platinga M et al, J. Immunol 2013).

4.6 Inflammatory monocytes in the airway and lung

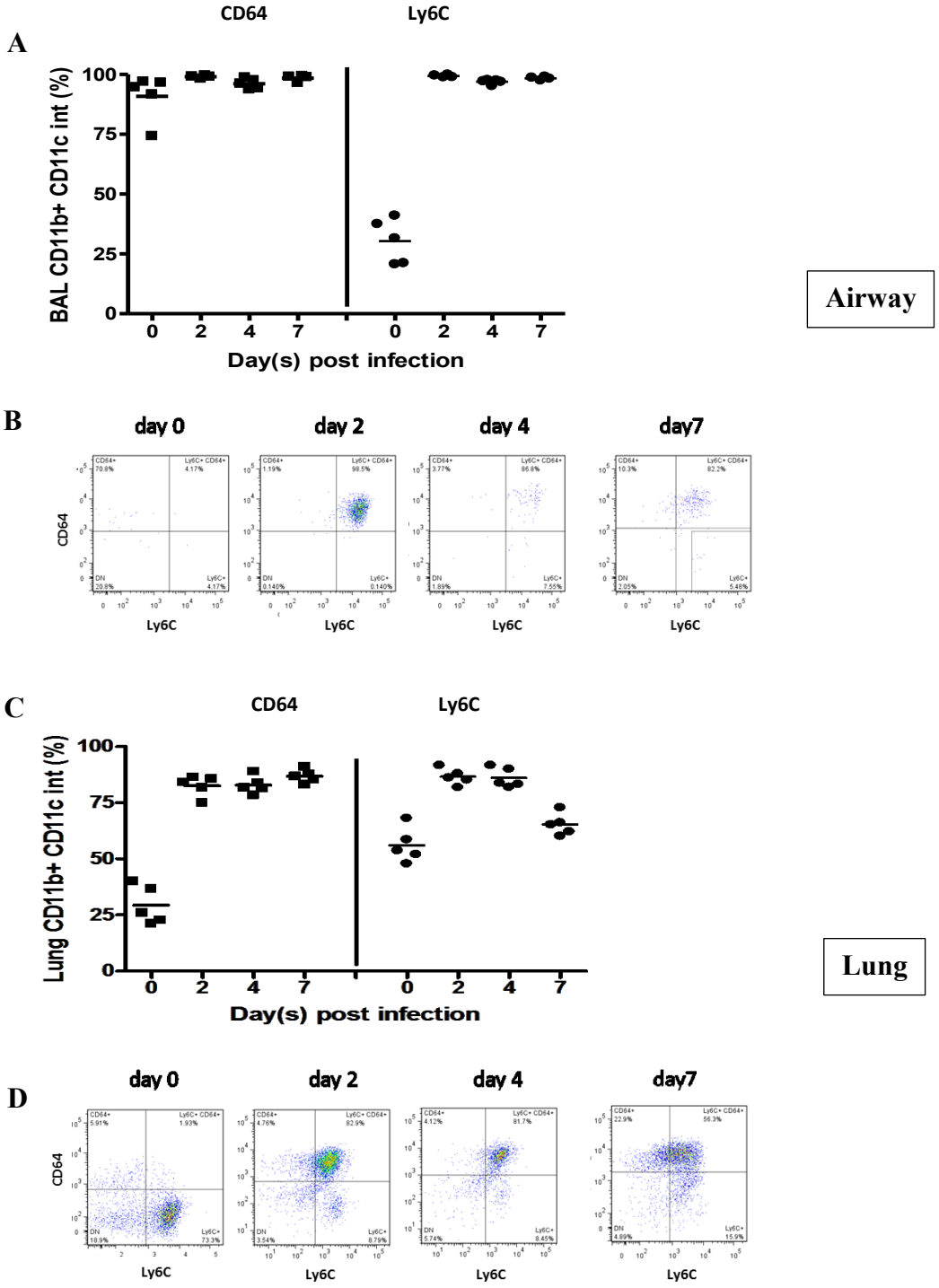


Fig 20 Confirming recruitment of inflammatory monocytes into the airway and lung Inflammatory F4/80^{pos} CD11b^{pos} CD11c^{int} Ly6C^{pos} cells recruited into the airway (A) and the lung (C) at day 2 post RSV challenge. Recruitment of these cells over a time course in the airway (B) and in the lung (D) were represented via flow cytometry plots. (n=5 mice per group; values represent ± SEM)

From this experiment, F4/80^{pos} CD11b^{pos} CD11c^{int} cells in the airway (Fig 20A) and lung tissues (Fig 20C) were found to be CD64^{pos}. The proportion of these cells that are Ly6C^{pos} were 100% in the airway at day 2, 4 and 7 post challenge (Fig 20A). The intensity of these cells were seen highest at day 2 post infection (Fig 20B), decreased at day 4 with a slight increase at day 7 post infection, which correlates positively with the percentage and cell number of F4/80^{pos} CD11b^{pos} CD11c^{int} cells in the airway (Fig 20A and 20C).

F4/80^{pos} CD11b^{pos} CD11c^{int} Ly6C^{pos} cells in the lung tissues post challenge showed similar trend to the ones seen in the airway. Majority of these cells are CD64^{neg} Ly6C^{pos} prior to infection. This may suggest the resident lung tissue macrophages are a different phenotype monocytic like F4/80^{pos} cells. After infection however, most of the F4/80^{pos} CD11b^{pos} CD11c^{int} cells were double positive for both CD64 and Ly6C. Similar to what was observed in the airway, the intensity of these cells (Fig 20D) correlates positively with the percentage and cell number of F4/80^{pos} CD11b^{pos} CD11c^{int} cells in the lung tissue (Fig 20B and 20D).

As mentioned previously, CD64 is a cell surface marker that is closely related to APCs like macrophages, dendritic cells and monocytes. The molecule is inherently expressed on alveolar macrophages, but only on some DC subsets. In this study, resident alveolar macrophages expressed CD64 on their surfaces throughout the time course of RSV infection. However, Ly6C was not expressed on alveolar macrophages (Fig 21A). Therefore, alveolar macrophages found in the airway during the time course of RSV infection are inherently of CD64^{pos} Ly6C^{neg} phenotype.

4.7 Resident alveolar macrophages remained Ly6C negative

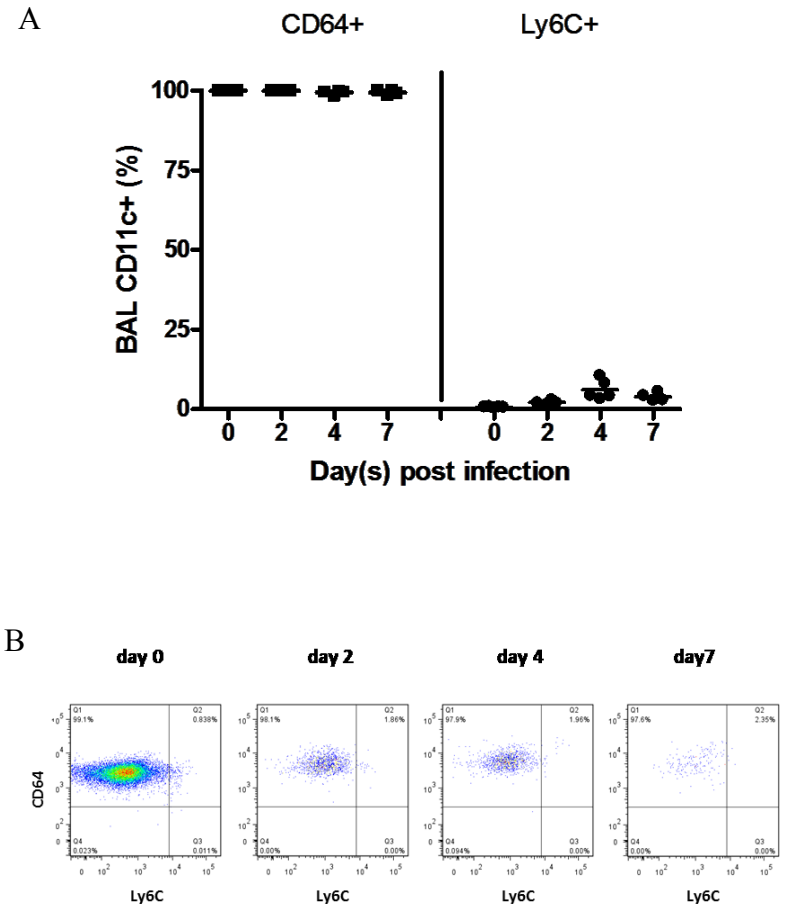


Fig 21 Resident alveolar macrophages remained Ly6C^{neg} in the airway Resident alveolar macrophages does not express Ly6C (A) throughout the course of RSV infection. Representative flow cytometry plots of the dynamics of these cells over a time course (B). Flow cytometry plots represent the populations (E) throughout the time course of RSV infection. (n=5 mice per group; values represent ± SEM)

Although the intensity of resident AMs decreased over time, they remained Ly6C^{neg} in the airway post RSV challenge (Fig 21B) making Ly6C a suitable marker to distinguish between alveolar macrophages and recruited inflammatory monocytes. Therefore, the CD64/Ly6C double positive cells found in the airway and lung suggested a recruited inflammatory phenotype of F4/80^{pos} CD11b^{pos} CD11c^{int} cells. This recruitment of inflammatory monocytes (F4/80^{pos} CD11b^{pos} CD11c^{int} Ly6C^{pos}) was markedly seen in the airway post RSV challenge.

4.8 Summary of Chapter 4

Macrophage sub-populations and monocytes have been characterised in Chapter 3 by establishing a flow cytometry panel. The staining panel has enabled the differentiation between resident macrophage populations in the lung tissues and airway, and the recruited monocytes in infected and non-infected lung.

In this chapter, the objective of the study is to determine the kinetics of monocyte recruitment during the early phase of RSV infection. In a time course study up to day 7 post RSV challenge, recruitment of F4/80^{pos} CD11b^{pos} CD11c^{int} cells were seen highest in both airway and lung at day 2 after RSV infection. These cells were described as recruited monocytes as per Section 3.7 in Chapter 3, which are believed to be the main contributors to excessive inflammation in RSV disease. These recruited monocytes has also been confirmed to be Ly6C^{pos} in the airway and lung, whereby proportion of the cells that are Ly6C^{pos} remained high; ~98-100% and ~75-85% in the lung. Resident AMs remained Ly6C^{neg} throughout the time course of infection, making Ly6C an ideal marker to distinguish recruited monocytes from resident AMs.

5.0 THE EFFECT OF BLOOD MONOCYTE DEPLETION IN MURINE MODEL OF RSV INFECTION

5.1 Introduction

In the last chapter, early recruitment of inflammatory monocytes was seen during RSV infection. The recruitment peaked at day 2 post infection making them a target as cells of interest for the study in this chapter. In this study, an intervention was introduced to the RSV murine model using clodronate liposomes (CL). Previously in this lab, $CD11c^{pos}$ $MHCII^{lo}$ cells (identified as alveolar macrophages) were depleted in RSV infection using CL administration via the intranasal route. Depletion of AMs resulted in significant reduction of cytokines and chemokines like TNF, IL-6, CCL3, IFN-α, and CCL5. The depletion significantly reduced the recruitment and activation of NK cells into the airway but did not influence the later T-lymphocyte response (Pribul et al., 2008) as summarised in figure 22.

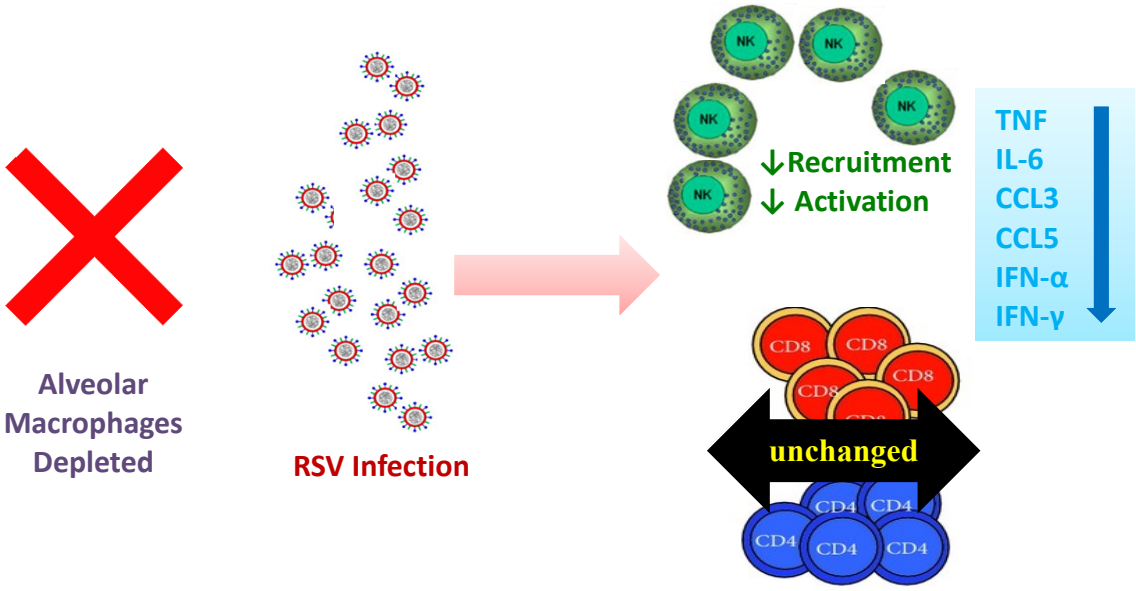


Fig 22 Macrophage depletion reduces early cytokine and chemokine production and , inhibited the activation and recruitment of natural killer (NK) cells Mice were treated with 30% CL 3 days prior to i.n. infection with 2×10^5 PFU of RSV. TNF, IL-6, CCL3, IFN-α, CCL5, and IFN-γ levels in BAL cells were measured by ELISA. NK cell recruitment and activation were reduced but does not alter the adaptive immune responses; percentages of CD4 and CD8.

5.2 Experimental Model

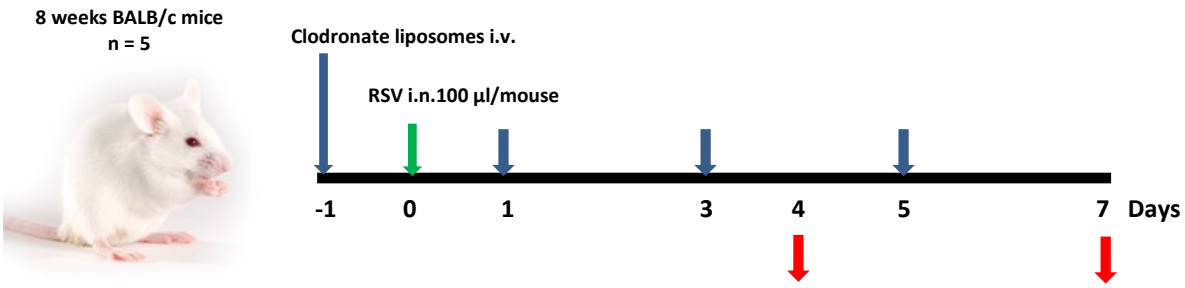


Fig 23: Experimental model for depletion of recruited monocytes. Clodoronate liposomes were administered via intravenous injection during RSV infection to address the importance of monocytes in RSV-induced inflammation. Clodronate liposomes were given 24 hours prior to infection and subsequent doses were administered every 48 hours. BALF and lung tissues were collected at day 4 and day 7 post RSV infection (n = 5 mice per group).

Circulating monocytes were depleted with intravenous (i.v.) injection of clodronate liposome (CL) during RSV infection to address the importance of recruited monocytes in RSV-induced inflammation. The first CL injection was given to 8 weeks old BALB/c mice one day prior to inoculation of RSV and subsequently, CL was given every 48 hours after the first dose throughout the time course of the experiment. This regimen was taken from a study which demonstrated that the increase in macrophage numbers in the pleural cavity in a helminth model of infection is due to local proliferation of resident macrophage and is independent of monocyte recruitment (Jenkins et al., 2011).

5.3 Experimental Aims

The aims of this study are

1. To deplete blood monocytes (BM) with i.v. injection of CL during RSV infection.
2. To monitor the effect of clodronate liposome administration on RSV infection.

5.4 Clodronate liposomes intravenous treatment depletes blood monocytes

These data demonstrate successful depletion of BM with i.v. injection of CL during RSV infection. Live blood cells were stained with CD115 also known as colony stimulating factor 1 receptor (CSF1R). In 2013, a work to standardize and optimised the identification and analysis of blood monocytes demonstrated that staining with CD115 produce a distinctive staining of blood monocytes compared to CD11b and CD14 as commonly used (Breslin et al., 2013). Although CD115 is expressed by monocytes, macrophages, osteoclasts, mononuclear phagocyte precursors and some epithelial cells, monocytes are the only cells in the peripheral blood that express CD115 (Auffray et al., 2009).

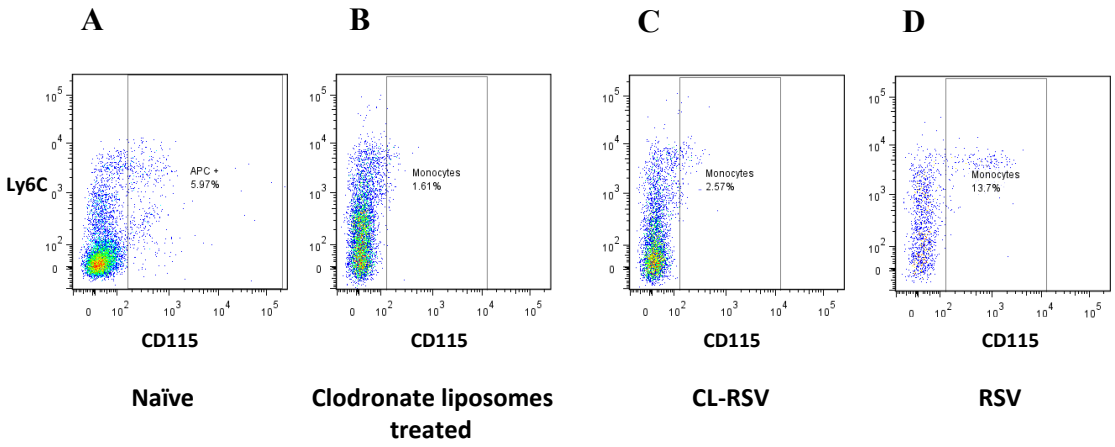


Fig 24 Clodronate liposomes intravenous treatment depletes blood monocytes Mice were treated with CL intravenously one day prior to i.n. infection with 100µl of 8.85 x 10^5 pfu/ml of RSV to each mouse. Monocytes were labelled in naïve blood (A), CL treated blood (B), CL treated with RSV (C) and with RSV infection alone. All cells were gated on single live $CD45^{pos}$ cells.

In this study, monocytes were seen in naïve blood (Fig 24A) and in the group with RSV infection. These were ablated in groups that received CL liposomes alone (Fig 24B) and together with RSV infection (Fig 24C) but not in RSV only controls. This confirms that BM monocytes were depleted by i.v. administration of CL.

5.5 Weight curve

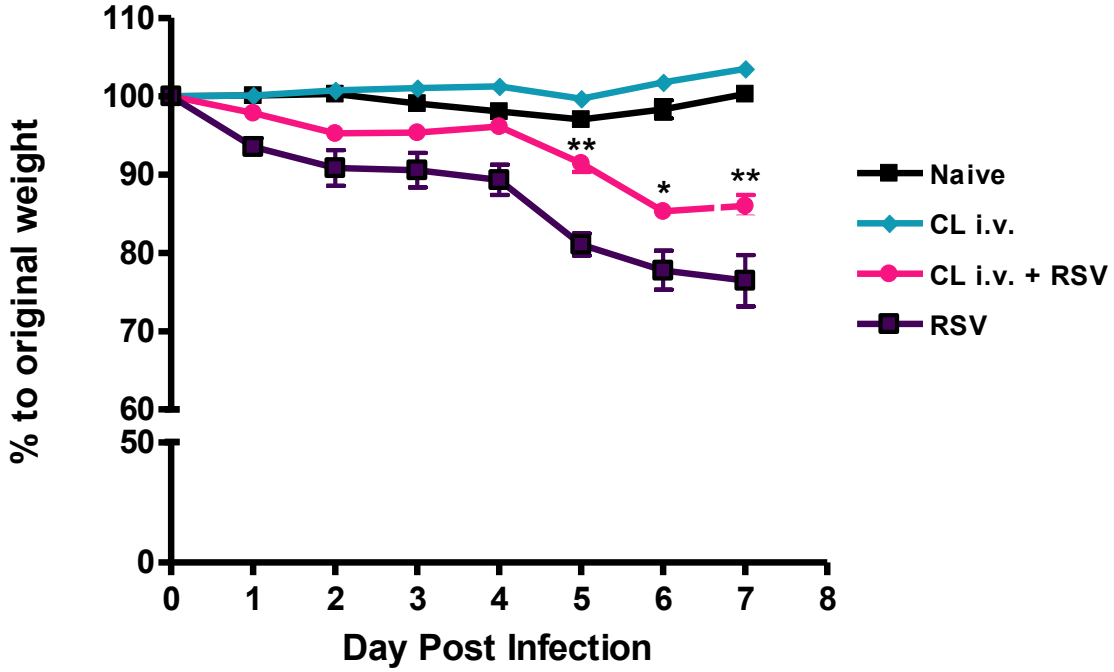


Fig 25: Clodronate liposomes treatment altered disease severity in RSV infection. Mice given clodronate liposomes with RSV infection were weighed daily and compared against naïve, clodronate liposomes only and RSV only controls. (n=5 mice per group; values represent ± SEM, * = p <0.05, ** = p <0.01, *** = p <0.001; data are otherwise not significant; comparison is between the group given RSV and CL i.v. administration with RSV infection)

In the murine model of RSV infection, disease severity is marked by weight loss which typically peaks at day 6 or 7 after RSV infection. Administration of CL alone has no effect on the weight, as there were no significant differences compared to the naïve group. However, CL administration during RSV infection reduced weight loss compared to normal RSV infection. The significant reduction was seen beginning at day 5 post infection, which was after the peak of viral replication and peak of inflammation in a normal RSV infection.

5.6 Viral load

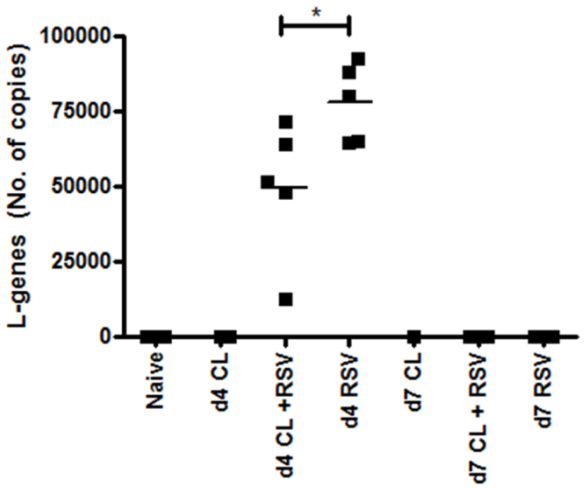


Fig 26: Clodronate liposomes treatment reduced viral load in RSV infection. Viral replication was measured by gene expression analysis of the RSV L-gene. RNA was isolated from lungs at day 4 and day 7 after RSV infection. After complementary DNA (cDNA) conversion, RSV L-gene mRNA copies were measured by qPCR. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$; data are otherwise not significant)

Viral load or viral replication normally correlates with weight loss in RSV infection. Therefore, the RSV L-gene copies were measured to demonstrate the effect of BM depletion on viral load. At day 4 after RSV infection with i.v. administration of CL, RSV L-gene mRNA copies were significantly reduced compared to normal RSV infection. (Fig 26). Non-infected (naïve) control and the group given CL without infection remained negative throughout the course of infection.

Reduction in the number of RSV L-gene mRNA copies in the group given clodronate liposomes with RSV infection compared to RSV only at day 4 (peak of inflammation) correlates positively with the weight loss observed between the two groups. Alteration of weight loss and the significant decrease in viral load suggests that depletion of monocytes via intravenous injection of clodronate liposomes may have a protective effect against RSV disease.

5.7 Total Cell Number

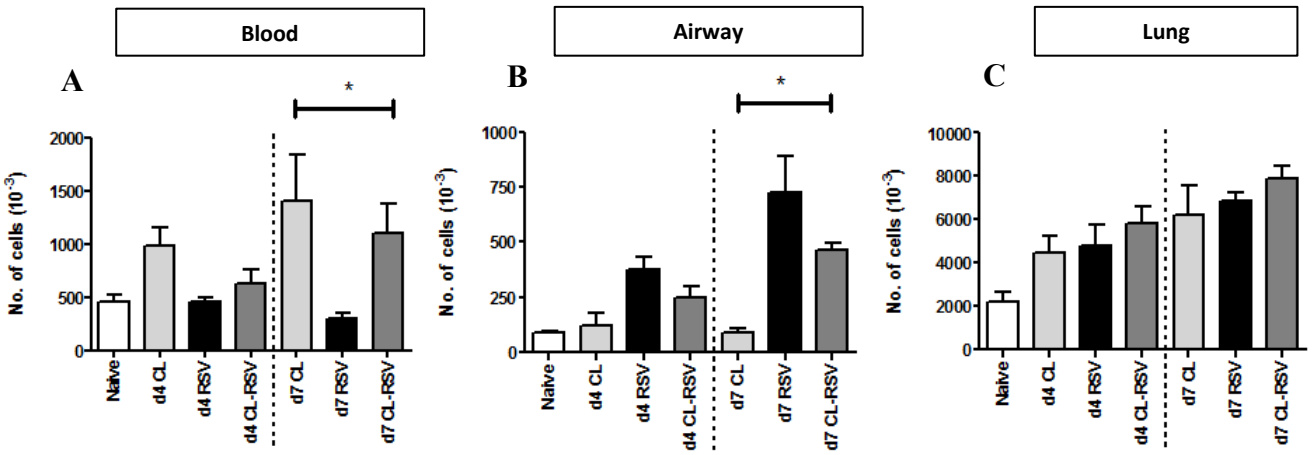


Fig 27: Cell number in the peripheral blood increased with clodronate liposomes treatment 7 days after RSV infection. Whole blood (A), BALF (B) and lung tissues (C) were collected at day 0, 4 and day 7 post infection. Total cell number in the peripheral blood, the airway (BAL) and in the lung tissues were determined using flow cytometry. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$; data are otherwise not significant)

Disease pathology, as measured by weight loss is often associated with cell recruitment into the site of inflammation. Following the effects of clodronate liposomes on the weight curve and viral replication, changes in blood and pulmonary cells were investigated during the time course of RSV infection. Collectively, there was no significant difference in total cell number between groups that were given CL alone, infected with RSV alone or in combination of both in all samples throughout the time course with the exception of the blood and the airway at day 7 post RSV infection (Fig 27A).

In the blood, RSV infection caused significant decrease in cell number in combination with i.v. injection of CL compared to CL administration alone at day 7 post infection. Conversely, the number of cells significantly increased in the airway when blood monocytes were depleted with RSV infection at day 7 post infection compared to depletion alone (Fig 27B).

5.8 Clodronate liposomes treatment reduced airway (BAL) CD11b^{pos} CD11c^{int} cell number and percentage

Intravenous injection of CL was used with the aim of depleting blood monocytes, with the expectation that this would decrease if not ablate recruitment of these cells into the airway and lung. The aim of this study is to determine the effect of CL on F4/80^{pos} subsets in the airway and lung throughout the time course of RSV infection.

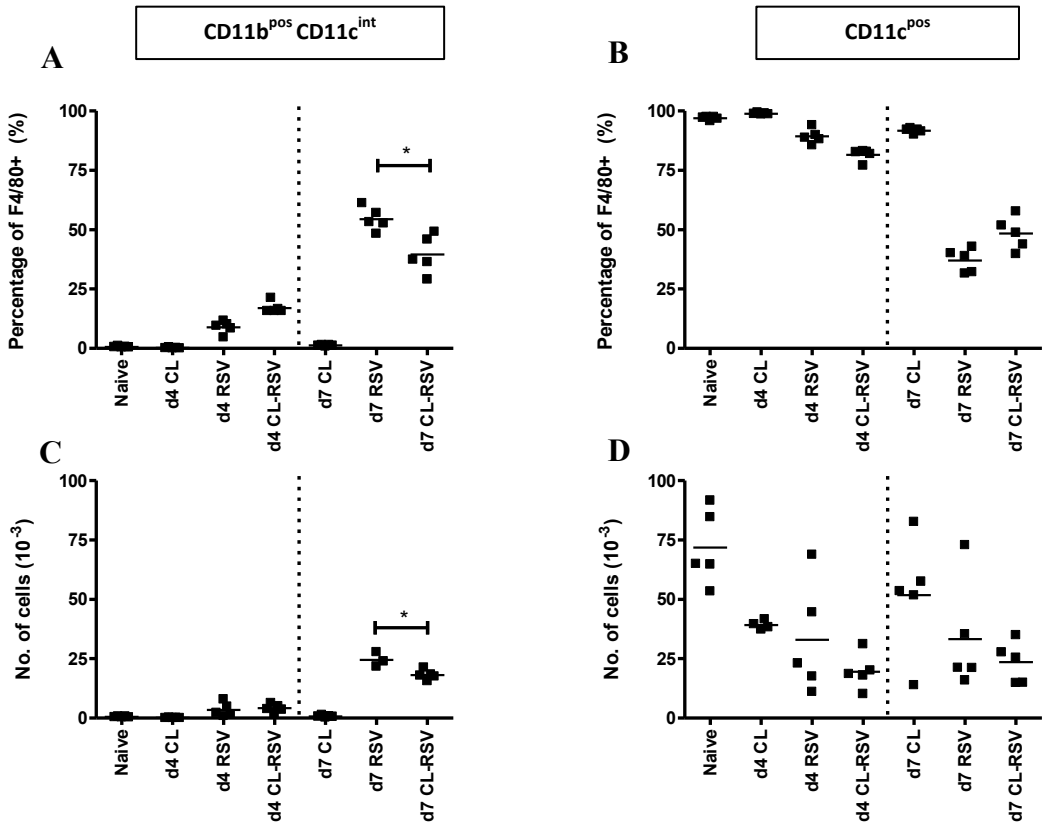


Fig 28: Depletion of blood monocytes reduced BAL CD11b^{pos} CD11c^{int} but did not change the CD11c^{pos} number and percentage. F4/80^{pos} BAL cells were stained for CD11b^{pos} CD11c^{int} (recruited monocytes) (A, C) and CD11c^{pos} (alveolar macrophages) (B, D) cells. Percentage (A, B) and cell number (C, D) of each cell population were determined using flow cytometry. Populations were gated on single live F4/80^{pos} CD45^{pos} Ly6G^{neg} cells. (n=5 mice per group; values represent ± SEM, * = p <0.05; data are otherwise not significant).

In the airway, the percentage (Fig 28A) and number (Fig 28C) of $CD11b^{pos}$ $CD11c^{int}$ cells significantly decrease with i.v. administration of CL at day 7 post RSV infection. There were no significant differences between the two groups at day 4 post infection, which supports the observation in chapter 4 whereby the percentage and cell number of $CD11b^{pos}$ $CD11c^{int}$ cells were not elevated day 4 post infection.

However, CL liposomes treatment did not cause any significant changes in the $CD11c^{pos}$ cells percentage (Fig 28B) and number (Fig 28D) with and without infection throughout the time course. This is an important observation to the model to ensure that any changes observed from this point onwards are due to depletion of blood monocytes and not local alveolar macrophages.

5.9 Clodronate liposomes treatment reduced lung CD11b^{pos} CD11c^{int} cell percentage

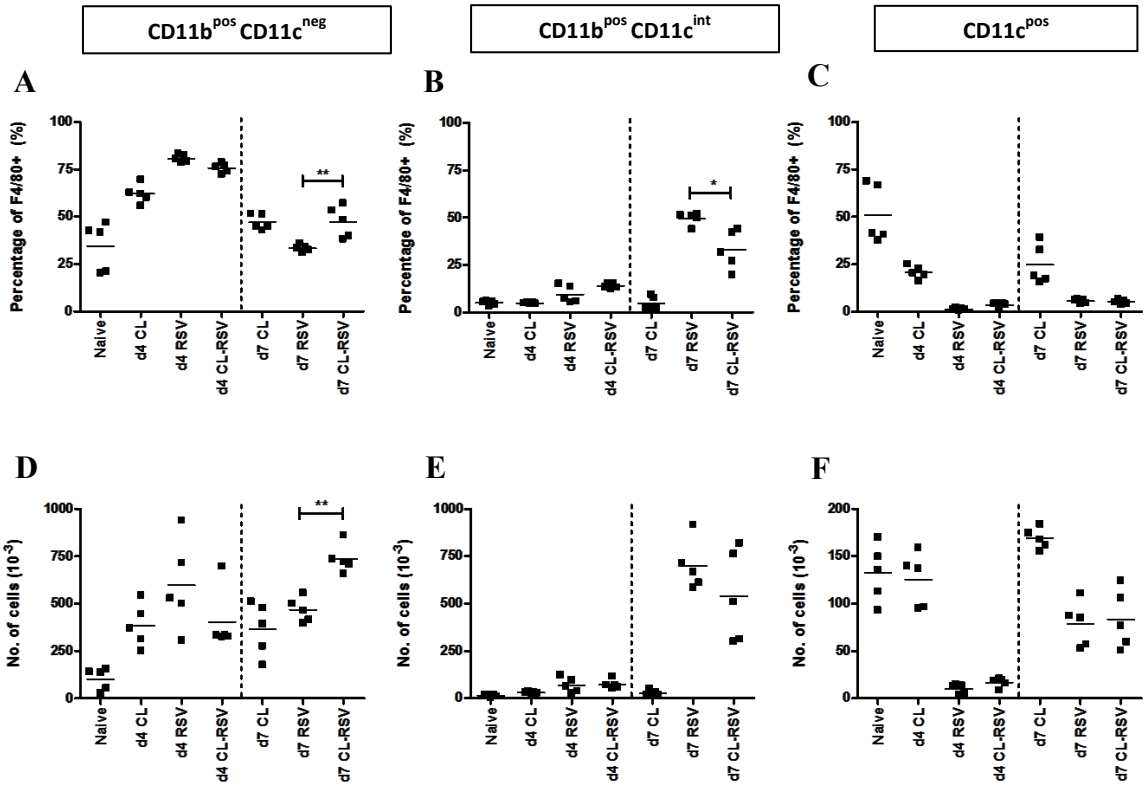


Fig 29: Clodronate liposomes treatment reduced CD11b^{pos} CD11c^{int} cell percentage in the lung. F4/80^{pos} lung cells were stained for CD11b^{pos} CD11c^{neg} (A, D), CD11b^{pos} CD11c^{int} (Fig B, E) and CD11c^{pos} (C, F) cells. Percentage (A, B, C) and cell number (D, E, F) of each cell population were determined using flow cytometry. Populations were gated on single live F4/80^{pos} CD45^{pos} Ly6G^{neg} cells. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$, ** = $p < 0.01$; data are otherwise not significant)

Similarly in the lung, CL treatment caused a significant decrease in $CD11b^{pos}$ $CD11c^{int}$ cell percentage at day 7 post RSV infection compared to the group given RSV only (Fig 29B). However, there was not a significant change in cell number between the two groups (Fig 29E). As seen in the airway, the $CD11c^{pos}$ cells percentage (Fig 29C) and cell number (Fig 29D) did not change between the groups. There was a significant increase seen in the percentage (Fig 29A) and cell number (Fig 29D) of the $CD11b^{pos}$ $CD11c^{neg}$ cells, which are the resident tissue macrophages or the interstitial macrophages in the CL treated group compared to RSV alone in the lung at day 7 post infection.

5.10 Clodronate liposomes treatment increased NK cell number in the airway at day 4 and 7 post RSV challenge

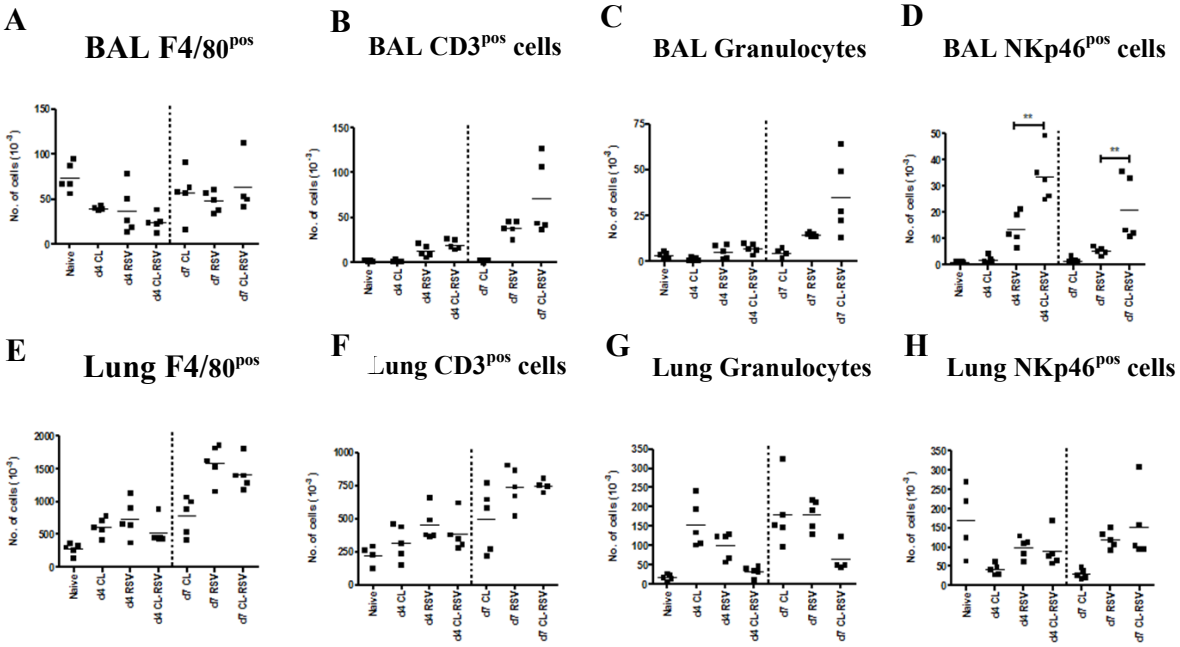


Fig 30: Clodronate liposomes treatment increased NK cell number in the airway. Number of different cells in the airway and lung was determined using flow cytometry. F4/80^{pos} populations were gated on single live F4/80^{pos} CD45^{pos} Ly6G^{neg} cells. CD3^{pos} populations were gated on single live CD3^{pos} NKp46^{neg} cells. Granulocytes were gated on single live CD45^{pos} Ly6G^{pos} cells. NKp46^{pos} populations were gated on single live CD3^{neg} NKp46^{pos} cells. (n=5 mice per group; values represent ± SEM, ** = p <0.01; data are otherwise not significant)

Intravenous administration of CL during RSV infection altered the percentage and/or number of the F4/80^{pos} sub-populations in the airway and lung. Using flow cytometry, number of different cells in the airway and lung were quantified. F4/80^{pos} cells are the macrophages and/or monocytes (Fig 30A, E), CD3^{pos} cells are the T-lymphocytes (Fig 30B, F), granulocytes are the Ly6G^{pos} cells (Fig 30C, G), which will be referred to as neutrophils and NKp46^{pos} cells will be referred to as the NK cells (Fig 30D, H).

Clodronate liposome treatment increased NK cell number in the airway (Fig 30D) with RSV infection compared to the group that were only infected with RSV at day 4 and day 7 post RSV infection. However, the increase in NK cell number did not influence the T-cell number in the airway or the lung. Increase in airway NK cell number in the clodronate liposomes treated group with RSV infection compared to RSV only group correlates positively with reduction in viral load seen at day 4 post RSV infection (Fig 26).

5.11 Clodronate liposomes treatment did not alter cell CD4 and CD8 T-cells number

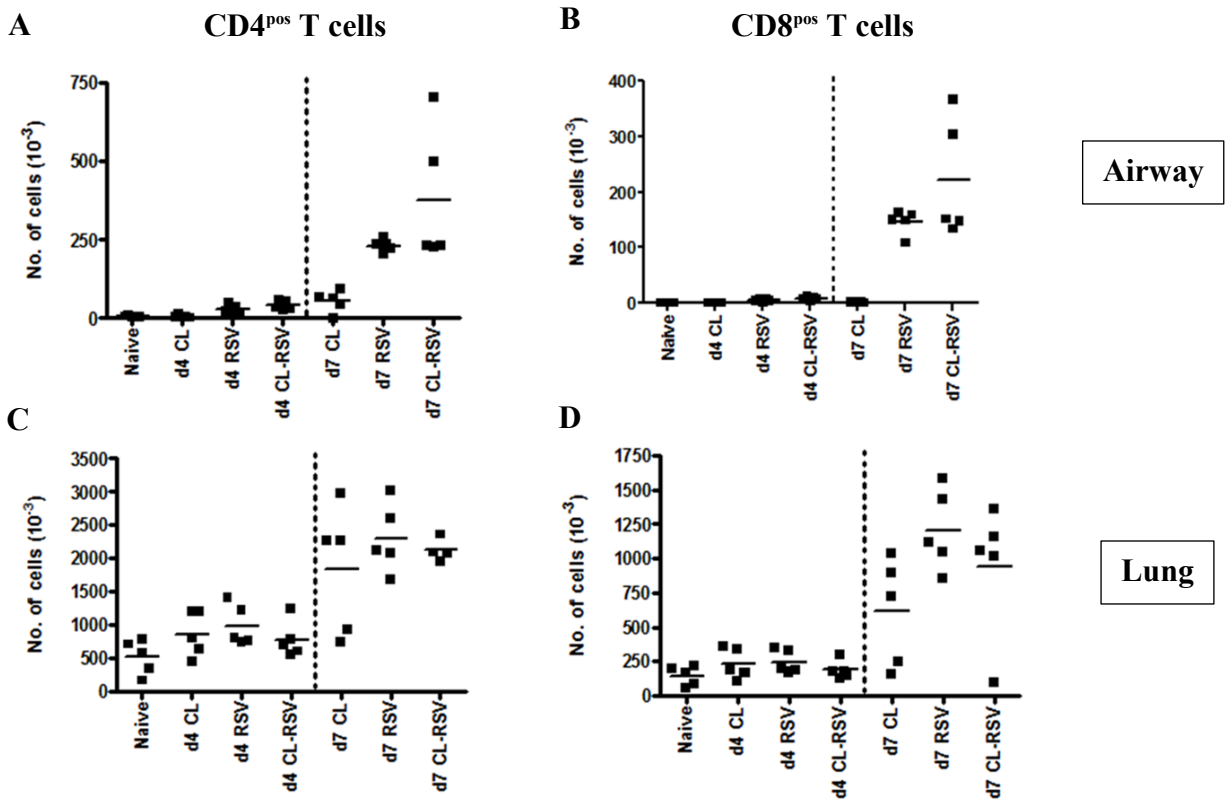


Fig 31: Clodronate liposomes treatment did not alter T-cell number in the airway and lung. Number of $CD4^{pos}$ and $CD8^{pos}$ T-cells in the airway (A, B) and lung (C, D) were determined using flow cytometry. Cells were gated on single live $CD3^{pos}$ $CD4^{pos}$ (A, C) and $CD8^{pos}$ (B, D) cells. (n=5 mice per group; values represent ± SEM)

Total T-lymphocytes did not show significant changes in terms of cell number in the airway or lung collectively as $CD3^{pos}$ cells. The number of $CD4^{pos}$ and $CD8^{pos}$ T-cells were determined to monitor whether CL treatment has any effect on the subsets throughout the time course of RSV infection. No significant changes observed in the lung tissues for both subsets (Fig 31C and D). This confirms that CL treatment had no effect on the T-lymphocytes with RSV infection.

5.12 Clodronate liposomes treatment reduced the number of neutrophils but did not alter lymphocyte number in the airway at day 7 after RSV infection

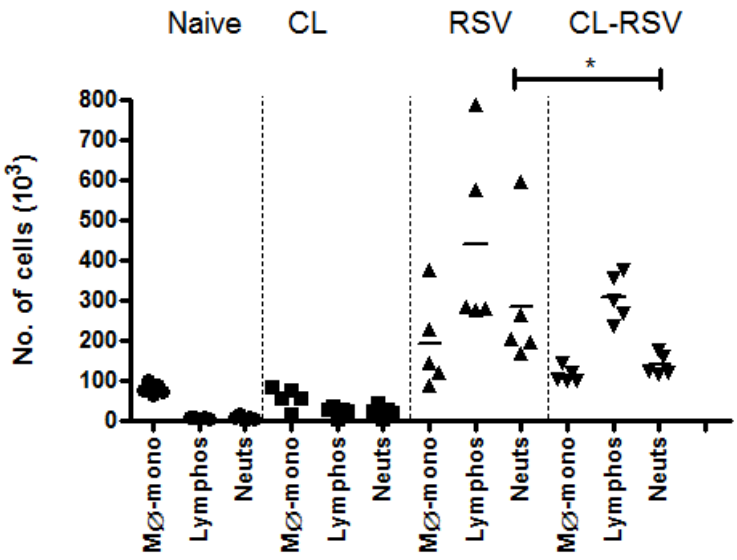


Fig 32: Clodronate liposomes treatment reduced neutrophil number at day 7 after RSV infection. BAL cells from day 7 post infection were stained with QuickDiff, which is a modification of Wright Stain for differential count of BAL cells. The number of neutrophils decreased with CL treatment during RSV infection. Lymphocyte and macrophages/monocyte cell number showed no significant changes in the airway at day 7. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$; data are otherwise not significant)

BAL cells collected on day 7 post RSV infection were differentially stained with QuickDiff to for differential count (Fig 32). QuickDiff is a modification of Wright stain which stains leukocytes. CL treatment alone did not alter the cell number compared to the group that did not receive any treatment (naïve). However, CL treatment with RSV infection significantly reduced neutrophil cell number compared to RSV infection alone. This observation did not correlate with the flow cytometry data.

CL treatment with RSV infection caused no significant differences in monocytes/macrophage and lymphocytes cell number from the cell count compared to the group infected with RSV alone. These observations correlated positively with the flow cytometry data.

5.13 Clodronate liposomes treatment reduced IFN-γ production at day 4 after RSV infection

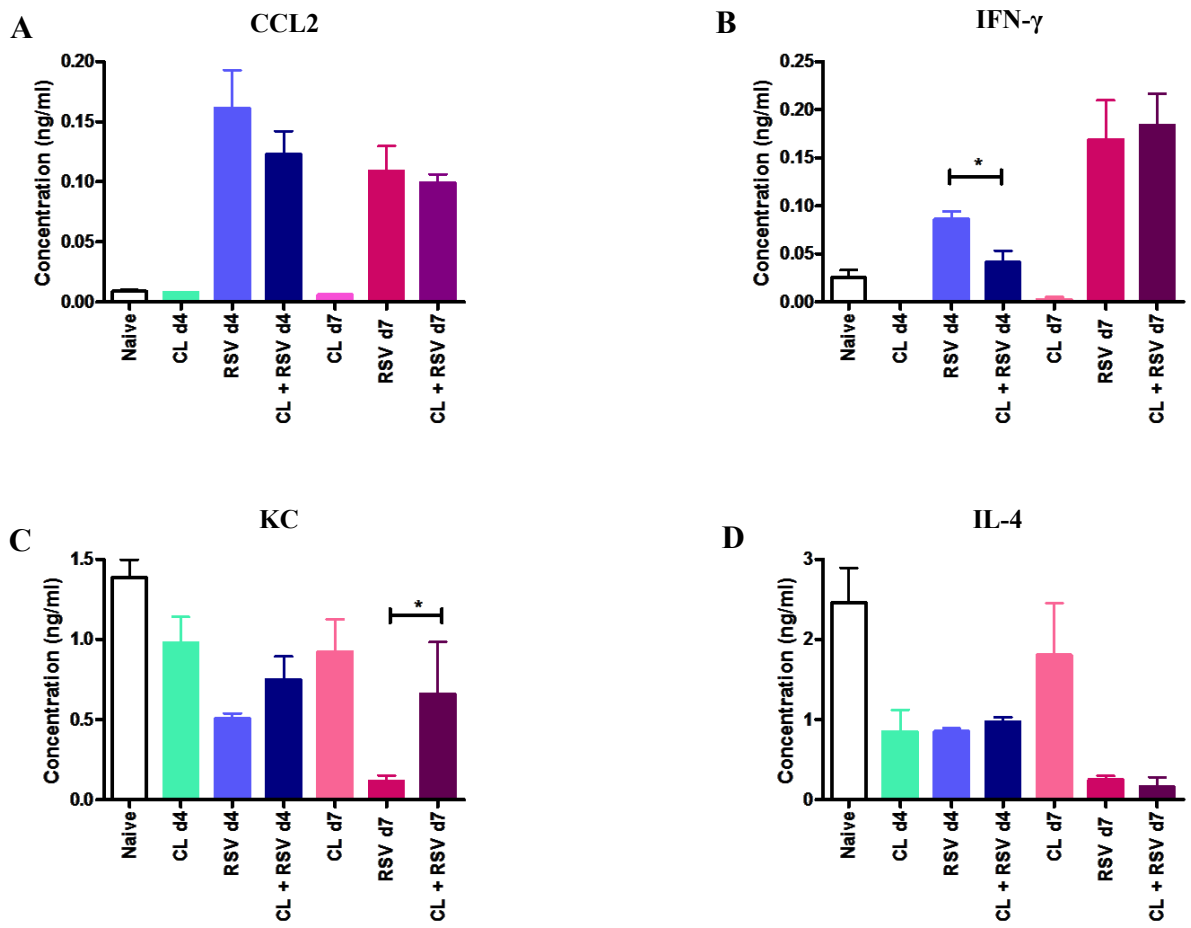


Fig. 33: Clodronate liposomes treatment reduced IFN-γ production at day 4 after RSV infection BAL supernatant obtained from BALF were used to measure cytokines and chemokines levels. CCL2 (A), IFN-γ (B), KC (C) and IL-4 (D) levels in BAL supernatant were measured by ELISA. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$; data are otherwise not significant)

Clodronate liposomes treatment with RSV infection altered the numbers of some cell populations and not the others compared to RSV infection alone. Cytokine and chemokine levels were measured by ELISA to determine the effect of CL. Amongst the cytokines and chemokines measured were CCL2, IFN-γ, KC and IL-4.

CCL2, also known as the monocyte chemo-attractant protein-1 (MCP-1) is the ligand to the CC-chemokine receptor 2 (CCR2), a receptor found on monocytes. KC or CXCL1 is a chemokine that has neutrophil chemo-attractant capacity. IFN-γ is a cytokine that is pivotal for viral clearance as it is able to directly inhibit viral replication. IL-4 is a cytokine that is associated with Th2 immunity whereby it can induce the differentiation of naïve T-helper cells to Th2 cells which antagonistically reduce the production of Th1 cells and Th1 response associated cytokine like IFN-γ.

NK cells have been known to be the main producer of IFN-γ early in RSV infection. CL treatment reduced IFN-γ production (Fig 33B) during RSV infection compared to RSV infection alone and this correlates negatively with increase in NK cell number but correlated positively with the reduced viral load seen at day 4 post infection. CL administration during RSV administration significantly increased the level of KC compared to RSV infection alone at day 7 which did not correlate positively with either the flow cytometry data or in cell differential count (Fig 33C). CCL2 (Fig 33A) and IL-4 (Fig 33D) however, did not show significant changes in production between the two groups at both day 4 and day 7 post infection.

5.14 Summary of Chapter 5

Intravenous treatment with CL depleted the blood monocytes. Total cell number in the blood decreased significantly with depletion of blood monocytes during RSV infection compared to RSV infection alone. However, there was an increase in the number of NK cells seen in the airway. In the airway, there was a decrease in the $CD11b^{pos}$ $CD11c^{int}$ cell (recruited monocytes) number but the $CD11c^{pos}$ cells (alveolar macrophages) were not affected. Similar observations were seen in the lung except that there was a significant increase in the $CD11b^{pos}$ $CD11^{neg}$ cells (tissue/ interstitial macrophages), suggesting that depletion of blood monocytes may influence proliferation of the interstitial macrophages population in the lung during RSV infection.

Differential counts of cells in the airway at day 7 showed a significant decrease in neutrophil cells number and this correlates negatively with the increase in the level of KC or CXCL1 in the BAL supernatant with blood monocyte depletion compared to RSV infection alone. Depletion of blood monocytes significantly increased the NK cell number in the airway and reduced the viral load, but did not correlate with the reduction in IFN- γ level in the BAL supernatant. However, the significant decrease of IFN- γ correlated positively with the reduction in weight loss. This suggests that depletion of blood monocytes may have a protective effect against RSV disease.

The results and findings from the study in this chapter are summarised in Figure 34.

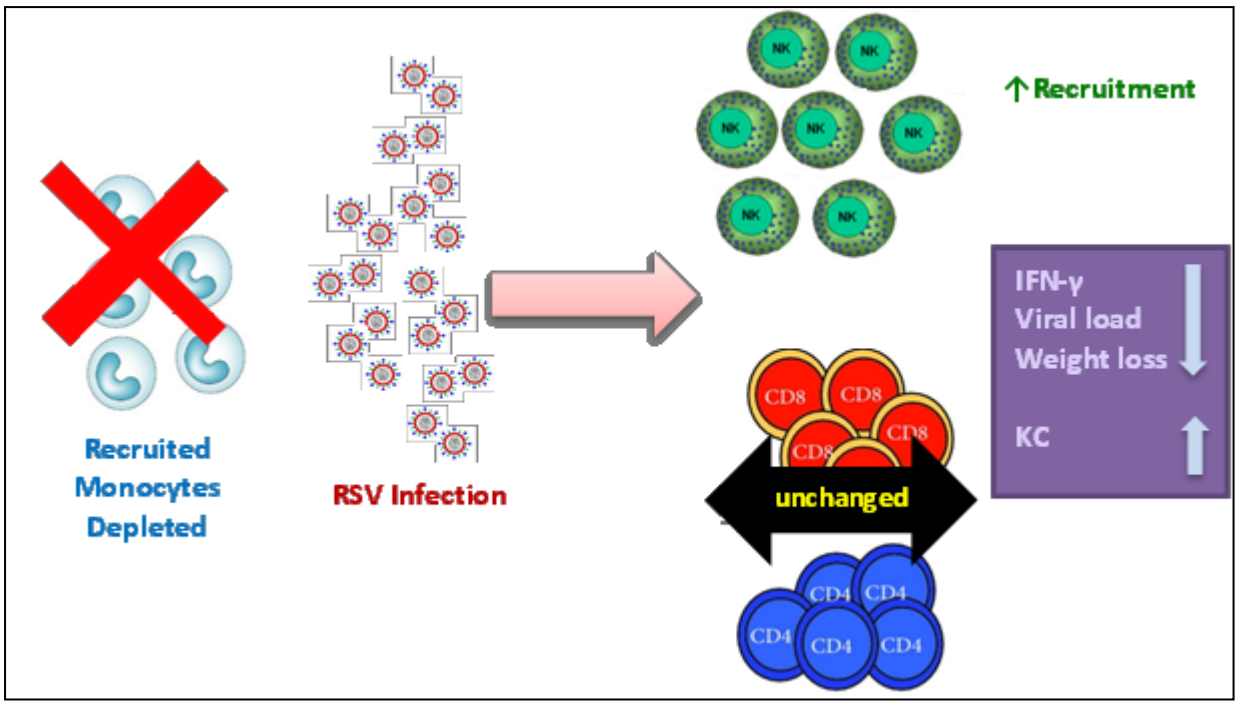


Fig 34 Summary figure of the results of recruited inflammatory monocyte depletion results in RSV infection Mice were treated with CL i.v. prior to i.n. infection with 100 µl/mouse of 8.85 x 10^5 pfu/ml of RSV. CCL2, IFN-γ, KC and IL-4 levels in BAL supernatant were measured by ELISA. Production of IFN-γ, viral genes and weight loss showed significant decrease. NK cell recruitment increased significantly in the airway but the percentages and number of CD4 and CD8 were not affected.

6.0 MACROPHAGE DIFFERENTIATION AND RSV IMMUNITY

6.1 Introduction

Macrophages can be classified based on their phenotype after activation. Macrophages differentiate along diverse lineages depending on the stimuli and the environment that they are exposed to (Stein and Keshav, 1992). A Th1 environment polarizes macrophages to an M1 phenotype, whilst Th2 and regulatory immunity give rise to M2 and "regulatory" macrophage phenotype respectively (Gordon, 2003; Gratchev et al., 2006; Stein and Keshav, 1992). Such differentiated macrophages have been identified using several methods; i.e. gene expression, intracellular and surface markers. (Gordon, 2003; Shirey et al., 2010).

It is plausible that macrophage differentiation plays a key role in determining the immunological phenotype of the anti-RSV response. The study described in this chapter provides preliminary findings on polarisation of macrophages, their differentiation and activation, using a recombinant RSV that abundantly expresses IL-4 to determine the effect on the outcome of RSV infection.

6.2 Aims

a) To determine phenotype markers on macrophages (i.e.: M1 vs M2) in mice challenged with RSV-WT vs rRSV-IL-4
b) To determine whether M1 vs M2 macrophages can alter anti-RSV responses *in vivo*.

6.3 Rationale of Study

One of the aims of this work is to polarise macrophages into M1 and M2 phenotypes by creating 'Th1' and 'Th2' environment during RSV infection. In the murine model of RSV, the immune response is normally Th1-biased. However, Th2 immunity has been associated with enhanced RSV disease in human (Hacking and Hull, 2002; Ogra, 2004).

To generate a 'Th2' response strong enough to enable macrophage differentiation to an M2 phenotype, a recombinant RSV expressing IL-4 was used to infect mice *in vivo*.

6.4 Construction of IL-4-expressing RSV

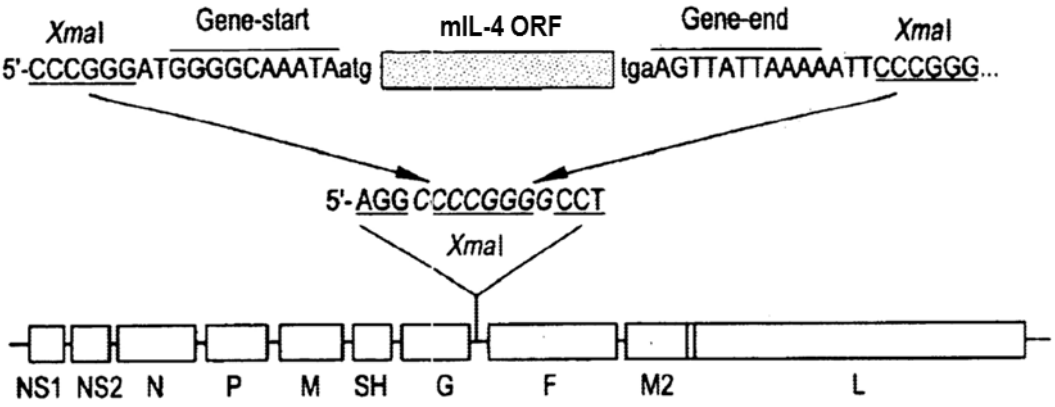


Fig 35: Construction of mIL-4-expressing RSV. Murine IL-4 (mIL-4) ORFs were modified into a RSV-specific gene which contains RSV gene sequences with *Xma*I sites flanked on each end. The *Xma*I-*Xma*I fragment was inserted between the G and F genes. Recombinant RSV expressing IL-4 was propagated in Hep-2 cells **(Adapted from Bukreyev *et al*, J Virol 1996; 70:6634-6641)**

The rRSV-IL-4 use in this study was grown from a stock batch available in the laboratory, originally a kind gift *from* Alex Bukreyev. The cDNA of a complete ORF of mIL-4 was modified by PCR to fit the gene-start and gene-end of the RSV-specific genome. This is the regulatory transcriptional sequences of the 5' and 3' end of the ORF respectively, which was then flanked by *Xma*I sites to produce an IL-4 transcriptional cassette. This cassette was inserted between the G and F genes (Bukreyev et al., 1996).

Gene insertion of the transcriptional cassette results in cytokine production by the virus during replication. However, this results in lower production of F, M2 and L proteins due to transcriptional polarity of the genome downstream from the site of insertion. Previously in this lab, Harker et al showed that co-expression of IL-4 by RSV using the rRSV-IL-4 promoted defective T-cell response (Bukreyev et al., 1996; Harker et al., 2010a).

6.5 IL-4 expression alters RSV disease

During primary infection with rRSV-IL-4 done previously in this lab, production of IL-4 is greatest at day 2 post infection but declines by day 4, further decreasing at day 8 and 12 post infection. Secondary challenge with WT RSV after priming with rRSV-IL-4 does not result in significant weight loss, but causes a reduction in total cell and lymphocyte percentages (Harker et al., 2010b).

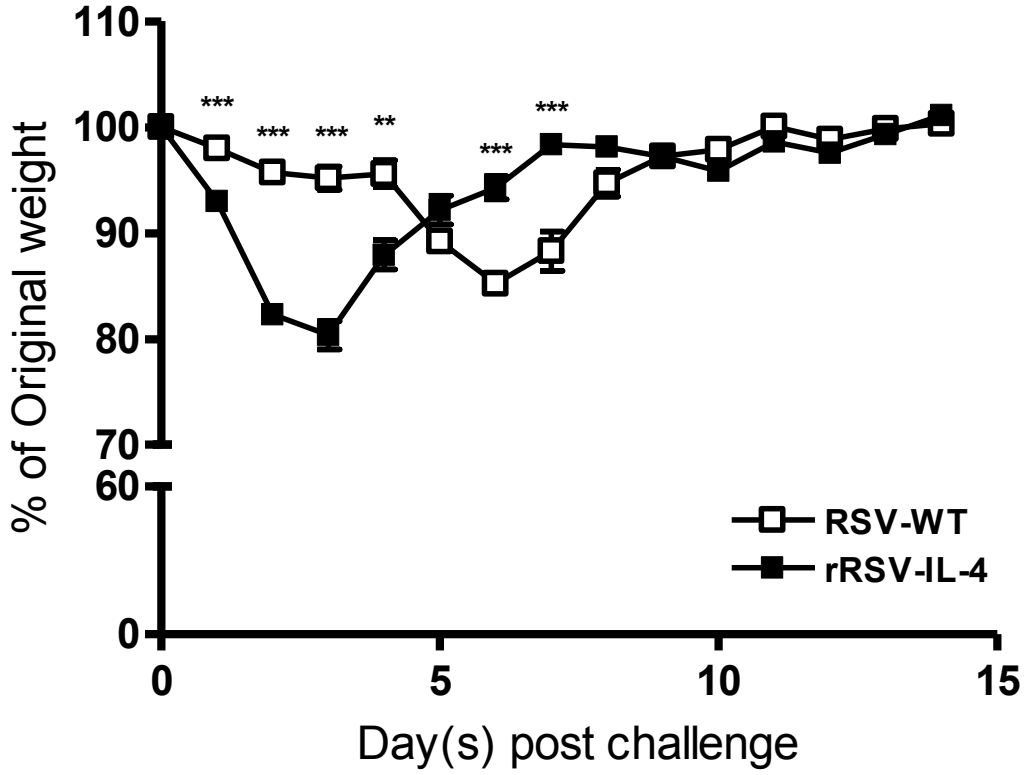


Fig 36: IL-4 expression by rRSV-IL-4 alters pathology in RSV disease. Mice were infected via the intranasal route with either RSV-WT or rRSV-IL-4. The weight of these mice was monitored for 15 days to obtain the percentage of the original weight. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$, *** = $p < 0.001$; data are otherwise not significant)

Disease severity in the murine model of RSV infection is marked by weight loss as previously mentioned in chapter 5. Mice were infected with rRSV-IL-4 and RSV-WT. Infection with RSV-WT induced the normal weight loss curve seen in a typical RSV infection in mice. However, IL-4 expression from infection with rRSV-IL-4 alters the weight loss curve. Weight loss was seen as early as day 1 post infection, peaked at day 4 post infection and started to recover at day 5 post infection (Fig 36) compared to peak of weight loss at day 6 post infection with RSV-WT. This suggests that RSV immune responses are affected by transgenic expression of IL-4.

6.6 IL-4 expression did not influence total cell number in the airway at day 7 post infection

Changes seen in weight loss are normally associated with changes in cell recruitment into the lung. In this study, day 7 post infection was chosen as a time point for a first look on the effect of IL-4 overexpression by rRSV-IL-4. Day 7 post infection is the peak of disease severity in normal murine model of RSV-WT infection. Typically, at day 7 post RSV-WT infection, cells were seen recruited into the lungs (Openshaw and Tregoning, 2005). Therefore, to assess the effects of IL-4 overexpression on RSV immunity, day 7 is believed to be the best time point to use.

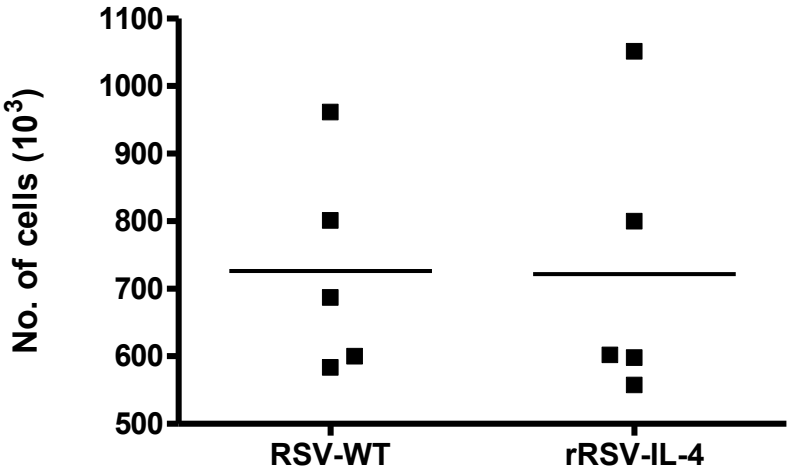


Fig 37: IL-4 expression did not change cell number in the airway 7 days after RSV infection. BALF was collected at day 7 post infection. Total cell number in the airway (BAL) was determined using flow cytometry. (n=5 mice per group; values represent ± SEM; data is otherwise not significant)

BALF was collected at day 7 post infection and single live BAL cells were measured by flow cytometry. At day 7, there were no significant changes seen in total cell number between the group infected with rRSV-IL-4 and RSV-WT in the airway.

6.7 IL-4 expression increased F4/80^{pos} cell activation in the airway

The main focus of this study was activation and differentiation of macrophages after infection with rRSV-IL-4 in comparison to RSV-WT. Although IL-4 expression during RSV infection at day 7 did not change total cell number in the airway, F4/80^{pos} cells in the group infected with rRSV-IL-4 had a higher MHCII expression measured in median fluorescence intensity (MFI) compared to the group infected with RSV-WT (Fig 38A).

MHCII is a cluster of cell surface molecules that presents antigen to CD4 T cells. MHCII is primarily expressed on APCs such as macrophages, DCs and B cells. MFI of the MHCII on the surface of F4/80^{pos} cells (herein referred to as macrophages) was measured. In mice infected with rRSV-IL-4, there was a significant increase in the MFI compared to mice infected with RSV-WT, suggesting an alteration in activation of macrophages at day 7 post infection in the airway. Such an increase in expression of MHC II molecule on macrophages is associated with classical activation of these cells, which normally occur in a Th1 immune environment.

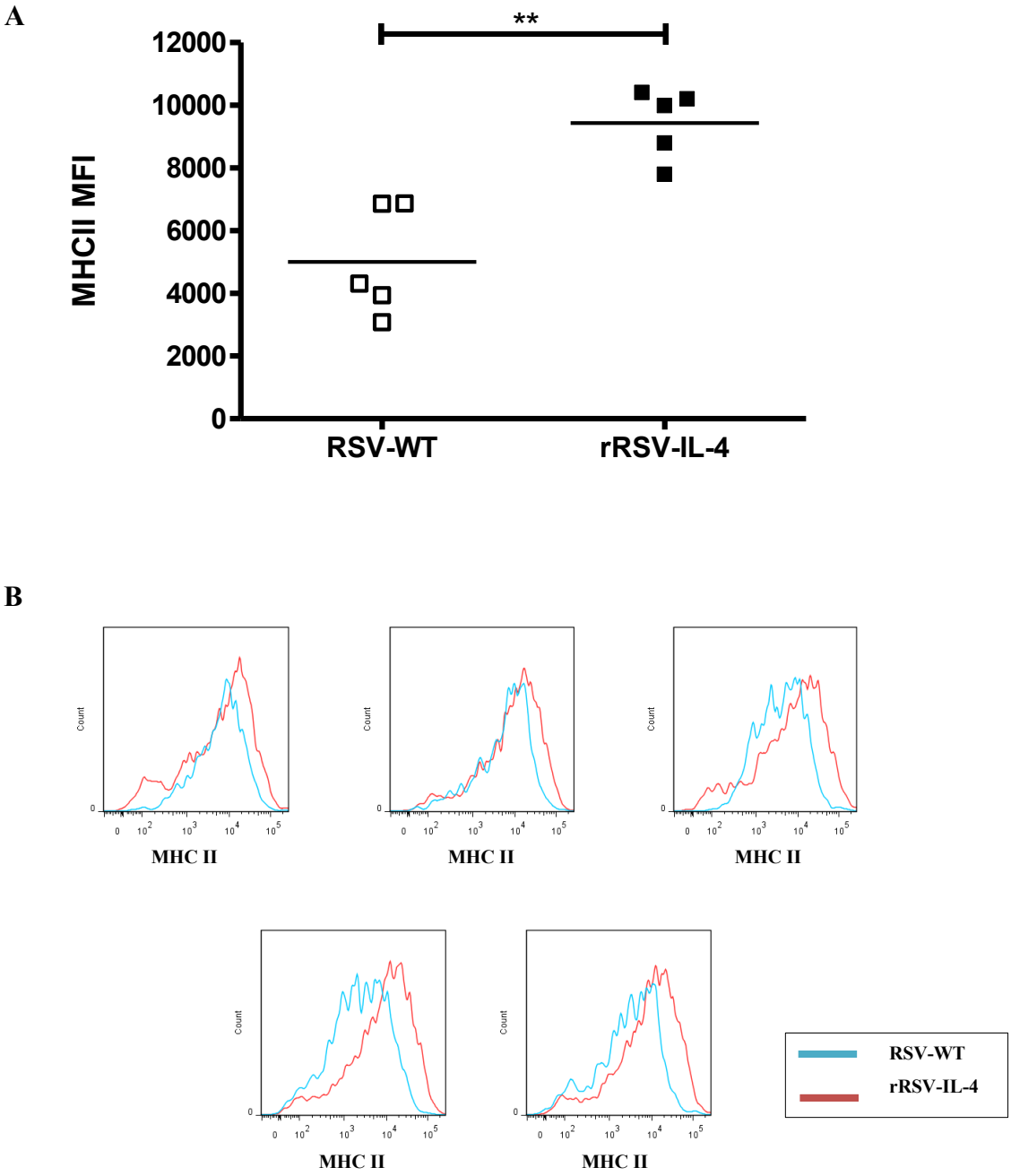


Fig 38: IL-4 expression increased F4/80^{pos} cell activation in the airway 7 days after RSV infection. The MHCII expression on F4/80pos BAL cells based on MFI. Total cell number in the airway (BAL) was determined using flow cytometry (A). Histogram representing the MFI of MHC II expression between the group infected with rRSV-IL-4 and the group infected with RSV-WT (B). (n=5 mice per group; values represent ± SEM, * = p <0.05, ** = p <0.01, *** = p <0.001; data are otherwise not significant).

6.8 BAL CD4^{pos} T cell recruitment increased with rRSV-IL-4 infection on day 7 post challenge

Macrophage activation during inflammation and/or infection leads to T-cell activation and recruitment. Macrophages stimulate naïve T-cells and not only effector cells. T-cell maturation and activation in return activates macrophages. More often, bone marrow-derived DCs are the only cell type that is able to prime T-cells.

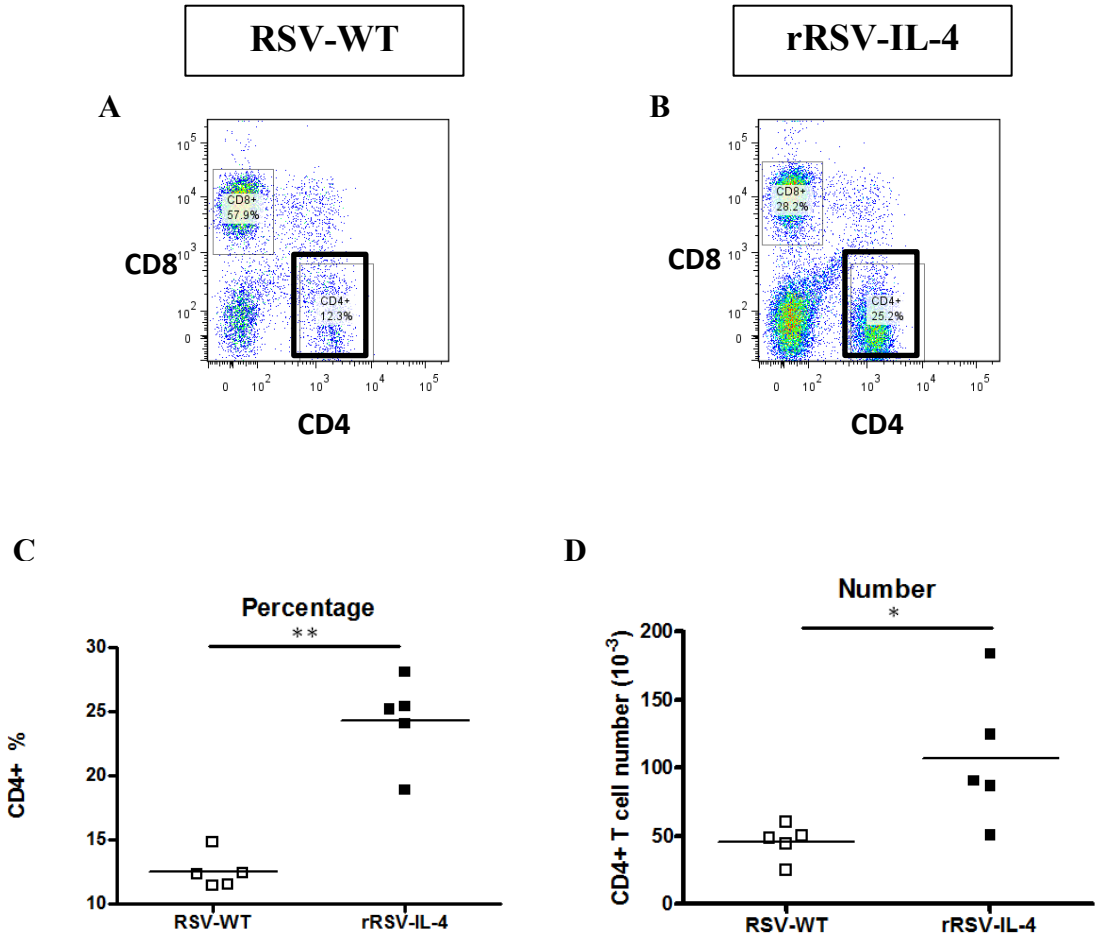


Fig 39: IL-4 expression increased CD4^{pos} T-cell percentage and number in the airway 7 days after RSV infection. CD4^{pos} T-lymphocytes in airway of mice infected with RSV-WT (30A) and rRSV-IL-4 (30B) were gated on live lymphocyte populations on forward and side scatter. The percentage (30C) and number (30D) of CD4^{pos} T-lymphocytes in the airway at day 7 post challenge was determined using flow cytometry. (n=5 mice per group; values represent ± SEM, * = $p < 0.05$, ** = $p < 0.01$; data is otherwise not significant)

From the measurement of $CD4^{pos}$ T-cell percentage (Fig 39C) and number (Fig 39D) in the airway at day 7 post infection, the significant increase in the proportion of CD4 T cells seen in the group infected with rRSV-IL-4 suggests that IL-4 co-expression during RSV infection may be skewing RSV T-cell immunity.

6.9 CD69 expression on CD4^{pos} T cells decreased with rRSV-IL-4 infection

CD69 is one of the earliest cell surface antigens expressed by T-lymphocytes. It is rapidly up-regulated on mature T-cells via TCR stimulation by pro-inflammatory stimuli (Yamashita et al., 1993). ICOS, also known as the inducible co-stimulatory molecule is mostly on activated T-cells. This molecule is widely accepted to be associated with Th2 cells (Dong et al., 2001; Rudd and Schneider, 2003). It is also has been shown to have importance in T-cell proliferation (Hutloff et al., 1999).

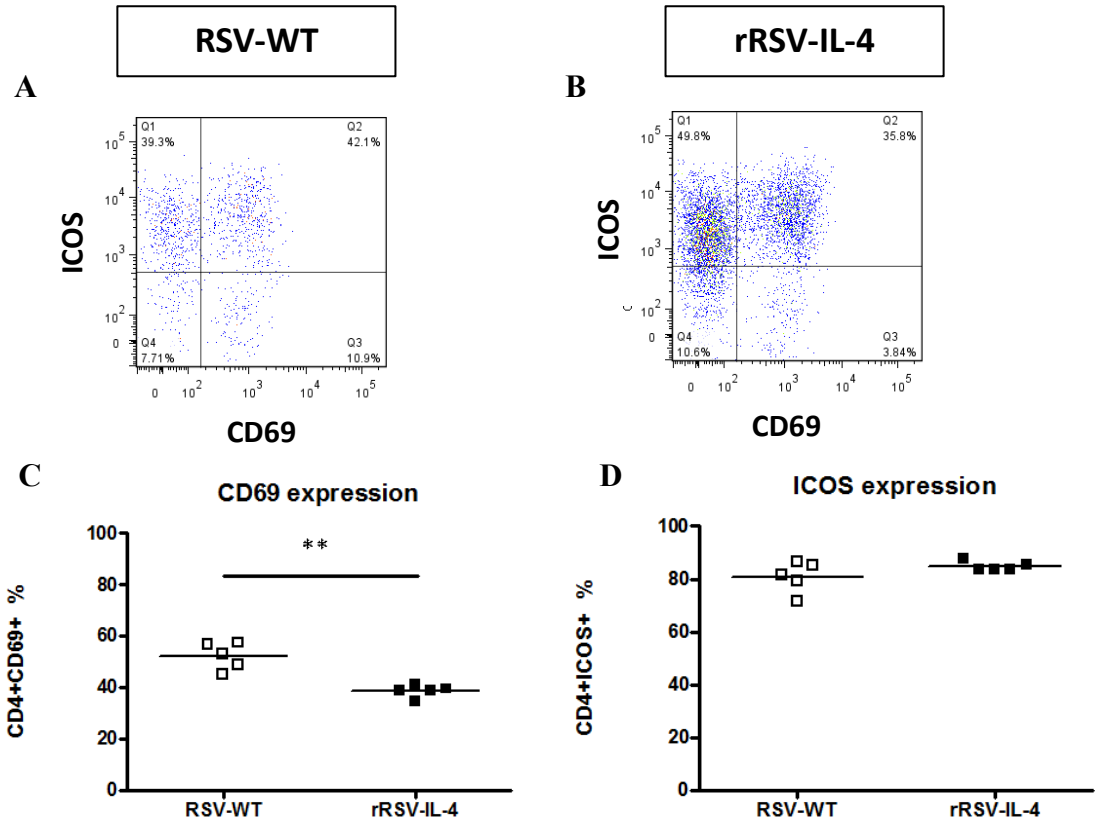


Fig 40: IL-4 expression reduced CD69 expression on CD4^{pos} T cells in the airway 7 days after RSV infection. CD69 and ICOS expression on CD4^{pos} T-cell were measured at day 7 post infection using flow cytometry. Airway cells of mice were infected with RSV-WT (A) and rRSV-IL-4 (B) were were gated on live CD4^{pos} lymphocyte populations. The percentage (C) and number (D) of CD4^{pos} T-lymphocytes in the airway at day 7 post challenge was determined using flow cytometry. (n=5 mice per group; values represent ± SEM, * = p <0.05, ** = p <0.01, *** = p <0.001; data are otherwise not significant)

To determine whether co-expression of IL-4 with RSV has any effect on the activation of $CD4^{pos}$ T-cell, CD69 and ICOS expression on $CD4^{pos}$ T-cell were measured.

Although IL-4 co-expression with RSV caused a significant increase in $CD4^{pos}$ T-cell number and percentage in the airway at day 7 post challenge, CD69 expression on these cells was significantly reduced with rRSV-IL-4 infection (Fig 40C). However, ICOS expression (Fig 40D) between the group infected with RSV-WT and rRSV-IL-4 did not change and remained high on $CD4^{pos}$ T-cells. This suggests that IL-4 expression may increase $CD4^{pos}$ T-cell number and percentage in the airway but may not necessarily induce activation nor does it suggest a Th2-biased response.

6.10 CD8 T cell recruitment decreased with rRSV-IL-4 infection

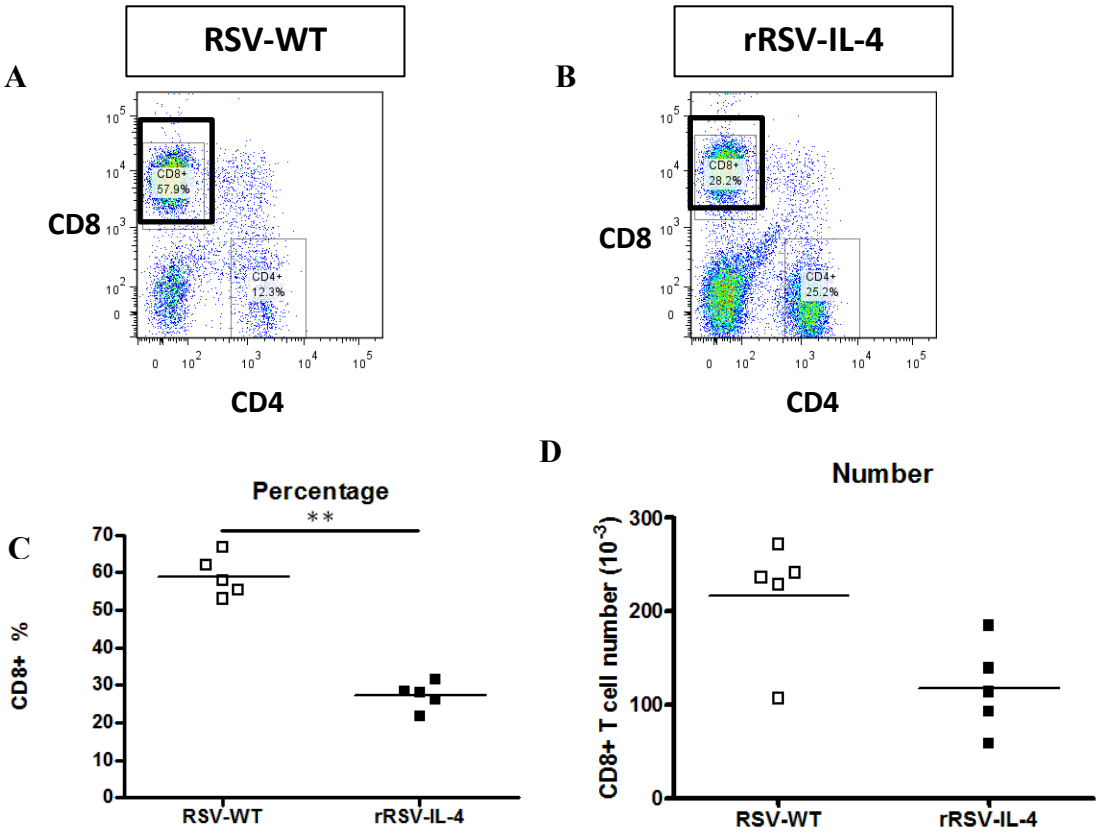


Fig 41: IL-4 expression decreased CD8^{pos} T-cell percentage in the airway 7 days after RSV infection. CD8^{pos} T-lymphocytes in airway of mice infected with RSV-WT (A) and rRSV-IL-4 (B) were gated on live lymphocyte populations on forward and side scatter. The percentage (C) and number (D) of CD8^{pos} T-lymphocytes in the airway at day 7 post challenge was determined using flow cytometry. (n=5 mice per group; values represent ± SEM,* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; data are otherwise not significant)

The percentage and number of $CD8^{pos}$ T-cells were determined in the airway at day 7 post challenge. $CD8^{pos}$ T-cell percentage (Fig 41C) and number (Fig 41D) were measured in the airway at day 7 post infection and a significant reduction in percentage was seen in the group infected with rRSV-IL-4 compared to RSV-WT which correlates negatively with the increase in $CD4^{pos}$ T-cell percentage. Although there seems to be a significant decrease in $CD8^{pos}$ T-cell number, statistical analysis showed no significance due to an outlier sample.

Nevertheless, the negative correlation between the two conventional T-cells supports the possibility of the T-cell immunity has been modified with rRSV-IL-4 infection.

6.11 ICOS expression on CD8 T cells decreased with rRSV-IL-4 infection

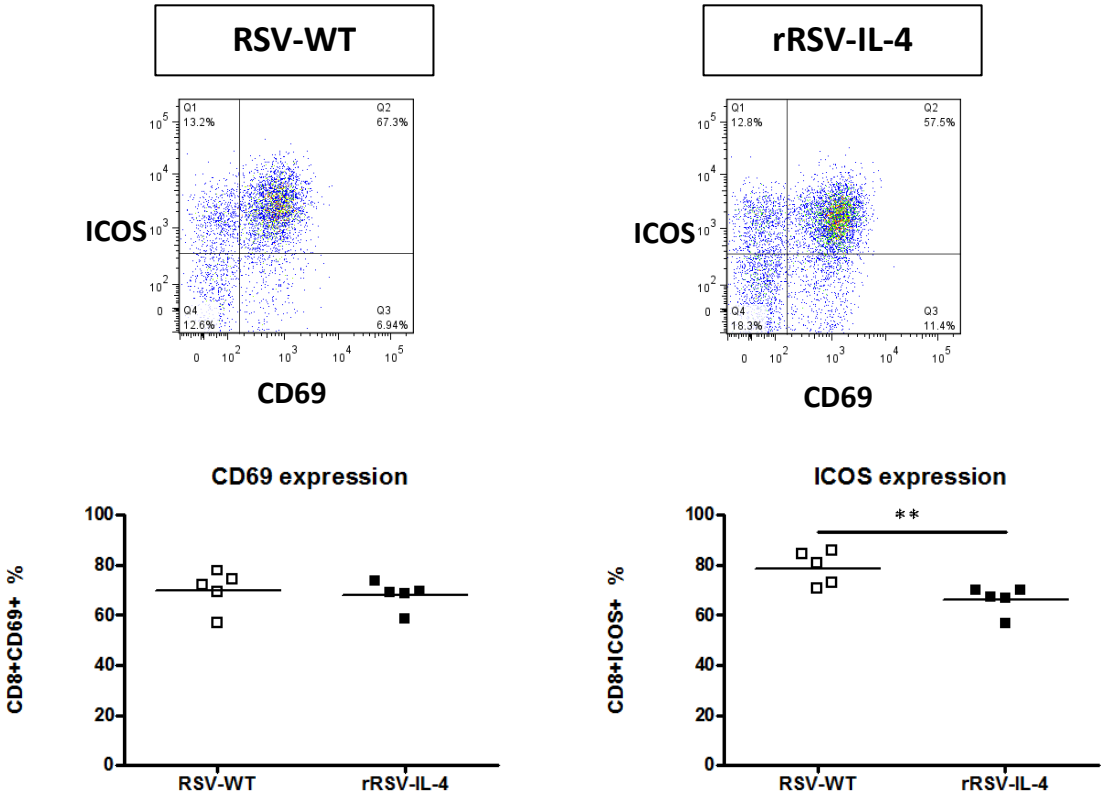


Fig 42 IL-4 expression reduced ICOS expression on CD8^{pos} T cells in the airway 7 days after RSV infection. CD69 and ICOS expression on CD8^{pos} T-cell were measured at day 7 post infection using flow cytometry. Airway cells of mice were infected with RSV-WT (A) and rRSV-IL-4 (B) were gated on live CD8^{pos} lymphocyte populations. The percentage (C) and number (D) of CD8^{pos} T-lymphocytes in the airway at day 7 post challenge was determined using flow cytometry. (n=5 mice per group; values represent ± SEM, * = p <0.05, ** = p <0.01, *** = p <0.001; data are otherwise not significant)

To determine whether co-expression of IL-4 with RSV has any effect on the activation of $CD8^{pos}$ T-cell, CD69 and ICOS expression on $CD8^{pos}$ T-cell were measured.

CD69 expression on $CD8^{pos}$ T-cells did not change between the groups. However, ICOS expression decreased significantly on $CD8^{pos}$ T-cells in the group infected with rRSV-IL-4. IL-4 expression did not alter $CD8^{pos}$ T-cell activation in the airway at day 7 post challenge. However, the decrease in ICOS expression on $CD8^{pos}$ T-cells correlates positively with the $CD8^{pos}$ T-cell percentage. These data suggests that ICOS induction may be of importance for $CD8^{pos}$ T-cell proliferation in RSV infection and co-expression IL-4 may have an effect on $CD8^{pos}$ T-cell maturation and proliferation rather than the possibility of Th2-skewed response.

6.12 Neutrophils dominate the granulocyte population recruited into the airway post challenge

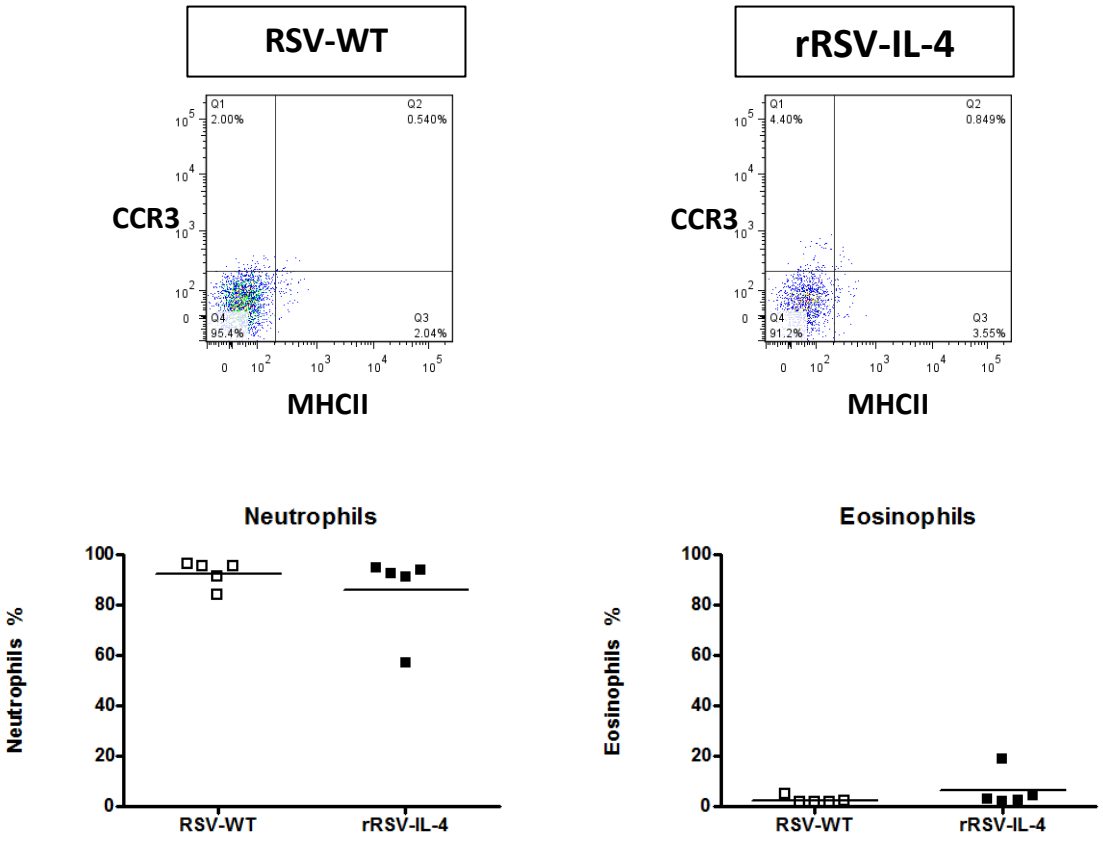


Fig 43: Neutrophils were the predominant granulocyte population in the airway 7 days after RSV infection. Granulocyte populations in the airway (BAL) were determined using flow cytometry. Cells were gated on single live cells. Neutrophils are $CCR3^{neg}$ $MHCII^{neg}$ cells and eosinophils are the $CCR3^{pos}$ cells. (n=5 mice per group; values represent ± SEM, * = p <0.05, ** = p <0.01, *** = p <0.001; data is otherwise not significant)

Neutrophils dominated granulocyte population recruited into the airway in both groups post challenge. Although the percentage was expected to be higher in the group infected with rRSV-IL-4 compared to RSV-WT, eosinophils remained low in both groups at day 7 post infection.

6.12 Summary of chapter 6

The main aim of this work is differentiate macrophages into an alternative phenotype, M2 and to observe any alterations in RSV disease *in vivo*. Recombinant RSV viruses that produce IL-4 (rRSV-IL-4) during viral replication were used. Expression of IL-4 with RSV infection altered the severity of the disease with an earlier weight loss compared to the normal curve seen in RSV-WT infection but did not affect total cell number in the airway.

The effects of IL-4 expression on various cells in the airway gave an impression of Th2 immunity, with significant increases in macrophage activation, $CD4^{pos}$ T-cell percentage and number, and reduction in $CD8^{pos}$ T-cell percentage. However, a significant decrease in $CD4^{pos}$ T-cell activation was seen and ICOS expression on $CD4^{pos}$ T-cells remained unchanged. Also, there were no significant changes seen in percentage of granulocyte populations in the airway.

To conclude, it seems that IL-4 expression in RSV infection may have only impaired the Th1 and the cytotoxic T-cell response rather than skewing the response to Th2.

7.0 DISCUSSION AND FUTURE WORK

RSV is the major cause of viral infantile bronchiolitis (McNamara and Smyth, 2002; Ogra, 2004). In recent years, RSV infection has also been shown to cause severe disease in the elderly and immunocompromised patients (Falsey et al., 2005; Habibi and Openshaw, 2012). Although symptoms and clinical outcomes of the disease vary between individuals, immunity to RSV does not provide solid protection against re-infection and recurrent infections with RSV strains of overlapping antigenicity occur throughout life (Glezen et al., 1986; Ogra, 2004). Currently, there is no vaccine against RSV but there are specific antiviral drugs available to treat those infected with it (Nair et al., 2010). Although Ribavirin is the only licensed RSV antiviral to date, there are very little evidence available to demonstrate its efficacy in bronchiolitis (Hall et al., 1983a; Hall et al., 1983b). It is strongly believed that lowering the viral load results in a reduction in clinical disease. The use of Palivizumab, an anti-RSV-F monoclonal antibody has shown to reduce viral load in ventilated infants with bronchiolitis but was not effective as a treatment in established RSV disease (Malley et al.,1998)

RSV disease has for some time been thought to be caused by excessive inflammation, resulting from immunological events triggered by infection (Kimpen and Simoes, 2001) . Upon infection, pro-inflammatory mediators are released which in turn recruit and activate inflammatory cells into the airway and lung (Openshaw and Tregoning, 2005; Tregoning and Schwarze, 2010). It is believed that innate immune responses in many respiratory diseases profoundly influence adaptive immunity (Braciale et al., 2012). Epithelial cells, macrophages, monocytes, DCs, γδ T-cells, NKT cells, and NK cells have crucial roles in this linked set of responses.

The work in this project focused primarily on innate immunity in RSV. Typically, during the early phase inflammation and/or infection, polymorphonuclear cells, and monocytes are amongst the first to get recruited into the site of inflammation. Since this study is focused on the lungs, macrophages and monocytes are the cells of particular interest. Macrophages are especially important because they are one of the first cells that encounter RSV in infection and can be infected by RSV (Midulla et al., 1993; Panuska et al., 1992). However, they are not the favoured cells to be infected compared to the airway epithelial cells, which are the initial site for the activation of immune response upon infection (Groskreutz et al., 2006; Liu et al.,2007). Macrophages and monocytes are significant sources of cytokines and chemokines (Gordon and Read, 2002). They also have the ability to activate multiple other cell types. However, they are understudied in the context of RSV infection.

In this project, we hypothesized that different pulmonary macrophage and monocyte populations have functionally distinct roles in regulating inflammation and pathology in RSV infection. We aimed to characterise different pulmonary macrophage population after RSV and to determine the roles of pulmonary monocytes/macrophages in terms of viral clearance, induction and resolution of inflammation.

7.1. Characterisation of recruited inflammatory monocytes and resident macrophages in RSV infection

In this project, the first aim was to establish a flow cytometry staining panel for characterization of macrophages and monocytes from naïve and infected lungs, and to distinguish resident from recruited cells. There many ways to characterise macrophages and monocytes phenotypically, and diverse phenotypes are associated with various functions.

Resident macrophages can be distinguished using PKH26PCL dye (Buntinx et al., 2008; Garn, 2006; Maus et al., 2001; Maus et al., 2003; Murphy et al., 2008; Silva et al., 2011), which remains stable in the AMs for at least 21 days (Murphy et al., 2008) and confirmed by my own studies. The dye was most stable in AMs compared to the other monocytic sub-populations. Another method of labelling that I tried was CFSE, another fluorescent staining dye. CFSE is mainly used in studies of lymphocytes and to monitor the proliferation and migration of T-cells (Carson and Wilson, 2013; Parish et al., 2009). Although the staining worked well on monocytes and macrophages, CFSE labelling was not able to provide a distinct difference between the resident and recruited populations. However, PKH26PCL has to be internalised by the cells and this may activate the macrophages; this was not therefore the ideal way to identify resting macrophages.

Other studies have used different combinations of surface markers to define lung macrophages and/or monocytes (Bedoret et al., 2009; Jenkins et al., 2011; Landsman and Jung, 2007; Shirey et al., 2010). In this study, it was found that the best way to distinguish recruited from resident macrophages is by using surface markers, which I therefore adopted for my studies and found that that the best markers were F4/80, CD11b and CD11c, and Ly6C was used to identify inflammatory monocytes. Resident AMs are $F4/80^{pos}$ $CD11b^{neg}$ $CD11c^{pos}$ and were $Ly6C^{neg}$ throughout the time course of infection, tissue monocytes were defined as $F4/80^{pos}$ $CD11b^{pos}$ $CD11c^{neg}$.

7.2 Recruitment of inflammatory monocytes to site of inflammation in RSV

Inflammatory monocytes, sometimes known as exudate macrophages are the $Ly6C^{hi}$ innate cells that are recruited during inflammation, and which can be responsible for disease pathology (Daigneault et al., 2012; Lin et al., 2008; Lin et al., 2009; Maus et al., 2001).

Ly6C^{pos} monocytes can express either high or low Ly6C on their surfaces (Geissmann et al., 2003; Lin et al., 2009; Shi and Pamer, 2011), but the most predominant subset during inflammation are of the Ly6C^{hi} phenotype.

In a time course of RSV infection up to day 7 post challenge, F4/80^{pos} CD11b^{pos} CD11c^{int} Ly6C^{hi} cells were seen in the airway. The number of these cells was highest at day 2 post infection in both airway and lung, but this was more apparent in the airway.

7.3 The importance of recruited inflammatory monocytes and RSV immunity

The importance of inflammatory monocytes has been demonstrated in many infectious diseases and non-infectious injuries like cigarette-induced lung emphysema (Lin et al., 2009; Xiong et al., 2011). They have been shown to be responsible for the pathology of HSV-1, pneumococcal bacterial infection and influenza (Conrady et al., 2013; Daigneault et al., 2012; Lin et al., 2008) and inflammation. Antibody blockade (Rosen et al., 1989) or the use of CCR2 KO mice (Kurihara et al., 1997; Lin et al., 2008; Wareing et al., 2007), which lack CCR2 expression in many cases leads to increased infection rate, reduced pathogen clearance, increased mortality rate and reduced recruitment of Ly6C^{hi} monocytes to infected tissues. However, the role of these inflammatory monocytes in RSV disease is not fully understood. Therefore, the focus of this study was to investigate the importance of recruitment of inflammatory monocytes in RSV-induced inflammation and immunity.

7.4 The role of recruited inflammatory monocytes in RSV immunity

Several methods were considered to demonstrate the role of macrophages in RSV disease. $CCR2^{-/-}$ mice (Kurihara et al., 1997; Lin et al., 2008; Wareing et al., 2007) and $CX3CR1^{GFP/+}$ mice (Gundra et al., 2014) have been used in other models. $CX3CR1^{GFP/+}$ mice would be a better option in this study, whereby the chemokine receptor CX3CR1 is expressed predominantly on monocytes, particularly the inflammatory subsets. However, these mice were not available to us.

Receptor blockade is another method that has been used in some studies. Blocking antibody against CD11b (Rosen et al., 1989) has been used in a bacterial infection model. Again, using the CD11b blocking antibody would block receptors of other CD11b expressing cells and would complicate the interpretation of results. Therefore, an invasive method to deplete monocytes was chosen using clodronate liposomes (CL) or dichloromethylene-bisphosphonate (Cl2MBP) (Van Rooijen and Sanders, 1994).

CL were previously used in the laboratory and administered intranasally to deplete macrophages in the lung (Pribul et al., 2008). Depending on the route of administration, such liposomes deplete different monocyte and macrophage subsets. Intraperitoneal administration depletes peritoneal macrophages (Biewenga et al., 1995) and intravenous injection depletes blood monocytes (Jenkins et al., 2011).

For this study, i.v. injection of CL was used to deplete blood monocytes to address their role in RSV immunity. Depletion of blood monocytes altered RSV measured as weight loss. Mice given CL i.v. had a significantly reduced weight loss compared to normal RSV infection, but viral load was significantly reduced in the lung at day 4 post infection as measured by

reduction in L-gene expression. There was a significant increase in NK cell recruitment into the airway and this negatively correlated with the reduced level of IFN-γ. NK cells are known to be one of the main producers of IFN-γ during inflammation. However, monocyte depletion study provided contradictory results, whereby IFN-γ was reduced. This may be due to the NK cells recruited into the airway not being the cytokine-producing subset, but may be cytolitic (Reefman et al., 2010). The increased KC in the airway correlates negatively with the reduction seen in neutrophil number. However, the depletion of blood monocytes had no effect on both $CD4^{pos}$ and $CD8^{pos}$ T cell number.

I conclude that inflammatory monocytes recruited early in RSV infection influence the NK cell response and viral clearance. Depletion of monocytes may be protective against RSV disease but had no effect on T-cell activity.

The results in this study do not strongly support the hypothesis that inflammatory monocytes recruited from the bloodstream into the lung during the innate stage of infection contribute to excess inflammation during the innate stage of RSV immunity. Instead, these results suggested a protective effect towards RSV disease.

However, there are still gaps in the understanding of the mechanisms involving monocyte depletion and the suggested protective effect towards RSV disease. Depletion of monocytes early in RSV infection influenced the NK cell response. The molecules and cell-to-cell interactions involved between NK cells and monocytes still need to be identified. This study has shown that monocytes may have an influence early in RSV immunity. Although direct mechanisms to address the direct relationship between recruited inflammatory monocytes and their effect on NK cells or possibly other innate cells remain to be addressed, the findings in

this study suggest monocyte depletion may be a possible therapeutic target for RSV disease by blocking monocyte recruitment.

The findings from the studies conducted in this project are summarized in the following schematic diagram.

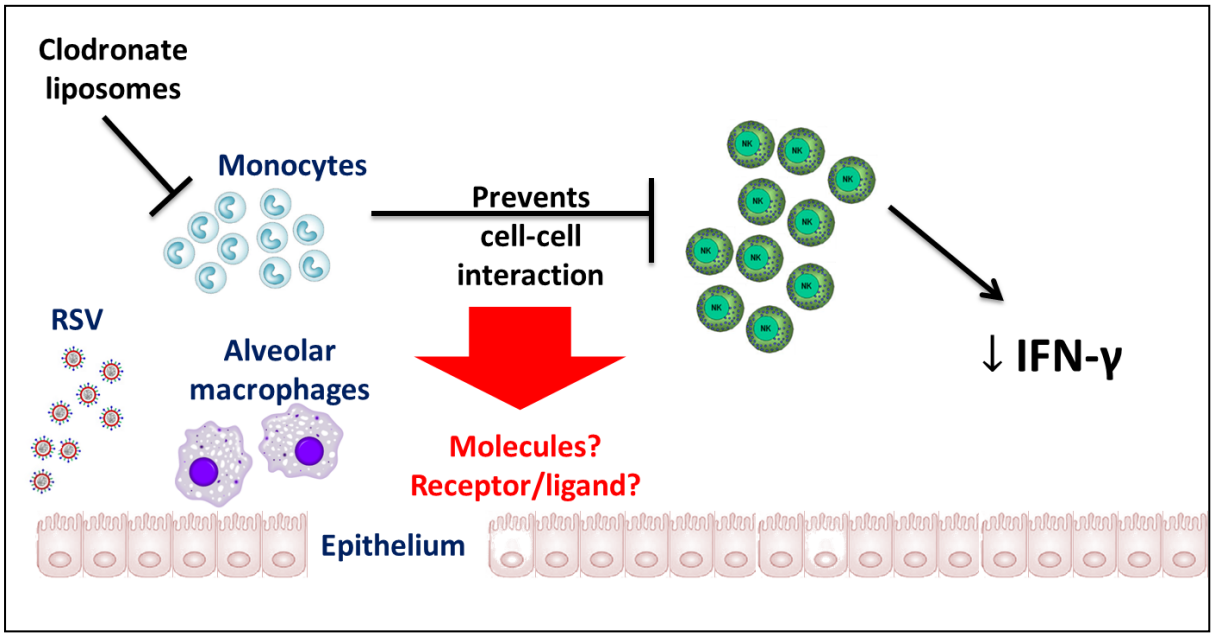


Fig 44 Possible role of inflammatory recruited monocytes in RSV infection RSV primarily infects epithelial cells and macrophages. NK cells are important for the control of viral infection. Early recruitment of inflammatory monocytes into the airway seems to have an influence towards the NK cells. Depletion of recruited monocytes may have increased the NK cell number, but reduced the level of IFN-γ which also reduced weight loss in mice.

7.4.1 Future Work

To further work on this study, a similar method of intervention should be used to determine monocyte-NK cell interaction and influence on RSV Immunity. CL will be administered via i.v route for monocyte depletion during RSV infection. The next practical investigation would be to determine the activation and the cytoxicity level of NK cells, and to measure intracellular cytokines produced by macrophages, monocytes, NK cells, NKT cells and

lymphocytes to determine the state of cell activation and source of cytokines during RSV infection with and without depletion of monocytes.

The biggest gap in this study was to demonstrate the mechanism behind the effects of depletion of monocytes early in RSV infection. One of the clear approaches is to address the existence of a crosstalk between NK cells and monocytes in which the molecules and direct and/or indirect involvement of cells in this interaction need to be determined.

In a study on myocardial infarction, cell to cell interaction between NK cells and monocytes via the T-bet/ IFN-γ/IL-12 signalling pathway leads to activation of NK cells which then promotes recruitment of inflammatory cells to the inflammation sites (Knorr et al., 2014). Although the interactions were not fully understood, findings from experimental studies lead to therapeutic possibilities in this disease.

In a human study, $CD14^{pos}$ cells that were co-cultured with NK cells collected from inflamed fluid samples and demonstrated the importance of monocytes towards IFN-γ production by NK cells *in vitro* compared to culture with NK cells alone. $CD56^{bright}$ NK cells were activated by monokines when co-cultured with $CD14^{pos}$ cells which in turn influenced production of IFN-γ (Dalbeth et al., 2004). These findings showed that monocytes have important role towards NK cell function and innate immunity.

7.5 Recombinant RSV expressing IL-4 and alternative activation of macrophages

Immunity in RSV disease has long been known to induce Th1, Th2 or cytotoxic CD8 T-cell responses depending on priming and challenge protocols. Infection with RSV-WT in a

murine model would normally elicit Th1 response (Hacking and Hull, 2002; Ogra, 2004). However, Th2 immune response can be achieved in secondary infection with RSV-WT and in some recombinant RSV model; rVV-F, rVV-G, rVV-βgal and rRSV-IL-18 (Culley et al., 2006; Harker et al., 2010a; Harker et al., 2010b; Openshaw, 1995).

Alternatively activated macrophages (AAM) or M2 have been shown to be responsible in resolution of inflammation and it is believed that they may have importance in RSV immunity. M2 macrophages are normally induced by Th2 cytokines like IL-4 and IL-13 (Gordon and Martinez, 2010; Stein and Keshav, 1992). Shirey KA and associates showed that RSV infection induces T- and B-cell-independent differentiation of M2 macrophages *in vivo* and *in vitro*. RSV infection in IL-4Rα$^{-/-}$ mice caused enhanced lung inflammation. Overall, their data demonstrated that RSV induces M2 macrophages to differentiate in response to IL-4 and IL-13, and mediated disease resolution via IL-4Rα/STAT6-, TLR4- and IFN-β-dependent signalling pathways (Shirey et al., 2010).

For my study, recombinant RSV expressing IL-4 or rRSV-IL4 was used to alternatively activate macrophage *in vivo* and to monitor the effect towards RSV immunity. From this first attempt at day 7 post infection, the data did not strongly reflect the expected Th2-biased response. The aim of the study was to polarise macrophage towards an M2 phenotype and to determine their role in RSV immunity. Although the number of CD4^{pos} T-cells increased with IL-4 expression with RSV infection at day 7 post challenge, ICOS expression on CD4^{pos} T-cells did not change and there was a significant decrease in CD4^{pos} and CD8^{pos} T-cell activation and reduction in CD8^{pos} T-cell percentage. This suggests that rRSV-IL-4 may have reduced the Th1 and cytotoxic T-cell response without the immune response being skewed towards Th2.

During acute inflammation, granulocytes are recruited in the site of inflammation. They are the most abundant type of leukocytes to respond and migrate to the site of inflammation. There is no exception in RSV disease, where neutrophils are the predominant airway leukocytes in RSV bronchiolitis (Bataki et al., 2005). Eosinophils have long been associated with Th2 immunity, particularly in helminthic infection and atopic diseases such as asthma and allergy. Although RSV disease is often claimed to be Th1-biased, there are cases in childhood bronchiolitis eliciting immune responses with the characteristics of Th2 immunity (Domachowske and Rosenberg, 1999) . Recent findings highlighted the role of eosinophils in the effector functions and initiating Th2 immune responses in RSV immunity (Spencer and Weller, 2010).

In my study, the percentages of both granulocyte types did not change between the groups infected with RSV-WT and rRSV-IL-4. This further supports the possibility that IL-4 expression in RSV may have reduced the cytotoxic T-cell response without the immune response being skewed towards Th2. This study has to be put on hold for various reasons. Our knowledge on polarization or differentiating macrophages towards an alternative phenotype is still limited, particularly in RSV disease. Also, the negative control used in this study against the recombinant RSV is not ideal. A virus construct with a similar gene modification without a cytokine expressing gene would be the best negative control for the recombinant cytokine expressing RSV.

7.6 Macrophage Polarization and RSV Immunity

Macrophages can be activated and the polarisation of the immune response is depending on the immunological environment and/or stimuli. Tissue or interstitial macrophages have been associated with alternative activation and resolution of inflammation (Johnston et al., 2012). IL-4 has long been associated as one of the stimuli that induces alternative activation of macrophages (Gordon, 2003; Gordon and Martinez, 2010; Mosser, 2003). In a 2013 study, IL-4 has been shown to have a major role in the proliferation of local tissue macrophages during a nematode infection via the IL-4Rα pathway.

In an RSV infection model, Shirey and colleagues proposed a model of alternatively activating macrophages using macrolides. These drugs are known to have the ability to polarise macrophage activation to the alternative phenotype (Shirey et al., 2014). To further the work on polarizing macrophage activation in RSV infection, these macrolides could be of use to induce alternative activation of macrophage *in vivo*. Once this alternative activation is demonstrated, identifying which population of the three; i.e. recruited inflammatory monocytes, tissue macrophages or alveolar macrophages were alternatively activated in RSV infection would be useful. Sorting these AAMs and adoptively transferring them into selected knockout mice to observe the immune response during RSV infection compared to WT-RSV would be informative.

Recently, Gundra and associates showed that the alternative activation of macrophages are distinctively different depending on the source macrophage population. They have shown that both tissue resident macrophages and recruited inflammatory monocytes can be alternatively activated. However, they found that these two populations were phenotypically different based on their transcriptional profile. Although both populations expressed high levels of

Arg-1, *Chi3l3* and *Retnla*, only inflammatory monocytes expressed high levels of *Raldh2*, PD-L2, CX3XR1 and CD206. Also, these monocytes had the ability to promote differentiation of $FoxP3^{pos}$ $CD4^{pos}$ T cells from their naïve state. In their studies, they have concluded that monocyte-derived AAM have properties that are associated with immune regulation (Gundra et al., 2014). To incorporate in this for future studies to address possible role of alternative active of macrophage/monocyte in RSV infection, a similar approach may be adopted. This could be the approach to narrow the existing gaps in the current study on the role of recruited monocytes in RSV infection.

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