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# Lung innate homeostasis and susceptibility to viral induced secondary bacterial pneumonias

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# STATEMENT OF ORIGINALITY

No part of this thesis has previously been submitted for a degree in any university and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgment is made in the thesis itself. All work contained within the thesis was performed by myself or in collaboration with members of the laboratory or Imperial College. See acknowledgments for details.

# ABSTRACT

Influenza A virus causes significant and well publicised morbidity and mortality as a single infection. However, in combination with a secondary bacterial super infection the resulting prognosis is worse and can result in hospitalisation or death. Despite extensive clinical and epidemiological evidence, the precise immunological mechanism(s) responsible for increasing susceptibility to secondary bacterial infections remain unknown. Possible mechanisms include disruption to the epithelial barrier, up-regulation of bacterial adhesion receptors, virus-induced immune suppression or a combination of all three.

In this thesis we examine a novel hypothesis that suggests influenza virus infection alters the lung homeostatic microenvironment resulting in a state of immune unresponsiveness that increases susceptibility to subsequent respiratory bacterial infections. This thesis demonstrates that respiratory bacterial complications only arise once influenza has caused significant respiratory damage and can occur many days after viral elimination. We also demonstrate that influenza infection results in a long term desensitisation of alveolar macrophage responses to subsequent bacteria and their products. Furthermore, in an attempt to resolve viral associated inflammation, the airway inadvertently over regulates by enhancing an innate immune negative regulator, CD200R, resulting in a transient state of immune hypo-responsiveness. Removal of this single receptor limits bacterial burden and completely prevents lethal bacteraemia. Finally, we provide preliminary data that suggests airway antimicrobial peptide expression is altered during an influenza infection and that innate immune status of the host can influence commensal bacteria communities of the upper respiratory tract.

This thesis highlights that infection history can significantly influence host immunity to subsequent infections and how an increased awareness of this could lead to more targeted use of existing antimicrobial therapies and the development of much needed novel therapeutics. Adjustment of the level of innate responsiveness may therefore provide a novel opportunity to prevent life-threatening consequences of lung influenza virus infection.

# DEDICATION

I would like to dedicate this thesis to my mother and father who have supported me in everything I have done over the last 27 years. I could not have asked for a better start to my life and I am forever indebted to them. I would also like to thank Tracy Hussell who not only gave me this fantastic opportunity but has also been there for me as a supervisor and also a friend. Furthermore, Tracy exudes inspiration and enthusiasm which is a testament to her world class teaching ability. I would also like to thank my closest friends for their patience and support they have given me over the last four years. I might not have made it to the end without you, thanks Mach, Phil, Aungy, Rhino and Charlie.

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Bobby $Th_{11}$

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# ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| AIDS | Acquired immunodeficiency syndrome |
| AMP | Anti-microbial peptide |
| AP-1 | Activating protein-1 |
| APC | Allophycocyanin |
| APC | Antigen presenting cell |
| BAL | Bronchoalveolar lavage |
| BALF | Bronchoalveolar lavage fluid |
| BM | Bone marrow |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CD200:Fc | Mouse CD200:mouse IgG1 fusion protein |
| CD200R | CD200 receptor |
| CFU | Colony Forming Unit |
| CGD | Chronic granulomatous disease |
| CNS | Central nervous system |
| COPD | Chronic obstructive pulmonary disease |
| CpG | Cytosine guanine |
| CR | Complement receptor |
| CRP | C – reactive protein |
| CTL | Cytotoxic T cell |
| CTLA-4 | Cytotoxic T lymphocyte antigen-4 |
| DALYs | Disability adjusted life years |
| DC | Dendritic cell |
| DMEM | Dulbecco modified eagle's mediun |
| DNA | Deoxyribonucleic Acid |
| ds | Double stranded |
| EAE | Experimental autoimmune encephalitis |
| EAU | Experimental autoimmune uveoretinitis |
| ELISA | Enzyme linked immunosorbent assay |
| FasL | Fas ligand |
| Fc | Fragment crystallisable |
| FCS | Foetal calf serum |
| FITC | Fluorescein Isothiocyanate |
| FCM | Flow cytometry |
| FSC | Forward scatter |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| HA | Haemagglutinin |
| H and E | Haematoxylin and eosin |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| HPLC | High performance liquid chromatography |
| iBALT | Inducible bronchial associated lymphoid tissue |
| ICOS | Inducible co-stimulatory molecule |

| | |
|---|---|
| IDO | Indoleamine-2,3-dioxygenase |
| IEC | Intraepithelial cell |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IgSF | Immunoglobulin superfamily |
| IL | Interleukin |
| IRAK | Interleukin 1 receptor associated kinase |
| IRF | Interferon regulatory factor |
| ITAM | Immunoreceptor tyrosine based activation motif |
| ITIM | Immunoreceptor tyrosine based inhibitory motif |
| JNK | c-Jun-N terminal kinase |
| kb | Kilobase |
| kD | Kilodalton |
| L | Ligand |
| LDL | Low density lipoprotien |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| LTK63 | Modified heat labile toxin of *Escherichia coli* |
| M | Molar |
| M cell | Microfold cell |
| MAMP | Microbe associated molecular pattern |
| MALT | Mucosal associated lymphoid tissue |
| MARCO | Macrophage receptor with collagenous structure |
| MBL | Mannose-binding lectin |
| MCP-1 | Monocyte chemotactic Protein-1 |
| M-CSF | Macrophage-colony stimulating factor |
| MDCK | Madine darby canine kidney |
| MDA-5 | Melanoma differentiation associated gene 5 |
| MHC | Major histocompatibility complex |
| MIR | Macrophage immunoglobulin-like receptor |
| MMP | Matrix metalloprotease |
| mRNA | Messenger ribonucleic acid |
| MW | Molecular weight |
| µg | Microgram |
| NA | Neuraminidase |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NALT | Nasal associated lymphoid tissue |
| NK cell | Natural killer cell |
| Ng | Nanogram |
| NK-κβ | Nuclear factor-κβ |
| NLR | NOD like receptor |
| NO | Nitric oxide |
| NOD | Nucleotide binding oligomerization domain |
| OD | Optical density |
| OX40L | OX40 ligand |
| PAFr | Platelet activating factor receptor |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| PercP | Peridinin chlorophyll protein |
| PFU | Plaque forming unit |
| Pg | Picogram |
| PG | Protoglandin |
| pIg | Polymeric immunoglobulin |
| pIgR | Polymeric immunoglobulin receptor |
| PMA | Phorbol-12-Myristate-13-Acetate |
| PRR | Pattern recognition receptor |
| PS | Phosphatidylserine |
| R | Receptor |
| RIG-I | Retinoic acid inducible gene I |
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RPMI | Roswell park memorial institute |
| RSV | Respiratory syncitial virus |
| SAP | Serum amyloid protein |
| SEM | Standard error of the mean |
| SH | Src homology |
| SHIP | SH2-containing inositol phosphatase |
| SIgA | Secretory immunoglobulin A |
| SIGIRR | Single immunoglobulin IL-1 related receptor |
| SIRP | Signal regulatory protein |
| SOCS | Suppressor of cytokine signalling |
| SR | Scavenger receptor |
| STAT | Signal transducers and activators of transcription |
| TCR | T cell receptor |
| TGF-β | Transforming growth factor-β |
| $Th_x$ | T helper subset |
| TIR | Toll interleukin receptor |
| TLR | Toll like receptor |
| TOLLIP | Toll interacting protein |
| TNF | Tumour necrosis factor |
| $T_{reg}$ | Regulatory T cell |
| TRAF | TNF Receptor Associated Factor |
| TREM | Triggering receptor expressed on myeloid cells |
| WHO | World health organisation |

# Introduction

# Chapter 1

## Foreword

This thesis examines how influenza virus infection enhances susceptibility to secondary respiratory bacterial pneumonias. To this end this introduction looks to describe basic immune functions of the respiratory tract and regulatory mechanisms involved in controlling innate immune responses.

## 1.0 Mucosal immunity

Throughout evolution, vertebrates have developed an elaborate collection of defense mechanisms that provide protection against the perpetual threat of invading micro-organisms. In mammals, the mucosal surfaces of the body, including the respiratory tract, form the largest surface that is exposed to opportunistic pathogens and exogenous antigen and as a result, contains the largest collection of lymphoid tissue in the body. Consequently, the respiratory tract has developed an array of intricate physiological systems to ensure both optimum conditions for gaseous exchange and protection against pathogen invasion. However, despite numerous mucosal defense systems, pathogens inevitably acquire virulence factors to target and subvert immune effectors and facilitate infection. This may result in inflammation within the delicate microenvironment of the lung which ultimately has serious consequences for the host. Respiratory infections can afflict anyone, but display an increased incidence when the immune status of the host is compromised or if the invading pathogen is highly virulent. In part, for these reasons respiratory infections are common and new respiratory infections are likely to emerge frequently.

## 1.1 Epidemiology of respiratory infections

The Burden of Disease data produced by the World Health Organisation (WHO) highlights that respiratory lung infections (excluding tuberculosis and pneumonias in patients with HIV/AIDS) cause more than 6 % of the global burden of disease [1;2;12]. This equates to an estimated 3.9 million annual deaths worldwide with at least 18, 000 in the USA alone [13]. The same WHO data also illustrates that respiratory

infections account for the highest burden of disease of all infectious diseases, responsible for an estimated 100 million disability adjusted life years (DALYs), exceeding that of HIV/AIDS, tuberculosis and other highly publicised infections (Figure 1.1). In addition to the significant mortality associated with such infections, is the substantial morbidity and subsequent economic disruption. Lung infections disproportionately burden low income countries where effective methods of prevention and primary care are underdeveloped or not available. Normalising DALYs against population size highlights a more than 20 fold increase in relative burden of disease in low income countries compared to the rest of the world [12;14]. Infectious diseases cause less of a burden in high income societies than do chronic lifestyle diseases such as cardiovascular disease, type II diabetes and gerontological diseases. However, in both the wealthiest and the poorest societies, lung infections are the most common, and perhaps severe, illness experienced by the general public, regardless of age or gender (Figure 1.2).

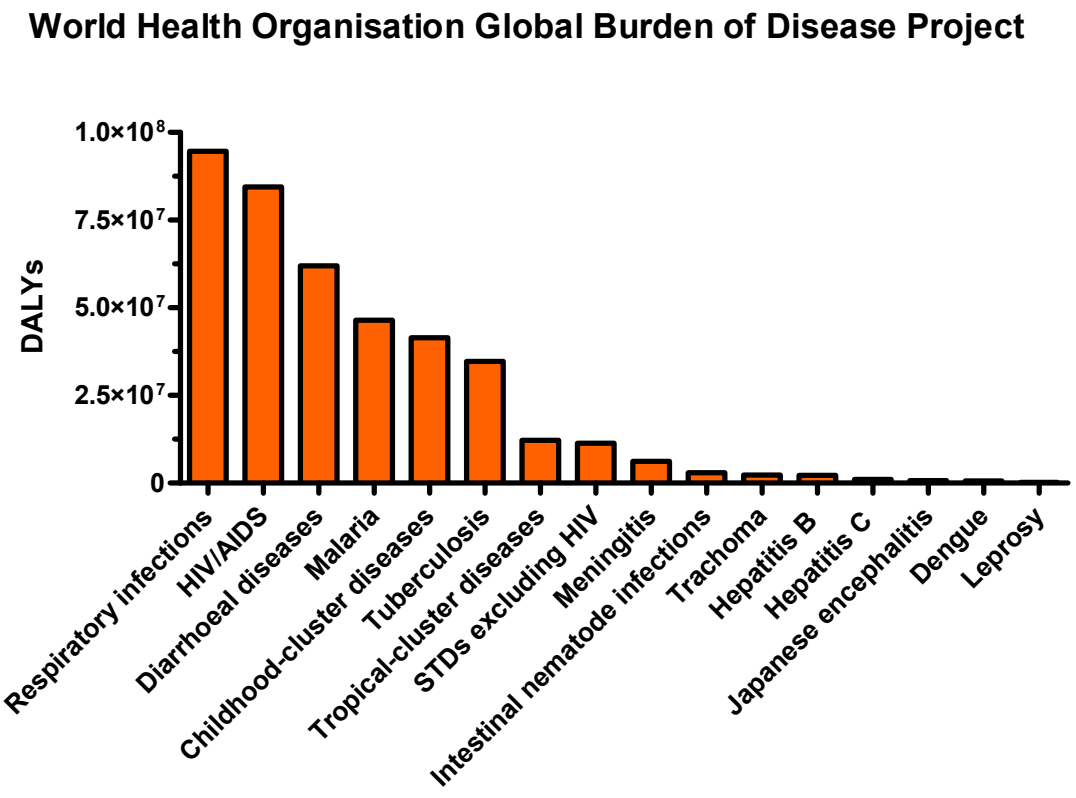



**Figure 1.1** The WHO Global Burden of Selected Diseases, as measured by Disability-Adjusted Life Years (DALYs) lost worldwide. DALYs take into account the amount of otherwise healthy life lost to morbidity and/or mortality. Data acquired from [1].

Furthermore, it is likely that the prevalence of lung infections will increase in high income countries, where an aging population is more susceptible to respiratory

infections [15]. The emergence of novel respiratory infections, such as the novel H1N1 swine influenza strain, SARS coronavirus and the continuing H5N1 influenza A virus epidemic in South East Asia, will contribute to the increasing burden of respiratory disease. A third and often neglected concern is the increasing number of common agents of community and nosocomial acquired pneumonias acquiring antibiotic resistance. All taken into account, global lung infections seem poised to become even more of a concern in the near future.

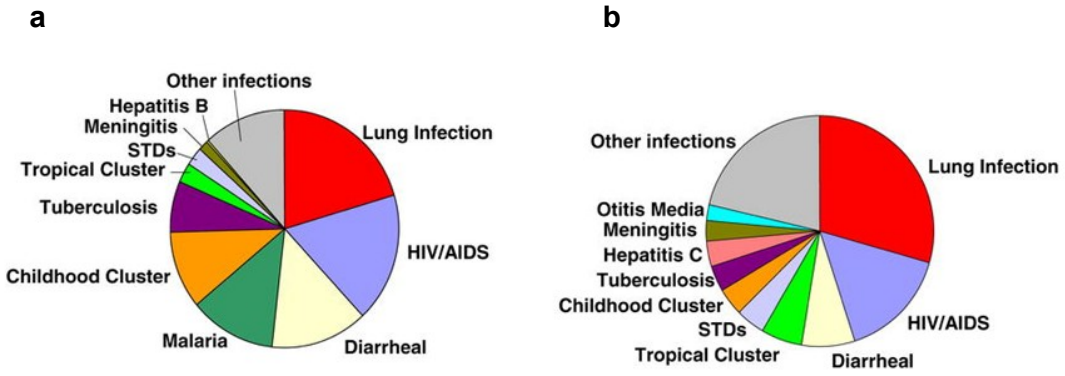



**Figure 1.2** The WHO Global Burden of Selected Diseases. The number of deaths attributed to infections in low (a) and high (b) socioeconomic regions of the world [2].

## 1.2 Innate immunity of the respiratory tract – The front line

Both the upper and lower respiratory tracts are protected against infection by many non specific innate and specific adaptive defences. A complex interplay between both innate and adaptive components results in the maintenance of a pathogen free environment throughout the lower respiratory tract that ensures optimal physiological function. Innate immunity fulfils a highly significant role early in infection against a diverse array of pathogens and is critical in the development of the more pathogen specific adaptive immunity.

### 1.2.1 Physical barriers

The initial obstacle to micro-organisms entering the respiratory tract is an array of mechanical barriers that act to limit pathogen invasion beyond the respiratory epithelium. Upper respiratory tract epithelial cells are covered with cilia that beat in a synchronised upward fashion to propel particles and bacteria toward the oropharynx. The ciliated epithelium is also coated with a layer of mucus that traps inhaled particles, prevents access to epithelial cells and aids their propulsion toward the

mouth. Further barrier defence is conferred by tight junctions between epithelial cells of respiratory mucosal surfaces, airflow pressure, differential temperature gradients and nasal passage and laryngeal particle size exclusion.

### 1.2.2 Chemical barriers – The role of antimicrobial peptides

Antimicrobial and immunomodulatory peptides produced by resident lung cells also play a major role in the rapid first line defence against respiratory infections [5]. Production of these soluble mediators may be constitutive or induced via recognition and detection of micro-organisms and can impart both direct and indirect immunity towards the invading micro-organism. A number of the most significant antimicrobial mediators and their proposed mechanisms of action are shown in Table 1.1 below.

**Table 1.1 Pulmonary antimicrobial peptides**

| Protein | Structure | Pulmonary Source | Antimicrobial activity | Immunomodulatory activity |
|---|---|---|---|---|
| Lactoferrin | Iron binding glycoprotein 80kDa | Neutrophil secondary granules & submucosal gland epithelial cells | Bactericidal/static, inhibits biofilm formation, anti-viral & anti-fungal | Binds LPS & CpG motifs preventing septic shock. Anti-oxidant |
| Secretory leukocyte proteinase inhibitor (SLPI) | 11.7 kDa non glycosylated protein | Macrophages, neutrophils & epithelial cells | Bactericidal/static & anti-viral | Potent anti protease. Inhibits LPS induced NF-κB activation. Attenuates neutrophil recruitment in sepsis models. Impairs monocyte activation. |
| Lysozyme | 14 kDa enzyme | Neutrophil secondary granules & submucosal gland epithelial cells | Bactericidal/static | Unknown |
| Human defensins | 3-5 kDa peptides | Neutrophil azurophil granules & pulmonary epithelial cells | Bactericidal/static, anti-viral, anti-fungal and anti-parasitic | Mitogenic and chemotactic activity. Adds to epithelium repair by enhancing epithelial proliferation. |
| LL-37 | Requires proteolytic processing to liberate functional antimicrobial peptide | Neutrophil secondary specific granules & submucosal gland epithelial cells | Bactericidal/static, anti-viral & anti-fungal | Reduces macrophage produced TNF after LPS stimulation. Also thought to have chemotactic activity. |
| Bactericidal permeability increasing protein (BPI) | 55 kDa protein | Neutrophil primary granules | Bactericidal/static & opsonises for neutrophil phagocytosis | Dampens LPS and endotoxin responses *in vivo*. |
| Surfactant proteins A & D | Lipoprotein complex | Type II pneumocytes & airway Clara cells | Bactericidal/static, anti-viral, anti-fungal & opsonin for phagocytosis of bacteria and viruses. | Modulate multiple leukocyte functions |
| Lactoperoxidase | Enzyme | Airway epithelium | Bactericidal/static, anti-viral & anti-fungal | |
| CCL20 | Chemokine | Airway epithelium | Bactericidal against Gram negative bacteria | B cell, immature DC and $T_{reg}$ chemoattractant. |

Information contained within Table 1.1 was adapted from [5].

### 1.2.3 Cells of the innate immune response

There are many effector cells that comprise the innate immune system, which exert potent antimicrobial activity and are fundamental in the establishment of a rapid response to invading micro-organisms. Some innate immune cells reside in the healthy lungs of uninfected individuals, but there is further recruitment in response to inflammatory signals generated during infection. Effector cells of the innate immune system are largely phagocytic. They recognise invading micro-organisms through detection of conserved proteins or polysaccharides uniquely expressed on their surface or contained within micro-organisms. This detection augments their activation resulting in increased antimicrobial capacity and release of chemokines and cytokines that promote inflammation [16]. Many of the proteins that are detected by the innate immune system are highly conserved and perform essential functions within micro-organisms, therefore allowing a limited number of host receptors to detect the vast array of micro-organisms that reside in the same environment as the host. Examples of such conserved microbial products include lipopolysaccharide (LPS), the cytosine guanine (CpG) dinucleotide DNA motif, peptidoglycan, flagellin and lipoteichoic acid (LTA). Toll-like receptors (TLRs) constitute an important family of host receptors that represent a significant mechanism for inducing production of antimicrobial products and in the establishment of a rapid and potent immune response to infection [16;17]. These are discussed in greater detail in the next section.

Macrophages are central cells of the innate immune system and fulfil a pivotal role in immunosurveillance and are consequently fundamental to the establishment of a protective inflammatory response to infection [18]. Alveolar macrophages are resident in the respiratory tract, comprising 90-95 % of all cells in the airways of uninfected individuals. Alveolar macrophages express a large number of receptors that facilitate recognition and phagocytosis of microbial pathogens, including TLRs, Fc, complement, mannose binding lectins and numerous scavenger receptors [19;20]. Phagocytosed micro-organisms are subsequently exposed to toxic mediators, such as reactive oxygen species (ROS) and proteases that can kill the engulfed micro organism [21]. The plethora of cytokines produced by activated macrophages is critical for the ensuing immune response and pulmonary defence. These include TNF-α, IL-12, IFN-γ, IL-10, IL-1 and a selection of chemokines that direct recruitment of cells [22;23]. These cytokines increase the production of additional chemokines and promote the up-regulation of adhesion molecules on lung endothelium and epithelium, facilitating the recruitment and diapedesis of neutrophils and lymphocytes into the lung.

Dendritic cells (DCs) are another important facet of the innate immune system that form a network within the submucosa of the nasopharynx, trachea and bronchial tree [24;25]. They constitutively express high levels of co-stimulatory molecules such as CD80, CD86 and MHC II. DCs engulf and process micro-organisms and other stimulatory antigens for presentation and are capable of migrating to draining lymph nodes to present antigen to effector lymphocytes which is critical for the development of an adaptive immune response and immunological memory [25;26].

Neutrophils, another cellular component of the innate immune response, which like macrophages display direct antimicrobial activity and release multiple cytokines. Neutrophils are the principal effector cells against many bacterial pathogens but also fulfil a significant role in protection against other microbial infections of the lung [27]. Neutrophils are recruited rapidly from the periphery to the site of infection in response to various stimuli such as chemotactic proteins KC (the murine homologue of IL-8) and MIP-2α produced by alveolar macrophages and airway epithelial cells [28]. Once at the site of infection, neutrophils are activated by local pro-inflammatory cytokines. Similar to macrophages, neutrophils are strongly phagocytic and exhibit a propensity for ingesting and killing invading pathogens [27]. They also produce a number of cytokines such as TNF-α, IL-1β, IL-6 and MIP-2α, which act to enhance and refine the pro-inflammatory response [29;30].

Finally, Natural Killer (NK) cells also respond rapidly to respiratory infections. They express a variety of both activator and inhibitory surface receptors that discriminate expression levels of MHC class I on infected and non infected host cells [31]. If the NK cell detects reduced expression levels of MHC I, as may be caused by viral infection, then it will proceed to kill the infected cell [32-34]. NK cell cytotoxicity is mediated by perforin/granzyme induced lysis and Fas-dependent apoptosis [35]. NK cells are activated by IL-12 derived from pulmonary macrophages and in turn generate IFN-γ, which further activates the macrophage and resident DC populations [35;36].

The immune system can be viewed as a co-ordinated interplay between innate/non-specific immunity and acquired/specific immunity. Through the production of cytokines and professional antigen presentation, the innate cellular response fulfils a fundamental role in orchestrating the development of an acquired immune response, crucial for specific cell mediated and humoral responses. However, there is growing appreciation for the intricate reciprocation that exists between the development and

maturation of the adaptive immunity and the termination and resolution of innate immune responses. This concept is revisited later in the introduction and throughout the thesis.

## 1.3 Detection, response and clearance of micro organisms

### 1.3.1 Pathogen recognition

To initiate the many effector functions of the innate immune system, described above, the recognition and detection of pathogen presence is essential. Despite the lack of precise specificity that is provided by the adaptive immune system there is a fundamental need for the host's innate immune system to decipher self from non self. The innate immune system harbours a family of cell surface and intracellular receptors, collectively referred to as pathogen recognition receptors (PRRs), which recognise pathogens directly and initiates signalling cascades that culminate in an 'appropriate' immune response. Each PRR has the ability to detect a broad specificity of ligands that have a common structural motif, such as regular patterns of lipids, matrix proteins, unmethylated dinucleotides, double stranded RNA or complex carbohydrates. These microbe associated molecular patterns (MAMPs) are present on or within all micro-organisms but absent from host cells. An important aspect of pattern recognition is that the PRRs cannot distinguish between pathogenic and non pathogenic micro-organisms as the conserved MAMPs are not uniquely expressed by pathogens. This fundamental predicament is essential for maintaining innate immune homeostasis, as demonstrated in the intestinal tract, and dysregulation of these interactions are thought to lead to inflammatory bowl diseases and other disorders [37]. A number of the major PRR family members are introduced below.

#### 1.3.1.1 Collectin family members

Not all PRRs are tethered to the host cell surface. Members of the collectin family, so called because they contain both collagen like and lectin biding domains, are humoral molecules of the innate immune system that interact with pathogens at mucosal surfaces such as the respiratory tract [38]. Eight members of the collectin family have been identified thus far, of which mannose binding lectin (MBL) and the surfactant proteins A and D are the most characterised [38]. They are present as free protein in blood plasma and respiratory epithelium exudate respectively. Collectins recognise a specific orientation of carbohydrates uniquely present on the surface of micro-organisms, resulting in the production of collectin - pathogen complexes that

highlight the presence of an invading pathogen and act to neutralise, agglutinate and opsonise pathogens for nearby professional phagocytes.

#### 1.3.1.2 Macrophage bound C-type lectin and scavenger receptors

Professional phagocytes also contain C-type lectin receptors that are expressed on their cell surface that act in a similar manner to secreted collectin family members. The macrophage mannose receptor is a cell bound C-type (calcium dependant) lectin that binds carbohydrates present on the surface of invading microorganisms. In addition to its recognition properties the macrophage mannose receptor can act as a putative phagocytic receptor due to its transmembrane domain [39]. Another well characterised surface bound C-type lectin is Dectin 1, a transmembrane receptor that binds β-glucan [40]. Dectin 1 contains an atypical immunoreceptor tyrosine based activation motif (ITAM) and has an important role in antifungal defence [41]. Another family of macrophage surface receptors that recognise various anionic polymers and acetylated low density lipoproteins are termed scavenger receptors (SRs) [42]. SRs can be characterised by their broad ligand specificity and are thought to have evolved as PRRs through gene duplication. Macrophage receptor with collagenous structure (MARCO) is one of five members of the SR-A subclass, expressed highly on human and mouse alveolar macrophage and has been shown to play an instrumental role in the detection and phagocytosis of respiratory bacterial pathogens [20]. Many of the members of the SR-A family are alternative splices of the same gene; MARCO is, however, a distinct gene product.

#### 1.3.1.3 Toll like receptors (TLRs) and NLRs family members

TLRs belong to the evolutionary conserved toll/interleukin-1 receptor (TIR) super family, which can be divided into two main subgroups: the IL-1 receptor and the TLR receptor family. The IL-1 receptor subgroup consists of at least 10 receptors including the IL-18 receptor and the orphan receptors ST2 and single immunoglobulin IL-1 related receptor (SIGIRR). The TLR subgroup is made up of 10 extracellular and intracellular receptors namely TLR 1 through 10 [43]. Figure 1.3 illustrates the cellular location of all TLRs and cytosolic pathogen recognition receptors (introduced below). All members of this family are transmembrane receptors, expressed by cells from the myeloid lineage but also select epithelium, that recognise MAMPs and signal through a common signalling TIR domain motif culminating in the activation of the transcription factor nuclear factor κB (NF-κB) and stress activated protein kinases [44;45]. Some TLRs utilise co-receptor complexes, for example TLR4 uses MD2 and CD14, which control receptor expression and increase

ligand binding avidity [46]. TLR intracellular signalling cascades are introduced in section 1.3.2.

The complete picture of TLR function in antimicrobial defence remains unresolved, however TLR ligation is essential in provoking inflammatory and innate effector responses and enhancing adaptive immunity against pathogens [47;48]. Furthermore, members of the TLR family have also been implicated in the pathogenesis of autoimmune, atherosclerosis, chronic inflammatory, asthma, rheumatoid arthritis and infectious diseases [49-57]. In reality, the host is constantly exposed to TLR ligands and stress induced proteins that if left unchecked would result in overwhelming inflammatory cytokine production and chronic myeloid cell activation. TLR function and signalling cascades are therefore tightly regulated during homeostasis and throughout ongoing innate inflammatory responses to limit gratuitous host immunopathology. The complexity of this regulation is introduced in section 1.3.2.

In addition to TLR family members expressed on the cell surface and in endosomal compartments, there is an additional family of cytosolic PRRs that detect the presence of pathogens that reside and replicate intracellularly. These include the nod like receptors (NLRs) and the intracellular sensors of viral nucleotides, retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and DNA dependant activator of interferon regulatory factors (DAI). The NLR family is composed of *circa* 20 intracellular proteins that share a common protein domain. All NLRs contain a nucleotide binding oligomerization domain (NOD) followed by a leucine rich repeat domain. NLRs can be further categorised in to three subfamilies, NOD, NALP and NAIP, by virtue of their amino terminus domains [58;59]. It is this amino terminus domain that determines ligand specificity and downstream functional properties. The proteins of the NOD family, NOD 1 and NOD 2, have been shown to sense bacterial peptidoglyans [60]. The NALP family members, of which there are 14, in combination with NAIP family members combine to form varying types of inflammasome that are responsible for cleaving the precursors of several inflammatory cytokines, such as IL-1β, IL-18 and IL-33, through the activation of a cascade of caspases [61]. Inflammasome generation can be induced by several bacterial infections but the precise mechanisms that control this remain unclear [62]. The involvement of caspase activation has suggested that NALPs may also contribute to antimicrobial defence by inducing apoptosis of infected cells.

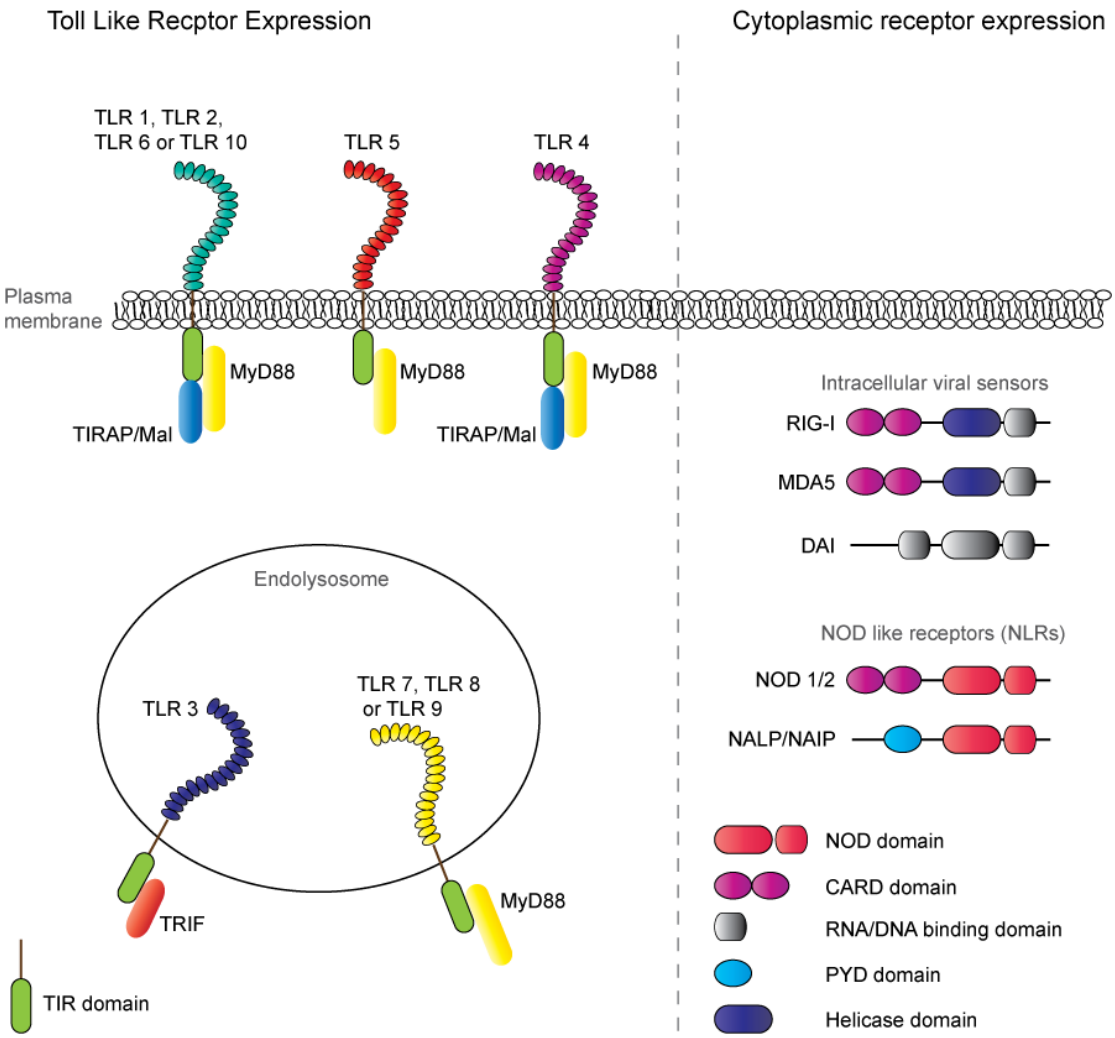



**Figure 1.3 TLR and NLR expression patterns.** The cellular location of TLR/NLR ligand determines the cellular location of the corresponding TLR/NLR. This acts as an additional regulatory mechanism that prevents unwarranted inflammation. Adapted from [9-11].

In addition to TLR 3 and TLR7/8 recognition, cytosolic recognition of viral RNA is mediated by the RNA helicase family proteins RIG-I and MDA5, whereas viral DNA has been recently been shown to be detected by DA [63,64]. These proteins recognise structural features of viral RNA that are absent from host RNA, therefore allowing discrimination and the rapid production of the type I interferons, IFN-α and IFN-β and thereby the induction of antiviral immunity [65-67]. The details of how DAI recognises viral DNA and its subsequent anti-viral effector function remains to be elucidated.

### 1.3.2 TLR signalling cascades

Upon ligand recognition TLR signalling is initiated by receptor dimerisation or hetero-dimerisation [68]. With the exception of TLR 3, which utilises the alternative signalling adapter TRIF, activation of the receptor recruits MyD88, an intracellular adapter protein, through its TIR domain which interacts with the TIR domain in the cytoplasmic tail of the TLR. This receptor-adapter complex promotes the recruitment and activation of several downstream kinases such as IL-1 receptor associated kinases (IRAK) 1, 2 and 4 [68]. The order in which these adapters are recruited is important with IRAK 4 recruited first that subsequently becomes activated and phosphorylates IRAK 1. The importance of IRAK 4 recruitment and involvement in TLR signalling has recently been exemplified in a clinical study that highlights IRAK 4's importance in childhood immunity to *S. pneumoniae* infection [69]. This receptor-adaptor complex then activates tumour necrosis factor associated factor 6 (TRAF 6) through death domain interactions common to MyD88 and IRAK adaptor proteins. Subsequently, the activation of additional kinases, including inhibitor of NF-κB (IκB) kinases (IKKs), results in the release of NF-κB from its inhibitory complex, allowing NF-κB to translocate to the nucleus and mediate inflammatory cytokine gene expression. A complex programme of cytokine production ensues resulting in a customised immune response relating to the initial TLR signal. Common TLR signalling cascades are depicted in Figure 1. 4.

In order to generate pathogen specific immune responses and diversity in TLR responses a selection of intracellular adaptor molecules are utilised [70;71]. For example TLR 3 uniquely uses TRIF to induce IFN-β synthesis through IRF-3 expression, a signalling pathway crucial for anti viral immunity [72;73]. TLR 4 can also activate IRF 3 through the use of TRIF, MAL and TRAM adaptor proteins [74;75]. Additional pathways also contribute to TLR function, such as Jun N terminal kinase (JNK) and the mitogen activated protein kinases (MAPKs) [76;77]. However, there remains a large void in our understanding in how a limited number of TLR can utilise a limited combination of adaptor signalling cascades to produce a highly specific and complex immune response ideally suited to the micro organism that was initially detected.

### 1.3.3. TLR negative regulators

As alluded to earlier, specific sites of the body, such as mucosal surfaces, are continuously exposed to a complex array of TLR ligands. Therefore, there must exist exquisite regulatory mechanism that determine when TLR signalling and effector functions are needed and when they are not. There exist several intracellular

negative regulators of TLR signalling that not only prevent unwarranted TLR responses during homeostasis but are also control the amplitude of TLR effector responses as a result of pathogen recognition. All TLRs, with the exception of TLR 3 and 6, utilise MyD88 in combination with other adaptor proteins to initiate their effector signalling cascades. Extensive investigation has demonstrated that inhibition of MyD88 abrogates NF-κB activation, and that other adapters such as MAL, TRIF and TRAM can only partially recover NF-κB activation. MyD88 is therefore an ideal target for negative regulation as it is critical for the initiation of TLR signalling cascades. In addition IRAK family members also play a fundamental role in initiating TLR signalling cascades and are also the target for several regulatory mechanisms. Alternatively spliced variants of both MyD88 and IRAK family members play a key role in negative feedback regulatory mechanisms to control excessive TLR mediated signalling and in the fine tuning of TLR responses [78-80]. This was demonstrated through over expression studies of MyD88s, an alternative spliced variant of MyD88 that lacks an interdomain critical for TLR and IRAK 4 binding. The study highlighted expression of MyD88s inhibited IL-1 and LPS but not TNF-α induced NF-κB activation [78]. Although MyD88 is ubiquitously expressed, expression of MyD88s has only been detected in the spleen and brain. Its expression has been shown in a human monocytic cell line THP-1 following a 16 hour stimulation with LPS [78]. Over expression of IRAK 2 splice variants have also been shown to inhibit LPS induced NF-κB activation in a dose dependant manner and have been shown to be up regulated in RAW 264.7 macrophage cells 3 hours after LPS treatment indicating a possible feedback effect on TLR signalling [81]. Figure 1.4 also highlights a number of negative TLR signalling regulators.

Additional TLR regulatory mechanisms are discovered at an ever increasing rate and for brevity a few of the most significant mechanisms will be introduced herein. IRAK M is a family member of the IRAK family and has been shown to have considerable regulatory properties on TLR signalling. Macrophages isolated from IRAK M deficient mice produce enhanced levels of inflammatory cytokines in response to TLR 4 and TLR 9 ligands [82]. IRAK M deficient mice also show increased *in vivo* inflammatory responses to bacterial infection compared with wild type mice, despite demonstrating equivalent bacterial load. Several members of the SOCS family have also been implicated in suppressing cytokine signalling [83]. Furthermore, SOCS-1 deficient mice are highly susceptible to sepsis compared with wild type mice, consistent with the belief that SOCS-1 is a non redundant negative regulator of TLR signalling [84;85]. However, two reports have indicated that the inhibitory effect of SOCS-1 on TLR

signalling is indirect and occurs by inhibiting type I IFN signalling pathways following IFN-β up regulation by TLRs and not the main NF-κB pathway [86;87].

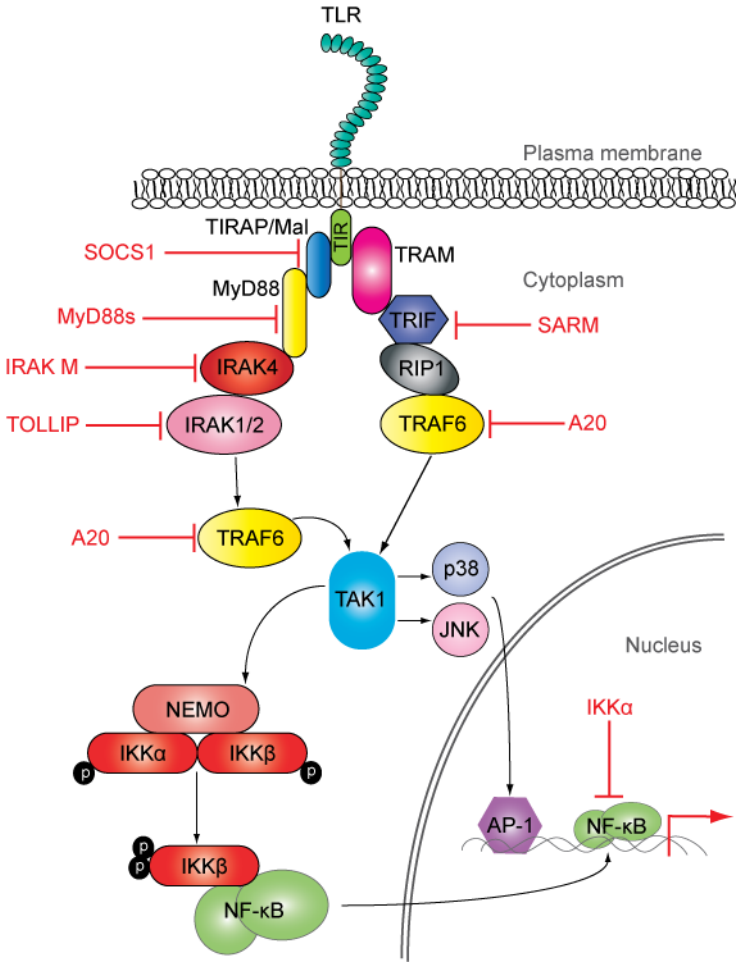



**Figure 1.4 TLR intracellular signalling cascades and negative regulation.** There exists a complex array of adapter proteins that inhibit and fine tune TLR receptor signalling in order to limit inflammation. Sterile alpha and armadillo motif containing protein (SARM). Adapted from [9-11].

Other important inhibitory regulators include toll interacting protein (TOLLIP) that has been shown to interact with several TLR members and can inhibit TLR 2 and TLR 4 medicated NF-κB activation through interacting with IRAK 1 [88;89]. In addition TOLLIP expression is elevated in intestinal epithelial cells which are hypo responsive to TLR 2 ligands [90]. A20 is another negative regulatory candidate that is rapidly induced by both LPS and TNF-α and is expressed by many cell types. A20 has been implicated in preventing the host from endotoxic shock and has been shown to cleave ubiquitin from TRAF 6 which is essential for TLR downstream signalling and NF-κB

translocation [91;92]. This suggests that A20 can control both MyD88 dependant and MyD88 independent TLR signalling pathways. Finally, TLR signalling can be controlled through reducing or compartmentalising the expression of TLRs themselves or even through the removal of TLR expressing cells through apoptosis. This can be achieved by the degradation of TLRs through ubiquitylation or inhibition of TLR expression by anti inflammatory cytokines such as IL-10 and TGF-β [93-95]. These mechanisms might play a physiological role in the tolerance to commensal bacteria. This concept is supported by the observation that intestinal epithelial cells (IECs) are non responsive to LPS stimulation but can mount a normal response to IL-1, this is thought to occur due to the low expression of TLR 4 levels and the lack of MD2, a co-receptor for LPS, which is indispensible for TLR 4 signalling [96]. Furthermore, there is growing evidence that pathogenic bacteria can regulate TLR signalling through the secretion of TIR domain homologues that directly bind to MyD88 and interfere with subsequent signalling cascades [97].

### 1.3.4 Phagocytosis – pathogen clearance and apoptotic cells

The eventual end point for the elaborate detection and recognition systems discussed above is ultimately to either tolerate or clear the micro-organism and develop immunological memory. In addition to the humoral aspects of both innate and adaptive immunity and the pleitropic activities of inflammatory cytokines, phagocytosis is necessary to ensure antigen presentation, pathogen clearance, resolution of inflammation and the initiation of tissue regeneration through the clearance of dead or apoptotic host cells from the site of infection.

Classic phagocytosis can be characterised by membrane extensions that require Syk tyrosine kinases resulting in the activation of NADPH oxidase and the production of pro-inflammatory cytokines [7]. However, there exist several forms of phagocytosis that do not require membrane extensions, do not involve Syk and are accompanied by the production of anti-inflammatory cytokines [7]. Despite over 100 years of research into this fundamental mechanism, phagocytosis remains an incredibly complex process that requires many parallel signalling pathways that collectively define the phagocyte's response and fate. As is seen with TLRs, phagocytes express a broad spectrum of receptors that participate in target recognition and internalisation. Some of these receptors transmit intracellular signals that trigger phagocytosis whilst others appear primarily to participate in binding or increasing the efficiency of internalisation. Furthermore, some receptors enable the recognition and internalisation of opsonised

pathogens whilst others detect and rapidly degrade apoptotic host cells. Several of the most characterised phagocytic receptors are illustrated in Table 1.2.

**Table 1.2 Phagocytosis receptors**

| Phagocytosis receptors | Ligands |
|---|---|
| *Micro-organism receptors* | |
| FcγRI (CD64) | IgG, CRP, SAP-opsonised particles |
| FcγRII (CD32) | IgG, CRP, SAP-opsonised particles |
| FcγRIII (CD16) | IgG, CRP, SAP-opsonised particles |
| FcεRI | IgE-opsonised particles |
| FcεRII (CD23) | IgE-opsonised particles |
| FcαRI (CD89) | IgA-opsonised particles |
| CR1 (CD35) | MBL, complement-opsonised particles |
| CR3 ($\alpha_M\beta_2$,CD11b/CD18, Mac1) | Complement-opsonised particles |
| CR4 ($\alpha_X\beta_2$,CD11c/CD18, gp150/95) | Complement-opsonised particles |
| SR-A | Bacteria, LPS, LTA |
| MARCO | Bacteria |
| Mannose receptor (CD206) | Mannan |
| Dectin-1 | B 1,3-glucan |
| CD14 | LPS, PDG |
| C1qR(P) | MBL, SAP and complement |
| *Apoptotic cell receptors* | |
| SR-A | LDL, PS |
| SR-B (CD36) | Collagen type I, PS, thrombospondin |
| Vitronectin ($\alpha_V\beta_3$) | Thrombospondin |

CR, complement receptor; SR, scavenger receptor; CRP, C-reactive protein; SAP, Serum amyloid P; PS, phosphotidylserine; LDL, low density lipoprotein. Adapted from [7;8].

All these receptors induce rearrangements in the actin-cytoskeleton that leads to the internalization of the micro-organism/apoptotic cell. However, fundamental differences in the underlying molecular mechanisms and ensuing response exist between the various receptors. For example, micro-organism phagocytosis through Fc mediated recognition results in pro-inflammatory cytokines production and assembly of the NADPH oxidase complex and super-oxide production, however, micro-organisms opsonised with complement do not initiate this inflammatory response [98]. It is unsurprising therefore that many pathogen associated virulence factors target phagocytosis and phagolysosome fusion mechanisms in order to

ensure pathogen survival [99;100]. On the contrary, phagocytosis of apoptotic cells is a process crucial in the development and homeostasis of all multi cellular organisms [101]; consequently it would be detrimental to the host if such phagocytic events invoked an inflammatory response. Indeed, it has been demonstrated that macrophages ingesting apoptotic neutrophils do not produce IL-1β, TNF-α, IL-8 and MCP-1, normally secreted by activated macrophages, instead they secreted anti-inflammatory compounds such as TGF-β, prostaglandin $E_2$ ($PGE_2$) and platelet activating factor (PAF) [102]. Furthermore, a recent study demonstrated that the phagocytosis of apoptotic lymphocytes by alveolar macrophage resulted in the production of $PGE_2$ that subsequently compromised anti bacterial capacity of the lung [103]. It is becoming increasingly apparent that the decision to mediate an inflammatory or anti-inflammatory response is regulated by the co-operation of other receptors that themselves are not involved in phagocytosis. For example, TLRs are recruited to the de-novo phagosomes providing a mechanism by which phagocytosis and inflammatory responses can be linked [104;105]. This suggests that the activation state of the macrophage might dictate the combination of receptors used, and this, in turn, could influence whether phagocytosis is accompanied by anti-inflammatory or pro-inflammatory mediators. This remains a very exciting and active area of research.

## 1.4 Innate immune regulation

### 1.4.1 Regulatory receptors and inflammatory activity of myeloid cells

As discussed above, the immune system must detect and quickly respond to the presence of an invading micro organism to ensure both host survival and pathogen clearance, but at the same time demonstrate a degree of refinement to minimize inappropriate or over-exuberant inflammation and immunopathology [106;107]. As previously mentioned, myeloid cells represent a population of immune cells with pleiotropic functions that utilise an array of potent effector functions that can result in unwarranted pathology to the host unless their activity is tightly controlled. Regulation can be achieved through cognate cell-cell interactions or via the secretion of soluble mediators [108;109]. Although the secretion of soluble factors can modulate a large number of cells over a large area of tissue, direct cell-cell contact mediated regulation provides a more specific and selective method of regulation. Investigation into the receptor interactions that mediate myeloid cell regulation identified members of the immunoglobulin superfamily (IgSF) as being important. The IgSF domain is the most abundant domain type in leukocyte surface proteins and receptors and has been shown to play a major role in regulating immune system function. Receptors

with IgSF domains that regulate myeloid cell activity include CD200R, CD172a (SIRP-α), triggering receptors expressed on myeloid cells (TREM1 and TREM2) and macrophage Ig-like receptor (MIR) [110-113].

### 1.4.2 CD200 – CD200R interaction and the regulation of myeloid cells

After CD200 (OX-2) was discovered in rat brain and thymus over 25 years ago it has now been shown to impart an inhibitory signal delivered to the myeloid cell through ligation with its receptor CD200 receptor (CD200R) [114]. CD200 was subsequently cloned and characterised in mice and humans [114-116]. Ensuing cloning analysis identified CD200 as a 41-47kDa transmembrane surface glycoprotein and a member of the IgSF with 2 extracellular IgSF domains [114;117]. In mice, it is a protein of 269 amino acids constituting a hydrophobic signal peptide, a membrane distal Ig-V-like domain, a membrane proximal Ig-CH2-like domain, a transmembrane segment and a short cytoplasmic tail [115;116]. It also possesses 6 N-linked glycosylation sites and shares structural homology to CD80/CD86. The cytoplasmic tail is only 19 amino acids long and shares no homology to known signalling kinases, which, combined with the lack of an ITIM/ITAM sequence or SH2/SH3 domain implies that it lacks any ability to induce an intracellular signal.

The cellular distribution of CD200 is very similar in mice, rats and human suggesting that it plays a fundamental role in the immune system of higher vertebrate [110;114;118]. To date CD200 expression has been reported on thymocytes, B cells, activated T cells, neurons, follicular dendritic cells, endothelium and cells in reproductive organs [110;114;118-121].

After the discovery of CD200, Barclay *et al* were also responsible for identifying and cloning its corresponding receptor, aptly named CD200R, and postulated its likely origin as a result of gene duplication of the CD200 gene [110]. The receptor has subsequently been independently characterised in humans and mice [122;123]. Similar to its ligand, CD200R is also an IgSF member, containing two extracellular IgSF domains, it also possesses an unusually high number of N-linked glycosylation sites, with 8 identified in rats and 10 in mice [110;123]. In addition, the cytoplasmic tail of CD200R contains 67 amino acids, substantially longer than the cytoplasmic tail of CD200. Further analysis identified the presence of three tyrosines residues as potential phosphorylation sites, therefore implying that the action of CD200 is solely delivered through the receptor and is not bi-directional [110;123]. One of these tyrosine residues is located within a NPXY motif, and a recent study in mast cells has

demonstrated this residue is phosphorylated upon ligation of the receptor [124]. Furthermore, this phosphoryation leads to the recruitment of adaptor proteins Dok-1 and Dok-2, which are in turn phosphorylated and associate with RasGAP and SH2-containing inositol phosphatase (SHIP). Ultimately, this was demonstrated to inhibit the phosphorylation of ERKs, p38 and JNK [124]. There have, however, been limited studies thus far citing the downstream signalling events from CD200R in other myeloid cells.

CD200R expression is far more restricted than that of the ligand being largely confined to myeloid cells. The expression profile of the receptor again appears to be conserved between mice, rats and humans and has thus far been described upon macrophages, dendritic cells, neutrophils, basophils and mast cells [110;123-125]. Though expression appears largely confined to myeloid cells, a number of studies describes expression of CD200R on peripheral blood $CD4^+$ T cells and B lymphocytes in mice and humans, and further identified high levels of CD200R mRNA expression in Th2 polarised T cells [123;126]. Furthermore, several more members of the CD200R family have recently been identified, taking the existing number to four isoforms of CD200R in mice (R1-4) and two in humans (R1-2), yet the exact role of these other receptors as well as their natural ligands remains ambiguous [123;127]. Table 1.3 highlights the expression profiles of CD200 and CD200R.

**Table 1.3 Cellular expression of CD200 and CD200R**

| Cell Type | CD200 | CD200R |
|---|---|---|
| T cells | -/+ | -/+ |
| Thymocytes | + | - |
| B cells | + | - |
| Monocytes/Macrophage | - | + |
| Dendritic cells | -/+ | + |
| Neutrophils | - | + |
| Mast cells | - | + |
| Basophils | - | + |
| Endothelium | -/+ | - |
| Epithelium | -/+ | - |

### 1.4.3 In vivo role for CD200 and CD200R – the knock out power of mice

The development of CD200 and CD200R knock out (KO) mice provided the first and best clues as to the physiological role for CD200 and CD200R [128]. Studies highlighted that the intrinsic defects demonstrated in naïve CD200 KO mice occurred in cells that expressed CD200R rather than the cell types expressing CD200. Naïve CD200 KO mice develop splenomegaly and display heightened numbers of splenic $CD11b^+$ myeloid cells, elevated numbers of splenic red pulp macrophages and a thicker marginal zone macrophage layer [128]. Furthermore, elevated levels of the immunotyrosine activating motif (ITAM) containing intracellular DAP-12 were detected within the marginal zone and on dendritic cells located within the red pulp. This implies an elevated level of myeloid cell activation. Lymph nodes are also slightly enlarged and exhibit an unusual tubular structure with no demarcation between nodes. Macrophage populations are again expanded and exhibit heightened activation. Microglia (macrophage-like cells of the CNS) also exhibit heightened activity, forming clumps reminiscent of that seen with inflammation or neural degeneration and express heightened levels of activation markers CD11b and CD45.

This apparent abnormality in myeloid homeostasis correlated with an increased propensity for the development of autoimmune conditions, showing increased susceptibility to collagen induced arthritis (CIA) [128]; experimental autoimmune uveoretinitis (EAU) [129;130], a model for retinal inflammation; and experimental autoimmune encephalitis (EAE), a model of multiple sclerosis [128] and a model for alopecia [131]. Symptoms of these diseases develop more rapidly in CD200 KO mice and lesions contain greater macrophage numbers in a heightened activation state. Collectively, these studies suggest that CD200 fulfils a significant role in regulating macrophage activation and ensuing inflammation, and that the CD200-CD200R interaction is important in down-regulating the activity of myeloid cells expressing CD200R [128;132]. CD200 has also been shown to be up-regulated on the surface of several tumours including chronic lymphocytic leukaemias [133-135]. Additional support to the concept of CD200 attenuating inflammatory and immune responses is provided by the recent identification of viral homologues of CD200, such as human herpesvirus 8, cytomegalovirus and myxoma virus, that transmit an inhibitory signal to tissue macrophages and dendritic cells, thereby reducing their ability to prime T cells and preventing viral clearance [136-139].

### 1.4.4 The CD200 – CD200R axis: A potential immunotherapeutic target

There is overwhelming evidence to suggest that CD200 has the capacity to modulate myeloid cell activity in an inhibitory manner. Manipulation of the CD200-CD200R interaction has clearly defined an important role for these molecules in dictating the phenotype of the myeloid response in conditions characterised by excessive immunity. Furthermore, it has become apparent that delivery of this inhibitory signal can be utilised immunotherapeutically to alleviate symptoms attributable to such excessive immunity or block inhibition in situations of tumour immune suppression. For example, the use of a blocking CD200 antibody, thereby inhibiting CD200-CD200R interaction, has been shown to exacerbate EAE and CIA, whilst the administration of an agonistic antibody to CD200R or a CD200:Fc fusion protein halts CIA disease progression and can reduce established arthritic disease [110;128;140;141]. Several of the studies described above also cited the potential for manipulating the CD200-CD200R axis during autoimmune conditions.

Finally, there is also a clear role for CD200 in governing alloimmunity. It has been demonstrated that blockade of CD200 signalling reduces graft survival [142] and that the administration of CD200:Fc results in prolonged graft survival in both allotransplants and xenotransplants [143]. Furthermore, the expression of CD200R on mast cells begs the question of the involvement of CD200 in regulating allergy and other atopic diseases [110;123]. Indeed, in a murine model of passive cutaneous anaphylaxis (PCA), the systemic administration of an agonistic antibody to CD200R inhibits FcR1-dependent responses [144].

### 1.4.5 The mechanism underlying CD200 mediated inhibition

The potential therapeutic applications for CD200-CD200R manipulation are clear, however, the molecular mechanisms that determine the inhibitory effect of CD200 when bound to CD200R remain ambiguous. A recent study demonstrates that CD200 signalling inhibits degranulation in mast cells and basophils and production of IL-13 and TNF-α by mast cells [125;144]. Furthermore, an agonistic antibody to CD200R was demonstrated to reduce IFN-γ and IL-17 induced production of IL-6 in murine resident peritoneal macrophages, and reduced cytokine and chemokine production of CD200R transfected U937 cells [145]. Therefore, it would appear that signalling through CD200R reduces the ability to produce pro-inflammatory cytokines. Finally, ligation of CD200R on plasmacytoid dendritic cells causes an up-regulation of indolamine-2,3-dioxygenase (IDO) expression and activity [146]. IDO mediates the catabolism of tryptophan leading to its immunosuppressive products being generated, and high

levels of IDO expression are affiliated with inhibition of alloreactivity and enhanced regulatory T cell function [147-149].

## 1.5 Adaptive Immune response to respiratory Infection

Fundamental to the resolution of infection and the development of immunological memory is the establishment of an acquired specific immune response, comprising both T cell mediated and humoral B cell responses.

### 1.5.1 T Cell mediated immunity

T cells are a subset of lymphocytes that originate from the bone marrow and mature in the thymus. They are identified by the expression of CD3 and can be further categorised into two main subsets: $CD4^+$ T helper (Th) lymphocytes and $CD8^+$ cytotoxic (CTL) lymphocytes. $CD4^+$ helper T cells exert their effector function primarily through the release of cytokines that activate and stimulate other cells. They also fulfil a significant role in antibody production by providing a critical signal for B cell activation through the CD40-CD40L interaction. $CD8^+$ cytotoxic T cells are proficient killers of infected and tumour cells, lysing infected cells via a perforin/granzyme or a Fas/FasL-dependent mechanism. They are also critical mediators in the resolution of viral infections [150].

Activation of both $CD4^+$ and $CD8^+$ T cell subsets requires interaction of the α and β chains, that make up the T Cell Receptor (TCR), in combination with CD3 and antigen peptide displayed on the major histocompatability complex (MHC) of the antigen presenting cell (APC). Antigenic products can be processed by distinct pathways and presented in conjunction with MHC class I or class II molecules. MHC I expression is universally observed on all nucleated cells and such presentation is important in the activation of $CD8^+$ T cells. Conversely, MHC II expression is restricted to APCs and such presentation of antigenic peptide is responsible for the activation of $CD4^+$ T cells. In addition to classic αβ TCR expressing T cells there exists a small number of T cells that utilise a γδ TCR. These γδ T cells are often associated with mucosal sites and anticipated to infer protection of epithelial surfaces. Furthermore, there is a population of unique intraepithelial T cells ($CD103^+$) in the respiratory tract, which recognize generic immunological distress signals rather than pathogen specific motifs, and fulfill a significant role in the recognition and eradication of infected epithelial cells [151].

In addition to TCR/MHC interaction a second co-stimulatory signal, provided by the APC, is needed to ensure activation of naïve T cells. The successful integration of two such signals results in the proliferation and differentiation of antigen specific T cells. The most well known co-stimulatory signal is the CD28/B7 pathway, which governs activation of naïve T cells [152]. This co-stimulatory signal up-regulates IL-4, IL-5, IL-8, IL-13, IFN-γ and TNF-α [153;154], and prevents T cell anergy [155;156] or cell death by inducing the expression of the anti-apoptotic molecule Bcl-$X_L$ both *in vitro* and *in vivo* [157;158]. Other co-stimulatory signals include OX40, 4-1BB, ICOS and CD27 [152;159;160].

### 1.5.2 T helper cell subset classification

The $CD4^+$ T helper subset can be further subdivided into $Th_1$, $Th_2$, T regulatory ($T_{reg}$) and $Th_{17}$ cells defined by the distinct array of cytokines each subset produces; IFN-γ, TNF-α and IL-2 from $Th_1$ cells; IL-4, IL-5 and IL-6 from $Th_2$ cells; IL-17 and TNF - α from $Th_{17}$ cells and IL-10 and TGF-β from $T_{reg}$ [161-166]. In recent years the addition of multiple $T_{reg}$ subsets has added complexity in understanding T cell immunology. $T_{reg}$ consist of four main subsets; natural $T_{reg}$ that express CD4, CD25 and CD152 as well as the transcription factor foxp3; Tr1 cells, that are induced $T_{reg}$ cells which do not express foxp3, these express high levels of CD152 and require IL-10 for their differentiation and suppression; and anergic $CD4^+$ and $CD8^+$ cells that are capable of suppression by competing for antigen and IL-2; they have a very high stimulation threshold and therefore do not become activated except in situations of very high antigen exposure [167]. Some studies also imply that $CD8^+$ T cells can also be subdivided on cytokine secretion profiles [168]. In spite of these concerted attempts to define each T helper subset by their individual cytokine and transcription factor expression profile, the distinction between the various subsets *in vivo* remains ambiguous. For example, some view the Th1/Th2 dichotomy not as discrete subsets, but rather as a continuum of different combinations of cytokines [169]. Figure 1.5 highlights the cytokine production profile from each subset and the transcription factor that is used as a marker for each subset differentiation.

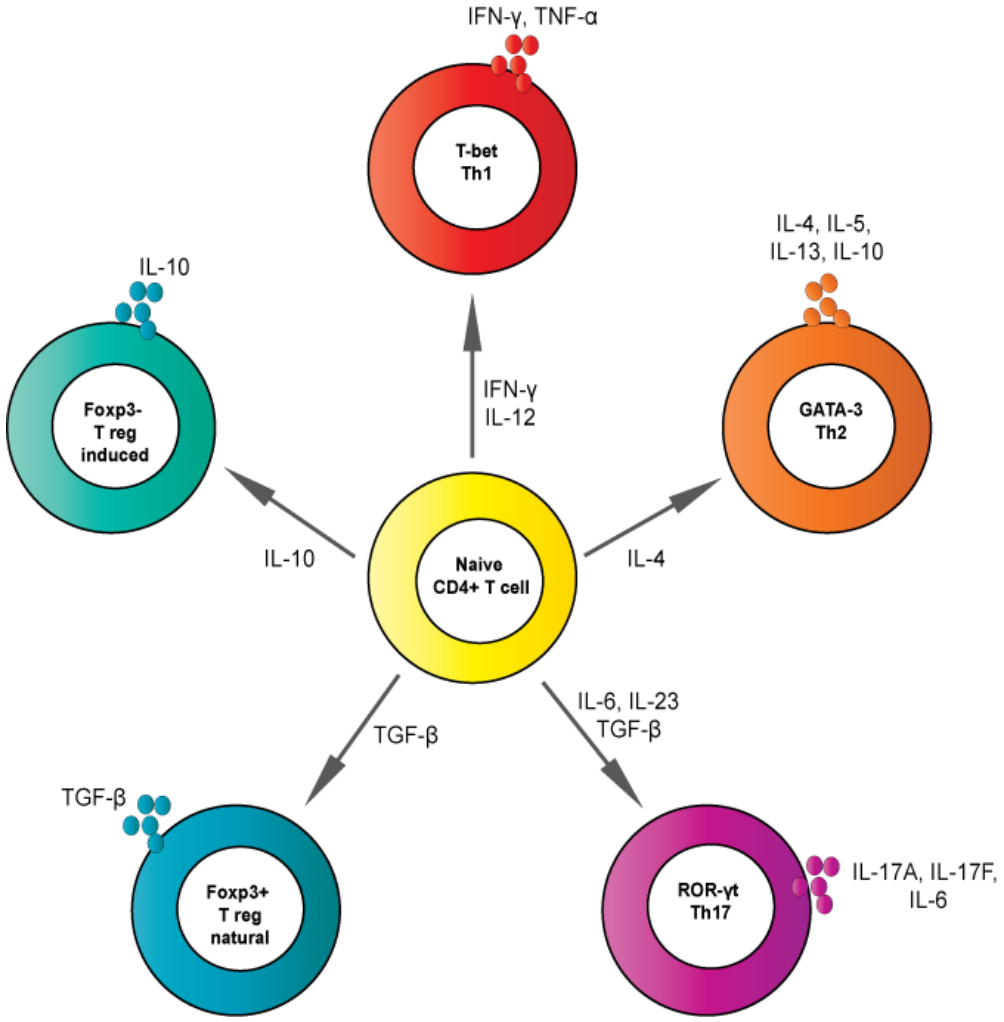



**Figure 1.5 CD4$^+$ T Cell subsets.** CD4$^+$ T helper subsets are derived from CD4$^+$ peripheral precursors under specific cytokine environments. The transcription factors that determine each subset and the cytokines that each subset produces are also shown. Reviewed in [3].

### 1.5.3 Subset differentiation and function

Peripheral T cells exists as a common naïve precursor that upon antigen stimulation secrete IL-2 before differentiating, via an intermediate ($Th_0$), into one or more distinct polarized subset [170-172]. The generation of a specific subset directed response is dictated by the cytokine milieu at the time of T cell activation [173;174], although the antigen dose, environmental conditions and transcription factor expression has also been shown to be important [175]. Longer contact of MHC with the TCR is required to

produce a stable $Th_2$ response than $Th_1$; it is also suggested that a higher degree of stimulation is required for $Th_1$ polarisation [176;177].

Another feature of T cell subset differentiation is the strong antagonism observed between different T cell subsets [178-181]. An example of these self-reinforcing gene expression patterns is highlighted by IFN-γ gene expression and concomitant silencing of IL-4 gene expression, while the opposite is seen on $Th_2$ differentiation [182]. Type I and II interferon signalling causes the STAT1-dependent expression of IL-12 receptor; ligation of this IL-12R leads to activation of T-bet, a transcription factor critical for the development of $Th_1$ responses, and STAT4-dependent potentiation of IFN-γ production. In a $Th_2$ polarised response, IL-4 signalling activates STAT6 and the transcription factor GATA-3 [182]. GATA-3 suppresses STAT4 while inducing positive epigenetic changes in the $Th_2$ cytokine cluster of IL-4, IL-5 and IL-13 genes [183;184]. Less is understood about how $Th_{17}$ and $T_{reg}$ subsets are polarised although IL-6 and TGF-β have been implemented in $Th_{17}$ polarisation (reviewed in [185]). Such antagonism reinforces development of distinct polarised T helper subsets and explains the strong bias observed in many infection models. $Th_{17}$ cells are entirely separate from the $Th_1$ and $Th_2$ lineages; cytokines from both $Th_1$ and $Th_2$ cells antagonise $Th_{17}$ generation, and it is likely that they have evolved to deal with different pathogens to those efficiently cleared by $Th_1$ and $Th_2$ responses [162]. IL-23 can polarise naïve cells into $Th_{17}$ cells in an ICOS and CD28-dependent manner ($Th_1$ cells are dependent on CD28 but not ICOS) [162]. Generation of $Th_{17}$ cells is independent of STAT1, STAT4 and STAT6 but requires TGF-β [162;186;187]. In APC-free culture conditions, $Th_{17}$ cells can be generated with the addition of only TGF- β and IL-6 [186].

During the generation of functional T cells, inappropriate self-directed effector responses are avoided primarily by deletion of self-reactive clones in the thymus. However, many antigens are not present in the thymus, and T cells are capable of T cell receptor (TCR) rearrangement in the periphery. To regulate effector responses, subsets of $T_{reg}$ have evolved, which are suppressive of activation of other cells in a contact-dependent mechanism. $T_{reg}$ are able to interact with and suppress both $Th_1$ and $Th_2$ effector T cells both through MHC-dependent mechanisms via DC, and directly through T cell-T cell interactions [161]. $T_{reg}$ can be either $CD4^+$ or $CD8^+$. There are four main $T_{reg}$ subsets; natural $T_{reg}$ are produced in the thymus and constitutively express CD4 and CD25 as well as the transcription factor foxp3 and surface CD152. Their generation is dependent on TGF- β. $Tr_1$ cells, by contrast, are induced $T_{reg}$,

which do not express foxp3 but express high levels of CD152 and require IL-10 for their differentiation and suppression. In addition to these $T_{reg}$ subsets, anergic CD4+ and CD8+ cells are capable of suppression by competing for antigen and IL-2; they have a very high stimulation threshold and do not therefore become activated except in situations of very high antigen exposure [167].

Cytokine expression profiles of each subset determine the discrete functions implemented by each population in an immune response. $Th_1$ cytokines enable a cell-mediated immune response to target intracellular pathogens by activating $CD8^+$ cytotoxic T cells (CTLs), macrophages and NK cells, whereas a $Th_2$ cytokine response induces a humoral response targeting extracellular pathogens – $Th_2$ cell cytokines activate mast cells and basophils through IL-4 and eosinophils through IL-5 [188;189]. This polarisation extends to the effect on B cell antibody production; $Th_1$ and $Th_2$ cells induce switching from IgM to IgG2a, but only $Th_2$ can induce production of IgG1, IgA and IgE [190].

## 1.6 Influenza A virus

### 1.6.1 Epidemiology of influenza infection

Influenza infection is an acute respiratory disease that is one of the most persistent and important infectious diseases that continues to afflict humans. Influenza is a global dilemma inducing seasonal epidemics that can affect 10 to 20 % of the population[191]. It is estimated that influenza A viral infections are responsible for between 10,000 to 20,000 deaths in the United Kingdom each year [192]. Furthermore, increasing numbers of human deaths attributed to the South Asian avian H5N1 strain and the novel H1N1 pandemic influenza strain are likely to increase influenza associated morbidity and mortality.

Influenza infection is typically self-limiting, culminating in a local and systemic reaction, whereby the afflicted individual may experience fever, chills, headaches, coughing and myalgia. However, there remain a significant proportion of patients who are considered to be at high risk of developing severe illness and complications, such as the elderly, the very young, pregnant women and the immunocompromised.

### 1.6.2 Classification and structure

Influenza viruses are enveloped, single stranded negative sense RNA viruses that belong to the family O*rthomyxoviridae.* Of the three types of influenza virus, types A

and B cause seasonal epidemics whilst C causes sporadic disease. Type A viruses can be further subtyped based on the haemagglutinin (HA) and neuraminidase (NA) surface-expressed glycoproteins. These outer membrane proteins are functionally significant and are essential to the viability and replicative potential of the virus. The HA protein is fundamental for binding and subsequent fusion to the host cell, whereas the NA protein fulfils a critical role in preventing viral aggregation and aiding release of viral particles from the host cell post-replication. Currently, 16 HA (H1-H16) and 9 NA (N1-9) subtypes have been identified in type A viruses. The RNA genome of influenza consists of 8-segmented genes, containing the genetic information for expression of 10 viral proteins, each encased by nucleoprotein (NP) [193].

### 1.6.3 Antigenic shift and drift

The ability of influenza virus to cause seasonal infection across the human population is attributed, in part, to its antigenic variability that enables it to successfully evade pre existing neutralizing antibodies of the host. As introduced above, influenza A viruses express HA and NA on its surface to enable the virus to gain access to and exit cells. Antigenic variation in these glycoproteins allows viral immune evasion and is largely responsible for the generation of novel viral strains responsible for epidemics and pandemics. There are to date at least 16 haemagglutinin subtypes and 9 neuraminidase subtypes. Human infection is commonly restricted to 3 HA subtypes (H1-3) and 2 NA (N1-2), with the remaining subtypes being seen in viruses that persist within avian reservoirs.

The antigenic variability that occurs within the influenza genome takes two forms: antigenic drift and antigenic shift. Antigenic drift occurs when genes encoding the surface glycoproteins undergo stepwise mutation, owing to the low fidelity of the influenza RNA-specific RNA polymerase (replicase) which lacks proof reading 5'-3' exonuclease activity. These mutations can lead to attenuated or non viable influenza, but can also result in immune evasion allowing the virus to continue to replicate. If the surface glycoproteins are sufficiently different that the host's antibodies are incapable of neutralizing it then the new strain is capable of causing disease.

Antigenic shift is a far less frequent form of antigenic change but can lead to the generation of a novel influenza viral strain capable of causing a large epidemic or pandemic. Antigenic shift occurs when two different viruses from distinct host's (possibly different species) co-infect a single host and exchange gene segments. The

ensuing re-assortment results in a new virus that possesses gene segments from each of the two original strains. This re-assortment can result in viral attenuation, but it can also result in a highly virulent viral strain due to expression of novel surface proteins derived from the animal host virus. In this scenario, the human host may possess little or no immunity to the novel surface proteins and the new variant can cause widespread disease through evasion of the host's immune response.

### 1.6.4 Influenza A viral replication cycle

Influenza virus is transmitted between individuals through droplet dispersion of viral particles from infected hosts [194;195]. The primary site of infection are epithelial cells of the upper respiratory tract, however alveolar macrophages and airway resident leukocytes have also been shown to be infected [194-196]. Influenza virus has the ability to infect any cell that possesses a suitable receptor, notably those expressing a specific form of sialic acid containing cell surface glycoprotein [195;196]. For example, HA molecules on avian viral strains preferentially bind to α 2,3 linked sialic acids, whereas the HA from human influenza viruses prefer α 2,6 linked sialic acids [197]. This difference, together with the occurrence of α 2,6 linked sialic acids on non ciliated human tracheal cells likely explains the difference in tropism between avian and human influenza viruses [198;199]. As introduced above, attachment and fusion to the host cell is mediated through HA. HA exists as a native precursor termed HA0, which attaches the virion to putative cell surface receptors containing sialic acid, and is subsequently cleaved through the action of extracellular host proteases to yield HA1 and HA2 [194;200]. The virion is subsequently endocytosed to form an endosome with HA2 mediating the fusion between virus membrane and host cell [194;195]. The low pH of the endosome induces a conformational change in the HA2 protein, culminating in enhanced proximity of viral and host cell membranes, and ultimately the fusion event [194;195;201]. Fusion subsequently leads to the liberation and nuclear import of viral nucleocapsids, which contains the crucial genetic code that dictates the amplification and generation of substantial viral progeny.

The viral RNA genome, once inside the host nucleus, is transcribed by viral RNA polymerases resulting in the synthesis of viral mRNAs. The mRNAs are subsequently transported to the cytoplasm of the host cell where they are translated to form viral nucleoproteins and polymerases [195;201]. The newly generated viral proteins are then re-directed towards the host nucleus where they assist in viral replication and further mRNA synthesis [195;201]. This positive feedback loop greatly amplifies the quantity of

viral RNA and proteins, which are subsequently packaged to form viral nucleocapsids that are exported from the nucleus before budding from the host plasma membrane to form active virions [195]. Hence the virus envelope is derived from host cell plasma membrane and the budding event is mediated via influenza neuraminidase protein. Figure 1.6 depicts the replication cycle of influenza A virus.

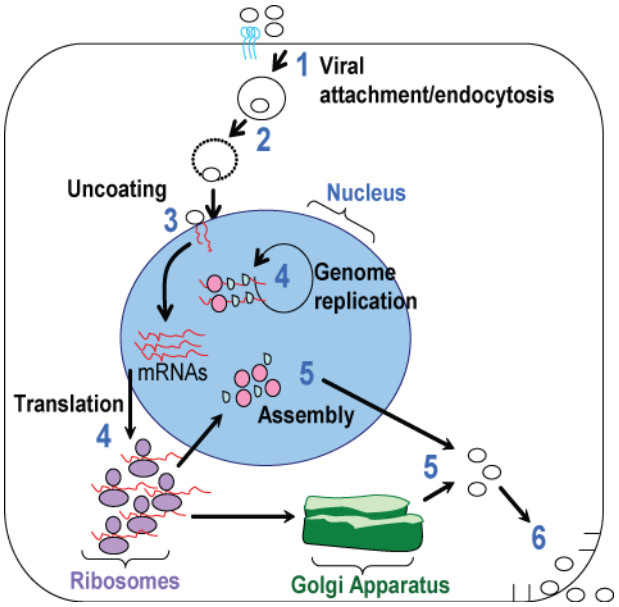



**Figure 1.6 Influenza A virus cycle**. Influenza virus binds to sialic acid containing receptors and enters the cell via an endocytic pathway (1). After fusion of viral and endosome membranes viral ribonucleoprotein complexes are transported into the nucleus (2-3). A series of primary and secondary transcriptions occur which produces viral transcription machinery and structural proteins (4-5). Viral nucleocaspsids are assembled in the nucleus, transported into the cytoplasm, where budding and release of new virions takes place (6).

### 1.6.5 Immunity to influenza

Succeeding influenza infection, the immune system of the host mounts an immediate response to restrict viral replication and limit the associated cytopathology. Infected cells activate a host of transcription factors, including NF-kB (nuclear factor-kB), IRFs (IFN regulatory proteins) and NF-IL6 (nuclear factor of interleukin-6), that co-ordinate the release of chemokines and cytokines that subsequently initiate antiviral defences [195]. Infected epithelial cells primarily release RANTES, MCP-1 and IL-8, whereas monocytes/macrophages secrete MIP-1α, MIP-1β, RANTES, MCP-1&3, IFNα/β, TNFα, IL-6 and IP-10 [202]. The plethora of chemokines released ultimately favours the recruitment of blood mononuclear cells to the site of infection. NK cells, in addition to their cytotoxic function, also contribute to the cytokine milieu releasing IFNγ, TNFα

and GM-CSF, all of which activate APCs [195]. Neutrophils are also prominent, and again, their numbers reflect the virulence and dose of virus. In the early phase of the response IFNα/β is critical in the containment and resolution of an influenza infection due to direct anti-viral effects and potential to mediate the further recruitment of inflammatory cells [203;204].

Ultimately, to resolve viral infection effectively, the induction of an adaptive immune response is a requirement and a combination of both cell-mediated and humoral components fulfil essential roles. Antibodies specific to HA and NA are fundamental in resistance to infection and subsequent recovery, as they act to neutralize viral infectivity and hinder viral release from infected cells. Cytotoxic $CD8^+$ T cells also confer a central role in resolution of viral infection via their cytopathic elimination of infected cells, whilst $CD4^+$ T helper cells also confer a significant role in resolution of influenza infection via the delivery of signals to the main effectors [194].

## 1.7 Group B Streptococcus

### 1.7.1 Epidemiology of Group B Streptococcus

Group B Streptococcus (GBS), also known as *Streptococcus agalactiae*, is the leading cause of invasive bacterial infections in human neonates and is increasingly recognised as a pathogen in adult populations including the elderly, pregnant women, diabetics and the immunocompromised [205;206]. GBS is an opportunistic pathogen that colonises the gastrointestinal and genitourinary tract of up to 50 % of healthy individuals [207]. Approximately 20-30 % of healthy women are colonised rectovaginally with GBS and a high percentage of neonates born to infected women will themselves become colonised with the bacterium [208]. The overall incidence of invasive neonatal GBS disease has been estimated to be 0.6 to 1.8 cases per 1000 live births in the USA with mortality rates between 4-6% [209].

Neonatal GBS infections can be characterised into two distinct forms, early-stage onset and late-stage onset. Early-stage onset infections are classified as occurring within one week of partum and present symptoms of respiratory pneumonia complicated by bloodstream septicaemia. This type of infection typically results from an ascending infection of the bacterium through the placental membrane *in utero,* or alternatively by aspiration of infected vaginal fluids during the birth process into the neonate lung [210]. In contrast, late-stage onset infection occurs in infants between 1 week and 4 months of age with gradual symptoms related to bacteraemia and a high

incidence of meningitis [210;211]. Late-onset is much less common than early-onset disease but is becoming increasingly important as its incidence is not declining as a result of prophylactic measures [211;212]. Little is known about the pathogenesis of late-onset infections, but vertical transmission from mother to baby probably explains most infections during this period. The major risk factors for GBS invasive infection include rupture of membranes before labour onset and increased interval between membrane rupture and delivery [210]. Importantly, a substantial proportion of neonates who survive GBS infection suffer from sequelae, cortical blindness, deafness, uncontrolled seizures and speech and language delay [210]. Nevertheless, despite the clinical importance of GBS our knowledge about the molecular events that lead to the development of invasive disease is very limited.

### 1.7.2 Classification and structure

GBS belongs to the family *Streptococcaceae* and is characterized by the Lancefield group B antigen. GBS is a Gram positive encapsulated bacterium possessing an array of immune resistance phenotypes and secreted toxins. Based on variations of the capsular polysaccharide (CPS), nine different serotypes have been described; serotypes Ia, Ib and II-VIII. Numerous experimental investigations have illustrated that only four serotypes, Ia, II, III and V, account for 80-90% of all clinical isolates [213]. GBS are commonly identified by colony phenotype and the presence of β-haemolysis when plated on sheep blood agar. In addition, a number of biochemical tests exist that can distinguish between the individual GBS serotypes [214].

### 1.7.3 GBS Virulence factors

The spectrum of GBS disease reflects a complex interplay of the host innate and adaptive immune systems with many surface associated and secreted bacterial components. Disease progression indicates that GBS has to adhere, invade and transcytose several epithelial and endothelial cell barriers to gain access to the bloodstream and cause invasive disease. GBS contains several virulence factors that contribute to disease at critical junctures of the infection process. The CPS is regarded as the most important virulence factor, as noncapsulated isogenic mutants are avirulent and a correlation was found between the lack of maternal anticapsular antibodies and invasive neonatal disease [215;216]. One mechanism by which CPS acts is by inhibiting the binding of the activated complement component C3b to its surface, preventing the activation of the alternative complement pathway [217].

In early-onset GBS disease, severe pneumonia and pulmonary damage is the major clinical presentation [218]. Investigations into the cause of this pathology identified the β-haemolysin/cytolysin (βH/C) surface associated toxin as having cytopathic effects on pulmonary epithelial cells [219]. The βH/C toxin is a surface associated molecule expressed by the majority of GBS strains and is encoded with in the GBS cyl operon [220]. The βH/C toxin is cytolytic for a broad range of cells including fibroblasts, lung epithelial cells, macrophage and neutrophils [219;221;222]. In addition, an interesting feature of the βH/C toxin is its close association to an orange-red carotenoid pigment co-expressed on the surface of GBS. The pigment has recently been found to play a role in pathogenesis by virtue of antioxidant properties. The pigment confers protection against antimicrobial effects of hydrogen peroxide, singlet oxygen and superoxide, all of which play a role in the oxidative burst killing mechanisms of host phagocytic cells [221].

GBS encodes several other virulence factors, including a C5a peptidase that cleaves the chemotactic complement component C5a causing impaired leukocyte recruitment to sites of infection [223]. A secreted hyaluronate lyase allows GBS to break down hyaluronic acid, a major component of the connective tissue, and has been postulated to facilitate spread through infected tissue [224]. Due to space limitation Table 1.4 summarises the key virulence factors of GBS.

**Table 1.4 Key virulence factors of Group B *Streptococcus***

| Virulence factor | Gene name | Chemical nature | Molecular/ cellular action(s) | Contribution(s) to disease pathology |
|---|---|---|---|---|
| Exopolysaccharide capsule | *cps*A-L, *neu*A-D | High molecular weight polymer with terminal sialic acid residues | Impairs complement C3 deposition. Decreases immune recognition through molecular mimicry of host sialic acid epitopes. | Blocks opsonophagocytic clearance. Delays neutrophil recruitment. |
| β – haemolysin/cytolysin | *cyl*E | CylE protein (79 kDa) | Forms pores in cell membranes. Induces apoptosis. Promotes cellular invasion. Triggers NOs and cytokine release. | Direct tissue injury. Penetration of epithelium. Induction of sepsis syndrome. Phagocytic resistance. |
| + linked pigment | *cyl* locus | Carotenoid | Antioxidant blocks $H_2O_2$ and singlet oxygen. | Impairment of oxidative burst killing. |
| Hyaluronate lysase | *hyl*B | HylB enzyme (110 kDa) | Cleaves hyaluronan and chondroitin sulphate. | Host dissemination. Impairs leukocyte trafficking. |
| C5a peptidase | *sap*B | ScpB protein (120 kDa) | Cleaves human C5a. Binds fibronectin. | Inhibits neutrophil recruitment. Epithelial adhesion and invasion. |
| CAMP factor | *cfb* | CAMP protein (24 kDa) | CAMP reaction (co-haemolysin). Binds to Fc portion of IgG & IgM. | Direct tissue injury. Impairment of Ab function. |
| Lipotechoic acid | Complex | Amphiphilic glycerol phosphate polymer of complex lipids and short chain fatty acid | Binds host cell surfaces and TLRs. Alanylation inhibits host anti-microbial peptide function. | Epithelial cell attachment. Activation of sepsis syndrome. Resistance of neutrophil killing. |
| C protein (α and β components) | *bca* (alpha) *cba* (beta) | Alpha: protein with multiple identical tandem repeats (14-145 kDa). Beta 84-94 kDa. | Blocks intracellular killing by neutrophils. | Epithelial cell attachment and invasion. Resistance to phagocytic clearance. |
| Serine protease | *csp*A | CspA protein (142 kDa) | Cleaves fibronectin to fibrin like fragments. | Resistance to phagocytic clearance? Promotes tissue spread. |
| Fibrinogen receptor | *fbs*A | FbsA protein (44.2 kDa) | Binds fibronectin through repetitive structure motifs. | Epithelium adherence. Resistance to opsonophagocytic clearance. |

Adapted from [6].

### 1.7.4 Immunity and host interactions

As previously mentioned, during the course of infection GBS encounters a number of different barriers. GBS attaches and gains access to target cells using a combination of membrane bound and secreted factors, illustrated in Table 1.2 above. Nonetheless, entry into the bloodstream, as occurs in invasive neonatal infections, requires the bacteria to not only traverse the pulmonary epithelial cell layer, the basement membrane and the vascular endothelial layer but simultaneously subvert ensuing innate defence mechanisms.

Effective phagocytosis of extracellular bacteria relies on efficient opsonisation by compliment and serotype specific antibodies. The importance of these mechanisms are illustrated by increased GBS susceptibility in neonates deficient of maternal antibody and a reduction of $LD_{50}$ in C3 or C4 knockout mice [215;225]. GBS employs several mechanisms that act to impair opsonophagocytosis and as a result remain undetected by resident phagocytes. The ability to resist immune detection is also demonstrated by GBS ability to cleave C5a, a chemotactic component of the compliment system that plays a key role in neutrophil recruitment [223]. Even phagocytosis of GBS does not guarantee bacterial death as the presence of the CPS and the orange-red carotenoid permits GBS to survive within macrophage for more than 24 hours [226]. Conversely experimental animal models have illustrated pulmonary surfactant, in particular surfactant protein A, can reduce haemolysin associated epithelial cell damage and inhibit bacterial proliferation [227;228].

GBS remains a major cause of invasive bacterial infection in neonates, although extensive research is continuing to elucidate its virulence factors and mechanisms of immune suppression, the complex interaction between host and bacterium remain to be fully understood.

## 1.8 *Streptococcus pneumoniae*

### 1.8.1 Epidemiology of *Streptococcus pneumoniae*

*Streptococcus pneumoniae*, commonly referred to as the pneumococcus, is the leading cause of morbidity and mortality among children worldwide, particularly in developing countries where it is responsible for approximately 25 % of all preventable deaths in children under the age of 5 and more than 1.2 million infant deaths per year [229;230]. It is estimated that 10.6 million children less than 5 years of age present with *S. pneumoniae* infections per year [229]. However, the true burden of *S. pneumoniae* infection is likely to be much higher as disease surveillance systems and diagnostic facilities are limited in developing countries. In the developed, world *S. pneumoniae* is responsible for approximately 30 % of all hospitalised cases of community acquired pneumonia (CAP) which can have mortality rates of more than 20 % and an incidence of 18.3 cases per 100, 000 elderly persons [231-233]. In countries with high HIV prevalence, there has been a significant increase in the incidence and prevalence of *S. pneumoniae* pneumonia and associated bacteraemia [234;235].

*S. pneumoniae* colonises the mucosal surfaces of the upper respiratory tract and can readily be cultured from the naso-oropharynx of healthy individuals. Once acquired, *S. pneumoniae* can be carried for weeks or months before its eventual clearance. The rates of carriage and distribution of disease causing serotypes vary considerably across geographical regions and age cohorts. The most prevalent serotypes worldwide are 6, 14, 19 and 23 but serotypes 1, 5 and 8, contribute significantly to invasive pneumococcal disease in young children in developing countries [236]. The majority of those healthy individuals colonised with *S. pneumoniae* appear to be asymptomatic, however if bacteria gain access to sterile parts of the respiratory tract a rapid inflammatory response ensues that results in disease. The most common clinical manifestations of *S. pneumoniae* infection include acute otitis media (middle ear infection) or bacterial pulmonary pneumonia. In a small proportion of those that develop pneumonia, invasive bacteraemic pneumococcal pneumonia can precede which is associated with increased morbidity and mortality. The mortality rates of patients that develop invasive bacteraemic pneumococcal pneumonia range from 10-30 % in adults and around 3 % in children [237]. Furthermore, of those that survive invasive pneumococcal disease around 25-50 % suffer from long term sequelae [238].

### 1.8.2 Classification and structure

*S. pneumoniae* is an encapsulated facultative Gram positive diplococcus which grows in pairs or chains and belongs to the group of α-streptococci. Based on variation within the capsule polysaccharide (CPS) 92 separate serotypes of *S. pneumoniae* have been identified. *S. pneumoniae* produces several virulence factors that have a role in colonisation and invasive pneumococcal disease. These include the CPS, the cell wall and several protein factors such as pneumolysin, neuraminidase and autolysin. Figure 1.7 shows the basic structure and virulence factors that are expressed by *S. pneumoniae*. These and there impact on host physiology is introduced below.

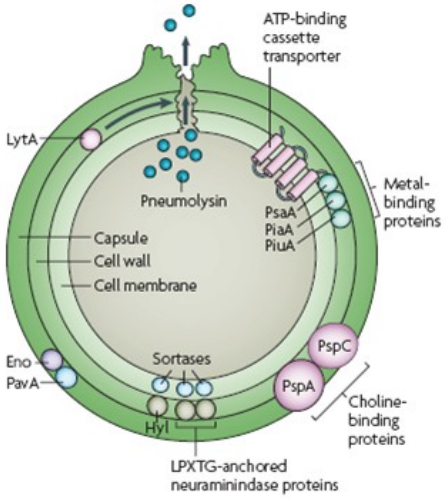



**Figure 1.7 *S. pneumoniae* structure and virulence factors.** Adapted from [4].

### 1.8.3 *S. pneumoniae* virulence factors and colonisation

A prerequisite for the development of carriage, invasive bacteraemic pneumococcal pneumonia and meningitis is adherence and colonisation of the nasopharyngeal mucosal epithelium. The implementation of an array of virulence factors (highlighted in Figure 1.7 and Table 1.5) allows *S. pneumoniae* to resist initial innate immune defences and gain a foothold within the host. The expression of a CPS is essential for *S. pneumoniae* virulence as strains that are CPS deficient are avirulent in mice and humans. CPS expression is thought to reduce entrapment within sialic acid rich mucopolysaccharides, therefore allowing access to the underlying epithelium, and also prevent opsonisation by complement, IgM and collectin binding proteins resulting in the avoidance of phagocytosis from sentinel phagocytes [239;240]. CPS expression variants are also more resistant to opsonophagocytic killing and are selected for during transition from the mucosal surface to the bloodstream [241;242]. All *S. pneumoniae* strains express a range of surface proteins that facilitate pathogen host cell interaction. The expression of phosphorylcholine (ChoP), a homologue of platelet activating factor (PAF), is common to several other microorganisms that reside in the upper respiratory tract. In *S. pneumoniae*, ChoP allows bacterial adherence to the receptor for platelet activating factor (rPAF) culminating in host cell signalling and subsequent inflammation [243]. rPAF is widely distributed on the mucosal surfaces of both the upper and lower respiratory tract epithelium. Other studies have identified additional adhesin interactions with host cell glycoconjugates, such as the choline binding protein A (CbpA) and the expression of surface associated

exoglycosidases; a neuraminidase (NanA), a β-galactosidase (BgaA) and a β-*N* acetylglucosaminidase (StrH) that act to remove terminal sugars from host glycoconjugates thereby unmasking putative receptors for adherence or providing a nutrient source [244;245]. The surface expression of pneumococcal adhesion and virulence A (PavA) and enolase (Eno) have also been shown to enhance *S. pneumoniae* adhesion through binding to the extracellular matrix components fibronectin and plasminogen respectively [246;247].

*S. pneumoniae* also produces the thiol-activated cytotoxin pneumolysin. Pneumolysin is a member of a family of cholesterol dependant cytolysins, such as streptolysin and listeriolysin produced by *Streptococcus pyogens* and *Listeria monocytogens* respectively, that are synthesised by Gram positive bacteria. Pneumolysin is produced as a 52 kDa soluble protein that oligomerizes in the membrane of target cells to form a large ring shaped transmembrane pore. Multiple studies have demonstrated that pneumolysin is a potent, wide ranging virulence factor and can be isolated in virtually all pneumococcal isolates [248-251]. Pneumolysin has been shown to contain multiple activities including; inhibition of ciliary beating, inhibition of phagocyte respiratory burst, induction of cytokine synthesis through ligation of TLR 4 and in $CD4^+$ T cell activation and chemotaxis [252;253]. Pneumolysin has also been shown to be essential for the survival of *S. pneumoniae* in both the upper and lower respiratory tract and has been implicated as a requirement for bacterial invasion from the lungs to the bloodstream [254;255]. The expression of hyaluronidase is also thought to facilitate bacterial spread through the extracellular matrix and connective tissue that is rich in hyaluronan containing polysaccharide components [256].

There are a number of additional secreted and expressed factors that are thought to add to the virulence of *S. pneumoniae* but have yet to be defined as true virulence factors, these include; sIgA protease, cleaves host mucosal IgA reducing their opsonising capabilities; autolysin (LytA), an additional cytotoxic protein that increases the secretion of pneumolysin through self lysis [257]; PspA, inhibits complement C3 component from attaching to the pneumococcal cell surface [258]; divalent metal ion binding lipoproteins, PpmA and SlrA and several LPXTG anchored proteins for example StrA [259-261]. This list is by no means exhaustive and many *S. pneumoniae* virulence factors remain undefined. However, despite a growing appreciation and understanding of how the various virulence factors of *S. pneumoniae* function in concert to allow colonisation and subvert host innate immunity, there is little

understanding of how host epithelial and tissue barriers are breached during progression from colonisation to invasion.

**Table 1.5 *S. pneumoniae* virulence factors and their role in disease**

| ***S. pneumoniae* virulence factors and disease** | **Role in colonisation** |
|---|---|
| *Upper airway colonisation* | |
| Capsule | Prevents entrapment in mucus, thereby allowing access to epithelial surfaces. Also inhibits opsonophagocytosis. |
| ChoP | Binds to rPAF on the epithelial surface of the nasopharynx. |
| Cbp A | Binds to human secretory component on polymeric Ig receptors during first stage of translocation across the epithelium. |
| NanA, BgaA and StrH | Act sequentially to cleave terminal sugars from human glycoconjugates which might reveal putative binding sites. |
| Hyl | Breaks down hyaluronan-containing extracellular matrix. |
| PavA | Binds to fibronectin. |
| Eno | Binds to plasminogen. |
| *Competition in upper airway* | |
| Bacteriocin (pneumocin) | Small antimicrobial peptide that targets other bacteria. |
| *Respiratory tract infection and pneumonias* | |
| Ply | Cytolytic toxin that also activates complement. Critical virulence factor *in vivo*. |
| PspA | Prevents binding of C3 onto pneumococcal surface. Also binds lactoferrin. |
| LytA | Digests the cell wall which results in the release of Ply. |
| PsaA | Component of the ABC transport system which is involved in resistance to oxidative stress. |
| PiaA andPiuA | Component of the ABC transport system. |
| NanA and NanB | Aid colonization by revealing binding sites, modifying the surfaces of competing bacteria in the same niche and/or modifying the function of the host clearance glycoproteins. |
| IgA | Cleave human IgA |

A non exhaustive list of *S. pneumoniae* virulence factors adapted from [4].

### 1.8.4 Innate immunity and host inflammatory response

Research into the host immune response to *S. pneumoniae* respiratory infection has mainly focussed on the role humoral components of the innate and adaptive immune systems play in first line defence against *S. pneumoniae*. It is clear that complement pathways are fundamental to *S. pneumoniae* resistance as mice deficient in C1q or secretory IgM demonstrate rapid progression to invasive bacteraemia and a reduction in macrophage activation and phagocytic function [262]. A recent case controlled clinical study has added credence to the significance of the classical complement pathway in *S. pneumoniae* host defence by demonstrating a significant

association between MBL mutant genotypes and increased susceptibility to *S. pneumoniae* infection in adults [263]. AMPs have also been implicated in host immunity toward *S. pneumoniae* infection. SP-D is present at high levels in the fluid lining the epithelium of the alveoli and is produced by alveolar type II cells and non ciliated Clara cells. Several studies have implicated SP-D in aggregating and opsonising *S. pneumoniae* resulting in the enhancement of neutrophil phagocytosis [264;265].

Despite extensive research into multivalent conjugate vaccine development and the role complement plays in immunity to *S. pneumoniae* infection, there is little examination of the cellular immune response elicited in response to *S. pneumoniae* infection [266-268]. As introduced above, TLRs are central in the recognition of micro-organism presence and in the initiation of subsequent cellular immune responses. A number of recent publications have demonstrated both TLR 2 and TLR 4 to be important in protection against invasive *S. pneumoniae* infection [269-271]. Epithelial and alveolar macrophage expressed TLRs respond to pneumolysin and bacterial cell wall components to produce inflammatory cytokines and chemokines, such as IL-β, TNF-α, IL-6, KC and MIP-2, that activate nearby macrophages and recruit neutrophils to the site of infection [272;273]. Nonetheless, despite mounting evidence supporting the importance of neutrophils in *S. pneumoniae* immunity, there exists a number of other studies that suggest an over exuberant neutrophil responses can increase pathology, resulting in increased mortality without affecting bacterial clearance [274]. The dependence on neutrophils for clearance may depend on the serotype and the infection/colonisation site of the host. Protection has also been associated with a specific induction of IL-17 and MCP-1 in BALB/c mice, which in a recent study have been shown to enhance the recruitment and killing capacity of neutrophils in a nasopharyngeal *S. pneumoniae* colonization model [275].

The role the adaptive immune system plays in a primary *S. pneumoniae* remains to be fully elucidated. A significant role for T cells has been suggested in MHC II KO mice that effectively lack $CD4^+$ T cells and are significantly more susceptible to *S. pneumoniae* pneumonia and bacteraemia than isogenic wild type controls [276]. $CD4^+$ T cell help has also been shown to be required for stimulating IgG responses to pneumococcal phosphorylcholine, surface protein A (PspA) and polysaccharide antigens. There is also accumulating evidence that $Th_1$ cytokines are crucial for bacterial clearance as IL-12 deficient patients develop severe *S. pneumoniae* infections. Inflammatory cytokine production in response to an *in vivo S. pneumoniae* infection is not only important in initiating protective innate immune function but also

in regulating the development of adaptive immunity and immunological memory [277;278]. A recent study has illustrated that IL-1β, IL-6, TNF-α, IL-12 and IFN-γ individually regulate the induction of both protein and polysaccharide specific Ig isotypes and that IL-10 down regulated Ig responses in an isotype non specific manner whereas IL-4 appeared to regulate Ig class switching [273]. However, due to the extensive numbers of pneumococcal serotypes and the pathophysiological differences between colonisation and invasive disease there remains a large gap in our understanding of *S. pneumoniae* infection.

## 1.9 Immunological interactions between respiratory viral infections and secondary bacterial super infections

It is commonly accepted that outside highly controlled laboratory settings, the clinical and pathophysiological characteristics of most infectious diseases are much more complex than implied by a simple one pathogen one disease model. Unlike most animal models used to study infectious diseases, humans are never free of previous or concurrent infections. This is particularly prominent with upper respiratory tract viral infections and their associated bacterial complications. The earliest suggestion that respiratory viral infections can predispose to bacterial disease has been accredited to R.T Laennec in 1803. He observed the prevalence of pneumonia increases following an epidemic of influenza virus ('la grippe') [279]. Although influenza virus is most commonly thought of in this context, many others respiratory viruses including respiratory syncytial virus (RSV), measles virus and rhinovirus may also predispose to secondary bacterial infections [280]. It was not until 1945 that the first well controlled study of secondary bacterial infections was conducted by M. V. de Torregosa et al [281]. They reported that *Haemophilus influenza*, *Streptococcus pneumoniae* and *Staphylococcus aureus* all have the ability to cause fatal pneumonia in an intranasal co-infection model with the mouse adapted influenza virus A/Puerto Rico/8/34 (PR8).

Clinically, with the exception of highly pathogenic strains such as H5N1 and H1N1 (1918 pandemic strain), influenza viral infection is not usually sufficient to cause death from pulmonary causes alone. Although the primary viral infection that killed many healthy adults during the 1918 pandemic was the most prominent clinical feature of the pandemic, it appears secondary bacterial pneumonia was the more common cause of death [282]. In addition, the majority of hospitalisations and deaths caused by more recent influenza outbreaks of 1957, 1968 and 1995 were more

frequently due to secondary bacterial pneumonia or exacerbations of underlying conditions [283-286]. Numerous *in vivo* animal models have provided further evidence supporting these clinical observations, highlighting the synergistic interactions between influenza virus and respiratory bacterial pathogens [287-289].

Despite the considerable clinical and experimental evidence, there remains relatively little knowledge regarding the immunological and molecular basis surrounding the interactions between influenza virus and respiratory bacterial pathogens. Initial studies during the 1918 influenza pandemic produced the concept of 'viral' induced epithelial damage that permitted bacterial access to otherwise inaccessible lung compartments. This mechanism of viral-bacterial synergism was strengthened by histopathological studies during the 1957/59 Asian influenza pandemic which highlighted bacterial adhesion to areas of viral cytotoxicity in the trachea and bronchi [290]. Further investigations proposed influenza virus mediated dysfunction of immune effectors as a mechanism for secondary bacterial infections. Numerous animal models have provided evidence of virus induced leukopenia and granulocytopenia in bacterial co-infection experiments [291-293]. Conversely, other experimental models demonstrate leukocytosis as the prominent clinical presentation of secondary bacterial infection. They argue that large numbers of activated neutrophils, macrophage and inflammatory mediators in the lung exacerbate tissue damage, particularly in combination with bacterial toxins and although the cellular infiltrate is increased the functional capabilities necessary for the clearance of bacteria may be altered [294;295]. A third principal concept for viral induced bacterial secondary infection concentrates on the alteration of cell surface receptor profile and adhesion molecules in response to viral infection. Influenza viruses possess NA which acts to cleave terminal sialic acid residues on host cell glycoproteins allowing the release of viral particles. Exogenously administered bacterial NA has been shown to increase adherence of pneumococcus *in vitro* to tracheal epithelial in an organ perfusion model [296]. It has therefore been postulated that NA contributes to bacterial adherence to epithelial tissue by exposing receptors for bacterial adherence and invasion. Administration of an influenza virus NA inhibitor prevented secondary bacterial pneumonia, thus suggesting that the combination of bacterial and viral NA at the site of infection is an important mechanism in synergism between the organisms [297].

The increasing incursions of highly pathogenic H5N1 influenza virus into humans in Southeast Asia are an eminent warning that we are overdue the next influenza pandemic. It is therefore critical that identification and exploration of the underlying

mechanisms of viral bacterial synergism are revealed in order to provide targets for prevention and treatment using immune therapies, drugs and vaccines.

## 1.10 Thesis objectives

Despite extensive epidemiological and clinical evidence, there remains relatively little knowledge regarding the immunological and molecular basis surrounding the interactions between influenza virus and secondary bacterial super infections. Previous work within the laboratory has demonstrated that a preceding respiratory inflammatory insult can alter subsequent immune responses to a secondary heterologous infection that cannot be explained through cross reactive T cells [298-301]. In reality, each individual has unknowingly acquired his/her unique history of respiratory infections and how this subsequently impacts on their immune responsiveness remains to be determined. We hypothesise that a preceding influenza viral infection induces a transient state of hypo-responsiveness that hinders the lungs capacity to respond and protect against respiratory bacterial pathogens.

In the following thesis I will seek to address the following aims:

1. Develop and characterise an *in vivo* secondary bacterial super infection model.
2. Investigate the temporal relationship between influenza viral infection and the susceptibility to secondary bacterial super infection.
3. Investigate the effects of influenza viral infection on the functionality of alveolar macrophages.
4. Analyse nasopharyngeal commensal flora communities before and after influenza viral infection.

# Materials & Methods

# Chapter 2

## 2.1 Laboratory Animals

### 2.1.1 Mice strains

Eight to twelve week old female BALB/c and C57BL/6 mice were purchased from Harlan Olac Ltd, Bicester UK. CD200R $^{-/-}$ were a kind gift from R. Williams (Kennedy Institute of Rheumatology, Imperial College, London). All mice were maintained in specific pathogen free conditions at Bio Safety Level 2 and kept in accordance with institutional and UK Home Office guidelines.

## 2.2 Pathogen Stocks

### 2.2.1 Influenza A virus

Recombinant influenza A strain A/HK/X31 (haemagglutinin [HA] titre 8192) was a kind gift from Dr Alan Douglas (National Institute for Medical Research, London, UK). The virus was titrated by haemagglutination assay. Human group 'O' red blood cells (RBC) were collected in Alsever's solution to prevent clotting (ratio 1:1). Cells were washed three times in Alsever's solution, each time centrifuging for 5 min at 240 g and re-suspended as a 10 % stock solution in PBS. Influenza stock solution was doubly diluted in PBS in a round-bottom plate. An equal volume (50 µl) of 0.5 % RBC/PBS was added and incubated at room temperature for 1 hour, or until RBCs had settled at the bottom of the plate. The HA titre was defined as the highest dilution of virus capable of causing agglutination, i.e. inhibit RBC precipitation into a defined button at the bottom of the well. The titre was expressed as the reciprocal of the highest dilution of virus showing agglutination, and represents 1 HAU/50 µl of virus.

### 2.2.2 *Streptococcus agalactiae* (Group B Streptococcus - GBS)

Human clinical isolate NCTC10/84 serotype V was a kind gift from Dr Toby Lawrence (Cancer Research, UK). GBS were grown at 37 $^{0}$C to mid logarithmic phase in Todd Hewit Broth (THB) (Sigma, UK) then mixed (1:1) with 80 % glycerol and frozen at -80 $^{0}$C until required. For murine inoculation, fresh THB was mixed (1:10) with an

overnight GBS culture and allowed to grow until mid logarithmic phase was reached. The culture was centrifuged for 10 min at 5000 g and washed twice with 1 ml sterile LPS $^{-/-}$ phosphate buffered saline (PBS). Inoculum dose was calculated by diluting the GBS culture in LPS$^{-/-}$ PBS to an $OD^{600}$ 0.4 which correlates to 1 x $10^8$ colony forming units (cfu) per ml. Recovered bacterial cfu was determined by manually counting colonies of serial tenfold dilutions plated on THB agar plates. GBS colonies were identified by their β–haemolysis when plated on sheep blood agar, their distinct bright orange pigment and colony morphology.

### 2.2.3 *Streptococcus pneumoniae* (D39)

Wild type *S. pneumoniae* (serotype 2) strain D39 (NCTC 7466, National Collection of Type Cultures, London, UK), a kind gift from Dr Jeremy Brown (Centre for Respiratory Research, UCL, London) and a transformant expressing GFP (D39 pGFP $^{302}$ ), a kind gift from Professor P. Andrews (Nottingham, UK) were used. Bacteria were cultured at 37 °C $^{o}$ in 5 % $CO_2$ on blood agar plates or in Todd-Hewitt broth (Sigma) supplemented with 0.5% yeast extract (OXOID) (THY broth) to an $OD^{600}$ of 0.4 (approximately 1 x $10^8$ cfu/ml) and stored at -80°C in 10% glycerol as single-use aliquots. Exact inoculum cfu used in each experiment and the recovered tissue cfu were determined by manually counting colonies of serial tenfold dilutions plated on Columbia agar supplemented with 5 % defibrinated horse blood. Bacteria were further identified by Gram stain, colony morphology, α–haemolysis on blood agar plates and by optochin sensitivity. GFP pneumococcal transformants (pGFP) were checked by UV microscopy for fluorescent activity following culture on blood agar plates and in THY broth.

### 2.2.4 *Pseudomonas aeruginosa* (PAK)

Wild type *Pseudomonas aeruginosa* PAK strain, an exceptionally kind gift from Dr Stephen Lory (Boston, USA), was grown at 37 $^{0}$C to mid logarithmic phase in LB and stored at -80°C in 10 % glycerol as single-use aliquots. For infection, mice were challenged with mid log phase 5 x $10^6$ cfu intranasally. Exact inoculum cfu used in each experiment and the recovered tissue cfu were determined by manually counting colonies of serial tenfold dilutions plated on LB agar.

## 2.3 Reagents used for *in vivo* studies

### 2.3.1 *In vivo* use of TLR ligands

Mice were intranasally (i.n.) challenged with 1 μg flagellin FliC (Apotech, Switzerland), 1 μg LPS (Sigma), 25 μg LTA or 1 μg recombinant chemokines KC or MIP-2α (R & D systems). All reagents, except LPS, were free of physiologically significant levels of endotoxin as measured by the LAL system, 6.4 endotoxin U/mg.

## 2.4 Mouse infection models

### 2.4.1 Single infection model

On day 0, BALB/c or C57BL/6 mice were anaesthetised with isoflourane and intranasally inoculated with either 50 HAU influenza A virus, which equates to 1.25 x $10^5$ pfu, or a specified dose of bacterial pathogen, unless otherwise stated. Infectious agents were diluted in endotoxin free sterile PBS and administered i.n. in a volume of 50 μl to mice held in an upright position. In some experiments, infected mice were re-infected with an identical titre of homologous virus or bacteria 2-6 weeks after the primary infection. Groups of 4-5 mice were weighed and followed daily for illness and mortality. Mice found to be moribund were culled and considered to have died on that day. Mice were harvested at predetermined time points post infection by injection of 3 mg pentobarbitone and exsanguinated via the femoral vessels.

### 2.4.2 Co-infection model

On day 0, BALB/c or C57BL/6 mice were inoculated with 50 HAU influenza A virus as previously described in 2.4.1. At the same time as, or at varying time points post influenza infection mice were inoculated with a pre specified dose of a bacterial pathogen, unless otherwise stated. Groups of 4-5 mice were weighed and followed daily for illness and mortality. Mice were culled at predetermined time points post GBS infection by injection of 3 mg pentobarbitone and exsanguinated via the femoral vessels.

### 2.4.3 Resolved heterologous infection model

On day 0, BALB/c or C57BL/6 mice were inoculated with either 50 HAU influenza A virus or a specified dose of bacterial pathogen, unless otherwise stated, as previously described in 2.4.1. Mice were allowed to resolve the primary infection as

shown by viral plaque assay or bacterial culture and were subsequently infected with either influenza virus or a specified bacterial pathogen. Groups of 4-5 mice were weighed and followed daily for illness and mortality. Mice were culled at predetermined time points post secondary infection by injection of 3 mg pentobarbitone and exsanguinated via the femoral vessels.

## 2.5 Sample recovery and cell preparation

### 2.5.1 Tissue, serum and nasal wash recovery

Bronchoalveolar lavage (BAL), lung tissue, spleen, serum and nasal wash were harvested by methods described previously [303]. Blood was first harvested by exsanguination via the femoral vessel and, after clotting at 4 $^0$C, centrifuged for 8 minutes at 5000 x g. The serum was isolated and frozen at -20 $^0$C for analysis of antibody and cytokines. BAL was obtained by inflating the lungs of each mouse 6 times with 1.5 ml 1 mM EDTA in minimal essential media (MEM) and placed in sterile tubes at 4 $^0$C. BAL was centrifuged for 5 minutes at 240 x g and the supernatant removed and stored at - 20 $^0$C for analysis of cytokines by ELISA. The pellet was re-suspended in RPMI-1640 supplemented with 10 % fetal calf serum (R10F) at a final concentration of 1 x $10^6$ cells per ml for further analysis. Lung tissue and spleen were disrupted to a single cell suspension by passage through a 100 µM sieve (BD labware, New Jersey, USA). The cell suspension was subsequently spun for 5 minutes at 240 x g rpm and red blood cells lysed by resuspending pellets in ACK buffer (0.15 M ammonium chloride, 1 M potassium hydrogen carbonate and 0.01 mM EDTA, pH 7.2) for 3 minutes at room temperature before centrifugation for 5 minutes at 240 x g and washing in R10F. Cell viability in all tissues was assessed using Trypan Blue exclusion and subsequently re-suspended in R10F to a final concentration of 1 x $10^6$ cells/ml. Nasal wash was obtained by washing the nasopharyngeal cavity with 200 µl sterile PBS, collected and frozen at -20 $^0$C for antibody ELISA analysis.

### 2.5.2 Isolation of lung of Type II epithelial cells

Lung type II epithelial cells were isolated as described previously [304]. Briefly, lungs were perfused by the infusion of 10 ml PBS into the right ventricle of the heart. Lungs were subsequently inflated with 2.5 ml of 5 mg/ml dispase II solution (Roche, Basel, Switzerland) and then allowed to collapse naturally. 0.5 ml of 1 % low melting point agarose was then slowly injected into the lungs and immediately allowed to solidify by

packing the lungs in ice. Lungs were then removed and incubated for 40 minutes in dispase solution before being transferred to DMEM containing 50 µg/ml of DNAse I solution (Roche, Basel, Switzerland) and the digested tissue teased away from the upper airways. Digested lung tissue was disrupted to a single cell suspension by passage through a 100 µM sieve (BD Labware, New Jersey, USA). Epithelial cells were enriched by negative selection, with removal of hematopoietic and endothelial cells by MACS separation. Briefly, lung homogenate was incubated with rat anti-mouse CD45 or CD90 antibodies (BD Pharmingen, Heidelberg, Germany), followed by anti-rat Dynabeads (Invitrogen) and applied to an MS column (Miltenyi Biotec, Gladbach, Germany) in the presence of a magnetic field. Unlabelled epithelial cells were washed through with buffer (PBS containing 0.5 % BSA and 2 mM EDTA).

### 2.5.3 Generation of bone marrow (BM) derived macrophages and DCs

Femurs were removed from BALB/c or C57BL/6 mice and bone marrow extracted by flushing extensively with RPMI-1640 media. RBC depleted bone marrow cells were washed and counted by Trypan blue exclusion. To generate BM macrophages, 2 x $10^5$ cells/ml were plated out with 20 ng/ml M-CSF in 10 mls RPMI with 20 % FCS, 0.1 U/ml penicillin / 0.1 µg/ml streptomycin and 10 % HEPES, and incubated at 37 °C for 72 hours. At this time, a further 5 mls of medium containing M-CSF (20 ng/ml; PeproTech Inc., Rocky Hill, NJ) was added on top of the existing 10 mls and incubated at 37 °C for another 72 hours. Media was subsequently removed and cells incubated in versene for 20 minutes at room temperature. Cells were then scraped from the plates, washed and re-suspended in R10F and counted. To generate BM dendritic cells, 2 x $10^5$ cells/ml were plated out in 10 mls RPMI with 10 % FCS, 0.1 U/ml penicillin / 0.1 µg/ml streptomycin and 10 % J558 supernatant (containing GM – CSF), and incubated at 37 °C for 72 hours. At this time, 5 mls of media was carefully removed and replaced with 5 mls of fresh media with 10 % J558 supernatant and incubated at 37 °C for another 72 hours. Floating and weakly adherent cells were collected by gentle pipetting, washed and re-suspended in R10F and counted.

### 2.5.4 Stimulation of BM macrophages/DCs

BM derived macrophages and DCs were cultured at 2 x $10^6$ cells/ml in 200 µl R10F in 96 well flat bottomed plates. Cells were stimulated with IFN-γ (10 ng/ml or 100 ng/ml), LPS (10 ng/ml or 100 ng/ml) or influenza virus (8 HA or 16 HA), *S. pneumoniae* (MOI 10, 5, 2) and GBS (MOI 10, 5, 2). Some cells were also cultured with OX110 (CD200R agonistic antibody) to a final concentration of 10µg/ml. Cells

were incubated at 37 °C and at varying times after stimulation (as indicated in the text), supernatants were removed for cytokine/chemokine analysis and cells stained for surface markers by flow cytometry as described below.

## 2.6 Flow Cytometric Analysis

### 2.6.1 Cell surface antigens

Cells were stained for surface markers and analysed by flow cytometry as described previously [303]. All antibodies were purchased from BD Pharmingen (Heidelberg, Germany) unless otherwise stated (See Table 2.1). Briefly, a minimum of 2 x $10^5$ or maximum 1 x $10^6$ BAL, lung or spleen derived cells were stained using various combinations of FITC, PE, PerCP (Pe-Cy5.5), Pe-Cy7 APC or biotin conjugated anti-mouse antibodies. All antibodies were diluted in PBS containing 1 % BSA/0.05 % sodium azide (PBA). Cells were stained for 30 minutes on ice, washed with PBA and spun for 5 minutes at 240 x g. For biotin conjugated antibodies streptavidin constructs conjugated to appropriate fluorophores were used. After washing, cells were then fixed for 20 minutes at room temperature with 2 % formaldehyde/PBS. Cells were then washed in PBA, data acquired on a BD FACS Canto II or LSR II and 30,000 lymphocyte or myeloid events analysed with CellQuest Pro software (BD Biosciences, Belgium) or FloJo analysis programme. Forward scatter and side scatter gates were used to exclude debris in organ homogenates.

### 2.6.2 Intracellular cytokine expression

To detect intracellular cytokines, 1 x $10^6$ cells/ml were incubated with 50 ng/ml PMA, 500 ng/ml ionomycin (Calbiochem, Nottingham, UK), and 10 µg/ml brefeldin A for 3 hours at 37 °C. Cells were then stained for external markers such as anti-CD4-APC or anti-CD8-PerCP and fixed as described above. After permeabilisation with PBS containing 1 % saponin / 1 % BSA / 0.05 % azide (saponin buffer) for 10 minutes, cells were stained with anti-TNF-α FITC, anti-IFN-γ-Biotin. 30 minutes later cells were washed once in saponin buffer and once in PBA. Data was then acquired as described above.

### 2.6.3 Apoptosis Analysis

#### 2.6.3.1 Annexin V staining

For apoptosis analysis, binding of FITC or PE-labelled Annexin V was detected according to manufacturers' instructions (BD Pharmingen, Heidelberg, Germany).

Briefly, cells were washed in PBS and re-suspended in Annexin-binding buffer containing Annexin V-PE or FITC and other extracellular markers of interest. After washing in binding buffer, 7-AAD was added to determine dead cells. Apoptotic cells were identified as 7-AAD$^-$/Annexin V$^+$. Data was then immediately acquired, collecting 30,000 lymphocyte events.

2.6.3.2 TUNEL assay

Apoptotic cells were detected using the *in situ* cell death detection kit (Roche). Briefly, cells were stained for differential surface markers and subsequently fixed in 2 % PFA, as described in section 2.6.1. Cells were then made permeable with 0.1 % Triton X-100 and 0.1 % sodium citrate. Cells were incubated for 60 minutes at 37 $^0$C in the TUNEL reaction mixture. Cells were subsequently washed twice in PBS and re-suspended in PBA for cytometric analysis

Table 2.1 – Antibody reagents

| Product Name | Clone | Catalogue Number | Company |
|---|---|---|---|
| CD31 | MEC 13.3 | 553371 | BD |
| CD45 | C363.16A | 553100 | BD |
| CD45 | RM4-5 | 553052 | BD |
| CD8 | 53.6.7 | 11-0081-82 | BD |
| CD11B | m1/70 | 01714D | Pharmingen |
| CD11C | HL3 | 550261 | BD |
| MHC II | M5/1/4.15.2 | 11-532-81 | BD |
| CD86 | GL1 | 553692 | BD |
| CD3 | 17A2 | SC18843 | Santa Cruz |
| F4/80 | BM8 | 13481/85 | BD |
| CD45rb | RA3-6B2 | 553093 | BD |
| CD200 | OX-90 | MCA1958 | Serotec |
| GR-1 | RB6-8C5 | 558111 | BD |
| LY-6G | RB6-8C5 | ab25024 | Abcam |
| B220 | RA3-6B2 | 553093 | BD |
| IFN γ | XMG1.2 | 554411 | BD |
| TNF α | MP6-XT22 | 554419 | BD |
| CD200R | OX-110 | MCA2281F | Serotec |
| CD4 | RM4-5 | 553051 | BD |
| TCR αβ | H57-957 | HM3601 | Caltag |

## 2.7 Tissue Imaging

### 2.7.1 Haematoxylin and Eosin staining of lung tissue

The azygous lobe was inflated and fixed with 2 % formalin in PBS. The tissue sample was subsequently embedded in paraffin wax and 4 µM sections were stained with haematoxylin and eosin (David Essex, Imperial College, UK).

### 2.7.2 Immunofluorescent staining for CD200

Lungs from C57BL/6 mice, at days 0, 3 and 7 after influenza infection, were inflated with 1.5 ml OCT (Tissue-Tek) and snap frozen in OCT freezing medium by liquid nitrogen flotation. All frozen tissues were stored at -80 °C. Cryostat sections (5-8 µm) were fixed in cold 80 % acetone and 20 % methanol, air dried, then blocked with 15 % normal goat serum for 30 minutes at room temperature. Sections were then incubated in primary mAbs to murine CD200 (OX90) (3 µg/ml) for 2 hours at room temperature, extensively washed in PBS, and then incubated for 1 hour in Alexa-488 conjugated goat anti-rat IgG (Molecular Probes). Sections were then washed three times in PBS and wet mounted for fluorescent microscopy. Sections were examined under a Nikon ECLIPSE TE2000-U fluorescence microscope.

### 2.7.3 Immunofluorescent staining for p65 translocation

For p65 translocation analysis, AM were fixed in 4 % PFA, permeabilized in 0.2 % Triton-X and stained with an rabbit-anti-p65 antibody (Santa Cruz) and Alexa 488-anti-rabbit antibody (Molecular Probes). Propidium iodide was used for nuclear staining.

## 2.8 Determination of pathogen load in tissues

### 2.8.1 Influenza-specific plaque assay

Lung homogenates were freeze thawed three times, centrifuged at 5000 x g and supernatants titrated in doubling dilutions on Madine Darby canine kidney (MDCK) cell monolayers in flat bottomed 96 well plates. After incubation at room temperature for 3 hours, samples were over-layed with 1 % methycellulose and incubated for 72 hours at 37 $^{0}$C. Cell monolayers were washed and incubated with anti-influenza antibody (Serotec) followed by anti-mouse-HRP (Dako) and infected cells detected using 3-amino-9-ethylcarbazole substrate. Infectious units were then enumerated by light microscopy and total plaque forming units per lung quantified (number of plaques × dilution factor × total volume of lung homogenate).

### 2.8.2 Enumeration of recovered bacterial cfu from infected tissues

Group B Streptococcus, *S. pneumoniae* and *P. aeruginosa* titre was determined by serial dilution of 20 µl aliquots from single cell suspensions of each tissue in sterile PBS. Serial dilutions were plated on THB agar (GBS), Columbia blood agar (*S. pneumoniae*) or LB agar (*P. aeruginosa*) and incubated overnight at 37 $^0$C. The total cfu per tissue was then determined manually by counting evident bacterial colonies. (Number of colonies x dilution factor x original cell suspension volume).

## 2.9 Soluble mediator detection

### 2.9.1 Cytokine detection using ELISA

IFN-γ, TNF-α, IL-6, IL-10, KC, and MIP-2α in BAL fluid and lung homogenate were quantified using OptEIA kits and following manufacturer's instructions (Pharmingen). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 µl of capture antibody diluted in the recommended buffer and incubated according to the manufactures instructions. After 5 washes with PBS containing 0.5 % Tween-20, plates were blocked with 200 µl PBS containing 10 % FCS and left for 1 hour at room temperature. Samples and standards (diluted in PBS + 10 % FCS) were then incubated for a further 2 hours at room temperature. After 5 washes, bound cytokine was detected using biotinylated antibodies pre-mixed with avidin-HRP followed by tetramethylbenzidine and hydrogen peroxidase. Optical densities were read at 450 nm using 570 nm as a reference. The mean optical density of wells containing no cytokine was subtracted from the results obtained for samples and standards. The concentration of cytokine in each sample was calculated from a standard curve.

### 2.9.2 Nitric oxide (NO) detection

The concentration of nitrite in BAL and lung homogenate was used as a measure of NO production and was quantified using the Greiss reagent kit. Briefly, samples and standards (50 µl) were added to a microtiter plate and treated with 1 % sulphanilamide for 10 minutes at room temperature. Subsequent addition of 0.1 % naphthylethylenediamine (NED) in 2.5 % $H_3PO_4$ yielded a magenta colour in the presence of NO. Optical densities were read at 550 nm. The mean optical density of wells containing media alone was subtracted from the results obtained for samples and standards. The concentration of NO in each sample was calculated from a standard curve.

## 2.10 Quantitative RT-PCR

### 2.10.1 Cytokine mRNA analysis

RNA was extracted from frozen lung lobe using the nucleospin RNA kit (Macherey & Nagel, Switzerland) and cDNA generated for each sample using the superscript II reverse transcriptase using random hexamers (Invitrogen). Real time PCR was performed using platinum SybrGreen super mix (Invitrogen) and the Rotor-gene 6000 (Corbett). The copy number of the gene of interest was calculated as arbitrary units using two standard curves method and normalized to 18S. Similar results were obtained using the GAPDH or β-actin as housekeeping genes. Primers used: MIP-2α (CXCL2) forward 5'-ATCCAGAGCTTGAGTGTGACGC-3' and reverse 5'-AAGGCAAACTTT TTGACCGCC-3', KC (CXCL1) forward 5'-GCCAATGAGCTGCGCTGT-3' and reverse 5'-CCTTCAAGCTCTGGATGTTCTTG-3', TNF-α forward 5'- CCTCCACTTG GTGGTTTGCT-3' and reverse 5'-CATCTTCTCAAAATTCGAGTGACAA-3, β-actin forward 5'-GCTTCTTTGCAGCTCCTTCGT-3' and reverse 5'-CGTCATCCATGGCG A ACTG-3' and 18S forward 5'-ACATCCAAGGAAGGCAGCAG-3' and reverse 5'-TTTTCGTCACTACCTCCCCG-3'

## 2.11 BAL proteomic analysis

### 2.11.1 Protein concentration analysis

BAL fluid protein concentration was determined using manufactures instructions provided with the Pierce BCA protein assay kit (Rockford, Illinois). In brief, individual BAL was pooled within each group to be analyzed. Pooled sample was centrifuged for 10 minutes at 15700 x g and subsequently filtered through a 0.2 µm sieve to ensure removal of any resident cell debris. Working reagent was added to samples and standards at a 4:1 ratio and incubated at 37 °C for 30 minutes. Absorbance was measured at 490 nm, and protein concentration calculated by comparison with an albumin standard.

### 2.11.2 SDS Page protein separation

1.5 mm 15 well NuPage 4-12 % Bis-Tris gels were prepared for use as described in the manufactures instructions (Invitrogen, UK). BAL samples were prepared as described in section 2.11.1 and diluted 1:1 in 2 x loading buffer (1 M Tris pH 6.8, 20 % glycerol, 10 % SDS, 0.1 % Bromophenol and 20 % DTT). Loading samples were

heated to 80 $^0$C for 5 minutes and centrifuged at 3000 x g for 1 minute before gel loading. SeeBlue pre-stained standard was used as a molecular weight marker in all experiments (Invitrogen, UK). After 2 hrs at 100 V gels were placed in Coomassie stain (40 % MeOH, 10 % Acetic Acid, 50 % $dH_20$ mQ, 0.2 g Coomassie Brilliant Blue) and shaken overnight at room temperature. Gels were then de-stained in 10 % Acetic Acid, 30 % MeOH and 60 % $dH_20$ until protein containing bands were clearly visible. After sufficient de-staining gels were rehydrated in $dH_20$ and stored at 4 $^0$C until further use.

### 2.11.3 Mass spectrometry and protein elucidation

Proteins bands of interest were subjected to in gel digestion with trypsin as described previously [305]. Samples were analysed by liquid chromatography coupled to tandem electrospray mass spectrometry (LC-ESI-MS/MS). Spectra were recorded using a Q-Tof spectrometer (Micromass, Manchester, UK) interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 0.1 % formic acid and aliquots were injected onto a 300 μm x 5 mm Pepmap C18 column (LC Packings, Amsterdam, NL) and eluted with an acetonitrile/0.1 % formic acid gradient. The capillary voltage was set to 3,500 V. A survey scan over the *m/z* range 400-1300 was used to identify protonated peptides with charge states of 2, 3 or 4, which were automatically selected for data-dependent MS/MS analysis, and fragmented by collision with argon. The resulting product ion spectra were transformed onto a singly charged *m/z* axis using a maximum entropy method (MaxEnt3, Waters) and proteins were identified by correlation of uninterpreted spectra to entries in SwissProt/TrEMBL, using ProteinLynx Global Server (Version 1.1, Waters) [306]. The database was created by merging the FASTA format files of SwissProt, TrEMBL and their associated splice variants (1,768,175 entries at the time of writing). No taxonomic or protein mass and *pI* constraints were applied. One missed cleavage per peptide was allowed, and the initial mass tolerance window was set to 100 ppm. In parallel, the spectra were also searched against the NCBI nr database (3,879,234 sequences at time of search) using Mascot (www.matrixscience.com). For an identification to be considered valid we required that two or more peptides independently matched the same protein sequence, that the peptide score was significant (typically greater than 55 ($p<0.05$), and that manual interpretation confirmed agreement between spectra and peptide sequence. In addition Mascot searches of all spectra were performed against a randomised version of the NCBI database using the same parameters as in the main search. In no case did this

search retrieve more than a single peptide, and in all instances the peptide score was below the 0.05 significance level.

## 2.12 16S rRNA analysis

### 2.12.1 DGGE gel casting and electrophoresis

Denaturing gradient gels (DGGE) were produced using a GM-100 Gradient Maker (CBS Scientific, Del Mar, USA) and run using a Protean II vertical electrophoresis system (Bio-Rad, Hercules, USA). In brief, an 8 % polyacrylamide gel (0.75 mm thick) with a gradient of 60- 30 % of denaturants (urea and formaldehyde) was cast and placed in 0.5 x Tris-acetate borate. All gels were run at 100 v for 16 hours at 60 ºC. Gels were analysed by incubating in $H_2O$/ethidium bromide for 60 minutes at room temperature and subsequently read using a UV illuminator.

### 2.12.2 16S rRNA amplification and DGGE analysis

For DGGE analysis, products were amplified using a two step, nested PCR approach. For the first round, 16S rRNA genes were amplified using the primers 339F-5'-ACTCCTACGGGAGGCAGCAGT-3' and 907R-5'-CCGTCAATTCMTTTGAGTTT-3' [307;308]. 25 µl PCR reactions were set up containing 2 µl of template, 2.5 µl 10 x buffer (Roche Applied Biosystems), and final concentrations of 1.5 mM Mg, 6 % DMSO, 0.5 µM primers, 0.8 mM total dNTP (0.2 mM each) and 1U HotStart Polymerase (Roche Applied Biosystems). Cycling conditions were one cycle of 95 °C for 5 minutes, then 20 cycles of 95 ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 60 seconds followed by a final extension of 72 ºC for 5 minutes. PCR products were diluted (1:10) and 2 µl used as template for the second round of amplification. Primers 339F (with a GC-clamp) 339F-GC-5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3' and 539 R 5'-GTA TTA CCG CGG CTG CTG GCA C-3' [309] were used. Reaction and cycling conditions were the same as used in the first round except for the number cycles which was incremented to 32. Two rounds of PCR were used to minimize the risk of unspecific and maximise the sensitivity of bacterial amplification. 10 µl of product and 2 µl of loading dye were loaded onto the gels and after electrophoresis for 17 hours at 100 V at 60ºC using 0.5 TAE buffer, gels were stained with ethidium bromide solution (2 µl EtBr in 200 ml Water) and photographed using the Biotech-IT Imaging System.

### 2.12.3 DNA Cloning and sequence analysis

16S rRNA gene sequences were initially amplified in duplicate 25 µl of PCR reaction which included (final concentration): 2 µl of sample, 2.5 µl 10 x HotStart buffer (Roche Applied Biosystems), 1.5 mM $MgCl_2$, 6 % DMSO, 0.8 mM total dNTPs, 1U HotStart polymerase (Roche Applied Biosystems) and 0.5 µM of primers 339F – 5'-ACTCCTACGGGAGGCAGCAGT-3' and 907R-5'CCGTCAATTCMTTTGAGTTT-3'. Reaction mixtures were amplified with a 5 minute initial denaturation step at 95 $^{o}$C followed by 32 cycles at 95 $^{o}$C for 30 seconds, 55 $^{o}$C annealing for 30 seconds and 72 $^{o}$C extension for 60 seconds. A final extension step of 5 minutes followed at 72 $^{o}$C to ensure all products contained intact ends for cloning. After the initial PCR the duplicated samples were pooled and run on a 1.5 % agarose gel for 1 hour at 100 V. Subsequent DNA bands were excised and purified according to QIAquick Gel Extraction Kit. Subsequent DNA product was cloned into *E. Coli* using a pGEM-T easy vector (Promega) as described in the manufacturer's instructions. White colonies were picked, solubilised in 35 µl of liquid LB and arrayed on a 96 well plate and sent off for sequencing. Plasmid DNA was purified using the Qiagen Plasmid Miniprep Kit according to the manufacturer's instructions and sequenced using the primer SP6.

### 2.12.4 Phylogenetic analysis and tree generation

DNA sequence chromatograms were uploaded to the ribosomal database (http://rdp.cme.msu.edu/), vector sequences trimmed (using the LUCY program) and remaining sequences quality control checked (using PHRED). Sequences of high quality were downloaded, primer regions additionally trimmed and only full length sequences ranging from 359 until 906 (*E. coli* numbering of 16S rRNA gene) were included for further analysis. No chimeric sequences were detected using Bellerophon (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi).

Briefly, 295 (including 52 from a human COPD patient) high quality sequences were aligned with NAST (http://greengenes.lbl.gov) and manually curated. A distance matrix calculating the pair wise distances of the high quality sequences was created on the green genes website (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), masking the hyper-variable regions with the lane mask utility. Operational taxonomic units (OTUs) were designated using DOTUR (Distance based OTU and richness determination) by the furthest-neighbour algorithm. For the creation of the phylogenetic tree, a cut off of 99 % was set and each OTU was then assigned an

organism name, based on the phylogenetic placement using sequence match of the ribosomal database (20 best match sequences define taxanomic rank), and compared to NCBI BLAST results for the percentage of sequence identity. The numbers of *16S rRNA* gene phylotypes were calculated at 99 % sequence identity with the furthest neighbour clustering in the program DOTUR. Evolutionary distances were calculated with the Jukes-Cantor (tree-Markov) model and phlyogenetic trees determined by the Neighbour-Joining method, with 1000 trees generated using mega (http://www.megasoftware.net/). Bootstrap confidence levels are shown at the tree nodes for all values. OTU designations are located at the termination of each branch and represent potential organism names. Abundance data for each OTU were calculated and annotated using treedyn (http://www.treedyn.org/).

## 2.13 Statistics

All experiments analysed contained 4-5 individual mice per group unless stated in the text. GraphPad Prism software was used for all statistical calculations. Depending on the experiment and the datasets to be compared several statistical tests were used: Non parametric Mann Whitney *t* test, unpaired two-tailed *t* test assuming unequal variance and non Gaussian distribution, non parametric Kruskal Wallis with Dunns post test and Log rank Mantel-Cox test to determine significance in survival curve analysis. The Bonferroni correction was applied when needed. Data are presented as the mean ± standard error of the mean (SEM). p vales < 0.05 were considered significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

# RESULTS

# Chapter 3

## 3.0 Bacterial infection model development

### 3.1 Introduction

#### 3.1.1 The need for robust bacterial infection models

Up until the advent of molecular biology and the genomics era, very little progress was made in understanding bacterial pathogenesis since the discovery of antibiotics by Sir Alexander/ra Fleming in the 1940's [310-312]. The development of efficacious vaccines against many of the important childhood bacterial infections added to the common misconception that the unrelenting struggle against bacterial infections had been won. However, despite extensive vaccination programmes in both the developed and developing world and the ubiquitous use of antibiotics, bacterial infections alone still account for 7.6 % of global deaths annually [313]. This figure excludes the number of deaths caused by respiratory bacterial pathogens that are categorised within the same Global Burden of Disease (GBD) code (W038) as respiratory infections [313]. The addition of this data would significantly increase the proportion of global deaths attributed to bacterial pathogens. In the UK, the most commonly acquired bacterial infection is *S. pneumoniae*. It is responsible for the majority of community acquired pneumonia cases and can result in invasive pneumococcal septicemia in more than 20 % of patients [314;315]. Worldwide, *S. pneumoniae* is responsible for more deaths from invasive infections than any other bacterium and is responsible for 25 % of all preventable deaths in children under the age of 5 and more than 1.2 million infant deaths per year [316-318]. In addition, there is increasing concern over the emergence of multi drug resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* (MDRTB), *S. pneumoniae* (DRSP) and Group A streptococcus amongst others and the increasing prevalence of serotypes of *S. pneumoniae* and *H. influenzae* that are not contained within the existing multi-valent vaccines [319-322]. This reality highlights the need for reliable and physiologically relevant models of infection to investigate the mechanisms and genetic basis of bacterial pathogenesis.

### 3.1.2 Existing respiratory bacterial infection models

As a result of these growing concerns, there is a need for renewed interest into advancing our understanding of the mechanisms of bacterial pathogenesis in the quest to develop novel anti microbial therapeutics. The majority of bacterial pathogens colonise and infect the body's mucosal surfaces, such as the respiratory or gastrointestinal tract. However, respiratory infections, including viral/bacterial bronchiolitis and bacterial pneumonia, account for almost 20 % of annual deaths worldwide in children under the age of 5 years and are responsible for the highest burden of disease of all infectious diseases, exceeding that of HIV/AIDS, Tuberculosis, Malaria and other highly publicised infectious diseases [2;12;313]. This has lead to the development of a plethora of both *in vivo* and *in vitro* models that investigate the pathogenesis of the major respiratory bacterial infections including, *Mycobacterium tuberculosis*, Streptococcal species (particularly *S. pneumoniae, S. pyogens and S. agalactiae)*, *Haemophilus influenzae*, *Staphylococcus aureus, Bordetella petussis* and *Pseudomonas aeruginosa* [323-330]. Despite this heightened interest and the large number of models used, several caveats and limitations remain when investigating bacterial pathogenesis in *in vivo* animal models. The major limitations reflect the physiological relevance to human clinical disease; this can be affected by the particular bacterial strain/serotype used, the route of infection and the strain of inbred mouse utilised. The huge numbers of possible combinations of these parameters result in confusing conclusions and difficulty in interpreting results across similar infection models. However, despite these problems, the use of forward genetic studies in inbred mice strains has led to the discovery of key innate response pathways that validate genetic determinants of susceptibility *in vivo* [331;332].

*S. pneumoniae* has been studied extensively in both *in vitro* and *in vivo* models. Nonetheless, due to historical reasons the majority of *in vivo* models concentrate on investigating the microbiology of the pathogen and the roles its virulence factors play in pathogenesis. Despite a large proportion of research focussing on multivalent conjugate vaccine development and the role complement plays in immunity to *S. pneumoniae* infection, there is little examination of the cellular immune response elicited in response to *S. pneumoniae* infection [266-268;333]. Furthermore, the existence of over 90 distinct serotypes of *S. pneumoniae* and the use of intra venous inoculation to study endothelial invasion, which occurs during pneumococcus meningitis, results in a lack of consistency and standardisation across the *S. pneumoniae* infection field. Research into *Streptococcus agalactiae* pathogenesis,

generally referred to as Group B streptococcus (GBS), follows a similar pattern. GBS is the leading cause of invasive bacterial infections in human neonates and is an emerging disease in immunocompromised adults [212]. Little is known about the pathogenesis of GBS infection in adults and is thought to infect neonates *in utero* or by aspiration of infected vaginal fluids into the neonate lung during *partum* [205;207;210]. As with *S. pneumoniae*, the majority of research into GBS investigates the importance of its virulence factors and the mechanisms by which GBS invades the meninges resulting in meningitis. There is a lack of investigation into the significance of cellular immunity to GBS and the mechanisms by which GBS can asymptomatically colonise mucosal surfaces, such as the upper respiratory tract, and occasionally result in invasive disease.

### 3.1.3 Genetic basis to respiratory bacterial susceptibility

Genetic epidemiology, including twin studies, provides robust evidence that genetic variation in human populations contributes to susceptibility to infectious disease. Even with today's medicine, infectious disease remain a major selective pressure and the genes involved in the immune response are the most numerous and diverse in the human genome, indicating the evolutionary advantages of a varied immunological response to a wide range of infectious pathogens [334]. However, despite the discovery of innate immune genes, such as those encoding Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain-like receptors (NLRs), there remains a large gap in our understanding of the huge variation in individual outcomes following exposure to potentially life threatening pathogens [335]. Susceptibility to infection and many other human diseases arises from the complex interaction of environmental and host genetic factors. This can involve the interaction of many genetic loci or can be the result of a single Mendelian gene disorder. An example of a single gene disorder that influences infection specific susceptibility is the protection afforded by sickle cell heterozygosity against falciparum malaria [336;337].

Another example in which a single gene mutation can result in increased susceptibility to an infectious pathogen is the susceptibility to *Mycobacterium bovis* (BCG) replication in mice that carry a single G169D mutation in the *Nramp1/Slc11a1* gene [338-340]. Nramp1 is responsible for the depletion of divalent cations in the phagolysosome that results in the inhibition of intracellular replication [341]. Experiments *in vitro* have demonstrated its role in the intracellular replication of *Salmonella typhimurium* in macrophage and it has been shown that polymorphic

variants at or near the human *NRAMP1* gene have been identified to associated with susceptibility to a number of infections and inflammatory conditions [342;343].

By linking specific genotypes to disease susceptibility phenotypes we can better understand the genetic basis for inter-individual differences in disease susceptibility as well as gain insight into how gene-environment interactions influence infection outcomes. By using genetically distinct and specific knockout mouse strains one can dissect interactive pathways and molecular events modulating host resistance or susceptibility to specific pathogens.

### 3.1.4 Aims and Hypothesis

Human studies on the pathogenesis of respiratory bacterial infections are invaluable, but animal models provide an unbiased tool for discovering novel mechanisms and genes contributing to disease outcomes under controlled laboratory environments. Therefore we wished to develop a robust and reproducible model of respiratory bacterial infection that is physiologically significant but is non lethal. To fulfil this aim we will:

1. Determine a non lethal infective dose of Group B streptococcus in commonly used mouse strains.
2. Characterise the immunological response needed for clearance of Group B streptococcus infection and identify histopathological consequences of infection.
3. Translate technical expertise developed using the Group B streptococcus infection model to characterise a more clinically relevant *S. pneumoniae* mouse infection model.

## 3.2 Results

Before investigating the influence respiratory viral infections have on immunity and pathology to a subsequent bacterial infection, a clear understanding of a common respiratory bacterial infection must first be examined. We selected Group B streptococcus (GBS) and *S. pneumoniae* as two relevant respiratory commensals that infect the host via the respiratory tract. The rational for using two separate bacterial species was to determine whether immunological and pathological parameters are shared between closely related respiratory pathogens or are species specific. A significantly different response would inform us which strain is more appropriate to use in our secondary bacterial infection model. The first part of this

results chapter will explain the development and characterisation of a GBS infection model before moving on to examine a *S. pneumoniae* infection model.

### 3.2.1 Group B streptococcus infection model characterisation

GBS is a leading cause of invasive bacterial infection in human newborns and is increasingly recognized as a pathogen in adult populations. To date, no suitable murine respiratory infection model for GBS exists. The majority of existing GBS infection models utilise high doses of bacteria that cause systemic toxic shock resulting in death within 24 to 48 hours. This is an unlikely situation in man; rather, bacteria asymptomatically colonise the upper respiratory tract and when presented with an opportunity, such as a virus infection or an altered state of homeostasis, invade surrounding tissues and result in infection. The response to GBS will clearly depend upon bacterial dose and mouse strain employed. Therefore, both BALB/c and C57BL/6 mouse strains were initially used in a comparative study to investigate immune responses to varying doses of GBS.

### 3.2.2 GBS administered at 5 x $10^6$ cfu induces a strong inflammatory response in the airway and lung of BALB/c mice that is non lethal

Both strains of mice were intranasally inoculated with varying doses of GBS as described in Figure 3.1. Mice were weighed daily over the course of 7 days and at specified time points total lung and airway cellular infiltrates were enumerated and analysed by flow cytometry (FCM). In both strains of mice there was no statistically significant difference in weight loss across all inoculum doses (Figure 3.2 a & b). However as a general trend, increasing the inoculum dose increased weight loss in C57BL/6 mice and slowed weight gain in BALB/c mice during the first three days of GBS infection. In both strains of mice 5 x $10^7$ colony forming units (cfu) (similar to the dose used by others) of GBS resulted in systemic sepsis, as defined by the presence of cfu in the peripheral blood (data not shown), and 100% mortality by day 1 (C57BL/6) and day three (BALB/c) post infection. It has been demonstrated previously, in respiratory infection models, that pathogen induced weight loss and clinical illness often reflects the amplitude of cellular infiltrate into both the lung and airway compartments. All GBS inoculum doses induced a rapid cellular infiltrate into the lung tissue in both C57BL/6 and BALB/c mice by day one post infection (Figure 3.2 c & d). In contrast, the airway cellular response observed in BALB/c mice was more than twice the amplitude of that seen in C57BL/6 mice across all doses 24 hours post infection (Figure 3.2 e & f). Therefore, 5 x $10^6$ cfu GBS was chosen to

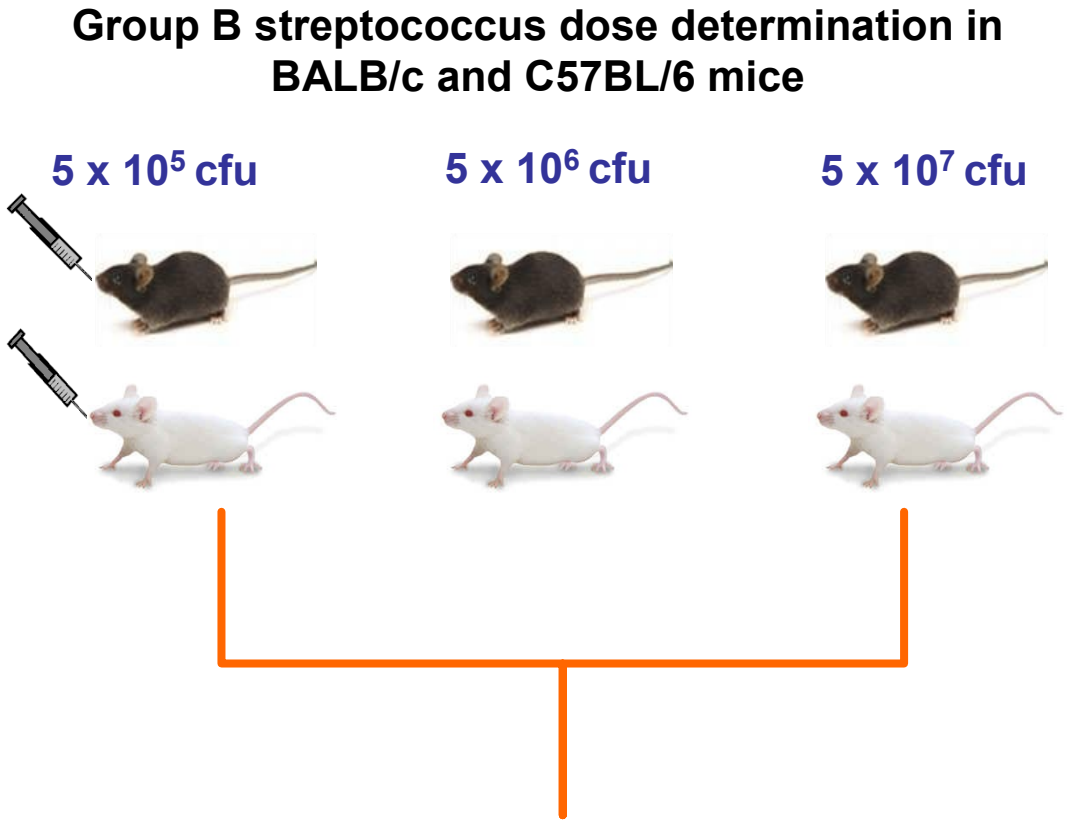



**Figure 3.1 An illustration of Group B streptococcus (GBS) dose determination protocol.** BALB/c and C57BL/6 mice were intranasally inoculated with varying doses of mid log phase GBS. Mice were followed over a period of 7 days and harvested on the days specified in the results. Designated tissues were removed and analysed subsequently.

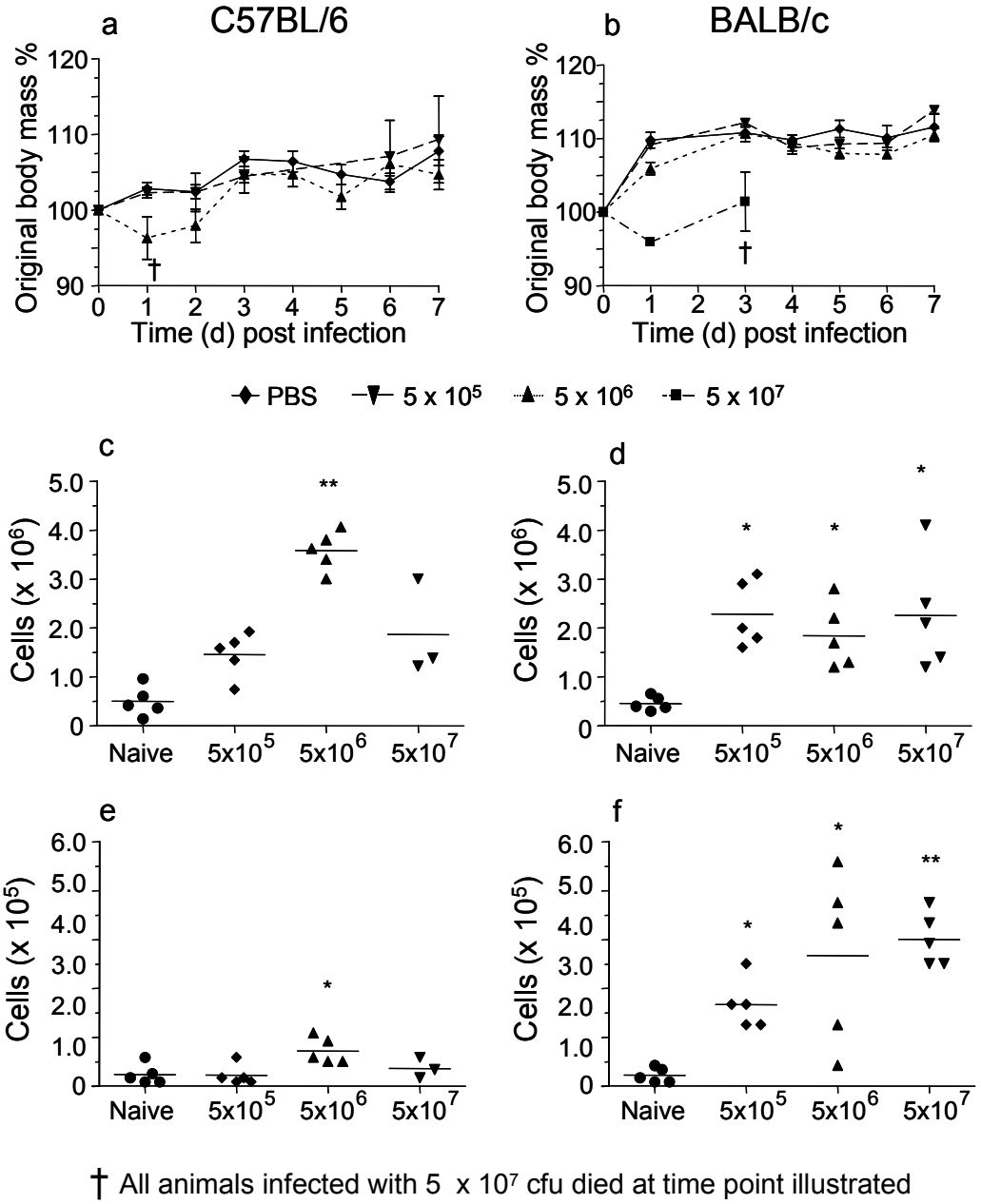
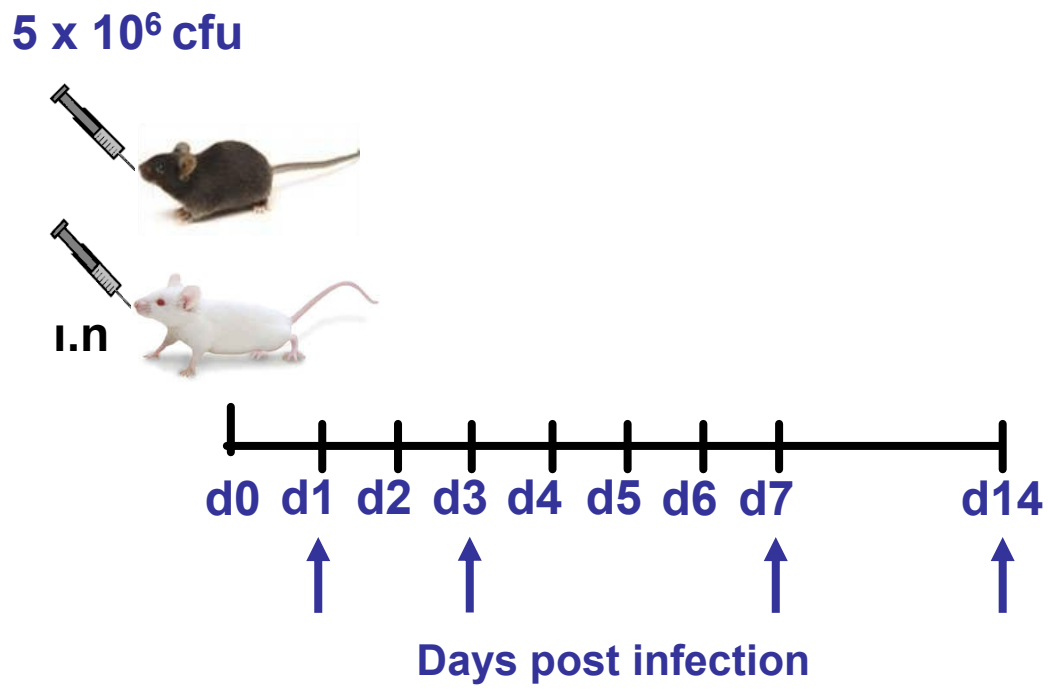



**Figure 3.2 GBS intranasally administered at 5 x $10^6$ cfu induced a strong non lethal phenotypic response in both airway and lung compartments.** C57BL/6 and BALB/c mice (n=5) were infected i.n. with either 5 x $10^5$, 5 x $10^6$, 5 x $10^7$ GBS colony forming units (cfu) or PBS control on day 0. Mice were subsequently harvested on days 1, 3 and 7 days post GBS infection. Individual mouse weight was monitored daily and expressed as a mean percentage of original weight (a & b). BAL was performed and lung tissue removed, homogenised and total viable cell counts were determined using Trypan Blue exclusion. Lung (c & d) and airway (e & f) total viable cell numbers 24 hours post infection are illustrated. Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

## Standard Group B streptococcus infection protocol




**Tissue harvest and analysis:**

- Daily weight loss (% day 0 weight)
- BAL
  - Cell phenotype – Flow cytometry
  - Bacterial cfu
  - Cytokines/chemokine analysis
- Lung
  - Cell phenotype – Flow cytometry
  - Bacterial cfu
  - Snap frozen lobe(s) – Cytokine analysis
- Nasal Wash – Bacterial cfu

**Figure 3.3** An illustration of the standard single GBS infection protocol. BALB/c mice were inoculated intranasally with 5 x $10^6$ cfu mid log phase GBS. Mice were then followed over a period of 14 days and harvested on the days specified in the results. Designated tissues were removed and analysed subsequently.

continue the characterisation of GBS respiratory infection because of its good cellular recruitment and its non lethal outcome.

### 3.2.3 BALB/c mice elicit a more vigorous immune response than C57BL/6 mice whilst ensuring bacterial elimination by day 14

After identifying 5 x $10^6$ cfu as a non lethal dose of GBS we next compared the cellular response profile and ability to resolve GBS in both BALB/c and C57BL/6 mice strains. Mice were infected with 5 x $10^6$ cfu GBS and followed over a period of 14 days. Figure 3.3 illustrates a standard GBS infection kinetic protocol. At chosen time points, lung and airway cellularity was enumerated, as previously described, and tissue specific clearance of GBS determined by plating 10 fold dilutions of single cell suspensions on Todd Hewitt Broth (THB) agar. As before, BALB/c mice elicited a greater cellular response in both the lung and airway compartments compared to that observed in C57BL/6 mice. In both strains this was rapid with the maximal response occurring three days post infection (Figure 3.4 a & b). In C57BL/6 mice, cellularity returned to naïve levels in both the lung and airways by day 14. However, cellularity in the lungs of BALB/c mice remained elevated over naïve levels up to and including day 14 post infection. On comparison of the GBS cfu titre between BALB/c and C57BL/6 mice, both strains demonstrated identical titres with no statistically significant difference between them in any tissue analysed (Figure 3.4 c-f). In both mouse strains GBS titre significantly increased from day one to day three, with the highest titres observed in the airways. Nevertheless, no detectable GBS cfu were recorded at any site 14 days post infection in either mouse strain (Figure 3.4 c-f).

### 3.2.4 GBS induces a transient myeloid response containing an early neutrophil and a later macrophage response

Due to the large, but transient, cellular response observed and the visible disease induced by influenza virus infection, we adopted BALB/c mice to dissect further the cellular response to a GBS infection. It is known that extracellular bacteria induce a strong and rapid innate immune response consisting of professional phagocytes, such as macrophage and neutrophils. Taking this into consideration, we used flow cytometric analysis to determine the proportion of specific innate cell subsets and determine their spatiotemporal relationship during a single GBS infection. Myeloid cell populations were primarily based upon differential expression of CD11b and CD11c as described by Gonzalez-Juarrero *et al* [344]. CD11b and CD11c staining of a naïve mouse lung, excluding lymphocytes by their forward and side scatter properties, revealed four distinct populations: $CD11b^{lo}$ $CD11c^{hi}$ (R1), $CD11b^{hi}$

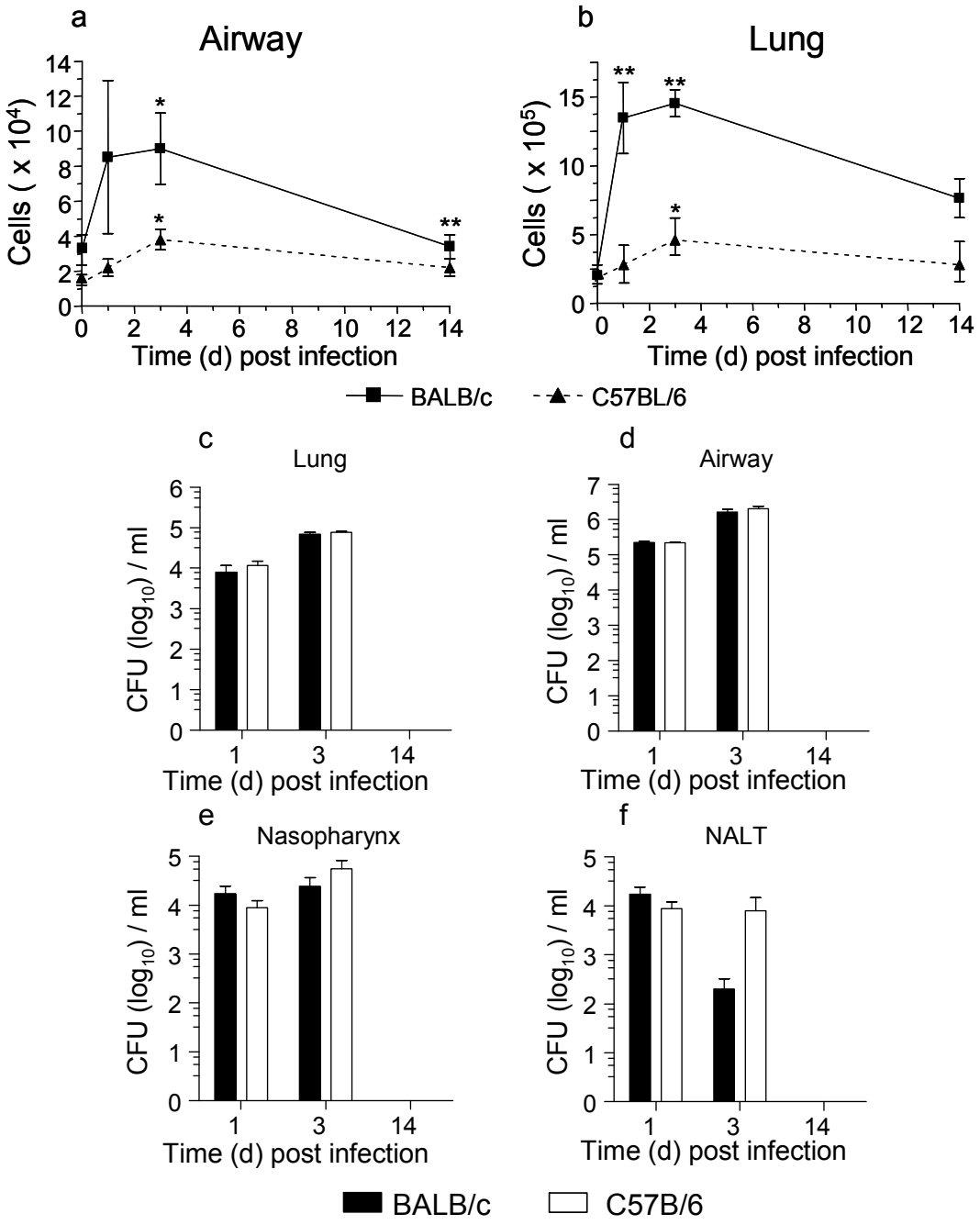



**Figure 3.4 BALB/c mice elicited a larger immune response than C57BL/6 mice whilst ensuring bacterial clearance by day 14 post infection.** C57BL/6 and BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ cfu of GBS on day 0. Mice were subsequently harvested on days 1, 3 and 14 days post infection. Airway (a) and lung (b) total viable cells were determined using Trypan Blue exclusion. CFU titres from the lung (c), airway (d), nasal wash (e) and nasal associated lymphoid tissue (NALT) (f) were calculated using serial dilutions of single cell suspensions of each tissue and plated on Todd Hewitt Broth (THB) agar. Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

$CD11c^{hi}$ (R2), $CD11b^{lo}$ $CD11c^{lo}$ (R3) and $CD11b^{hi}$ $CD11c^{lo}$ (R4) (Figure 3.5 a & b). Cells in the region R1 exhibited the same CD11b/CD11c profile as the majority of cells isolated from the airways of naïve mice (Figure 3.5 a) and appeared morphologically like alveolar macrophages (data not shown). Cells in R2 also expressed low levels of Gr1/Ly6g and high MHC II levels. They also appeared morphologically similar to activated macrophage or dendritic cells, with cytoplasmic processes (data not shown). Cells in R3 are difficult to distinguish due to their autofluorescence level. They express low levels of Gr1 and have small, horse-shoe shaped, nuclei resembling monocytes (data not shown). Fibroblasts and other connective cells are also likely to fall within this gate. Finally cells in R4 expressed high levels of Gr1, Ly6g and CD11b and were negative for MHC II. The majority appeared morphologically to be neutrophils as they possessed multilobed nuclei, however there was also the presence of small monocytes. This differential expression profile was subsequently utilised to distinguish distinct myeloid populations during single GBS infections and all subsequent infection models investigated throughout this thesis. However, during the course of many respiratory infections macrophage and monocytes up regulate CD11b and other cell markers upon activation, therefore the combination of alternative cell markers can be utilised to further scrutinize specific cell types.

GBS infection transiently increased the total number of myeloid cells in the lung peaking on day three before decreasing by day 14 post infection (Figure 3.5 c). Further dissection of the lung innate response highlighted a significant population of neutrophils, peaking on day one before being replaced by macrophages on day three post infection (Figure 3.5 d & e). These observations were mirrored in the airway (Figure 3.6 a-c). NK cells also play an important role in innate defence against invading bacterial pathogens [30;345-347]. NK and NK T ($CD3^+$) cells followed similar kinetics to that of neutrophils with the percentage and cell number peaking on day one p.i (Fig. 3.7 a). NK cells also play a key role in early IFN-γ production in the lung (Fig. 3.7 b). This was also seen in the airway (data not shown). Myeloid cells are not only central to the establishment and maintenance of the inflammatory response to infection by secreting cytokines and chemokines but also play a key role in presenting antigen to recruited T cells, which consequently ensures their full activation. GBS infection elicits a $CD4^+$ T cell dominated response in the lung by day three post infection (Figure 3.8 a & b). Further analysis of this lymphocyte response highlights a large percentage of activated $CD4^+$ T cells (Figure 3.8 c).

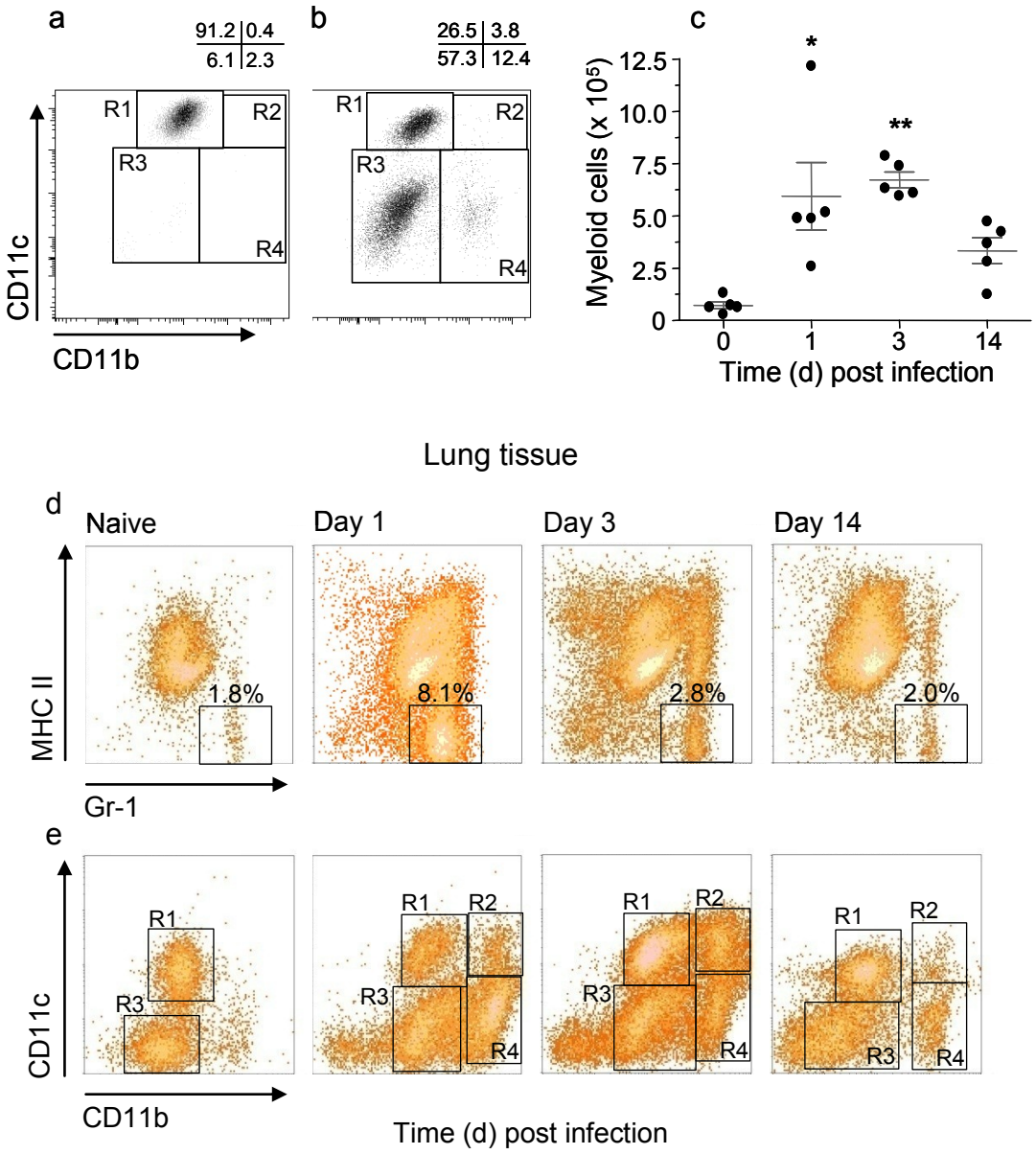



**Figure 3.5 5 x $10^6$ GBS induced a transient myeloid response containing an early neutrophil and a later macrophage response.** BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ GBS cfu. Mice were subsequently harvested on days 1, 3 and 14 days post infection. BAL was performed and lung tissue removed, homogenised and total viable cell counts determined as previously described. Subsets of myeloid cells were determined from naïve airway (a) and lung (b) populations. Total myeloid cells were determined by their relative forward and side scatter appearance (c). Lung neutrophils (d) were discriminated as Gr-1 $^{hi}$ MHC II $^{lo}$ CD11c $^{lo}$ CD11b $^{hi}$ and followed over the course of infection. Lung macrophage (e) populations were defined as R1, R2 & R4 (that are not neutrophils). Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

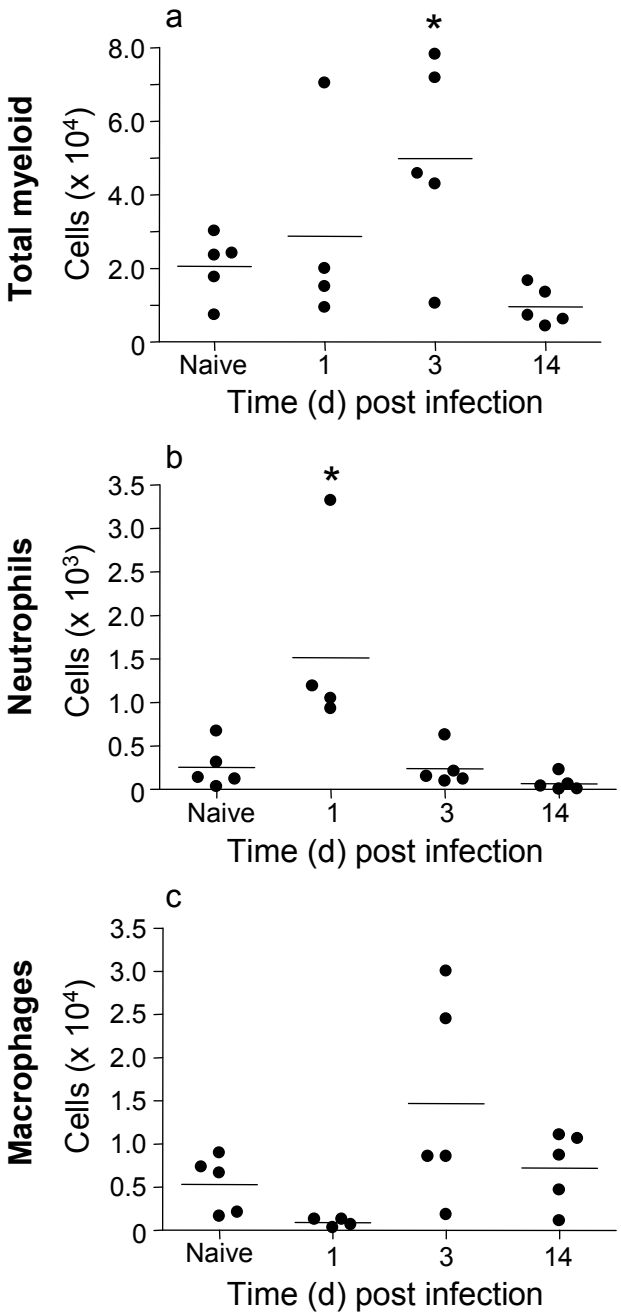



**Figure 3.6 Airway recruitment of neutrophils and macrophage in response to GBS infection follows similar kinetics to that observed in the lung tissue.** BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ GBS cfu. Mice were subsequently harvested on days 1, 3 and 14 days post infection. BAL was performed and total viable cell counts determined using Trypan Blue exclusion. Total airway myeloid cells (a) were determined by their relative forward and side scatter appearance. Recruited neutrophils (b) and macrophage (c) were also followed over the course of infection. Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

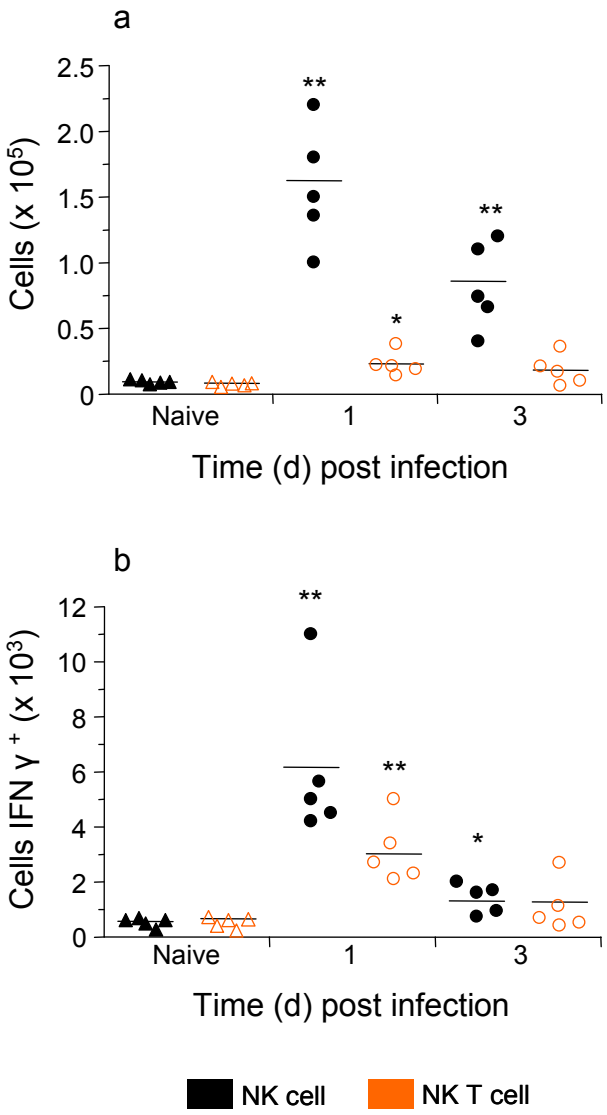



**Figure 3.7 GBS infection activates a rapid NK and NK T cell response that contributes to IFN-γ cytokine levels.** BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ GBS cfu. Mice were harvested on specified days post infection at which BAL was performed and lung tissue removed, homogenised and total viable cell counts determined using Trypan Blue exclusion. Lung NK (closed) and NK T (open) cell subsets were also quantified (a) and the total number expressing IFNγ (b) was determined by intracellular staining. Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

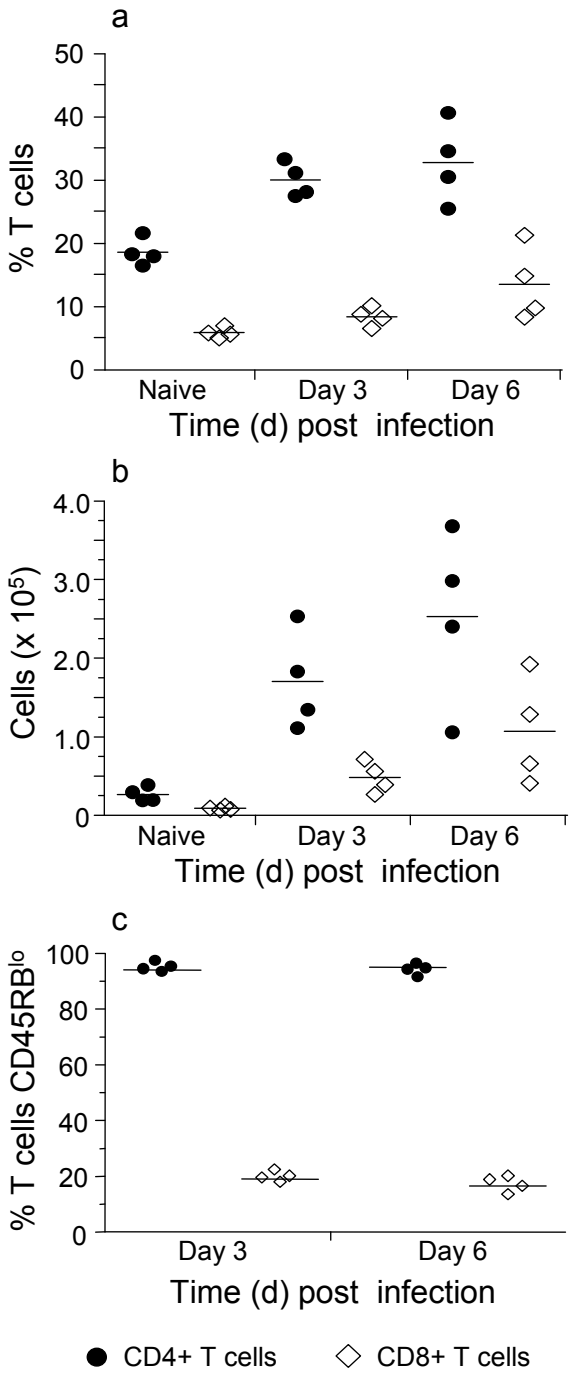



**Figure 3.8 GBS infection elicited a CD4$^+$ dominated T cell response.** BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ GBS cfu. Mice were harvested on specified days post infection at which BAL was performed and lung tissue removed, homogenised and total viable cell counts determined using Trypan Blue exclusion. The percentage (a) and cell numbers (b) of lung CD4 $^+$ and CD8 $^+$ T cells were determined using flow cytometry. Activated T cells were determined as CD45rb$^{lo}$ expressing cells (c). Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

A similar response was observed in the airway, but due to a lack of total lymphocyte numbers the results were insignificant (data not shown).

### 3.2.5 GBS causes extensive lung inflammation and injury that is resolved by day 7 post infection

To further investigate pathological changes to the lung in response to GBS infection, the right azygous lobe was processed for histology at days 1, 3 and 7 days post infection (Figure 3.9). Perivascular and peribronchiolar infiltrates were clearly visible on both 1 and 3 days post infection. The extent of this infiltrate was most evident on day 1 post infection. There was also evidence of bronchiolar wall hypertrophy, bronchiolar oedema, significant vascular leakage and the presence of cellular debris in the larger airways. By day 3 post infection, the cellular infiltrate showed a more diffuse and patchy distribution within the lung parenchyma tissue, with a reduction in both the perivascular infiltrate and vascular leakage. There was also a slight improvement in alveoli architecture by this time point. A significant improvement of lung histology was observed by day seven post infection. This was highlighted by the remnants of a small number of isolated foci of peribronchiolar infiltrate and a marked improvement in alveoli architecture.

### 3.2.6 GBS re-challenge elicits a robust memory immune response resulting in reduced bacterial titre in all compartments analysed

Having demonstrated that GBS infection elicits a robust innate immune response followed by the presence of activated $CD4^+$ T cells we next investigated the ability of GBS to induce immunological memory by looking at the cellular infiltrate, bacterial clearance and T cell memory subsets in response to a GBS re-challenge. Upon re-challenge with $5 \times 10^6$ cfu GBS, 14 days after a primary infection, a rapid cellular response was again observed in both lung and airway compartments. The re-challenge response was significantly greater in amplitude in the lung compared to that seen in the initial primary response. This trend was also observed in the airway but failed to reach significance (Figure 3.10 a & b). The greater amplitude and quality of cellular recruitment observed in response to a GBS re-challenge resulted in reduced GBS cfu titres observed across all tissues analysed in comparison to the levels measured in a primary GBS infection (Fig. 3.10 c). Further investigation into a possible mechanism for the observed increase in immunity highlighted an increase in the proportion and total number of lung $CD4^+$ effector ($CD62L^{lo}$ $CD44^{hi}$) and $CD4^+$ central ($CD62L^{hi}$ $CD44^{hi}$) memory T cells (Fig. 3.11 a & b). This significant difference was not observed in the airway (data not shown).

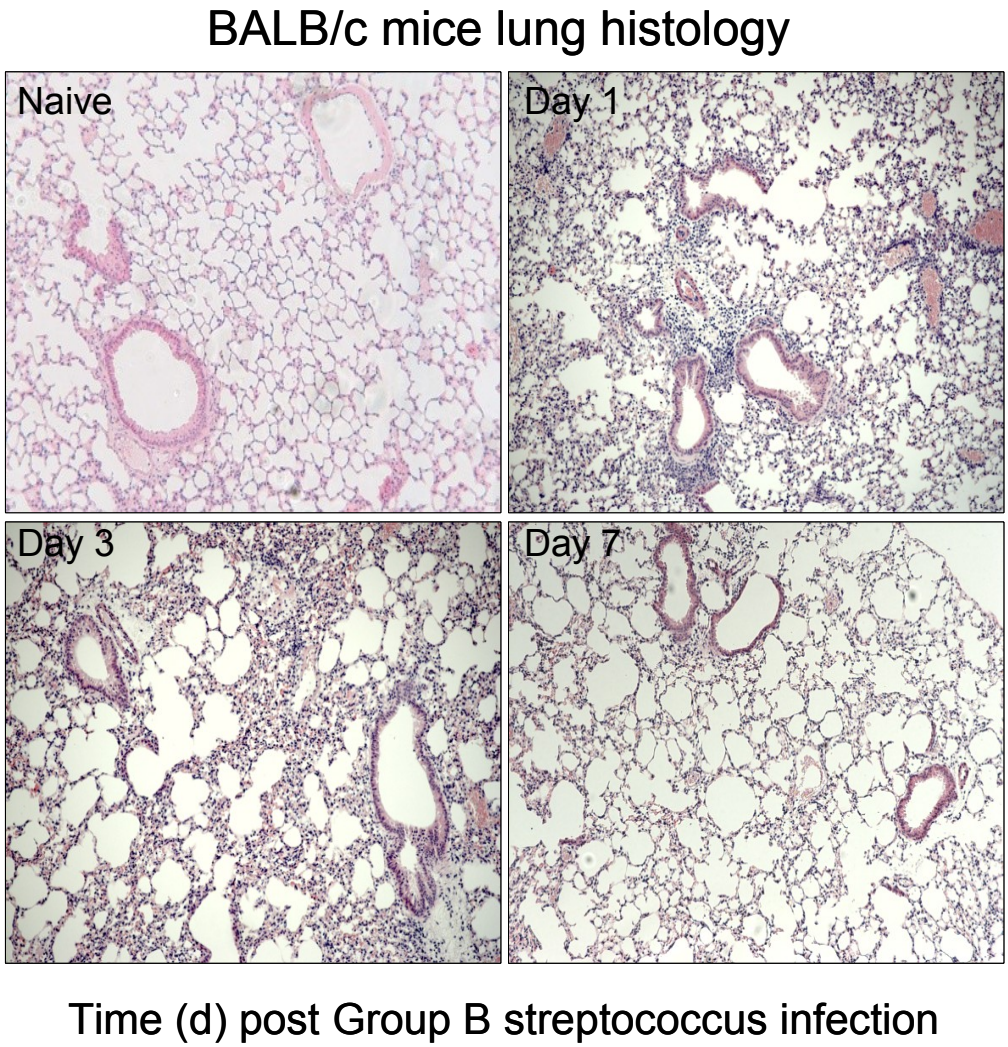



**Figure 3.9 Progressive lung pathology and injury in the lungs of BALB/c mice during GBS infection.** BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ cfu GBS on day 0. Mice were subsequently harvested on days 1, 3 and 7 days post infection. Processing of mouse lungs for histology and light microscopy of H & E stained sections were carried out as described in section 2.7.1. Magnification was x 10. Sections are representative of five mice studied at each time point.

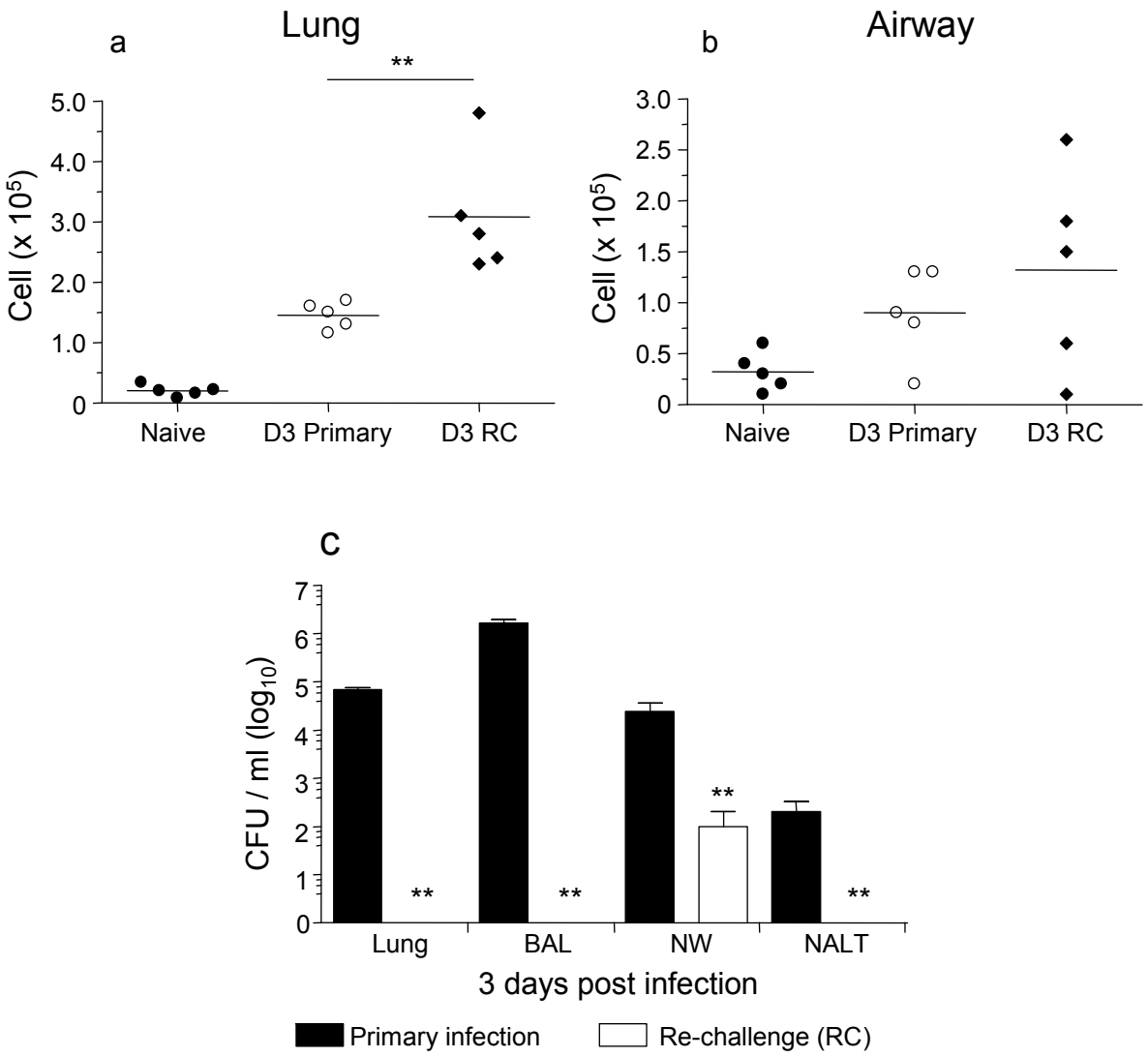



**Figure 3.10 GBS re-challenge elicited a robust memory immune response resulting in reduced bacterial titre in all compartments analysed.** BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ GBS cfu on day 0. 14 days later mice were re-challenged with 5 x $10^6$ cfu GBS. Mice were harvested 3 days post re-challenge on which BAL was performed and lung tissue removed. Total viable cell counts in lung (a) and airway (b) was enumerated using Trypan Blue exclusion. Bacterial cfu (c) from lung, airway (BAL), nasopharynx (NW) and nasal associated lymphoid tissue (NALT) were calculated using serial dilutions of single cell suspensions and plated on Todd Hewitt Broth (THB) agar. Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

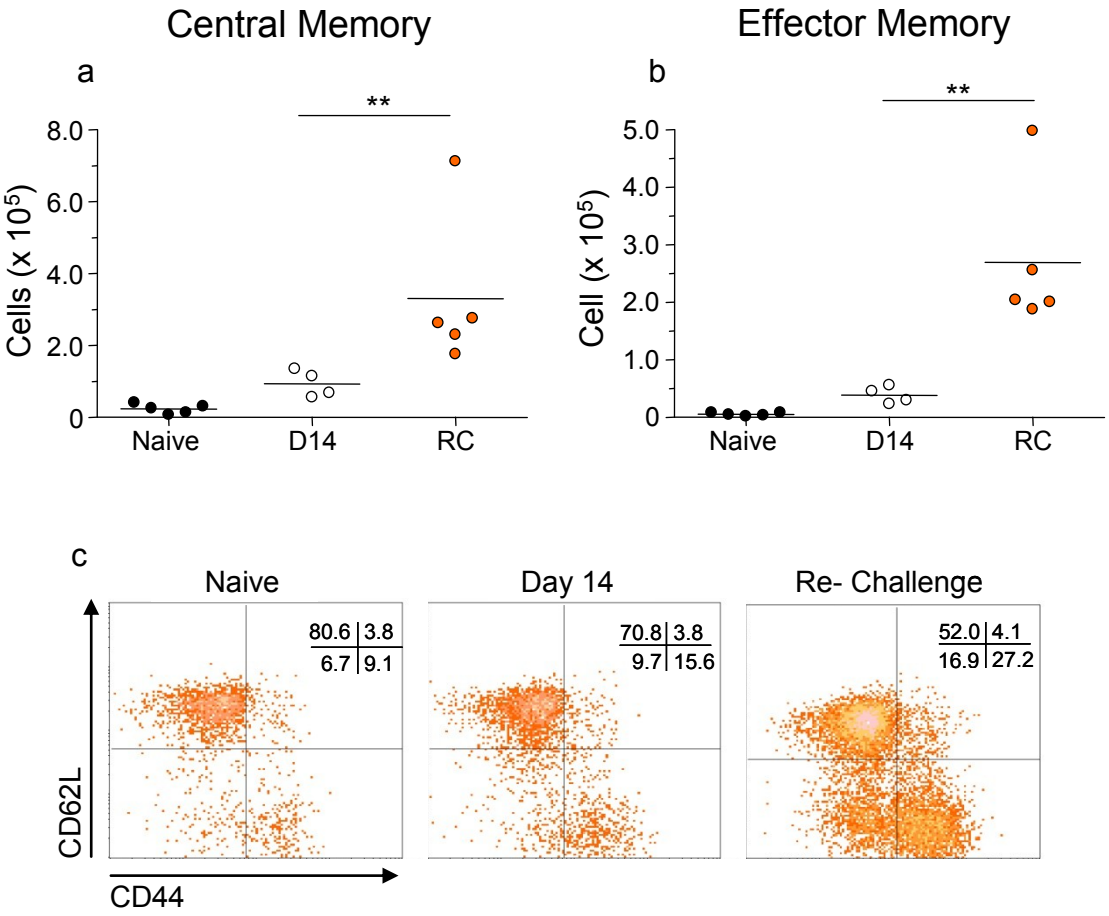



**Figure 3.11 The percentage and number of CD4⁺ effector and central memory T cell subsets are increased upon GBS re-challenge.** BALB/c mice (n=5) were infected intranasally with 5 x $10^6$ GBS colony forming units on day 0. 14 days later mice were re-challenged (RC) with 5 x $10^6$ CFU GBS and mice harvested 3 days later. Lung tissue was removed, homogenised and total viable cell counts were determined using Trypan Blue exclusion. Total number of CD4⁺ central (a) and effector (b) memory T cells were enumerated from naïve, day 14 primary (D14) and re-challenged (RC) mice. Representative dot plots of CD4⁺ lung $CD62L^{hi}$ $CD44^{hi}$ (central memory) and $CD62L^{lo}$ $CD44^{hi}$ (effector memory) are shown (c). Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

### 3.2.7 *Streptococcus pneumoniae* infection model characterisation

*Streptococcus pneumoniae* is a commensal of the human nasopharynx and is a common cause of lobar pneumonia, otitis media, septicaemia and meningitis [324]. It causes more deaths from invasive infections than any other bacterium and is the fifth leading cause of death worldwide [318]. Attention has therefore been focused on the mechanisms of its pathogenesis and the role of its virulence determinants. Additionally, the recent emergence of antibiotic resistance and the growing recognition for its role in viral induced secondary bacterial pneumonia has increased the need for robust laboratory based animal models. Using the same approach used to investigate a GBS infection, we wished to determine a non lethal infectious dose and characterise the immunological response to *S. pneumoniae* in both BALB/c and C57BL/6 strains of mice.

### 3.2.8 *Streptococcus pneumoniae* administered at doses higher than 1 x $10^5$ cfu is lethal to C57BL/6 mice within 48 hours of infection

Both strains of mice were intranasally inoculated with varying doses of D39, a historically important and commonly used serotype 2 strain of *S. pneumoniae*, as described in Figure 3.12. Mice were weighed daily over the course of 7 days and at specified time points total lung and airway cellular infiltrates were analysed along with the bacterial titres in specific tissue compartments. In C57BL/6 mice there was no statistically significant weight alteration compared to PBS infected controls (Figure 3.13 a), however, as a general trend, increasing inoculum dose slowed weight gain over the course of infection. In contrast, infection of BALB/c mice with the two highest doses of *S. pneumoniae* resulted in a transient weight loss on days 3 and 4 post infection (Figure 3.13 b). Most strikingly, high doses of *S. pneumoniae* infection resulted in the death of C57BL/6 mice whilst BALB/c mice survived. No BALB/c mice died after infection with any dose over the course of the infection whereas C57BL/6 mice demonstrated 50 % mortality by day 3 post infection with 1 x $10^6$ cfu and 25 % mortality by day 7 with 1 x $10^5$ cfu (Figure 3.13 c & d). The increase in lethality observed in C57BL/6 mice with increasing inoculum dose reached significance using the log rank Mantel-Cox test (p 0.03).

We also determined the recoverable bacterial titres from specified tissue compartments in both strains of mice at 1, 3 and 7 days post *S. pneumoniae* infection. Bacterial titres recovered from the airways of C57BL/6 mice and BALB/c mice infected with 1 x $10^6$ cfu were equivalent on day 1 post infection but are significantly reduced on days 3 and 7 in BALB/c mice compared to the levels

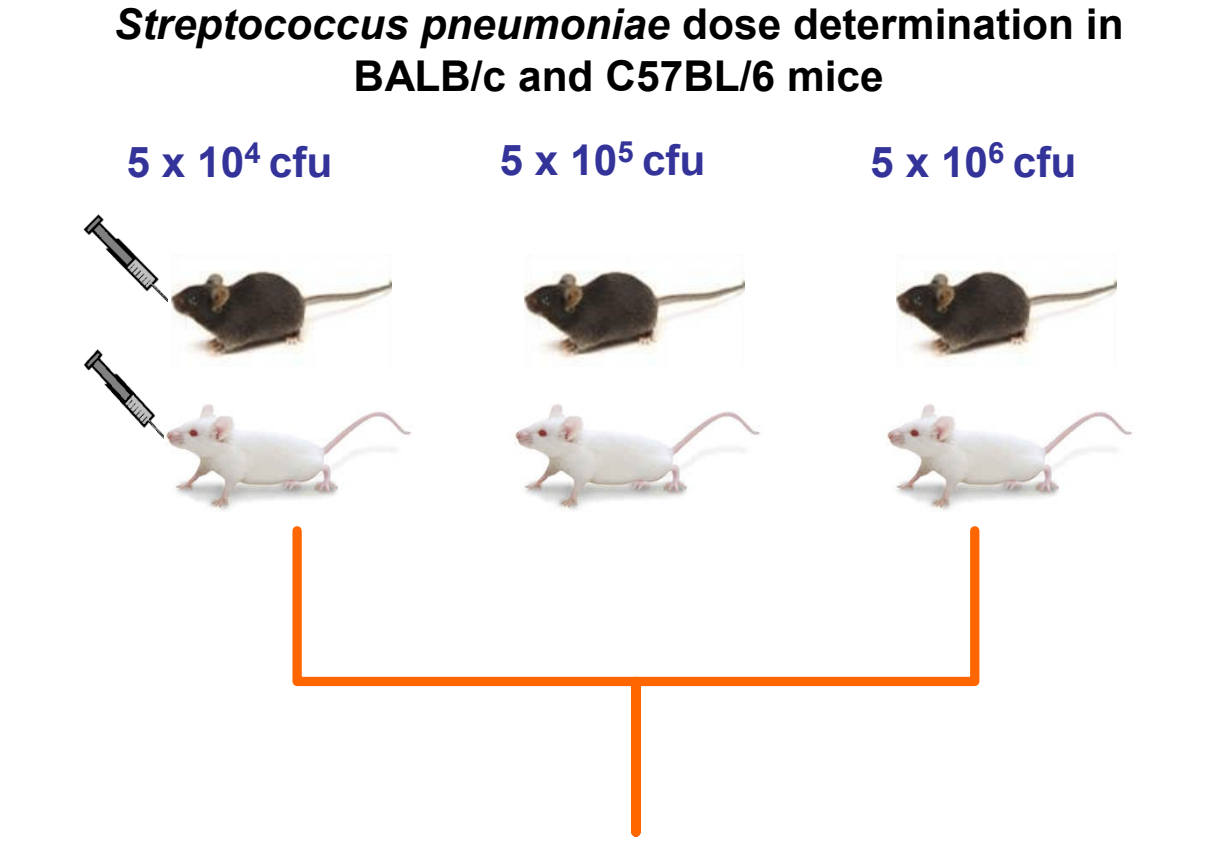



**Figure 3.12 *S. pneumoniae* dose determination protocol.** BALB/c and C57BL/6 mice were intranasally inoculated with varying doses of D39, a commonly used clinical isolate of serotype 2 *S. pneumoniae*. Mice were followed over a period of 7 days and harvested on the days specified in the results. Designated tissues were removed and analysed subsequently.

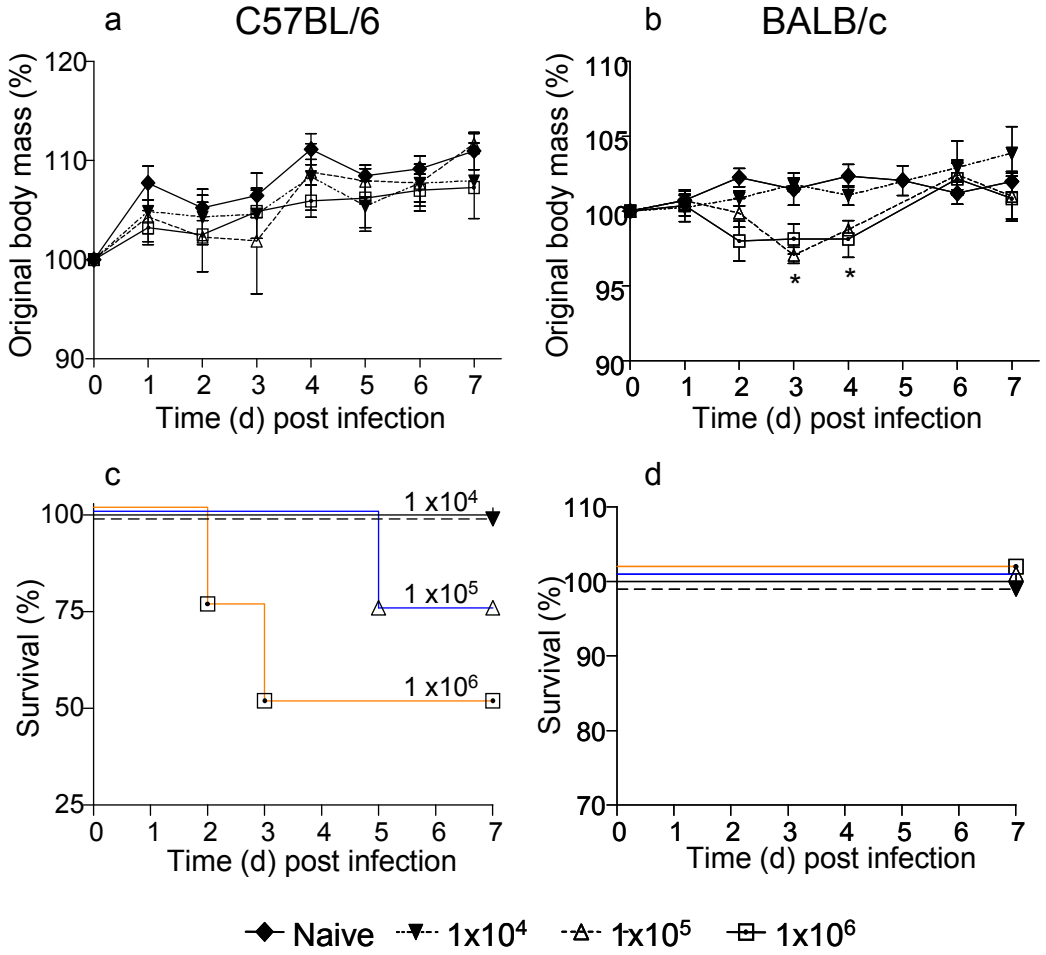



**Figure 3.13 *S. pneumoniae* administered at 1 x $10^5$ cfu or higher is lethal to C57BL/6 mice within 48 hrs of infection.** BALB/c and C57BL/6 mice (n=4) were infected intranasally with 1 x $10^4$, 1 x $10^5$ or 1 x $10^6$ *S. pneumoniae* cfu on day 0. Mice were subsequently harvested on days 1, 3 and 7 days post infection. Individual mouse weight was monitored daily and expressed as a mean percentage of original weight (a & b). Moribund mice, determined by home office guidelines, were culled and recorded as deceased using a Kaplan Meier survival curve (c & d). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$ Log rank Mantel - Cox test was used to determine significance in survival.

observed in C57BL/6 mice (Figure 3.14 a & b). Infection with 1 x $10^5$ cfu produced a similar trend, again with a significant reduction in bacterial load measured in BALB/c mice on days 3 and 7 post infection. Both strains of mice clear 1 x $10^4$ cfu bacteria from the airways by day 7 p.i (Figure 3.14 a & b). Intriguingly, no bacterial cfu were recovered from the lung tissue of BALB/c mice in response to all inoculum doses at any time point analysed (data not shown). In contrast, 1 x $10^5$ and 1 x $10^6$ cfu infectious doses resulted in a 3 log increase in bacterial cfu from day 1 to day 3 in the lung tissue of C57BL/6 mice (Figure 3.14 c). Bacteria were still present at day 7 at these two doses whilst there was no detectable presence with the 1 x $10^4$ dose from day 3 onward (Figure 3.14 c). Nasopharyngeal carriage also differed significantly between C57BL/6 and BALB/c mice strains. As a general trend, there was a 2 log increase in bacterial titre at each infectious dose when comparing C57BL/6 to BALB/c mice (Figure 3.14 d & e). The presence of bacterial cfu in the nasopharynx occurred at all time points analysed and showed a dose response with regards to the initial infectious dose. As was seen with the difference in mortality between the two mice strains, the presence of bacterial cfu in the peripheral blood was also uniquely seen in C57BL/6 mice (Figure 3.14 f). Although one mouse infected with 1 x $10^4$ cfu recorded the presence of bacteraemia by day 1 post infection, 50 % of both 1 x $10^5$ and 1 x $10^6$ CFU infected mice recorded very high levels of blood bacteraemia. There were no peripheral bacterial cfu detected at any time point analysed across all inoculum doses used in BALB/c mice (data not shown).

### 3.2.9 *S. pneumoniae* elicited a rapid cellular response in the airway and lungs of BALB/c mice that was absent in C57BL/6 mice

As was seen with a GBS infection, there was little airway or lung cellular recruitment in C57BL/6 mice on days 1 and 3 post *S. pneumoniae* infection (Figure 3.15 a & c). However, by day 7 there was a small, yet significant, increase in the total airway cell numbers in response to both the 1 x $10^6$ and 1 x $10^4$ cfu in C57BL/6 mice (Figure 3.15 a). Once more, mirroring that observed with a GBS infection, BALB/c mice elicited a robust cellular response in both the airway and lung in response to the highest dose of *S. pneumoniae*, the amplitude of which was approximately 3 times that observed in response to an identical inoculum dose in C57BL/6 mice (Figure 3.15 b & d). In contrast to C57BL/6 mice, low doses of *S. pneumonia* elicited a cellular response in the lungs of BALB/c mice by days 3 post infection (Figure 3.15 d). This was resolved by day 7 with the total cell number returning to that seen at day 0. In contrast, infection with 1 x $10^6$ cfu resulted in the total number of cells in both

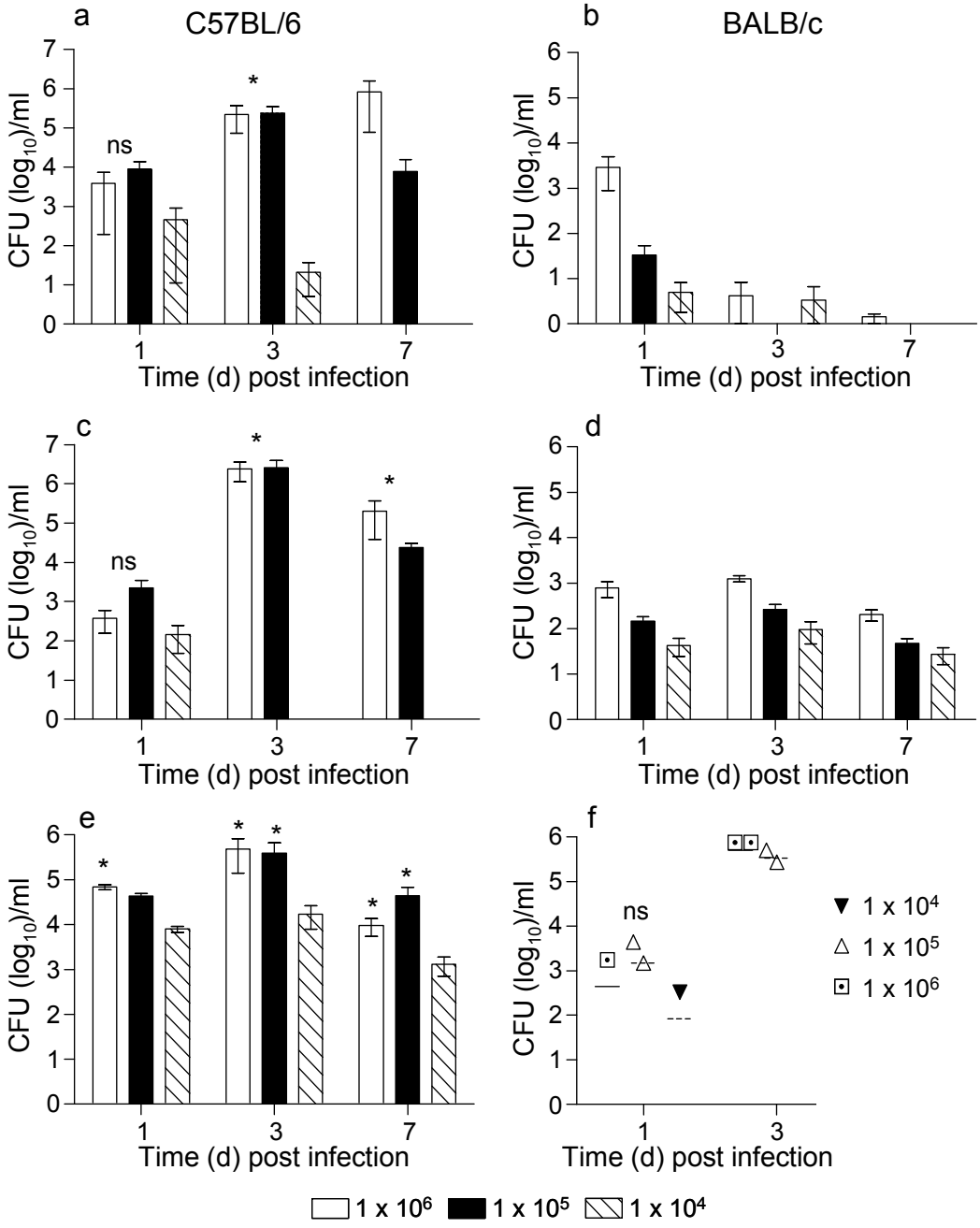



**Figure 3.14 Bacterial titres recovered from C57BL/6 mice were elevated in all tissues analysed compared to BALB/c mice.** C57BL/6 (left) and BALB/c (right) mice (n=4) were infected intranasally with 1 x $10^4$, 1 x $10^5$ or 1 x $10^6$ *S. pneumoniae* cfu on day 0. On specified days post infection BAL and nasal wash was performed and lung tissue was removed. Airway (a & b), lung (c) and nasal wash (d & e) bacterial titres from C57BL/6 (left column) and BALB/c (right column) were determined by serial dilutions on Columbus blood agar. Peripheral blood bacterial titres from C57BL/6 mice were also analysed (f). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

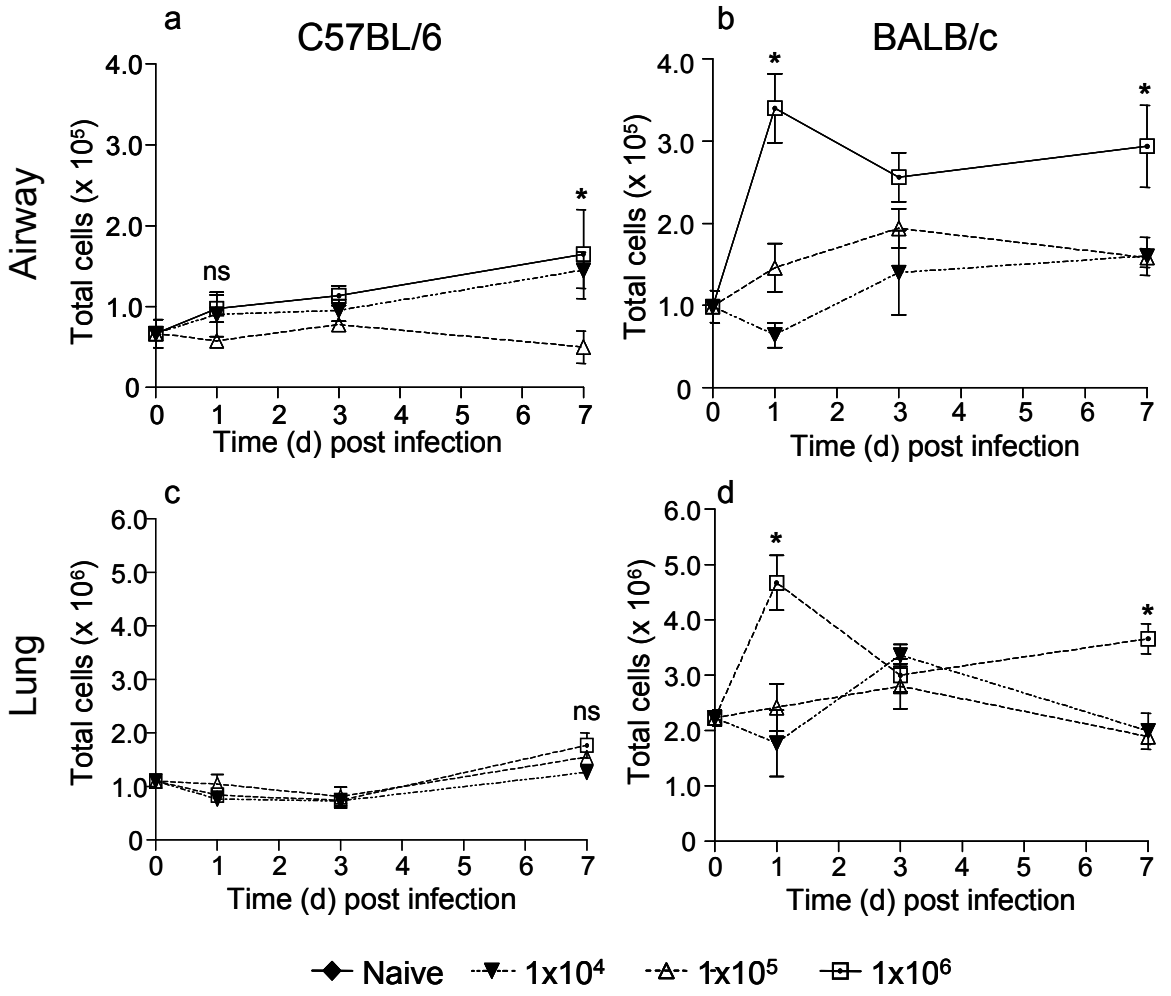



**Figure 3.15 *S. pneumoniae* elicit a rapid cellular response in the airway and lungs of BALB/c mice that was absent in C57BL/6 mice.** C57BL/6 (left) and BALB/c (right) mice (n=4) were infected intranasally with 1 x $10^4$, 1 x $10^5$ or 1 x $10^6$ cfu *S. pneumoniae* on day 0. Total airway cells (a & b) and lung cells (c & d) were harvested on specified days and total cells enumerated using Trypan blue exclusion. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

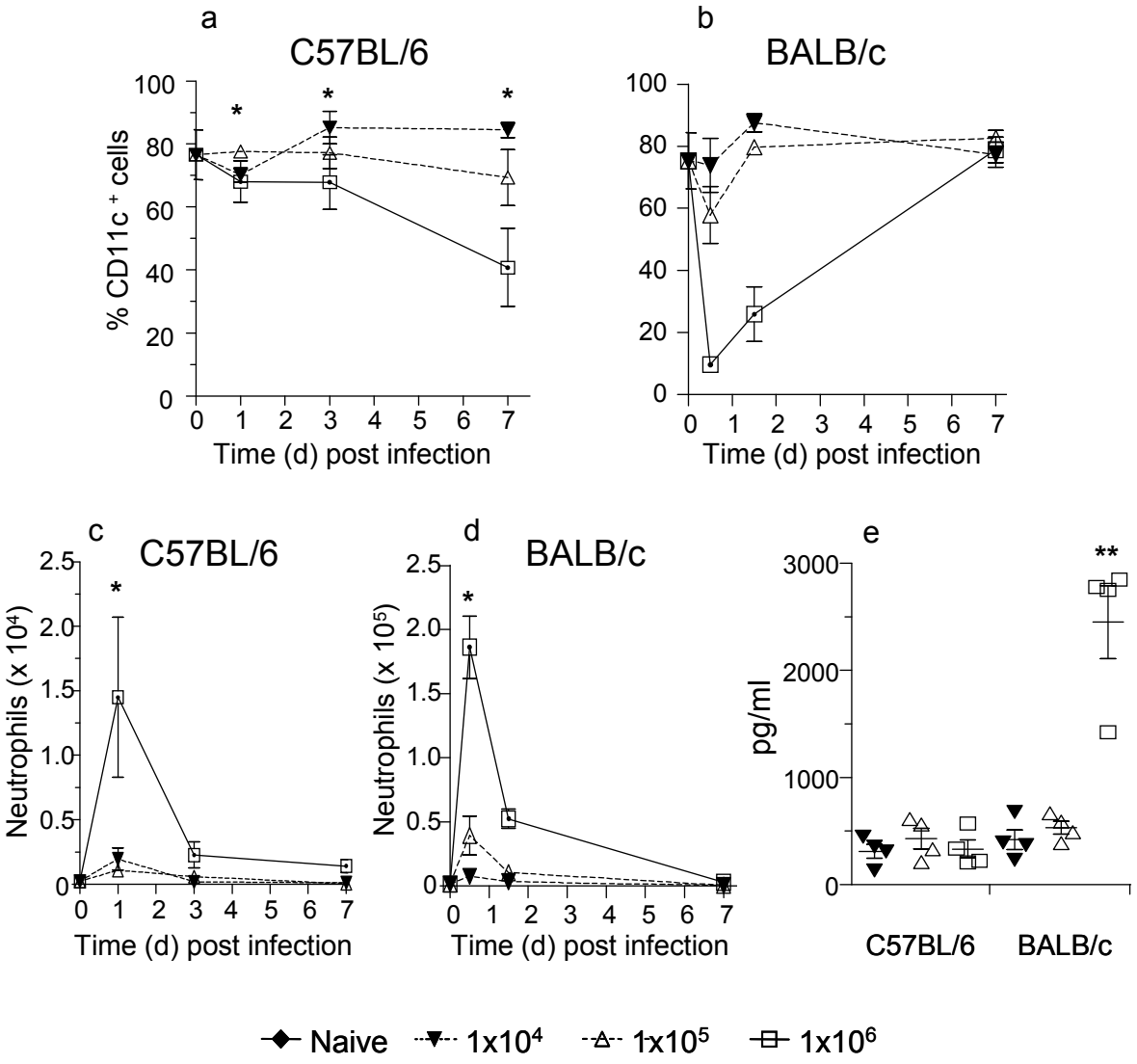



**Figure 3.16 *S. pneumoniae* infection results in a robust neutrophil recruitment to the airway.** C57BL/6 and BALB/c mice (n=4) were infected intranasally with 1 x $10^4$, 1 x $10^5$ or 1 x $10^6$ cfu of *S. pneumoniae* on day 0. BAL was used to recover airway cells at specified time points post bacterial infection. Alveolar macrophage proportions (a & b) and neutrophil total numbers (c & d) were determined using differential FCM staining as previously described. Lung tissue levels of KC 12 h post infection (e) were also analysed by ELISA. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

the airway and lungs of BALB/c mice remaining elevated up to and including day 7 post infection. Further investigation into the composition of the cellular response to *S. pneumoniae* highlights that, at low doses of infection, alveolar macrophages constitute the major cell type present within the airway (Figure 3.16 a & b). However, as the inoculum dose of *S. pneumoniae* is increased, a correlated increase in neutrophil recruitment to the airway is observed in both C57BL/6 and BALB/c mice (Figure 3.16 c & d). This enhanced airway recruitment of neutrophils observed in BALB/c mice is more than 10 times the number seen in C57BL/6 mice. This is reflected by a significant elevation in the levels of KC, a murine homolog of IL-8, detected in the lung tissue of BALB/c mice in response to 1 x $10^6$ cfu of *S. pneumoniae* compared to C57BL/6 mice (Figure 3.16 e).

## 3.3 Discussion

### 3.3.1 BALB/c mice demonstrate increased resistance to respiratory Streptococcal infections

We demonstrate that BALB/c and C57BL/6 mice differ significantly in their susceptibility, as represented by survival time and numbers of bacteria present in peripheral blood, to both GBS and *S. pneumoniae* respiratory infections. Despite recovering comparable levels of bacteria from C57BL/76 and BALB/c mice following a non lethal dose of GBS, C57BL/6 mice succumbed when infected with a larger dose of GBS. The relative resistance to respiratory bacterial infection observed in BALB/c mice was more evident with a *S. pneumoniae* infection. The level of *S. pneumoniae* recovered from BALB/c mice was consistently lower, compared to C57BL/6, in all tissues analysed and was never detected in the lung tissue regardless of inoculum dose used. This result most likely explains the absence of bacteraemia in BALB/c mice and the 100 % survival regardless of *S. pneumoniae* dose used. These findings are largely supported by a number of recent studies that investigated the genetic resistance of some commonly used inbred mouse strains in response to *S. pneumoniae* infection [348;349]. Using the exact strain of *S. pneumoniae* used in our study, Gingles *et al* demonstrate a large variation in *S. pneumoniae* resistance across several commonly used inbred mouse strains. The capacity to limit the development and progression of bacteraemia, specifically in BALB/c mice, is thought to be strongly dependent upon the ability to control the levels of bacteria in the lung and that this is affected by the amplitude of cells recruited to the site of infection. These observations are supported in another study that used a lobar pneumonia infection model, in which BALB/c mice demonstrated increased

resistance against a serotype 3 strain of *S. pneumoniae* compared to C57BL/6 mice [325]. Resistance in this case again depended on the intensity of cellular recruitment but also highlights a role for enhanced phagocytic activity and oxidative burst in alveolar macrophages and neutrophils. Similar innate defects in macrophage bactericidal capacity also contribute to the enhanced susceptibility of DBA/2 mice to *Pseudomonas aeruginosa* infection [350]; though in the case of this organism the infiltration of neutrophils may play an important role in immunity [351]. Our results also indicated that enhanced cellularity in BALB/c mice to both GBS and *S. pneumoniae* correlates with resistance and likely explains the reduction in bacterial titres and possibly the transient weight loss observed in BALB/c mice following *S. pneumoniae* infection. However, despite a more robust cellular response observed in BALB/c mice, there was no significant difference in GBS bacterial load observed between C57BL/6 mice and BALB/c. Assuming the absence of pre-existing anti-pneumococcal polysaccharide capsule antibodies, this suggests that both defects in the innate immune system that determines cellular recruitment and bactericidal capabilities play a critical role in determining mouse strain differences in susceptibility to respiratory bacterial infection.

### 3.3.2 BALB/c mice elicit an enhanced neutrophil response to respiratory streptococcal infections

Neutrophils were a dominant feature of both GBS and *S. pneumoniae* infection most notably in BALB/c mice following *S. pneumoniae* infection. This is particularly apparent at a high inoculum dose and correlates with elevated levels of KC, a neutrophil chemoattractant and murine homolog of human IL-8. Heightened recruitment may explain the control of *S. pneumoniae* infection in BALB/c mice, though at present we cannot rule out enhanced cellular anti-bacterial activity in BALB/c mice. An enhanced neutrophil response is consistent with the studies of Gingles *et al* and Preston *et al*, where both postulate that bacteraemia is avoided by neutrophil phagocytic and anti-bacterial activity. BALB/c mice also show greater recruitment of neutrophils into the airway in response to *P. aeruginosa* compared to susceptible DBA/2 mice that are unable to clear the infection [351;352]. Protection has also been associated with a specific induction of IL-17 and MCP-1 in BALB/c mice, which in a recent study have been shown to enhance the recruitment and killing capacity of neutrophils in a nasopharyngeal *S. pneumoniae* colonization model [275]. Furthermore, neutrophils are essential for the clearance of *Staphylococcus aureus* [353]. There is a swath of evidence highlighting the importance of the effector function of neutrophils in the clearance of bacteria rather than their mere presence alone.

Mice deficient in cathespin G, a serine protease produced by neutrophils, are more susceptible to infection with the gram positive bacteria *Staphylococcus aureus* and a deficiency of neutrophil elastase renders mice more susceptible to gram negative bacteria [354;355]. Nonetheless, despite mounting evidence supporting the importance of neutrophils in anti-bacterial defence, there are a number of other studies that suggest an over exuberant neutrophil response can increase pathology to *S .pneumoniae* infection, resulting in increased mortality without affecting bacterial clearance [274]. The dependence on neutrophils for clearance may depend on the respiratory site colonised. *S. pneumoniae* infection does not cause differential neutrophil recruitment in the nasal cavity of C57BL/7 and BALB/c mice [356]. It would be interesting to test whether neutrophil depletion using an anti Ly6G, a neutrophil specific surface marker, antibody makes BALB/c mice as susceptible to *S. pneumoniae* as C57Bl/6 mice or alters their nasopharyngeal commensal burden.

Our observations, and those of others, raise a number of intriguing questions about what determines the amplitude and quality of a bacteria induced neutrophil response in an inbred mouse strain. For example, are there subtle differences in TLR expression in the respiratory tract that could in turn affect the production of neutrophil chemotactic factors? Is there a variation in the downstream signalling events and the machinery needed to produce neutrophil chemoattractants? A recent study highlights variations in chemokine production in C57BL/6 and BALB/c mice in response to infection with murine gammaherpesvirus-68 [357]. Are there innate differences in the migratory ability of neutrophils or even the ability to produce or release neutrophils from the bone marrow? Is a neutrophil response critical for all *Streptococcaceae* family members or even all *Lactobacillales* bacteria?

### 3.3.3 MHC associations and bacterial susceptibility

Attempts to map disease susceptibility to individual genes have been made for many years, however due to the complexity of the host's genetic components and the important role played by environmental factors, such as pathogenicity determinants of the microbe itself, progress has been slow. It was shown that mice with a tendency toward a $Th_1$ or $Th_2$ lymphocyte response manifest different reactions to inoculation with the parasite *Leishmania major* for example, however it is rare that susceptibility to an infectious agent is due to a single gene alone [358-360]. Marley *et al* clearly show that the mouse *H2* haplotype is responsible for the recruitment and function of neutrophils following a *S. pneumoniae* infection but that other non MHC genes were

also required [361]. Furthermore, within the *H2*$^{k}$ haplotype, CBA/Ca mice are significantly more susceptible to *S. pneumoniae* infection than *H2*$^{k}$ AKR mice and *H2*$^{d}$ BALB/c are more resistant than *H2*$^{d}$ DBA/2 strain. Thus *H2* genes alone do not cause susceptibility to *S. pneumoniae* infection but may contribute with other susceptibility alleles [348]. More recently, using linkage analysis of an F2 cross between resistant BALB/c and susceptible CBA/Ca strains, a region close to the locus *D7Mit7*, on chromosome 7, has been described that increases survival to *S. pneumoniae* infection. The identified locus lies within a region of chromosome 7 that includes several genes involved in inflammation and innate immunity, such as tyrosine kinase-binding protein (*Tyrobp*), the platelet activating factor acetylhydrolase 1B gamma subunit gene (*Pafah1b3*) and the gene for nuclear factor–κB inhibition (*IKB-b*) amongst others [362]. Evidence that inbred mouse strain differences in neutrophil recruitment explain innate susceptibility to infection also comes from other models of infection. For example, the differences in response of resistant BALB/c *H2*$^{d}$ and susceptible CBA/CaH mice to *Candida albicans* lies in short-lived cellular responses derived from the bone marrow, predominantly neutrophils [363]. Furthermore, a recent investigation using a systems genetics approach identified a quantitative trait locus on chromosome 2 of mice that implicated the interleukin 1-α and prostaglandin E synthases pathways as key networks involved in modulating Group A streptococcus sepsis [364].

By virtue of the variation in the distribution of phenotypes observed across commonly used inbred mouse strains, growing evidence from systems genetic linkage studies and the enormous complexity of host-pathogen interactions it is highly unlikely that a single gene will explain susceptibility or resistance to common respiratory bacterial pathogens such as GBS or *S. pneumoniae*. One can imagine a situation whereby a polymorphism in any of the detection and effector mechanisms involved in inflammation or resolution toward respiratory bacterial pathogens could provide a unique response that may or may not confer resistance.

### 3.3.4 Alternative mechanisms in determining resistance to respiratory Streptococcal infections

Having discussed the major phenotypic differences observed between C57BL/6 and BALB/c in response to two common respiratory bacterial infections, our results provided a number of other intriguing insights that warranted a brief discussion. We show that a GBS infection provokes a rapid infiltration of both NK and NK T cells into the airway and lung tissue. We also observe the development of a $CD4^{+}$ dominated T

cell response late in infection that could provide B cell help or seed a memory T cell population that provides enhanced immunity upon a GBS re-challenge. The role NK cells play in bacterial infection is not entirely clear, though once activated they release IFN-γ which may enhance APC activation and $Th_1$ T cell development [35;365]. The presence of NK cells may therefore explain the development of the $CD4^+$ $Th_1$ T cell dominated response observed during a primary and secondary GBS infection. The presence of $CD4^+$ T cells in response to a GBS re-challenge enhanced bacterial clearance, however, whether this was due to T cell dependent antibody production or the T cells themselves remains to be elucidated. The presence of NK T cells during a primary GBS infection also raised some interesting questions. Although NK T cells become activated early after challenge with both viral and bacterial pathogens, they are mainly thought to be critical for the clearance of viral infections. However, recent studies show that NK T cells play a critical role in both *S. pneumoniae* and *P. aeruginosa* infections [366;367]. Invariant NK T cells (iNK) secrete cytokines, such as IL-4, IFN-γ, TNF-α, IL13 and GM-CSF [368;369]. Furthermore, a recent study investigating novel NK T cell populations shows that $NK1.1^{neg}$ iNK T lymphocytes represent a new population of IL-17 producing cells that contribute to neutrophil recruitment through preferential IL-17 secretion. Induction of these cells could therefore provide a quick response to infection and contribute anti-*S. pneumoniae* immunity. In addition to KC and MIP-3, TNF-α, together with IL-1 also stimulates neutrophil recruitment [370]. NK T cells may therefore facilitate this in experimental *S. pneumoniae* models. Indeed rapid TNF-α activity within the airways has been shown to protect BALB/c mice from *S. pneumoniae* infection [371]. The status of innate immunity prior to lung infection is also likely to determine the outcome of infection. A recent study demonstrates that protection against *S. pneumoniae* infection is achieved if the mice were previously primed using UV killed *Haemophilus influenza*e [372]. This prior, irrelevant, bacterial challenge is likely to have primed the anti microbial lung microenvironment such that it responds better to subsequent unrelated organisms.

The role of T cells in respiratory bacterial infection remains unclear and is highly dependant on the species of bacterial pathogen investigated. Mice that do not express MHC II, and thus fail to select $CD4^+$ T cells in the thymus, show prolonged *S. pneumoniae* carriage [276]. However, the $CD4^+$ T cell dependent effector functions responsible are yet to be identified and as discussed above, there could also be other important immunity genes present within the mouse *H2* locus that confers resistance to *S. pneumoniae* infection. One likely role of $CD4^+$ T cells in mediating protection against *S. pneumoniae* is the assistance of antigen specific antibody

responses or the activation of macrophages via IFN-γ production. γδ T cells are prevalent in the murine intraepithelial compartment of the lung and it is postulated that they regulate pulmonary dendritic cells and alveolar macrophage and confer protection against *S. pneumoniae* bacteraemia by resolving over exuberant inflammation [373].

## 3.4 Conclusion

Animal models clearly establish a role for genetic factors in the predisposition to respiratory bacterial infections. There is also extensive data from humans that suggest the involvement of complex genetic and host/environment interactions. Understanding the molecular mechanisms responsible for successful protection against *S. pneumoniae,* and other common respiratory pathogens, is critical for the development of effective vaccines, but may also provide novel targets for immunomudulation and antimicrobial drug discovery. We have developed a GBS and a *S. pneumoniae* infection model in both C57Bl/6 and BALB/c mice within the laboratory. Both strains of mice become infected but differ in their susceptibility. Relative resistance is associated with increased neutrophil and NK T cell responses.
For the purposes of the following chapter we will utilise BALB/c mice to develop a secondary bacterial infection model due to its inherent resistance to respiratory bacterial pathogens.

# Chapter 4

## 4.0 Influenza virus induced secondary bacterial pneumonias

### 4.1 Introduction

#### 4.1.1 Infection history determines immune responsiveness

Although continuously exposed to environmental antigens, the mucosal surfaces of the respiratory tract must restrain excessive inflammatory responses in order to fulfil its role of gaseous exchange and prevent bystander tissue damage. Powerful mechanical and immunosuppressive mechanisms protect the lungs against most external aggressions, yet repeat infections with variable immunopathological outcomes are common. In recent years, epidemiological and animal model data demonstrated that one respiratory tract infection alters immunity and pathology to a second unrelated pathogen, even long after the resolution of the first pathogen and in the absence of cross-reactive immunity [299;374]. The outcome of successive infections may be beneficial for the host, as shown for some respiratory virus combinations, or significantly worse as illustrated by the increased susceptibly to life-threatening bacterial pneumonia in patients previously infected with influenza virus [299;375-377]. Altogether, these data suggest that the lungs undergo some form of education process or maturation that alters the way it responds to subsequent challenges.

In the absence of shared T and B cell epitopes what does this maturation or adaptation involve? Bystander activation of unrelated memory T cells, known to be retained in the lungs long after resolution of respiratory viral infection, may modify the local cytokine balance and influence immunity and pathology to subsequent infections [299;378;379]. Regulatory T cells and their associated immune suppressive cytokines, such as IL-10, may also be initiated by the first infection and reduce the amplitude of immunity to the next. We often attribute immune adaptation to acquired T and B lymphocytes and assume that innate immune compartments return to their pre-infection state even after multiple episodes of infection and inflammation. Nevertheless, the number of dendritic cells and their ability to prime T cells remain elevated in the murine lungs long after the resolution of an influenza or respiratory syncytial virus (RSV) infection [380;381]. Influenza infection also leads to the long term

formation of inducible bronchus-associated lymphoid (iBALT) tissue in the lungs where these dendritic cells are located [382].

### 4.1.2 Bacterial recognition and defence in the respiratory tract

Conserved microbial associated molecular patterns (MAMPs), expressed on the surface of pathogens, are recognized by mucosal sentinel cells via pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs). The engagement of TLRs results in the activation of nuclear factor (NF)-kB and the production of pro-inflammatory and antimicrobial signals (as described in section 1.3) [16]. An alteration in the level of PRR expression or cellular re-localization of these receptors as the result of an inflammatory episode can lead to abnormal innate signalling and associated inflammatory responses. For example, TLR expression and response are compartmentalized in the gut epithelium to prevent continual recognition of commensal flora although such compartmentalization has not been described thus far in the respiratory tract [383;384]. Lung epithelial cells do, however, up-regulate TLRs, in particular TLR3 and TLR2 in response to respiratory syncitial virus (RSV) and influenza infection, which may be mediated by the release of interferons (IFN) by infected macrophages [385-387]. Whether such up-regulation has an impact on secondary infection is unclear. Since many of these receptors share components of their signalling pathways, changes in the expression level or phosphorylation status of molecules within these pathways may also interfere with the recognition of subsequent pathogens.

Enhanced adherence of bacteria in the context of viral infection has also been extensively studied [388]. In the case of influenza and pneumococcus synergism, increased bacterial adherence is proposed to be mediated by influenza expressed neuraminidase which exposes cryptic receptors via the cleavage of sialic acids from surface glycoconjugates [389]. Adenoviruses also enhance bacterial adherence [390]. Various antimicrobial molecules are present in the lumen of the gut (beta-defensins) or in the airways (collectins) that have multifunctional properties, including the ability to suppress macrophage responses and to target different types of micro-organisms [391-393]. Most of them are produced at steady state but can also be up-regulated by TLR ligands [394;395]. Altered expression of such molecules with a broad range of specificity could also explain how one infection can impact on multiple secondary pathogens independent of antigenic cross-reactivity.

Furthermore, it is well established that viral infection can affect the function and viability of host cells at the site of infection. Neutrophil apoptosis occurs during lympho choriomeningitis virus (LCMV) infection and suppression of monocyte and neutrophil chemotaxis occurs in humans and animal models of influenza and herpes simplex virus infection [396-399]. Secondary infections may be further affected by the inhibition of macrophage activation and phagocytosis by influenza [400]. It has been shown that RSV infection induces cytokine production and the up-regulation of co-stimulatory molecules by alveolar macrophages, but also reduces their antimicrobial function upon bacterial challenge [401]. In addition, innate cells, including macrophages and epithelium are susceptible to tolerance, which in the short term compromises subsequent inflammatory responses. A good example of this phenomenon is cross-tolerance between TLR agonists, which could explain why patients with sepsis develop an immunosuppressive state characterized by the hypo-responsiveness of their monocytes and increased susceptibility to pulmonary bacteria [402-404].

### 4.1.3 Hypothesis and aims

Human epidemiological and clinical data clearly demonstrates a relationship between a primary influenza virus infection and the development of secondary bacterial pneumonias. Despite the existence of a number of possible explanations, the field remains ambiguous and the precise mechanism(s) by which influenza virus increases susceptibility to respiratory bacterial pathogens remains to be elucidated. On the basis of previous work within the laboratory, illustrating how a previous infection or inflammatory insult can modulate immunity to a second unrelated infection [298;299;405], we hypothesise that influenza virus infection pre-disposes to secondary bacterial pneumonia by modulating the capacity of the airway to detect and mount a sufficient innate cellular response. We investigate this hypothesis via the following aims:

1. Develop a robust and reproducible model of influenza induced secondary bacterial infection that is physiologically significant but is non lethal.
2. Determine the temporal relationship between the two disparate infections, and at what point the combination of virus/bacteria becomes more detrimental than either alone.
3. Assess specific innate pathways and their role in controlling secondary bacterial pneumonia following influenza infection.

## 4.2 Results

As discussed above, the precise mechanism(s) by which influenza increases susceptibility to respiratory bacterial infections remain to be fully elucidated. We therefore sought to develop a sequential secondary bacterial infection model and use this to investigate the temporal relationship that exists between the two infections and shed light on how influenza modulates immunity to increase susceptibility to a subsequent bacterial infection. Having previously developed and characterised both a GBS and *S. pneumoniae* single bacterial infection model, it was also necessary to understand a typical influenza virus infection in mice.

### 4.2.1 Influenza infection induces weight loss and pulmonary inflammation in BALB/c and C57BL/6 mice

A typical influenza virus infection protocol is illustrated in Figure 4.1 panel a, both BALB/c and C57BL/6 mice infected with 50 HAU of influenza virus, equivalent to 1.25 x $10^5$ pfu, experienced acute weight loss that began on day 2-3 post infection and peaked on days 6-7. C57BL/6 mice lost less weight and recovered weight significantly quicker than BALB/c mice (Figure 4.1 b). The peak airway infiltrate coincided with maximal weight loss implying that illness severity, measured in part by weight loss, correlated directly with the exuberance of the host's immune response (Figure 4.1 b & c). By day 14 the observed pulmonary inflammation had largely subsided and mice had returned to their original body weight. Illness associated with influenza infection is also attributed to viral cytopathology. Analysis of influenza virus replication highlighted a rapid phase of viral replication with titres peaking on day 1-2 post infection before declining gradually becoming undetectable by day 7 (Figure 4.1 b). Peak viral load directly coincided with a transient neutrophil response observed in both the airway and lung tissue. Neutrophilia was more protracted in the lung tissue with levels elevated above naive levels up to and including day 14 post infection (Figure 4.1 d). T cells are also involved in viral clearance and are a significant component of the observed pathology, causing airway occlusion and producing inflammatory mediators that are responsible for the observed cachexia and fever. The extent of this cellular response is clearly evident with histology (Figure 4.2). The right azygous lobe was removed at days 2, 7 and 14 post influenza infection and examined using light microscopy. Perivascular and peribronchiolar infiltrates were visible 2 days after influenza infection. However, the extent of this infiltrate was most evident on day 7 post infection. At this time point there was also evidence of bronchiolar wall hypertrophy, bronchiolar oedema, epithelium hyperplasia and the

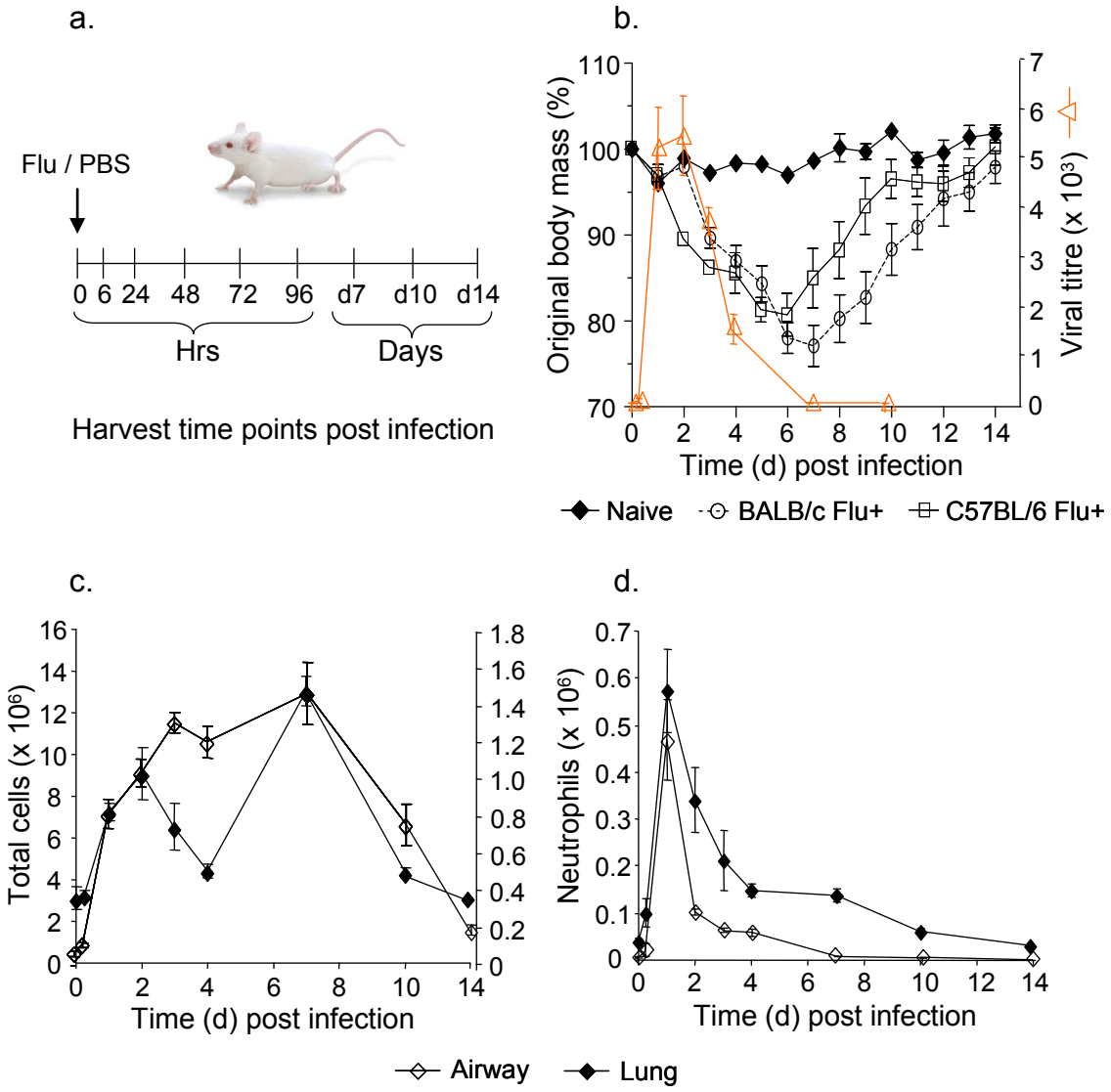



**Figure 4.1 Influenza infection induces weight loss and pulmonary inflammation in BALB/c and C57BL/6 mice.** A typical infection protocol uses BALB/c or C57BL/6 mice (n=4) infected intranasally with 50 HAU HK/X31 influenza A virus or PBS naïve control (a). Weight loss (b, left axis) was monitored daily and expressed as mean % of original body mass. BAL was performed and lung tissue removed at 6 hrs, 1, 2, 3, 4, 7 and 14 days after infection. Total viable airway (c - right axis) and lung (c - left axis) cells were enumerated using Trypan blue exclusion. Flow cytometry was utilised to determine the number of neutrophils present in the airway and lung tissue (d). Viral titres were also analysed from snap frozen BALB/c lung lobe (b, right axis). Data are presented as the mean ± SEM of 4 mice per group.

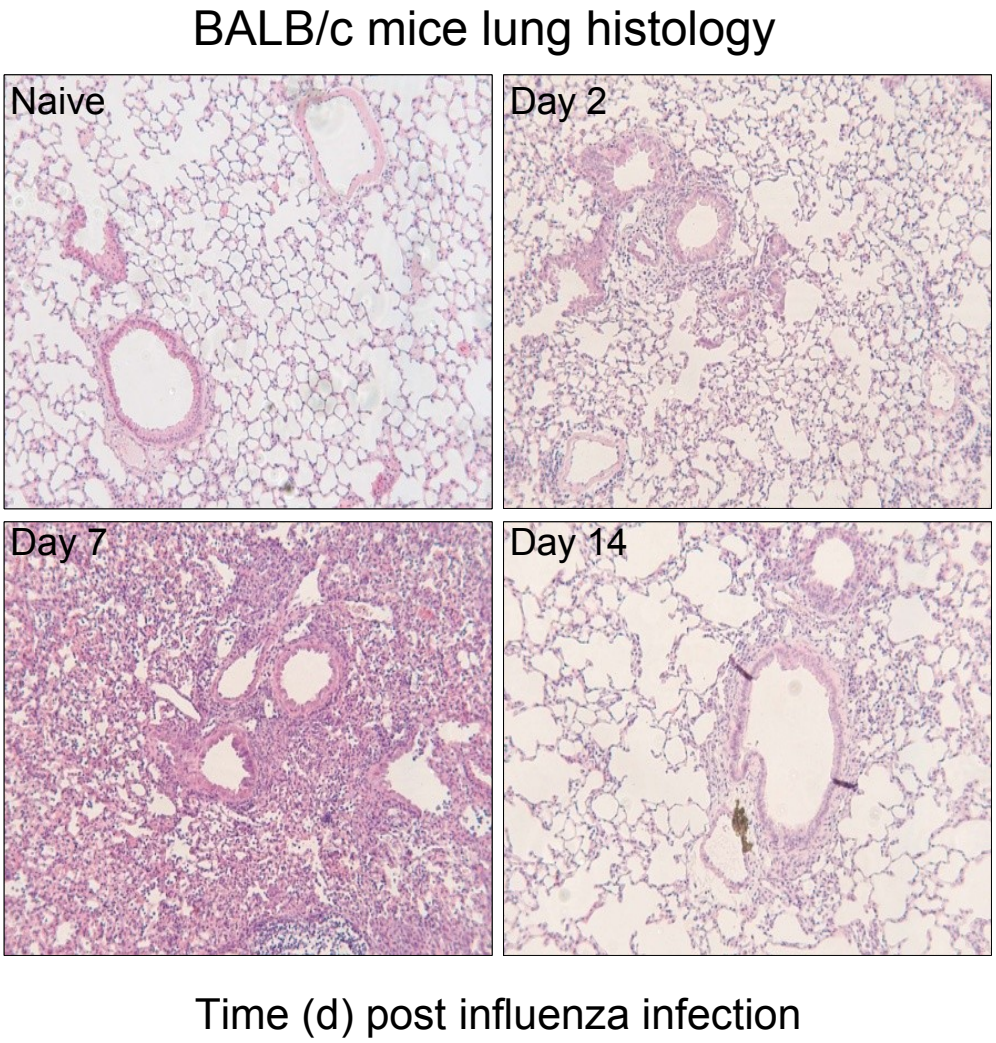


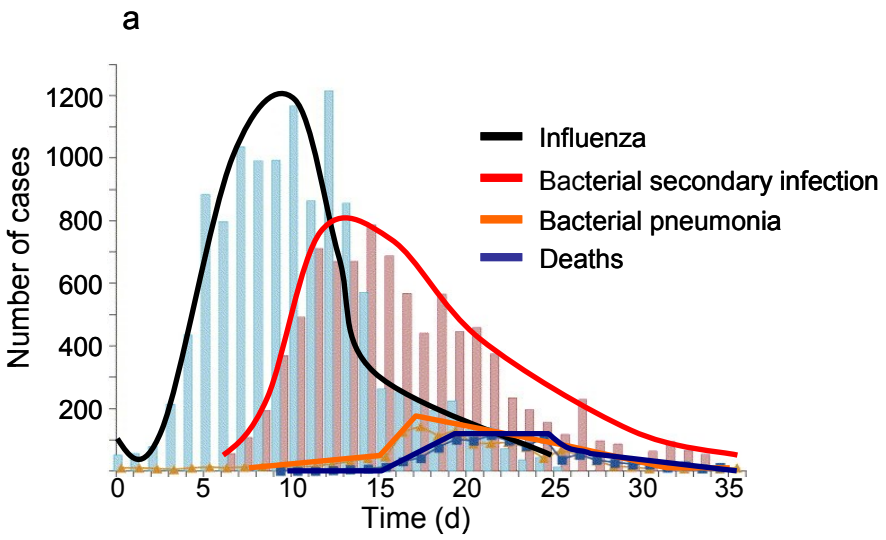

**Figure 4.2 Progressive lung pathology and injury in the lungs of BALB/c mice during an influenza infection.** BALB/c mice (n=5) were infected intranasally with 50 HAU HK/X31 influenza A virus on day 0. Mice were subsequently harvested on days 2, 7 and 14 days post infection. Processing of mouse lungs for histology and light microscopy of H & E stained sections were carried out as described in section 2.7.1. Magnification was x 10. Sections are representative of five mice studied at each time point.

presence of cellular debris in the larger airways. By day 14, the excessive cellular infiltrate had subsided leaving a small number of isolated foci of peribronchiolar infiltrate surrounding the larger airways. At this time point an improvement in alveoli architecture was also clearly visible (Figure 4.2).

### 4.2.2 Simultaneous infection of BALB/c mice with influenza virus and GBS results in reduced associated viral weight loss

Extensive clinical evidence suggests that during, or shortly after, an influenza infection a 'window of opportunity' exists in which the lung becomes highly susceptible to invasive bacterial infection. Epidemiological evidence from the 1918 influenza pandemic clearly demonstrates this temporal relationship (Figure 4.3 a). The appearance of secondary bacterial pneumonias begins approximately 7 days after the first cases of influenza virus infection. To investigate this temporal relationship an awareness of the distinct phases of an influenza infection in mice is necessary (Figure 4.3 b). Using this as a rudimentary guide, we developed a secondary bacterial infection model in which a pre-determined non lethal dose of GBS was intranasally administered at various time points post influenza infection (Figure 4.4). Bacterial titres in the nasopharyngeal cavity, distal airway, lung tissue and peripheral blood were measured along with other immune parameters to determine whether or not a concurrent or previous influenza infection affects the host's susceptibility to a respiratory bacterial infection.

Initially, BALB/c mice were simultaneously infected intranasally with 50 HAU of influenza virus and 5 x $10^6$ cfu GBS. Mice were subsequently followed over 14 days and at specified time points post GBS infection, harvested and analysed. As was described previously, mice infected with GBS alone showed no outward signs of clinical illness and followed the same weight profile as vehicle control mice (Figure 4.5 a). Both groups of mice infected with influenza followed a typical weight loss profile, however mice co-infected with influenza and GBS recovered weight significantly faster than those infected with influenza alone (Figure 4.5 a). On comparison of viable bacterial titres recovered from co-infected and GBS alone infected mice, no significant difference was observed in the airway, lung tissue and nasopharyngeal cavity up to and including day 3 post GBS infection. However, on day 5, viable GBS bacterial titres were observed in the lung tissue of 50 % of those mice simultaneously infected with influenza and GBS (Figure 4.5 c). GBS cfu also




Adapted from: Brundage JF. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. Lancet Infect Dis. 2006, 6 (5):303-12

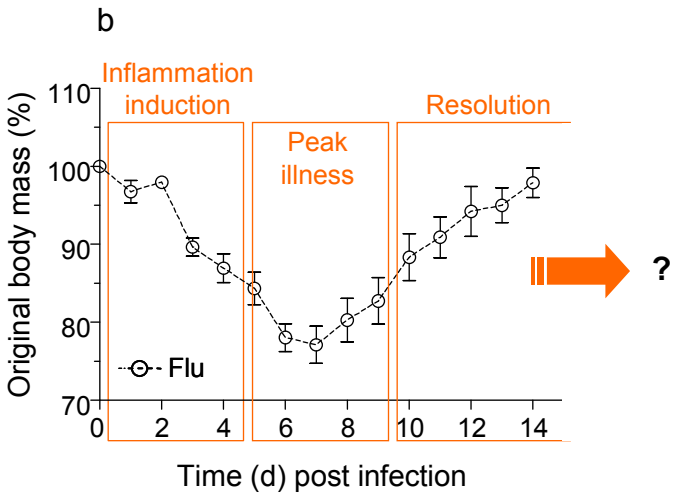
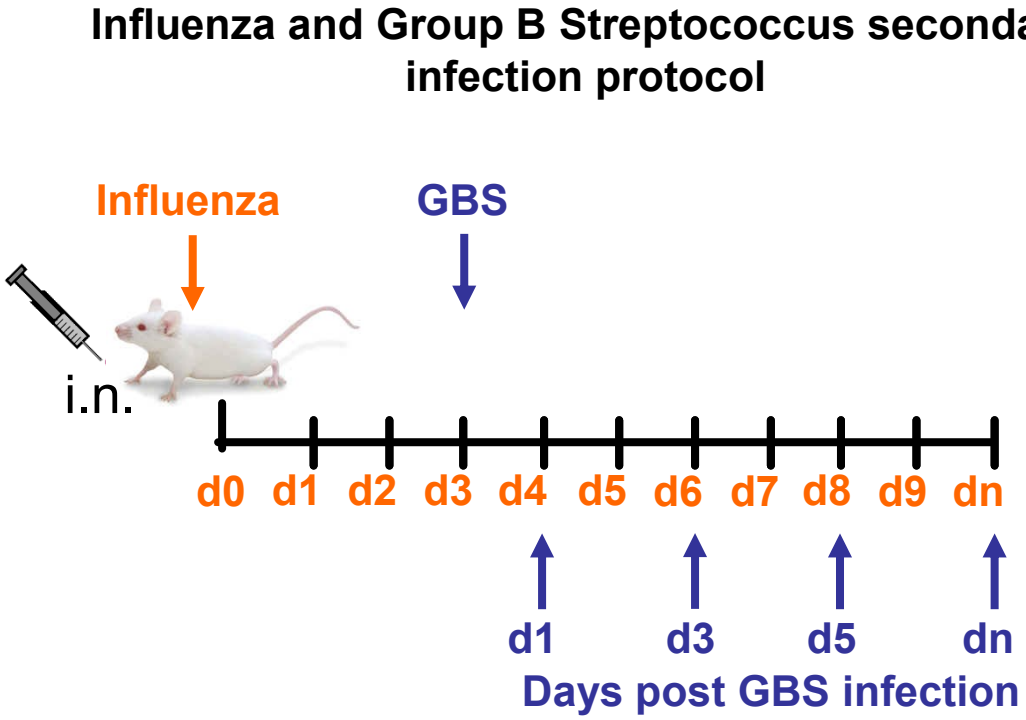



**Figure 4.3 Epidemiological data suggests that secondary bacterial infections occur within a certain time frame after an initial influenza virus infection.** (a) Human epidemiological data from the 1918 Spanish Flu pandemic highlighting the temporal relationship between influenza virus infection and a wave of secondary bacterial infections. (b) A representation of a typical murine influenza infection using weight loss to demonstrate three distinct phases of infection.




**Tissue harvest and analysis:**

- Daily weight loss (Original body mass %)
- BAL
  - Cell phenotype – Flow cytometry
  - Bacterial cfu
  - Cytokines/chemokine analysis
- Lung
  - Cell phenotype – Flow cytometry
  - Bacterial cfu
  - Snap freeze lobe(s) – Cytokine analysis
- Nasal Wash – Bacterial cfu/IgA antibody measurement

**Figure 4.4 An illustration of a standard secondary bacterial infection protocol.** BALB/c mice (n=4) are inoculated intranasally (i.n.) with 50 HAU HK/X31 influenza A virus. On a pre specified day after influenza infection mice are infected i.n. with 5 x $10^6$ cfu mid log phase Group B streptococcus (GBS), unless otherwise stated. Mice are followed over the course of the infection and harvested on days specified in the results. Designated tissues were removed and analysed subsequently.

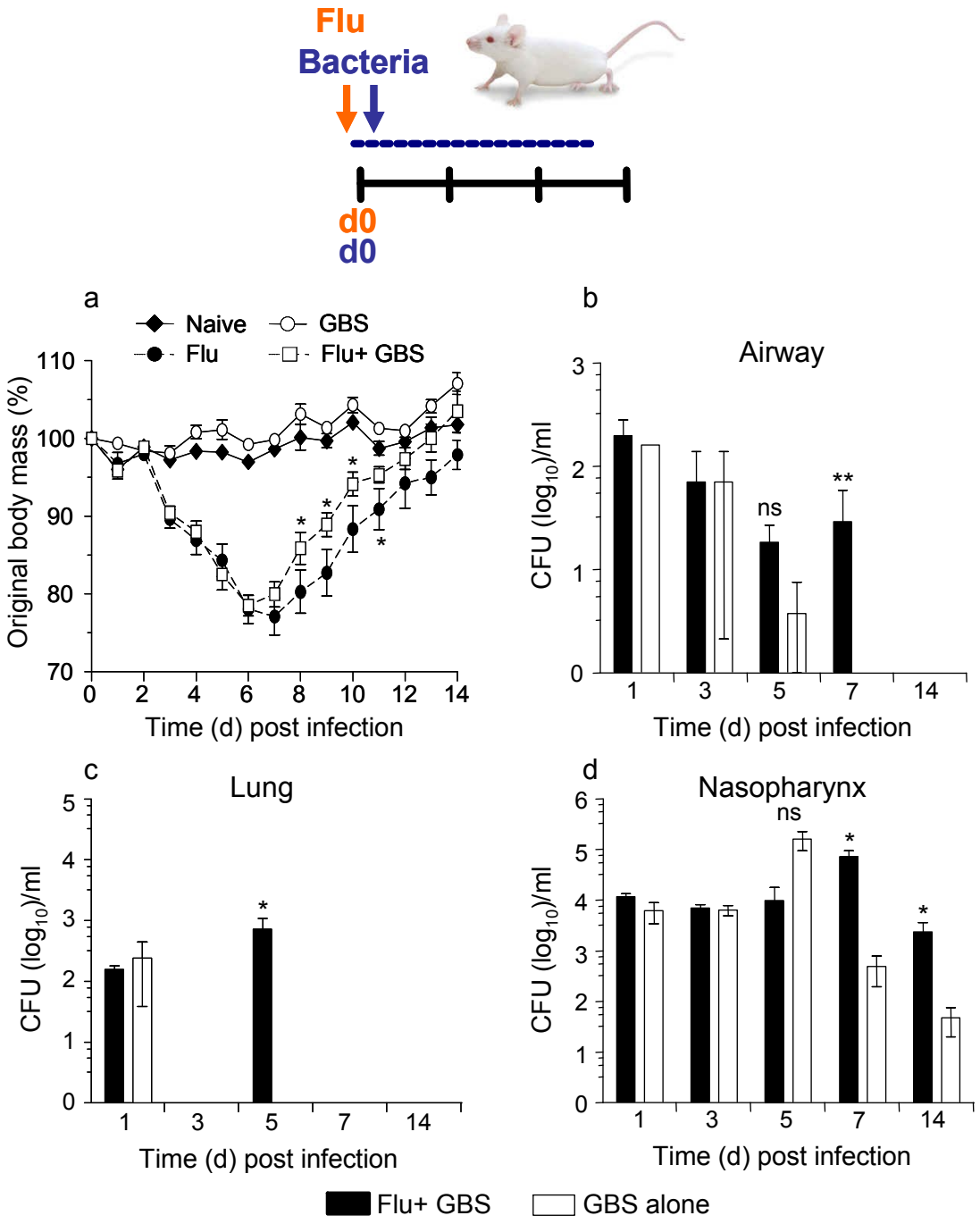



**Figure 4.5 Simultaneous infection of BALB/c mice with influenza and GBS alleviates viral induced weight loss but reduces bacterial clearance.** BALB/c mice (n=4) were intranasally infected simultaneously with 50 HAU HK/X31 influenza A virus and 5 x $10^6$ cfu GBS or GBS and influenza on there own. Weight loss (a) was monitored daily and expressed as mean % of original body mass. On days post GBS infection lung tissue was removed and BAL and nasal wash was performed. Colony forming units (cfu) were calculated using serial dilutions of single cell suspensions of each tissue sample and plated on THB agar. GBS cfu titre recovered from airway (b), lung (c) and nasopharynx (d). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

remained in the airway until day 7 and were significantly higher in the nasopharyngeal cavity of mice simultaneously infected with influenza and GBS (Figure 4.5 b & d). The level of GBS recovered from the nasopharynx was significantly greater than observed in the airway or lung at all time points analysed. There was no recorded difference in the outward appearance and clinical illness score of mice simultaneously infected with influenza and GBS compared to mice infected with GBS alone (data not shown).

### 4.2.3 Mice infected with GBS 3 days after influenza virus infection contain heightened bacterial titres in all tissues analysed

We have previously shown that infection of BALB/c mice with 5 x $10^6$ cfu of GBS resulted in the clearance of bacteria in all tissues by day 5 post infection, with no associated weight loss. This is driven by a transient neutrophil and macrophage response which effectively primes an adaptive response capable of establishing immunological memory. We have also demonstrated that a simultaneous infection of influenza and GBS resulted in reduced influenza induced weight loss and a small, yet significant, delay in bacterial clearance from the airway and lung tissue. Therefore, we next investigated whether a previous influenza virus infection alters the susceptibility to a GBS infection. BALB/c mice were intranasally infected with influenza or vehicle control, as previously described, and subsequently infected with 5 x $10^6$ cfu of GBS 3 days later. Mice were harvested on days 1, 3, 5, 7 and 11 days post GBS infection. Mice infected with GBS after a primary influenza infection did not demonstrate additional weight loss compared to mice infected with influenza alone (Fig. 4.6 a). The absence of weight loss to GBS infection alone was again confirmed. Existing theories suggest the increased susceptibility to bacterial infections after a primary influenza infection can be attributed, in part, to a reduction in respiratory epithelial integrity. We previously demonstrated that mice infected with GBS alone had no detectable cfu in the airway or lung tissue after 3 days. In contrast, mice infected with GBS 3 days after an influenza virus infection demonstrated a significant increase in bacterial titre recovered from both the airway and lung, when compared to GBS infected alone mice, and a reduction in the rate of bacterial clearance (Figure 4.6 b & c). The levels of GBS recovered from the nasopharynx were also elevated in mice infected with influenza virus 3 days prior to GBS infection (Figure 4.6 d). Interestingly, although bacterial titres were increased in mice previously infected with influenza virus, the administration of GBS 3 days after a primary influenza infection reduced viral load compared to mice infected with influenza virus alone (Figure 4.6 e).

### 4.2.4 GBS infection during peak influenza illness results in additional weight loss and a significant increase in bacterial susceptibility

After demonstrating that BALB/c mice have enhanced susceptibility to a GBS infection 3 days after infection with influenza virus, coinciding with peak viral load, we next investigated the effects on bacterial susceptibility during the peak illness phase of an influenza infection. As previously described, BALB/c mice were infected with 50 HAU of influenza or vehicle control and subsequently infected with 5 x $10^6$ cfu of GBS 5 days later. The addition of GBS on day 5 of an influenza infection resulted in additional weight loss (Figure 4.7 a). The additive effect of a GBS infection on weight loss was accompanied by a substantial increase in GBS cfu recovered from the airway, lung tissue and nasopharynx (Figure 4.7 b-d). The bacterial titres recovered from all three compartments were 10 times greater than those recovered from the corresponding tissue at the same time post GBS infection when GBS was administered 3 after influenza infection. However, despite these significant differences bacteria were cleared from the airway and lung tissue by day 7 and had returned to levels observed in GBS alone infected mice in the nasopharynx (Figure 4.7 b-d). Additionally, mice infected with GBS on day 5 of an influenza infection appeared more sedentary and hunched compared to mice infected with GBS alone (data not shown).

### 4.2.5 Influenza induced susceptibility to respiratory GBS infection remains for several weeks

After demonstrating that susceptibility to a respiratory GBS infection increases as an influenza illness proceeds, we next examined whether the enhanced susceptibility continues or abates on resolution of influenza illness. BALB/c mice were again infected with 50 HAU of influenza virus, or vehicle control, and allowed to recover for 14, 28 or 42 days. All mice returned to day 0 weight by day 14-16 post infection and total airway and lung cellularity was equal to naive levels by day 28 post influenza infection (data not shown). All recovered mice were subsequently infected with 5 x $10^6$ cfu of GBS and harvested at specified time points post GBS to compare bacterial susceptibility. Mice infected with influenza and allowed to recover for 14 days demonstrated elevated GBS bacterial titres in both the airway and lung 3 days post GBS infection compared to vehicle (no influenza virus) control mice (Figure 4.8 a). There was no significant difference observed in the nasopharyngeal bacterial load. Mice allowed to recover from influenza infection for 28 days also showed enhanced susceptibility to GBS infection, with elevated GBS bacterial titres recovered from both the airway and lung compartments (Figure 4.8 b). Once more there was no difference

observed in the nasopharyngeal carriage rates between the two groups. Finally, mice infected with influenza virus 42 days (6 weeks) prior to infection with GBS also displayed enhanced GBS susceptibility (Figure 4.8 c).

Before investigating possible mechanisms for how a primary influenza infection increased susceptibility to a subsequent bacterial infection, we investigated whether a primary GBS infection could alter subsequent influenza immunity and whether GBS can colonise the nasopharynx and upon subsequent influenza infection propagate and develop into an invasive bacterial infection. To examine this, we infected BALB/c mice with GBS and allowed them to resolve the infection for 28 days. Mice were subsequently infected with influenza virus, as previously described, and harvested at specified time points post influenza to investigate influenza immunity and GBS titre (Figure 4.9 a). No recoverable GBS cfu were detected in any tissue at the time of influenza infection (data not shown). A previous GBS infection did not affect total cell recruitment in response to influenza (Figure 4.9 b & c). However, despite the presence of a residual $CD4^+$ T cell population in the airway of mice infected with GBS 28 days previously (Figure 4.9 d), there was no significant difference in the proportion or total number of $CD8^+$ or $CD4^+$ T cells in response to influenza infection between the two groups (data not shown). No detectable GBS cfu were recovered from any tissue at all time points analysed post influenza infection (Figure 4.9 e).

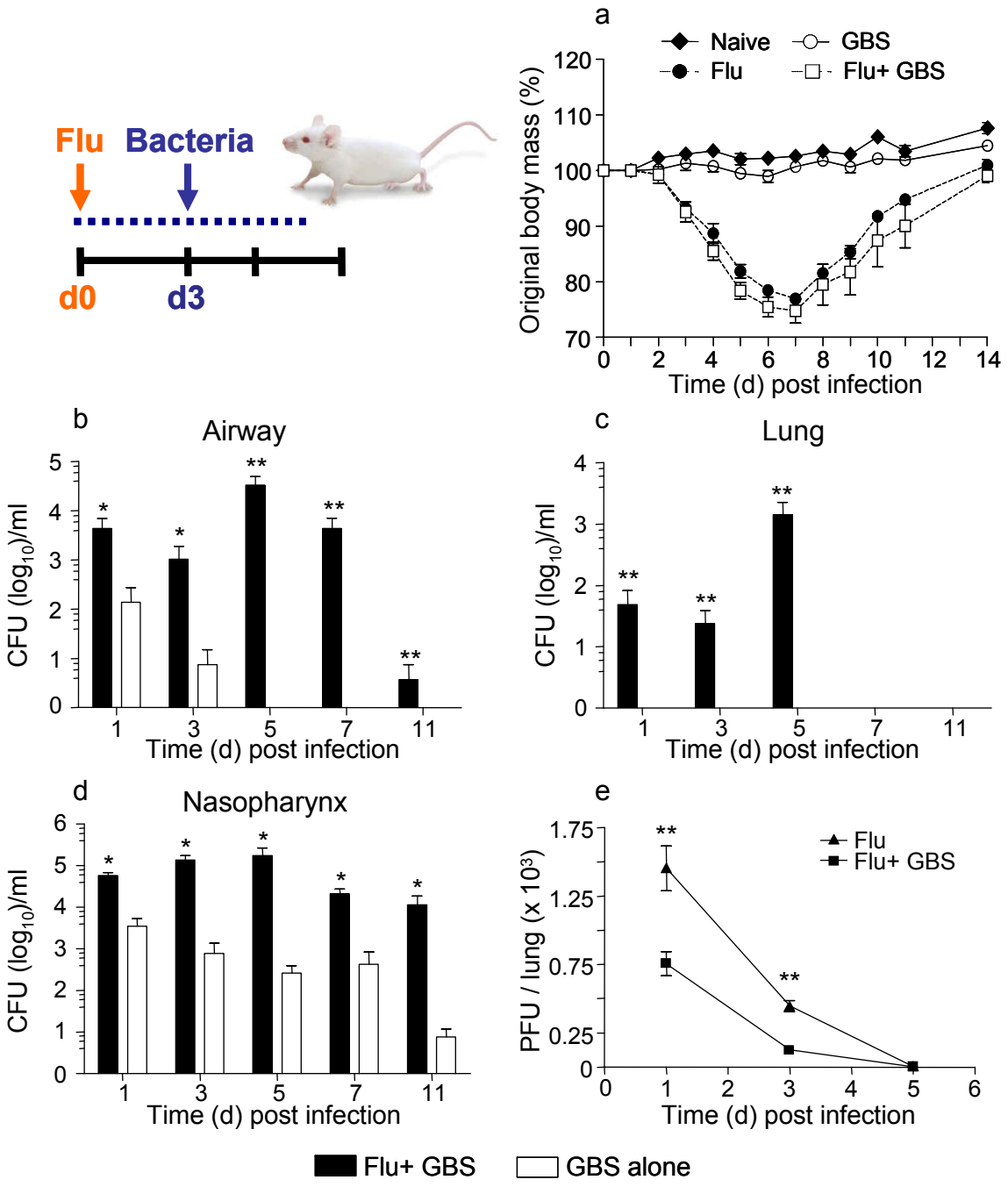



**Figure 4.6 BALB/c mice infected with GBS 3 days after influenza virus have a reduced ability to clear bacteria.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. After three days, mice were challenged with either 5 x $10^6$ cfu GBS i.n or PBS control. Weight loss (a) was monitored daily and expressed as a % of original body mass. On days post GBS infection lung tissue was removed and BAL and nasal wash was performed. Airway (b), lung (c) and nasopharynx (d) bacterial titres were calculated using serial dilutions of single cell suspensions of each tissue sample and plated on THB agar. Viral titre (e) was also analysed from snap frozen lung lobe. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

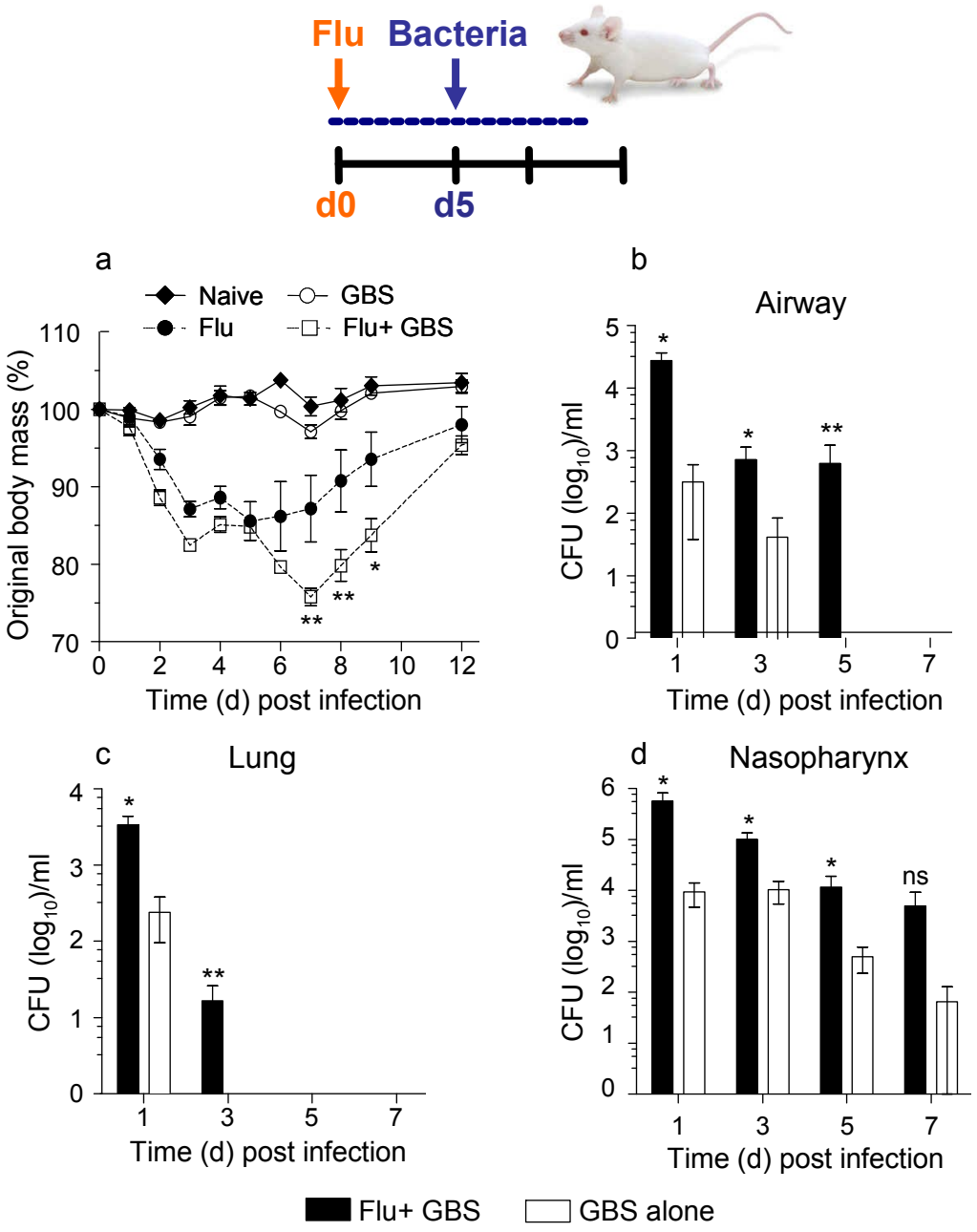



**Figure 4.7 GBS infection during peak influenza illness results in increased weight loss and bacterial burden.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. Five days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS or PBS control. Weight loss (a) was monitored daily and expressed as % of original body mass. On days post GBS infection lung tissue was removed and BAL and nasal wash was performed. CFUs were calculated using serial dilutions of single cell suspensions of each tissue sample and plated on THB agar. GBS cfu titre recovered from airway (b), lung (c) and nasopharynx (d). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

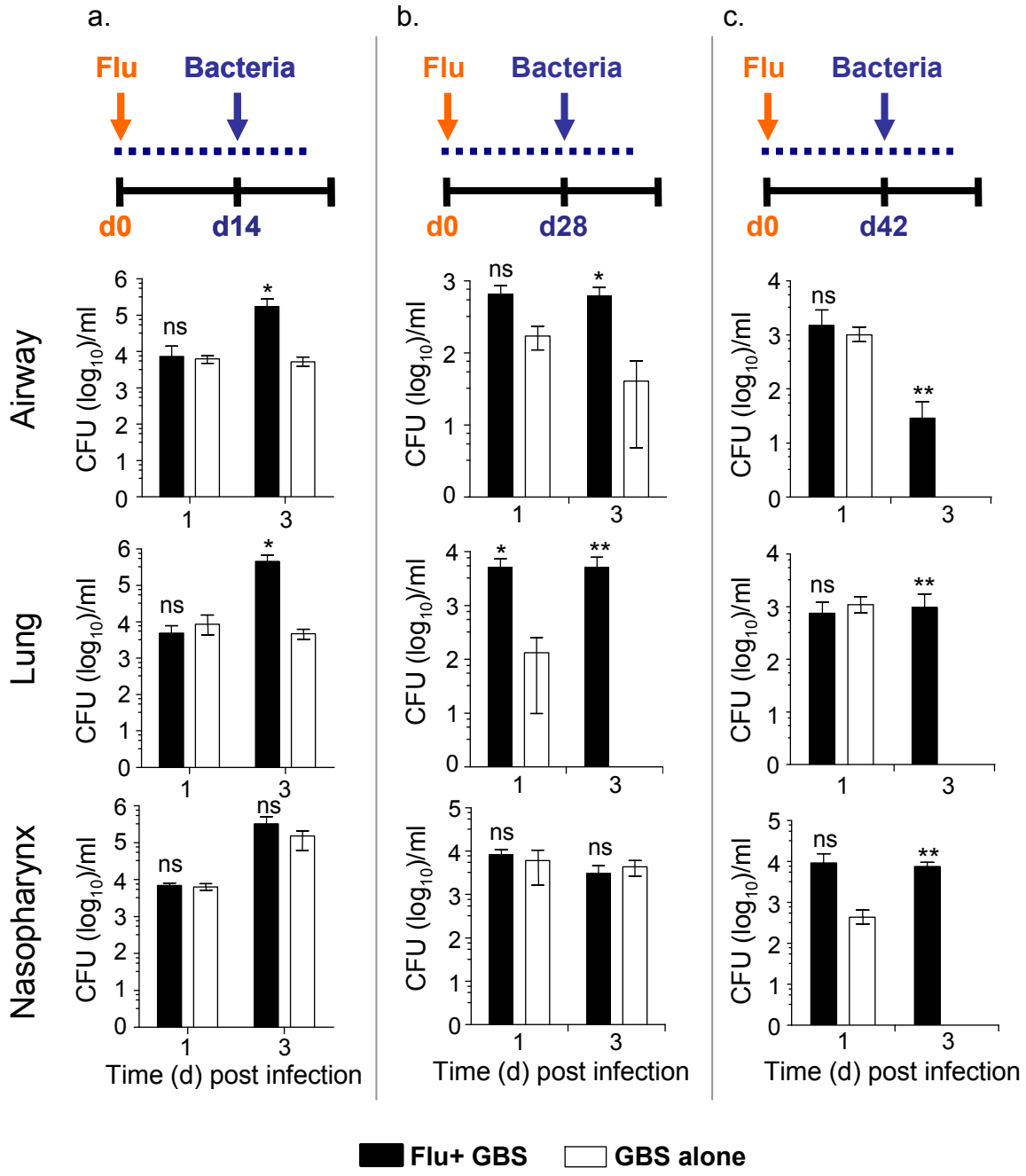



**Figure 4.8 Influenza virus infection has a long lasting effect on the lung's ability to clear a GBS infection.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. 14 days (a), 28 days (b) or 42 days (c) after influenza infection all mice were challenged with 5 x $10^6$ cfu GBS. On days specified, post GBS infection, BAL, lung and nasal wash was performed and collected. CFUs were calculated using serial dilutions of single cell suspensions of each tissue sample and plated on THB agar. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

### 4.2.6 Co-infection with influenza virus and GBS does not significantly increase airway cellularity but enhances total lung cell numbers

We have demonstrated that susceptibility to a respiratory GBS infection begins as early as 3 days after the primary influenza infection and continues for at least 6 weeks. To investigate possible mechanism(s) responsible for this phenomenon, we next examined differences in the innate cellular response to a GBS infection in mice previously infected with influenza virus in comparison to naive mice. By analysing the total cellular response at time points after GBS infection it was apparent that a GBS infection at the same time as, or up to 5 days after, an influenza infection resulted in an additive increase in the total number of cells present in the airway 12-24 hours post GBS infection (Figure 4.10 a, c & e). This increase was most evident at 24 hours, in mice infected with GBS at the same time as or 5 days after influenza infection, and at 72 hours when mice were infected with GBS 3 days after the primary influenza infection. However, due to a large standard deviation, there was no statistically significant increase at any time point. However, at all time points analysed, mice co-infected with GBS and influenza consistently contained more total cells in the airway compared to mice infected with GBS alone. On comparison of total lung cellularity, there was a significant increase in total numbers 24 hours after GBS infection in mice simultaneously infected with influenza and GBS (control, 1.7 ± 0.4 x $10^6$; + influenza 4.6 ± 0.6 x $10^6$; n=4; p 0.03) and on day 3 in mice infected with GBS 3 days after the primary influenza infection (control; 5.4 ± 0.7 x $10^6$; post influenza 1.0 ± 0.3 x $10^7$; n=4; p 0.02) (Figure 4.9 b & d). However, this significant increase in cellularity was not observed in mice infected with GBS on day 5 of an influenza infection compared to mice infected with influenza alone (Figure 4.10 f).

In contrast, mice allowed to completely recover from the primary influenza infection for 28 and 42 days displayed a reduction in cellular recruitment to the airway in response to a GBS infection (Figure 4.11 a ). This was statistically significant in mice allowed to recover from influenza for 42 days. There is no statistical significance when comparing lung cellular recruitment between influenza recovered mice and PBS control mice (Figure 4.11 b). Table 4.0 summarises the outcome of a GBS respiratory infection when administered at varying times post influenza virus infection.

### 4.2.7 A previous influenza virus infection reduces the number of airway neutrophils present following a respiratory bacterial infection

Investigating the total cellular infiltrate elicited in response to GBS only provides a small insight into the cellular responsiveness of naive and influenza infected/resolved mice. Further interrogation of the composition of the cellular response was needed to determine the potential effects influenza infection had on specific leukocyte subsets. We have previously demonstrated that neutrophils are rapidly recruited to both the airway and lung tissue during an influenza infection (Figure 4.1 d). Their precise function during an influenza infection remains open to debate, however the evidence for their role in anti-bacterial defence is much more compelling. There is abundant *in vitro* evidence highlighting synergistic effects of influenza virus and bacteria in detrimentally affecting both the function and viability of neutrophils [287;406]. Therefore, we next examined the exact composition of the cellular response to GBS in naive mice and mice previously infected with influenza. Using FCM and differentially expressed surface antigens, the proportions of neutrophils, macrophages and their activation status were measured. Mice simultaneously infected with influenza and GBS showed similar proportions of neutrophils in the airway at all time points analysed as those mice infected with GBS alone (Figure 4.12 a).

On calculating the actual numbers of neutrophils in the airway there appeared to be an increase in total numbers on day 1 post infection in mice simultaneously infected with influenza and GBS compared to mice infected with GBS alone, however this increase was statistically insignificant (Figure 4.12 b). There was an increase in the number of neutrophils in the lung tissue on day 1 in mice simultaneously infected with influenza and GBS compared to mice infected with GBS alone (p 0.025 data not shown). In contrast, mice infected with GBS on day 3 of an influenza infection displayed a reduction in the proportion and total numbers of neutrophils 1 day post GBS infection compared to mice infected with GBS alone (Figure 4.12 c & d). This reduction was also observed in the lung tissue of mice infected with GBS on day 3 of an influenza infection (Figure 4.13 a-c). Mice infected with GBS on day 5 of an influenza infection also showed a significant reduction in total neutrophil numbers and proportion in the lung tissue (data not shown). In mice allowed to recover from an influenza infection for 42 days, a similar reduction in total number and the proportion of airway neutrophils 24 hours post GBS infection was observed (Figure 4.12 e & f). This trend was also seen in the lung tissue but was statistically insignificant (data not shown).

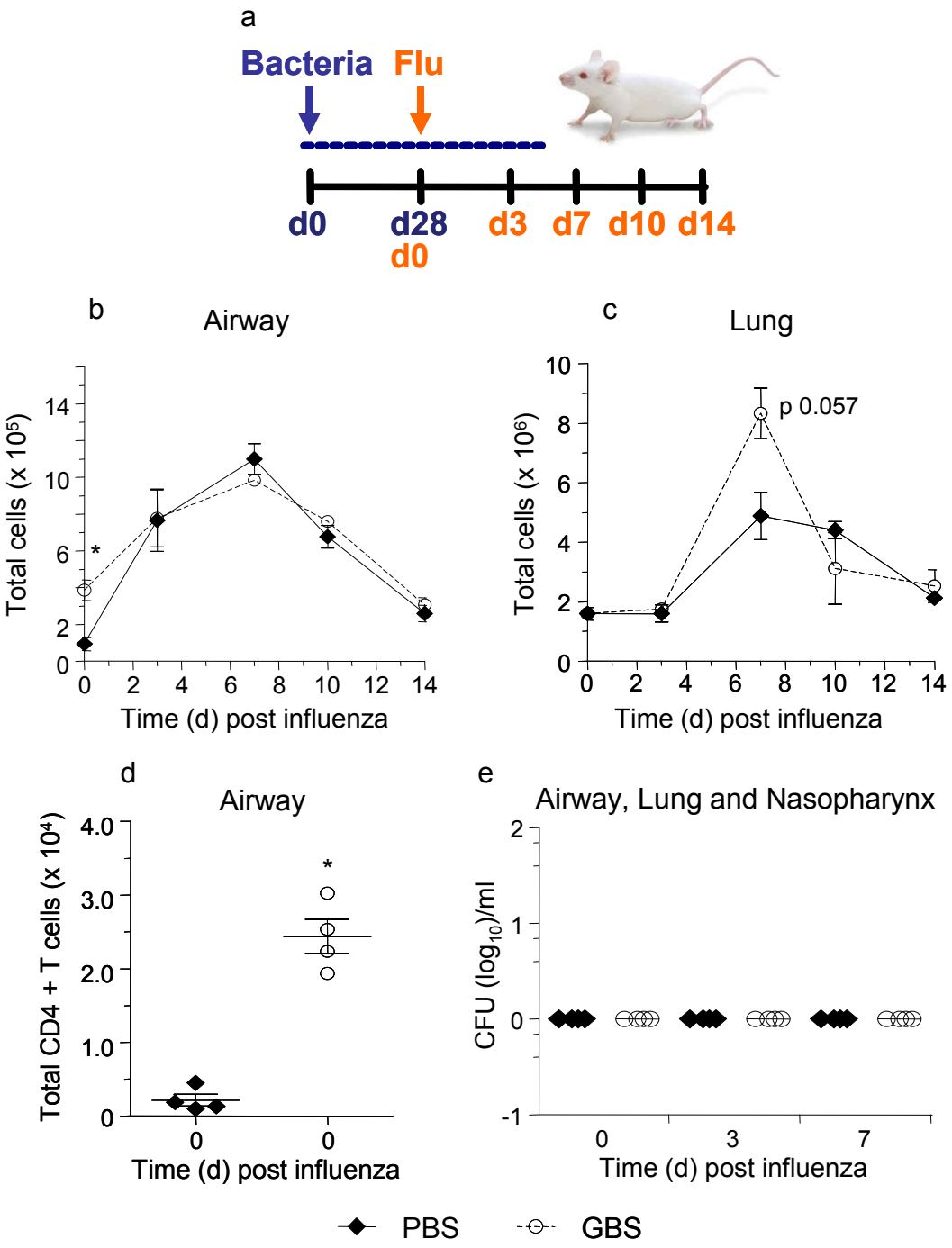



**Figure 4.9 Influenza infection does not reactivate a previous GBS infection.** BALB/c mice (n=4) were infected intranasally (i.n.) with 5 x $10^6$ cfu GBS or PBS control. Mice were subsequently infected i.n. with 50 HAU HK/X31 influenza A virus 28 days later (a). On days specified, post influenza infection, airway cells (b) and lung cells (c) were removed and total viable cell counts were determined using Trypan Blue exclusion. Flow cytometry was used to determine the total number of airway $CD4^+$ T cells at the time of influenza infection (d). CFUs were also measured in the airway, lung and nasopharynx by using serial dilutions of single cell suspensions and plated on THB agar (e). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

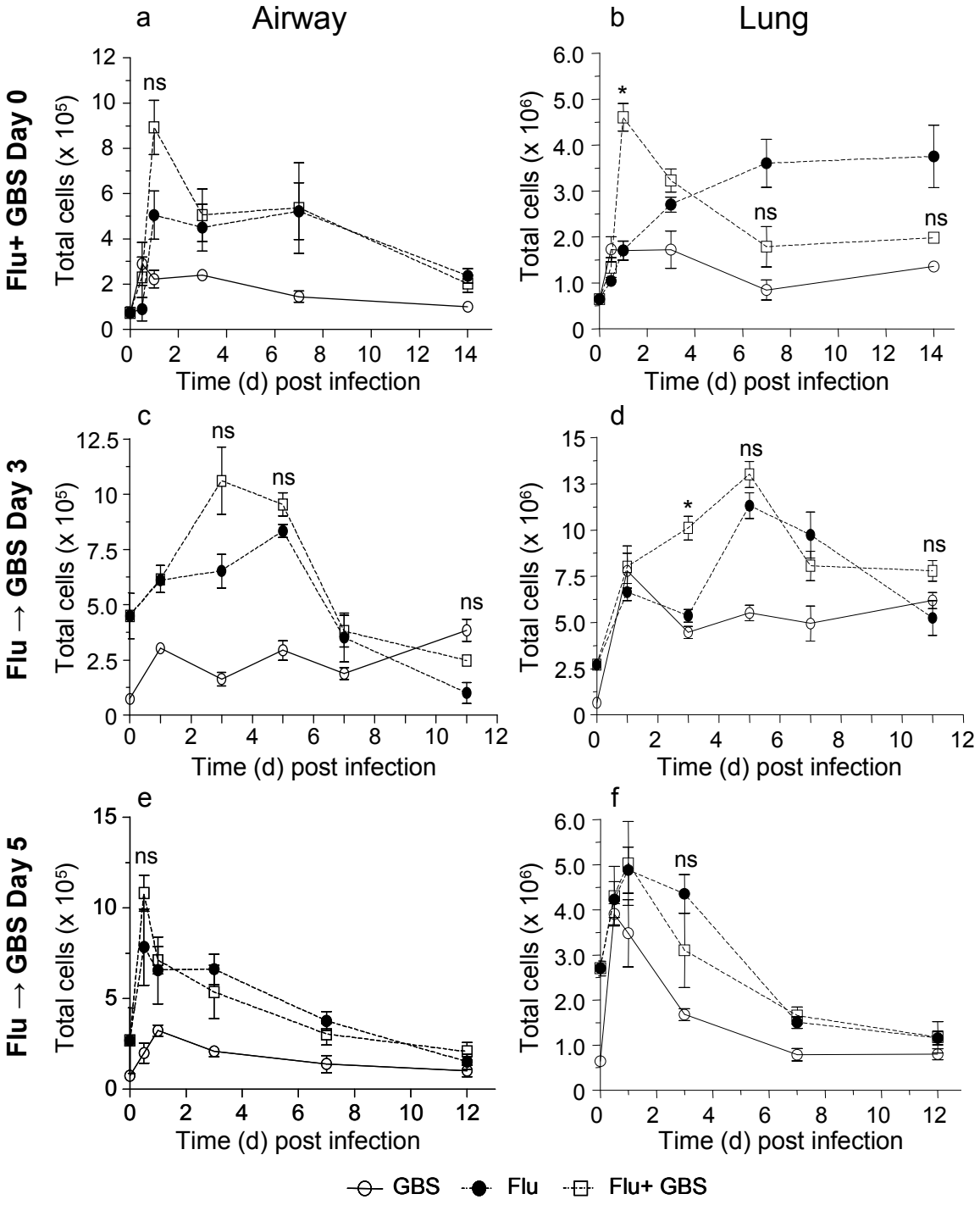



**Figure 4.10 Co-infection with influenza virus and GBS does not significantly increase airway cellularity but enhances lung total cell numbers.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. 5 x $10^6$ cfu GBS was intranasally administered at the same time (a-b), 3 days after (c-d) or 5 days after (e-f) the primary influenza infection. On days specified, post GBS infection, BAL was performed and lung tissue was removed, homogenised and total viable cell counts were determined using Trypan Blue exclusion. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$ ns = no significance

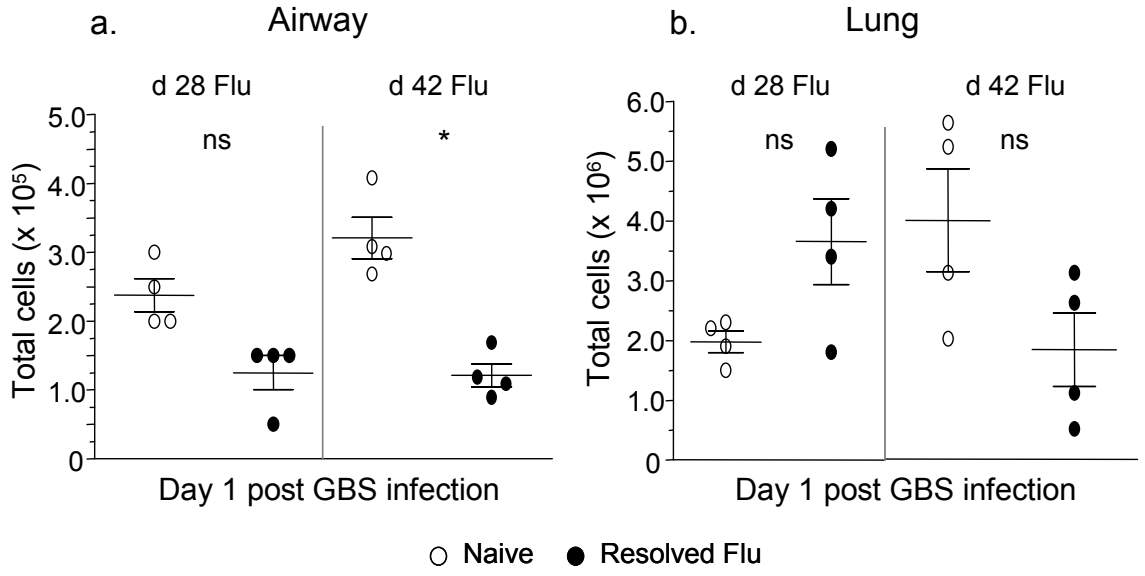



Table 4.0.

| Day of influenza infection on which GBS is administered | Influenza illness stage | Airway CFU | Lung CFU | NW CFU | Airway Cellularity | Lung Cellularity |
|---|---|---|---|---|---|---|
| Day 0 | Induction | ↔ | ↔ | ↔ | ↔ | ↑ |
| Day 3 | Peak Illness | ↑ | ↑ | ↑ | ↔ | ↑ |
| Day 5 | Peak Illness | ↑ | ↑ | ↑ | ↔ | ↔ |
| Day 28 | Resolved | ↑ | ↑ | ↔ | ↓ | ↔ |
| Day 42 | Resolved | ↑ | ↑ | ↑ | ↓ | ↔ |

**Figure 4.11 Infection with GBS after influenza resolution results in reduced cell recruitment to the airway.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. 5 x $10^6$ cfu GBS was administered 28 or 42 days after the primary influenza infection. On days specified, post GBS infection, airway cells (a) and lung cells (b) were removed and total viable cell counts were determined using Trypan Blue exclusion. Table 4.0 summarises the cfu and cellularity data from different secondary infection protocols Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

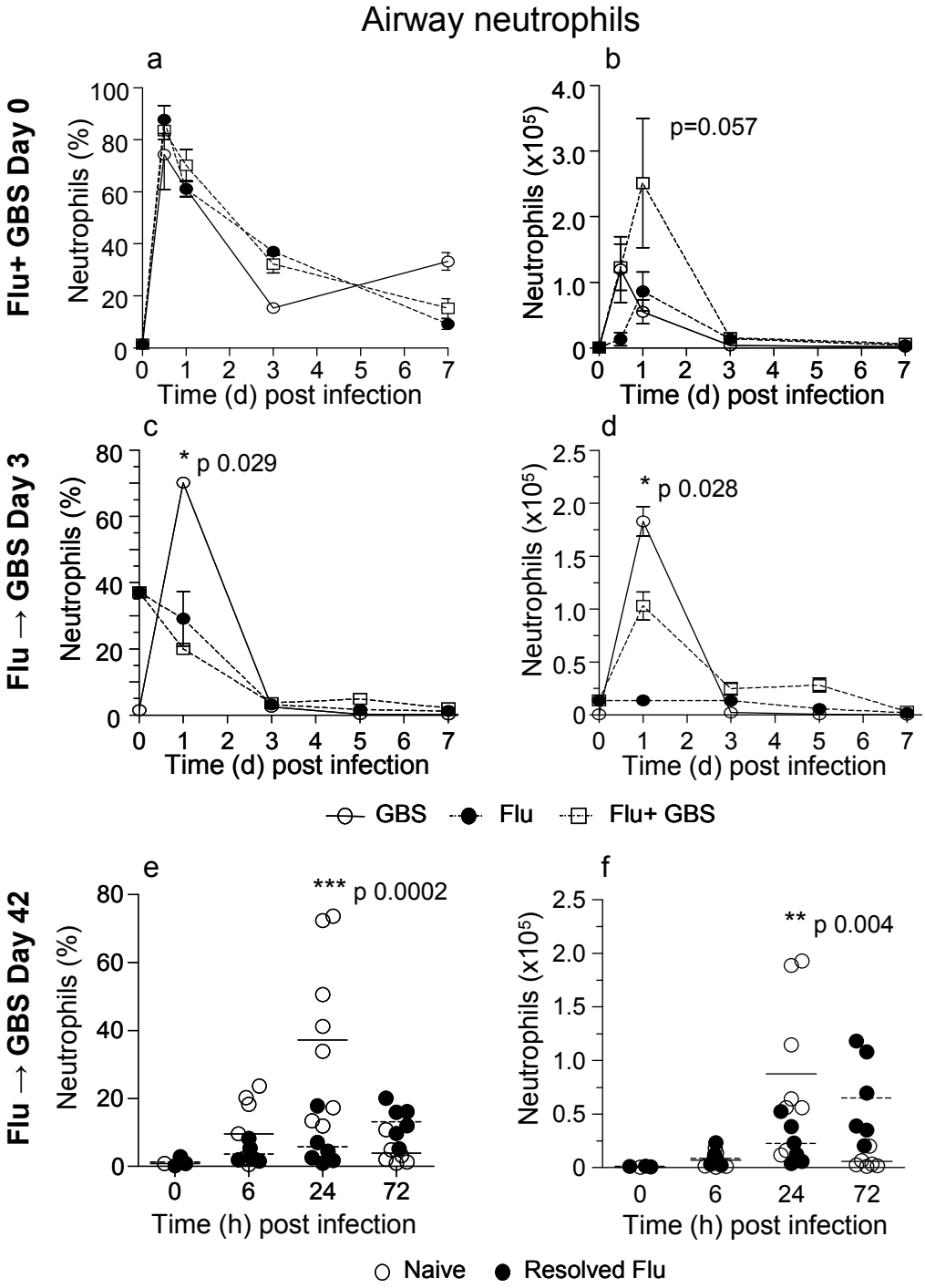



**Figure 4.12 Influenza infection reduces airway neutrophil recruitment to a bacterial infection that remains for at least 6 weeks.** BALB/c mice (n=4) were infected i.n with 50 HA HK/X31 influenza A virus or PBS control. 5 x $10^6$ cfu GBS was administered i.n. at day 0 (a-b), 3 days (c-d) or 42 days (e-f) after influenza infection. Mice were harvested on specified days after GBS infection and BAL was performed to quantify total viable cells using Trypan blue exclusion. Total neutrophils were determined from the % of $CD11b^+$ $Ly6G^+$ MHC II low cells multiplied by the total airway viable cell count. Data are presented as the mean ± SEM of 4 mice per group and represent two separate experiments. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$ *** = $p<0.001$

Despite seeing a reduction in innate cellular responses in influenza recovered mice following a GBS infection, there was no comparable decrease in adaptive immunity. A comparison of the lymphocyte response to GBS in influenza resolved mice demonstrated a significant increase in percentage and total number of lymphocytes in the lung compared to vehicle infected controls. This was also reflected with increased levels and activity of $CD4^+$ T cells and $CD8^+$ T cells in terms of percentage and total number 3 days post GBS infection. This increase in lymphocyte numbers observed in influenza recovered mice was also seen in the airway (data not shown).

### 4.2.8 TLR receptor expression is transiently altered in mice infected with influenza

All cells that have the ability to migrate from one site of the body to another accomplish this by responding to chemotactic gradients setup by the release of cytokines and chemokines from cells at peripheral effector sites. However, the production of chemoattractant mediators is dependent upon the detection of the presence of an object or organism foreign to the host. Multiple cells at mucosal sites of the body express a wide variety of PRRs that are responsible for detecting the presence of invading pathogens through interactions with MAMPs. Two such PRRs, TLR 2 and MARCO have been suggested to play critical roles in bacterial detection and defence in numerous respiratory infection models [407;408]. TLR 2 is responsible for the detection of peptidoglycans (PGN) and lipoteichoic acids (LTA) both of which are present in the cell wall of Gram positive bacteria such as GBS. However, their involvement in respiratory viral and secondary bacterial infection models has not been comprehensively elucidated. Therefore we next examined the total number of TLR2 and MARCO positive cells and their respective expression level in our secondary infection model. Analysis of myeloid cell subsets highlighted that although the proportion of $TLR2^+$ $CD11c^+$ macrophages was significantly reduced in the airway and lungs of mice infected with influenza virus 3 days prior to GBS, their total numbers were elevated (Figure 4.14 a+c). There was no significant difference in total numbers of lung $TLR2^+$ $CD11c^+$ macrophages at any time point analysed (Figure 4.14 d). In contrast, the proportion and total number of $TLR2^+$ $CD11c^-$ cells, comprising of both neutrophils and infiltrating exudate monocytes, was significantly greater in both the airway and lung tissue of mice infected with influenza virus compared to GBS alone infection (Figure 4.15 a-d). Analysis of TLR 2 expressed on the surface of myeloid subsets showed no alteration over the course of both influenza and GBS infection.

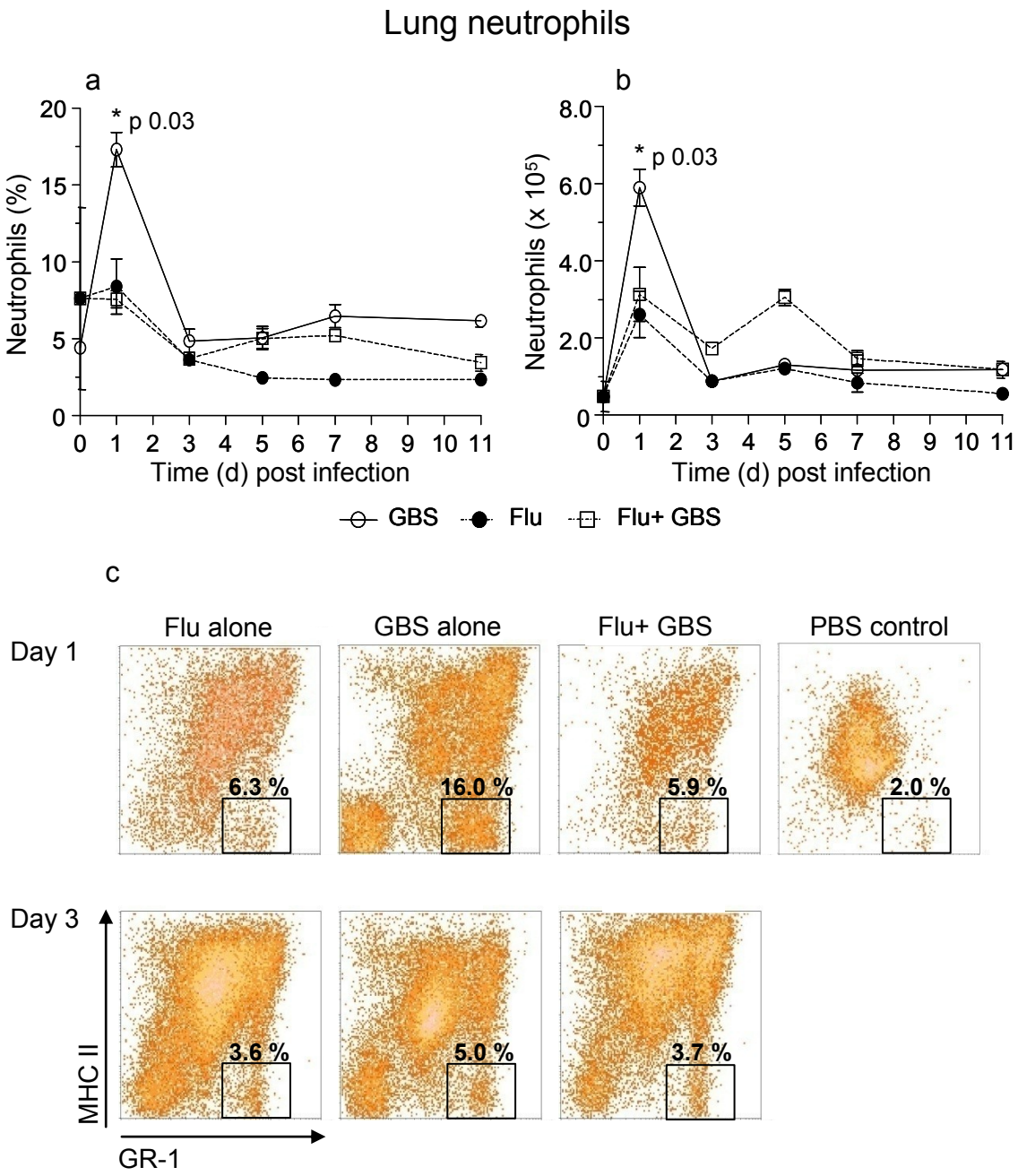



**Figure 4.13 Influenza infection reduces neutrophil numbers and percentage in the lung in response to bacterial infection.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. Three days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS or PBS control. Mice were subsequently harvested on days 1, 3, 5, 7 and 11 days post GBS infection. Lung tissue was removed, homogenised and total viable cell counts were determined using Trypan Blue exclusion. Percentage (a) and total number (b) of lung neutrophils were discriminated as $CD11b^+$ $Ly6G^+$ $MHCII^{lo}$. (c) represents relative lung neutrophil percentages in all groups on day 1 and day 3 post GBS infection. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

Influenza virus infection also altered the proportion and total numbers of $MARCO^+$ cells in both the airway and lung and was greatest in mice infected with GBS 3 days after infection with influenza virus (Figure 4.16 & 4.17). The geometric mean of intensity MARCO expression on airway $CD11c^+$ macrophages also did not alter significantly over the course of infection, but peaked in the lung tissue on day 8 post influenza infection (peak illness) (Figure 4.16 d).

### 4.2.9 Mice infected with GBS on day 3 of an influenza infection elicit a normal chemokine response but have heightened airway apoptosis

The reduction in neutrophils in mice infected with GBS following influenza virus could be the result of:

1. A reduced ability of neutrophils to migrate to the airway and lungs from the peripheral blood or the bone marrow. This could be due to a reduction in neutrophil chemotactic factors, such as KC or MIP-2α, or a reduced ability of neutrophils to extravasate from peripheral blood and into the lung tissue and subsequently the air spaces.
2. The apoptosis or necrosis of neutrophils recruited as a result of the primary influenza infection, therefore reducing the apparent proportions and total numbers of neutrophils in response to a secondary bacterial infection.

To investigate these two possible scenarios we measured the levels of neutrophil chemotactic factors and the proportions of apoptotic cells in the airway and lungs of mice infected with GBS alone and on day 3 of an ongoing influenza infection. There was no significant differences in the level of lung (data not shown) or airway KC and MIP-2α between the two groups (Figure 4.18 a & b). However, a greater proportion and total number of $CD11b^+$ $F480^-$ $TUNEL^+$ (neutrophils) apoptotic cells was observed in the co-infected group compared to GBS alone. Thus the fewer neutrophils observed may be due to enhanced cell death (Figure 4.18 c &d).

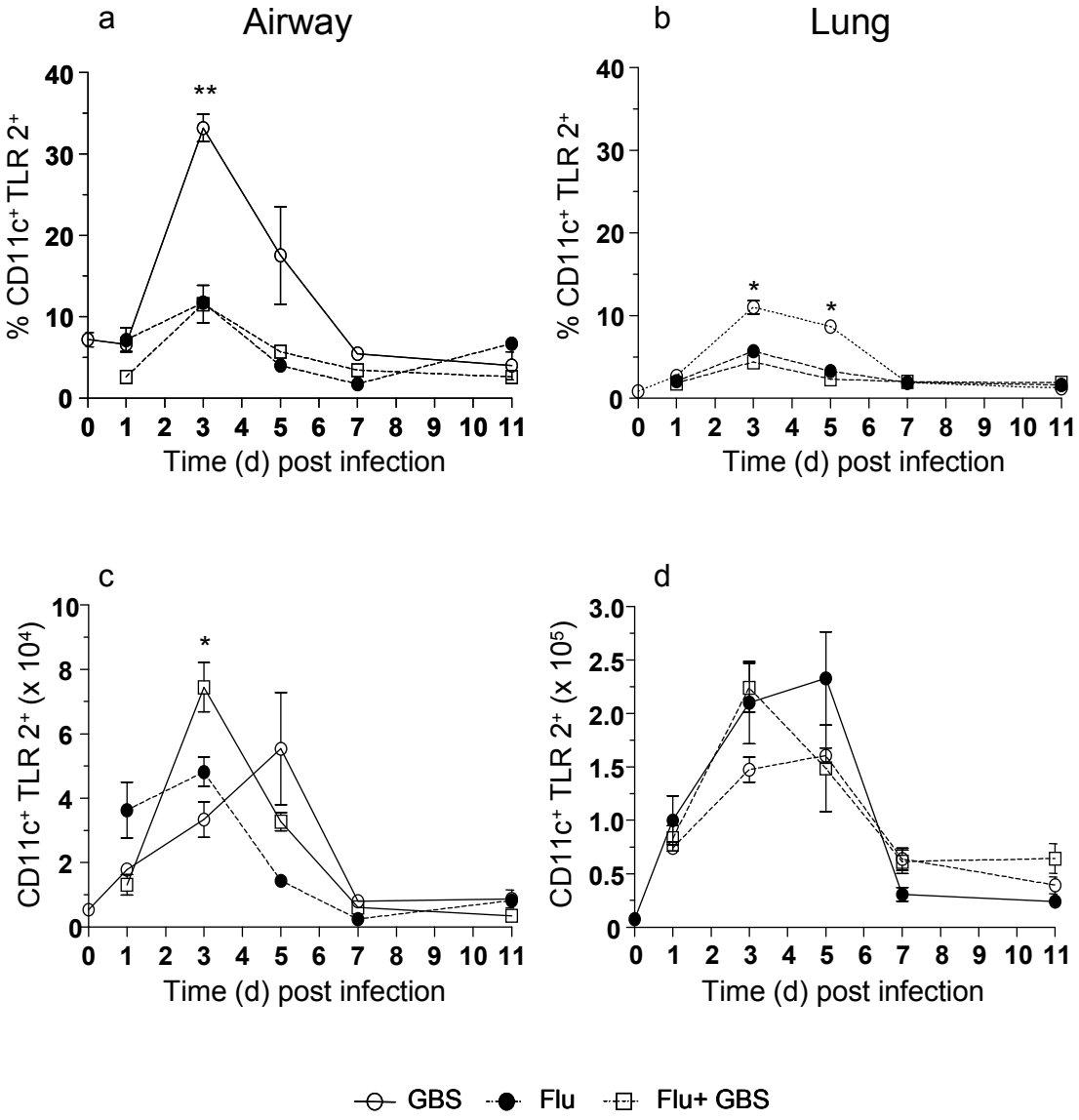



**Figure 4.14 Influenza virus alters the proportion of TLR 2⁺ myeloid cells.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. Three days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS or PBS control. Mice were subsequently harvested on specified days post bacterial infection. BAL was performed and lung tissue was removed, homogenised and total viable cell counts enumerated. Differential antibody staining was used to derive the proportions of airway (a & c) and lung (b & d) $CD11c^+$ $TLR2^+$ cells. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

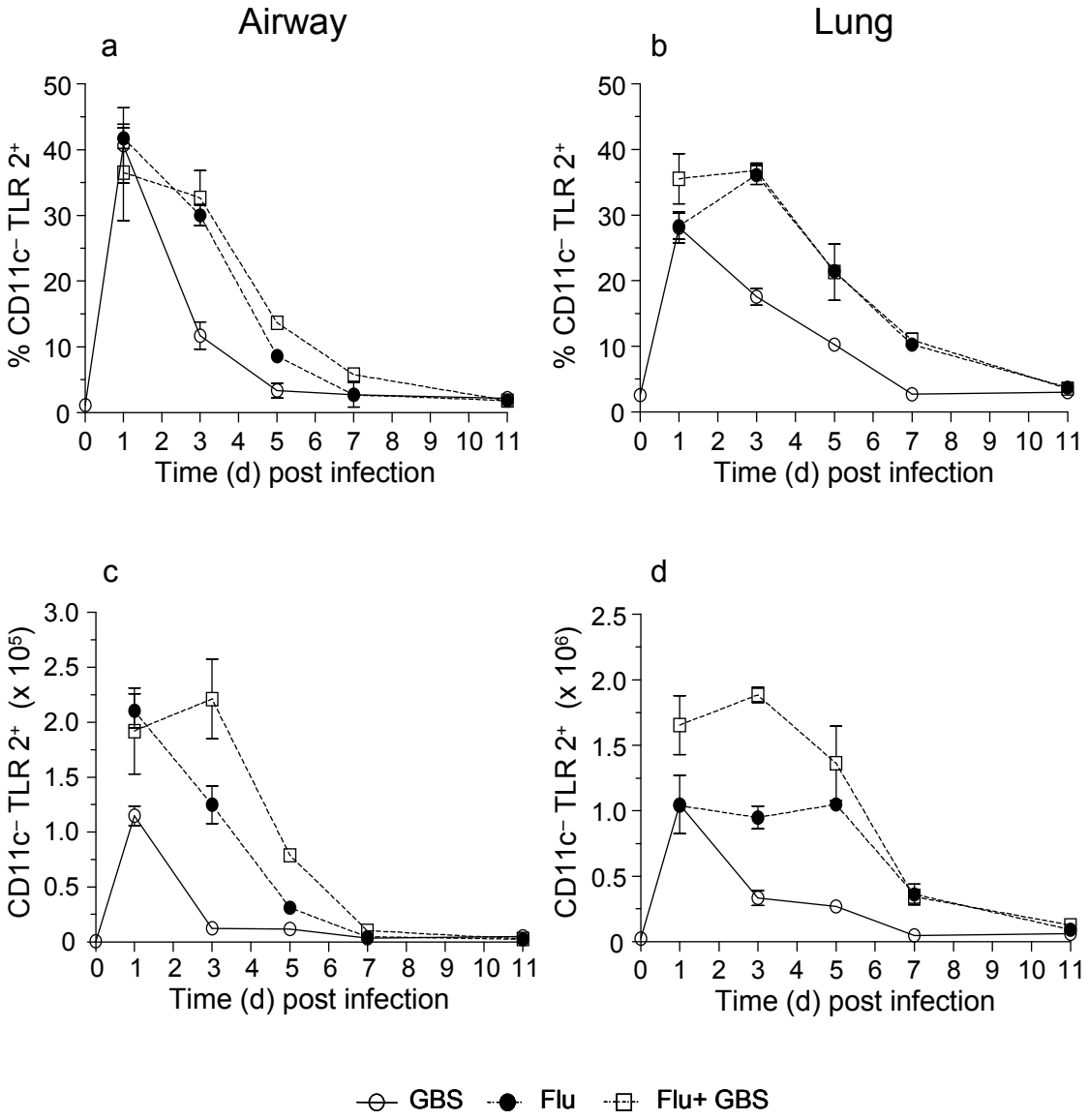



**Figure 4.15 Influenza increases the number of CD11c⁻ TLR2⁺ cells in both the lung and airway.** BALB/c mice (n=4) were infected intranasally (i.n.) with 50HA HK/X31 influenza A virus or PBS control. Three days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS i.n or PBS control. Mice were subsequently harvested on specified days post GBS infection. BAL was performed and lung tissue was removed, homogenised and total viable cell counts were determined using Trypan Blue exclusion. A differential stain was used to derive the total numbers of airway (a & c) and lung (b & d) CD11c⁻ TLR2⁺ cells. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

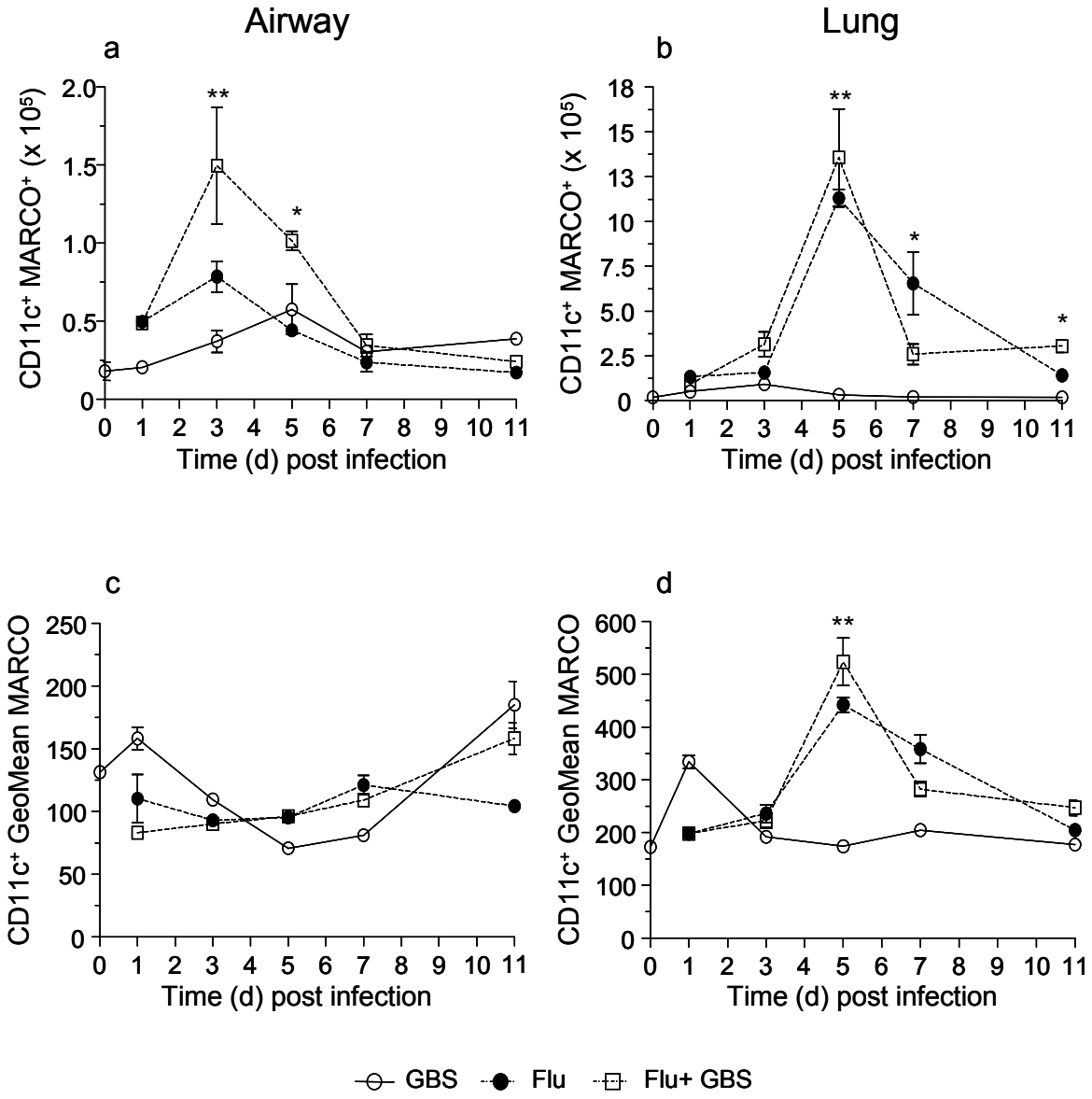



**Figure 4.16 Influenza increases the total number of MARCO expressing macrophage in the airway and lung compartments.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. Three days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS or PBS control. Mice were subsequently harvested on specified days post bacterial infection. BAL was performed and lung was removed, homogenised and analysed using flow cytometry (FCM). Myeloid cell types were discriminated using forward and side scatter. Total number of airway (a) and lung (b) cells expressing $CD11c^+$ $MARCO^+$ were enumerated. The geometric mean of MARCO expression on airway (c) and lung (d) cells was also determined. Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

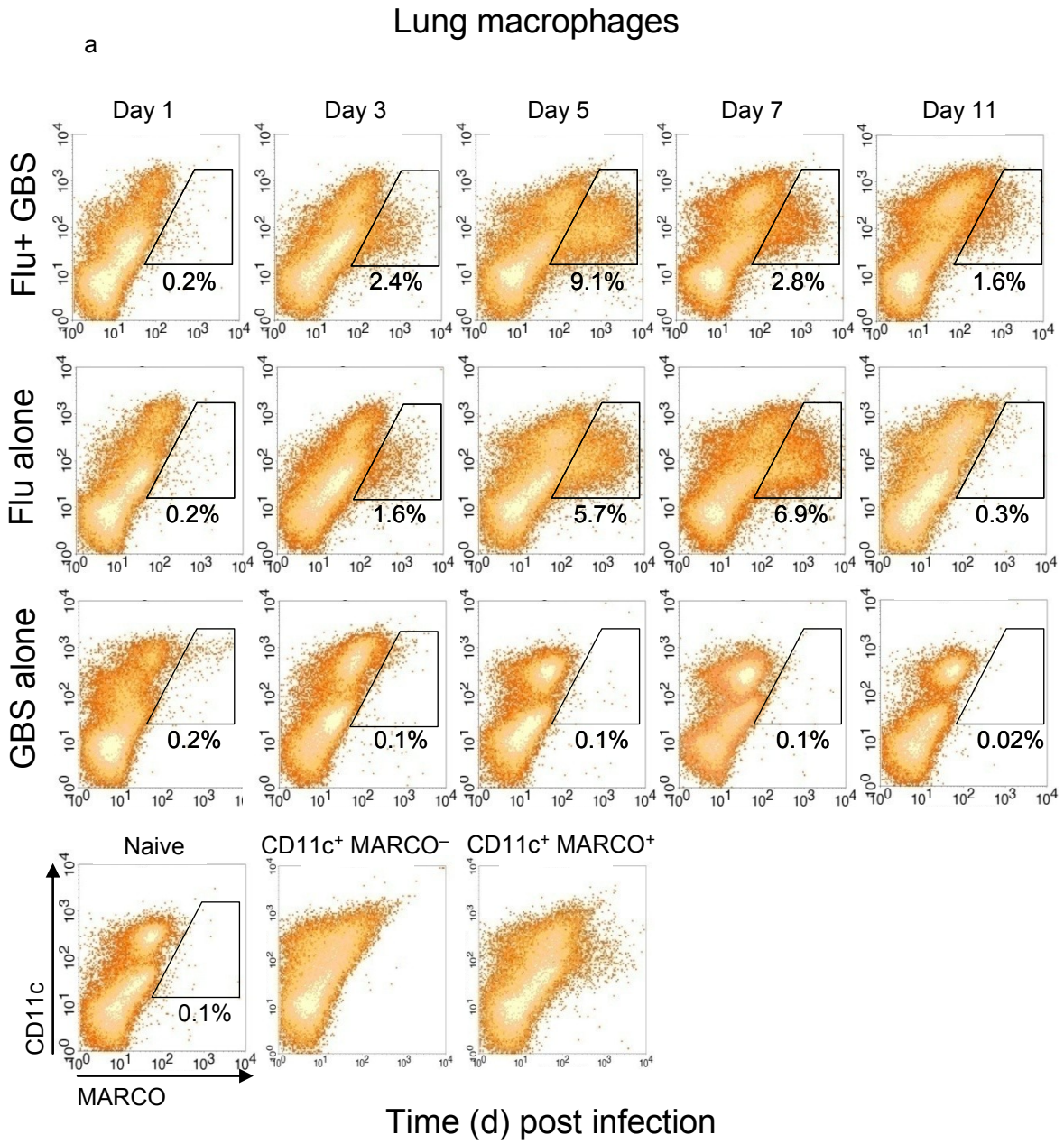



**Figure 4.17 Influenza increases the proportion of lung macrophage expressing MARCO.** BALB/c mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza A virus or PBS control. Three days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS or PBS control. Mice were subsequently harvested on specified days post GBS infection. Lung was removed, homogenised and analysed using flow cytometry. Myeloid cell subtypes were discriminated using forward and side scatter. A comparison of the proportion of lung myeloid cells positive for MARCO and CD11c between the different infection states are represented in (a). Representative plots for naïve and negative stained samples are also shown.

### 4.2.10 The long term effect on airway cellular recruitment observed in mice previously infected with influenza is not GBS specific

We next examined how specific, in terms of bacterial species and TLR ligands, the detrimental effect of influenza viral infection had on the host's anti bacterial defence. Utilising mice that had been allowed to recover from an influenza infection for 42 days (6 weeks) and vehicle matched controls; we challenged mice with the Gram positive bacterium *Streptococcus pneumoniae* and the Gram negative bacterium *Pseudomonas aeruginosa*. As before a previous influenza virus infection significantly increased *S. pneumoniae* and *P. aeruginosa* titres 4 and 24 hours post infection (Figure 4.19 a & b). These results coincided with a significant reduction in recruited airway neutrophils (Figure 4.19 a & b). To investigate whether this decrease in neutrophil numbers was a consequence of bacterial virulence factors, we next intranasally challenged mice, previously infected with influenza virus or vehicle control 42 days previously, with a selection of purified recombinant bacterial toll receptor ligands. Again reduced neutrophils were observed (Figure 4.19 c).

### 4.2.11 The influenza induced defect in neutrophil recruitment is associated with impaired TLR induced cytokine production

Results so far suggest a reduction in neutrophils may reflect a reduction in chemotactic signals. Indeed this was the case 42 days post influenza infection when mice were challenged with bacterial TLR ligand flagellin (FliC) neutrophils were reduced (Figure 4.20 a). This did not reflect a reduction in the ability of neutrophils to migrate since intranasal administration of KC (CXCL1) and MIP-2α (CXCL2) resulted in equivalent numbers of neutrophils being recruited to the airway and lung tissue of mice previously infected with influenza compared to vehicle controls (Figure 4.20 a). In addition, both KC and MIP-2α protein levels were reduced in the airway and lung tissue of mice previously infected with influenza compared to vehicle controls (Figure 4.20 b). KC and MIP-2α mRNA transcripts were also reduced in mice previously infected with influenza (Figure 21 a). Thus, chemotactic signals are reduced in the influenza experienced lungs possibly via a desensitisation to TLR signalling.

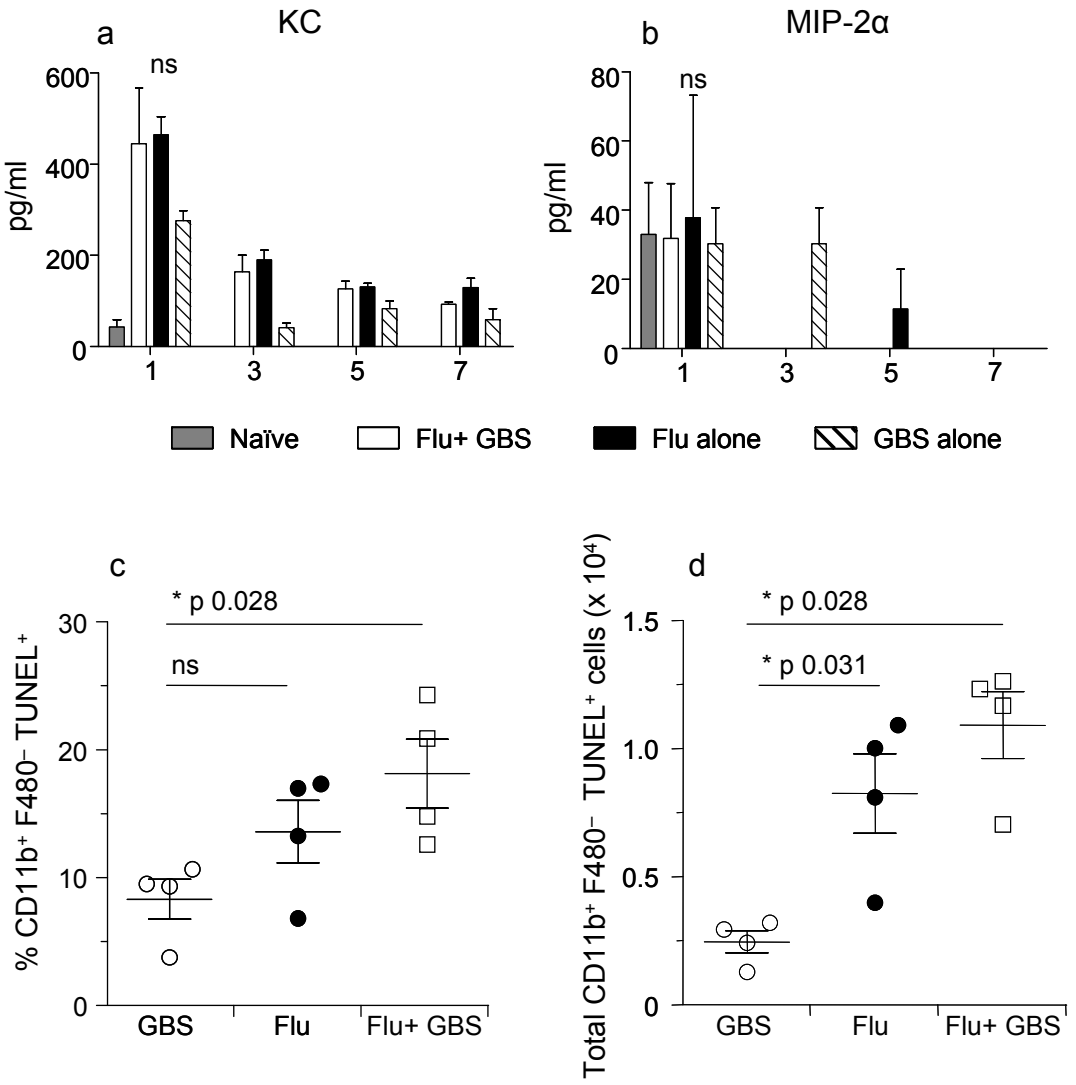



**Figure 4.18 Influenza infection 3 days prior to GBS infection does not alter neutrophil chemoattractant levels but enhances apoptosis.** BALB/c mice (n=4) were infected intranasally with 50HAU HK/X31 influenza A virus or PBS control. Three days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS or PBS control. Mice were subsequently harvested on specified days post GBS infection. BAL was performed and subsequently purified using centrifugation and analysed for levels of murine KC (a) and MIP-2α (b) using ELISA. Airway cells recovered 24 hours after GBS infection were assayed for apoptosis using TUNEL staining (c-d). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

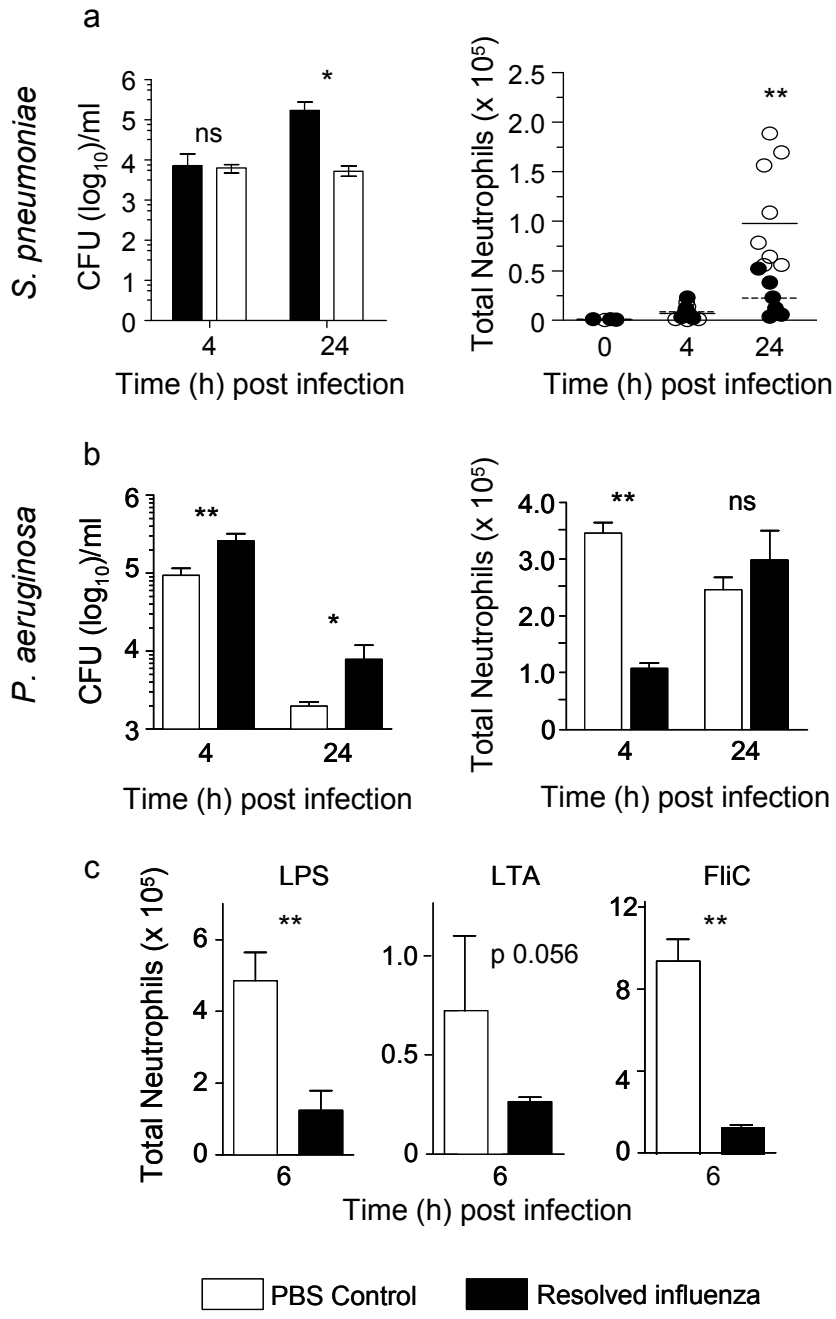



**Figure 4.19 Influenza induced defect in airway neutrophil recruitment is not GBS specific.** BALB/c mice (n=4) were infected intranasally (i.n.) with 50HAU HK/X31 influenza A virus or PBS control. 42 days after influenza infection mice were challenged i.n. with 1 x $10^4$ cfu *S. pneumoniae* (a), 5 x $10^6$ cfu *P. aeruginosa* PAK strain (b), 1µg LPS (c), 25µg LTA (c) or 1µg flagellin FliC (c). The total number of neutrophils in the airways at specified time points after stimulation was determined by flow cytometric analysis. Bacterial cfus were determined by serial dilution of BALF in PBS and plated on non selective agar. Data are presented ± SEM as the combination of 2 separate experiments. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

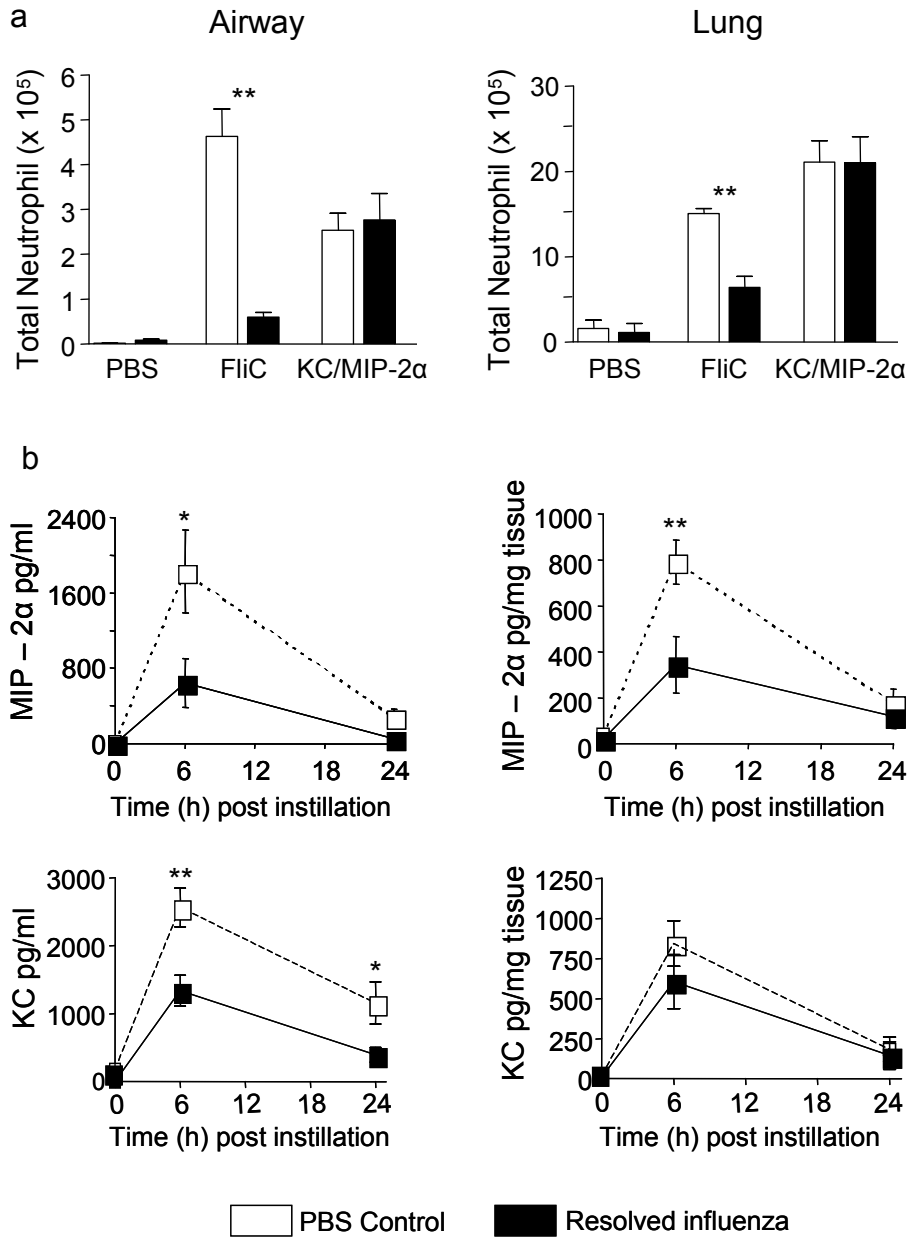



**Figure 4.20 Defective neutrophil recruitment in post influenza infected mice is associated with impaired TLR mediated cytokine production.** Airway and lung neutrophil recruitment in BALB/c mice intranasally (i.n.) infected with 50HAU HK/X31 influenza or PBS 42 days previously were compared 6 hours after i.n administration of 1μg flagellin FliC, or co-instillation of 1μg of recombinant KC and MIP-2α (a). MIP-2α and KC levels were measured in the BAL fluid and lung homogenates of post influenza or control mice 6 and 24 hrs post FliC administration (b). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

### 4.2.12 Translocation of NF-κβ subunits by TLR stimulation is inhibited in alveolar macrophage isolated from influenza resolved mice

Both alveolar macrophages and non-haematopoietic resident cells, such as type 1 and type 2 epithelial cells are instrumental in inducing TLR dependant signals early during infection. Mice were infected with influenza or vehicle control and allowed to recover for 42 days. FliC was then intranasally administered and alveolar macrophage collected and analysed. Alveolar macrophages isolated from influenza recovered mice 1 hour after intranasal FliC challenge displayed reduced levels of KC, MIP-2α and TNF-α mRNA transcripts (Figure 4.21 a); a reduction consistent across varying doses of FliC (Figure 4.21 b). Importantly we also observed a reduction in p65, a sub unit of NF-κβ, translocation to the nucleus after FliC stimulation (Figure 4.21 c). This reduction in NF-κβ signalling activation was also observed in isolated epithelium cells from influenza exposed mice (data not shown). However, we could only purify them to 65 %. They were difficult to isolate and the difference was only 2-4 fold, therefore we cannot say with certainty that TLR responsiveness by these cells was also affected.

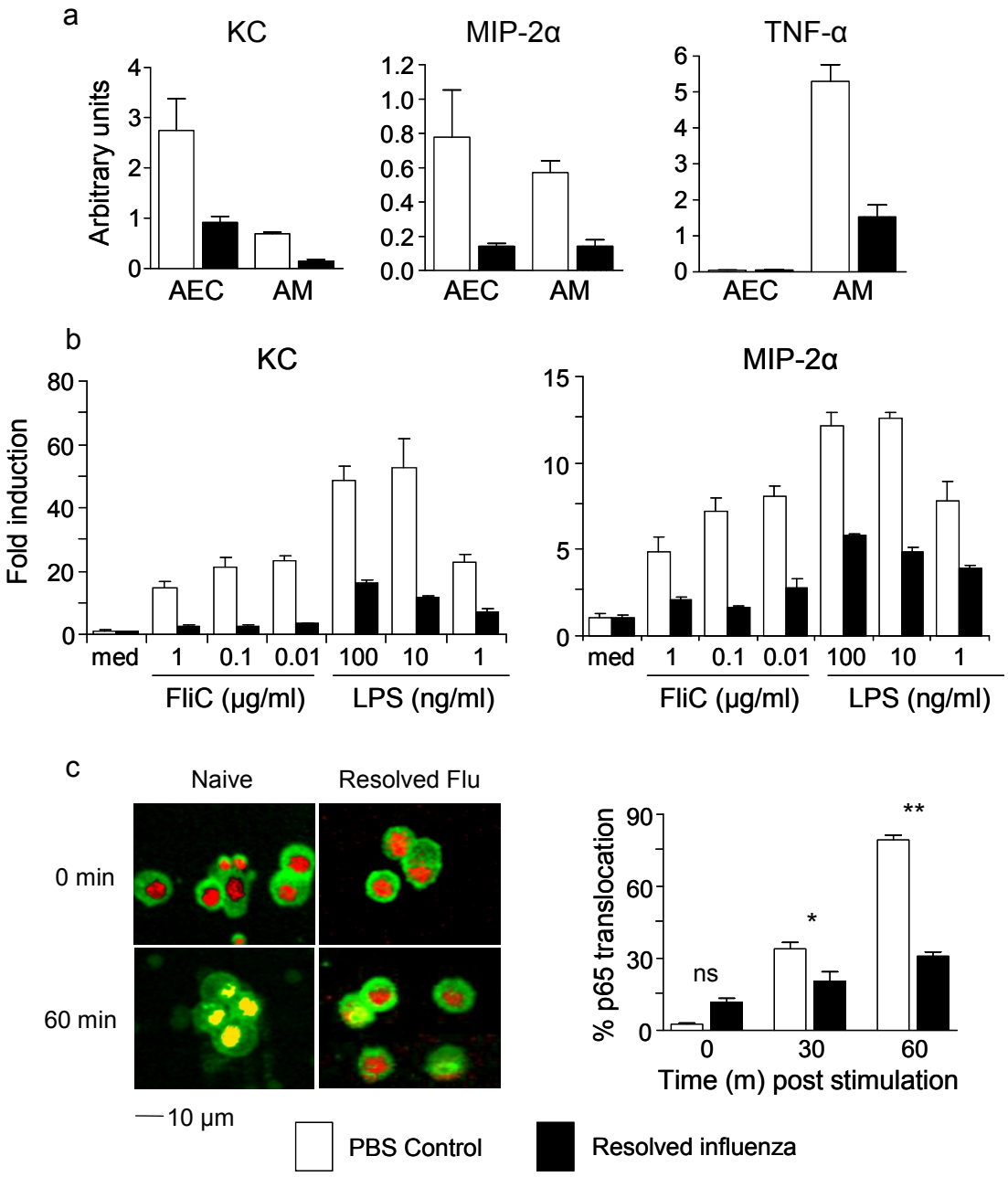



**Figure 4.21 Alveolar macrophage NF-κβ signal transduction is altered after influenza infection.** BALB/c mice (n=5) intranasally (i.n.) infected with 50 HAU HK/X31 influenza or PBS 42 days previous were challenged i.n. with 1µg FliC. Alveolar macrophage (AM) were purified after 1 h and RNA transcript level was measure by real time PCR. Results are expressed as a ratio between the gene of interest and 18s rRNA (a). AM were also stimulated overnight with Flic and LPS and resulting supernatant KC and MIP-2α levels were measured (b). Results are expressed as fold increase compared with stimulation with medium alone (n = 10). AM isolated from control and post-influenza mice were stimulated with 1 µg/ml FliC and stained for p65 (green). A nuclear stain is shown in red (c). Data are presented as the mean ± SEM of 4 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

## 4.3 Discussion

### 4.3.1 The timing of a secondary bacterial infection is critical in determining illness outcome

Susceptibility to a secondary GBS infection begins as early as 3 days after the primary influenza infection and continues for at least 6 weeks. We show that maximal susceptibility, measured by bacterial outgrowth and outward signs of disease occur during peak illness and early resolution phase of an influenza infection. We also demonstrate that the mechanisms leading to secondary bacterial infection are dependent on the time at which GBS infection occurs after the primary influenza infection. Existing *in vivo* mouse models, investigating the mechanisms involved in influenza induced secondary bacterial infections, also demonstrate peak susceptibility to bacterial infection between 7 and 14 days post influenza infection [279;409-411]. Furthermore, epidemiological and clinical evidence also suggests that susceptibility to secondary bacterial infection is greatest 5-10 days post the onset of influenza symptoms [375;412]. Assuming an incubation period of 2-4 days this would equate to the same period of susceptibility determined in our secondary infection model.

As described earlier in this thesis, the majority of existing concepts surrounding mechanisms of virus induced bacterial susceptibility centre around respiratory epithelial damage, effector cell dysfunction and enhanced bacterial adhesion. Previous studies highlight enhanced bacterial titre and mortality in secondary infection models in which bacteria are administered during peak viral illness [413;414]. Heightened bacterial adherence and invasion at sites of viral induced cell death is also observed in human specimens obtained from post mortems [290;415;416]. We however demonstrate that susceptibility to bacterial infection remains long after the virus has been cleared and is associated with a reduction in airway cell recruitment, in particular neutrophils. Pulmonary epithelial damage from primary influenza infection peaks around day 5, and is restored by day 12-14 post influenza, however this is likely to be determined by viral strain, viral replication propensity and amplitude of host cellular response. Susceptibility to secondary bacterial infection does therefore not entirely depend on epithelial damage. The extent to which each of these mechanism influences susceptibility is likely to be dependent upon the precise phase of influenza infection at which the bacteria gain access to the airways. For example, the ability of influenza virus associated neuraminidase to expose putative bacterial binding sites most likely contributes to bacterial infection susceptibility

during peak viral load [25]. We show that the greatest susceptibility to GBS occurs from 5-14 days post influenza infection suggesting epithelial damage, immune suppression and/or alternative unknown mechanisms are responsible. Recent evidence highlights tumour necrosis factor-related apoptosis inducing ligand (TRAIL), produced by airway infiltrating exudates monocytes, as a key mechanism involved in epithelial leakage that is maximal on day 7 of a murine influenza infection. However, this evidence was obtained using the A/PR/8 strain of influenza and was less evident when A/HK/X31 was used [417]. Alternative experimental models utilising ozone and bleomycin, also highlight the importance of epithelial integrity in protecting against opportunistic respiratory bacterial pathogens [418-420]. Nevertheless, the loss of respiratory epithelium integrity is less likely to explain the log increase in airway and nasopharynx GBS titres at the longer time points after an influenza infection.

A long term increase in susceptibility to subsequent bacterial infections is also observed with meningococcal bacteria several weeks after the resolution of an influenza virus infection [421;422]. Some infections improve host immunity to a second unrelated pathogen through cross reactive T cell and antibody epitopes whilst others attenuate excessive inflammation and pathology [423-426]. For example RSV or *Cryptococcus neoformans* infection is attenuated in mice previously infected with influenza virus, the effect is independent of cross-reactive adaptive immunity and occurs up to 6 months after influenza infection [299]. These data taken together with a large amount of evidence from invertebrate infection models allude to long term modifications in innate immune pathways [427;428].

Alternative explanations for increased bacterial susceptibility include modifications to the overall structure of the lung, such as collagen and fibronectin deposition, which may provide additional binding sites for bacteria [429]. Airway remodelling, common in chronic conditions such as asthma or fibrosis, also occurs after acute viral infection. These effects are mediated in part by matrix metalloproteinases (MMPs), induced by infection or injury, that are important mediators of resolution of infection. Indeed, they play a dual role by participating in repair mechanisms, for example by activating TGF-β but also by modulating inflammatory responses [430;431]. For instance, matrilysin (MMP7) is necessary for the regeneration of the airway epithelium after mucosal injury but also directly activates defensins and modulates the chemokine gradient and neutrophil transmigration. Whether MMP expression is sustained after resolution of one infection in order to modulate successive infections remains to be elucidated [432-434]. Respiratory infection can also induce lymphatic vessel hyperplasia [435].

*Mycoplasma pulmonis* infection induces robust lymphangiogenesis driven by vascular endothelial growth factor (VEGF) produced by infiltrating inflammatory cells. After inflammation resolution, the network of newly synthesized lymphatic vessels persists in the airways for several weeks. Therefore an alteration in the lymphatic network could alter subsequent immunity to infection as this provides conduits for enhanced drainage and migration of activated APCs to the draining lymph nodes. Further studies are needed to look at the role of angiogenesis and lymphangiogenesis in sequential infection models. Furthermore, the maintenance of minor populations of $CD8^+$ T cells, $CD11c^+$ cells and isolated lymphoid aggregates (iBALT) in the influenza resolved lung could also contribute to altered immunity to subsequent infections [436;437].

Finally, we also observed that mice infected with GBS simultaneously or 3 days after influenza infection showed accelerated weight recovery and a reduction in influenza viral load, respectively. This is an intriguing result that is difficult to explain but warrants a brief discussion. Our data is contrary to existing *in vitro* and *in vivo* evidence that postulate the presence of bacterial proteases, produced by bacteria such as GBS, increase the cleavage of influenza HA0 to HA1 resulting in increased viral infectivity and load [279;438;439]. It is likely that increased levels of IFN-γ producing NK and NK T cells observed in co-infected mice account for the improved viral clearance in our study. Anti viral properties of NK cells in influenza virus clearance is well documented and essential for viral eradication [440-442]. Further investigation in alternative antiviral mechanisms, such as IFN responses, is needed to shed more light on this affect.

### 4.3.2 Desensitization to TLR mediated signals represents a novel long term modification to innate immune responsiveness

In this study we show that a previous influenza virus infection reduces neutrophil numbers and enhances bacterial titer. The reduction in neutrophil numbers results from two mechanisms depending on what stage of influenza illness bacteria gain access to the lungs. Apoptosis accounts for the early reduction whereas a recruitment problem dominates later [287]. A similar defect in neutrophil recruitment occurs in response to *S. pneumoniae* and *P. aeruginosa* infection following influenza infection. Previous studies have implicated altered chemotaxis of neutrophils in mice previously infected with influenza [443;444]. Furthermore, IFN-γ produced in response to many viral infections can result in increased apoptosis of neutrophils in the bone

marrow and could therefore result in reduced neutrophil numbers at the site of infection [445].

In spite of these suggestions, the long term decrease in neutrophil transmigration requires a different mechanism as influenza virus is no longer present in the host. Long term functional alteration in neutrophils was ruled out by showing unimpaired neutrophil airway recruitment in influenza recovered mice after intranasal administration of the neutrophil chemoattractants, KC (CXCL1) and MIP-2α (CXCL2). Furthermore, neutrophil recruitment is reduced in response to purified TLR agonists in influenza resolved lung. Therefore, it appears that the cells that produce neutrophil chemoattractants are impaired and indeed isolated cells stimulated *ex vivo* also produce less neutrophil chemoattractants. However, removal and analysis of cells *ex vivo* does not indicate whether transcriptional regulation occurs independently in this cell type or as a result of co-operation with others. Cross talk between alveolar macrophage and alveolar epithelial cells (AECs) has been shown to occur *in vivo* [446]. By isolating these populations from naïve and influenza recovered mice we show *in vitro* that nuclear translocation of the p65 subunit of NF-*k*B in response to flagellin is inhibited in alveolar macrophage isolated from mice previously infected with influenza, which is associated with a defect in cytokine production. We therefore propose a model whereby sustained reduction of NF-*k*B activation in alveolar macrophage after the initial viral infection leads to reduced inflammatory response and neutrophil recruitment. Though we have examined macrophages we cannot exclude an indirect role for other cell populations such as DCs, exudate monocytes and tissue (interstitial) macrophages. It remains to be determined whether alveolar macrophages are directly affected by infection, or their phenotype altered by interaction with recruited inflammatory cells.

Furthermore, whether the pool of lung macrophages that give rise to alveolar macrophages is also affected by previous infection warrants further investigation [447]. The molecular mechanisms responsible for prolonged TLR desensitization remain to be resolved. Because many of these receptors share components of their signalling pathways, changes in the expression level or phosphorylation status of molecules within these pathways may also interfere with the recognition of subsequent pathogens. TLR signalling is inhibited by several negative regulators downstream of the receptor [448]. For example, increased expression of IL-1 receptor associated kinase M (IRAK-M) correlates with the unresponsiveness of alveolar macrophages to LPS in a mouse model of abdominal sepsis [449]. Intestinal commensals also inhibit

TLR responses by directly interfering with the signalling pathways in epithelial cells [450;451]. Lung epithelial cells do, however, up-regulate TLRs, in particular TLR3 and TLR2, upon respiratory syncitial virus (RSV) and influenza infection, which may be mediated by the release of IFN by infected macrophages, whether such up-regulation has an impact on secondary infection is unclear [387;452]. We do show an increase in the number of TLR $2^+$ myeloid cells in the airway and lungs of influenza infected mice. Clearly, a previous infectious event could impact significantly on responsiveness to the next, depending on how long the first influence lasts and whether the subsequent pathogen triggers similar signalling pathways.

The pertinent question is why would the lung leave itself vulnerable to a secondary bacterial infection in this manner? In certain compartments, such as the lung, inflammatory cascades need to be regulated in order to prevent bystander tissue damage, which itself can be life threatening to the host. The lung contains a heavy microbial load in the upper respiratory tract and certain species in the lower respiratory tract (see Chapter 6) but is devoid of any significant resident organized lymphoid tissue. Pathogens bypassing passive anti-microbial strategies can cause significant indirect pathology in the lungs due to the recruitment of excessive numbers of immune cells that occlude the airspaces. An alteration in TLR responsiveness therefore is beneficial for those infections associated with excessive immunity but at the expense of other pathogens such as bacteria. This phenomenon, though detrimental in a sub-population of co-infected individuals, is likely to represent an evolutionary advantage for the respiratory tract and shares interesting similarities with immunosuppressive mechanisms operating at other mucosal sites.

## 4.4 Conclusion

Many mechanisms can explain why the host responds differently to heterologous pathogens, from a simple breach of the mucosal barrier to subtle alterations in immune modulatory mechanisms. However, evidence produced herein reveals that a crucial pathway of innate immunity does not return to a pre-infection state therefore providing a novel mechanism which may contribute to increased susceptibility to bacterial infection observed after respiratory viral infection. The challenge in this field is to investigate the cellular and molecular mechanisms at mucosal sites, where the education of the immune system is crucial for efficient protection against pathogens. In particular, identifying the molecules involved in increasing susceptibility or protection against subsequent infection may have a significant impact on therapeutic

strategies to tackle infectious diseases. To summarise our findings Figure 4.22 highlights how the mechanism(s) responsible for enhancing susceptibility to secondary bacterial infection alters depending on the phase of influenza infection the bacteria gain access to the distal airways.

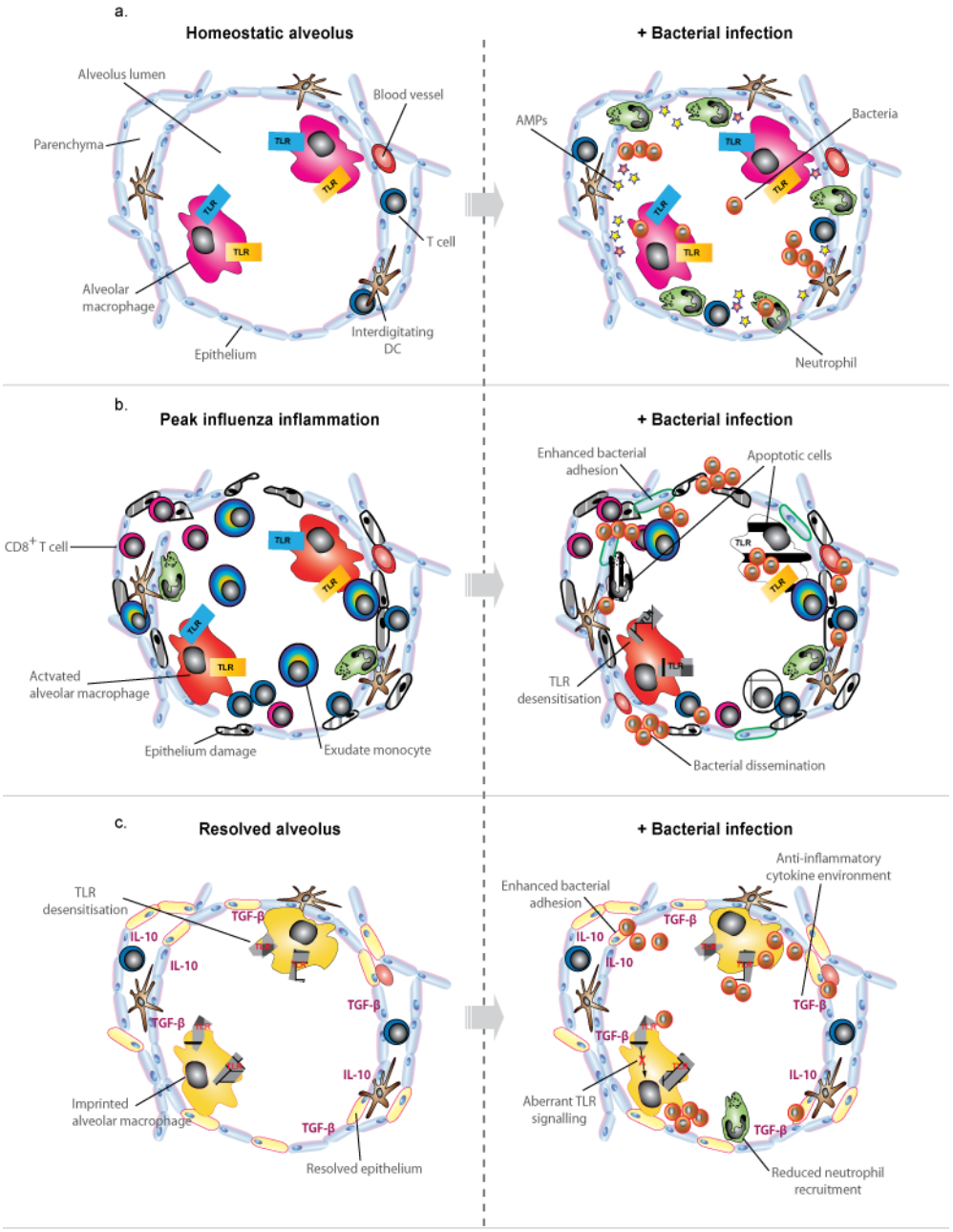



**Figure 4.22 Model of secondary bacterial infection at different stages after influenza infection.** In homeostasis, bacteria are cleared from the airway by alveolar macrophages, recruited neutrophils and AMPs. Epithelium integrity is intact and prevents bacterial invasion and dissemination (a). During influenza infection (day 5-8 post infection), epithelial damage, exposure of bacterial binding sites and apoptosis of infiltrating neutrophils facilitates bacteria tissue invasion and subsequent dissemination (b). After resolution of influenza infection, alveolar macrophages and epithelial responsiveness is attenuated, resulting in impaired neutrophil recruitment and consequently decreased antibacterial capacity. In addition the presence of IL-10 and TGF-β produced during inflammation resolution maintains an anti-inflammatory environment.

# Chapter 5

## 5.0 Innate immune homeostasis and inhibitory myeloid receptors

### 5.1 Introduction

In the previous chapter, we developed a robust *in vivo* secondary bacterial infection model that highlighted the temporal relationship between influenza infection and secondary bacterial pneumonias. Furthermore, we described a novel viral mediated immune suppression mechanism in which alveolar macrophages become hypo-responsive to bacterial Toll ligands and consequently less capable of initiating subsequent immune responses. This evidence demonstrates that a self limiting acute inflammatory insult can alter tissue-specific immune responsiveness over a prolonged period of time, suggesting that infection history can influence the way in which a specific tissue responds to subsequent immune stimulation.

The lung, similar to the intestinal tract, must maintain a high threshold of ignorance to innocuous antigen to avoid unwarranted inflammation and bystander tissue damage [169;453-455]. This high level of immune regulation depends upon the delicate balance between positive inflammatory signals, such as Toll-like receptors, and regulatory repressor pathways. An example of this is the restraint of alveolar macrophages by epithelial $\alpha_v\beta_6$ integrin tethered TGF-$\beta$ [456;457]. Progression of an inflammatory response requires down regulation of the $\alpha_v\beta_6$ integrin, which is initiated by exposure to Toll-like receptor ligands [456]. Equally important, especially within mucosal sites, is resolution of the immune response, without which excessive tissue damage can occur. The mechanisms mediating resolution are likely to be similar to those that maintain homeostasis. TGF-$\beta$ mediated suppression of alveolar macrophages, for example, is restored by cleavage of latent TGF-$\beta$ by matrix metalloproteinase (MMP)-9 produced by activated alveolar macrophages [456]. Recent work within the laboratory has uncovered a novel regulatory mechanism that suppresses alveolar macrophages activity in homeostasis and during resolution of acute inflammation [458]. Further details of this mechanism and other integral regulatory pathways are described below.

### 5.1.1 Paired activating and inhibitory myeloid receptors

Lung and airway resident myeloid cells are not only critical effector cells involved in protecting the host from invading pathogens, but also play an integral part in governing the initiation, maintenance and resolution of inflammation. The development of an efficient and robust immune response is critical to the successful resolution of infection; however, a degree of control and tolerance is needed to prevent bystander tissue damage and to distinguish self from non-self [107;459]. This control is achieved through receptor ligation of soluble mediators as well as cognate cell-cell interactions [108;460-462]. The role of such signals in dictating immunity and tolerance at the level of the T cell are well characterised, but comparatively little is known regarding the interactions that govern cells of the myeloid lineage, despite their central role in immunity and immunopathology [463-465].

Myeloid cells are an extremely heterogeneous population and can be individually defined by their complex combinations of surface receptors that determine specific phenotype and function [108]. These receptors include pattern recognition receptors (PRR), cytokine, complement and Fc receptors, which facilitate pathogen clearance and phagocytosis, accessory molecules that promote T cell activation and those that dictate the migration of cells from the circulation and into tissues. An important family of receptors that dictate myeloid cell function are paired activating and inhibitory receptors. These include paired immunoglobulin-like receptors (PIRs), signal regulatory proteins (SIRPs), triggering receptors expressed by myeloid cells (TREMs) and the CD200 receptor family [107;108;110;111;466;467]. There remains a large gap in our knowledge of these receptors and in many cases the physiological ligand remains unknown. However, the CD200 (OX 2)-CD200R (OX2-receptor) interaction has recently gained considerable interest in infectious disease models with a growing appreciation of its inhibitory effects on the myeloid immune compartment. Furthermore, continued research into this myeloid inhibitory interaction has highlighted clear immunotherapeutic potential in the treatment of tumours and in alleviation of autoimmunity, allergy and immunopathology [111;128;142;144;145]. However, much of the existing knowledge is contradictory and the role they play in human disease remains to be revealed.

### 5.1.2 CD200-CD200R and their role in respiratory tract homeostasis

CD200 is a type 1 transmembrane glycoprotein with two immunoglobulin superfamily (IgSF) domains [114;468]. The expression of CD200 is varied, being detected in

thymocytes, recirculating B cells, some peripheral T cells, follicular dendritic cells, neurons in the central nervous system, endothelium, trophoblasts and smooth muscle [114;118;119;121;128]. This unusual and specific distribution was found to be highly conserved in mice and humans [118;131;469]. This conserved expression of CD200 on specific cellular populations suggests a significant biological role for this molecule. Conversely the expression of CD200 receptor (CD200R) is limited to myeloid cells, including macrophages, dendritic cells, neutrophils, basophils and mast cells [110;123-125;144] although expression on peripheral $CD4^+$ T cells in mice and humans and high levels of CD200R mRNA expression in $Th_2$ polarised T cells has been described in one study [123].

Further investigation into the structure of CD200R demonstrated high homology to its ligand CD200, but in addition, the existence of a cytoplasmic tail with signalling capacity [110;122;123]. The generation of CD200 and CD200R KO mice by Sedgwick *et al* illustrated that the vast majority of the altered phenotypic traits present in these mice were focused in the leukocyte compartments, for example the enlarged spleens present in CD200 KO mice contain twice the number of $CD11b^+$ myeloid cells and have elevated numbers of macrophages and granulocytes [128]. Marginal zone macrophages and dendritic cells within T cell areas of the white pulp express higher levels of the ITAM containing DAP12 molecule, implying heightened cellular activation. Distinct phenotypes are also observed in peripheral lymph nodes. The fact that the phenotype is observed in those cells that express the CD200R rather than those that express CD200 led Hoek *et al* to postulate that the CD200 KO phenotype was a consequence of a loss in an inhibitory signal to the myeloid cells [128]. Additional studies added strength to this hypothesis by illustrating that CD200 ligation of CD200R imparts a uni-directional negative signal to cells that express CD200R [110;114;125;132;144]. The absence of an effect on CD200-expressing cells is likely due to the lack of intracellular signalling motifs or docking domains on the short cytoplasmic tail of CD200 [115;116]. All cells expressing CD200 ligand can therefore potentially negatively regulate innate myeloid responses. Subsequently, CD200 knock out mice have been demonstrated to exhibit a more severe or accelerated disease phenotype in a variety of conditions where macrophage function are critical elements of the disease process, such as experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA) [128]. However, much of the evidence concerning the role of CD200 in immune regulation are observational and there is little in the literature to divulge the mechanisms by which CD200 exerts a suppressive signal to the myeloid compartment. A recent study demonstrates the potential of signalling through

CD200-CD200R to suppress cytokine production in CD200R transfected U937 cells, and the potency of this suppression correlates with density of CD200R expression [470]. Furthermore, the same study demonstrates CD200R ligation suppresses IFN-γ and IL-17 induced secretion of IL-6 by peritoneal macrophages. Similarly, engagement of CD200R in murine bone marrow derived mast cells over-expressing the receptor reduces degranulation and secretion of IL-13 and TNF [144]. Similar findings were subsequently confirmed in human mast cells. Finally, some evidence supports a role for CD200R ligation in up-regulating indoleamine 2,3-dioxygenase (IDO) in plasmacytoid dendritic cells resulting in heightened tryptophan catabolism and tolerance [146].

### 5.1.3 Hypothesis and Aims

In this chapter, we investigate the impact of manipulating the CD200-CD200R axis during influenza virus infection and its impact on susceptibility to secondary bacterial pneumonias. We postulate that in some circumstances, an attempt to restore the lung to homeostasis after an acute inflammatory insult leads to a period of immune over regulation that results in immune hypo-responsiveness and hence susceptibility to bacterial infection. To address this we will:

1. Demonstrate that a previous influenza virus infection increases susceptibility of C57BL/6 mice to *S. pneumoniae* infection.
2. Determine the expression and role of CD200R in lung immunity during homeostasis and in response to single influenza and *S. pneumoniae* infection.
3. Investigate the role of CD200R in a secondary bacterial infection model.

## 5.2 Results

### 5.2.1 Influenza significantly increases *S. pneumoniae* susceptibility and results in lethal bacteraemia

Before investigating whether the CD200R-CD200 axis plays a role in susceptibility to bacterial infections following influenza virus infection, we first needed to examine a *S. pneumoniae* infection in C57BL/6 mice as the CD200 and CD200R KO mice are bred onto this mouse background. Utilising both the non lethal intranasal dose of *S. pneumoniae*, determined in section 3.2.8 and the secondary bacterial infection protocol developed in Chapter 4, we examined the effects of a preceding influenza virus infection on *S. pneumoniae* clearance in wild type C57BL/6 mice. As with

BALB/c mice, intranasal infection with 1 x $10^4$ cfu *S. pneumoniae* does not result in significant weight loss in C57BL/6 mice compared to vehicle infected control mice. However, C57BL/6 mice infected with 1 x $10^4$ cfu *S. pneumoniae* three days after an influenza virus infection demonstrated enhanced weight loss compared to mice infected with influenza alone (Figure 5.1 a). In addition to the observed weight loss, mice infected with both influenza and *S. pneumoniae* appeared less mobile, hunched and developed hypothermic shock 48-72 hours post *S. pneumoniae* infection. This was not observed in mice infected with *S. pneumoniae* alone (data not shown). These clinical manifestations were highlighted by a significant increase in mortality observed in mice infected with *S. pneumonia* and influenza virus compared to those infected with *S. pneumoniae* alone (Figure 5.1 b). Similar to a secondary GBS infection, *S. pneumoniae* infection in mice previously infected with influenza resulted in a significant increase in bacterial titres recovered from the airways, lung tissue and nasopharynx compared to mice infected with *S. pneumoniae* alone (Figure 5.1 c-e). Systemic bacteraemia in influenza infected mice occurred 48 hours after *S. pneumoniae* infection, demonstrated by the presence of viable bacteria in peripheral blood (Figure 5.1 f).

We next established whether cellular recruitment was altered in C57BL/6 mice, as was seen in BALB/c mice, when challenged with *S. pneumoniae* following influenza infection. Infection of naive C57BL/6 mice with 1 x $10^4$ cfu of *S. pneumoniae* elicited a modest, but statistically insignificant, increase in the total number of cells observed in the airways 48 hours post infection compared to naive and vehicle infected control mice (Table 5.1). Infection with *S. pneumoniae* on day 3 of an influenza virus infection also failed to increase total cellularity in the airway and lung tissue compared to mice infected with influenza alone. Of the cells recruited in response to *S. pneumoniae* a small number of neutrophils were observed in the airway and lung tissue of naive mice (Figure 5.2 a-d). In contrast to BALB/c mice, a preceding influenza infection in C57BL/6 mice resulted in enhanced total neutrophil numbers in response to *S. pneumoniae* infection compared to mice infected with *S. pneumoniae* alone (Figure 5.2 b & d). This is likely a consequence of the elevated bacterial titres observed in both the airway and lung tissue of co-infected mice.

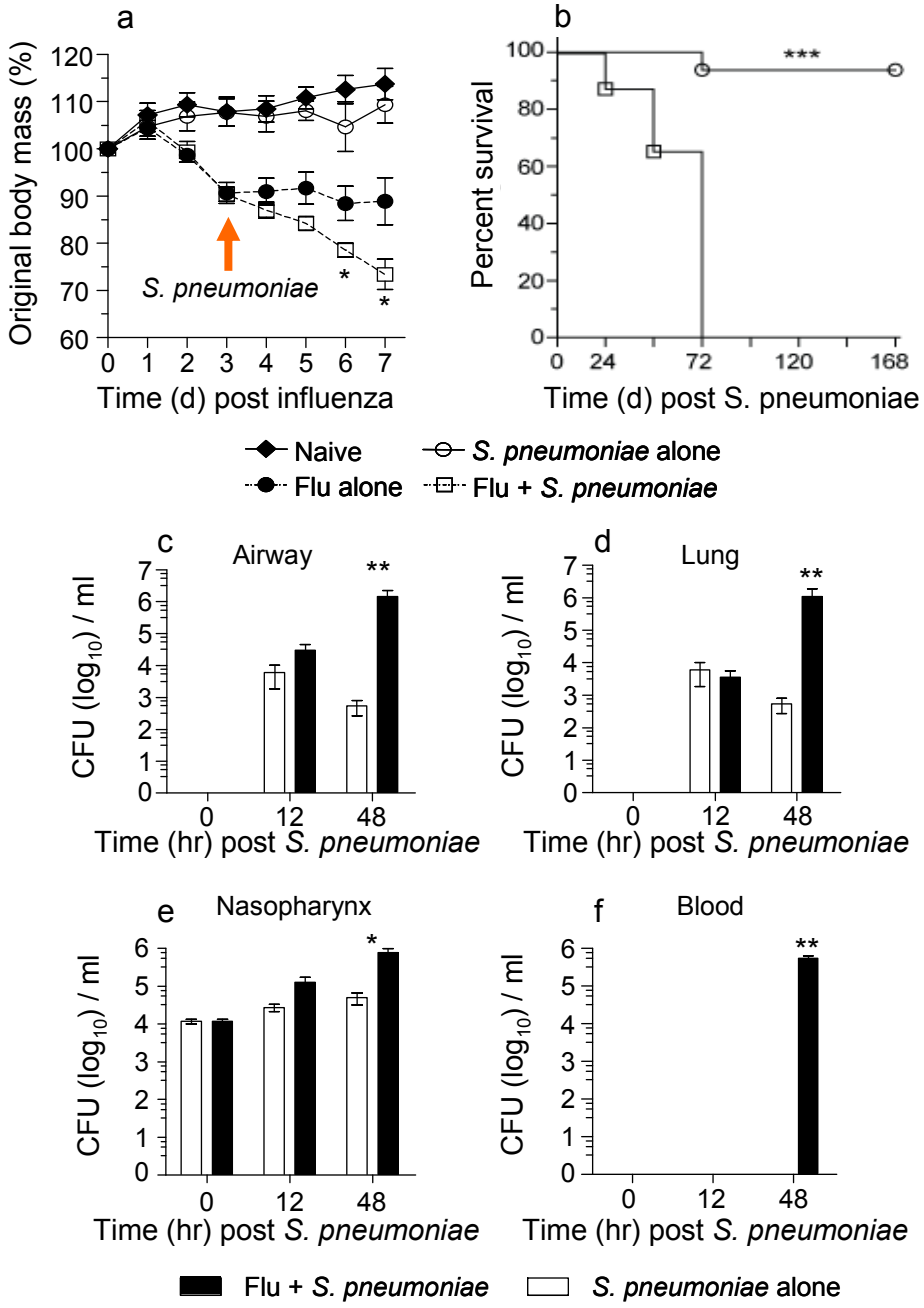



**Figure 5.1 Influenza significantly increases susceptibility to *S. pneumoniae* and results in bacteraemia.** C57BL/6 mice (n=5) were intranasally (i.n.) infected with either 50 HAU HK/X31 influenza A virus or PBS control on day 0. Three days later 1 x $10^4$ cfu *S. pneumoniae* were administered i.n. Weight loss (a) and survival (b) was monitored daily and expressed as mean % of original body mass and % survival, respectively. BAL (c), lung (d), nasal wash (e) and peripheral blood (f) samples were collected at specified time points and cfu calculated using serial dilutions of single cell suspensions of each tissue sample plated on blood Columbia agar. Data represent the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

**Table 5.1** Total cells 48 hours post *S. pneumoniae* infection

| Condition | Airway cellularity | Lung cellularity |
|---|---|---|
| **Vehicle control/Naive** | 1.7 ± 0.7 x$10^5$ | 1.0 ±0.4 x $10^6$ |
| ***S. pneumoniae*** | 3.2 ± 3.9 x $10^5$ | 8.0 ±3.1 x $10^5$ |
| **Flu d3 + PBS** | 9.4 ± 3.9 x $10^5$ | 6.8 ±4.2 x $10^6$ |
| **Flu d3 + *S. pneumoniae*** | 8.9 ± 2.5 x $10^5$ | 7.0 ±1.8 x $10^6$ |

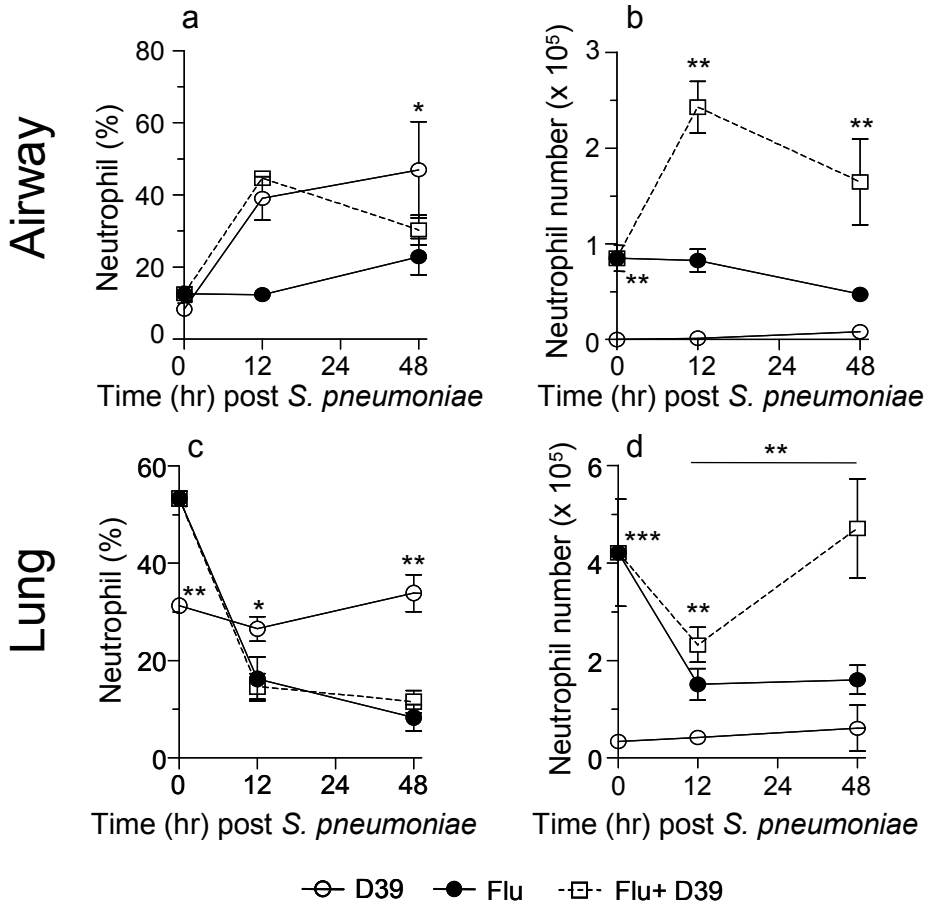



**Figure 5.2 *S. pneumoniae* induced a modest recruitment of neutrophils to the airway and lungs.** C57BL/6 mice (n=5) were intranasally (i.n.) infected with either 50 HAU HK/X31 influenza A virus or PBS control on day 0. Three days later 1 x $10^4$ cfu *S. pneumoniae* (D39) were administered i.n. BAL and lung tissue were removed and total cells were quantified using trypan blue to differentiate between viable and non viable cells. Total airway and lung cell numbers 48 hours post *S. pneumoniae* infection are illustrated in Table 5.1. Neutrophil % and total numbers in both the airway and lung compartments were quantified using differential flow cytometric staining (a – d). Data represents mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

### 5.2.2 CD200R and CD200 expression during homeostasis and influenza infection in C57BL/6 mice

After demonstrating that a prior influenza infection increases susceptibility to *S. pneumoniae* infection in C57BL/6 mice we next investigated the distribution of CD200R and CD200 expression on specific cell subsets in homeostasis and during an influenza virus infection in C57BL/6 mice. An in depth understanding of CD200R expression profile may provide information on its role during homeostasis and infection. We revealed that alveolar macrophages expressed unusually high basal levels of CD200R compared to non mucosal splenic macrophages, lung interstitial monocytes and macrophages (Figure 5.3 a). In addition, it was noted that BALB/c alveolar macrophages expressed significantly lower levels of CD200R than alveolar macrophages isolated form C57BL/6 mice during homeostasis, though this was still higher than splenic counterparts (Figure 5.3 a upper left panel-purple). Dendritic cells are not present in the airways but those in the lung interstitium have been shown to sample airway lumen content using dendrites [471]. We observed bimodal expression of CD200R expression on lung DCs, the reason for this may represent different DC populations or differences depending on their location within the lung parenchyma, for example adjacent to the respiratory epithelium or contained within iBALT. Interstitial neutrophils expressed low amounts of CD200R (Figure 5.3 a-bottom right panel). Therefore, high CD200R expression was observed on myeloid cells in contact or within the airway lumen at this mucosal site. For CD200R to be functional in the airway and lung however, cells expressing CD200 are required. In addition to previously described expression on hematopoietic cells, including B cells and some $CD4^+$ and $CD8^+$ T cells (Figure 5.3 b), lung endothelial cells, identified as $CD45^-$ $CD31^+$, also exhibited high CD200 expression (Figure 5.3 c). Importantly, type II epithelial cells that dominate in the lung alveoli, isolated from collagenase/dispase digested lungs as described in section 2.5.2, were also positive for CD200 (Figure 5.3 d). Immunohistology on OCT inflated lungs further verified CD200 expression on the luminal aspect of epithelial cells (Figure 5.3 e). The demonstration of CD200 expression on epithelial and endothelial cells was done in conjunction with Dr R. Snelgrove (see acknowledgements).

After illustrating that alveolar macrophages expressed high levels of CD200R in homeostasis and that CD200 is expressed on T cells and respiratory epithelium, we hypothesised that CD200R may limit the amplitude of innate immunity through interaction of recruited leukocytes needed to clear influenza infected cells. We therefore investigated the temporal and spatial expression profile of CD200R and

CD200 during an influenza infection. Despite high basal expression levels of CD200R on alveolar macrophages, influenza virus infection exponentially increased CD200R expression levels on alveolar macrophages and lung monocytes/macrophages (Figure 5.4 a & b). The level of CD200R expression remained elevated above basal levels up to and including 14 days post infection. Influenza virus infection also increased the % and total number of $CD200^+$ $CD4^+$ and $CD8^+$ T cells in the lung tissue (Figure 5.4 c & d). However, CD200 intensity of expression was reduced on both $CD4^+$ and $CD8^+$ T cells recruited to the airway (Figure 5.4 e & f). Furthermore, endothelial and epithelial expression of CD200 remained constant throughout the course of infection, although the intensity of expression was reduced on endothelial and epithelial cells at day 7, the time of peak inflammation (data not shown). The temporal expression of CD200 and CD200R during an influenza infection was also investigated in conjunction with Dr R. Snelgrove (see acknowledgements).

### 5.2.2 A lack of CD200R increases resistance to a *S. pneumoniae* infection

We next investigated whether a lack of CD200R altered innate immunity against an intranasal *S. pneumoniae* infection. To investigate this we intranasally infected CD200R KO mice and wild type controls with 1 x $10^4$ cfu *S. pneumoniae* and evaluated survival and bacterial clearance. As previously demonstrated, no significant weight loss was observed over the course of infection (data not shown). There was no significant difference in survival rates between the two groups (Figure 5.5 a). However, CD200R KO mice had reduced bacterial titres in both the airway and lung compartments at 12 hours post *S. pneumoniae* infection compared to wild type controls (Figure 5.5 b & c). No *S. pneumoniae* was detected in peripheral blood of either CD200R KO or wild type mice at any time point analysed (data not shown). Interestingly the bacterial titres observed in the nasopharyngeal cavity were identical between both groups of mice at all time points analysed (Figure 5.5 d). In a separate experiment looking at later time points (day 7), no bacteria were observed in wild type or CD200R KO mice in the lung or airways though they remained in the nasopharynx (data not shown). This implies that an absence of CD200R limits the extent of bacterial load but does not affect its ultimate clearance.

CD200R KO mice had greater airway cell recruitment to *S. pneumoniae* compared to wild type controls (Figure 5.6 a). Despite a significant increase in lung cellularity over the course of infection in both wild type and CD200R KO, there was no difference

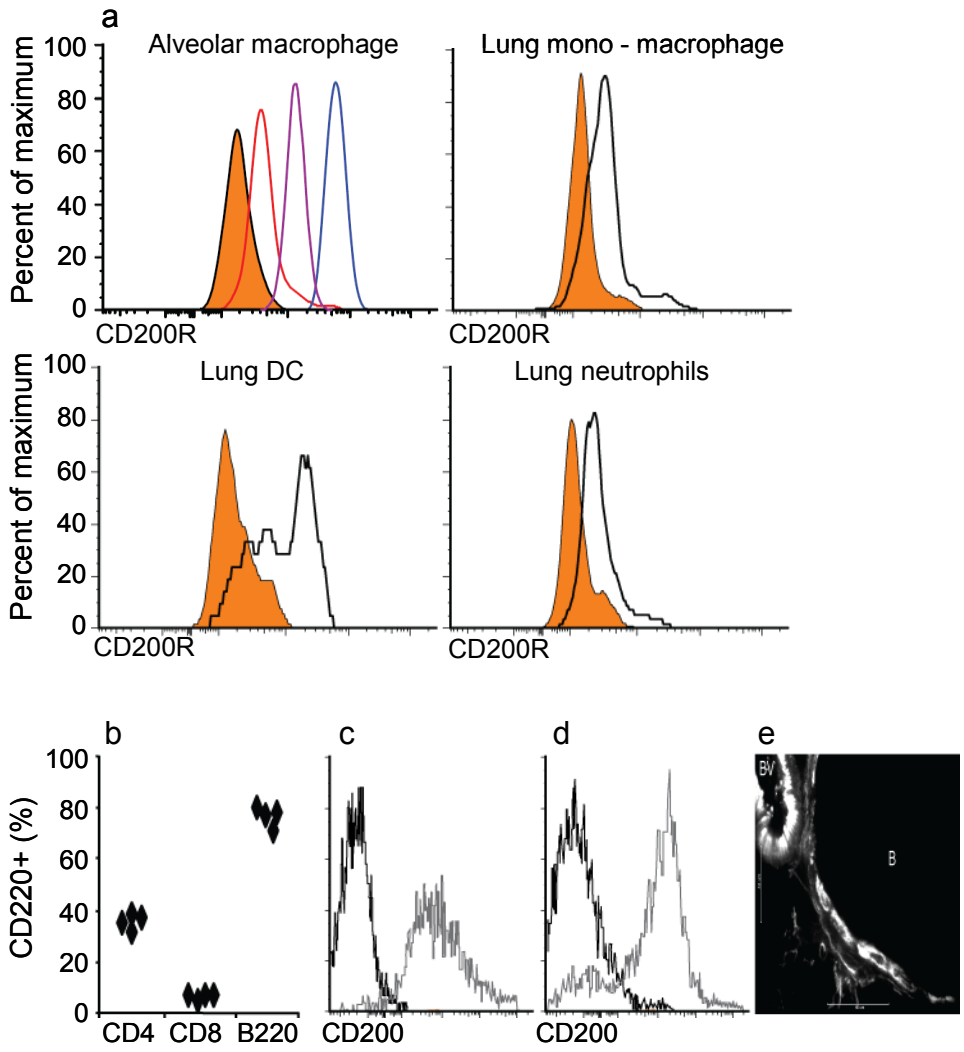



**Figure 5.3 Airway and lung expression of CD200R and CD200.** Lung and BAL cells from naïve C57BL/6 mice (n=5) were stained for CD200R expression (a). Representative histogram overlays of CD200R expression on alveolar (blue), splenic (red), isotype control (orange) and BALB/c alveolar macrophages (purple) (upper left). Upper right and lower panels show CD200R expression on other lung myeloid populations (orange, isotype control; black, anti-CD200R). The % CD200 expression on lung $CD4^+$, $CD8^+$ and $B220^+$ cells was assessed by flow cytometry (b). CD200 expression on lung endothelium ($CD31^+$, $CD45^-$, pro-surfactant protein $c^-$) (c) and lung epithelium (CD31+, $CD45^-$, pro-surfactant protein $c^+$) (d) is shown in representative histogram overlays (black, isotype control; grey, anti-CD200). OCT inflated lungs were sectioned and stained for CD200 as described in section 2.7.2 (e). Original magnification, x 400; BV, blood vessel; B, bronchiole. Representative of 3 experiments.

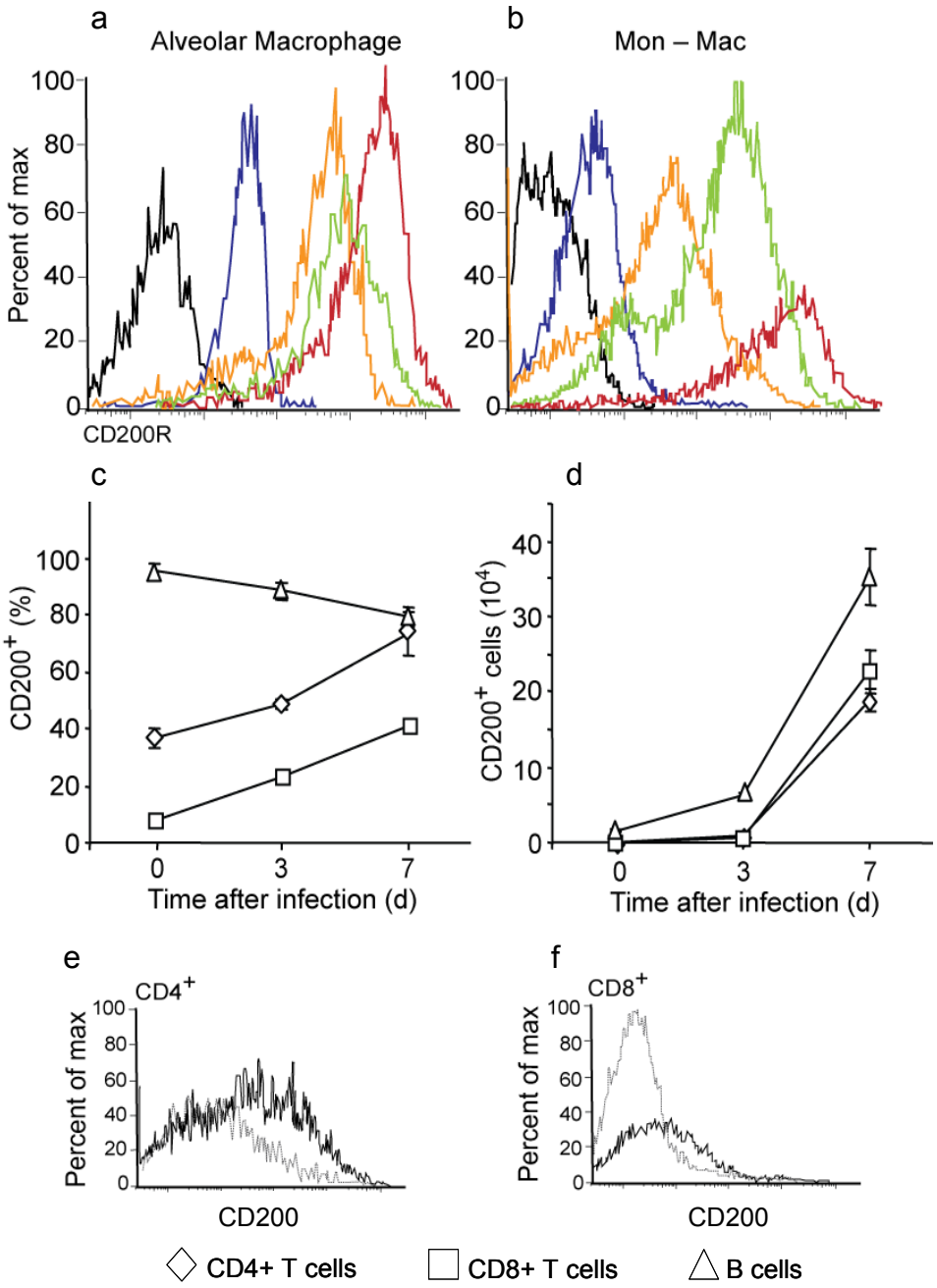



**Figure 5.4 CD200R and CD200 expression alters during an influenza virus infection.** C57BL/6 mice (n=5) were infected intranasally with 50 HAU HK/X31 influenza A virus on day 0. BAL and lung tissue was removed at specified time points post infection. CD200R expression on alveolar macrophages (a) and lung monocyte/macrophage (b) was determined using flow cytometry (black, isotype control; blue, day 0; yellow, day 4; green, day 7 and red, day 14 post influenza infection). The percentage (c) and total number (d) of $CD200^+$ $CD4^+$ T cells (diamonds), $CD8^+$ T cells (squares) and B cells (triangles) in lung tissue was also determined. The difference in CD200 expression on airway (grey) and lung (black) T cells 7 days post infection is shown in (e). Data represents mean ± SEM and representative of three independent experiments.

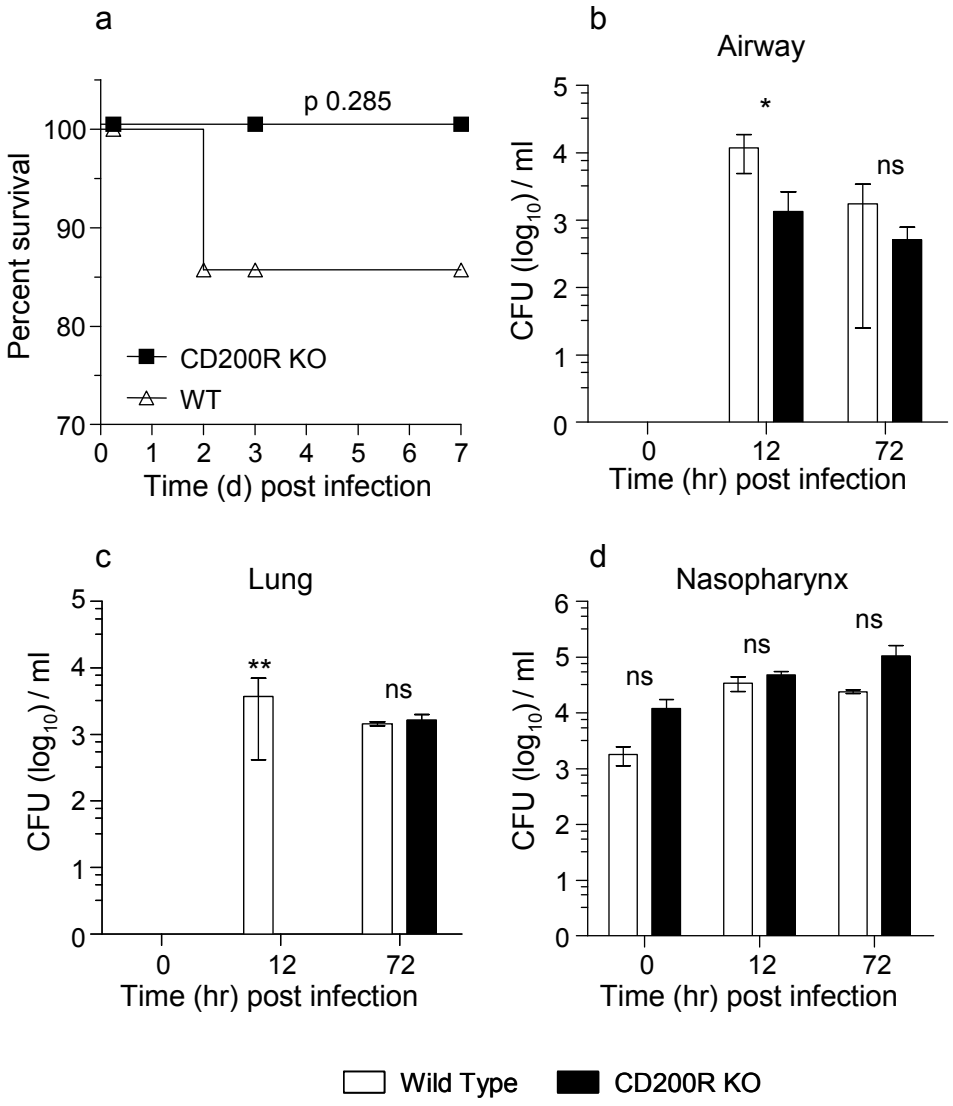



**Figure 5.5 CD200R KO mice limit bacterial titres in both the airway and lung compartments.** CD200R KO and wild type C57BL/6 (WT) litter mates (n=4) were intranasally (i.n.) infected 1 x $10^4$ cfus *S. pneumoniae* and subsequently harvested 12, 72 hours and 7 days post infection. Survival (a) was monitored daily. BAL (b), lung (c), nasal wash (d) samples were collected at specified time points cfu calculated using serial dilutions of single cell suspensions of each tissue sample plated on blood Columbia agar. Data represent the mean ± SEM of two separate experiments. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

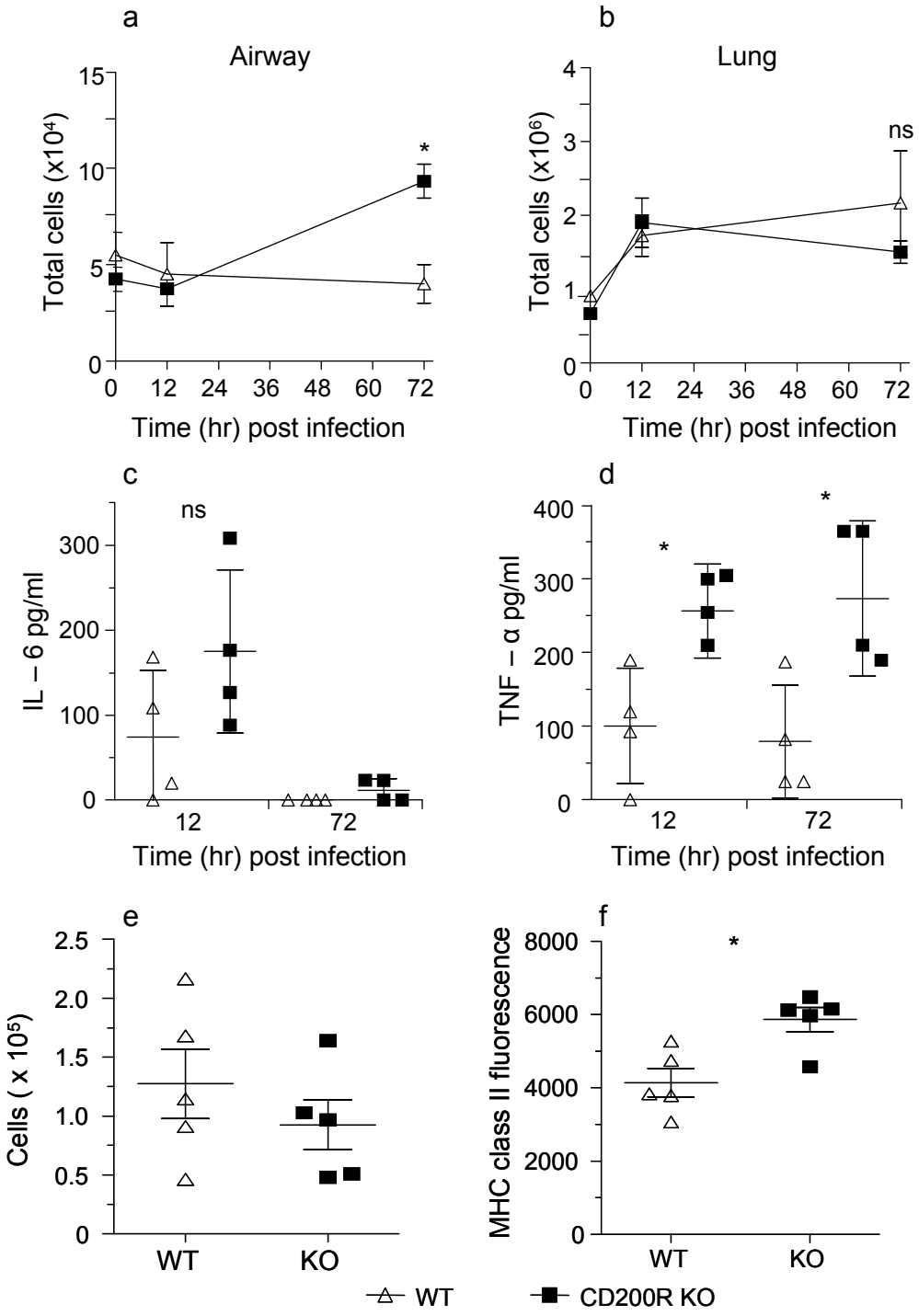



**Figure 5.6 CD200R KO demonstrate heightened airway cellular recruitment and inflammatory cytokine production.** CD200R KO and WT controls (n=4) were intranasally (i.n.) infected with 1 x $10^4$ cfu *S. pneumoniae* and subsequently harvested 12 and 72 hours post infection. Total airway (a) and lung (b) cell numbers were enumerated using Trypan blue exclusion. Airway IL-6 and TNFα cytokine levels were analysed using ELISA. Alveolar macrophage from naive wild type and CD200R KO mice were enumerated (e) as described and MHC II expression was determined using flow cytometry (f). Data represent the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

between the two groups (Figure 5.6 b). The constituents of the airway and lung infiltrates did not differ between the two groups other than in amplitude, comprising of neutrophils and MHC II expressing $F480^{+}$ monocytes (data not shown). The proportions and total numbers of $CD4^{+}$ T cells did not significantly alter over the course of infection in either the airway of lung tissue (data not shown). There was, however, an increase in inflammatory cytokine levels measured in the airways of CD200R KO mice (Figure 5.6 c & d). This was statistically significant for TNF-α levels at 12 and 72 hours post infection.

### 5.2.3 CD200R KO alveolar macrophage show heightened activity during homeostasis

Cellular analysis of BALF during homeostasis revealed no inherent difference in the proportion or total number of alveolar macrophage between CD200R KO and wild type mice (Figure 5.6 e). However, their expression of MHC II was elevated suggesting at least partial activation (Figure 5.6 f). To further interrogate the role CD200R plays in determining alveolar macrophages responsiveness, we isolated alveolar macrophages from wild type and CD200R KO mice and exposed them to various stimuli, *ex vivo,* and measured their inflammatory response. Alveolar macrophages lacking CD200R demonstrated enhanced production of both TNF-α and IL-6 in response to LPS and *S. pneumoniae* stimulation (Figure 5.7 a-d). In order to confirm these findings, we stimulated wild type, CD200R expressing, bone marrow (BM) derived macrophages in the presence or absence of an agonistic antibody to CD200R (OX110). The results demonstrated that signaling through CD200R reduces inflammatory cytokine and nitric oxide production by BM macrophages in response to IFN-γ, LPS and influenza virus (Figure 5.8 a-d).

### 5.2.4 CD200R KO mice are protected against influenza induced secondary bacterial pneumonia

After demonstrating that influenza virus infection increased CD200R expression levels on myeloid cells and that signalling through CD200R regulated inflammatory cytokine production, we hypothesised that increasing CD200R expression over the course of an influenza infection inhibits macrophage responsiveness to bacterial infection. In order to test this hypothesis we infected wild type and CD200R KO mice with a non lethal dose of *S. pneumoniae* at varying time points after influenza virus infection and measured survival, bacterial clearance, inflammatory cytokine production and cellular responses.

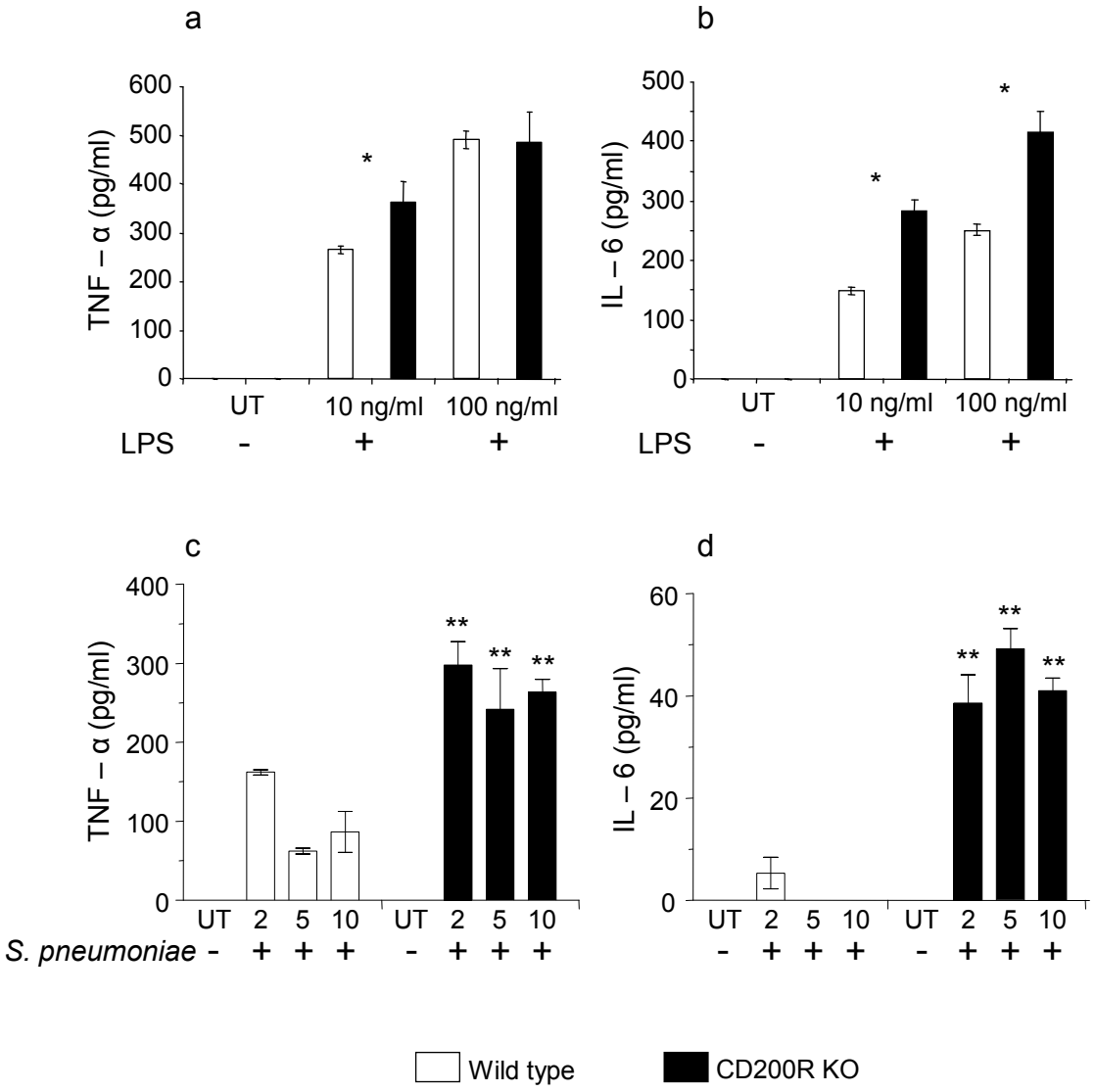



**Figure 5.7 Alveolar macrophage from CD200R KO mice show enhanced inflammatory cytokine production in response to stimuli *ex vivo*.** Alveolar macrophages were isolated from CD200R KO and WT controls (n=4) by adherence of BALF to plastic for 1 h at 37 $^0$C in 5% $CO_2$. 5 x $10^4$ of purified alveolar macrophage were then stimulated with medium alone, LPS (a & b) and varying multiplicity of infection (MOI) of *S. pneumoniae* (c & d). The supernatants were removed 24 hours later and analysed for TNF-α and IL-6 by ELISA. Data are presented as the mean ± SEM and are representative of three separate experiments. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

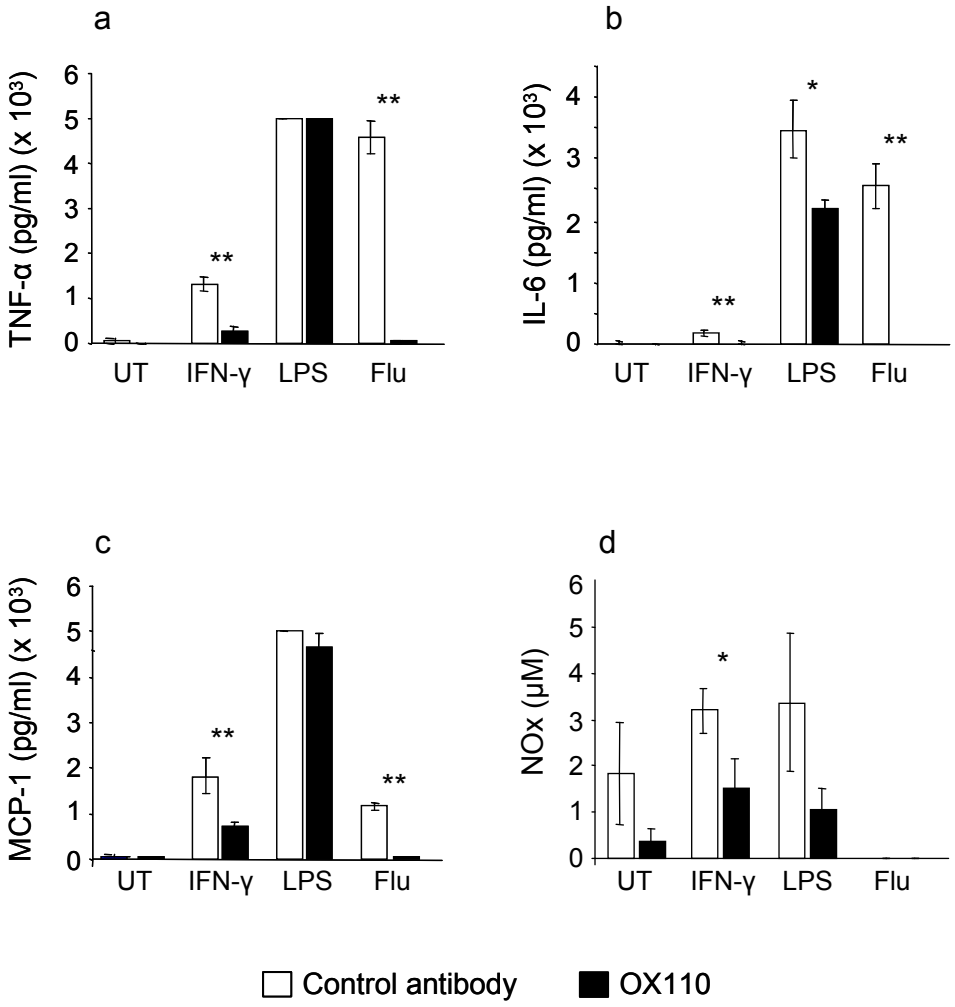



**Figure 5.8 Signaling through CD200R reduces pro-inflammatory cytokine production by BM macrophages.** WT bone marrow (BM) derived macrophages were stimulated with 10 ng/ml IFN–γ, 100 ng/ml LPS or 5 HAU influenza in the presence of 10 μg/ml of an isotype control antibody (open) or a CD200R agonistic antibody (OX110; closed). At 48 hours after stimulation, culture supernatants were removed and TNF-α (a), IL-6 (b) and MCP-1 (c) cytokine concentrations as indicated were measured by ELISA. Supernatant nitric oxide concentrations were also measured using Greiss reaction (d). Results in each panel represent mean values ± SEM from triplicate samples and are representative of 3 separate experiments. *=p<0.05, **=p<0.01.

CD200R KO mice infected with *S. pneumoniae* three days after influenza virus infection displayed reduced weight loss 48 hours post *S. pneumoniae* infection compared to WT controls (Figure 5.9 b). In addition, wild type mice appeared physically more ill, displaying the characteristic hunched and immobile phenotype described earlier (data not shown). More significantly CD200R KO mice contained significantly less bacterial titres in both airway and lung compartments at 6 and 48 hours post *S. pneumoniae* infection (Figure 5.9 c & d). CD200R KO mice also failed to develop systemic bacteraemia detected in wild type mice 48 hours post *S. pneumoniae* infection (Figure 5.9 e). Interestingly, as was observed in the single *S. pneumoniae* infection (section 5.2.2.), the levels of bacteria recovered from the nasopharyngeal cavity increased over the course of infection but did not significantly differ in titre between the two groups at all time points analysed (Figure 5.9 f).

This large reduction in bacterial titre in CD200R KO mice was also observed when *S. pneumoniae* was administered 7 days after influenza infection. Once more, CD200R KO mice lost significantly less weight in response to *S. pneumoniae* infection, appeared less ill and displayed a large reduction in bacterial titres recovered from airway and lung compartments compared to wild type mice (Figure 5.10 b-f). CD200R KO mice again failed to develop systemic bacteraemia observed in wild type mice 48 hours after *S. pneumoniae* infection (Figure 5.10 e). Infection with *S. pneumoniae* 14 days after influenza produced the same results. However, at this time point wild type mice did not exhibit any significant weight loss upon *S. pneumoniae* infection (Figure 5.11 b). In addition to the differences in bacterial susceptibility, CD200R KO mice recovered from the primary influenza virus infection more rapidly (Figure 5.11 b). Nevertheless, CD200R KO mice again displayed reduced bacterial titres recovered from the airway and lung compartments and again failed to develop systemic bacteraemia (Figure 5.11 c-f). Regardless of when *S. pneumoniae* was added and the time point harvested there was no differences in nasopharyngeal bacterial titres.

Closer inspection of the data revealed that wild type mice were least able to contain *S. pneumoniae* when bacteria were administered 7 days after the initial influenza infection (Figure 5.12 a-d). This was demonstrated by calculating the fold increase of *S. pneumoniae* titres recovered 48 hours after infection from mice infected with *S. pneumoniae* alone and mice previously infected with influenza virus and comparing this value across the differing time points of infection after influenza infection (Figure

5.12 a-d). In addition to the significant differences in bacterial titres, 100 % of CD200R KO mice infected with *S. pneumoniae* following influenza virus survived regardless of when they are infected with bacteria (Figure 5.12 e).

### 5.2.5 The lack of CD200R provides enhanced antiviral immunity

It is likely that alteration in immunity and pathology to an influenza infection in CD200R KO mice contributes to the reduced susceptibility to secondary bacterial pneumonia. Wild type mice recruited significantly more cells to the airways (p 0.04) in response to influenza infection on day three and seven post influenza infection compared to CD200R KO mice (Figure 5.13 a & b). This was also confirmed by H & E histology (Figure 5.14 b). In both wild type and CD200R KO mice the cellular infiltrate subsided to naïve levels by day 14. The enhanced airway cellularity in wild type mice was mirrored in the lung tissue on day 7 of an influenza infection (Figure 5.13 b). We next examined viral clearance kinetics in wild type and CD200R KO mice. A lack of CD200R enhanced anti-viral ability (Figure 5.13 c). This may result from the increased percentage and total numbers of airway NK cells in CD200R KO mice (Figure 5.13 d & e). There was no increase in NK number observed in the lung tissue of CD200R KO mice (data not shown).

There were no significant differences in the proportions of alveolar macrophages, recruited $F480^+$ macrophage or neutrophils at any time point analysed between WT and CD200R KO mice (data not shown), however there was a general increase in the total number of myeloid subsets in both the airway and lung tissue of wild type mice at all time points analysed. This failed to reach significance at any time point analysed. Analysis of macrophage activation also highlighted no significant difference in the expression of PRRs, such as TLR2 and MARCO, or the percentage of TNF-α and IFN-γ producing macrophages in the airway or lung. However, there was a significant increase in the intensity of MHC class II expression on alveolar macrophages in CD200R KO mice 7 and 14 days post influenza infection (p 0.0286 and p 0.0159 respectively) (data not shown).

Analysis of lymphocyte populations showed a general increase in the proportion of $CD4^+$ and $CD8^+$ T cells in CD200R KO mice over the course of influenza infection. This trend was mirrored in the lung tissue. The only significant difference was an elevated proportion of $CD8^+$ T cells in the airway of CD200R KO mice on day 7 post influenza infection (p 0.029). Despite this, the total number of $CD4^+$ and $CD8^+$ T cells

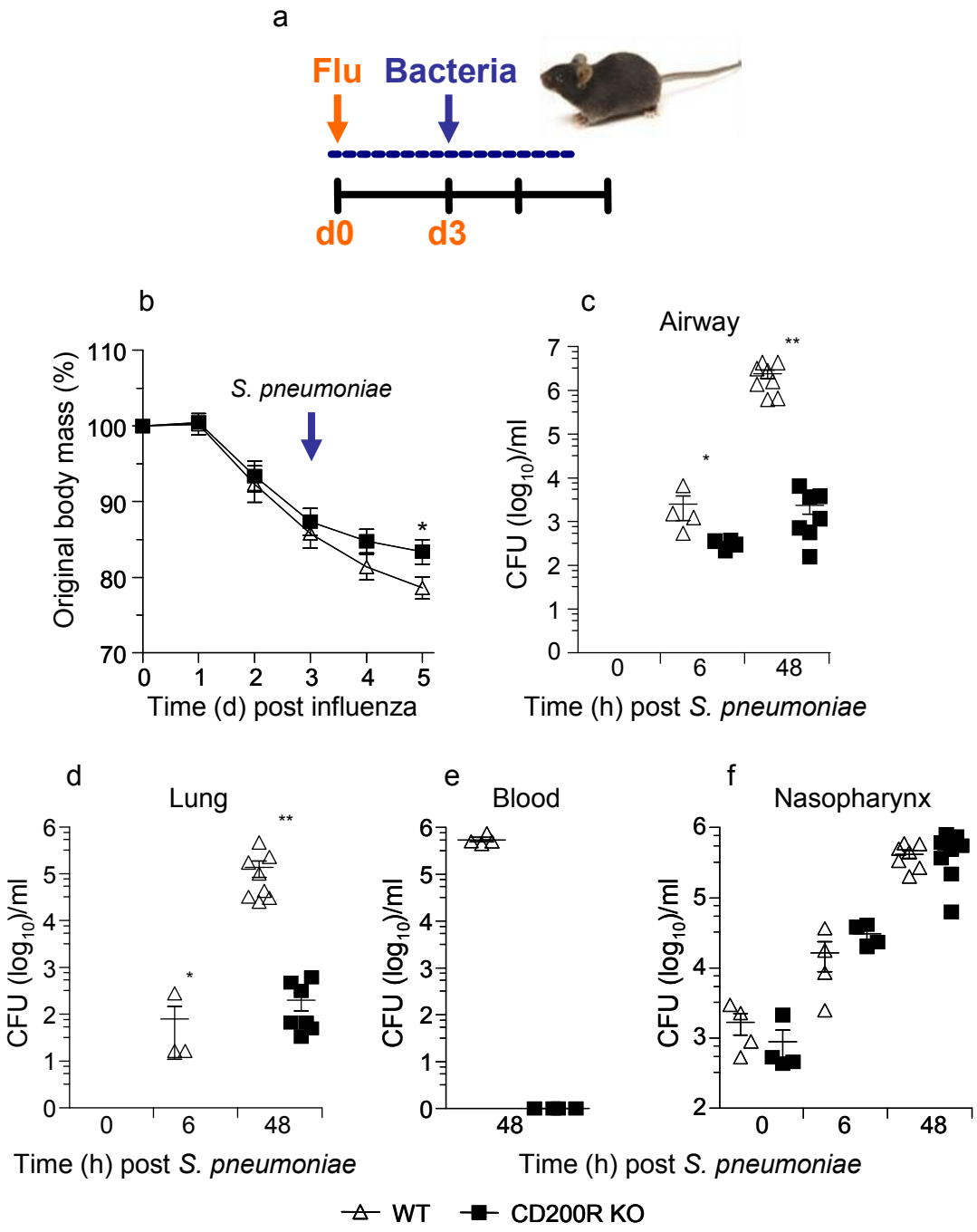



**Figure 5.9 A lack of CD200R protects mice from a secondary *S. pneumoniae* infection.** CD200R KO and WT controls were infected intranasally (i.n.) with 50 HAU HK/X31 influenza A virus. After three days, mice were infected i.n. with 1 x $10^4$ cfu *S. pneumoniae* (a). Weight loss (b) was monitored daily and expressed as a mean % of original body mass. 6 and 48 hours post *S. pneumoniae* infection BAL and nasal wash was performed and lung tissue removed. Airway (c), lung (d), nasopharyngeal (e) and peripheral blood (f) bacterial titres were calculated using serial dilutions of single cell suspensions plated on blood supplemented Columbia agar. Data are presented as the mean ± SEM of 4 - 8 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

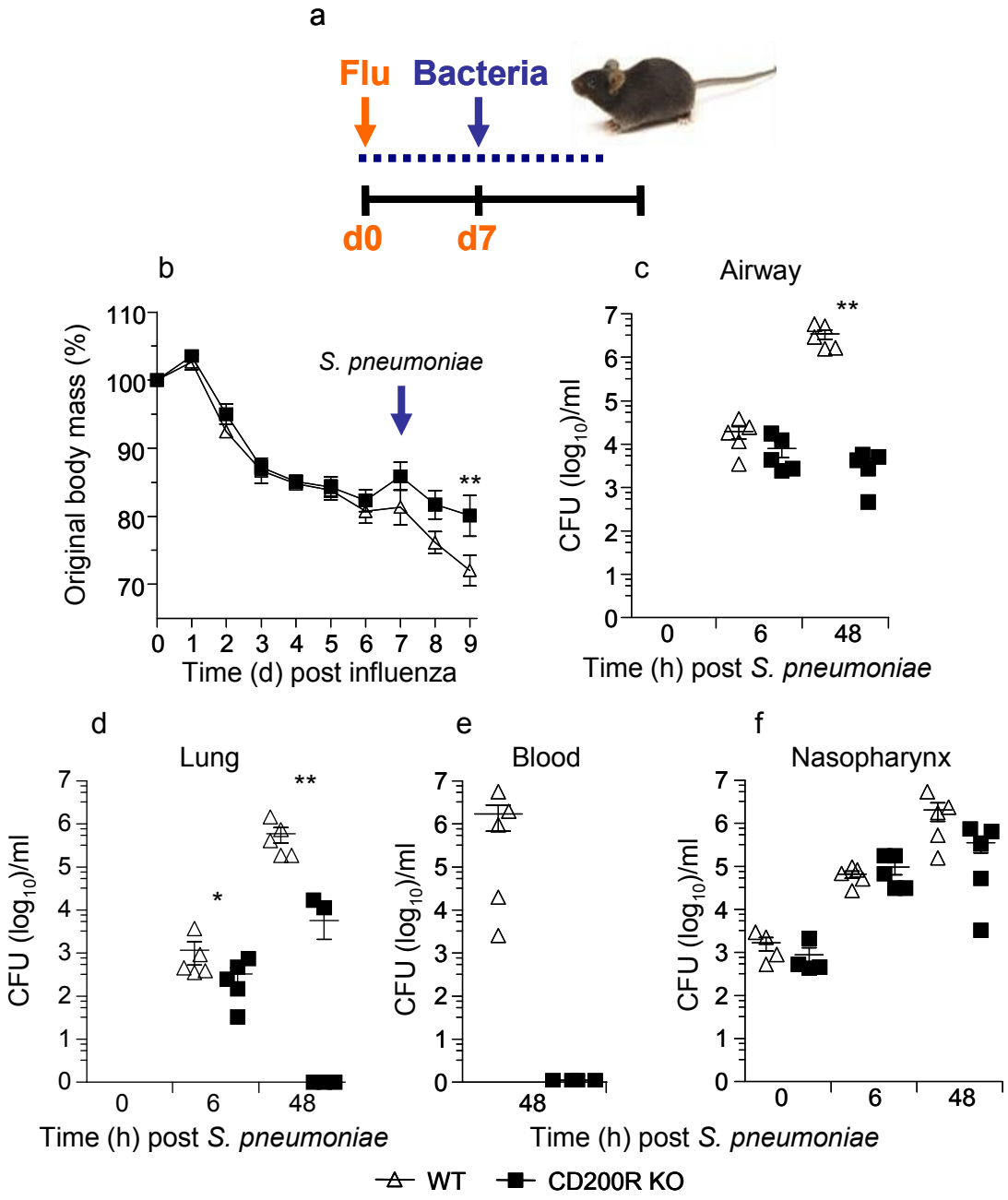



**Figure 5.10 A lack of CD200R protects mice from a secondary *S. pneumoniae* infection on day 7 of an influenza infection.** CD200R KO and WT control mice (n=5) were infected intranasally (i.n.) with 50 HAU HK/X31 influenza A virus. After seven days, mice were infected i.n. with 1 x $10^4$ cfu *S. pneumoniae* (a). Weight loss (b) was monitored daily and expressed as a mean % original body mass. 6 and 48 hours post *S. pneumoniae* infection BAL and nasal wash was performed and lung tissue removed. Airway (c), lung (d), nasopharyngeal (e) and peripheral blood (f) bacterial titres were calculated using serial dilutions of single cell suspensions of each tissue sample and plated on blood supplemented Columbia agar. Data are presented as the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

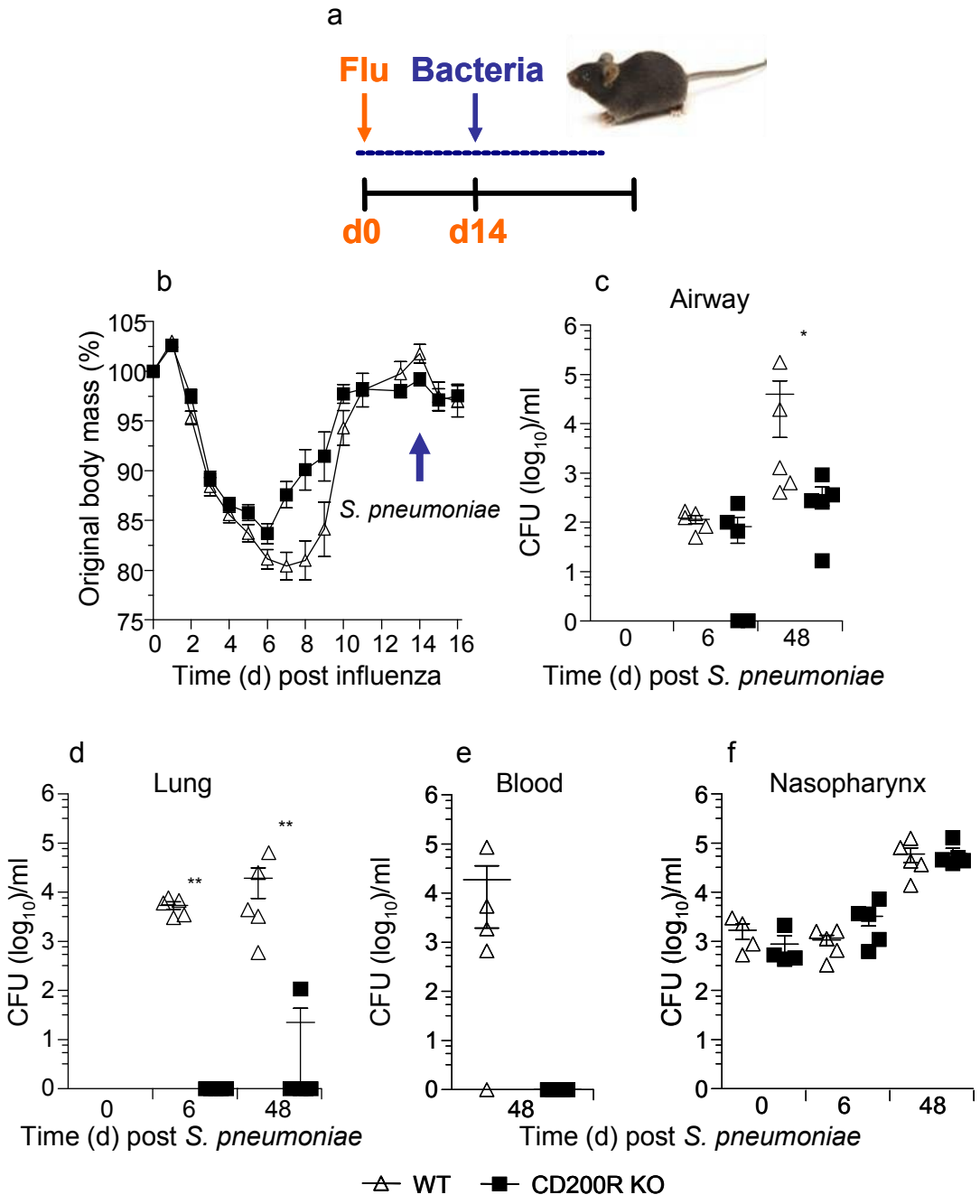



**Figure 5.11 A lack of CD200R protects mice from a secondary *S. pneumoniae* on day 14 of an influenza infection.** CD200R KO and WT mice (n=5) were infected intranasally (i.n.) with 50 HA HK/X31 influenza A virus. After fourteen days, mice were infected i.n. with 1 x 104 cfu *S. pneumoniae* (a). Weight loss (b) was monitored daily and expressed as a mean % original body mass. 6 and 48 hours post *S. pneumoniae* infection BAL and nasal wash was performed and lung tissue removed. Airway (c), lung (d), nasopharyngeal (e) and peripheral blood (f) bacterial titres were calculated using serial dilutions of single cell suspensions of each tissue sample and plated on blood supplemented Columbia agar. Data are presented as the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

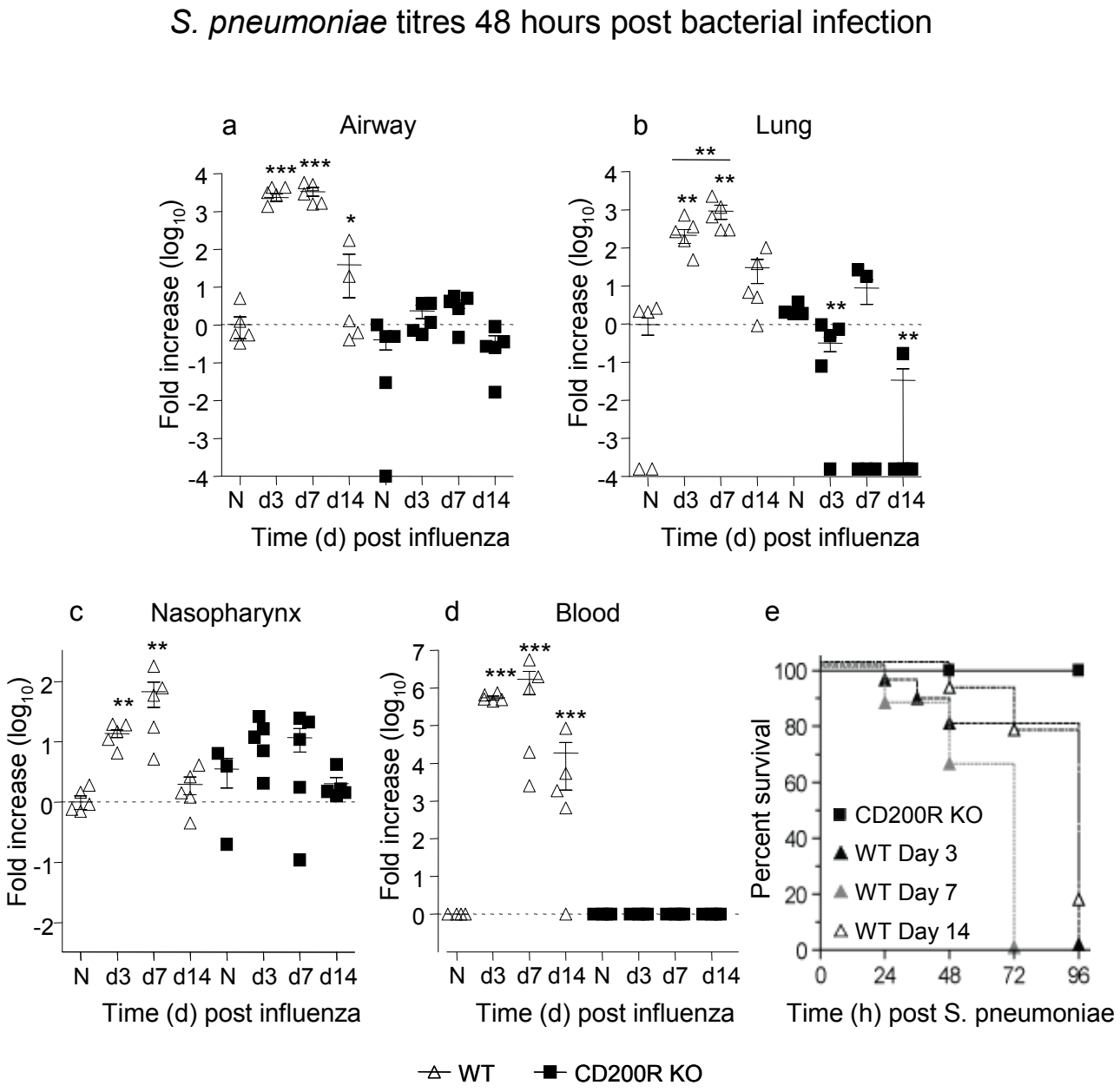



**Figure 5.12 Susceptibility to a secondary bacterial infection is greatest 7 days after influenza virus infection.** Normalised bacterial titres recovered from the airway (a), lung tissue (b), nasopharyngeal cavity (c) and peripheral blood (d) 48 hours after *S. pneumoniae* infection in mice infected 3, 7 or 14 days previously with influenza virus. Recovered bacterial titres are expressed as the fold increase over the bacterial titres recovered 48 hours after infection of naïve mice with *S. pneumoniae*. Survival (e) at each time point post influenza infection was also measured. Data represent the mean ± SEM n = 5.

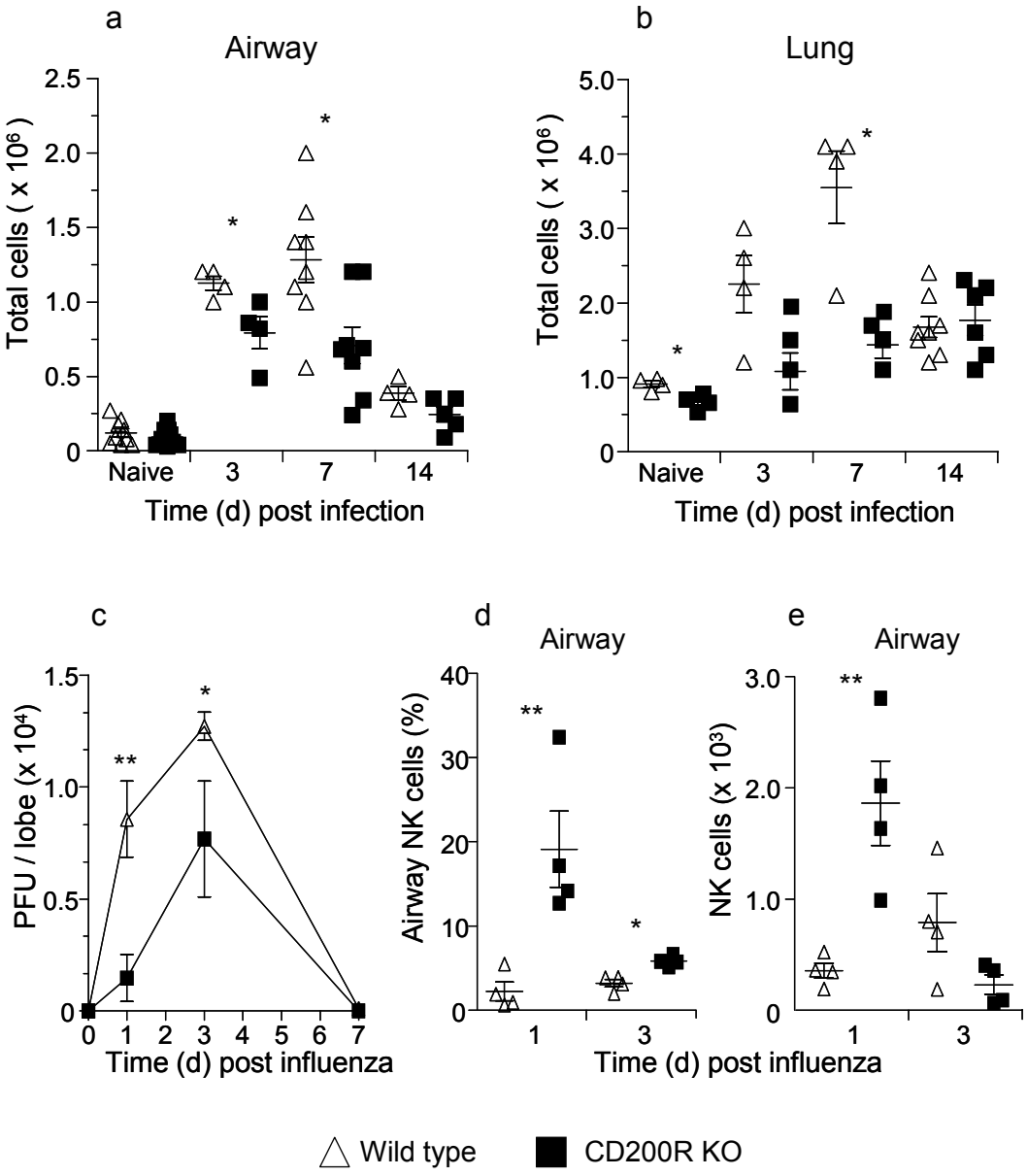



**Figure 5.13 A lack of CD200R reduced cellular recruitment and influenza viral titres.** CD200R KO and WT controls (n=4-8) were intranasally (i.n.) infected with 50 HAU HK/X31 influenza A virus on day 0. BAL (a) and lung (b) cells were quantified, at specified time points, using Trypan blue exclusion. Influenza viral titres (c) were determined from frozen lung lobes. The % (d) and total number (e) of airway NK cells were determined using flow cytometry. Data are presented as the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

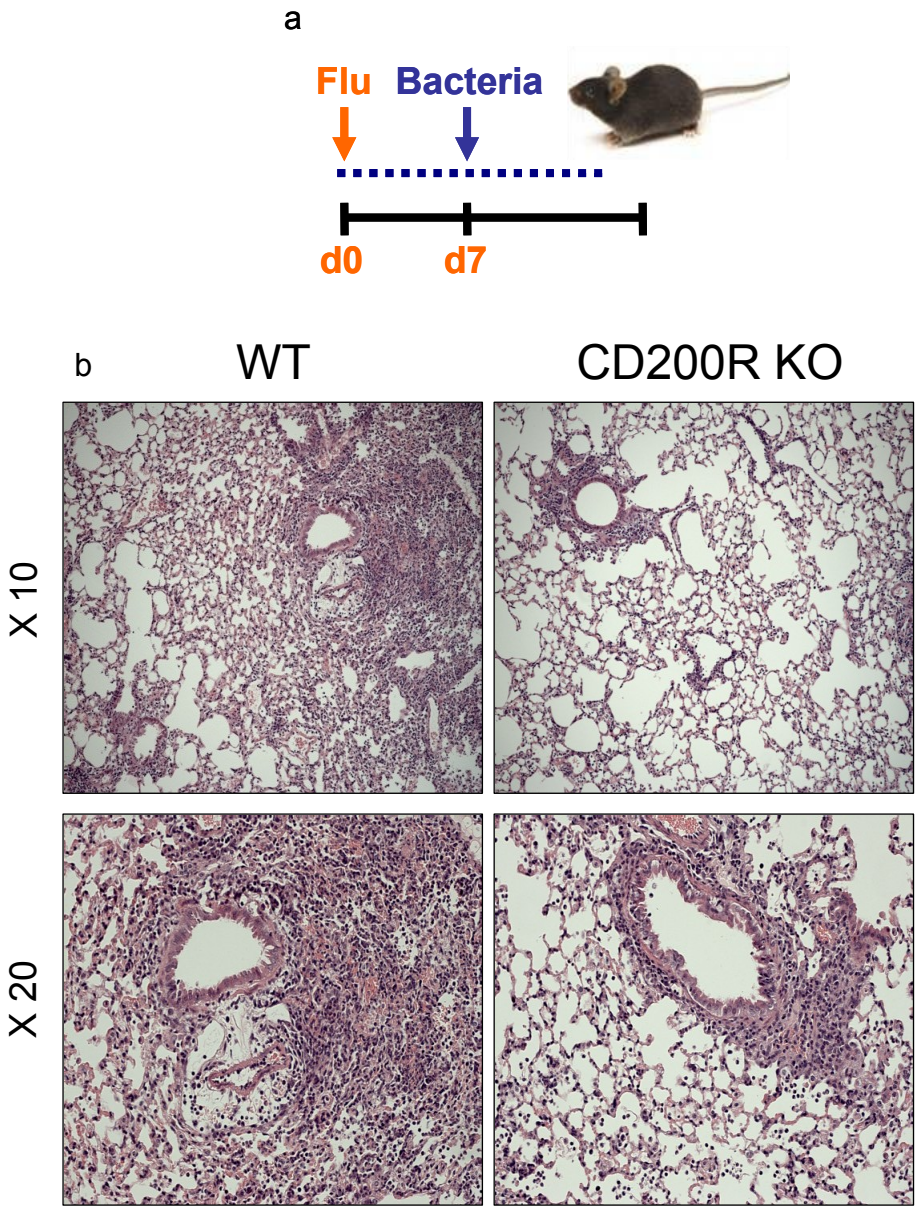



**Figure 5.14 Lung pathology in WT and CD200R KO mice infected with *S. pneumoniae* on day 7 of an influenza infection.** Wild type and CD200R KO mice (n=4) were infected intranasally with 50 HAU HK/X31 influenza virus on day 0. 7 days later the mice were intranasally infected with 1 x $10^4$ cfu *S. pneumoniae*. Mice were subsequently harvested 48 hours post *S. pneumoniae* infection. Processing of mouse lungs for histology and light microscopy of H & E stained sections were carried out as described in section 2.7.1. Magnification was x 10. Sections are representative of four mice studied at each time point.

and the proportion that were $CD69^+$ and TNF-α and IFN-γ producing were, in general, elevated in wild type mice on day 7 post infection. However, none of these differences were statistically significant (data not shown).

### 5.2.6 Cytokine environment of the airway and lung at time of *S. pneumoniae* infection

Since many cytokines are also anti-bacterial we examined whether there were any differences between wild type and CD200R KO mice at the time of bacterial administration. Wild type mice demonstrated elevated levels of TNF-α and IL-6 in the airway at 3 and 7 days post influenza (Figure 5.15 a & e). Airway IFN-γ levels were only elevated in wild type mice 7 days after influenza infection (Figure 5.15 c). On analysis inflammatory cytokine levels measure from lung tissue a similar pattern was observed. IFN-γ and IL-6 levels were elevated on day 7 whilst TNF-α was higher at day 3 post influenza in wild type mice (Figure 5.15 b, d & f). Therefore, mice that survive the bacterial secondary infection had less inflammatory cytokines present at the time of bacterial infection.

### 5.2.7 Airway cellular differences between wild type and CD200R mice 7 days after influenza infection

Having demonstrated that wild type mice recruit a greater number of cells to the airway and lung and have elevated levels of inflammatory cytokine 7 days after influenza infection, we next investigated what influence this may have on susceptibility to bacterial infection. Administration of *S. pneumoniae* to influenza infected mice at day 7 resulted in 100 % mortality in wild type mice by day 4 post infection (Figure 5.16 a). *S. pneumoniae* infection also caused a further increase in airway cellularity that was more pronounced in wild type compared to CD200R KO mice (Figure 5.16 b). This difference was less apparent in the lung but still significant at early time points (data not shown). Of these additional cells neutrophils dominated in wild type mice (Figure 5.16 c & d). This was accompanied by an increase in the expression OX40L, an immune potentiator on alveolar and tissue macrophages at 48 hours post *S. pneumoniae* infection (Figure 5.16 e & f). Furthermore, we observed higher levels of IL-10R 48 hours post *S. pneumoniae* infection on alveolar macrophages from CD200R KO mice (p 0.0159) (Figure 5.16 g). Having previously demonstrated that TLR and MARCO expression was increased on innate immune cells during an ongoing influenza infection, we also compared the expression levels of TLR2, TLR4 and MARCO on alveolar macrophage in wild type and CD200R KO mice, however no significant differences were detected (data not shown).

### 5.2.8 CD200$^+$ leukocytes are present in the airway during influenza infection and secondary bacterial infection

Recent literature proposes that phagocytic function of alveolar macrophages can be altered by the presence of inflammatory cytokines produced in response to influenza infection and through cognate interactions with infiltrating cells [472-475]. We hypothesised that the negative influence of CD200R on macrophages following influenza infection may play a role in reducing their anti-bacterial function. To support this idea CD200$^+$ T cells were present in both the airway and lungs of wild type and CD200R KO mice during a secondary bacterial infection (Figure 5.17 a-d). The intensity of CD200 expression was also elevated on wild type CD4$^+$ and CD8$^+$ T cells with higher levels observed in the lung (Figure 5.17 e & f). The presence of apoptotic CD200+ expressing monocytes in the airway was also observed in both wild type and CD200R KO 7 and 14 days after influenza infection (Figure 5.18 a-e).

To investigate whether the presence of CD200 expressing leukocytes can reduce the anti-bacterial function of alveolar macrophages, we next examined, *in vitro,* the effects of CD200R ligation on macrophage phagocytic functionality. After demonstrating that alveolar macrophages engulfed GFP expressing *S. pneumoniae ex vivo* (Figure 5.19) we stimulated wild type and CD200R KO naive alveolar macrophage with OX110, a CD200R agonistic antibody, for 30 minutes prior to exposure to *S. pneumoniae*. Our findings demonstrated that *in vitro* ligation of CD200R does not alter the phagocytic function of alveolar macrophage (Figure 5.19 b & c).

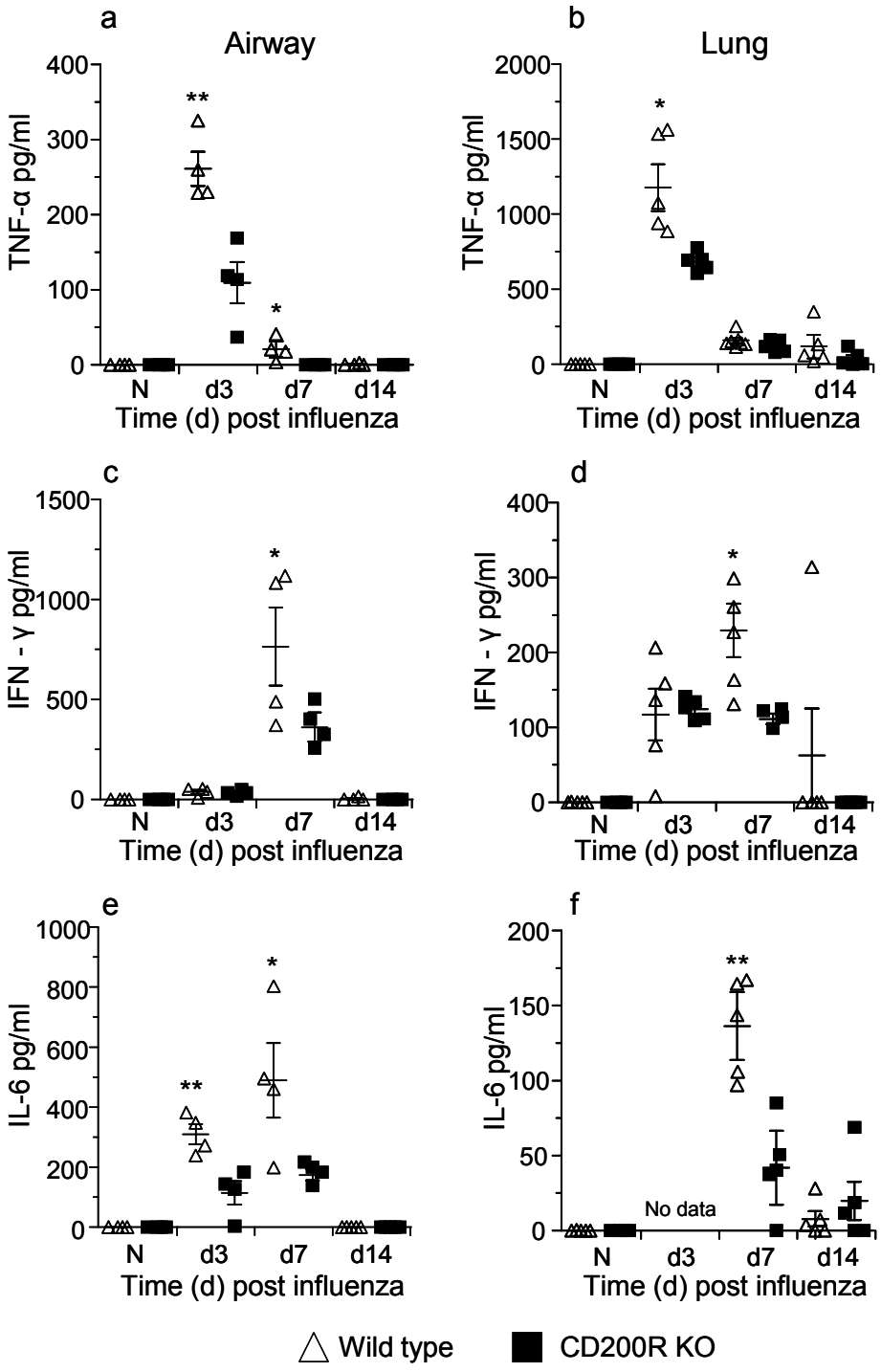



**Figure 5.15 Wild type mice show elevated inflammatory cytokine production in response to influenza.** CD200R KO and WT controls were intranasally (i.n.) infected with 50 HAU HK/X31 influenza A virus on day 0. Airway levels of TNF-α (a), IFN-γ (c) and IL-6 (e) were quantified, using ELISA, at time point specified. Lung tissue was removed, at specified time points, homogenised and assayed for TNF-α (b), IFN-γ (d) and IL-6 (f) using ELISA. Data represent the mean ± SEM n = 4 (airway) or 5 (lung). $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

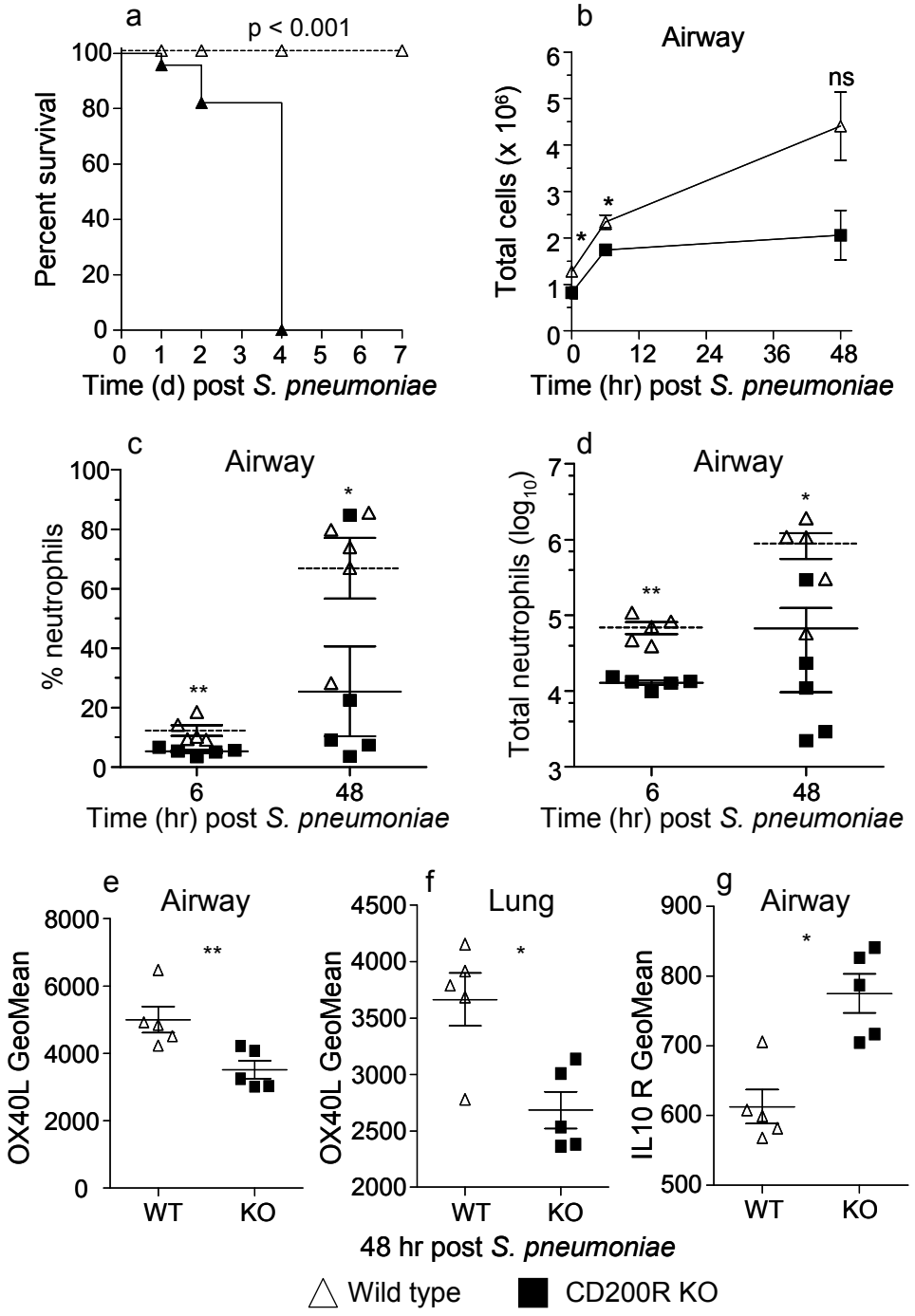



**Figure 5.16 CD200R KO mice recruit less neutrophils and activated alveolar macrophages.** WT and CD200R KO mice (n=4) were intranasally (i.n.) infected with either 50 HAU HK/X31 influenza A virus on day 0. Seven days later 1 x $10^4$ cfu *S. pneumoniae* was administered i.n. BAL (a) and lung (b) total cells were enumerated at specified time points using Trypan blue exclusion. Neutrophil % (c) and total numbers (d) were quantified using FCM. OX40L expression was also analysed on $CD11c^+$ cells in airway and lung. Data are presented as the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

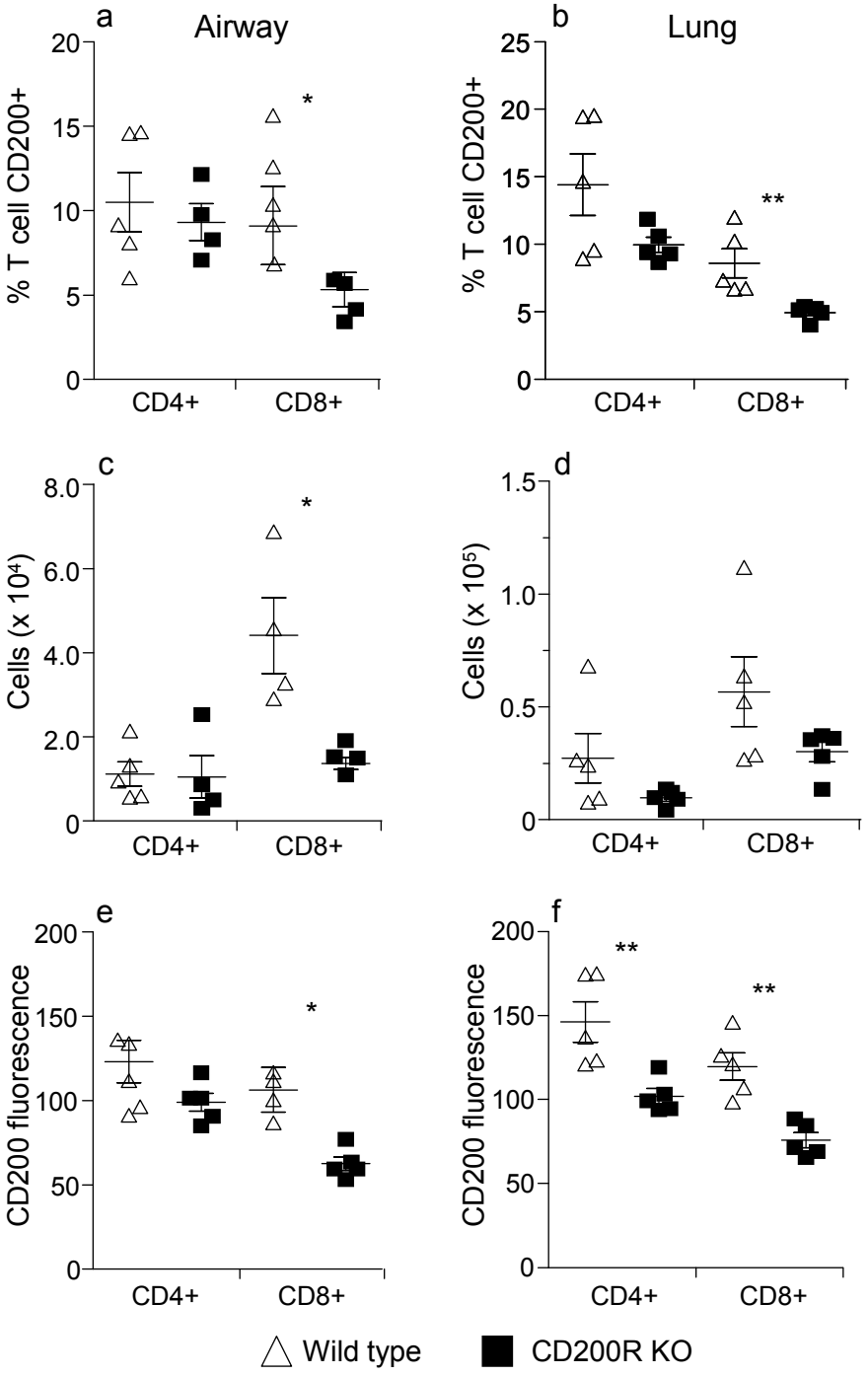



**Figure 5.17 The presence of CD200⁺ T cells in the airways and lung of co-infected mice.** WT and CD200R KO mice (n=5) were intranasally (i.n.) infected with 50 HAU HK/X31 influenza A virus on day 0. Seven days later 1 x $10^4$ cfu of *S. pneumoniae* was administered i.n. Mice were culled 48 hours post *S. pneumoniae*. The proportion (a & b) and total number (c & d) of CD200⁺ CD4⁺ and CD8⁺ T cells were determined using flow cytometry. Mean fluorescence intensity of CD200 on T cell subsets was also determined (e & f). Data are presented as the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

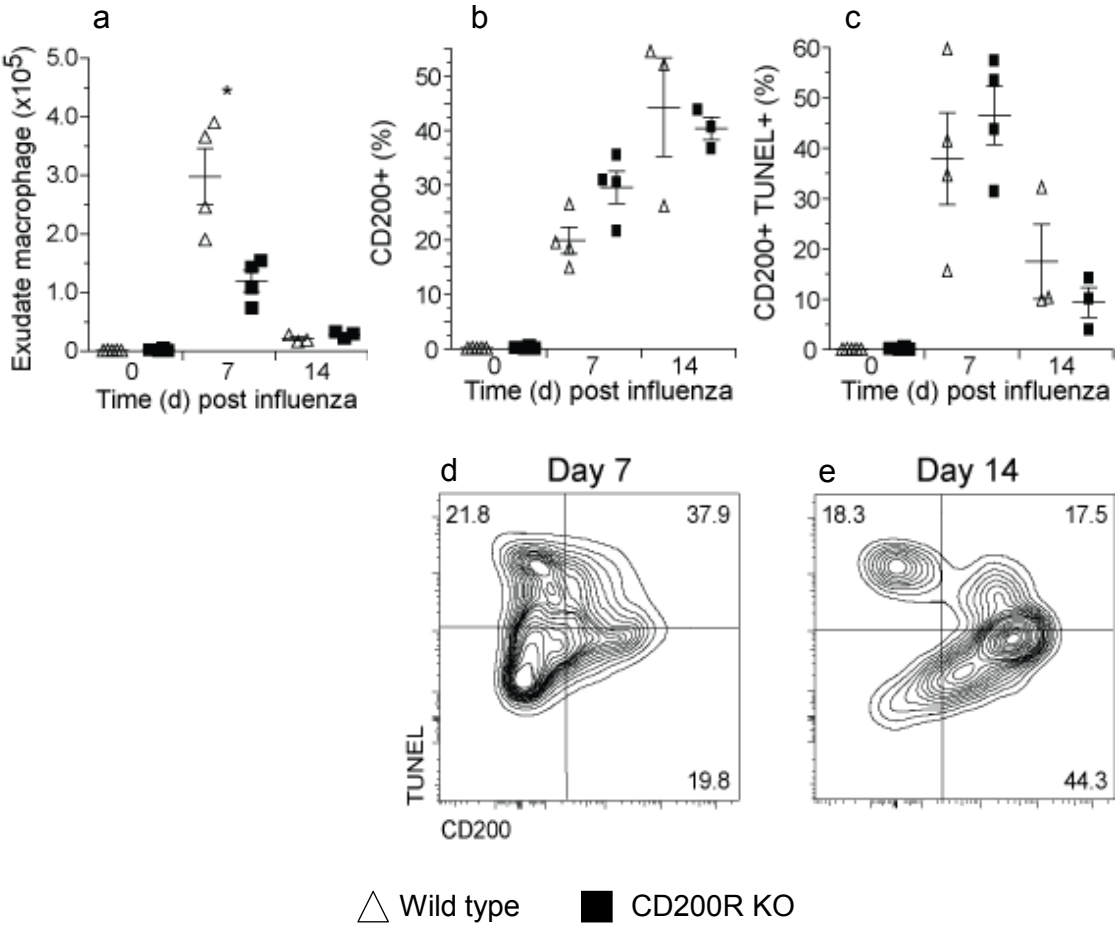



**Figure 5.18 CD200 is expressed on apoptotic exudate monocytes in the airway during influenza infection.** WT and CD200R KO mice (n=4) were intranasally (i.n.) infected with 50 HAU HK/X31 influenza A virus on day 0. BAL was performed at specified time points after influenza infection. The total number (a) of exudate monocytes were determined by flow cytometry and shown to be $CD11b^+$ $F480^+$ as described in section 2.6.1. % $CD200^+$ (b) and $CD200^+$ $TUNEL^+$ (c) was determined. A representative flow cytometric plot from day 7 (d) and day 14 (e) WT mice is shown. Data are presented as the mean ± SEM. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

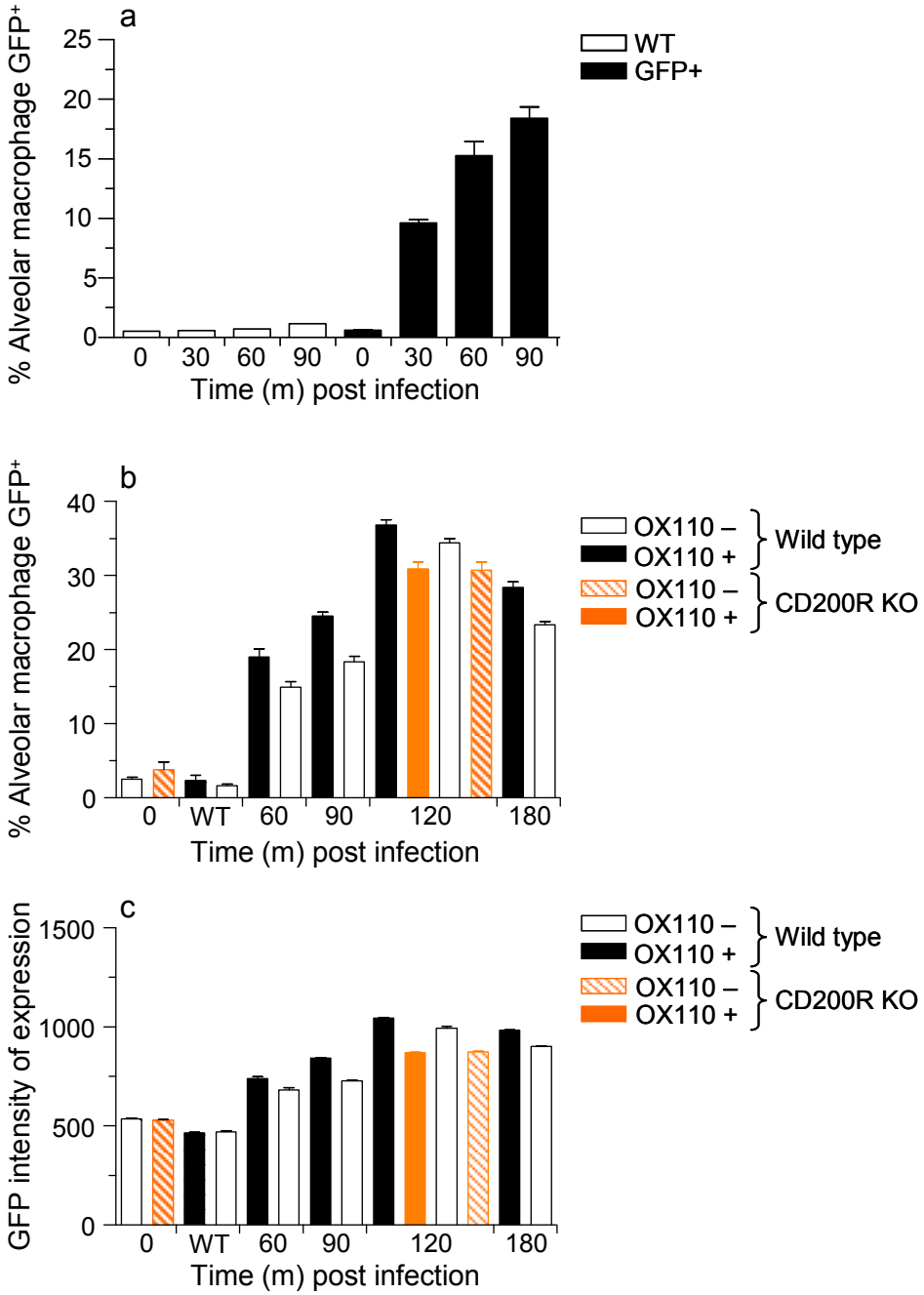



**Figure 5.19 *In vitro* CD200R ligation does not alter alveolar macrophage phagocytic function.** Alveolar macrophages were isolated from naive WT mice (n=5) and incubated at a density 5 x $10^4$ cells per well with opsonised WT or pGFP expressing *S. pneumoniae* (MOI 5). The % GFP+ alveolar macrophages were subsequently determined using FCM (a). In a separate experiment, 5 x $10^4$ of WT and CD200R KO alveolar macrophages were pre-stimulated for 30 mins with or without 10µg/ml of OX110 before the addition of pGFP$^+$ *S. pneumoniae* (b & c). Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

## 5.3 Discussion

### 5.3.1. Susceptibility to *S. pneumoniae* is increased in C57BL/6 mice following influenza virus infection

In Chapter 3 we showed that C57BL/6 mice demonstrated a dose related increase in susceptibility to a *S. pneumoniae* infection, here we demonstrate that a preceding influenza virus infection significantly reduces the dose of *S. pneumoniae* that results in lethal systemic bacteraemia. Using the secondary bacterial infection model developed in Chapter 4 we illustrate that 1 x $10^4$ cfu of *S. pneumoniae*, a non lethal dose when given to naïve mice, results in lethal bacteraemia when administered 3, 7 or 14 days after an influenza infection. We also show that peak susceptibility to a secondary bacterial super infection, determined by total bacterial load and time to mortality, coincides with peak influenza-associated illness, 7 days after an influenza infection. Furthermore, the development of a secondary bacterial super infection significantly enhances weight loss and results in a hypothermic syndrome characteristic of systemic septic shock. We therefore highlight that the clinical manifestation of a *S. pneumoniae* secondary bacterial infection is considerably more severe than what is observed during a GBS secondary bacterial infection. This increase in severity of bacterial infection is likely a consequence of the niche adaptation of *S. pneumoniae* to inhabiting and infecting respiratory mucosa and its associated virulence factors, such as its polysaccharide capsule, pneumolysin and hyaluronidase that allows tissue invasion and dissemination [4;255;259;476]. Our findings are similar to existing epidemiological evidence, with regards to the temporal relationship between influenza and secondary bacterial pneumonias, and existing animal models of influenza induced *S. pneumoniae* super infections, therefore validating both our experimental setup and results [412;472;477-480]. However, it is important to remember, as discussed in Chapter 3, the strain of mouse, the infective dose of both influenza and *S. pneumoniae* used and timing of the secondary bacterial infection post influenza infection all independently affect the outcome of a model and should be taken into consideration when interpreting secondary bacterial infection model results.

Further comparison of the GBS and *S. pneumoniae* secondary infection models highlights interesting differences including the lack of synergistic cellularity and the enhancement of neutrophil numbers observed when *S. pneumoniae* is administered on day 3 of an influenza infection. However, due to the extensive discussion on contrasting neutrophil responses in BALB/c and C57BL/6 mice in Chapter 3 and the

clear difficulties in comparing the two different secondary bacterial infection models there is no further discussion concerning these differences.

### 5.3.2. The role of CD200R and CD200 during homeostasis and Influenza virus infection

There is growing evidence that myeloid cell immune homeostasis, in the absence of antigen, is an active process mediated by tissue specific micro-environmental factors that define a threshold for responsiveness [169]. The maintenance of this threshold is likely to represent an evolutionary strategy that limits inflammation, particularly in mucosal sites that are exposed to an abundance of innocuous antigen and opportunistic micro-organisms. We show that CD200R is highly expressed by alveolar macrophages at homeostasis and that expression on pulmonary dendritic cells is bi-modal, therefore suggesting the existence of two distinct dendritic cell populations during homeostasis. Intriguingly, only myeloid cells within (alveolar macrophages), or in contact with (dendritic cells dendrites), the airway lumen express high levels of CD200R at homeostasis, compared to macrophages resident in the lung parenchyma or at distant sites that express significantly lower levels of CD200R during homeostasis. However, in order for them to be restrained, CD200 is required. In homeostasis we detect CD200 expression on airway epithelium, lung endothelial, T lymphocytes and B cells, similar to that described in other sites [114;118-120]. However, haematopoietic cells are of low abundance in the non-inflamed airway and luminal myeloid cells are unlikely to be influenced by CD200 expressed on endothelial cells. We therefore propose that CD200 expressed on the luminal aspect of airway epithelial cells is the most likely mechanism for controlling alveolar macrophages that express abundant CD200R levels. This suggests the existence of a dynamic homeostatic regulatory system that is determined at the level of the tissue compartment.

It has been previously shown that naive CD200 KO mice display elevated numbers of activated macrophages in their spleen, mesenteric lymph nodes and CNS, compared to wild type controls, and that alveolar macrophages from aged CD200 KO mice respond more vigorously to inflammatory stimuli *ex vivo,* thus suggesting that a lack of CD200R ligation intrinsically alters alveolar macrophages in a manner that makes them more primed to respond to activating stimuli [458]. This novel mechanism of maintaining a hypo-responsive state of alveolar macrophages is akin to other important lung specific homeostatic pathways for example: 1) epithelial $\alpha_v\beta_6$ integrin bound TGF-$\beta$ [456;457] and 2) binding of surfactant proteins to the inhibitory receptor

signal regulatory protein-α (SIRP-α)[481]. Furthermore, we show that the homeostatic level of CD200R on BALB/c derived alveolar macrophages is significantly lower than that detected on alveolar macrophage from C57BL/6 mice. Could this difference in CD200R expression levels reduce the level of homeostatic regulation placed upon BALB/c alveolar macrophage, thereby reducing their threshold of activation and thus explaining the robust innate cellular responses we observe in BALB/c mice in all infection models used in this thesis? Could this observation also explain the differences in neutrophil responses observed between C57BL/6 and BALB/c mice? It is fascinating to postulate that a loss of CD200R, as is the case in CD200R KO mice, pushes the CD200R KO (C57BL/6 background) toward a BALB/c phenotype that elicits rapid innate immune responses to respiratory bacterial and viral infection such as *S. pneumoniae* and influenza virus.

If CD200 determines myeloid regulation during homeostasis how would immune responses be initiated in tissues that exhibit high CD200 and CD200R expression, such as the lung or CNS? One could assume that for an immune response is to occur, CD200 or its receptor would have to be shed or their inhibitory function reduced. SIRP-$\alpha$ (another inhibitory myeloid regulator) on macrophages is lost in response to LPS stimulation to permit an inflammatory response and a re-introduction restores macrophage homeostasis [482]. Similarly, microbial products cause the loss of $\alpha v\beta 6$ on airway epithelial cells, removing the TGF-$\beta$ mediated suppression of alveolar macrophages [456]. We fail to see any such loss of CD200R expression on lung and airway myeloid populations but rather an increase in its intensity throughout an influenza infection. Furthermore, we show an increase in the number of $CD200^+$ leukocytes in the lung when infected with influenza. However, closer analysis reveals CD200 expression is reduced on airway infiltrating $CD4^+$ and $CD8^+$ T cells. Therefore, in homeostasis airway epithelial CD200 signals to the myeloid cell through CD200R; not to prevent an inflammatory response but rather to raise the threshold required for activation, thus preventing an immune response to innocuous antigen. During heightened inflammation however, leukocytes enter the airways, lose CD200 expression and compete with epithelial cells for myeloid cell interaction, thus allowing inflammation to proceed with vigour. It is also possible that, in the course of infection, other molecules are up-regulated on the surface of ligand and receptor bearing cells that may impede a meaningful interaction. Conversely, not only is CD200 lost on many cells entering the airways but the scarcity of any

structural organization may limit the close association of infiltrating cells and the potential to impart an inhibitory signal through CD200R to myeloid cells at this site.

### 5.3.3. A loss of CD200R protects against lethal bacteraemia during secondary bacterial pneumonias

As discussed above, the dynamic interaction of CD200R, expressed on alveolar macrophages, and CD200, expressed on the luminal aspect of respiratory epithelium, determines a level of immune hypo-responsiveness during homeostasis that prevents unwarranted inflammation in response to innocuous antigen. Pathways governing immune homeostasis are also likely to restrain the amplitude of inflammation and ultimately return the system to a state of homeostasis. Toll-like receptor signalling marks the presence of an invading pathogenic microorganism, but ensuing inflammatory cascades must be moderated to prevent unnecessary inflammatory disease [483;484]. We show that a loss of CD200-CD200R homeostatic regulation, utilising CD200R KO mice, results in an enhanced ability to initially contain a non lethal *S. pneumoniae* infection in both the airway and lung tissue. This intervention also results in enhanced basal activation of alveolar macrophages, shown by increased MHC II expression, an increase in airway cellularity and an increase in inflammatory cytokine production in response to *S. pneumoniae* infection in both *in vivo* and *in vitro* circumstances. These results provide evidence that CD200-CD200R interaction imparts a hypo-responsive immune state, present in homeostasis that increases the level of activation needed to initiate anti-bacterial immunity. CD200R ligation has previously been shown to suppress TNF-α and IL-6 production by mouse peritoneal macrophages, IFN-γ induced IL-8, MIG and IP-10 by human U937 monocytes, IgE mediated degranulation and IL-13 and TNF-α production by mouse and human mast cells [125;144]. These findings are analogous to our *in vitro* studies that highlight ligation of CD200R reduces pro-inflammatory cytokine production from stimulated bone marrow derived macrophages. The precise downstream signalling events following CD200R ligation are not currently known, however, Zhang *et al* utilized an *in vitro* mast cell based system to show that phosphorylation of a tyrosine residue within an NPXY signature in the cytoplasmic tail of CD200R resulted in the inhibition of downstream RAS/MAPK pathways [124].

As previously alluded to, pathways that maintain immune homeostasis and limit the amplitude of ongoing inflammation are also likely to restore it to its original resting state. It has been previously postulated that ROS produced by myeloid cells act as pro-inflammatory mediators during early inflammation, but ultimately also facilitate

resolution of inflammation by inducing apoptosis of the very cells that produce them [130;485]. Similarly, the transcription factor NF-κB is critical for the production of many inflammatory mediators, but also ultimately fulfils an active role in the resolution of inflammation and lung epithelial tethered TGF-β acts to prevent initial inflammation but also restores homeostasis [456;486]. We have previously shown that CD200 KO mice display prolonged illness and pulmonary cellularity in an influenza infection model, highlighting a potential role for CD200-CD200R in tempering innate immunity and shaping the resolution of acute inflammation [458]. In addition, the increase in CD200R expression on myeloid populations during influenza induced lung inflammation might increase the sensitivity of macrophages to CD200 mediated suppression, as has been shown on basophils in alternative viral infection models [137]. This led us to the hypothesis; in an attempt to temper innate immunity and to initiate resolution of influenza induced inflammation, the lung inadvertently imposes a state of macrophage hypo-responsiveness that renders the host susceptible to secondary bacterial infection. To support this hypothesis, we show that mice lacking CD200R are able to limit *S. pneumoniae* infection and prevent the development of a lethal bacteraemia at all time points post influenza infection. These data suggest a novel mechanism explaining the increase in susceptibility to opportunistic bacterial pathogens observed shortly after influenza viral infections. However, there remain several caveats that might also explain the enhanced resistance to secondary bacterial pneumonia observed in CD200R KO mice. Our data illustrates that CD200R KO mice limit influenza viral replication and contain lower total cell and inflammatory cytokine levels in the airway at 3 and 7 days post influenza infection relative to WT controls. Could this reduction in viral titres and attenuated inflammatory response result in less pathology, such as epithelial damage, in CD200R KO mice, thus accounting for the resistance to secondary *S. pneumoniae* infection? Could the lack of CD200R expression on other myeloid cells, such as neutrophils and monocytes, alter their phenotype or function that could also impact on resistance to *S. pneumoniae* infection? Our investigations revealed no differences in TLR or MARCO expression levels between CD200R KO and wild type mice. Another potential caveat surrounds the enhanced NK cell responses observed in response to influenza virus infection in CD200R KO mice. As discussed in Chapter 3, the role NK cells play in bacterial infections remains controversial but must also be considered in this model system.

An additional observation that warrants a brief discussion was the similar titres of *S. pneumoniae* recovered from the nasopharyngeal compartment of wild type and CD200R KO mice in both the *S. pneumoniae* single infection model and the influenza secondary infection model. This observation adds validity to our suggestion of tissue-specific regulatory mechanisms that determine innate responsiveness and function. This distinction in immune responsiveness between the lower and upper respiratory tract is discussed further in Chapter 6.

### 5.3.4. CD200 expressing cells and alveolar macrophage anti-bacterial functionality

We postulate that luminal expression of CD200 on epithelial cells plays an important role in myeloid homeostasis and its potential role in mediating resolution of inflammation once the TLR burden has been reduced sufficiently. However, the concept that CD200 expressing leukocytes can also regulate myeloid immune responsiveness is also an intriguing possibility. The very cells activated by innate immunity may actually in turn mediate myeloid immune regulation and suppression. The potential of T cells to temper initial innate inflammatory responses has been reported previously although the precise mechanism involved remains elusive [487]. More recently Guarda *et al* describe a novel mechanism of macrophage inflammasome inhibition by effector and memory T cells that suppress potentially damaging inflammation yet leave primary innate responses intact [488].

As discussed previously, efferocytotic uptake of apoptotic cells by macrophages leads to a reduced microbicidal state that prevents long term tissue damage by chronic inflammation, but may also inadvertently reduce responsiveness to a subsequent infection through the production of prostaglandin E2 ($PGE_2$) and inhibition of NADPH oxidase [489;490]. However, in addition we now postulate that clearance of apoptotic inflammatory cells, during the resolution of influenza infection, mediates alveolar macrophage immune suppression. Phagocytosis can be inflammatory if the ingested particle is pathogenic; however the ingestion of apoptotic host cells is not, a mechanism critical in distinguishing between harmless and harmful antigens. CD200 is a p53 gene target and is up-regulated on apoptotic cells, thereby reducing the inflammatory response to self antigen [491]. This begs the question; does CD200, up regulated on apoptotic leukocytes, interact with elevated CD200R, highly expressed on alveolar macrophage after influenza infection, affect their bacterial phagocytic capacity or intracellular killing mechanisms? Unfortunately our *in vitro* attempts to produce data to support this concept were unsuccessful;

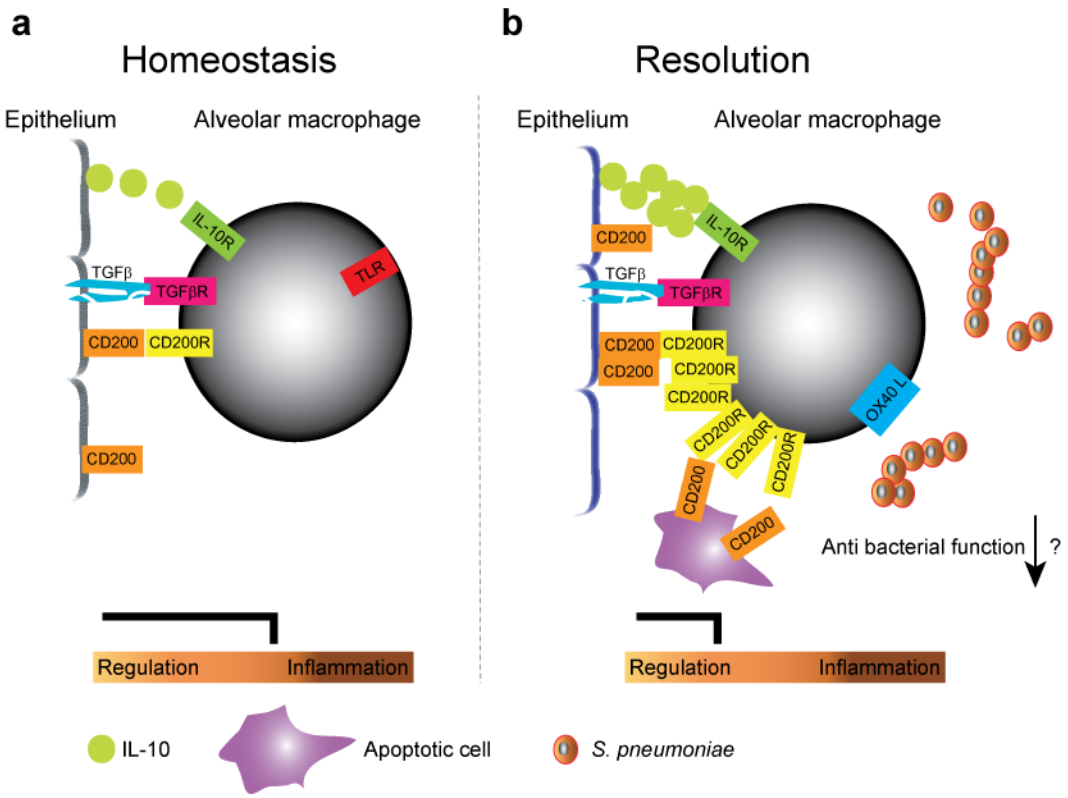
however this may be due to the existence of additional *in vivo* soluble or cognate inhibitory signals that in combination with CD200R ligation detrimentally affect alveolar macrophage anti-bacterial function. Furthermore, there may exist unknown ligands for CD200R? As surfactant protein A and D have been shown to induce inhibitory effects via the ligation of SIRP-α [492].

There are a growing number of mechanisms, such as terminating lymphocyte survival, induction of programmed cell death by apoptosis and the reprogramming of pro-inflammatory monocyte macrophages toward alternative phenotypes that continue to add complexity to how innate and adaptive immune systems reciprocally regulate one another [493;494]. These and many more questions remain unanswered and further explanation is needed in order to further understand the role inhibitory paired receptors play in resolution of acute inflammation and their role in secondary bacterial pneumonias.

## 5.4 Conclusion

The precise mechanisms that explain how respiratory viral infections, in particular influenza virus, result in increased susceptibility to secondary bacterial pneumonias remain elusive. In this study we shed light on a novel mechanism whereby influenza virus causes an overshoot in the expression of an innate immune negative regulator, CD200R, which essentially raises the level above which subsequent bacteria are recognised and inadvertently imposes a state of macrophage hypo-responsiveness that renders the host susceptible to secondary bacterial infection. If we remove such pathways using genetically modified mice then pathology to influenza and secondary bacterial infection is reduced. Furthermore, this study highlights an important quandary regarding the use of CD200R as an immunotherapeutic target. CD200R has been proposed as a promising target for alleviating the excessive inflammation associated with autoimmune, allergic and excessive respiratory inflammatory diseases. A complete understanding of the potential therapeutic risk, with regards to bacterial susceptibility, is needed when developing such immunotherapeutic intervention strategies [140;141;143;144]. Figure 5.20 schematically represents our novel mechanism of how influenza virus increases susceptibility to secondary bacterial pneumonias.

**Figure 5.20 Influenza induced macrophage hypo-responsiveness**




During homeostasis alveolar macrophage are maintained in a state of immune homeostasis through interaction of CD200, expressed on respiratory epithelium, and CD200R, expressed on alveolar macrophages (a). In an attempt to temper and resolve innate inflammation CD200R levels are increased and interact with CD200 expressed by airway lymphocytes, monocytes and apoptotic cells. The presence of yet unidentified CD200R ligands might also be elevated during inflammation resolution (b).

# Chapter 6

## 6.0 Proteomic landscape and the bacterial microbiota

The preliminary data sets in this results chapter were produced from proof of principal experiments. All work contained herein, unless stated in the acknowledgement section, was carried out by myself, under the supervision of Dr Robin Wait and Dr Markus Hilty. This results chapter is divided into two sections;

1. Proteomic analysis of murine airway anti-microbial proteins.
2. Characterisation of the respiratory tract commensal flora during homeostasis (naïve mice) and an ongoing influenza virus infection.

## 6.1 Introduction

### 6.1.1 Antimicrobial peptides and their role in respiratory tract immunity

The respiratory mucosa is constantly exposed to a myriad of potential pathogens, pollutants and inflammatory stimuli. However, these exogenous particles rarely cause problems in a healthy lung owing to the existence of an array of innate defence proteins, commonly referred to as antimicrobial peptides (AMPs), present in the mucous that cover the respiratory epithelium. A detailed classification and description of AMPs mechanisms of action can be found in section 1. 2. AMPs are constitutively produced by respiratory epithelium and resident alveolar macrophages and provide early, broad spectrum, protection against invading pathogens [5]. In vertebrates, basal expression of AMPs by gut epithelial cells also plays a critical role in dictating the density and composition of the resident gut commensal flora [495]. Although numerous AMPs are constitutively expressed, they are also regulated by inflammatory cytokines produced in response to MAMP recognition by PRRs. The best known example of this type of regulation is the induction of β-defensins and lipocalin-2, an iron sequestering AMP, in response to IL-1β stimulation of respiratory epithelium cells [496;497]. Furthermore, IL-17A and IL-22, produced by $T_h17$ T cells, can induce the expression of human β-defensin 2 (HBD2), HBD3 and the chemokine CCL20 in bronchial epithelium. IL-22 and IL-17 can also synergistically induce lipocalin-2

expression in mouse tracheal epithelium cells, which is indispensable for antimicrobial activity against *Klebsiella pneumoniae* [498-501]. AMP expression is also altered during infection. Chong *et al* demonstrate that the expression of β-defensin 3 and 4 is enhanced in the lungs, trachea and sinonasal mucosa of mice infected with influenza virus [502]. The respiratory fungal pathogen, *Aspergillus fumigates,* also augments HBD2 and HBD9 gene expression in A549 pneumocyte cells [503]. Nonetheless, it is difficult to ascertain from *in vivo* experiments whether a pathogen, such as influenza virus, itself directly augments AMPs expression or indirectly via the action of ensuing inflammatory cytokines. This quandary is highlighted in a recent study that shows LPS indirectly augments β-defensin transcription levels in A549 pulmonary epithelial cells via the production of IL-β and TNF-α by mononuclear phagocytes [504]. Furthermore, AMPs themselves can regulate the expression of other AMPs. Zhang *et al* highlights the role of human cathelicidin LL37 and its synergistic interaction with IL-1β to increase the production of IL-8, IL-10 and the chemokine CC-chemokine ligand 2 (CCL2) as well as increasing local concentrations of α-defensin [505].

AMPs also play a pivotal role in providing feedback regulation that can limit and resolve inflammation. Surfactant protein A (SP-A) inhibits downstream signalling via Akt from TLR2 and TLR4, which decreases pro-inflammatory cytokine production from stimulated human airway macrophages [506]. In a similar fashion, α-defensins can block the secretion of IL-1β by LPS activated monocytes. Furthermore, SP-A, along with a related family member SP-D, reduces the activity and phagocytic capacity of alveolar macrophages by binding to and activating the signal inhibitory regulatory protein alpha (SIRP-α), an inhibitory receptor highly expressed on these cells [492].

There is growing evidence that suggests that AMPs play a complex role in host defence of the airway providing a self contained response to extracellular microbial stimuli that encompasses pathogen recognition, inflammation, pathogen clearance and resolution of inflammation. However, there remains a lack of knowledge surrounding how infection history affects AMP expression and whether the loss of respiratory epithelium and other cells responsible for AMP production, observed during respiratory viral infections, results in a distorted AMP repertoire that could have important consequences for the host's anti microbial competence. AMPs and their mechanism of action have attracted growing interest in light of increasing numbers of multiple drug resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA), and the decline in the development of new

antibacterial therapies [507-509]. Research into the modulation of AMP gene and protein expression in animal models, as well as increased investigation into peptide-based therapeutics could provide novel therapeutics for the treatment and prevention of respiratory infections.

### 6.1.2 The respiratory tract commensal flora

Much of what is known regarding the diversity and function of the commensal microbiota of the respiratory tract is based on data derived from traditional culture-based techniques [510]. However, these methods are limited and only detect a small fraction of the bacterial diversity therefore providing only a selective and invariably biased analysis on bacterial diversity. Consequently, little is known about the specific composition and dynamics of the respiratory commensal flora in disease and health. Although the mucosal surfaces of the upper respiratory tract harbour a complex mixture of microbial species, the relative paucity of cultivated bacteria and the necessity for efficient gaseous exchange has led to the common assumption that the lower respiratory tract is devoid of a complex microbial biota. Molecular methods of bacterial detection have the potential to offer more detailed insights into the complex microbial communities present in the entire respiratory tract. 16S rRNA sequencing has been used to characterize the microflora of the human oral cavity, oesophagus, colon and stool, revealing many novel uncultivable bacterial species [511-515]. However, despite the routine use of molecular diagnostic testing to detect causative pathogens of community acquired pneumonia and the molecular identification of bacteria present in the airways of patients with cystic fibrosis, COPD and ventilated associated pneumonia, these methods have not been utilised to characterise the resident microflora of the upper and lower respiratory tract of mice, or humans, during homeostasis and infection with influenza virus [516-519].

As is observed in the gut, the density and complexity of the respiratory tract commensal flora provides an important layer of defense against invasion by pathogenic microbes. However, the challenge for the innate immune system of the host is to discriminate between inactivating pathogens whilst tolerating colonization by commensal organisms. Calibrating this immune response to ‘commensal’ microbes, however, is complex, as microbial virulence is often context dependent, being influenced by the homeostatic immune status of the host and the presence of co-inhabiting micro-organisms [520;521]. *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Haemophilus influenzae* commonly reside

asymptomatically in the nasopharyngeal cavity of healthy individuals [522;523], however despite growing understanding and identification of pathogen virulence factors that are responsible for colonization and invasive disease, the mechanisms behind how the host's immune response tolerates the presence of 'commensal' microbes and how the *status quo* is altered that allows 'commensal' pathogens to outgrow and cause disease remains to be elucidated.

### 6.1.3 Proteomic analysis of airway anti-microbial proteins aims and hypothesis

As discussed above, AMPs are abundant at the respiratory mucosal surface and play an essential role in antibacterial immunity and immune modulation. Epithelial cells and airway macrophages contribute to this diverse array of AMPs and some even suppress airway macrophage function. It therefore follows that any alteration of epithelia or macrophages will impact on the anti-microbial protein repertoire and subsequent control of secondary bacterial species. We therefore initiated a global screen of BAL specimens from naive and influenza infected mice to ask:

1. Can qualitative alterations in antimicrobial peptide expression by identified using mass spectrometry to determine the proteomic landscape?
2. Does influenza infection alter the repertoire of AMPs expressed in the airway mucosal surface?

### 6.1.4 Characterisation of the commensal landscape in naive and influenza infected mice aims and hypothesis

Many bacterial species cultured from patients with severe influenza induced pneumonia are present in the nasopharynx of healthy individuals, therefore suggesting influenza infection provides an opportunity for certain bacterial species to gain access to and colonise the lower respiratory tract [523]. Though the precise composition of respiratory microbiota may alter between species, the innate pathways that limit their load and location, during health and disease, are likely to be conserved. Therefore, we wished to investigate two hypotheses;

1. The level of innate immune cell responsiveness determines the composition of respiratory commensal flora during homeostasis.
2. Infection with influenza virus alters the diversity of resident bacterial species in both the upper and lower respiratory tract.

To test these hypotheses we will determine the bacterial species present in the nasopharynx, distal airways and lungs of naive and influenza infected mice using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene identification. We will also examine the role CD200R plays in influencing the commensal bacterial landscape during homeostasis and during an ongoing influenza virus infection.

## 6.2 Results

### 6.2.1 A secondary bacterial infection significantly reduces total airway protein compared to mice infected with influenza virus alone

Before analysing the precise protein constituents of bronchoalveolar lavage fluid (BALF), we first investigated the total protein concentration found in the airways of mice infected with each pathogen independently and in combination (see section 2.11.1 for method details). At day six of a single influenza infection there was more than six times the total protein present in the BALF compared to that of naive uninfected controls (Figure 6.1 a). However, the intranasal administration of Group B streptococcus (GBS) on day three of an influenza infection significantly reduced the amount of total protein present in the BALF 3 days after GBS infection compared to mice infected with influenza alone. GBS infection alone resulted in a small, yet significant, increase in the total protein constituent of BALF compared to naive controls. However, this was significantly less than that measured in mice infected with both influenza and GBS (Figure 6.1 a).

After quantifying the differences in BALF total protein content in mice infected with both influenza and GBS compared to those infected with GBS alone, we next investigated the qualitative differences in individual protein constituents of each infection group. Total airway protein from each infection group was separated by molecular weight using gel electrophoresis (Figure 6.1 b) and after in-gel protein band trypsin digestion the resulting peptides were subjected to liquid chromatography and electrospray mass spectrometry (LC-ESI-MS/MS), as described in section 2.11.3. An example of how this technique can be used to determine the presence of a protein in a tissue sample is demonstrated in Figure 6.2 a & b. Utilising this mass spectrometry technique and subsequent downstream bio-informatic processing, Table 6.1 compares, between all infection groups, the total readout of proteins that

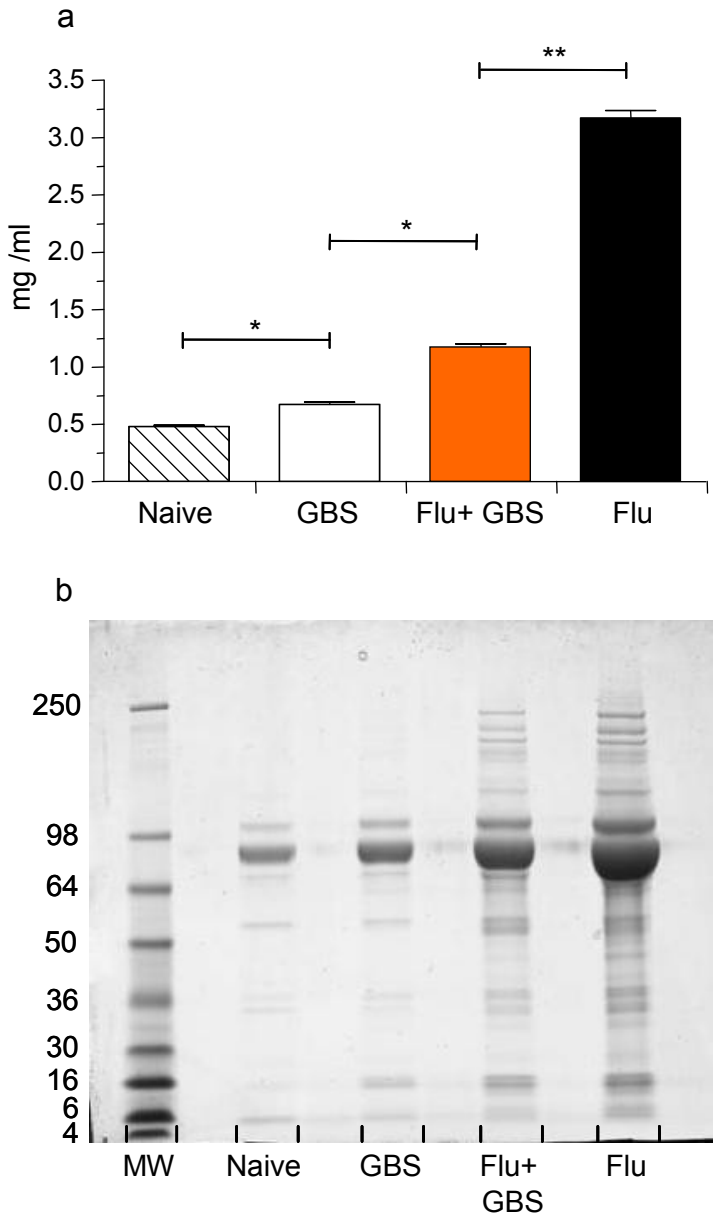
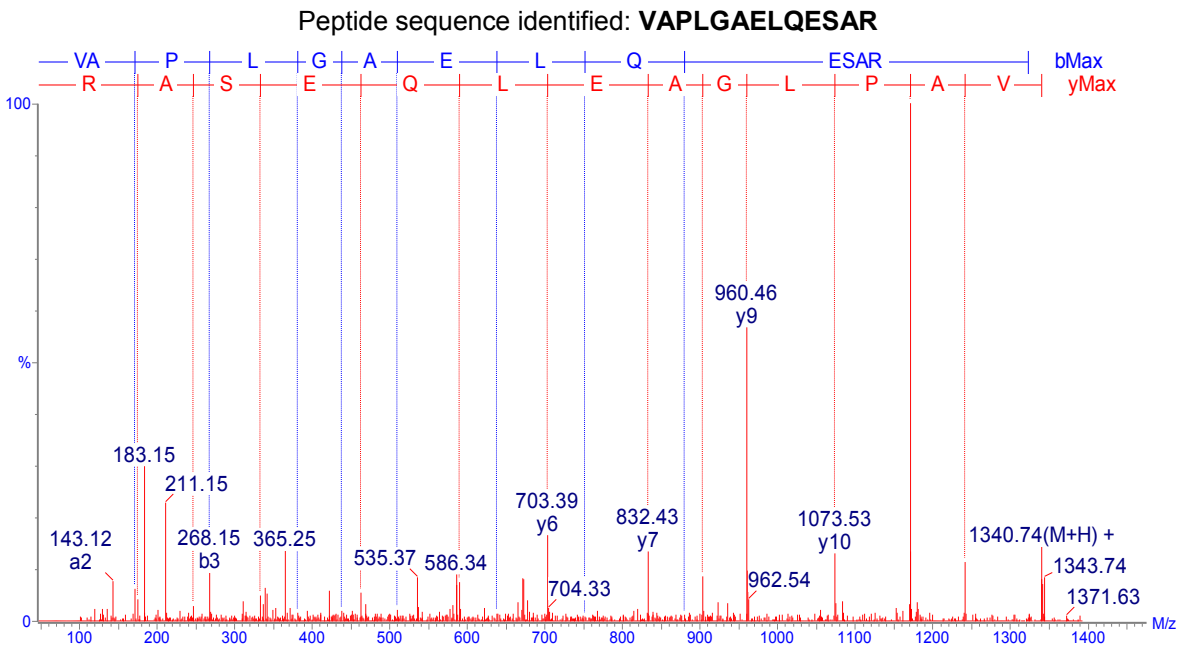



**Figure 6.1 Influenza and GBS co-infection reduces total protein concentration in BALF.** BALB/c mice (n=4) were infected intranasally (i.n.) with 50 HA HK/X31 influenza A virus (Flu) or PBS control. Three days after influenza infection mice were challenged i.n. with 5 x $10^6$ cfu of Group B streptococcus (GBS) or PBS control. All mice were harvested 3 days post GBS or PBS infection. BAL was performed on each mouse and subsequently pooled within each group. BAL supernatant was purified, by passage through a 0.2 µm sieve and centrifugation, to remove remaining cells and cell debris. Total protein concentration (a) was determined using Pierce BCA Protein assay kit. Equal volumes of BALF were prepared, as described in section 2.11, and loaded onto a 4-12% graded Bis-Tris SDS Page gel (b). Resolved protein bands were stained with Coomassie Blue for visual inspection and proteomic analysis. Data are presented as the mean ± SEM of 4 mice per group. Significance was determined by using Mann Whitney T test and Kruskal Wallis ANOVA with Dunns multiple comparison post test . * = $p<0.05$ ** = $p<0.01$

a




b

SwissProt complete amino acid sequence for Apolipoprotein A1 (Mus musculus)

```
          10         20         30         40         50         60
MKAVVLAVAL VFLTGSQAWH VWQQDEPQSQ WDKVKDFANV  YVDAVKDSGR DYVSQFESSS
          70         80         90        100        110        120
LGQQLNLNLL ENWDTLGSTV  SQLQERLGPL TRDFWDNLEK ETDWVRQEMN KDLEEVKQKV
         130        140        150        160        170        180
QPYLDEFQKK WKEDVELYRQ KVAPLGAELQ ESARQKLQEL QGRLSPVAEE FRDRMRTHVD
         190        200        210        220        230        240
SLRTQLAPHS EQMRESLAQR LAELKSNPTL NEYHTRAKTH LKTLGEKARP ALEDLRHSLM
         250        260
PMLETLKTKA QSVIDKASET LTAQ
```

**Figure 6.2 Mass spectra of an Apolipoprotein A1 peptide fragment and the coverage attained of the complete amino acid sequence.** BALB/c mice (n=4) were infected intranasally (i.n.) with 50 HA HK/X31 influenza A virus or PBS control. Three days after influenza infection mice were challenged with 5 x $10^6$ cfu GBS i.n or PBS control. All mice were harvested 3 days post GBS or PBS infection. Bronchoalveolar lavage (BAL) was performed on each mouse and subsequently pooled within each group. BAL supernatant was purified, by passage through a 0.2µm sieve and centrifugation, to remove cells and cell debris. Protein bands were subjected to in gel trypsin digestion. Peptides were subsequently analysed using liquid chromatography coupled to tandem electrospray mass spectrometry (LC-ESI-MS/MS). Spectra were recorded using a Q-Tof spectrometer interfaced to a Micromass CapLC capillary chromatograph. Proteins were subsequently identified by correlation of uninterpreted spectra to entries in SwissProt/TrEMBL, using ProteinLynx Global Server (further methodology can be found in section 2.11). Panels a & b illustrate a representative mass spectrum from Apolipoprotein A1 recovered from a pooled BAL sample and its corresponding alignment to the complete amino acid sequence respectively.

were considered significantly valid. A significant protein sequence was identified when two or more peptide sequences, from the same excised protein band, independently matched a predicted protein sequence, the peptide sequence score was greater than 55 ($p<0.05$) and that manual interpretation of the peptide spectra confirmed agreement between spectra and protein sequence. Visual inspection of the significant protein constituents of BALF highlighted a large abundance of high molecular weight (250-298 kDa) proteins produced in response to single influenza infected and co-infected mice (Table 6.1). Murinoglobulins (IgG and IgA), ceruloplasmin, transferrin, serine protease inhibitors, serum albumin and other plasma proteins were also abundantly represented in BALF from mice infected with influenza virus. Interestingly, vitamin D binding protein was only detected in mice co-infected with influenza and GBS whilst being undetectable in mice infected with either influenza virus or GBS alone.

Anti-microbial proteins that are involved in innate defence and immunomudulation range in size from 20 kDa to approximately 3 kDa, with the exception of 80 kDa lactoferrin [5]. Comparison of significant proteins detected within this size range highlighted a number of differentially expressed proteins in mice previously infected with influenza and then infected with GBS compared to mice infected with GBS alone. Table 6.2 expands this interesting region to reveal several anti-bacterial proteins expressed in response to single GBS infection that are not present in mice infected with influenza and GBS. These proteins include: Chitinase 3 like protein, lysozyme C type M and surfactant protein B. Conversely, a large number of proteins of low molecular weight were only present in mice previously infected with influenza. These include several complement precursor components, anti oxidant proteins such as peroxiredoxin 2 and apolipoprotein A-1, a constituent of blood serum. The presence of lung carbonyl reductase (NADPH) was detected in both infection groups but not in the naïve uninfected group (Table 6.2). This preliminary screen has validated the use of this technique on BALF and demonstrates further detailed analysis and quantification is warranted.

### 6.2.2 Influenza virus infection increases the nasopharyngeal commensal burden and allows bacteria access to the distal airways

In recent years there has been an explosion of interest into the intestinal microbiota and its phylogenetic composition [524]. Many interesting results have shed light on the mechanisms by which the immune system tolerates the presence of certain microorganisms and how the microorganisms themselves play a vital role in the

## Table 6.1 Total unique BALF protein expression

| | Naive | | | GBS alone | | | Flu+ GBS | | | Flu alone | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MW | Entry Name | Name | Classification | Entry Name | Name | Classification | Entry Name | Name | Classification | Entry Name | Name | Classification |
| 250-98 | | | | gels_Mouse | Gelsolin (Precursor). Actin depolymerising factor | | MUG1_Mouse | Murinoglobulin 1 (Precursor) | Plasma Protein | MUG1_Mouse | Murinoglobulin 1 (Precursor) | Plasma Protein |
| | | | | | | | cfah_Mouse | Complement factor H (Precursor) | Complement | Unidentified | | |
| | | | | | | | A2MG_Mouse | Alpha 2 Macroglobulin 1 (Precursor) | Plasma Protein | A2MG_Mouse | Alpha 2 Macroglobulin 1 (Precursor) | Plasma Protein |
| | | | | | | | Q6P5C8_Mouse | Ceruloplasmin | | Q6P5C8_Mouse | Ceruloplasmin | |
| | | | | | | | SPA3K-Mouse | Serine protease inhibitor A3K (Precursor) | Plasma Protein | SPA3K-Mouse | Serine protease inhibitor A3K (Precursor) | Plasma Protein |
| | | | | | | | SPA3K-Mouse | Serine protease inhibitor A3K (Precursor) | Plasma Protein | SPA3K-Mouse | Serine protease inhibitor A3K (Precursor) | Plasma Protein |
| | | | | | | | ITIH3_Mouse | Inter alpha trypsin inhibitor heavy chain H3 (Precursor) | Serum Protein | ITIH3_Mouse | Inter-alpha-trypsin inhibitor heavy chain H3 [Precursor] | Plasma Protein |
| | | | | | | | | | | COTR_Mouse | Serine protease inhibitor A3K (Precursor) | |
| | | | | | | | SPA3C_Mouse | Serine protease inhibitor A3C (Precursor) | | SPA3K-Mouse | Serine protease inhibitor A3K (Precursor) | Plasma Protein |
| | | | | | | | CO3_Mouse | Complement C3 (Precursor) | Complement | CO4_Mouse | Comlement C4 precursor | Complement |
| | | | | | | | | | | IC1_Mouse | Plasma protease C1 Inhibitor (Precursor) | Complement |
| 98-64 | Albumin / COTR_Mouse | | Serum Protein | SPA3C_Mouse | Serine proteases inhibitor A3C (Precursor) | | | | | | | |
| | | | | A1AT2_Mouse | Alpha 1 antitrypsin 1-2 (precursor) a.k.a Serine protease inhibitor 1-2 | | A1T2_Mouse | Alpha 1 antitrypsin 1-2 precursor. (Serine protease inhibitor 1-2) | | | | |
| 64-50 | | | | ch3l1_Mouse | Chitinase 3 like protein 1 (Precursor) | Remodelling and Defense | VTDB_Mouse | Vitamin D Binding Protein | Serum Protein | A1AT2_Mouse | Alpha 1 antitrypsin 1-2 (precursor). Serine protease inhibitor 1-2 | |
| | | | | | | | A2HS_Mouse | Alpha 2 HS Glycoprotein precursor a.k.a Fetuin A Countertrypin | | A2HS_Mouse | Alpha 2 HS Glycoprotein precursor a.k.a Fetuin A Countertrypin | |
| | | | | | | | A2HS_Mouse | Alpha 2 HS Glycoprotein precursor a.k.a Fetuin A Countertrypin | | HPT_Mouse | Haptoglobin (Precursor) | Plasma Protein |
| | | | | | | | | | | CO3_Mouse | Complement C3 (Precursor) | Complement |
| 50-36 | | | | | | | Q80XP1_Mouse | Complement Component 3 | Complement | Q80XP1_Mouse | Complement Component 3 | Complement |
| | | | | | | | HPT_Mouse | Haptoglobin (Precursor) | Plasma Protein | CO3_Mouse | Complement C3 (Precursor) | Complement |
| | | | | | | | HPT_Mouse | Haptoglobin (Precursor) | Plasma Protein | A2MG_Mouse | Alpha 2 macroglobulin (Precursor) | Plasma Protein |
| | | | | | | | CO3_Mouse | Complement C3 (Precursor) | Complement | | | |
| | | | | | | | | Unidentified | | SAMP_Mouse | Serum Amyloid P-Component (Precursor) | |
| 36-30 | Unidentified | | | INMT_Mouse | Indolethylamine N-methyltransferase | | CBR2_Mouse | Lung carbonyl reductase [NADPH] | | INMT_mouse | Indolethylamine N-methyltransferase | |
| | | | | CBR2_Mouse | Lung carbonyl reductase [NADPH] | | CO3_Mouse | Complement C3 (Precursor) | Complement | CO3_Mouse | Complement C3 (Precursor) | Complement |
| | | | | | | | APOA1_Mouse | Apolipoprotein A-1 (Precursor) | Serum Protein | | | |
| | | | | | | | | | | FRIL1_Mouse | Ferritin light chain 1 | Plasma Protein |
| | | | | | | | PRDX2_Mouse | Peroxiredoxin 2 | Anti Oxidant | PPIA_Mouse | Peptidyl-prolyl cis-trans isomerase A | |
| 16 | | Albumin | Serum Protein | HBB1_Mouse | Haemoglobin subunit beta-1 | Serum Protein | Hbb1_Mouse | Haemoglobin subunit beta 1 | Serum Protein | Hbb1_Mouse | Haemoglobin subunit beta 1 | Serum Protein |
| | | | | Lyscm_Mouse | Lysozyme C type M (Precursor) | Anti Bacterial | hbb2_Mouse | Haemoglobin subunit beta 2 | Serum Protein | HBA_Mouse | Hemoglobin subunit alpha | Serum Protein |
| | | | | | | | | | | H2A2_Mouse | | |
| 6-4 | Q5SX22_Mouse | Ubiquitin B | | gi22296601 | Surfactant Protein B | | Q5SX22_Mouse | Ubiquitin B | | | | |
| | | | | Q5SX22_Mouse | Ubiquitin B | | | | | APOA2_Mouse | Apolipoprotein A 2 (Precursor) | Serum Protein |
| | uter_Mouse | Uteroglobin (Precursor). Clara cell phospholipid binding protein | | uter_Mouse | Uteroglobin (Precursor). Clara cell phospholipid binding protein | | | | | uter_Mouse | Uteroglobin (Precursor). Clara cell phospholipid binding protein | |

A comparative table illustrating the unique proteins found within each group. Mass spectrometry and bioinformatics were utilised to determine the proteomic composition of airway exudate during each infection. (Further methodology can be found in section 2.11).

**Table 6.2 Unique anti-bacterial protein expression**

| GBS alone | | | | Flu+ GBS | | | |
|---|---|---|---|---|---|---|---|
| MW | Entry Name | Name | Classification | MW | Entry Name | Name | Classification |
| 64-50 | ch3l1_Mouse | Chitinase 3 like protein 1 (Precursor) | Remodelling & defense | 64-50 | VTDB_Mouse | Vitamin D Binding Protein | Serum Protein |
| | | | | 64-50 | A2HS_Mouse | Alpha 2 HS Glycoprotein precursor a.k.a Fetuin A Countertrypin | |
| | | | | 64-50 | A2HS_Mouse | Alpha 2 HS Glycoprotein precursor a.k.a Fetuin A Countertrypin | |
| | | | | 50 | Q80XP1_Mouse | Compliment Component 3 | Compliment |
| | | | | 50 | HPT_Mouse | Haptoglobin (Precursor) | Plasma Protein |
| | | | | 50-36 | HPT_Mouse | Haptoglobin (Precursor) | Plasma Protein |
| | | | | 50-36 | CO3_Mouse | Compliment C3 (Precursor) | Compliment |
| 36 | CBR2_Mouse | Lung carbonyl reductase [NADPH] | | 36 | CBR2_Mouse | Lung carbonyl reductase [NADPH] | |
| | | | | 36 | CO3_Mouse | Compliment C3 (Precursor) | Compliment |
| | | | | 36-30 | APOA1_Mouse | Apolipoprotein A-1 (Precursor) | Serum Protein |
| | | | | 36-30 | PRDX2_Mouse | Peroxiredoxin 2 | Anti Oxidant |
| 16 | Lyscm_Mouse | Lysozyme C type M (Precursor) | Anti Bacterial | 16 | hbb2_Mouse | Haemoglobin subunit beta 2 | Serum Protein |
| 6-4 | gi22296601 | Surfactant Protein B | | | | | |

**Peptide/protein present in:**

Single GBS infection only | Single Flu infection only | Both infections

**Airway expression of anti-microbial proteins is altered by influenza virus infection.** BALB/c mice (n=4) were infected intranasally (i.n.) with 50HA HK/X31 influenza A virus (Flu) or PBS control. Three days after influenza infection mice were challenged i.n. with 5 x $10^6$ cfu GBS or PBS control. All mice were harvested 3 days post GBS or PBS infection. Bronchoalveolar lavage (BAL) was performed on each mouse and subsequently pooled within each group. BAL supernatant was purified, by passage through a 0.2µm sieve and centrifugation, to remove cells and cell debris. Protein bands of interest were subjected to in gel trypsin digestion. Peptides were subsequently analysed using liquid chromatography coupled to tandem electrospray mass spectrometry (LC-ESI-MS/MS). Spectra were recorded using a Q-Tof spectrometer interfaced to a Micromass CapLC capillary chromatograph. Proteins were subsequently identified by correlation of uninterpreted peptide spectra to entries in SwissProt/TrEMBL, using ProteinLynx Global Server (further methodology can be found in section 2.11). Table 6.2 identifies proteins uniquely expressed in each infection group.

health and function of the intestinal tract [524]. However, the precise mechanisms that determine which micro-organisms are allowed to colonise a mucosal surface and how the immune status and health of the host determines the specific diversity and density of the microbiota has received less attention. The upper respiratory tract (defined as proximal to the larynx), similar to the intestine, has a complex milieu of commensal microflora [525;526]. In contrast, the lower respiratory tract (defined as distal from the larynx) is assumed to have no resident commensal flora, due to its vital physiological function of efficiently exchanging gases (Figure 6.3 a & b). We therefore investigated the total bacterial load present in the nasopharyngeal cavity and distal airways in naive mice and at specific time points after influenza virus infection. We observed that bacterial colonies can be cultured from the nasopharyngeal cavity of naïve mice but not from the distal airways (Figure 6.3 c & d day 0). Nevertheless, mice infected with influenza virus alone (note; no exogenous bacteria were administered) demonstrated a significant increase in total bacterial load present in the nasopharyngeal compartment between 2 and 6 days after influenza infection (Figure 6.3 c). The total bacterial load returned to naïve levels by day 14 post influenza infection. In contrast, despite there being no culturable bacteria in the airways of a naïve mice, bacteria were cultured from the distal airway 2 days post influenza and remained, in 50 % of the mice, until day 14 post influenza (Figure 6.3 d).

### 6.2.3 Qualitative comparison of tissue specific bacterial species in naïve wild type and CD200R KO mice

Aerobic culture techniques, although quantitative, can not determine the presence of unculturable bacterial species, the dominant bacterial species present or the range of bacterial diversity within the tissue of interest. We therefore wanted to use a culture independent method that could provide a more comprehensive view of bacterial diversity and could be utilised to evaluate complex bacterial community dynamics, particularly during an ongoing influenza infection. Utilising primers to highly conserved regions of the 16S rRNA we amplified the intervening variable region and discriminated between closely related bacterial species. After demonstrating that the total bacterial load in both the nasopharyngeal and distal airway compartments is augmented during an influenza infection, we next identified the bacterial species present in these tissue compartments during homeostasis (naïve mice) and an ongoing influenza infection. We utilised two techniques, described in detail in section 2.12, to illustrate the qualitative differences in bacterial species recovered from three

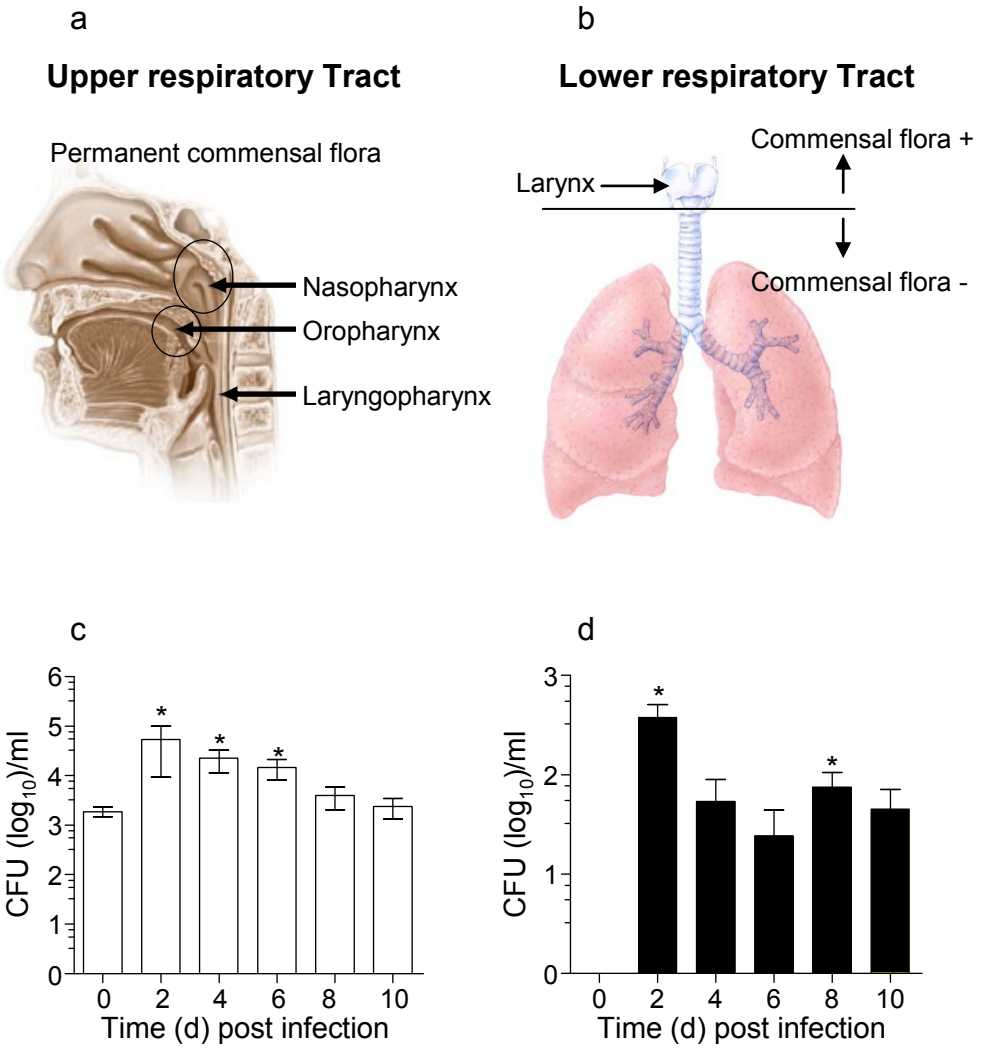
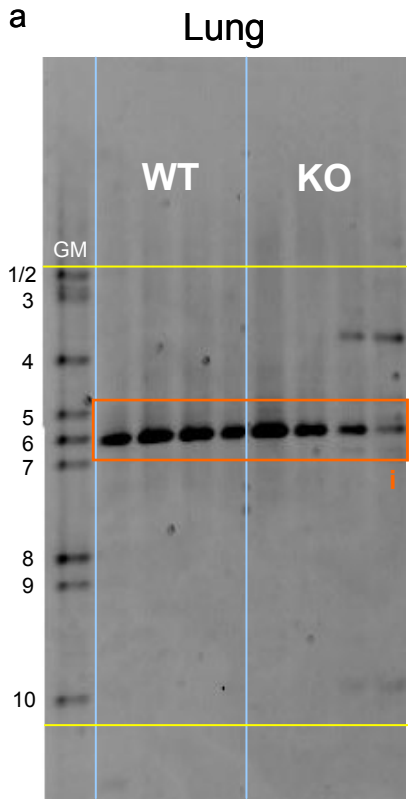



**Figure 6.3 Influenza virus infection increases nasopharyngeal bacterial burden and results in the appearance of unidentified bacteria in the distal airways.** Naïve C57BL/6 wild type mice (n=5) were infected intranasally (i.n) with 50 HA HK/X31 influenza virus on day 0. On specified days post influenza infection bronchoalveolar lavage (BAL) and nasal wash was preformed. Panels a & b highlight the sites of the upper and lower respiratory tract, respectively, that have or do not have permanent resident commensal flora. Total bacterial titres from the nasopharyngeal cavity (c) and airways (d) were calculated by serially diluting the respective samples and plated on non selective Columbia blood agar overnight at 37°C. Data are presented as the mean ± SEM of 5 mice per group. $p<0.05$ was taken to be significant. * = $p<0.05$ ** = $p<0.01$

Panels a & b adapted from http://en.wikibooks.org/wiki/Human_Physiology/The_respiratory_system Dated 04/06/2009

distinct compartments of the respiratory tract. We also compared the differences in commensal diversity between wild type C57BL/6 (WT) and CD200R (KO) mice. The specificity of this technique was confirmed with the detection of *S. pneumoniae* in the lung tissue of mice 48 hours after *S. pneumoniae* infection (Figure 6.4 a). Using denaturing gradient gel electrophoreses (DGGE), our initial findings demonstrated the presence of multiple bacterial species (using a bacterial genus marker as a rudimentary guide) in both the distal airway and the lung tissue of naive WT and CD200R KO mice. By visually comparing the relative positions of the resulting cDNA bands on the denaturing gradient gel, which represent distinct groups of bacterial species, there was a clear distinction between both the WT and CD200R KO mice and between different respiratory tract locations (Figure 6.5 a-c). Despite variations between individual mice within each group, the reliability of the technique was validated by the presence of uniform bacterial bands observed in all individual mice within a specific group (see white boxes highlighting consistent bacterial DNA bands present across individual groups. Figure 6.5). Note that exogenous administration of *S. pneumoniae* in Figure 6.4 dramatically reduced the repertoire of commensal bacteria found in uninfected mice (Figure 6.5). This suggests a dominant bolus of bacteria can outgrow those already present.

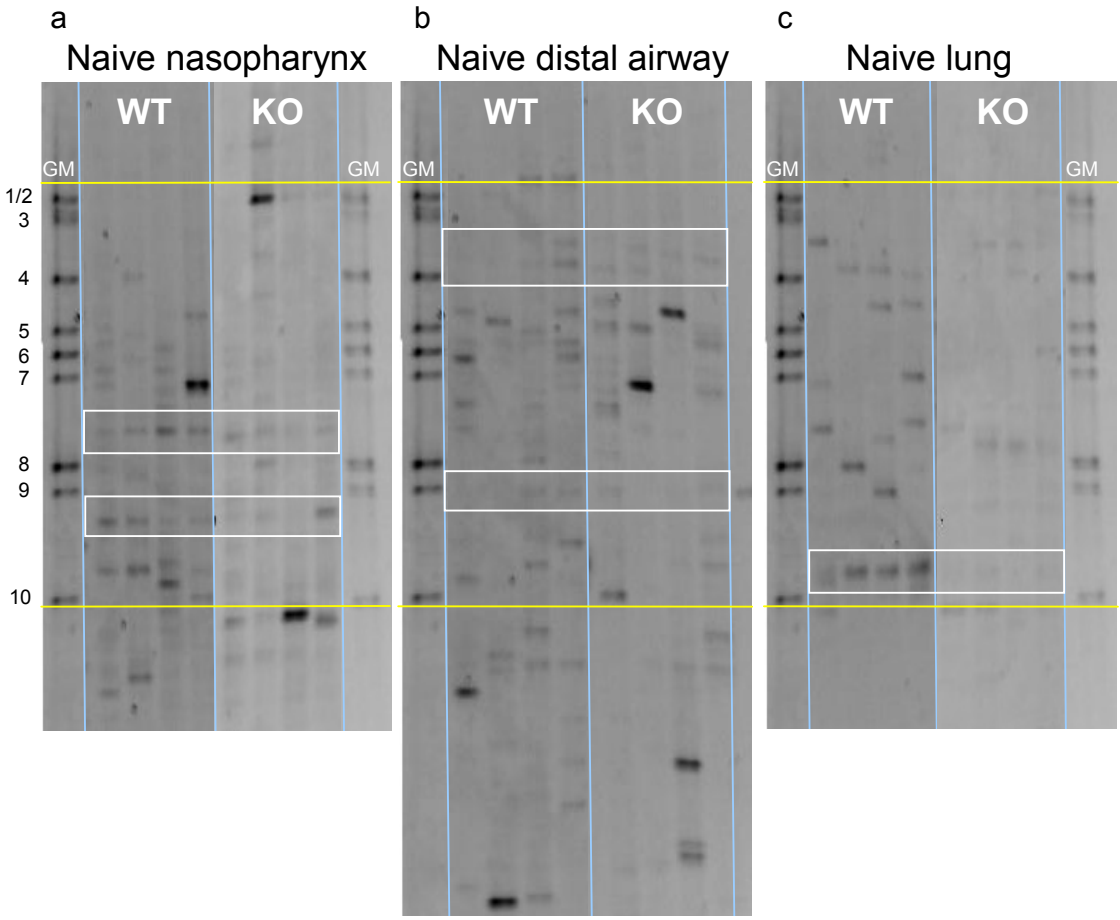
To investigate further the precise bacterial species present within each compartment, we cloned and subsequently sequenced the extracted and purified cDNA from a selection of individual mice (see section 2.12 for method details). We wanted to identify the bacterial species present in the naive distal airway of WT and CD200R KO mice before investigating whether bacterial diversity is altered during an influenza virus infection. Figure 6.6 demonstrates the presence of both *Acinetobacter sp.* and *Prevotella sp.* in the naive distal airways of both WT and CD200R KO mice. There are several other interesting bacterial species present in both the WT and CD200R KO distal airway, however *Brevundimonas sp.*, *Rhizobium sp.*, *Stenotrophomonas sp.*, *Moraxella sp.* and *Pseudomonas sp*. were only identified in the airway of naive WT mice. On comparison of the bacterial landscape in the nasopharyngeal compartment, several species were present in both WT and CD200R KO mice, including *Prevotella sp., Lachnospiraceae sp.*, *Porphyromonadaceae sp.* and *Streptococcus sp.* species (Figure 6.6 b). The presence of *Staphylococcus sp. and Alphaproteobacteria sp.* was unique to the nasopharyngeal compartment of CD200R KO mice. The results also highlighted a disparity of species present between the nasopharynx and the distal airways. *Lachnospiraceae sp.*, *Lactobacillus sp* and *Porphyromonadaceae sp.* were found exclusively in the nasopharyngeal cavity of WT




Bacterial genus marker (GM)

| | | | |
|---|---|---|---|
| **1** | *Bacteroidales sp* | **6** | *Prevotella sp. & S. pneumoniae* |
| **2** | *Staphylococcus sp* | **7** | *Prevotella sp* |
| **3** | *Megaspaera sp* | **8** | *Neisseria sp* |
| **4** | *Porphyromonas sp* | **9** | *Veillonella dispar* |
| **5** | *Porphyromonas sp* | **10** | *Veillonella sp.* |

**Figure 6.4 Specific bacterial species can be identified *ex vivo* using culture independent 16S rRNA sequencing.** Naïve C57BL/6 wild type (WT) and CD200R KO mice (n=4) were infected intranasally (i.n) with 50 HA HK/X31 influenza A virus. After three days, mice were challenged with 1 x $10^4$ colony forming units *S. pneumoniae*. Mice were culled 48 hrs post *S. pneumoniae* infection and lung tissue (a) was removed. Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. 10 µl of 16S rRNA product was then loaded onto a 30-60% denaturant DGGE gel and run at 100 v for 16 hours. A more in depth description of the protocol used can be found in section 2.12. A bacterial genus marker was used to allow comparison of band migration between gels and to quickly identify the presence of specific bacterial species (see table). i = *Streptococcus pneumoniae*

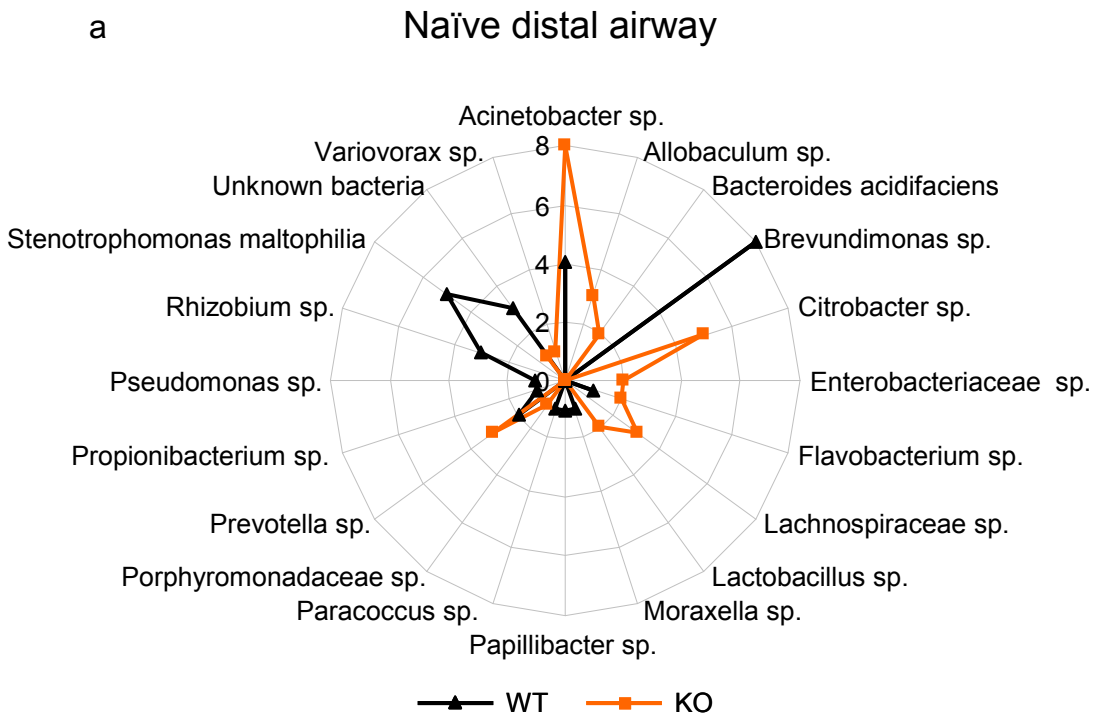



Bacterial genus marker (GM)

| | | | |
|---|---|---|---|
| **1** | *Bacteroidales sp* | **6** | *Prevotella sp. & S. pneumoniae* |
| **2** | *Staphylococcus sp* | **7** | *Prevotella sp* |
| **3** | *Megaspaera sp* | **8** | *Neisseria sp* |
| **4** | *Porphyromonas sp* | **9** | *Veillonella dispar* |
| **5** | *Porphyromonas sp* | **10** | *Veillonella sp.* |

**Figure 6.5 Qualitative comparison of tissue specific bacterial species present in naïve wild type C57BL/6 and CD200R KO mice.** Naïve wild type (WT) and CD200R KO (KO) mice (n=4) were culled and nasal wash (a), BAL (b) and lung tissue (c) were removed. Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. 10 µl of 16S rRNA product were then loaded onto a 30-60% denaturant DGGE gel and run at 100 v for 16 hours. A more in depth description of the protocol used can be found in section 2.12. A bacterial genus marker was utilised to allow comparison of band migration between gels and to quickly identify the presence of specific bacterial species (see table). White boxes highlight uniform bacterial bands observed within individual groups of mice.

a




b

Naïve nasopharynx

| Species | WT n number | KO n number |
|---|---|---|
| Alphaproteobacteria sp. | 0 | 1 |
| Bacteroidales. Sp | 3 | 0 |
| Bradyrhizobium sp | 1 | 0 |
| Lachnospiraceae sp. | 12 | 3 |
| Lactobacillus sp. | 2 | 1 |
| Mesorhizobium sp | 1 | 0 |
| Microbacteriaceae sp | 1 | 0 |
| Porphyromonadaceae sp | 9 | 7 |
| Prevotella sp. | 1 | 2 |
| Rhodobacteraceae sp. | 0 | 1 |
| Staphylococcus sp. | 0 | 6 |
| Streptococcus sp. | 1 | 10 |
| Uncultured cyanobacterium sp. | 1 | 0 |
| Unknown bacteria | 1 | 0 |

Present in both WT & KO  Present in WT only  Present in KO only

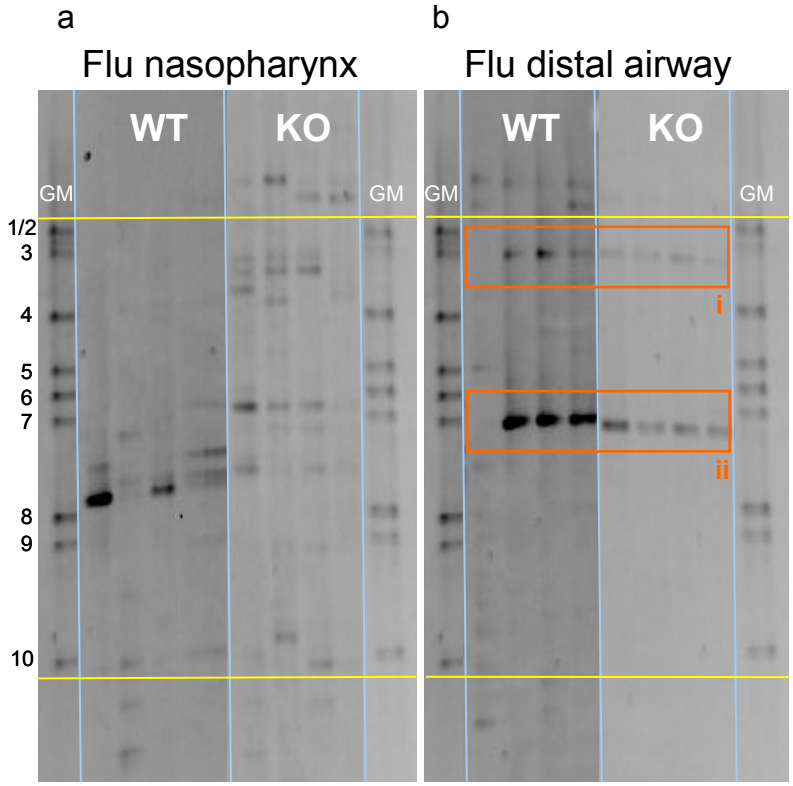
**Figure 6.6 Qualitative comparison of tissue specific bacterial species in naïve wild type C57BL/6 and CD200R KO mice.** BAL (a) and nasal wash (b) was performed on naïve wild type (WT) and CD200R (KO) mice (n=4). Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. Individual 16S rRNA products were subsequently cloned using a pGEM-T easy vector into *Escherichia coli*. Resulting colonies were sequenced and subsequently designated as belonging to a particular bacterial species. A more in depth description of the protocol used can be found in section 2.12. The axis in panel a and the frequency in panel b represents the n number of individual 16S rRNA sequences of a given bacterial species isolated from a selected mouse within each group.

mice, whilst they were present in both the nasopharyngeal cavity and airway of CD200R KO mice.

### 6.2.4 Influenza virus infection qualitatively alters the commensal landscape

After identifying the bacterial species present in the nasopharyngeal cavity and distal airways of naive WT and CD200R KO mice, we next wanted to ascertain whether an influenza virus infection alters the diversity of bacterial species present in each of these compartments. As was seen in the distal airway of naive mice, *Acinetobacter Sp.* was also detected in the airway at day 7 of an influenza infection in both WT and CD200R KO mice (orange box ii Figure 6.7 b and 6.8 a). Both *Moraxella sp.* and *Prevotella sp.*, which were detected in the naive airway, remained in the airway at day 7 of an influenza virus infection in WT mice. These species were not detected in the airway of CD200R KO mice during an influenza infection, *Moraxella* was also absent from the airways of naive CD200R KO mice (Figure 6.6 a). Nevertheless, *Staphylococcus sp.* were detected in the airway of both WT and CD200R KO mice during an influenza infection despite being absent from the naive airways of both mice strains (orange box i Figure 6.7 b and 6.8 a). *Staphylococcus sp*. were also detected in the nasopharynx of naive CD200R KO mice (Figure 6.6 b). We also observed the emergence of *Streptococcus sp.* and *Haemophilus parahaemolyticus* in the airway of WT mice infected with influenza virus (Figure 6.8 a). These bacterial species were absent from the airways of naive WT mice (Figure 6.6 a). Interestingly these species were not detected in the airways of CD200R KO mice either during naive homeostasis or at day 7 of and influenza infection (Figure 6.6 a & 6.8 a). Despite this, the appearance of *Proteus mirablis* in the airway of CD200R KO mice was detected during an ongoing influenza virus infection (Figure 6.8 a & b). Due to time constraints the precise elucidation of bacterial species in the nasopharyngeal cavity of mice infected with influenza was not analysed, however DGGE analysis highlighted an alteration in the diversity of bacterial commensal composition as seen in the distal airway in both WT (Figure 6.9 a) and CD200R KO (Figure 6.10 a) mice. Furthermore, it is interesting to note that the number of unique bacterial species present in the distal airways during homeostasis (n=20) was greater than the number present during an ongoing influenza infection (n=17). This was also apparent in the nasopharyngeal cavity upon visual inspection of the DGGE gels (Figures 6.9 & 6.10).

Utilising the sequence data generated a phylogenetic tree was constructed, as described in section 2.12, to highlight the prevalence of specific bacterial species

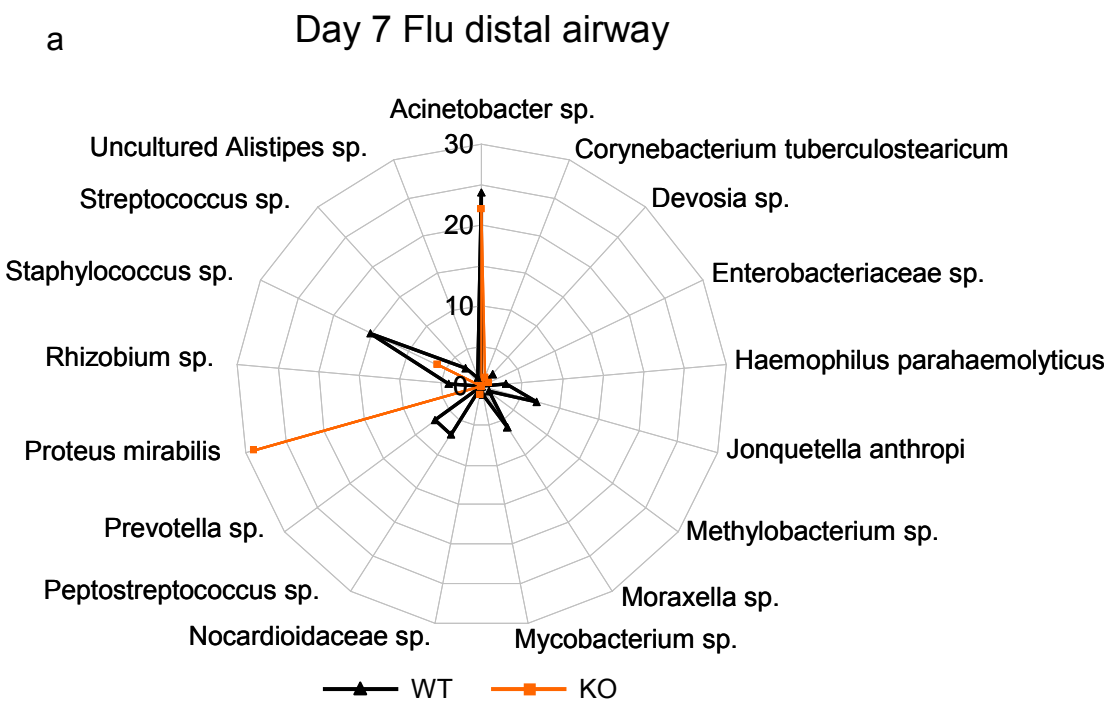



Bacterial genus marker

| | | | |
|---|---|---|---|
| **1** | *Bacteroidales sp* | **6** | *Prevotella sp. & S. pneumoniae* |
| **2** | *Staphylococcus sp* | **7** | *Prevotella sp* |
| **3** | *Megaspaera sp* | **8** | *Neisseria sp* |
| **4** | *Porphyromonas sp* | **9** | *Veillonella dispar* |
| **5** | *Porphyromonas sp* | **10** | *Veillonella sp.* |

**Figure 6.7 Qualitative comparison of nasopharyngeal and airway bacterial flora during an influenza infection in wild type C57BL/6 and CD200R KO mice.** Wild type (WT) and CD200R (KO) mice (n=4) were infected intranasally (i.n) with 50 HA HK/X31 influenza A virus on day 0. 7 days later nasal wash (a) and BAL (b) was performed and subsequently used to identify bacterial specie presence. Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. 10 µl of 16s rRNA product was then loaded onto a 30-60% denaturant DGGE gel and run at 100 v for 16 hours. A more in depth description of the protocol used can be found in section 2.12. A bacterial genus marker was used to allow comparison of band migration between gels and to quickly identify the presence of specified bacterial species (see table). i = *Staphylococcus sp*. and ii = *Acinetobacter sp*.

a



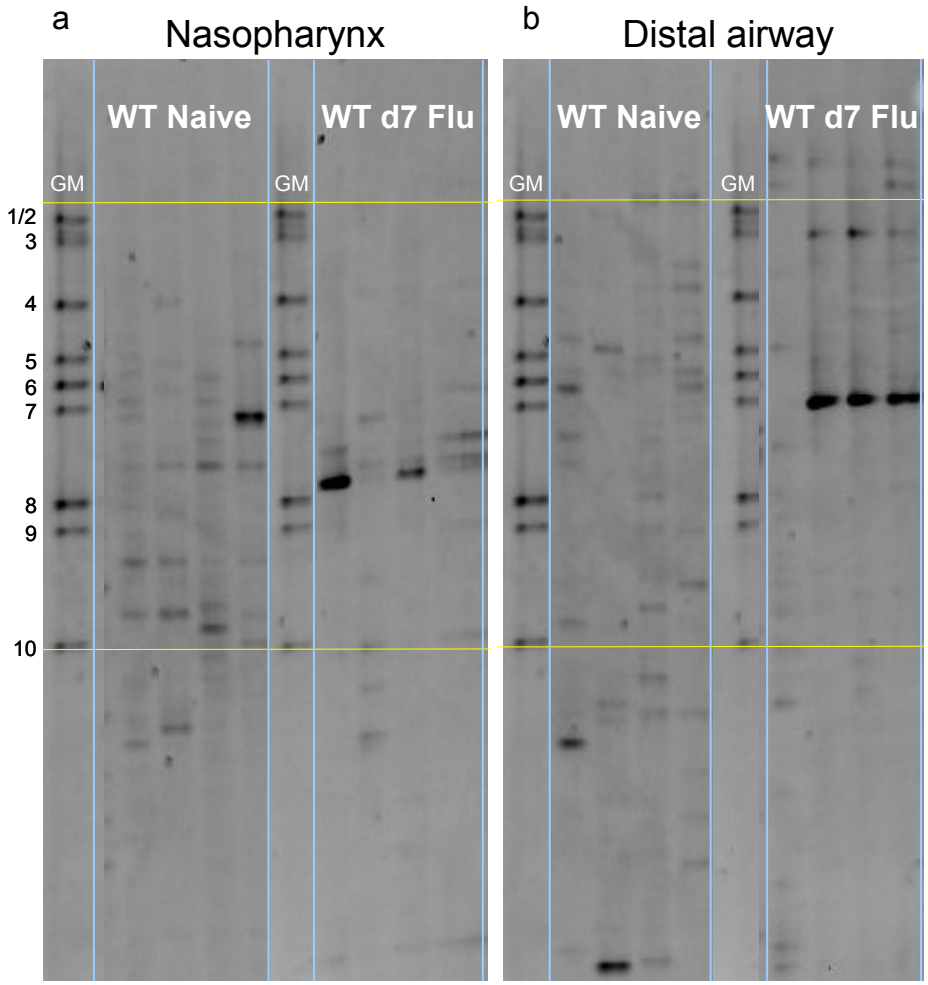

b

| Species | WT n number | KO n number |
|---|---|---|
| Acinetobacter sp. | 24 | 22 |
| Corynebacterium tuberculostearicum | 0 | 1 |
| Devosia sp. | 2 | 0 |
| Enterobacteriaceae sp. | 0 | 1 |
| Haemophilus parahaemolyticus | 3 | 0 |
| Jonquetella anthropi | 7 | 0 |
| Methylobacterium sp. | 1 | 0 |
| Moraxella sp. | 6 | 0 |
| Mycobacterium sp. | 1 | 0 |
| Nocardioidaceae sp. | 0 | 1 |
| Peptostreptococcus sp. | 7 | 0 |
| Prevotella sp. | 7 | 0 |
| Proteus mirabilis | 0 | 29 |
| Rhizobium sp. | 4 | 0 |
| Staphylococcus sp. | 15 | 6 |
| Streptococcus sp. | 3 | 0 |
| Uncultured Alistipes sp. | 1 | 0 |

Present in both WT & KO  Present WT only  Present in KO only

**Figure 6.8 Qualitative comparison of airway bacterial flora during an influenza infection in wild type C57BL/6 and CD200R KO mice.** BAL (a & b) was performed on wild type (WT) and CD200R (KO) mice (n=4) infected with 50HA HK/X31 influenza A virus 7 days previously. Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. Individual 16S rRNA products were subsequently cloned using a pGEM-T easy vector into *Escherichia coli*. Resulting colonies were sequenced and subsequently designated as belonging to a particular bacterial species. A more in depth description of the protocol used can be found in section 2.12. The axis in panel a and the frequency in panel b represents the n number of individual 16S rRNA sequences of a given bacterial species isolated from a selected mouse within each group.

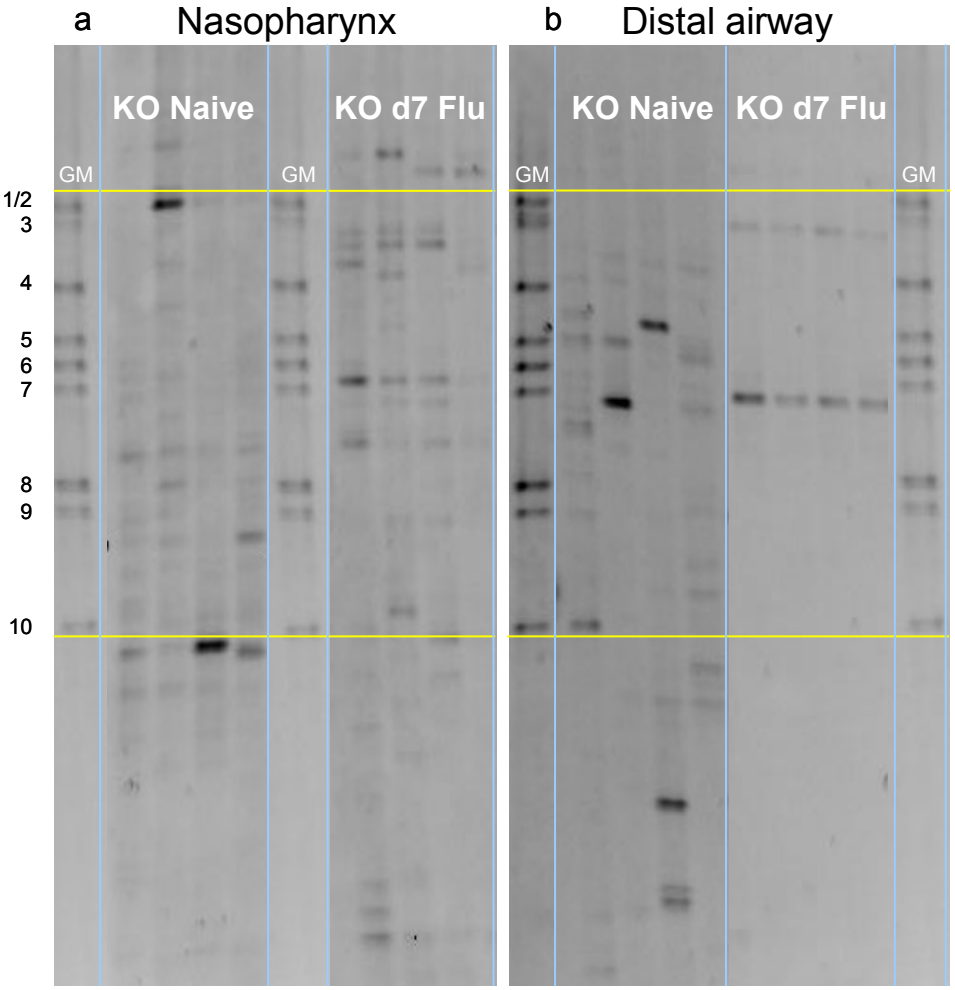
present at a specified anatomical location during homeostasis and during an influenza infection (Figure 6.11). The tree demonstrates more clearly the reduction in bacterial diversity observed in the distal airway and nasopharyngeal cavity during an ongoing influenza infection in both WT and CD200R KO mice. It also clearly highlights the bacterial species that become dominant (dark blue and black squares) during an ongoing influenza infection, namely *Staphylococcal sp.*, *Acinetobacter lwoffi*, *Proteus mirabilis* and *Streptococcus sp*. Interestingly, a bronchiole brush sample from a patient suffering from chronic obstructive pulmonary disease (COPD) also highlighted the presence and dominance of *Streptococcus sp.* and *Acinetobacter sp* (lane 7 Figure 6.11).




Bacterial genus marker

| | | | |
|---|---|---|---|
| **1** | *Bacteroidales sp* | **6** | *Prevotella sp. & S. pneumoniae* |
| **2** | *Staphylococcus sp* | **7** | *Prevotella sp* |
| **3** | *Megaspaera sp* | **8** | *Neisseria sp* |
| **4** | *Porphyromonas sp* | **9** | *Veillonella dispar* |
| **5** | *Porphyromonas sp* | **10** | *Veillonella sp.* |

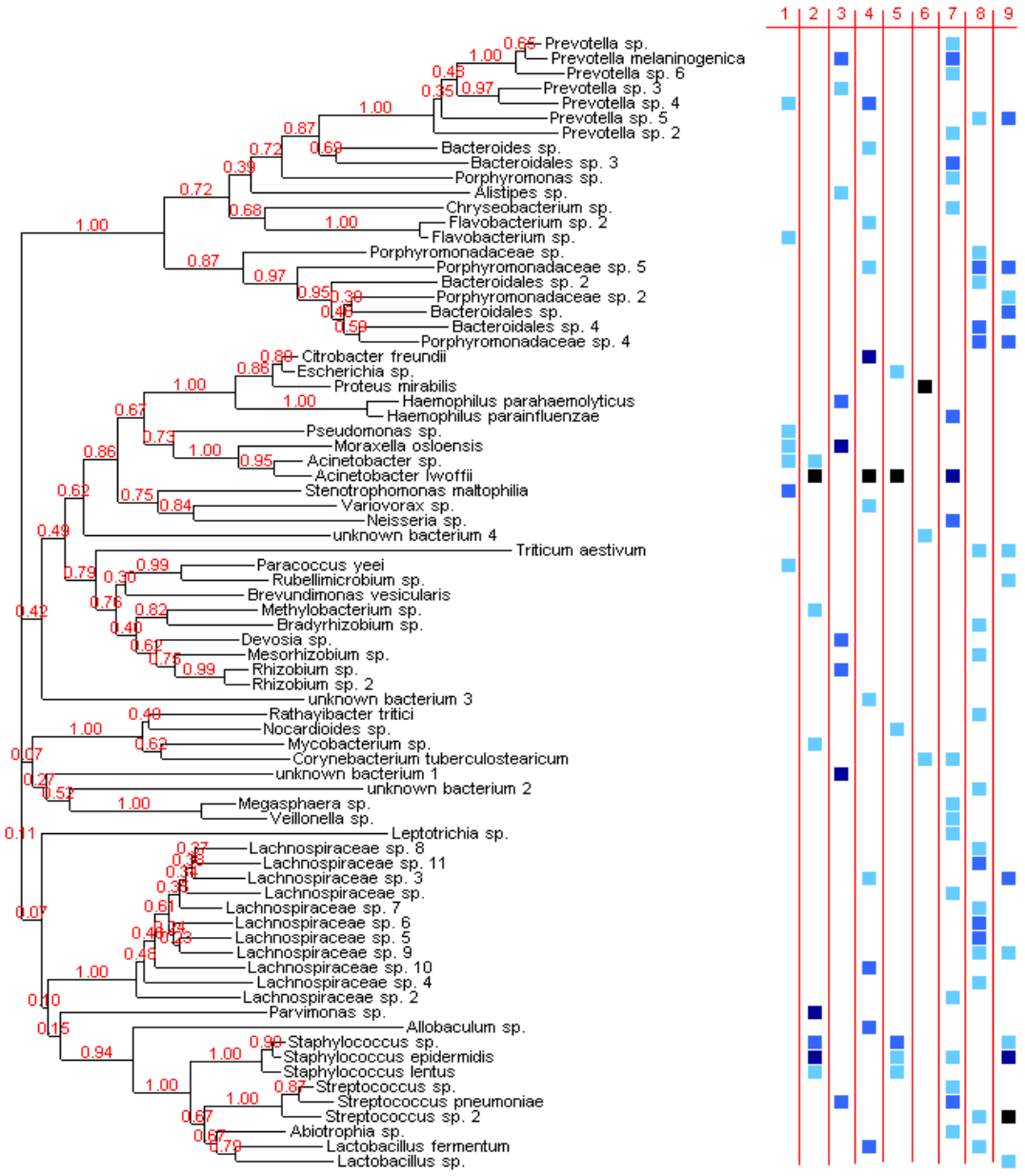
**Figure 6.9 Influenza virus infection qualitatively alters the bacterial landscape in both nasopharyngeal and airway compartments in WT C57BL/6 mice.** Wild type (WT) mice (n=4) were infected intranasally (i.n) with either 50HA HK/X31 influenza A virus or PBS on day 0. 7 days later nasal wash (a) and BAL (b) was performed and subsequently used to identify bacterial specie presence. Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. 10 µl of 16S rRNA product were then loaded onto a 20-80% denaturant DGGE gel and run at 100 v for 16 hours. A more in depth description of the protocol used can be found in section 2.12. A bacterial genus marker was used to allow comparison of band migration between gels and to quickly identify the presence of specified bacterial species (see table).




Bacterial genus marker

| | | | |
|---|---|---|---|
| **1** | *Bacteroidales sp* | **6** | *Prevotella sp. & S. pneumoniae* |
| **2** | *Staphylococcus sp* | **7** | *Prevotella sp* |
| **3** | *Megaspaera sp* | **8** | *Neisseria sp* |
| **4** | *Porphyromonas sp* | **9** | *Veillonella dispar* |
| **5** | *Porphyromonas sp* | **10** | *Veillonella sp.* |

**Figure 6.10 Influenza virus infection qualitatively alters the bacterial landscape in both nasopharyngeal and airway compartments in CD200R KO mice.** CD200R KO mice (n=4) were infected intranasally (i.n) with either 50HA HK/X31 influenza A virus or PBS on day 0. 7 days later nasal wash (a) and BAL (b) was performed and subsequently used to identify bacterial species presence. Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. 10 µl of 16S rRNA product were then loaded onto a 30-60% denaturant DGGE gel and run at 100 v for 16 hours. A more in depth description of the protocol used can be found in section 2.12. A bacterial genus marker was used to allow comparison of band migration between gels and to quickly identify the presence of specified bacterial species (see table).




**Lane legend**

| | | |
|---|---|---|
| 1 = Naive WT airway | 4 = Naive KO airway | 7 = Human COPD sample |
| 2 = Flu WT airway Exp.1 | 5 = Flu KO airway Exp.1 | 8 = Naive WT nasal wash |
| 3 = Flu WT airway Exp.2 | 6 = Flu KO airway Exp.2 | 9 = Naive KO nasal wash |

0.1 – 5 % 5 – 15 % 15 – 25 % > 25 % of total sequences

**Figure 6.11 Phylogenetic analysis of bacterial 16S rRNA detected during homeostasis and peak influenza infection.** Bronchoalveolar lavage and nasal wash was taken from naive and influenza infected (day 7) wild type (WT) C57BL/6 and CD200R KO (KO) mice (n=4). Total RNA was purified from each tissue sample and 16S rRNA was amplified using a nested PCR approach. Individual 16S rRNA products were subsequently cloned using a pGEM-T easy vector into *E. coli*. Resulting colonies were sequenced and subsequently designated as belonging to a particular bacterial species. A more in depth description of the protocol used can be found in section 2.12. Colour represents the prevalence of a specific species within each sample.

## 6.3 Discussion

### 6.3.1 A reduction in total protein concentration in the airway of co-infected mice

In this study we utilised an innovative proteomic screening platform to investigate the protein composition of BALF from BALB/c mice infected with either influenza or GBS alone or in combination. We demonstrated that the amount of total protein detected 3 days after a GBS infection was marginally higher than that detected in a naive airway, whilst the total protein concentration 6 days after an influenza virus infection was six times greater than that detected during homeostasis. Furthermore, administration of GBS three days after an influenza infection significantly reduced the concentration of total protein compared to the level detected in response to a single influenza infection. We also showed preliminary evidence that suggests a prior influenza infection alters the expression of several anti-bacterial proteins that are produced in response to a GBS infection.

The large disparity observed in airway protein concentration most likely reflects the inflammatory nature of the respective infectious agents or the presence of pathogen associated virulence factors that limit immune responses and inflammation. Total airway protein concentration is commonly used as a surrogate for respiratory epithelial damage and airway inflammation [527]. Influenza virus is cytopathic in nature and principally infects respiratory epithelial cells resulting in the secretion of a plethora of inflammatory mediators and other proteins that, in turn, recruit additional leukocytes to the site of infection [195]. Upon arrival, these recruited leukocytes add to the milieu of secreted proteins by releasing further inflammatory mediators and effector proteins [195;202-204]. A recent study highlights tumour necrosis factor-related apoptosis inducing ligand (TRAIL), produced by infiltrating monocytes, as a key mechanism involved in alveolar leakage and mortality in a murine influenza infection model [417]. These mechanisms, in combination with virus induced epithelial cell necrosis, likely explain the enhanced total protein concentration observed in the airway during an influenza virus infection. In contrast, we have previously demonstrated that a non lethal dose of GBS elicits a transient neutrophil and macrophage cellular response that results in the elimination of bacteria from the airway and lungs by day seven post infection (see results Chapter 3.0). Despite the presence of cytolytic GBS virulence factors, the presence of additional virulence factors, such as the C5a peptidase, that cleaves the chemotactic complement component C5a, inhibits innate immune responses and impairs leukocyte recruitment

to the site of infection [217;219;221-223]. GBS also expresses β-haemolysin/cytolysin (βH/C), a toxin that is co-expressed with an orange-red carotenoid pigment on the bacterial cell surface [220]. This pigment contains potent antioxidant properties, which confers protection against antimicrobial effects of hydrogen peroxide, singlet oxygen and superoxide [221]. Could the expression of this antioxidant pigment scavenge reactive oxygen and nitrogen species produced by infiltrating leukocytes that could in turn reduce the collateral damage caused by ROS commonly attributed to influenza infection? Recent evidence highlights the damaging effects of ROS produced in response to influenza infection. The administration of a superoxide dismutase mimetic significantly reduces influenza associated pathology and peak viral load [528]. Taken together, the presence of immune suppressive virulence factors, produced by GBS, might explain the reduction in total protein concentration measured in the airway of mice co-infected with influenza and GBS.

An alternative explanation for the reduction in total airway protein observed in co-infected mice could involve GBS administration enhancing the quality of the immune response elicited or even increasing the rate at which influenza induced inflammation is resolved. In Chapter 4, we demonstrate that administration of GBS on day three of an influenza infection results in a significant reduction in viral titre compared to mice infected with influenza alone. Amelioration of influenza induced pathology and vascular permeability is also observed during co-infection with *Trichinella spiralis*, a parasitic worm [529]. It is plausible that GBS may therefore actually prove to be beneficial in controlling viral replication. However, there exists substantial evidence that bacterial co-infection results in enhanced inflammation and pathology in response to influenza virus infection. Bacterial proteases have been shown to enhance influenza viral load by increasing the rate of HA0 to HA1 cleavage that results in enhanced viral infectivity [530-532]. Furthermore, neuraminidase, present on the surface of influenza virus, increases adherence of *S. pneumoniae* to tracheal epithelial in an organ perfusion model [296]. There are likely to be many interconnected factors that contribute to reducing the amount of protein present in the airway in co-infected mice. This is a puzzling result that may provide interesting evidence for unknown *in vivo* pathogen-pathogen interactions that contribute to disease pathology and progression.

### 6.3.2 Influenza virus infection alters anti microbial protein (AMP) expression

Despite the intriguing observation that the administration of GBS reduced total airway protein levels in response to an influenza infection, our original question was to

investigate whether damage to respiratory epithelium, caused by influenza virus infection, resulted in a reduction in AMPs expression. Our preliminary findings illustrate that surfactant protein B, lysozyme C type M and chitinase 3 like protein 1 were expressed in the airway three days after a GBS infection. However, in mice that were previously infected with influenza virus they were not. Again, these findings could have many possible explanations, however, before speculating it is imperative to consider the limitations and potential caveats of analysing proteomic data producing using mass spectrometry. All existing proteomic interrogation techniques contain several inherent weaknesses that mainly arise due to the complexity of the proteome. The major considerations surround the huge dynamic range of the proteins under interrogation and the contamination of tissue samples by highly abundant serum proteins. The latter problem is of particular significance when looking for very low abundant proteins such as AMPs. There are also many limitations in the peptide sequencing methodological itself that must be considered, such as which algorithm or combination of algorithms to use when searching public protein databanks, the level of statistical stringency applied and the quality of expert interpretation of the resulting data set. Nevertheless, these problems exist when analysing any data produced using these techniques and despite ones caution in interpreting these preliminary data the results remain intriguing. An additional caveat present in this study was the different amounts of protein loaded into the gel electrophoresis, therefore potentially introducing quantitative errors into the interpretation of our data.

Our findings are similar to that observed by another study that utilised HPLC and mass spectrometry to investigate AMPs expression in response to UV killed nontypeable *H. influenzae* (NTHi) [533]. They show that lysozyme, surfactant protein D and chitinase 3-like protein 1 expression in response to UV killed NTHi protects mice against a lethal *S. pneumoniae* infection. Existing evidence implicates both surfactant protein B and lysozyme C type M in having direct anti-bacterial and immunomodulatory effector function against both GBS and *S. pneumoniae* in *in vivo* infectious models [5;534-537]. Despite the lack of a comprehensive proteomic analysis of AMP expression during an influenza virus infection, there is evidence highlighting that beta defensins and surfactant protein D expression are altered [538]. We do not provide any potential mechanism for the observed reduction in surfactant protein B, lysozyme C type M and chitinase 3 like protein 1 in our study, however, potential explanations could involve the apoptosis and necrosis of the cells involved in

producing AMPs or local inhibition by IFN produced in response to influenza virus infection interfering with AMP transcription and translation.

Although we postulate the absence of specific AMPs in influenza infected mice could explain the increase in susceptibility to bacterial infection, we cannot rule out the contribution of immunomodulatory proteins produced in response to influenza virus infection that are absent from the airway in a single GBS infection. Several proteins were observed in the co-infected mice that were absent from mice infected with GBS alone. These include vitamin D binding protein, several complement precursor components, peroxiredoxin 2 and apolipoprotein A1 (Apo-A1). Apo-A1 is the major constituent of high density lipoprotein (HDL) and binds to LPS *in vivo* and reduces acute lung injury and sepsis in mice treated with LPS and LTA [539;540]. Indeed Apo-A1 increases inflammatory cytokine production and cellular apoptosis during influenza infection in one study but others suggest Apo-A1 suppresses inflammation and inhibits the production of inflammatory cytokines from influenza infected type II pneumocytes [24;541]. Thus increased apoptosis of professional phagocytes and reduced inflammatory cytokine production by Apo-A1 may be required to resolve influenza induced inflammation but enhance susceptibility to subsequent bacterial infection. A suppressive role for Apo-A1 is supported by mimetic peptides that protect against atherosclerosis, asthma, diabetes and renal inflammation *in vivo* [542;543].

This proteomic screen produced preliminary data that sought to identify potential AMPs and/or immune modulatory proteins that are differentially expressed in the airways of mice infected with bacteria alone or bacteria following an influenza infection. The investigation produced many intriguing results that warrant further investigation and validation. Exploration of the expressed proteome of an influenza infected lung is an extremely novel and topical subject. To our knowledge this is the first attempt at analysing and comparing the proteomic signature of an influenza infected airway to that of a bacteria infected airway.

### 6.3.3 The commensal microbiota of the respiratory tract during homeostasis

Using traditional microbiological techniques we show that bacteria can be cultured from the nasopharynx of naive mice but not from the distal airway and lung tissue. However, upon infection with influenza virus, culturable bacteria appear in the distal airways and the number of bacteria present in the nasopharynx increases significantly. Furthermore, the use of a more sensitive detection technique highlights

the existence of a diverse assortment of bacteria present in the lung, distal airway and nasopharynx of naive mice and that the composition of this flora differs between different anatomical sites of the respiratory tract and under different homeostatic conditions. It is commonplace that the upper respiratory tract, including the nasopharynx, contains a complex commensal population and increasing evidence indicates the existence of intricate host-microbe and inter microbial interactions [521;544]. In contrast, little is known about the presence of 'commensal' bacteria in the lower respiratory tract as until recently it was assumed to be sterile and contain no resident microflora. Nevertheless, inhaled air is not sterile and microbe bearing secretions from the upper respiratory tract can aspirate into the lower airways. Thus the lower respiratory tract faces the same challenges confronted by other mucosal surfaces, balancing inflammatory responses to levels that prevent microbial invasion without compromising vital organ function.

As with the proteomic profiling of innate anti-bacterial proteins discussed above, this study contains preliminary data that does not allow sufficient statistical interrogation, therefore appropriate consideration is needed when interpreting and discussing the results. The approach used in this study also contains several limitations such as the risk of generating artefacts from contaminating environmental bacterial when using samples with very low bacterial DNA content, as is the case when using murine tissue samples. We also only selected one representative mouse from each specified infection group to determine the specific bacterial species present. However, taking these limitations into consideration, there are several intriguing findings of which the most salient will be discussed here.

There are obvious anatomical and physiological features of the respiratory tract that may give particular bacterial species a growth advantage, such as airflow pressure, temperature and oxygen gradient, the presence of respiratory cilia and the existence of competing microflora. However, we have shown that status of innate immunity also plays an important role. We found similarities between WT and CD200R KO mice, such as the presence of both *Acinetobacter sp* and *Prevotella sp* in the naive airway, and differences, such as the presence of *Staphylococcus sp.* in the nasopharynx of CD200R KO mice whilst being absent in WT mice. These findings are consistent with human studies, in that *Acinetobacter sp.* are commonly found in the nasopharyngeal cavity of healthy individuals but are implicated in a variety of diseases including pneumonia and serious blood infections [545]. Interestingly, we also observed an apparent increase in the prevalence of *Acinetobacter sp*, amongst others, in the

airway of both WT and CD200R KO mice during an ongoing influenza infection. The disparity in commensal diversity between WT and CD200R KO mice in homeostasis suggests that CD200R bearing cells such as macrophages may be required to limit nasal commensals in homeostasis. In this way, innate immunity trims the density of commensals most likely via receptors that detect and phagocytose specific bacteria and that these may be enhanced in CD200R KO mice. A recent paper highlights TREM2 as an important receptor for bacterial phagocytosis [546]. Is this, or are receptors involved in phagocytosis, differently expressed in WT and CD200R KO mice? Neutrophil NADPH oxidase plays a crucial role in the defence against *Acinetobacter baumannii* infection [547]. It is likely that these are altered in CD200R KO cells. TLR expression may also be adjusted and more ready to respond. This suggests that the readiness of innate immune inflammation may dictate the composition of bacterial flora and even mediate competitive interactions between competing commensal species. This is highlighted by Lysenko *et al* that show that neutrophil recruitment and activation may underlie the *H. influenza* induced clearance of *S. pneumoniae* [548].

### 6.3.4 Influenza virus infection alters the composition of commensal bacteria in the distal airways and nasopharynx

Perhaps the most significant finding in this preliminary investigation is that, despite an increase in total bacteria in the distal airway and nasopharynx during an influenza virus infection, the diversity of the species of bacteria detected is not only reduced but largely consists of bacteria that are implicated in respiratory disease. The appearance of *Staphylococcus*, *Streptococcus* and *Haemophilus* species in the distal airway of wild type mice infected with influenza virus is striking. We also observe the appearance of *Proteus mirabilis* in the distal airway of KO mice in response to influenza virus infection. These findings are consistent with a large body of evidence investigating the presence and composition of bacterial flora in the lungs of cystic fibrosis (CF) and COPD patients [549-551]. These patients have altered airway physiology and likely altered inflammatory homeostasis, which may be responsible for the increased colonisation of respiratory opportunistic pathogens such as *P. aeruginosa*, *S. pneumoniae* and *S. aureus*. A recent study investigating the bacterial diversity in endotracheal aspirates from intubated patients colonised with *P. aeruginosa* shows that bacterial diversity decreases following the administration of antibiotics, but surprisingly, results in *P. aeruginosa* dominating the community of bacteria that remain [552]. This occurred despite *P. aeruginosa* demonstrating sensitivity to the antibiotics used *in vitro*. The loss of bacterial diversity may therefore

contribute to pathogen selection and persistence. It was previously assumed that an increase in the diversity of bacterial species would enhance virulence of individual species [553]. However, mounting evidence now suggests that in polymicrobial infections, less-virulent strains are often favoured; suggesting that increased diversity may in fact reduce virulence [554]. These observations beg the question of how and why do certain bacterial species gain access to the distal airways during respiratory viral infections or during times of 'altered' homeostatic states and what causes the loss of bacterial diversity? Other than those reasons discussed in Chapters 4 and 5 for why bacteria may gain access to the airway during influenza infection, one potential explanation through which a reduction in bacterial diversity might lead to increased dominance of specific pathogenic species is quorum sensing. The altered airway microenvironment caused by influenza infection may result in altering gene expression of bacteria such as *Staphylococcus sp., H. influenzae* and *Streptococcus sp.*, normally found in the nasopharyngeal cavity. This may result in the production of virulence factors that can interfere or even kill surrounding bacterial species therefore allowing these bacteria to outgrow and resist immune effector functions.

Another explanation to why specific bacterial species appear to dominate during times of altered homeostasis could be related to their ability to survive during times of enhanced inflammation. As discussed previously, influenza infection provokes an inflammatory response consisting of many antimicrobial mediators such as interferons, TNF-α and ROS. It is known that the expression of catalase by *S. aureus* contributes significantly to the survival of this pathogen in environments high in oxidative stress and allows it to survive in the presence of other pathogenic bacteria such as *S. pneumoniae* [555;556]. Other explanations for the observed alteration of bacterial communities associated with CF and Crohns sufferers involves alterations in mucin glycosylations that allow certain bacterial species to dominate over another [557;558]. This may also occur during an ongoing influenza infection?

## 6.4 Conclusion

These preliminary studies highlight the immense complexity of how one respiratory infection can affect the host's immunity to a subsequent infection. It also highlights the importance of recognizing and understanding the complex bacterial community that exists within the respiratory tract and how infection and/or therapeutic intervention can have untold consequences to its dynamic composition. Further studies investigating the contribution of bacterial dynamics to the pathogenicity in

health and disease will lead to more targeted use of existing antimicrobial therapies and the development of much needed novel therapeutics.

# Discussion

# Chapter 7

## 7.0 General Discussion

Influenza A virus causes significant and well publicised morbidity and mortality as a single infection. However, in combination with a secondary bacterial super infection, a common complication following influenza infection, the resulting prognosis is worse and can result in hospitalisation or death. Despite extensive clinical and epidemiological evidence, the precise immunological mechanism(s) responsible for increasing susceptibility to secondary bacterial infections remain to be fully elucidated. In this thesis we developed a robust *in vivo* experimental model to investigate host innate immunity against respiratory bacterial pathogens and how a preceding influenza viral infection might modify this. We revealed two novel mechanisms explaining how a preceding influenza viral infection influences innate immunity resulting in a period of increased susceptibility to bacterial pathogens. Our data show that a previous viral infection desensitises the airway in its ability to detect and respond to bacterial ligands. Furthermore, in an attempt to resolve viral associated inflammation the airway inadvertently over regulates, by enhancing an innate immune negative regulator, CD200R, resulting in a transient state of immune hypo-responsiveness. Removal of this single receptor limits bacterial burden in the airway and lung and completely prevents bacterial dissemination and bacteraemia. Finally, we provide preliminary data that suggests antimicrobial peptide expression is altered during an influenza infection and that innate immune status can influence the bacterial commensal community of the upper respiratory tract.

There are many concepts arising from this thesis that merit further consideration, but for brevity I will concentrate on the following topics for further discussion:

1. Macrophage homeostasis and infection history determine airway responsiveness.
2. A chronology of mechanisms enhance susceptibility to secondary bacterial pneumonias.
3. The contribution from commensal bacteria in inflammatory lung disease.
4. Antibiotic usage in inflammatory lung disease – a clinical case.

## 7.1 Macrophage homeostasis and infection history determines airway responsiveness

Our data show that host genetic factors influence resistance and responsiveness to both respiratory bacterial and viral infections. We show that BALB/c ($H2^d$) mice consistently elicit an enhanced innate cellular response to all model pathogens used in this thesis (GBS, *S. pneumoniae* and influenza virus) and demonstrate increased resistance to respiratory bacterial infections compared to C57BL/6 ($H2^b$) mice. However, despite these differences in the vigour of immunity during a single bacterial infection, the outcome with regards to bacterial load, phenotype of cells recruited and final endpoint, i.e. lethal bacteraemia, is consistent across the two mouse strains in a secondary bacterial infection model. This suggests that an environmental insult, such as a viral infection, can override pre-existing genetic resistance, thereby influencing the immune responsiveness of the host. For the purposes of this discussion innate immune responsiveness is defined as the ability of tissue specific macrophages to detect and respond to a bacterial pathogen. How a preceding environmental insult or viral infection achieves this remains unknown, however we discuss a number of concepts herein that attempt to provide insight into these underlying mechanisms.

### 7.1.1 Tissue specific regulation establishes macrophage responsiveness

The threshold above which a threat is perceived by innate immune cells is established through a dynamic interaction that exists between host genetic determinants and the local microenvironment. At sites of high antigenic burden, such as mucosal surfaces, resident innate immune cells require an additional degree of 'tolerance' compared to cells that inhabit less environmentally exposed sites of the body. We have previously demonstrated and discussed in Chapters 4 & 5 several mechanisms that actively regulate the 'threshold of activation' of innate immune cells resident in the airway during homeostasis [446;458]. We now suggest that the threshold of innate responsiveness can be determined by past inflammatory experiences thus explaining why some individuals, such as asthmatics, respond inappropriately to environmental allergens; in this case the 'threshold of activation' is set too low resulting in unwarranted inflammation. On the other hand a high 'threshold of activation' can be equally damaging as we show in this thesis for lung bacterial secondary infections during the resolution of a respiratory viral infection. One can imagine a scenario in which macrophages, resident in the upper and lower respiratory tract, play an important role in limiting commensal microbial burden, even in the absence of exogenous infection. If the threshold for activation is set or driven too high then commensal or environmental micro-organisms could potentially be

'ignored' and subsequently given the opportunity to invade host tissue compartments. The concept of commensal opportunism is discussed in greater detail later in this discussion.

This high threshold found at mucosal surfaces, or following influenza virus infection, may occur spontaneously, through as yet unidentified polymorphisms in either CD200R or its ligand CD200, or as a consequence of an attempt to return the lung to its pre-infection state. The belief that inflammation recedes once the antigen is eliminated is, to some extent, redundant. Instead resolution is an active process and involves analogous pathways that limited innate immune cell reactivity during homeostasis. Following severe, life threatening inflammation, such as that experienced in a small number of individuals during an influenza infection, these regulatory pathways are over-exuberant as previously exemplified by the excess production of IL-10 and enhanced CD200R expression on airway antigen presenting cells [458;480]. We are not suggesting that this post-influenza deleterious state occurs in all those infected with influenza, but that the activation threshold in resolution is similarly affected, in some, by the parameters described earlier during homeostasis. We believe it is in these subsets of patients that bacterial complications, hospitalisation and mortality may occur. Indeed an overshoot in innate immune regulation with untoward consequences is not unique to inflammatory lung disease as the CD200/CD200R axis is already thought to contribute to the suppressive tumour microenvironment either directly [559-562] or via the induction of $T_{reg}$ cells [563;564].

Resolution not only requires a cessation of inflammation but also the clearance of the inflammatory infiltrate; a large proportion of which undergoes programmed cell death via apoptosis. As discussed in Chapter 5, efferocytotic uptake of apoptotic cells by macrophages leads to a reduced microbicidal state that prevents long term tissue damage by chronic inflammation, but may also inadvertently reduce responsiveness to a subsequent bacterial infection. The influenza induced desensitisation of airway macrophages to respiratory bacteria we discussed in Chapter 4 may thus be an indirect effect of the clearance of inflammatory cells rather than direct viral effects *per se*. Efferocytosis induced production of prostaglandin E2 ($PGE_2$) alone suppresses the anti-microbial activity of alveolar macrophages by inhibiting NADPH oxidase [489;490]. The role of CD200 in this process is not currently known, but is possible since it leads to an inhibition of inflammation in the CNS by reducing microglial cytokine expression and we report a large number of CD200 expressing cells present in the airway during the time of peak susceptibility to a secondary bacterial super-infection

[565]. Apoptotic cell expression of CD200 may thus signal to CD200R expressing macrophages and prevent presentation of self antigen. Therefore, in an attempt to return to homeostasis CD200 expressing apoptotic cells ingested by CD200R lung myeloid cells may reduce their anti-bacterial capability allowing subsequent bacteria, if present, to outgrow. A similar macrophage dysfunction is also observed in severe sepsis, however the link with apoptotic cells remains unverified providing another potential role for CD200-CD200R interaction and reduced macrophage functionality [566-568]. An absence or blockade of CD200R signalling may therefore release airway myeloid cells, or other myeloid phagocytes, from apoptotic cell-mediated suppression and enhance their bactericidal activity.

### 7.1.2 Infection history modulates the innate immune system

As discussed above, infection history can transiently alter lung innate responsiveness through enhancing regulatory pathways involved in tempering indiscriminate innate immune responses and restoring 'homeostasis'. However, a return to pre-infection homeostasis may never actually occur. Our data show that an influenza viral infection reduces the ability of alveolar macrophages to detect and respond to the presence of bacterial Toll ligands. This evidence demonstrates that a self limiting acute inflammatory insult can alter tissue specific immune responsiveness over a prolonged period of time. Infection history may therefore influence the way in which a specific tissue responds to subsequent immune stimulation. We often attribute such immune adaptation to acquired T and B lymphocytes and assume that innate immune compartments return to their pre-infection state even after multiple episodes of infection and inflammation. However, there is a growing body of evidence to suggest that we all develop a unique but subtle inflammatory profile. This uniqueness is determined by the sequence of infections or antigenic insults encountered that 'permanently' mould our lungs through experience [377;569;570].

Influenza viral infection, for example, protects against respiratory syncytial virus (RSV) induced immunopathology and lymphocytic choriomeningitis virus (LCMV)-infected mice exhibit heightened clearance of the unrelated vaccinia virus [570-572]. The outcome of infection history depends on the precise sequence of pathogens encountered by the host. Influenza, for example, inhibits vaccinia virus replication but enhances LCMV and murine cytomegalovirus (MCMV). The critical determinant appears to be the amplitude of cellular infiltrate in response to the second infection [572]. In most, but not all, cases, the improved outcome to the second infection can not

be explained through cross reactive T or B cell epitopes. Rather, we believe that the microenvironment of the lung stromal or innate immune cells is 'permanently' altered. Evidence for long term modification of the innate immune compartment is provided by studies where microbial products such as unmethylated CpG dinucleotides or a modified bacterial labile toxin (LTK63) afford protection against an array of subsequent respiratory pathogens [298;573;574]. This phenomenon has been coined 'innate imprinting' and can be defined as "the long term modification of a microenvironment, which will consequently lead to a reduced, and in some cases more protective, immune response to a subsequent pathogen". Myeloid inhibitory molecules such as CD200 and its receptor may also contribute to this 'matured' or 'educated' environment.

Experiments in invertebrate models of infection add credence to this concept of long term innate immune adaptation [427]. Injection of the bacterial cell wall component LPS provides long-lasting antimicrobial resistance in mealworm beetles when subsequently challenged with a heterologous natural fungal pathogen [428]. Copepods, which are miniature crustaceans, infected with a natural parasitic tapeworm are capable of specific memory responses and react more efficiently upon subsequent rechallenge with antigenically similar pathogens [575]. It appears that innate immunity not only contains mechanisms that calibrate its own responsiveness but also, more intriguingly, remember. Whether this is inherent or a consequence of additional regulatory pathways remains to be determined. This learning process, in the absence of adaptive immunity, also occurs with homologous or closely related pathogens. This innate imprinting, by sequential waves of inflammation or infection, may be beneficial, as shown for successive viral infections, or detrimental, as illustrated by the increased susceptibly to life threatening bacterial pneumonia in patients infected with seasonal and pandemic influenza. It is important to remember that in all of these situations, we are not describing cross-reactive acquired immunity but nonspecific influences from either what has gone before or what is currently present. We are therefore all unique because prior local or distal antigenic experiences have shaped our responsiveness to future antigenic insults, all in the absence of cross-reactive acquired immunity.

The mechanisms underlying this educated innate immunity are currently unclear but are believed to require phagocytes and the Toll like receptor pathway [576]. If this can happen in invertebrates, there is a high probability it will also happen in vertebrates. Speculation into the potential mechanisms that may explain this desensitisation

include augmented expression or regulation of intracellular TLR signalling adapters such as TOLLIP, A20 and IRAK M [9;10;577]. The induction of IL-10 producing $T_{reg}$ cells may also explain how one inflammatory event can impact on subsequent heterologous infections in a bystander fashion. IL-10 levels are enhanced in the lung after secondary pneumococcal or meningococcal challenge in mice that have previously been infected with influenza virus, this in turn reduces antibacterial effector functions of neutrophils and other myeloid cells [410;480;578]. Alternative ideas also include epigenetic modification of lung stromal cells or even the local stem cells responsible for renewing airway epithelium [579]. The concept of stromal cell adaptation is expanded in the following section.

### 7.1.3 Is there a role for resident stromal cells in tissue imprinting and bacterial susceptibility?

The long term adaptation and altered responsiveness of the airway epithelium has been extensively studied in other inflammatory lung disease models [580-586]. Airway metaplasia can continue in ex-cigarette smokers for more than two years after they have stopped smoking [587]. Such long term changes are likely to affect innate immune responsiveness to subsequent infections. Further evidence that epithelial cells are modified for prolonged periods is provided by the development of resistance to peroxide exposure through long-term changes in epithelial cytoskeletal structure, molecular forms of p53 and heat shock proteins and genome-wide reprogramming of gene expression [588]. If this occurs *in vivo*, then epithelial cell heterogeneity would be extensive and depend on the type of prior inflammatory insult and precise composition of the ensuing cellular response. The longevity of these alterations is assumed to be transient but has never been investigated. It is feasible that this influence may occur during resolution of the first inflammatory insult when epithelial integrity is regenerated by bone marrow progenitors or local stem cells in a process controlled by interaction with underlying sentinel mesenchymal cells, such as fibroblasts [589]. These mesenchymal cells may be altered by the primary inflammatory insult and maintain the 'memory' of these inflammatory events to educate newly regenerated epithelium. The transformation of airway epithelial cells into secretory epithelium containing mucus-secreting cells (mucous cell metaplasia) is a feature of many bronchial diseases in humans and is also observed in numerous rodent experimental systems.

Finally, we cannot rule out indirect effects on epithelial permeability. Loss of epithelial integrity is a key step in the pathogenesis of acute respiratory distress syndrome

whether caused by viral infection, pneumonia, aspiration, sepsis or trauma [590-592]. Reduced integrity results in the transition of airway bacteria into the parenchymal tissues and blood and allows direct contact with antigen presenting cells that are less restrained by the local microenvironment compared to the airways [458]. In CD200R KO mice influenza viral load is blunted from the outset, which will prevent the extent of epithelial damage and expression of putative receptors for bacterial adhesion. IFN-γ expression is also reduced and so in turn will impact less on bacterial scavenger receptor expression such as MARCO [593]. Furthermore, during infection, epithelial cells secrete a plethora of chemokines, such as CCL2, that recruit inflammatory leukocytes that impact on epithelial integrity [417]. A decreased viral load in CD200R KO mice may therefore liberate less chemokines causing the recruitment of fewer apoptosis-inducing immune cells or their release from the bone marrow [594;595]. Figure 7.1 combines all of these concepts to illustrate how an infection or inflammatory insult can modify airway responsiveness over time.

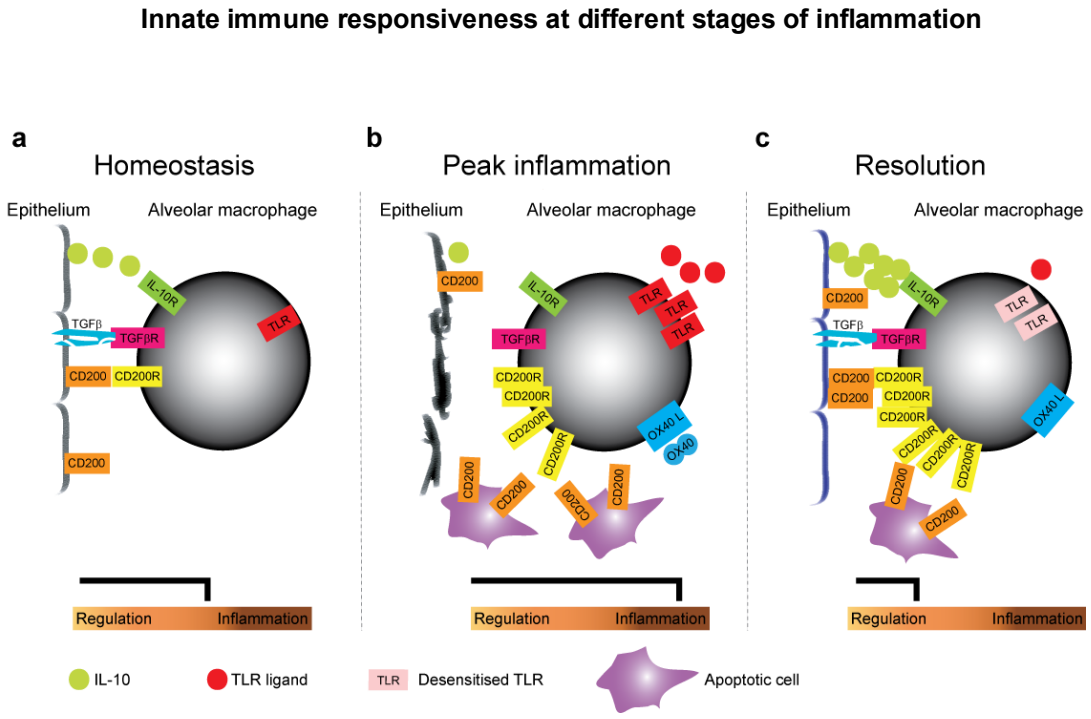



**Figure 7.1 The concept of altered innate immune responsiveness.** Alveolar macrophages are tightly regulated during homeostasis through intimate interactions with respiratory epithelium (a). Upon influenza virus infection, epithelium integrity is lost allowing TLR and OX40L signalling to potentiate inflammation (b). Apoptotic cells expressing CD200 bind to elevated CD200R levels on alveolar macrophage resulting in a reduced inflammatory capacity (b). Altered epithelium, elevated CD200R levels, heightened IL-10 levels and aberrant TLR signalling induce a hypo -responsive state (c).

### 7.2 A chronology of mechanisms enhance susceptibility to secondary bacterial pneumonias

There clearly exists a temporal relationship between influenza viral infection and the occurrence of secondary bacterial pneumonias [412]. As previously discussed, there exist an array of mechanisms that explain the increase in bacterial susceptibility following an influenza viral infection, these include: viral mediated epithelium damage, mucociliary dyskinesia, phagocyte apoptosis, neutrophil dysregulation and aberrant chemotaxis and IFN-γ mediated phagocyte dysfunction [279;593;596]. This list is by no means exhaustive. In addition to these we now add two novel mechanisms, proposed above, providing further insight into this complex conundrum. It is highly likely that a specific combination of several mechanisms results in the development of lethal bacteraemia. This combination of mechanisms might be strongly influenced by; the precise influenza viral strain and its associated virulence and replicative ability, exemplified by PB1-F2 expressing strains; initial host genetic susceptibility to respiratory viral and or bacterial infections; polymorphisms in CD200 and CD200R and their down stream signalling adapters; pre-existing nasopharyngeal colonisation with bacterial species capable of culminating in invasive pneumonia and also the health and fitness of the host which can be determined by other underlying medical conditions or the environment in which the host lives [597-599]. To try and determine the relative contribution of each individual mechanism would be extremely difficult and possibly meaningless in the context of actual clinical disease. It is known, and we have shown, that bacterial secondary infection is most commonly observed after viral elimination [417;600]. Therefore it is likely that influenza virus infection sets up a chain of events that causes direct pathology itself but also indirect long term consequences, through regulating innate immune responsiveness, that collectively determine bacterial susceptibility. Figure 7.2 attempts to highlight this multifactoral process of influenza induced bacterial susceptibility.

**The chronology of mechanisms that enhance bacterial susceptibility**

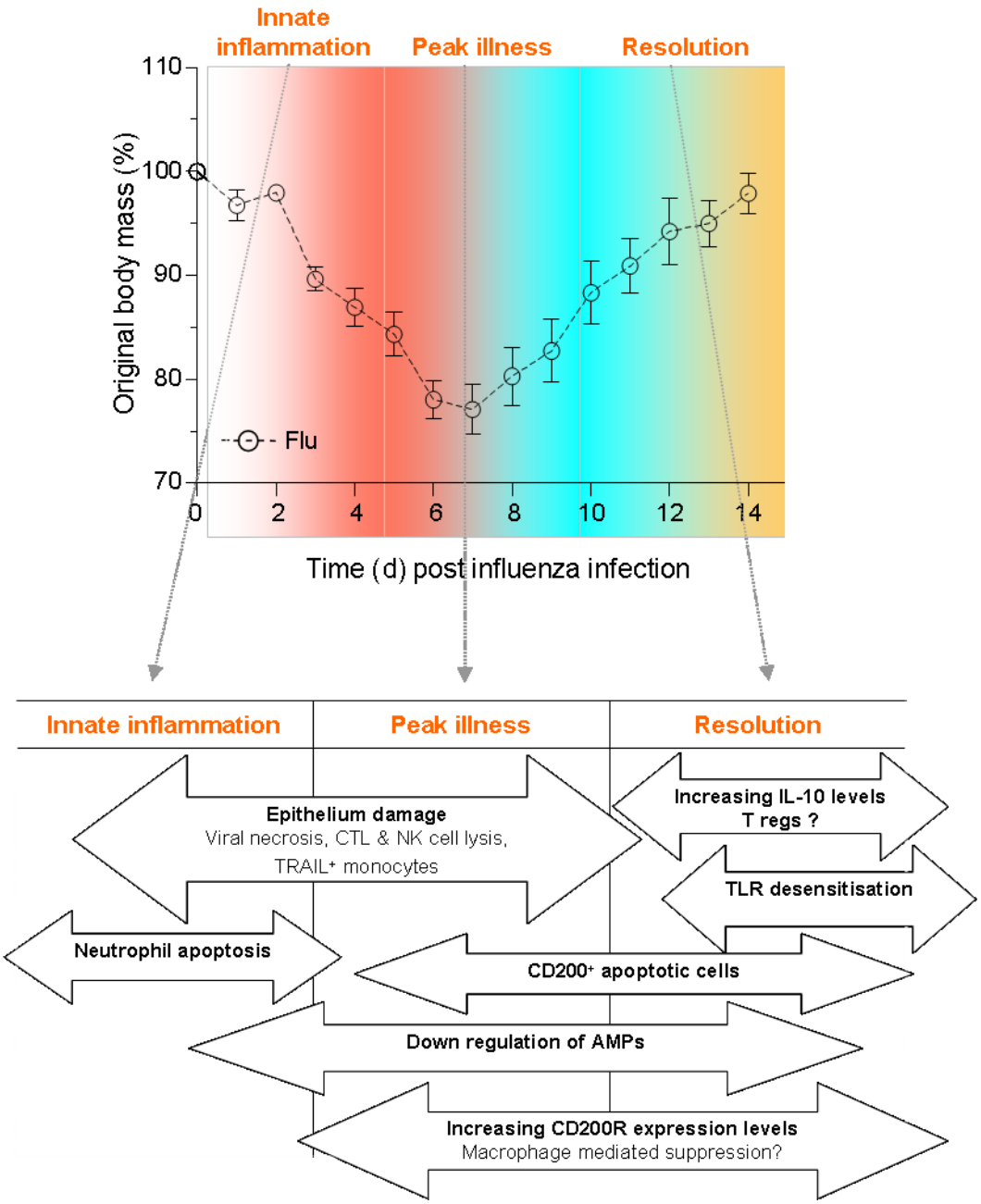



**Figure 7.2 Susceptibility mechanisms and their temporal relationships.** The various mechanisms that enhance susceptibility to secondary bacterial pneumonias occur in a temporal fashion each collectively contributing to reducing the host's capacity to protect against bacterial pathogens. The longevity of a number of these mechanisms remains unknown.

**7.3 The contribution from commensal bacteria in inflammatory lung disease**

Despite extensive research into the fundamental mechanisms that enhance the susceptibility to respiratory bacterial infections following influenza viral infection, there has been less interest in understanding why specific bacterial species gain access to the lung and from where do they originate. Extensive epidemiological and clinical data identify *S. pneumoniae*, *S. aureus* and *H. influenzae* as the three major bacterial pathogens responsible for invasive secondary bacterial pneumonias in individuals previously infected with influenza virus [412]. A common theme shared between these bacterial pathogens is there ability to asymptomatically colonise the nasopharyngeal cavity of healthy individuals. It is highly unlikely that these bacteria simultaneously co-circulate with seasonal or pandemic influenza viral strains, thus providing a hypothesis that commensal bacteria, residing in the nasopharyngeal compartment, gain access to the lower respiratory tract during times of innate homeostasis disruption, as is evident during a severe influenza viral infection.

Our data show that during an ongoing influenza viral infection there is an increase in the number of commensal bacterial present within the nasopharyngeal compartment and also the appearance of unknown commensal bacteria in the distal airways. An outgrowth of particular commensals almost certainly contributes to the severity of many inflammatory pathologies modelled in animals and human disease. To date there is more evidence for this in the intestinal tract where symbiotic bacteria provide tonic signals that contribute to 'normal' physiological functions such as epithelial homeostasis and the exclusion of pathogenic micro-organisms through binding site competition [601-603]. Mucosal epithelial cells in particular play a pivotal role in discriminating between pathogens and commensals [604]. However they may also contain the ability to sense an increase or alteration in commensal density, which we have shown occurs in the nasopharynx during an ongoing influenza infection. The change in the local microenvironment by influenza is likely to benefit certain commensal species over others. Influenza causes cytopathology of epithelial cells and could effectively reduce key bacterial sensors such as TLR2, TLR4, NOD1 and MyD88 as has been shown in alternative infection models in the intestinal tract [605-607]. Influenza neuraminidase also cleaves sialic acid residues, which was originally thought to contribute to secondary bacterial susceptibility by revealing putative bacterial binding sites. Recent evidence now suggests that cleaved sialic acid actually signals to bacteria to increase their number and act in a feedback loop to promote pneumococcal neuraminidase and virulence factor expression [608]. This increase in bacterial titre combined with a reduction in mucociliary clearance,

enhanced aspiration of nasal mucus, a reduction in macrophage recognition and responsiveness is likely to result in their passage to the lower respiratory tract, effectively providing an opportunity for these otherwise asymptomatic commensals to cause invasive lung disease.

Viral mediated effects on respiratory epithelium will also impact on other innate and adaptive immune cells (DCs and intraepithelial lymphocytes) to which they are intimately associated [609;610]. Which bacterial species outgrow will also depend on the inciting agent and original immune status of the host [611]. A lack of IgA (or its hypermutation) causes the selective expansion of particular commensal species and the depletion of specific T cell subsets, using T bet deficient mice, leads to a bacterial driven immune pathology reminiscent of ulcerative colitis [612-614]. A loss of certain commensals at the expense of others may also be equally important since many encode virulence proteins that interfere with TLR and NF-κB signalling or its cellular location that may play unknown roles in maintaining homeostasis or immune tolerance [604;615].

As discussed in Chapter 6, the local commensal community can also be selectively altered by a change in anti-microbial peptide (AMPs) expression during influenza-induced cytopathology [616]. An alteration in AMPs expression as a result of a primary bacterial infection enhances resistance to a subsequent malarial parasite infection in drosophila [617]. AMPs (abundant in the respiratory tract) also alter with age, with older flies producing higher diptericin levels after live bacteria infection than young adult flies [618]. Although effects that alter with age are attributed to senescence, another interpretation is that components of innate immunity are more tightly regulated as time progresses due to the experience of a greater number of infections. Thus, the older flies have a more experienced innate immune system, and therefore more focused production of diptericin, responding vigorously only in the presence of an infectious pathogen. Similar alterations in innate AMPs may also occur in vertebrates, mammals and even humans. In addition to lysing or opsonising bacteria, some AMPs synergise with cytokines to enhance other pro-inflammatory cytokines and chemokines that recruit bactericidal neutrophils and macrophages [619]. In turn cytokines further enhance AMPs including beta defensins and lipocalin 2 [620;621]. We have shown that a preceding influenza infection might alter the expression pattern of a number of AMPs that in turn could impact on the complex mechanisms involved in determining commensal dynamics and tolerance in the upper respiratory tract. Therefore, the elevated cytokine responses we observe in CD200R KO mice

together with the reduced viral load may therefore improve anti-microbial immunity and explain the deceased susceptibility to subsequent bacterial super-infection following influenza.

Finally, does any alteration in innate immune responsiveness, be that in response to a respiratory viral infection or through chronic inflammation, allow commensal bacteria to outgrow and therefore contribute to inflammatory lung disease? We show that during an acute inflammatory episode bacterial species implicated in human respiratory disease settings dominate the bacteria that appear in the distal airways [545;549;551]. It is known that asthmatics and COPD patients experience periods of disease exacerbation that are associated with bacterial co-pathologies [622-625]. However, it is not known whether the disease exacerbations enhance susceptibility to bacterial infection or that an underlying pre-existing bacterial infection (asymptomatic commensal carriage) proceeds, during periods of innate immune dysregulation, and exacerbate existing inflammatory lung disease. Figure 7.3 highlights this concept with reference to a threshold over which commensal bacteria burden contributes to inflammatory lung disease. There is much data highlighting that a threshold exists in the intestinal tract in order to minimise potential immunopathology. If these intricate host-pathogen mechanisms were to be disrupted in the airway, for example during an acute or chronic lung inflammatory episode, one could envisage a situation in which commensal community dynamics would be altered leading to the outgrowth or dominance of pathogenic species that could lead to or contribute to the inflammatory situation.

**Does commensal burden contribute to inflammatory lung disease?**

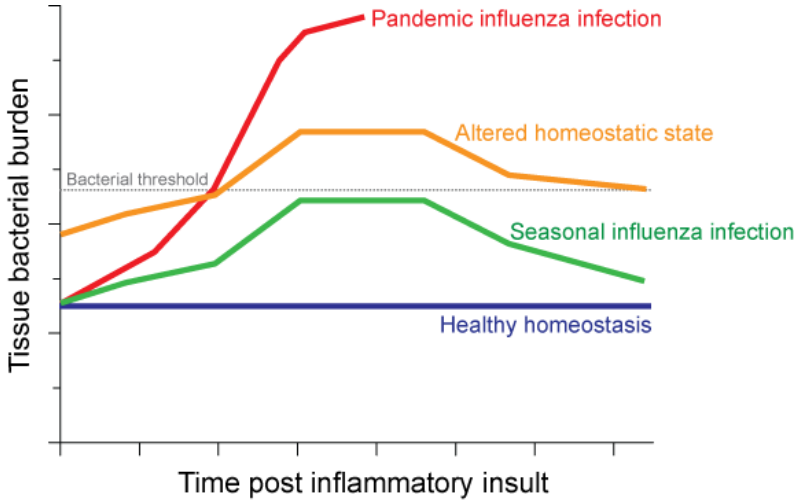



**Figure 7.3 The clinical bacterial threshold.** During homeostasis bacterial commensal burden is kept in equilibrium by host innate immune responses (blue). In times of aberrant innate homeostasis, for example during an influenza infection (green) or an asthmatic/COPD exacerbation (orange), the burden of commensal flora increases and can result in clinical manifestation and contribution to clinical disease. In time of severe innate homeostasis disruption, such as during infection with highly virulent influenza (red), commensal burden increases substantially and can result in invasive bacterial disease.

### 7.4 Antibiotic usage in inflammatory lung disease settings – a clinical case

Based on the data in this thesis one might envisage that antibiotics should routinely be administered during periods of inflammatory lung disease. Unfortunately, it is unlikely to be this straightforward as antibiotic usage increases the dominance of pathogenic species, as is seen with *Clostridium difficile*, and there remains the serious concerns regarding the increasing antibiotic resistance amongst common respiratory bacterial pathogens such as *S. aureus* and *S. pneumoniae* [626-631]. There is also the issue of which antibiotic regimen to give and at what time they should be administered during an inflammatory lung event. This is currently of high interest as the numbers of H1N1 swine influenza infections and attributed deaths continue to rise [632]. McCullers *et al* have show that ampicillin (a β-lactam antibiotic that lyses bacteria) completely clears *S. pneumoniae* from influenza co-infected mice however it also enhances inflammatory cytokine levels and associated mortality. The release of large quantities of bacterial TLR ligands by the antibiotics is thought to initiate and enhance the ongoing innate inflammation. In comparison clinidamycin (a macrolide that inhibits protein synthesis but does not lyse bacteria) treatment reduces TNF-α

levels, weight loss and increased survival despite less effective bacterial clearance [633]. The use of azithromycin, another macrolide protein synthesis inhibitor that also contains anti-inflammatory properties performs even better, but does not fully clear the bacteria from the host [633]. This suggests that a certain level of bacteria can be tolerated without contributing to clinical disease but if a specific threshold of bacterial burden is reached (as described in Figure 7.3) or they are lysed through the use of β-lactam antibiotics then worse inflammation ensues resulting in enhanced mortality. The best strategy therefore may be to use bacteriostatic antibiotics followed by a bactericidal one when bacterial levels are low.

An understanding of how endogenous commensal bacteria contribute to inflammatory disease phenotypes and how combinations of existing anti-microbial therapies might be used to increase host tolerance to bacterial co-pathologies could be an interesting alternative approach to alleviate clinical disease. To date the incidence of secondary bacterial pneumonias following H1N1 swine and H5N1 avian influenza infections remains unknown, however, existing epidemiological studies from previous influenza pandemics suggest we may need to rethink the usage of antiviral and antibiotic combinations in the event of the appearance of secondary bacterial pneumonias [634;635].

### 7.5 Final conclusions

In this thesis, we show how infection history can determine the responsiveness of the airway to subsequent antigen challenge. This is achieved, in part, through long term alterations in regulatory mechanisms that ensure tissue resident macrophages exists in a state of hypo-responsiveness. Whether this is beneficial or detrimental to the host is determined by the severity of the first infection and the precise chronology of pathogens encountered by the host. Furthermore it also highlights the importance of recognizing and understanding the complex bacterial community that exists within the respiratory tract and how infection and/or therapeutic intervention can have unknown consequences to its dynamic composition. Further studies investigating the contribution of commensal bacterial dynamics to the pathogenicity in health and disease will lead to more targeted use of existing antimicrobial therapies and the development of much needed novel therapeutics. Adjustment of the level of innate responsiveness may therefore provide a novel opportunity to prevent life-threatening consequences of lung influenza virus infection.

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Ref Type: Report

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## 9.0 PUBLICATIONS

1. A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. Snelgrove RJ, Goulding J, Didierlaurent AM, Lyonga D, Vekaria S, Edwards L, Gwyer E, Sedgwick JD, Barclay AN, Hussell T. Nat Immunol. 2008 Sep;9(9):1074-83.

2. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M, Lawrence T, van Rijt LS, Lambrecht BN, Sirard JC, Hussell T. J Exp Med. 2008 Feb 18;205(2):323-9. Epub 2008 Jan 28.

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6. The impact of successive infections on the lung microenvironment. Didierlaurent A, Goulding J, Hussell T. Immunology. 2007 Dec;122(4):457-65. Review.

1. CD200R plays a critical role in influenza induced secondary bacterial infections. Goulding J, Snelgrove R & Hussell T. Promega Young Immunologist of the Year 2008 Finalist. British Society of Immunology, Glasgow , November 2008. Oral Presentation.

2. Lung innate immune homeostasis and susceptibility to viral-induced secondary bacterial pneumonia. Goulding J & Hussell T. 3rd Steering and Scientific Committee INCO Meeting. SavinMucoPath. Montevideo, Uruguay. September 2008. Oral Presentation.

3. Influence of respiratory viral infection on secondary bacterial pneumonia. Goulding J & Hussell T. Post Graduate Mucosal Innate and Adaptive Immunity Course, La Plata, Argentina. September 2007. Oral Presentation.

4. Influence of co-infections on immunity and pathology in the lung. Goulding J & Hussell T. 2nd Steering and Scientific Committee INCO Meeting. SavinMucoPath, La Plata, Argentina. August 2007. Oral Presentation.

5. Influenza modulates anti-bacterial immune defences which contribute to post influenza secondary bacterial infection. Goulding J, Snelgrove RJ, Didierlaurent A, Wissinger E & Hussell T. Imperial College Graduate Symposium, July 2007. Poster Presentation.

6. Influenza virus immunity in the face of concurrent respiratory bacterial infection. Goulding J, Gwyer E, Snelgrove R & Hussell T. European Congress of Immunology, Paris. September 2006. Poster Presentation.