The immune response in Influenza A-*S. pneumoniae* coinfection

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I, Gregory Thomas Ellis, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Streptococcus pneumoniae coinfection is a major cause of influenza-associated mortality. In this thesis the underlying disease mechanisms and the role of the immune response are investigated in a mouse model. Coinfection with otherwise mild influenza and *S. pneumoniae* strains is shown to synergistically cause mortality and severe disease. Loss of bacterial but not viral control, and subsequent outgrowth, is identified as the main driver of mortality.

Influenza-mediated immune impairment and lung damage have been proposed as mechanisms of coinfection. Here the aspects of the immune response profiled are not impaired; in contrast, coinfection induces a strong proinflammatory cytokine response and an influx of functional neutrophils. Depletion of neutrophils or TNF-α blockade exacerbates disease and bacterial outgrowth, showing these aspects of the immune response are protective. In addition to profiling the downstream response to bacterial outgrowth, the upstream causes of bacterial colonization are investigated. CCR2$^{-/-}$ mice are shown to be more resistant to coinfection. Influenza-infected CCR2$^{-/-}$ lungs lack inflammatory monocytes and exhibit reduced damage prior to coinfection. How inflammatory monocyte derived damage is mediated is investigated. Blockade of TRAIL - a cell-death inducing ligand - during the viral phase prior to coinfection ameliorates disease. Inflammatory monocytes are shown to comprise the majority of TRAIL-expressing cells during influenza infection, and TRAIL expression is largely absent in CCR2$^{-/-}$ mice. Therefore a mechanism is proposed for coinfection where influenza-induced TRAIL-expressing inflammatory monocytes cause lung

damage, allowing bacterial colonization, while neutrophils and TNF-α counter subsequent bacterial outgrowth. Other aspects of coinfection, such as bacterial spread to the brain and other facets of the immune response, are also investigated.

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Abbreviations

AP-1	Activator protein 1
APC	Allophycocyanin
BAL	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CFU	Colony forming units
cRNA	Complementary RNA
Cy5.5	Cyanine 5.5
Cy7	Cyanine 7
DC	Dendritic cell
dpi	Days post infection
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein
GCSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte macrophage colony stimulating factor
H+E	Hematoxylin and eosin
HA	Hemagglutinin
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
IAV	Influenza A
IFN	Interferon
IFNαβ	Interferon alpha beta receptor
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T cell
IP10	Interferon-gamma induced protein 10

IRF	Interferon regulatory factor
KC	Keratinocyte-derived chemokine
LDH	Lactate dehydrogenase
LIF	Leukaemia inhibitory factor
LIX	Lipopolysaccharide induced CXC chemokine
LPS	Lipopolysaccharide
Ly6(C/G)	Lymphocyte antigen 6(C/G)
M1	Matrix protein 1
M2	Matrix protein 2
MACS	Magnetic activating cell sorting
MCP1	Monocyte chemoattractant protein 1
MCSF	Macrophage colony stimulating factor
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gamma
MIP	Macrophage inflammatory protein
MPO	Myeloperoxidase
NA	Neuraminidase
NEP	Nuclear export protein
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer (cell)
NP	Nucleoprotein
PAFR	Platelet activating factor receptor
PDBu	Phorbol 12,13-dibutyrate
PE	Phycoerythrin
PerCp	Peridinin-chlorophyll
Poly I:C	Polyinosinic:polycytidylic acid
R848	Resiquimod
Rag	Recombination activating gene
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid-inducible gene 1
RNP	Ribonucleoprotein

ROS	Reactive oxygen species
rRNA	Ribosomal RNA
Strep	*Streptococcus pneumoniae*
TCID$_{50}$	Tissue culture infectious dose 50
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor
vRNA	Viral RNA

Chapter 1. General Introduction

1.1. Project overview: Influenza A - *S. pneumoniae* coinfection

Influenza A virus (IAV) is a negative sense RNA virus and major human respiratory pathogen, with recent pandemics in 1918, 1957, 1968 and 2009 (Palese *et al.*, 2004; Nicholls, 2013). *Streptococcus pneumoniae* (*S. pneumoniae/Strep*) is a gram-positive extracellular bacterium that frequently asymptomatically colonizes the human upper respiratory tract. It is one of the most common bacterial respiratory pathogens and can result in complications such as pneumonia and septicaemia (Kadioglu *et al.*, 2008; Henriques-Normark *et al.*, 2013).

Although these pathogens individually are problematic, the serious danger of coinfection has long been recognised. Louis Cruveilhier commented following the 1918 IAV pandemic: "If grippe condemns, the secondary infections execute" (Cruveilhier, 1919). Secondary bacterial coinfections are implicated as the cause of death in the majority of fatal cases in the 1918 IAV pandemic, with *S. pneumoniae* as the most commonly identified secondary infection (Morens *et al.*, 2008). Understanding why IAV promotes susceptibility to *S. pneumoniae* infection is therefore a crucial public health question.

In this thesis the immune response to IAV-*Strep* coinfection is investigated in a mouse model. Crucial roles for neutrophils, tumour-necrosis factor-alpha (TNF-α) and inflammatory monocytes are described. In this general introduction the

biology of both IAV and *Strep*, and of neutrophils, TNF-α and inflammatory monocytes, will be outlined. The substantial medical evidence for coinfection will be described, and the existing literature on coinfections comprehensively summarized.

1.2. Influenza A biology

1.2.1. Viral genome, structure and classification

IAV is one of three influenza types belonging to the *Orthomyxoviridae* family. These types - A, B and C – have common ancestry, but are genetically divergent to the extent where exchange of genetic material cannot occur. Of these types IAV is the most common respiratory pathogen, and is typically restricted to infection of respiratory epithelial cells, although influenza B also causes human disease (Julkunen *et al*., 2000). IAV virions are comprised of eight single-stranded, negative sense RNA segments, which are coated with with nucleoprotein (NP) and the trimeric viral RNA polymerase (PB1, PB2 and PA proteins) to form ribonucleoprotein (RNP) complexes. This virion core is encased in matrix protein 1 (M1), which is further enveloped by a lipid layer containing the surface glycoproteins hemagglutinin (HA) and the neuraminidase (NA) (Bouvier *et al*., 2008). Although there are 16 known HA and 9 known NA subtypes, only H1-3 and N1-2 have caused human pandemics. The ion channel matrix protein 2 (M2) traverses the lipid envelope, with a ratio of M2 to HA ranging from 1:10 to 1:100 (Palese, 2004).

IAV nomenclature is defined by the type, location, isolate number, year of isolation, and subtype; for example A/Puerto Rico/8/1934 (H1N1) indicates type A, isolate number 8 from Puerto Rico, isolated in 1934, with hemagglutinin subtype 1 and neuraminidase subtype 1 (Bouvier *et al.*, 2008).

IAV RNP segments are numbered in order of decreasing length. PB2, PA, HA, NP and NA are encoded by segments 1, 3, 4, 5 and 6 respectively. Segment 2, which codes for PB1, has an alternative reading frame in some IAV strains which encodes PB1-F2, a small pro-apoptotic protein. Segment 7 encodes for both the M1 protein, and, by alternative splicing, the M2. Segment 8 encodes for the non-structural protein 1 (NS1), an antagonist for anti-viral interferon (IFN), and, again due to alternative splicing, the nuclear export protein (NEP) (also referred to as NS2), which is required for viral RNA export from the host cell nucleus (Bouvier *et al.*, 2008).

As the genome is segmented, viral subtypes can "reassort" different segments. Reassortment of segments between different virus strains can lead to major changes in surface glycoproteins in a process known as "antigenic shift". Furthermore the poor fidelity of viral RNA transcription leads to the accumulation of mutations in a process known as "antigenic drift". Although the selective pressure exerted by host anti-HA monoclonal antibodies means the main mutation target for antigenic drift is the stem region of HA, other viral proteins can also be subject to this process. These viral mutations can enable immune evasion and reinfection of a previously IAV-exposed population, and are a major challenge for IAV vaccination (Doherty *et. al.*, 2006; Bouvier *et al.*, 2008).

1.2.2. Viral life cycle

Viral infection of target cells is initiated by the recognition of sialic acid moieties on the surface of upper respiratory tract epithelial cells by HA. Viral HA molecules have differing affinities for α-2,3 or α-2,6 linkages between the terminal sialic acid and galactose, and the distribution of these linkages affects the species preference of different viruses. In human respiratory epithelia α-2,6 linkages are more common overall, although α-2,3 linkages are frequent in the lower respiratory tract (Julkunen *et al.*, 2000; Bouvier *et al.*, 2008).

Viral binding leads to endocytosis by both clathrin-mediated and clathrin-independent mechanisms, and cleavage of the HA by host proteases (Steinhauer *et al.*, 1999; Lakadamyali *et al.*, 2004). The requirement for these proteases and their distribution typically limits the tissue tropism of IAV to respiratory epithelial cells, although macrophages and leucocytes may also be infected; it is technically challenging to assess whether phagocytic and antigen presenting cells are productively infected or have simply ingested virions (Manicassamy *et al.*, 2010). Following endocytosis the reduction in pH causes a conformational change in the HA, which mediates the fusion of the viral and endosomal membranes. Endosomal hydrogen ions are also pumped into the viral core by the M2 protein, weakening protein-protein interactions and leading to release of viral RNPs into the cytoplasm. Viral RNPs are then transported to the nucleus by host proteins due to their nuclear localisation signals. Within the nucleus the viral RNA polymerase, using the viral RNA (vRNA) as a template, synthesises mRNA, for the production of viral proteins, and complementary RNA (cRNA), which serves

as a template for production of more vRNA copies. RNA export from the nucleus is mediated by the association of M1 and NEP. Upon synthesis of viral proteins in the cytoplasm, viral particles are packaged; it is not clear whether this process is random (generating a small number of complete infectious particles) or regulated. NA acts as a sialidase, cleaving surface moieties on the host cell, allowing budding of virus particles; this protein is also subject to "antigenic drift" (; Julkunen *et al.*, 2000; Bouvier *et al.*, 2008, Doherty *et. al.*, 2006).

1.3. *S. pneumoniae* biology

1.3.1. *S. pneumoniae* epidemiology

S. pneumoniae (Strep) – the "pneumococcus" - is a gram-positive extracellular bacterium that is the primary cause of bacterial pneumonia in developed countries, and can result in complications such as meningitis, otitis media and septicemia. It is spread by aerosol and is commonly found residing asymptomatically in the human nasopharynx, although spread to other, previously sterile sites can lead to the complications described. Asymptomatic carriage peaks at approximately 60% in early childhood at around 2-3 years of age, and diminishes to around 10% in adults. There are at least 93 different serotypes of *S. pneumoniae*, defined by their capsular structure. Rates of carriage and virulence vary between different serotypes. The wide variety of serotypes is a major difficulty for vaccination; current vaccines such as the widely used "23-valent" only contain polysaccharides from certain serotypes. Polysaccharide vaccines are poorly immunogenic, and more recently protein-polysaccharide conjugate

vaccines have been introduced. Typically penicillin is used to treat *Strep*, although antibiotic resistance is increasing (Kadioglu *et al.*, 2008; Henriques-Normark *et al.*, 2013).

1.3.2. *S. pneumoniae* microbiology and colonization

Strep has a 2.16 mbps genome, sequence analysis of which indicates it is a facultative anaerobe that depends on the fermentation of 14 different carbohydrates. The surface of *Strep* is comprised of a polysaccharide capsule, overlaying a peptidoglycan and lipoteichoic acid cell wall, which in turn encases the plasma membrane (Henriques-Normark *et al.*, 2013). The capsule can vary between two states - "opaque" and "transparent" - of different thickness, distinguishable by their distinct colony morphologies. During the early stages of colonization, transparent variants are favoured. The capsule has several roles in promoting colonization: it hinders access of leucocytes to complement attached to the underlying cell wall, reduces phagocytosis, and decreases mucosal entrapment (Kadioglu *et al.*, 2008).

In addition to the capsule, *Strep* expresses a number of virulence factors that are involved in colonization, whether through promotion of adhesion, exposure of binding sites, or modulation of the immune response. ChoP promotes bacterial adhesion by binding to human platelet activating factor receptor (PAFR), which is commonly found on human epithelial tissues such as the nasopharynx (Bogaert *et al.*, 2004). Some strains express a pilus like structure, which may bind to an unknown epithelial receptor (Henriques-Normark *et al.*, 2013). CbpA (also known

as PspC) binds to human secretory component, an epithelial glycoprotein that is involved in the transport of immunoglobulin (Ig) across the epithelial surface. Furthermore it binds the complement component Factor H, reducing bacterial opsonisation. PavA and Eno bind to the extracellular matrix components plasminogen and fibronectin respectively. Neuraminidases such as NanA cleave terminal sialic acids found on the human epithelium and therefore may expose binding sites. Other proteins counter aspects of the immune response – for example, PspA interferes with complement deposition and phagocytosis. Secreted pneumolysin (Ply) forms transmembrane pores on the surface of target cells, reduces ciliary beating, and inhibits the phagocytic respiratory burst (Kadioglu *et al.*, 2004; Kadioglu *et al.*, 2008).

1.4. Neutrophil biology

Neutrophils have been shown to play a role in both influenza and *Strep* infections (Sun *et al.*, 2007; Tate *et al.*, 2009), and therefore are of substantial interest in coinfection. Neutrophils are granular, short-lived, terminally differentiated, polymorphonuclear phagocytic cells generated in large numbers in the bone marrow. Neutrophils circulate in the bloodstream and provide one of the first lines of defence against microbes. Production of neutrophils is regulated by granulocyte-colony stimulating factor (G-CSF). During development from hematopoietic stem cells a range of granules are formed, classified by the presence of certain proteins and their order of formation: primary (azurophil) granules containing myeloperoxidase (MPO), secondary (specific) granules containing lactoferrin, and tertiary granules containing gelatinase. These granules

also contain a wide range of other antimicrobial effectors (Borregaard *et al.*, 2010).

Recruitment of circulating neutrophils to sites of infection is mediated by inflammatory stimuli such as Interleukin-1β (IL-1β) and TNF-α, which stimulate the upregulation of P and E-selectin on endothelial cells and lead to circulating neutrophils "rolling" along the vascular endothelium. Firmer attachment is mediated by the interaction of neutrophil integrins with ICAM. Interactions during the attachment process result in activation and cytoskeletal rearrangement within the neutrophil. A complex process mediated by junction-adhesion-molecule A (JAM-A) and other proteins allows neutrophil transendothelial migration into the infected tissue (Borregaard *et al.*, 2010).

Once within the tissue, neutrophils have a wide range of antimicrobial effects. Neutrophils directly phagocytose microbes, creating a phagocytic compartment. This process is greatly augmented by opsonisation of bacteria with antibody - which is recognised by Fc receptors on activated neutrophils - and complement (Lee *et al.*, 2003). A major mechanism of bacterial control is the "respiratory burst", undertaken by Phox (phagocyte oxidase/NADPH oxidase) at the phagocytic membrane, which results in the release of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide, which can directly react with and kill microbes (Segal, 2005). Furthermore, neutrophils release granules containing multiple antimicrobial effectors into the phagocytic compartment and the extracellular space. First discharged are the secondary and tertiary granules, which contain many antimicrobial peptides such as LL37, a potent pore-forming

protein (Duplantier *et al*., 2013), and tissue remodelling factors such as matrix metalloproteinase 9 (gelatinase), which by digesting the extracellular matrix disrupts the vascular basement membrane, aiding further neutrophil influx. Primary granule contents are then released, which contain many components related to digestion and microbial killing. These include myeloperoxidase, which catalyses the formation of potent halide species such as hypochlorous acid from hydrogen peroxide generated by the respiratory burst, and other antimicrobial agents such as pore-forming defensins and neutrophil elastase, a multifunctional serine protease that can degrade bacterial virulence factors (Borregaard *et al.*, 1997; Faurschou *et al.*, 2003; Nathan, 2006).

Another more recently described mechanism of neutrophil killing is neutrophil extracellular traps (NETs), where neutrophil nuclei release their chromatin into the extracellular space. Chromatin directly traps microbes, and is coated in antimicrobial proteins including histones and effectors from azurophillic granules such as myeloperoxidase and elastase (Urban *et al*., 2006). In addition to their described killing functions, neutrophils can produce cytokines such as TNF-α, and generate chemotactic factors such as chemerin; however, on a per-cell basis the capacity of neutrophils to produce cytokine is less than other immune cells such as monocytes (Nathan, 2006).

1.5. TNF-α biology

Tumour necrosis factor alpha (TNF-α) was originally identified as an endotoxin-induced glycoprotein that induced necrosis of sarcomas transplanted into mice. It is now characterised as a canonical pro-inflammatory cytokine primarily produced by immune cells, including macrophages and activated T cells. It is typically only detected in the tissue or serum during infectious or autoimmune conditions (Bradley, 2008). It has a wide range of roles in many different diseases, both protective and pathogenic, including during influenza and *Strep* infection (Takashima *et al*., 1997; Hussell *et al*., 2001), and is therefore of significant interest during coinfection.

TNF-α signals through two distinct receptors – tumour-necrosis factor receptor-1 (TNFR1) and TNFR2, which are widely expressed on most tissues, although hematopoietic cells may preferentially express TNFR2. TNFR1 signals via TNF-receptor associated death-domain (TRADD). This leads to the formation of the TRADD-RIP-1-TRAF2 complex. This complex has two possible downstream activities – activation of a large number of cellular signalling kinases, including p38 and P13K, resulting in the activation of transcription factors such as activator-protein-1 (AP-1) and "nuclear factor kappa-light-chain-enhancer of activated B cells" (NF-κB); or promotion of apoptosis by the binding of "Fas-associated protein with death-domain" (FADD) to TRADD, leading to cleavage of pro-caspase 8. The function of TNFR2 is less well studied; however, as this receptor lacks an intracellular death domain, it is believed to primarily contribute to proinflammatory signalling such as NF-κB activation (Bradley, 2008).

TNF-α signalling promotes a range of biological functions. TNF-α induces upregulation of adhesion molecules such as E-selectin and ICAM1 on endothelia, leading to recruitment of leucocytes such as neutrophils. TNF-α can promote classical inflammatory effects such as vasodilation through induction of cyclooxygenase 2 (COX2). TNF-α has been demonstrated to be a component in endotoxin-induced sepsis, and is essential for protection against bacterial infections such as *S. aureus* (Bradley, 2008). TNF-α promotes the respiratory burst in neutrophils, raises levels of intracellular calcium, and causes actin reorganisation (Nathan, 2006).

1.6. Inflammatory monocyte biology

Monocytes are a mixed population of circulating hematopoietic cells that play many roles in different infections, including influenza and *Strep* (Lin *et al.*, 2008; Davis *et al.*, 2011), that may be of interest during coinfection. Monocytes originate from a common myeloid progenitor, and there are various monocyte subsets in both humans and mice. "Inflammatory" monocytes, as opposed to "resident" monocytes, are typically defined in mice by the strong expression of lymphocyte antigen 6C (Ly6C) and are believed to be equivalent to human CD14^{high} "inflammatory" monocytes (Gordon *et al.*, 2005). Egress of Ly6C^{+} inflammatory monocytes from the bone marrow is dependent on C-C chemokine receptor type 2 (CCR2). CCR2 is the receptor for monocyte-chemoattractant protein 1 (MCP-1/CCL2) and other ligands (Serbina *et al,* 2006, Serbina *et al.*, 2008).

Inflammatory monocytes contribute to innate immunity through a range of mechanisms. Monocytes differentiate into various macrophage and dendritic cell (DC) populations, although the fate of each monocyte subset and their specific role in different tissues is complex. Stimulation of "pattern recognition receptors" (PRRs) - which recognise common motifs in microbial species termed "pathogen associated molecular patterns" (PAMPs) - on monocytes leads to production of cytokines such as TNF-α and IL-1β (Serbina *et al.*, 2008). Inflammatory monocytes also produce reactive nitrogen intermediates through inducible nitric oxide synthase (iNOS). During infections, inflammatory monocytes can develop into a TNF-α and inducible nitric oxide synthase (iNOS)-producing DC (termed "tipDC") phenotype (Aldridge *et al.*, 2009). The crucial role of inflammatory monocytes in some bacterial infections has been clearly demonstrated by the susceptibility of CCR2$^{-/-}$ mice to the intracellular bacterium *L. monocytogenes* (Serbina *et al,* 2006; Serbina *et al.*, 2008).

1.7. The immune response to Influenza A

1.7.1. Overview of the adaptive response

It has long been established that the adaptive response is required for the control of IAV. The adaptive response is believed to be primarily stimulated by the migration of antigen-presenting dendritic cells from the respiratory tract to the lymph nodes or spleen in the first 36 hours following infection. The subsequent response is comprised of two crucial elements – neutralising antibody and cytotoxic T cells. Neutralisation of HA and to a lesser extent NA by B-cell

produced systemic IgG and locally produced IgA can provide sterile immunity to IAV virus. CD8 cytotoxic T cells play a crucial role by recognition of infected cells via major histocompatibility complex class I (MHCI) and subsequent elimination through perforin or Fas-dependent lysis. CD8 T cells tend to recognise more highly conserved elements of the IAV virus such as NP (Doherty *et al.*, 2006). CD4 T cells may play a complementary role through promoting the CD8 response, and by providing cognate T-cell help to B-cells through TCR:MHCII interactions and costimulation via CD40L:CD40 in the spleen and lymph nodes, which promotes antibody production and class switching (Swain *et al.*, 2012). Both CD4 and CD8 T cells, as well as neutralising antibodies and memory B cells, contribute to immunological memory against IAV. Different studies have assessed the role of these adaptive responses during IAV using mice deficient in or depleted of these cells. No individual aspect of the adaptive response is capable of controlling IAV alone; B, CD8 T and CD4 T cell responses are required for clearance and survival (Brown *et al.*, 2004). IAV evades adaptive responses by antigenic drift, which can lead to reduced affinity antibodies and less sensitive CD8 responses. Also, evasion through antigenic shift can lead to a complete lack of pre-existing antibodies, allowing potentially severe infection and rapid spread throughout the population, resulting in pandemics (Schmolke *et al.*, 2010).

1.7.2. Overview of the innate response

Although the adaptive response is critical for control of IAV, the innate response also plays a substantial role, both in priming the adaptive response and in viral control. During influenza, T cells reach the lung after approximately 5 days, and therefore, in a novel infection, early viral replication must be controlled by the innate response. The role of innate immunity in IAV is complex, as viral growth must be controlled without causing excessive immune-mediated damage to the respiratory epithelium (Tripathi *et al*., 2013).

Mucins and soluble mediators

Upon infection of the respiratory tract IAV encounters mucins and soluble mediators. Mucins, which entrap water and create a viscous layer, act as a physical barrier to infection and may entrap viruses, particularly - as mucins are predominately α-2,3 sialylated - influenza strains with affinity for α-2,3 linkages (Nicholls, 2013). Soluble mediators such as such as surfactant-protein A may reduce viral entry by acting as decoy sialic acid receptors for the HA. Other mediators such as surfactant protein-D and mannan-binding-lectin bind directly to other carbohydrate moieties on the HA (Tripathi *et al*., 2013).

Recognition of influenza

IAV induces a cellular immune response dependent on its recognition through either toll-like-receptors (TLRs) or cytoplasmic receptors. In IAV-infected

respiratory epithelial cells, cytoplasmic retinoic acid-inducible gene 1 (RIG-I) recognises the 5'-triphosphate of viral RNA. Recognition leads to a conformational change allowing interaction between the CARD domains of RIG-I and mitochondrial adaptor protein "mitochondrial antiviral-signaling protein" (MAVS). MAVS signals through the kinase TBK1/IKKε, activating the transcription factors interferon regulatory factor 3/7 (IRF3/7). MAVS also activates NF-κB via the kinase RIP1 (Kawai *et al*., 2006). Activation of these transcription factors leads to the subsequent immune response, including cell recruitment and production of cytokines, notably type 1 (IFNα and IFNβ) and type III (IFNλ) interferons (Schmolke *et al*., 2010; Nicholls *et al*., 2013).

In addition, several TLRs have been described to be involved in recognition of influenza. In plasmacytoid dendritic cells, major producers of type 1 interferon, TLR7 recognises IAV single-stranded RNA in the endosome (Diebold *et al*., 2004). Sensing of influenza by TLR3 in human respiratory epithelial cells has been described (Le Goffic *et al.*, 2007). TLR4 deficient mice show reduced lung injury following severe influenza infection, suggesting TLR4 is in involved in influenza recognition (Imai *et al*., 2008). Furthermore, inflammasome-activating NOD-like-receptors (NLRs) have also been described to play a role during influenza (Allen *et al*., 2009).

Activation of the cellular immune response

RIG-I signalling leads to the production of type 1 interferons (IFN), which signal via the IFNαβ receptor. This causes formation of STAT1/2 heterodimers, which migrate to the nucleus and associate with IRF9. This complex binds interferon-

stimulated regulatory elements (ISREs), leading to the transcription of many interferon-stimulated genes (ISGs). ISGs include antiviral proteins such as Mx and IFITM3, and mediators of downstream immune responses (Trincheri *et al*., 2010; Iwasaki *et al*., 2014). To counter this, IAV NS1 protein antagonises the type 1 - and potentially type III - interferon response at multiple levels. These include associating with RIG-I to prevent signalling, reducing host gene expression by preventing 3' polyadenylation of pre-mRNA, and directly interfering with antiviral ISG function (Schmolke *et al*., 2010). RIG-I signalling also leads to the activation of NF-κB, which contributes to the induction of various proinflammatory cytokines. Cytokines such as TNF-α, IL-6 and IFN-γ are secreted in response to IAV infection (Julkunen *et al*., 2000; Wack *et al*., 2011).

The cellular response to IAV is initially mediated by lung-resident alveolar macrophages, which upon activation become highly phagocytic and produce proinflammatory cytokines. Infected epithelial cells secrete chemokines such as MCP-1, leading to monocyte recruitment and differentiation into effector cells such as monocyte-derived DCs (Schmolke *et al*., 2010). Activation of dendritic cells leads to migration to the lymphoid tissues and antigen-presentation, prompting the adaptive immune response. IAV infection also results in the recruitment of natural killer (NK) cells and neutrophils, and prompts an IL-17 response from γδ T cells (Nicholls *et al*., 2013; Tripathi *et al*., 2013).

1.7.3. Neutrophils in Influenza A infection

The role of neutrophils in IAV infection is not yet clear, and it may be dependent on the severity of the influenza infection. Mouse models clearly show recruitment of neutrophils to the lung early during influenza infection (Tate *et al.*, 2009). Several studies have used mouse models to investigate the role of neutrophils during influenza.

The protective role of neutrophils during IAV

Several studies have attempted to directly increase or decrease the neutrophil response to assess their role. RB6 - a monoclonal antibody against Gr-1 – is commonly used for depletion. Gr-1 is an epitope found on both the cell surface molecules Ly6G (specific for neutrophils) and Ly6C (highly expressed on inflammatory monocytes and at various lower levels on other cells, including neutrophils, dendritic cells and lymphocyte subsets) (Daley *et al.*, 2008), and therefore this antibody has poor specificity. RB6 treatment exacerbates both severe (Tumpey *et al.*, 2005; Fujisawa, 2008) and mild (Tate *et al.*, 2008, Tate *et al.*, 2011) IAV infection. However, due to the low specificity of neutrophil depletion, these results are difficult to interpret.

The monoclonal antibody 1A8, which targets Ly6G, has also been used to more specifically deplete neutrophils. In some studies 1A8 treatment exacerbates mild (Tate *et al.*, 2009; Tate *et al.*, 2011) and severe (Dienz *et al.*, 2012) IAV infection. Gain of function approaches have also been taken - mice overexpressing the

neutrophil promoting cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) have increased resistance to severe influenza (Huang *et al.*, 2011). However, GM-CSF has many roles, so it is not clear whether this increased protection is due to greater neutrophil numbers. Overall these studies suggest that neutrophils play a protective role in influenza, possibly limiting viral replication during the early phase of infection. One study shows neutrophil depletion is only deleterious if performed early in influenza (Tate *et al.*, 2011), which would support this hypothesis.

The harmful role of neutrophils during IAV

However, other studies, albeit generally using more indirect readouts, suggest that neutrophils may be detrimental and result in tissue damage and pathology during influenza infection. In contrast to the previous results, one study did not observe a significant effect of 1A8 treatment during mild IAV infection, and furthermore suggested neutrophil NETs may contribute to lung damage (Narasaraju *et al.*, 2011). Severe pathogenesis induced by an IAV strain bearing the 1918 pandemic HA and NA was characterised by massive neutrophil recruitment to the lungs (Kobasa *et al.*, 2004). Furthermore IFNαβR$^{-/-}$ mice were more susceptible to severe influenza infection, which is attributed to reduced inflammatory monocyte and increased neutrophil recruitment (Seo *et al.*, 2011). This suggested neutrophils are harmful, although the lack of type I interferon signalling in this study may lead to deficiencies in other aspects of the immune response, so it is difficult to draw clear conclusions. Also notably, mice unable to produce reactive oxygen species were better protected during influenza, although the total number

of neutrophils in these mice was increased (Snelgrove *et al*., 2006). Mice lacking the receptor for IL-17 (a potent neutrophil chemoattractant) recruited less neutrophils and had reduced disease severity during severe IAV infection (Crowe *et al*., 2009), although there are other possible interpretations of this study, such as a detrimental role for Th17 cells during influenza. In summary, neutrophil may contribute to pathology during influenza, possibly through ROS production and NET formation.

Other aspects of neutrophils during IAV

Other studies suggest neutrophils may affect the downstream adaptive response. Neutrophils may contribute to antigen presentation to CD8 T cells during IAV infection (Hufford *et al*., 2012). Neutrophils are a component of the myeloid-derived-suppressor-cell (MDSC) population, cells that can suppress T-cell proliferation through arginase and nitric oxide synthase 2 activity. This can cause harmful immunosuppression during IAV infection. This is ameliorated by invariant-natural killer T cells (iNKTs), which reduce MDSC proliferation and arginase/nitric oxide synthase 2 activity (De Santo *et al*., 2008).

Overall the role of neutrophils in influenza is not yet clear, and in mouse models may be highly dependent on the experimental context, particularly the severity of the influenza virus infection. It is likely they help control viral replication, but in a setting inducing excess inflammation can contribute to pathology.

1.7.4. TNF-α in Influenza A infection

Mouse models show that TNF-α is produced in the lungs within 2-4 days following IAV infection (Vacheron *et al.*, 1990; Hennet *et al.*, 1992). However, the role of TNF-α in influenza is not clear, and appears complex - possibly causing excess pathology during severe infections, but protective in others.

TNF-α has been shown to reduce viral replication in a porcine epithelial cell line (Seo *et al.*, 2002), and increase expression of signalling proteins such as RIG-I crucial to viral recognition in a human epithelial cell line (Matikainen *et al.*, 2006). Both TNFR1 and TNFR2 signalling has been shown to regulate the number of effector CD8 T cells in the lung (Turner *et al.*, 2004; DeBerge *et al.*, 2014). These results suggest that TNF-α is antiviral and has a role in regulating the adaptive response.

In vivo mouse models of influenza investigating TNF-α give a range of results. Several severe disease models attempt to abrogate TNF-α signalling to reduce immunopathology. Anti-TNF-α treatment slightly reduced weight loss or mortality in two severe IAV models (Peper *et al.*, 1995; Hussell *et al.*, 2001;). TNFR1$^{-/-}$ mice exhibited slightly reduced weight loss in severe influenza models using H5N1 avian IAV strains or reconstructed 1918 pandemic virus (Szretter *et al.*, 2007; Belisle *et al.*, 2010). However, somewhat surprisingly, TNF-α$^{-/-}$ mice exhibit slightly increased weight loss and more severe inflammation during mild IAV infections (Damjanovic *et al.*, 2011; DeBerge *et al.*, 2014). The somewhat marginal weight loss differences observed in these studies suggest that TNF-α

deficiency alone does not greatly affect influenza infection. The contradictory results suggest that the beneficial or harmful role of TNF-α in influenza may depend on disease severity.

Other studies have given further information on the role of TNF-α by attempting to block multiple proinflammatory cytokines induced upon influenza infection. Combined blockade of TNF-α, IL-6 and IL-1β reduced weight loss in a severe IAV model (Swiergel *et al*., 1999), while mice deficient in both TNFR1, 2 and IL-1 receptor had marginally delayed mortality during lethal H5N1 infection, although not during another severe IAV strain (Perrone *et al*., 2010). Furthermore, another study showed that TNF-$\alpha^{-/-}$, TNFR1$^{-/-}$ or TNFR1$^{-/-}$TNFR2$^{-/-}$ mice were not better protected against a lethal H5N1 infection (Salomon *et al*., 2007). Mice lacking the IL-17 receptor had lower levels of TNF-α, but were better protected from severe IAV (Crowe *et al*., 2009). Taken together, these studies suggest that TNF-α does increase pathology during severe IAV infections, but this effect is relatively small and somewhat variable depending on influenza severity. Therefore the role of TNF-α in IAV as protective or pathogenic during influenza is not yet clearly defined.

1.7.5. Inflammatory monocytes in Influenza A infection

The role of inflammatory monocytes in IAV is complex; although they appear to promote early immunopathology, they may be required for full adaptive responses, depending on the infection context. Inflammatory monocyte migration to the lungs during IAV infection is CCR2 dependent (Herold *et al*., 2006), and

therefore CCR2 deficient mice are frequently used to investigate the role of inflammatory monocytes.

Inflammatory monocyte mediated lung damage during IAV

Many studies suggest that inflammatory monocytes cause lung damage during IAV infection. CCR2$^{-/-}$ mice were shown to have reduced lung damage during severe IAV infection (Lin *et al*., 2008; Herold *et al*., 2008), while a CCR2 small molecule inhibitor reduced damage and mortality (Lin *et al*., 2011). Further studies have supported these findings, but also reveal a role for inflammatory monocytes in promoting T cell responses to IAV. CCR2$^{-/-}$ mice have reduced early pathology but somewhat reduced T cell expansion in the lymph nodes, leading to slightly increased viral titres (Dawson *et al*., 2000). A subsequent study shows inflammatory-monocyte derived tipDCs cause immunopathology during severe IAV infection, but were required for a full CD8 T cell response (Aldridge *et al*., 2009).

Other studies use alternative methods to CCR2 deficiency to assess the monocyte response to influenza. Absence of the CCR2 ligand MCP1 has only modest effect - MCP1$^{-/-}$ mice or mice treated with anti-MCP-1 exhibit similar disease (Dessing *et al*., 2007; Narasaraju *et al*., 2010), although anti-MCP-1 may reduce inflammation in mild influenza (Damjanovic *et al*., 2011). Another study implies monocytes are protective using treatment with muramyl dipeptide. Muramyl dipeptide is a PAMP that stimulates NOD2, an intracellular receptor that recognises bacterial peptidoglycan. Muramyl dipeptide treatment increases MCP1

levels and monocyte recruitment during IAV, and is protective (Coulombe *et al*., 2012). However stimulation of NOD2 may have other effects, making this study somewhat difficult to interpret. Overall inflammatory monocytes appear to cause early damage during IAV, but can also play a role in downstream T cell responses, and therefore whether in a given context they are protective or harmful is not yet clear.

TRAIL as a mechanism of inflammatory-monocyte mediated damage

Other studies have focused on the molecular mechanisms of inflammatory monocyte mediated damage. One molecule that has been heavily implicated in inflammatory-monocyte mediated damage during influenza infection is TNF-related apoptosis-inducing ligand (TRAIL) (Herold *et al*., 2008). TRAIL was originally identified genetically due to its homology to TNF superfamily ligands, and was initially mainly studied in the context of tumour cell apoptosis. More recently, roles for TRAIL in the immune system and during infection have been explored. TRAIL is a membrane bound or soluble ligand that induces apoptosis on target cells through two death receptors in humans and one in mice – death receptor 5 (DR5). Mice also encode decoy receptors that can increase resistance to TRAIL-mediated apoptosis by reducing ligand availability or activating cell survival pathways (Benedict *et al*., 2012).

In vitro studies show IAV induces TRAIL expression on immune cells, and demonstrate TRAIL-mediated lysis of various cell lines. IAV increases TRAIL expression on human peripheral blood mononuclear cells (PBMC), which leads to

killing of a melanoma-derived cell line. Furthermore IAV infection of a human lung epithelial cell line increases susceptibility to lysis mediated by recombinant TRAIL (Brincks *et al*., 2008). Human-monocyte-derived macrophages treated with influenza upregulate TRAIL, and can kill a human T cell line in a partially-TRAIL dependent manner (Zhou *et al*., 2006). In a human lung epithelial cell line TRAIL is directly induced by IAV and promotes increased viral replication (Wurzer *et al*., 2004).

In vivo studies have confirmed that TRAIL-mediated killing is a mechanism for inflammatory-monocyte mediated epithelial damage during severe IAV infection. TRAIL expressed by inflammatory monocytes (in one study cited here termed "exudate macrophages") leads to the apoptosis of DR5 expressing alveolar epithelial cells and pathology; and therefore anti-TRAIL treatment improves survival (Herold *et al*., 2008; Davidson *et al*., 2014). Inflammatory monocyte upregulation of TRAIL may be dependent on IFN-β derived from alveolar macrophages (Hogner *et al*., 2013). However, the role of TRAIL is still somewhat unclear, as TRAIL^{-/-} mice are more susceptible to severe IAV, as TRAIL may be involved in the CD8 T cell clearance of infected cells, and regulation of the overall CD8 T cell response (Brincks *et al*., 2008; Brincks *et al*., 2011). In summary TRAIL expressed by inflammatory monocytes causes damage during IAV infection; however, different studies have given contradictory results on whether the overall presence of TRAIL during influenza is beneficial.

1.8. The immune response to *S. pneumoniae*

1.8.1. Overview of the adaptive response

The adaptive response plays a crucial role in countering *Strep* infection. Antibody-mediated complement-dependent opsonophagocytosis is believed to be the primary mechanism of *Strep* control, and the presence of serotype-specific IgG against the bacterial polysaccharide capsule correlates with protection. Traditional "23-valent" polysaccharide vaccines attempt to elicit this antibody response. However, polysaccharide antigens are poorly immunogenic, and therefore more recently polysaccharide-protein conjugate vaccines, which elicit T cell help and lead to stronger antibody responses, have been developed (Casal *et al*., 2003).

Preformed "natural antibodies" - antibodies formed without prior *Strep* infection, produced in response to normal gut flora - against phosphocholine moieties in the bacterial cell wall have also been described to be protective (Mold *et al*., 2002). In addition to promoting humoral immunity, CD4 T cells may also play a further protective role. CD4 T cells infiltrate rapidly to *Strep* infected tissues in a pneumolysin-dependent manner, and may be required for bacterial clearance through induction of a Th1 response (Kadioglu *et al*., 2008).

1.8.2. Overview of the innate response

Innate immunity to *Strep* infection is essential for bacterial recognition and mediating - in conjunction with the adaptive response - opsonophagocytosis. Furthermore the innate response plays a role in activating adaptive immunity (Casal *et al.*, 2003).

Recognition of S. pneumoniae

Strep infection is detected through multiple pathways. TLR2 recognises streptococcal cell wall lipoteichoic acid and other lipopeptides. TLR4 - which senses lipopolysaccharide (LPS) from gram-negative bacteria - is also involved in the response to *Strep*, possibly through recognition of pneumolysin. Streptococcal DNA is sensed by TLR9, while both pneumolysin and streptococcal DNA can induce inflammasome activation. Streptococcal DNA may also induce type 1 IFN signalling via stimulator of interferon genes (STING) (Koppe *et al.*, 2012). In addition, the cytoplasmic receptor NOD2 senses cell wall peptidoglycan digested by lysozyme in phagocytes. (Davis *et al.*, 2011). In response to streptococcal recognition, many pro-inflammatory cytokines are induced and contribute to defence, including IL-6, IL-12, IL-17, IL-18 and TNF-α (Casal *et al.*, 2003; Bordon *et al.*, 2012).

Opsonophagocytosis of S. pneumoniae

Neutrophils and complement play, in conjunction with antibody, a crucial role in opsonophagocytosis of *Strep*. Neutrophil chemotaxis to *Strep* infected sites is promoted by both cytokines and bacterial components such as pneumolysin. Deposition of complement C3 on the surface of the bacteria is essential for protection against *Strep*. The relative contributions of the classical complement pathway, which is activated through antibodies bound to the surface of the bacterium and is considered an aspect of the adaptive response, or the alternative and lectin pathways, which are activated by direct binding of bacterial cell surface components and therefore are considered aspects of innate immunity, is still unclear. In addition to complement, soluble mediators such as the mucosal antimicrobial peptide surfactant protein D can promote opsonophagocytosis by binding streptococcal carbohydrates, causing bacterial aggregation and increasing neutrophil uptake (Casal *et al.*, 2003; Kadioglu *et al*., 2004).

Other aspects of the innate immune response to S. pneumoniae

Complement may also be involved in activating adaptive immunity, as splenic marginal zone B-cells and follicular dendritic cells both express high levels of complement receptors, and these receptors appear to be required for the full induction of humoral immunity to *Strep* (Casal *et al.*, 2003; Kadioglu *et al*., 2004). Macrophages also contribute to streptococcal control by cytokine production and phagocytosis (Weiser, 2010).

1.8.3. Neutrophils in *S. pneumoniae* infection

The antibacterial role of neutrophils during *Strep* infection has been extensively studied. Neutrophils migrate into *Strep* infected mouse lungs (Mizgerd *et al.*, 1996), and as previously outlined their role in opsonophagocytosis has been long established, as human neutrophils can kill *Strep in vitro* in an antibody and complement dependent manner (Janoff *et al.*, 1999).

Some *in vivo* mouse pneumonia models indirectly suggest that neutrophils are protective during *Strep* infection. Mouse strains that recruit more neutrophils during *Strep* infection are more resistant (Gingles *et al.*, 2001). Overexpression of the neutrophil-promoting cytokine GM-CSF two weeks prior to streptococcal infection is protective (Steinwede *et al.*, 2011), and neutrophils can also produce IFN-γ, which is shown to be protective, in response to *Strep* (Yamada *et al.*, 2011).

Studies more directly addressing the role of neutrophils give more mixed results. Poorly specific neutrophil depletion by RB6 treatment exacerbates moderate *Strep* pneumonia (Sun *et al.*, 2007; Zhang *et al.*, 2009) and meningitis (Mildner *et al.*, 2008), although another pneumonia study finds RB6 reduces bacterial loads during severe pneumonia (Marks *et al.*, 2007). Another study finds no effect of RB6-treatment during mild pneumonia (Stegemann *et al.*, 2009). These discrepancies may be due to the use of different streptococcal isolates in each study, as well as different mouse strains and pathogen doses, giving different disease severities. It may be that, as is the case during IAV infection, neutrophils

are protective or pathogenic depending on disease context. Furthermore, despite the clear relevance of neutrophils to *Strep* control shown in some mouse models and *in vitro* studies, a strong association has not yet been found between human phagocyte deficiencies and opportunistic *Strep* infection (Rosenweig *et al*., 2009).

Another notable study, although in a somewhat unusual context, shows specific neutrophil depletion using anti-Ly6G treatment increases bacterial loads in an infant mouse *Strep* carriage model (Short *et al*., 2012). This suggests that neutrophils help control bacterial load during *Strep* carriage. Notably, the higher bacterial loads result in greater transmission of *Strep* to cohoused "contact" mice infected with influenza, which is also relevant to coinfection.

Strep uses different mechanisms to evade and counter the neutrophil response. An *in vitro* study shows *Strep* promotes neutrophil cell death (Zysk *et al*., 2000). *Strep* expresses endonucleases that enable evasion of NET-mediated killing (Beiter *et al*., 2006). Furthermore the bacterial capsule helps evade the opsonophagocytic neutrophil response by reducing complement deposition, interfering with both the classical and alternative complement pathways (Hyams *et al*., 2010).

1.8.4. TNF-α in *S. pneumoniae* infection

TNF-α is one of many proinflammatory cytokines induced during *Strep*, and is believed to have a protective role. *Strep* cell wall components stimulate TNF-α production by human monocytes (Heumann *et al*., 1994). Mouse models have shown that TNF-α is upregulated during *Strep* pneumonia, and anti-TNF-α treatment increases bacterial loads and decreases survival (Van der Poll *et al*., 1997), as well as decreasing immune cell numbers (Takashima *et al*., 1997). Another study corroborated these findings, and identified Gr-1$^+$ cells as the major source of TNF-α (Hatta *et al*., 2010). Furthermore, TNFR1$^{-/-}$ mice have higher bacterial loads when challenged with *Strep* (Kerr *et al*., 2002).

In contrast, another study found that TNF-α was not required for protection or pulmonary inflammation during a mild *Strep* infection, but helped prevent systemic disease in a lethal setting (Kirby *et al*., 2005). This may suggest TNF-α may only be crucial in more severe *Strep* infections. There may be some redundancy between TNF-α and IL-1β, as IL-1R1$^{-/-}$ mice show increased susceptibility to *Strep* only when also treated with anti-TNF-α (Rijneveld *et al*., 2001). This is supported by the increased bacterial loads and reduced neutrophil infiltrate observed in TNFR1$^{-/-}$TNFR2$^{-/-}$IL-1R1$^{-/-}$ "triple mutant" mice (Jones *et al*., 2005); "single mutant" TNFR1$^{-/-}$, TNFR2$^{-/-}$ or IL-1R1$^{-/-}$ mice did not show an impairment in neutrophil recruitment. In summary the majority of studies suggest TNF-α plays a protective role promoting pulmonary inflammation during *Strep* infection, but this may be dependent on the severity of the bacterial strain used, and there may be some redundancy with other proinflammatory cytokines.

1.8.5. Inflammatory monocytes in *S. pneumoniae* infection

The role of CCR2-dependent inflammatory monocytes in *Strep* infection has not been extensively studied. In one study a protective monocyte/macrophage (in this case defined as F4/80$^+$CD11b$^-$, which may not be the cell population defined here as inflammatory monocytes) influx is reported to aid bacterial control. CCR2$^{-/-}$ mice have reduced monocyte/macrophage recruitment and moderately higher bacterial loads following *Strep* infection. The protective monocyte/macrophage influx is dependent on NOD2-sensing of bacterial peptidoglycan digested by lysozyme in phagocytes. NOD2 sensing also promotes downstream antibacterial antibody responses (Davis *et al.*, 2011). Consistent with this, overexpression of MCP-1 in the lung increased mononuclear phagocyte recruitment (here defined as F4/80$^+$CD11b$^+$CD11c^{low}) and reduced bacterial load during *Strep* pneumonia, although the danger of excessive immune responses was highlighted as MCP1 overexpressing mice developed bronchiolitis obliterans (an inflammatory or fibrotic narrowing of the bronchioles) (Winter *et al.*, 2007). In a different setting, CCR2 deficiency did not affect a mouse model of streptococcal meningitis (Mildner *et al.*, 2008). Overall CCR2 dependent monocyte recruitment appears to be moderately beneficial during *Strep* infection, although this is dependent on the experimental context, and excessive monocyte recruitment may lead to lung damage.

The role of TRAIL - a key inflammatory monocyte effector - in *Strep* infection has been investigated by two studies. Human monocytes stimulated with *Strep* or type 1 IFN *in vitro* upregulate TRAIL (Halaas *et al.*, 2004). TRAIL$^{-/-}$ mice are

more susceptible to a moderate severity strain of *Strep* (Steinwede *et al*., 2012). It is proposed that TRAIL-expressing cells promote apoptosis rather than necrosis of DR5 expressing macrophages, which promotes bacterial killing. The protective or pathogenic role of TRAIL has not been investigated in more mild or severe *Strep* disease contexts, and therefore remains to be determined.

1.9. Medical evidence for coinfection

Coinfections in the 1918 pandemic

There is a large body of medical evidence showing that IAV predisposes to secondary bacterial infections, particularly *Strep*. An analysis of bacterial culture records from autopsies in the 1918 "Spanish Flu" pandemic revealed that over 95% of fatal cases had bacterial colonization in the lung (Morens *et al*., 2008). *Strep* was the most common bacterium identified, present in approximately 24% of cases. This suggests that many or even the majority of those killed in this pandemic may have died of coinfection.

Coinfections in the 2009 pandemic

More recent evidence from the 2009 "swine flu" IAV pandemic, where coinfection rates are likely to be reduced due to widespread antibiotic use, has supported this view. However there is substantial variation in the reported proportion of coinfections, possibly reflecting the different treatment and screening protocols in different regions. Many studies were carried out in the

United States. A report on postmortem samples from eight different US states showed 29% had evidence of coinfection, with *Strep* again the most common (Centre for Disease Control, 2009). Another US study of 100 fatal cases found 25% showed evidence of coinfection, with *Strep* and *S. aureus* as the most common (Shieh *et al*., 2010). An analysis of medical records in New York found histological or microbiological evidence for coinfection in 55% of fatal cases (Gill *et al*., 2009). A screening of nasopharyngeal swab samples from pandemic patients most commonly detected *S. aureus*, *S. pneumoniae*, and *H. influenzae*, at approximately 15, 10 and 5% of patients respectively (Koon *et al*., 2010). Somewhat contradictorily, a study assessing the risk factors associated with death or hospitalisation in patients in California found bacterial coinfections in only 4% of patients (Louie *et al*, 2009).

Studies in other countries also show variable rates of coinfection. An observation of 337 patients on mechanical ventilation in Argentina showed 8.3% had *Strep* coinfection (Estenssoro *et al*., 2010), while another study on hospitalised patients in Spain found 62% had *Strep* coinfection (Cilloniz *et al*., 2012). An Australian analysis found a 25% coinfection rate in 2009 pandemic cases, with *S. aureus* as the most common (Blyth *et al*., 2013). A South African study of *Strep* bacteraemia from 2009-11 found prior influenza in 8% of patients, and a correlation with increased pneumococcal load (Wolter *et al*., 2013).

Whether coinfection is more common during pandemic or seasonal influenza, and whether this affects the severity of pandemics, is not yet clear. A study assessing seasonal versus pandemic IAV from 2005-2009 in Sweden found lower levels of

coinfection during the pandemic, although again identified *Strep* as the most common coinfection (Liderot *et al.*, 2013). In contrast to this, a study on American children found more cases of coinfection in pandemic than non-pandemic influenza, identifying *Strep* and *S. aureus* the most common (Dawood *et al.*, 2013). Furthermore incidence of invasive pneumoccal pneumonia was higher during 2009 than 2008 in Barcelona, correlating with the influenza pandemic (Pedro-Botet *et al.*, 2014). Overall, a wide range of evidence suggests *Strep* and to a lesser extent *S. aureus* coinfection was a widespread complicating factor during the 2009 IAV pandemic.

Coinfections from seasonal influenza

In addition to reports from IAV pandemics, another study has reported a correlation between seasonal IAV and the incidence of invasive pneumococcal pneumonia (Fleming-Dutra *et al.*, 2013). Mathematical models have reinforced this conclusion (Grabowska *et al.*, 2006), and proposed there is a short term "susceptibility window" following IAV infection (Shrestha *et al.*, 2013). Another model proposes an association between *Strep* meningitis and prior viral infection (Opatowski *et al.*, 2013). In summary, there is substantial evidence for both IAV-*Strep* coinfection and other secondary bacterial coinfections, both in pandemic and seasonal settings, and therefore investigating this phenomenon is highly relevant to public health.

1.10. *In vitro* models of coinfection

1.10.1. Influenza A-*S. pneumoniae* models

Studies of IAV-*Strep* coinfection generally attribute mortality to a failure to control the bacterial infection, and propose mechanisms that fit into two broad categories. Firstly, those suggesting prior influenza leads to immune impairment in the antibacterial response to *Strep*. Secondly, those proposing lung dysfunction - such as epithelial damage or increased bacterial adhesion - allows *Strep* colonization. Many studies have investigated these causes of coinfection *in vitro*.

Immune impairment

Several studies have investigated different aspects of immune impairment during coinfection. The combination of IAV and bacteria may lead to immune impairment through neutrophil dysfunction. IAV accelerates *E. coli* induced human neutrophil apoptosis (Colamussi *et al*., 1999), and a subsequent study reproduced this result with *Strep* (Engelich *et al*., 2001). However, IAV also caused increased neutrophil function such as ROS production and phagocytosis, contrary to the concept of "impairment". Combining these two concepts, excess apoptosis was later attributed to cooperative IAV-*E. coli* stimulation of the respiratory burst (Engelich *et al*., 2002). Therefore it appears that IAV can lead to hyperactivation and excess neutrophil death, although whether this would represent "impairment" *in vivo* is not clear.

Contrasting with the concept of immune impairment, two studies have observed greater release of proinflammatory cytokines from human monocyte-derived dendritic cells when stimulated with IAV and *Strep* than with each stimuli alone (Wu *et al*., 2011; Kuri *et al*., 2013). In summary although *in vitro* studies suggest IAV can lead to neutrophil dysfunction, it is not clear whether strong immune *in vivo* impairment occurs.

Epithelial adhesion

Other *in vitro* studies have suggested IAV allows greater epithelial adhesion by *Strep*. IAV and other viral infections increase the adherence of *Strep* and *H. influenzae* to primary and immortalised cell lines (Avadhanula *et al*., 2006). IAV also increases *Strep* adherence to a human alveolar cell line (McCullers, 2004). This effect is ameliorated by inhibition of the viral neuraminidase, which may expose bacterial binding sites on epithelial cells through sialic acid cleavage. However, in this instance it is not clear whether the protective effect of neuraminidase inhibition is due to prevention of pneumococcal binding site exposure or treatment of the underlying viral infection.

Secondary *Strep* infection may also have an affect on primary IAV infection. Blockade of influenza replication in a Madin-Darby canine kidney (MDCK) cell line by a viral neuraminidase inhibitor is rescued by addition of a streptococcal neuraminidase, suggesting some cooperation between pathogens in viral release (Nishikawa *et al*., 2012). In summary *in vitro* models suggest that IAV can increase *Strep* adhesion to epithelial cells, and may have effects on viral release.

1.10.2. Other *in vitro* coinfection models

Although *Strep* appears to be the most common secondary coinfection following IAV many other coinfections have been detected. This may indicate that IAV promotes susceptibility to secondary infections through a broad, non-specific mechanism, or through multiple different mechanisms. Other coinfections following IAV have also been investigated *in vitro*, and may also provide useful insights into IAV-*Strep* coinfection. As with IAV-*Strep* coinfection models, they generally suggest immune impairment or increased epithelial adhesion as mechanisms of coinfection.

Immune impairment

One possible mechanism of immune impairment is the antagonism of Type I IFNs with IL-1β production in human bone-marrow-derived macrophages (Guarda *et al*., 2011), which is of interest as type I IFNs are induced in influenza infection. Type I IFN-IL-1β antagonism may be mediated through the induction of nitric oxide, which inhibits the NLRP3 inflammasome (Hernandez-Cuellar *et al*., 2012). Components of the type I IFN signalling pathway may also inhibit other proinflammatory cytokine production. RIG-I stimulation in mouse peritoneal macrophages leads to activation of IRF3, which can bind to the IL-12 promoter and directly suppress IL-12p40 protein production, causing susceptibility to *L. monocytogenes* (Negishi *et al*., 2012). However, in contrast to effect of interferon alone in these studies, human peripheral blood leucocytes produce more TNF-α and IL-1β upon endotoxin treatment with prior IAV stimulation (Ludemose *et al*.,

1993). Overall, it is possible to speculate that IAV derived type 1 IFN may hinder aspects of the immune response, such as the inflammasome; however, a combined IAV-bacterial stimulus may also lead to more proinflammatory cytokine production.

Epithelial adhesion

Other *in vitro* coinfection models also show that influenza can promote bacterial epithelial adhesion. IAV increases adhesion of *S. aureus* to alveolar cell lines (Passariello *et al*., 2011), and promotes invasion by methicillin-resistant *S. aureus* into the MDCK cell line (Takayama *et al*., 2014). Furthermore, swine H1N1 IAV increases adhesion of *S. suis* (a streptococcus swine pathogen) to a swine tracheal epithelial cell line (Wang *et al*., 2013). Therefore IAV appears to increase bacterial adhesion to epithelial cells in multiple species.

1.11. Mouse models of coinfection

1.11.1. Characteristics of Influenza A-*S. pneumoniae* mouse models

There are many *in vivo* mouse models suggesting a range of mechanisms for coinfection. Similar to results from *in vitro* models, either IAV-mediated immune impairment or lung damage/dysfunction are generally proposed as mechanisms. The majority of models focus on acute coinfection in the first two weeks following influenza infection, and share several common characteristics. Typically a window of susceptibility to secondary infection from 3 to 14 days post

influenza is observed, with greatest susceptibility at approximately 7 days (McCullers *et al.*, 2002). Also observed is loss of bacterial control in the lung and subsequent dissemination to other organs (Goulding et al., 2011). In some models using severe influenza infections, coinfection delays viral clearance (Smith et al., 2013). Increases in many proinflammatory cytokines are also frequent (Smith et al., 2007).

1.11.2. Immune impairment in coinfection

Several different aspects of the immune response have been reported to be impaired during IAV-*Strep* coinfection. While most models focus on acute coinfection, influenza can lead to long-term desensitisation to TLR ligands, causing increased susceptibility to *Strep* up to 6 weeks following primary IAV infection (Didierlaurent *et al.*, 2008).

Alveolar macrophage impairment

In acute coinfection models alveolar macrophages have been reported to be impaired - decreased alveolar macrophage phagocytosis, changes in functional state and a reduction in total numbers have been observed. IFN-γ produced during the T-cell response to IAV impairs bacterial phagocytosis by reducing the expression of the scavenger receptor MARCO on alveolar macrophages (Sun *et al.*, 2008). The negative role of IAV-induced IFN-γ is supported by another study showing treatment with linezolid - which moderately reduces the IFN-γ response to influenza - improves survival during coinfection (Breslow-Deckmann *et al.*,

2013). CD200 is a cell-surface ligand expressed on apoptotic cells during influenza that negatively regulates alveolar macrophages via CD200R. CD200R^{-/-} mice have increased resistance to coinfection, suggesting that CD200 expression reduces the alveolar macrophage antibacterial response (Goulding *et al.*, 2011). It is also possible that macrophages are in the "wrong" functional state - the expression of genes associated with alternatively activated macrophage activation such as FIZZ-1 are elevated during influenza. As these macrophages may be less antibacterial than classically activated macrophages, this may contribute to coinfection (Chen *et al.*, 2012). Reduction in the total number of alveolar macrophages by influenza prior to coinfection has also been described (Ghoneim *et al.*, 2013). To summarize, alveolar macrophages may be depleted and impaired during influenza, causing susceptibility to secondary *Strep* infection.

Neutrophil impairment

Influenza may also impair neutrophil function although, unlike alveolar macrophages, total neutrophil numbers are increased during coinfection (LeVine *et al.*, 2001; Li *et al.*, 2013). One study reports greater neutrophil numbers and increased myeloperoxidase activity in the lung, but does show the phagocytic capacity of neutrophils from coinfected mice is marginally decreased *in vitro* (Damjanovic *et al.*, 2013). Another study reports reduced myeloperoxidase per neutrophil in the lung (LeVine *et al.*, 2001). The level of alpha-1-anti-trypsin, which may impair the activity of neutrophil proteolytic enzymes, is also increased in coinfection (Kosai *et al.*, 2008). Although influenza can induce neutrophil NETs, these do not appear to participate in subsequent *Strep* killing (Moorthy *et*

al., 2013). Overall whether neutrophils are impaired in coinfection remains unclear; total neutrophil numbers and some aspects of their function are increased, but others appear to be moderately impaired.

Two studies have attempted to deplete neutrophils in coinfection to further elucidate whether they are impaired, and to determine whether they are on balance protective or pathogenic. However, various issues with these studies means it is difficult to clearly assess the role of neutrophils. One study reports specific neutrophil depletion using 1A8 does not exacerbate bacterial load or pathology during coinfection (Damjanovic *et al.*, 2013). However, in this coinfection model all mice reach endpoint, and therefore only a very strong positive effect of depletion would be detectable; a negative effect would not be detected. Another study depletes neutrophils with low specificity using RB6 during *Strep* and coinfection. This depletion exacerbates *Strep* infection, and *Strep* coinfection performed 3 days after IAV; however, it had no effect when coinfection was performed 6 days after IAV (McNamee *et al.*, 2006). This study suggests this is due to progressively increasing neutrophil impairment during IAV infection, rendering them non-protective (and therefore depletion has no effect) at 6 days post infection (dpi). Somewhat confusingly given this premise, this study showed that ROS production and neutrophil-bacterial association *in vitro* is reduced in neutrophils extracted from influenza-infected mouse lungs at both 3 and 6dpi. Furthermore, to increase the number of neutrophils elicited for functional assays, this study simultaneously treated influenza-infected mice with LPS aerosolization. Therefore the poor specificity of *in vivo* depletion and potentially confounding factor of LPS aerosolization make these results hard to interpret. An alternative

interpretation of these results is that lower bacterial loads observed following *Strep* infection alone and during coinfection performed at 3dpi can be exacerbated by neutrophil depletion, while there is no capacity to exacerbate the already very high loads observed during coinfected performed at 6dpi. Therefore whether neutrophils are protective or pathogenic during coinfection remains unclear.

Impairment mediated by influenza induced cytokines

Cytokines produced during influenza may contribute to coinfection susceptibility through immune impairment. The anti-inflammatory cytokine IL-10 may promote coinfection by inhibiting the immune response, as anti IL-10 treatment ameliorates disease (Van der Sluijs *et al*., 2004), although this was not reproduced with IL-10$^{-/-}$ mice (Sun *et al*., 2008).

Several studies have implicated influenza-induced type I IFNs as detrimental to the antibacterial response – IFNαβR$^{-/-}$ mice are more resistant to coinfection, produce more of the neutrophil chemoattractants KC and MIP2, and recruit greater neutrophil numbers (Shahangian *et al*., 2009). Also of note is that this result also implies, but does not confirm, that neutrophils are protective during coinfection; this is reinforced as administration of recombinant KC and MIP2 increases protection in wild-type mice. Another study with a similar implication shows Type I IFN transiently reduces IL-17 production by γδ T cells, leading to moderately reduced neutrophil recruitment (Li *et al*., 2012); this effect may be mediated by IFN-induced IL-27 (Cao *et al*., 2014). Polyinosinic:polycytidylic acid (Poly I:C) – a TLR3 agonist that induces a type I IFN response – promotes

susceptibility to subsequent *Strep* infection (Tian *et al.*, 2012). Type I IFN can also exacerbate pre-existing pneumococcal carriage through the inhibition of CCL2 production and macrophage recruitment (Nakamura *et al.*, 2011). However, in contrast to these studies showing a detrimental role for type I IFN, expression of IFNα through treatment with an adenoviral vector increases survival and the neutrophil response in an otherwise lethal *Strep* infection (Damjanovic *et al.*, 2014). Therefore whether type I IFN derived from influenza promotes susceptibility to *Strep* infection is not clear, although the majority of studies suggest it plays a harmful role.

Not all influenza-induced cytokines necessarily have a detrimental role during coinfection; influenza induces IL-22, and IL-22$^{-/-}$ mice are more susceptible to coinfection, suggesting this induction is protective (Ivanov *et al.*, 2013).

Strong proinflammatory responses during coinfection

In contrast to the studies above, other investigations suggest that coinfection induces a strong inflammatory response. Multiple studies have observed increased levels of proinflammatory cytokines induced in coinfection relative to IAV or *Strep* alone, such as IL-1β and TNF-α (LeVine *et al.*, 2001), and IL-12 and IFN-γ (Seki *et al.*, 2004; Smith *et al.*, 2007; Kukavica-Ibrulj *et al.*, 2009). As previously stated, neutrophil numbers are also increased in coinfection (LeVine *et al.*, 2001; Li *et al.*, 2013). Given that many aspects of the immune response are increased in coinfection, one possible critique of studies suggesting immune impairment as a mechanism is that they focus strongly on one aspect of the

immune response that is decreased, while overlooking an otherwise greatly heightened proinflammatory response.

Interventions affecting the immune response and their implications

Interventions that promote aspects of the immune response are sometimes protective in coinfection. This implies that these components of the immune response in coinfection are often insufficient; however it does not confirm that these are impaired relative to the response to single *Strep* infection. Combination therapy following bacterial infection with intravenous immunoglobulin and P4 peptide - an immunomodulator that increases the activity of phagocytic cells - improves coinfection survival (Weeks *et al*., 2011). TLR4 agonistic antibody treatment during IAV and *Strep* infection improves coinfection survival and reduces bacterial loads (Tanaka *et al*., 2013). However, reduced levels of the proinflammatory cytokines TNF-α, IL-6, KC and MIP2 are observed following this treatment, which may be a consequence of reduced bacterial loads. Therefore whether this study "increases" the immune response is not clear.

In contrast some interventions dampening components of the immune response during coinfection are protective. This contrasts with the concept that these factors of the immune response are impaired, instead suggesting they may be excessively induced and contribute to immunopathology. Treatment with dexamethasone – an anti-inflammatory steroid – improves survival of severely ill coinfected mice (Ghoneim *et al*., 2013). Inhibition of indoleamine-2,3-dioxygenase (a tryptophan-catabolising enzyme, which, although canonically

associated with anti-inflammatory responses, can increase cytokine production in epithelial cells) marginally reduces bacterial outgrowth and the levels of TNF-α and IL-10 during coinfection (van der Sluijs *et al.*, 2006). Although this implies these cytokines are harmful, it is possible that the effect of indoleamine-2,3-dioxygenase inhibition is through another mechanism, and, similar to previous suggestions, reduction of cytokine levels is a consequence rather than a cause of reduced bacterial loads.

A study assessing the efficacy of different antibiotics during coinfection suggested that TLR2 recognition of *Strep* and the resultant excessive inflammation may be harmful. TLR2$^{-/-}$ mice are marginally more protected from coinfection, and ampicillin was shown to be a poor antibiotic, as it led to excess *Strep* lysis and TLR2 stimulation (Karlstrom *et al.*, 2009; Karlstrom *et al.*, 2011). However these results are somewhat in contrast to other studies where TLR2$^{-/-}$ mice did not exhibit any changes relative to wild type mice in susceptibility to coinfection (Dessing *et al.*, 2007), and where ST2 (a negative regulator of TLR2) deficiency has no effect (Blok *et al.*, 2013). Overall, it remains unclear whether the aspects of the immune response to coinfection are impaired, and whether decreasing or promoting these responses is protective.

1.11.3. Lung dysfunction or damage in coinfection

In addition to immune impairment, the other major proposed cause of susceptibility to IAV-*Strep* coinfection is lung damage or dysfunction caused by influenza infection. This can take a range of forms, such as exposure of bacterial

binding sites and increased adhesion, a gross reduction in integrity due to viral damage, or decreased mechanical bacterial clearance.

Increased epithelial adhesion

Several studies have suggested IAV increases streptococcal binding to the lung epithelium. IAV increases streptococcal adherence to mouse tracheal epithelial cells (Plotkowski *et al*., 1986). One proposed mechanism is for this exposure of bacterial binding sites due to sialic acid cleavage by viral neuraminidase. Supporting this, treatment with the neuraminidase inhibitor oseltamivir ameliorates coinfection (McCullers *et al*., 2003), although this may simply reflect treatment of the primary initial viral infection.

One bacterial binding site that may be exposed by influenza during coinfection is the epithelial receptor PAFR, which is bound by the streptococcal virulence factor ChoP. PAFR mRNA expression is highly upregulated in influenza infected and coinfected mouse lungs, and PAFR$^{-/-}$ mice are slightly more resistant to coinfection, with moderately reduced bacterial loads (van der Sluijs *et al*., 2006; Seki *et al*., 2009). Furthermore, in a model of single *Strep* infection, reduction of PAFR levels upon treatment with type I IFN reduced streptococcal binding and epithelial transmigration (LeMessurier *et al*., 2013). These studies suggest upregulation and exposure of PAFR during coinfection contributes to bacterial colonization. However another coinfection study shows that treatment with a competitive PAFR antagonist increases bacterial load (McCullers *et al*., 2002). Therefore whether PAFR binding is a significant contributor to coinfection

susceptibility remains unclear. In summary IAV increases streptococcal epithelial binding, and different binding sites have been implicated; however, it has not been shown conclusively that this is a mechanism of coinfection.

Lung damage and other lung dysfunctions

Influenza-induced lung damage may also promote coinfection, as demonstrated by several studies assessing the effect of the proapoptotic viral peptide PB1-F2. IAV strains expressing PB1-F2 and the resulting lung damage and inflammation cause more severe coinfections (McAuley *et al*., 2008). This may be dependent on a specific PB1-F2 sequence (Alymova *et al*., 2013), and different PB1-F2 variants have been correlated with coinfections of different severity (Weeks-Gorosope *et al*., 2012).

Influenza may also reduce mechanical clearance of streptococci. Influenza infection suppressed ciliary beating in an *ex vivo* trachea model, leading to reduced bacterial clearance (Pittet *et al*., 2010), and this may contribute to streptococcal colonization of the lung *in vivo*.

Most coinfection studies imply mortality is a consequence of bacterial outgrowth, and propose mechanisms that facilitate streptococcal outgrowth. However, another lung dysfunction that may contribute to mortality is a lack of lung repair. Transcriptional profiling in a model of 2009 pandemic IAV-*Strep* coinfection showed a lack of lung repair responses (Kash *et al*., 2011). However, these mice also exhibited bacterial outgrowth and bacteraemia, which may cause mortality.

Furthermore, lack of lung repair may be due to a loss of pathogen control, where inflammation rather than resolution and repair may be a more appropriate response.

1.11.4. Other aspects of coinfection mouse models

Many other models of IAV-*Strep* coinfection that do not focus on immune impairment or lung dysfunction also provide further insights into the synergy between infections. One study shows influenza may have a direct effect on *Strep* biology – damage signals induced by influenza may cause the transition of *Strep* from a biofilm-like to a potentially more infectious dispersed state (Marks *et al*., 2013). Another study focuses on aspects of *Strep* that promote colonization during coinfection. PspA - a virulence factor that interferes with complement deposition - deficient streptococci cause less severe coinfections (King *et al*., 2009). This indicates that intact streptococci expressing bacterial virulence factors are required for coinfection.

In addition to promoting coinfection within individuals, IAV also promotes transmission of *Strep*. IAV increases *Strep* transmission between infant mice (Diavatopoulous *et al*., 2010) and in ferrets (McCullers *et al*., 2010). Whether this is due to a defined mechanism or simply a consequence of increased bacterial loads in coinfected donor animals raising the probably of transmission is not clear.

There are various proposed interventions for coinfection, which may give further mechanistic information. Several quinolone antibiotics have been shown to be

protective (Hayashi *et al*., 2006), as is garenoxacin (Fukada *et al*., 2013), suggesting bacterial outgrowth is the primary driver of mortality in coinfection. Reducing the severity of prior influenza infection through vaccination with FluMist is protective (Sun *et al*., 2011). Live attenuated influenza vaccine is also protective, but not pneumococcal conjugate vaccine (Mina *et al*., 2013). These results hint that the key determinant of coinfection is the severity of primary viral infection rather than anti-streptococcal immunity, and therefore ameliorating influenza represents the best therapeutic strategy. However, somewhat countering this concept, immunization with pneumococcal PspA, whether alone (Seo *et al*., 2012) or with Poly I:C treatment (Ezoe *et al*., 2011) also ameliorates coinfection.

1.11.5. Mouse models of other influenza A coinfections

Mouse models of other IAV coinfections can also provide useful insights into possible IAV-*Strep* coinfection mechanisms, particularly where similar mechanisms are observed. The diversity of coinfections observed may indicate that IAV promotes susceptibility to secondary infections through a broad, non-specific mechanism, or through multiple different mechanisms.

Immune impairment during S. aureus coinfection

S. aureus is a common coinfection, and has been extensively investigated in mouse models. The majority of studies focusing on IAV-*S. aureus* coinfection show immune impairment, particularly of proinflammatory cytokines and phagocytes. Whether these mechanisms are directly applicable to IAV-*Strep*

coinfection is not clear, although some similarities are evident. Similarly to results from an IAV-*Strep* coinfection study (Li *et al.,* 2012), IAV reduced the IL-17 response to *S. aureus* (Kudva *et al.*, 2010; Robinson *et al.*, 2013). Inhibition of the IL-17 response can lead to reduced IL-1β production (Robinson *et al.*, 2013). Influenza induced IFN-γ can also reduce phagocytosis of *S. aureus* by alveolar macrophages (Hang *et al.*, 2011). This is similar to IFN-γ mediated suppression of the phagocytic receptor MARCO on alveolar macrophages during IAV-*Strep* coinfection (Sun *et al.*, 2008). Influenza also impairs the NK cell response to *S. aureus* in the lung (Small *et al.*, 2010).

Similarly to prior reports in IAV-*Strep* coinfection (McNamee *et al.*, 2006), influenza may also inhibit the neutrophil response to *S. aureus* coinfection. Influenza inhibits the NADPH oxidase involved in the neutrophil respiratory burst during IAV-*S. aureus* coinfection (Sun *et al.*, 2014), and promotes neutrophil death mediated by the *S. aureus* cytotoxic factor Panton-Valentine leukocidin (Niemann *et al.*, 2012). Mice overexpressing GM-CSF were protected against *S. aureus* coinfection due to greater numbers of alveolar macrophages and neutrophils (Subramaniam *et al.*, 2013). Depletion of alveolar macrophages, neutrophils or reactive oxygen species removes the protective effect of GM-CSF overexpression. These studies strongly suggest neutrophil death or reduced function contributes to bacterial coinfection. However, in another IAV-*S. aureus* coinfection model, depletion of neutrophils or alveolar macrophages had no effect (Iverson *et al.*, 2011). In summary, although not all results are completely consistent with this view, susceptibility to *S. aureus* coinfection is likely due to

suppression of proinflammatory cytokines and phagocytes by influenza, and this immune impairment may be relevant to IAV-*Strep* coinfection.

Immune impairment in other coinfections

Other coinfections have also been investigated, and may also provide mechanistic information that relates to IAV-*Strep* coinfection. Immune impairment mediated by influenza-derived cytokines has been investigated. Peak susceptibility to *N. meningitis* correlates with influenza-induced IFN-γ (Alonso *et al*., 2003). Although this specific study does not investigate immune impairment, it is notable that influenza-induced IFN-γ appears to increase susceptibility to several different bacteria, and IFN-γ mediated immune impairment may represent a general mechanism of coinfection.

Type I IFNs also appear to promote susceptibility to a range of bacterial infections through immune impairment. As type I IFNs are induced during influenza, this may be highly relevant to IAV-*Strep* coinfection, although most studies described here induce type I IFNs through other means than IAV infection. Type I IFNs can induce susceptibility to *L. monocytogenes* (O'Connell *et al*., 2004; Auerbuch *et al*., 2004; Carrero *et al*., 2004), possibly through suppression of protective IFNγ signalling in macrophages (Rayamajhi *et al*., 2010), or overactivation and death of bone marrow granulocytes (Navarini *et al*., 2006). This observation may be relevant to IAV-*Strep* models where IFNαβR$^{-/-}$ mice are more granulophillic and coinfection resistant (Shahangian *et al*., 2009). In addition, induction of type I IFNs by Poly I:C treatment appears to exacerbate *M. tuberculosis* infection

(Antonelli *et al*., 2010), and type I IFNs suppress protective IL-1 production (Mayer-Barber *et al.*, 2011). More directly linking type I IFNs to coinfection, one study shows prior influenza can exacerbate *M. tuberculosis* infection in a type 1 IFN dependent manner (Redford *et al.*, 2014).

Another aspect of influenza-mediated immune impairment is the production of glucocorticoids, which can lead to systemic suppression of the immune response and susceptibility to *L. monocytogenes* (Jamieson *et al*., 2010). However, it is notable that removal of the glucocorticoid response by adrenalectomy led to an excessive immune response and mortality, and therefore this highlights the delicate balance between bacterial control and immunopathology required to control coinfection.

Immunopathology and tissue damage in other coinfections

Some studies suggest immunopathology is the primary driver of mortality during coinfection, and attempt to ameliorate this. One study attempted to reduced IAV-immunopathology during IAV-*H. influenzae* coinfection, but disease severity was not ameliorated by CCR2 or TNFR1 deficiency (Lee *et al*., 2010). An IAV-*B. pertussis* model - although observing increased bacterial loads - attributed mortality to immunopathology, especially excessive neutrophil recruitment (Zavitz *et al*, 2010). However, this model gave somewhat contradictory results, as while blockade of the neutrophil chemoattractant MIP2 was protective, blockade of its receptor CXCL2 (which is also a receptor for the neutrophil chemokine KC) was harmful. This highlights the difficulty, in the context of bacterial outgrowth

during coinfection, in dissecting protective and pathogenic aspects of the immune response.

One study attempts to address this practical difficulty using a highly unusual IAV-*L. pneumophila* coinfection model. This study shows mortality even in the absence of bacterial outgrowth, which is attributed to an inability to tolerate tissue damage. This coinfection can be ameliorated by treatment with amphregulin - an epithelial growth factor - in TLR2 and 4 deficient mice (Jamieson *et al.*, 2013). Complicating these findings, tissue damage does not appear to be dependent on the immune response. Although a context where bacterial outgrowth does not occur is useful, it is arguable it renders this model somewhat unphysiological and difficult to compare to other mouse models.

Bacterial infections prior to influenza

Although almost all studies focus on bacterial coinfections following influenza, a small proportion test the effect of pre-existing bacterial colonization. As pre-existing carriage of *Strep* is common (Kadioglu *et* al., 2008), these models may have some relevance to IAV-*Strep* coinfection.

Interestingly, application of bacteria prior to influenza ("coinfection in reverse") can ameliorate the subsequent influenza infection. Exposure to a *H. influenzae* lysate (Tuvim *et al.*, 2009) or *L. rhamnosus* (Harata *et al.*, 2010; Youn *et al.*, 2012) increases the immune response against influenza. Priming with low-pathogenicity *S. aureus* can lead to alternative macrophage activation during

influenza, reducing lung injury (Wang *et al*., 2013). These protective effects, albeit with inactivated or non-pathogenic bacteria, may imply the timing of coinfection is crucial.

1.12. Project outline: Investigating the immune response to Influenza A-*S. pneumoniae* coinfection

Although there has been extensive research on the immune response to IAV-*Strep* coinfection, several aspects remain unclear, and there are limitations to many existing studies. Downstream consequences of bacterial outgrowth are frequently not separated from assessment of the underlying influenza-induced factors that allow initial bacterial colonization. Very severe coinfection regimes are used, which only give scope to perform strongly protective interventions, limiting what can be investigated. Furthermore, not all single infection controls are performed, leaving interpretation of interventions difficult. In this thesis I use a mouse model of IAV-*Strep* to investigate the underlying mechanisms involved in bacterial colonization, and investigate the subsequent immune response, while attempting to address these issues.

Several aspects of the immune response during coinfection merit further investigation. The protective role of neutrophils has been implied by previous studies (Shahangian *et al*., 2009) but has not been assessed with a specific depletion except in the context of a severe coinfection (Damjanovic *et al*., 2012). Furthermore neutrophil function has not been assessed using lung neutrophils without confounding factors such as LPS aerosolization (McNamee *et al*., 2006).

Although an increase in TNF-α during coinfection (LeVine *et al.*, 2001) has been observed previously, its role as beneficial or as a promoter of immunopathology is not clear. The role of CCR2 dependent inflammatory monocytes has only been studied in a pneumococcal carriage-IAV model (Nakamura *et al.*, 2011), not in acute coinfection.

Therefore in this thesis I investigate a mouse model of IAV-*Strep* coinfection. I use low virulence single infections to assess synergy rather than effects of the single infections themselves. I initially perform broad profiling of the immune response, and observe substantial upregulation of neutrophils and proinflammatory cytokines, including TNF-α. The coinfection model is then calibrated to approximately 50% mortality, allowing both positive and negative interventions to be detected. Using a range of interventions to investigate mechanisms, I propose a model where influenza induced TRAIL-expressing inflammatory monocytes mediate lung damage, allowing bacterial colonization and outgrowth. Outgrowth is subsequently countered by a protective TNF-α and neutrophil response, which does not appear to be functionally impaired, but can often be insufficient to prevent mortality. In addition to the main focus of my thesis, I also more briefly investigate other aspects of coinfection that have not been previously comprehensively studied, such as bacterial spread to the brain.

Chapter 2. Materials and Methods

Mice: All experiments unless otherwise specified used 6-10 week old C57BL/6 mice bred at the MRC-National Institute for Medical Research (NIMR) under specific pathogen–free conditions. Rag1$^{-/-}$ (C57BL/6 Jackson Rag1$^{-/- \cdot MOM}$) mice were bred at the NIMR under specific pathogen–free conditions. CCR2$^{-/-}$ (C57BL/6) mice were kindly provided by Dr. A. O'Garra (NIMR) and Dr. J. Langhorne (NIMR). IFNαβR$^{-/-}$ (C57BL/6) mice were kindly provided by Dr. A. O'Garra (NIMR). TNFR1$^{-/-}$ (p55) (C57BL/6 N6) and TLR2$^{-/-}$ (C57BL/6 N7) mice were kindly provided by Dr. J. Langhorne (NIMR). All protocols for breeding and experiments with animals were approved by the Home Office, UK, under the Animals (Scientific Procedures) Act 1986 and project licence 70/7643.

Clinical scoring and endpoints: Mice were deemed to have reached endpoint at 75% of starting weight or at a moderate severity clinical score of 5 or greater. Clinical scores were determined by (1 point each) piloerection, hunched posture, laboured breathing, decreased movement, movement only on provocation, absence of movement on provocation, hypothermia, partially closed eyes or evidence of middle ear infection (disrupted balance).

Preparation of pathogens and mouse infections: Influenza A virus strain X31 (H3N2) (a reassortment virus with the A/PR/8/34 (H1N1) backbone) was grown in day 10 embryonated chicken eggs (kindly performed by the Division of Virology, NIMR), stored at -80° and titrated on confluent monolayers of MDCK cells, with cellular lysis observed after 3 days. TCID$_{50}$ was calculated according

to the Spearman-Karber method. *S. pneumoniae* D39 (a kind gift from Dr. M. Coles, University of York) was stored at -80° on cryopreservative beads (Technical Services Consultants). Identity of *Strep* bead stocks was verified by observation of α-hemolysis and optochin-sensitivity upon plating on brain heart infusion agar plates supplemented with defibrinated horse blood, and using API Strep 20 kits (Biomerieux) as per the manufacturer's instructions. *Strep* for mouse infection was grown from bead stocks in brain-heart infusion broth under microaerophillic conditions at 37° for 16 hours to autolytic phase, then subcultured and grown to an optical density of 0.4, then centrifuged at 3000rpm for 5 minutes and room temperature, before resuspension in PBS immediately prior to infection. When described as "heat killed" the bacterial preparation was incubated 80° for 10 minutes, and killing of bacteria confirmed by plating. Mice were typically infected under light transient isoflurane-induced anaesthesia with a 30μl volume via the intranasal route (i.n.). Where specified, mice were infected under ketamine-induced anaesthesia with a 20μl volume via the intratracheal route (i.t.). Mice were awakened from ketamine anaesthesia using atipamezole.

Mouse Treatments: 15μg/30μl Pam3CSK4 (Enzo Life Sciences) was given i.n. under light transient isoflurane-induced anaesthesia at 5dpi. 50μg/30μl Poly I:C (Enzo Life Sciences) was given under light transient isoflurane-induced anaesthesia at 3 and 4 dpi. All antibody treatments were given via the intraperitoneal route (i.p.) in a 200μl volume. 150μg anti-Ly6G (clone 1A8) or isotype control (2A3) (BioXCell) were given every 24 hours from 4 to 12dpi. 500μg anti-Gr-1 (RB6-8C5) (BioXCell) or vehicle control (PBS) were given every 48 hours from 4 to 8dpi. 500μg anti-TNF-α (XT3.11) or isotype control

(HRPN) (BioXCell) were given on 5 and 7dpi. 150µg anti-TRAIL (Cambridge Bioscience) or vehicle (PBS) were given as either continuous treatment every 48 hours from 1 to 9dpi, early treatment at 1 and 3dpi, or late treatment at 6 and 8dpi.

Histology: Whole lungs were perfused with 10% neutral buffered formalin (NBF) in situ. Tissue was then fixed for 24 hours in 10% NBF, embedded in paraffin and sectioned. Each lung specimen was stained with hematoxylin and eosin (H&E) (performed with the assistance of Histology section, NIMR). Imaging of slides was performed on a VS120 slide scanner (Olympus) with a VC50 camera, a UPLSAPO lens, at magnification of 20x and a numerical apeture of 0.75. Images were analysed using OlyVia Image Viewer 2.6 (Olympus).

Quantification of live bacterial loads: Streptococcal loads were determined in mouse lung, spleen, brain, bronchoalveolar lavage fluid (BAL) and blood. Lung, spleen or brain tissue were homogenized in PBS through a 70µm filter prior to storage at -80°. BAL was centrifuged at 1400rpm for 5 minutes at 4° and supernatant stored at -80°. Blood was taken by cardiac puncture and clotting prevented by keeping samples on ice with heparin (20U/ml) (Sigma), before centrifugation at 1400rpm for 5 minutes at 4° and storage of supernatant -80°. Serial dilutions of single cell suspensions, BAL or blood supernatant were performed on brain heart infusion agar plates supplemented with defibrinated horse blood and the number of colony-forming units (CFUs) counted.

Quantification of live viral loads: Live viral loads were quantified in the lung and spleen. Lungs were excised from mice, digested with 20µg/ml Liberase TL

(Roche) and 50μg/ml DNAse 1 (Sigma), and homogenised using gentleMACS (Miltenyi), as per the manufacturer's instructions. Spleens were homogenized through a 70μm filter. All samples were stored at -80°. Samples were titrated on confluent monolayers of MDCK cells and cellular lysis observed after 3 days. $TCID_{50}$ was calculated according to the Spearman-Karber method.

Viral and bacterial RNA quantification: Viral and bacterial RNA levels were quantified in the lung and spleen. Lung or spleen were homogenised in PBS, passed through a 70μm filter and centrifuged at 1400rpm for 5 minutes at room temperature. RNA was extracted from lung or spleen homogenate pellet using TRI reagent (Ambion) as per the manufacturer's instructions. 400ng total RNA was reverse transcribed using the ThermoScript RT-PCR System kit (Invitrogen) as per the manufacturer's instructions. The cDNA served as template for quantitative PCR using TaqMan Gene Expression Assays (Applied Biosystems), universal PCR Master Mix (Applied Biosystems) and the ABI-PRISM 7900 sequence detection system (Applied Biosystems). IAV Matrix and *Strep* 16s rRNA were quantified relative to the housekeeping gene (*Hprt1*) as previously described (Ward et al., 2004; Kash et al., 2011). Primers for influenza matrix M1 gene were as follows: forward: 5'-AAGACCAATCCTGTC ACCTCTGA-3'; reverse: 5'-CAAAGCGTCTACGCTGCAGTCC-3'; and probe: 5'-TTTGTGTTCACGCTCACCGT-3'. Primers for Strep 16s rRNA were as follows: forward: 5'-GGTGACGGC AAGCTAATCTCTT-3'; reverse, 5'-AGGCGAGTTGCAGCCTACAA-3'; and probe, 5'-AAGCCAGTCTCAGTTCG-3'.

Preparation of single cell suspensions from the lung for flow cytometry: Lungs were excised from mice, digested with 20μg/ml Liberase TL (Roche) and 50μg/ml DNAse 1 (Sigma) for 30 minutes at 37° and 5% CO_2, and homogenised using gentleMACS (Miltenyi), as per the manufacturer's instructions. For analysis of epithelial cells, the gentleMACS homogenisation step was not performed. Lung suspension was then passed through a 70μm cell strainer and washed with PBS. Red blood cells were lysed using ammonium chloride and cells were seeded into a 96-well U-bottom plates.

Preparation of single cell suspensions from the brain for flow cytometry: Brains were excised from mice, homogenised through repeated passage into a 1ml syringe into a digest solution of Collagenase D (Roche) (0.5mg/ml), Dispase II (Roche) (2mg/ml) and DNAse 1 (Sigma) (3.5μg/ml). Following 30 minutes digestion at 37° and 5% CO_2, brain suspension was then passed through a 70μm filter and washed with PBS. The suspension was resuspended in 70% Percoll (GE Healthcare) and overlaid with 30% Percoll before centrifugation, retaining the fraction at the interface.

Flow cytometry: Lung or brain single cell suspensions or BAL fluid were preincubated with anti-FcγRIII/II (Fc block, to reduce non-specific Fc receptor binding of antibody constant regions) in PBS prior to 30 min incubation with one or more of the following fluorochrome labelled antibodies (Biolegend unless otherwise specified): FITC-conjugated anti-Ly6G; APCCy7 conjugated Ly6G; PerCpCy5.5-conjugated anti-Ly6C; FITC-conjugated anti-Ly6C; PECy7-conjugated anti-CD11b; APC-conjugated anti-CD11b; APCCy7-conjugated anti-

CD3; PE-conjugated anti-F4/80; PE-conjugated anti-TRAIL; PE-conjugated anti-DR5; APC-conjugated CD45; APCCy7-conjugated anti-EpCam; V450-conjugated anti-CD4; PE-Texas Red conjugated anti-CD8; PerCpCy5.5-conjugated anti-NKp46; FITC-conjugated anti-E-cadherin and V450-conjugated anti-CD11c (BD Biosciences). Cells were then washed with PBS and stained with LIVE/DEAD Fixable Dead Cell Stain (Life Technologies) prior to fixation in 4% formaldehyde, and were then assessed using a LSR II Fortessa or an LSR II (Becton Dickinson). Data analysis performed on FlowJo (Treestar). Cell counts were performed on a Brightline hemacytometer (Hausser Scientific) with Trypan blue exclusion.

Cytokine quantification: Cytokine levels were quantified in BAL fluid and from *in vitro* neutrophil culture supernatants. BAL fluid was recovered and centrifuged at 1400rpm for 5min at 4ºC and supernatant collected. *In vitro* neutrophil culture supernatants were taken after 5 hours following cell adhesion to the culture plate. Concentrations of cytokines were assessed by Milliplex Map Kit (Millipore) as per manufacturer's instructions and read on a Luminex 100 (BioRad). Concentrations of TNF-α and IL-22 (e-Bioscience), KC and MPO (R and D systems), and IFN-α and IFN-β (PBL Assay Science) were quantified by ELISA according to the manufacturer's protocol. ELISA plates were read on a Safire II plate reader (Tecan).

Neutrophil purification: Neutrophils were purified for *in vitro* assays from whole mouse lung by manual maceration followed by 20 minutes digestion at 37° and 5% CO_2, with Collagenase D (Roche) (0.5mg/ml), Dispase II (Roche) (2mg/ml)

and DNAse 1 (Sigma) (3.5μg/ml), with EDTA (Sigma) (10μM) added for the final 5 minutes (to chelate calcium, thus reducing integrin association and aiding digestion). Digested lung was mashed through a 70μm filter, and neutrophils separated from the single cell suspension by positive selection on two sequential MACS columns using anti-Ly6G-biotin and anti-biotin microbeads (Miltenyi Biotec), as per the manufacturers instructions. In some cases remaining dead cells were removed by overlaying cell suspension on a 40%/85% Percoll gradient, centrifuging for 20 minutes at 800xg and room temperature, and retaining the fraction at the 40/85% interface.

Other neutrophil purifications tested during optimization process: During optimization of the protocol above, other purification procedures were attempted. In place of manual maceration homogenization using gentleMACS (Miltenyi), as per the manufacturer's instructions, was attempted. In place of Collagenase D and Dispase II other digest mixes – containing Liberase (20μg/ml) (Sigma) or Collagenase IV (400μg/ml) (Sigma) (all digest mixes contained 3.5μg/ml DNAse 1 (Sigma)) were tested.

Reactive oxygen species: $5x10^4$ lung neutrophils were seeded on a white 96-well flat-bottomed plate in calcium and magnesium positive media and rested at 37° for 1 hour. Luminol (Sigma) (50μM) and Horseradish Peroxidase (Sigma) (1.2U/ml) were added followed by stimulation with PDBu (Sigma) (50nM) or media. Luminescence was immediately read on a Safire II plate reader (Tecan).

NET formation: 5×10^4 lung neutrophils were seeded onto a 24-well flat bottomed transparent plate in calcium and magnesium positive media supplemented with 3% mouse plasma and rested at 37° for 1 hour, followed by stimulation for 2 hours with 5×10^5 CFU *C. albicans* (clinical isolate SC 5314; a kind gift from Dr. J. Duarte) or media. After 2 hours incubation at 37° NET formation was visualized by addition of the DNA stain Sytox (Life Technologies) (8.3μM). Images were taken and analyzed on a DM IRB (Leica) microscope with an Orca-ER Digital Camera C4742 80 (Hamamatsu) camera, and an N PLAN PH1 lens (Leica), at magnification of 10x and a numerical aperture of 0.25. Image acquisition software was Micromanager 1.4 and processing was performed using ImageJ 1.64. NETs were defined as Sytox$^+$ areas >2000μm^2 (in collaboration with Ms. N. Branzk, NIMR).

Neutrophil culture: 1×10^5 (unless otherwise specified) lung neutrophils were seeded onto a 96-well flat bottomed plate in complete media (RPM1 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, glutamate, and β-mercaptoethanol) and rested for 1 hour, followed by stimulation with Pam3CSK4 (Enzo Life Sciences) (1μg/ml), LPS (Enzo Life Sciences) (1μg/ml), PDBu (Sigma) (50nM) or media. Culture supernatants were removed after 5 hours and protein concentrations assessed by ELISA as described above. Where specified prior to stimulation neutrophils were pretreated with recombinant mouse IFNα (PBL Assay Science) (10 or 100 U/ml) for 1 hour.

Quantification of lung damage: Lung damage was assessed in BAL fluid. Lactate dehydrogenase (LDH) activity was assessed using the enzymatic detection step of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. Protein levels were quantified by Pierce BCA protein assay (Thermo Scientific) as per the manufacturer's instructions. Assay plates were read on a Safire II plate reader (Tecan).

Statistics: All statistical comparisons were performed using Prism 6 (GraphPad). Specific statistical tests used for each experiment are specified in accompanying figure legends. A p value of <0.05 was considered significant. n.s. = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Chapter 3. Causes of mortality in a mouse model of Influenza A-*S. pneumoniae* coinfection

3.1. Background

Before investigating mechanisms of coinfection, it was necessary to establish a model, and determine what drives mortality. There are several possible drivers of mortality during IAV-*Strep* coinfection – a loss of viral control, a loss of bacterial control, or immunopathology due a strong immune response.

Many IAV-*Strep* mouse models do not quantify viral load in a coinfection setting. Where quantified, viral clearance does not appear to be impaired (McNamee *et al*., 2006), or is marginally delayed (Li *et al.*, 2012; Smith *et al*., 2013). In contrast, almost all models report increased streptococcal loads and bacterial spread to other organs during coinfection (for example, LeVine *et al*., 2001). Many studies report a very strong inflammatory response, with many proinflammatory cytokines induced (Smith *et al*., 2007), which may lead to immunopathology. It therefore is possible that each of these factors contributes to mortality, although it is likely one is the primary driver.

Recent studies testing different antibiotics in coinfection have given indirect information on the causes of mortality. These studies support the hypothesis that bacterial outgrowth is the primary driver of mortality. A recent study showed treatment with the immunosuppressive corticosteroid dexamethasone did not ameliorate coinfection, but treatment with the antibiotic azithromycin did

(Damjanovic *et al.*, 2013). This suggests bacterial outgrowth is more responsible for driving mortality than immunopathology. Combination therapy with dexamethasone and antibiotic was marginally more effective than antibiotic alone, suggesting once bacterial outgrowth is controlled, a modest improvement can be achieved by ameliorating any remaining immunopathology.

Another study demonstrates that, while all antibiotic treatments tested are somewhat protective, those that did not exacerbate immunopathology were superior; for example, azithromycin increased survival more than ampicillin. This study proposes that ampicillin promotes immunopathology due to excessive inflammation in response to bacterial lysis (Karlstrom *et al.*, 2009). This reinforces the concept of bacterial outgrowth as the primary driver of mortality, and immunopathology as a consequence of this.

Overall, these antibiotic studies suggest bacterial outgrowth is the primary driver of mortality. Immunopathology appears to be a secondary issue that does not appear to drive mortality alone; it may be a consequence of a strong immune response to bacterial outgrowth. A further complication in investigating this is that immunosuppressive therapy to reduce immunopathology may exacerbate bacterial outgrowth. Overall, further clarity on the drivers of mortality in coinfection are needed.

3.2. Hypotheses and Aims

I therefore aimed to establish a mouse model of IAV-*Strep* coinfection, with two key questions as a starting point. Firstly, is viral or bacterial outgrowth, or immunopathology, the primary driver of mortality during coinfection? Secondly, what are the mechanisms by which influenza promotes this mortality? I proposed to use influenza and *Strep* single infections of low or moderate severity, to allow focus on synergy between infections rather than their individual effects. In this chapter I will focus on the establishment of the model and the causes of mortality.

I hypothesised that:

- Influenza would lead to increased susceptibility to streptococcal infection.
- Viral clearance would either be unchanged or only marginally impaired by coinfection.
- Bacterial outgrowth would be observed during coinfection, and required for mortality.

3.3. Results

Selection of infectious agents and disease readouts

To establish a murine model of IAV-*Strep* coinfection, appropriate mild to moderate single infections and a suitable mouse strain were required. The majority of influenza research undertaken in mice uses inbred C57BL/6 or BALB/c mice infected intranasally with A/Puerto Rico/8/1934 (H1N1) (PR8),

although use of other strains such as A/WSN/1933 (H1N1) (WSN) is also common (Bouvier *et al.*, 2010). For consistency and to allow comparison with other studies, as well as the availability of many genetically modified mice, C57BL/6 mice were used.

PR8 is potentially useful as a model as, unlike most recent human isolates of influenza, it induces severe disease and substantial mortality in inbred mice. However, for the study of coinfection, a severe initial influenza infection may make it difficult to assess synergy with *Strep*, and therefore the milder X31 strain was used. X31 is a reassortant virus bearing the HA and NA of A/Hong Kong/1/1968 (H3N2), with all other viral genes from PR8. This strain is particularly useful as it induces moderate disease without - except at very high doses - causing excessive mortality (Bouvier *et al.*, 2010).

D39 is a common laboratory strain of *Strep* frequently used in mouse models (Chiavolini *et al.*, 2008), including coinfection (Goulding *et al.*, 2011). It was originally isolated in 1916 and was used in Avery's experiments demonstrating DNA is the genetic material (Avery *et al.*, 1944; Lanie *et al.*, 2007). While commonly referred to as virulent, its pathogenicity in mice shows substantial variation depending on mouse strain, dose, route of administration, and volume administered. Furthermore due to the age of the strain, different divergent stocks of D39 now show substantial variation in virulence. D39 was selected as a potentially mild strain of *Strep* that would allow comparisons with other studies. Although some coinfection studies administer bacteria via the intratracheal route

(for example, Shahangian *et al.*, 2009), here more physiological intranasal administration was used.

To assess coinfection, appropriate disease readouts were required. Weight loss is a common feature of mouse models of many viral infections, including influenza and coinfection, and relates closely to disease outcome; is has been shown during influenza to not be caused by dehydration or malnutrition (Sanders *et al.*, 2012). It is therefore a good readout for disease severity. In contrast, studies on *Strep* infections rarely assess weight loss to determine disease severity, and therefore clinical scores are useful as a supplementary readout. Mice were therefore defined to have reached endpoint when they reached 75% of starting weight, or a high clinical score (defined as 5 or greater, assessed as described in materials and methods). Weight loss, clinical score and survival therefore are reliable measurements for disease severity during coinfection.

Coinfection causes synergistic disease in a murine model

I established an infection regime for IAV-*Strep* coinfection five days apart (shown in Fig. 1A, all days post infection hereafter specified refer to the primary influenza infection). A previous study had reported a peak in susceptibility at 7dpi (McCullers *et al.*, 2002), leading to mortality under 24 hours, which is somewhat impractical for further investigations. Therefore 5dpi - where mice were reported to survive at least 3 days – was selected as a more appropriate timepoint for secondary infection.

In this model weight loss (Fig. 1B; for clarity, in all weight figures, mice euthanized after reaching endpoint are included at their final weight in the calculation of subsequent means), mouse mortality (Fig. 1C) and disease severity as assessed by clinical signs (Fig. 1D; again, for clarity, mice euthanized after reaching endpoint are included at their final clinical score in calculation of the mean) were observed. Intranasal administration of 8×10^3 TCID$_{50}$ X31 (hereafter referred to simply as IAV unless otherwise specified) induced moderate weight loss of approximately 15% of body weight in C57BL/6 mice (Fig. 1B), and very mild clinical symptoms - typically piloerection and decreased movement - in a small proportion of mice (Fig. 1D), with only a small number of mice reaching endpoint (Fig. 1C). Intranasal administration of 2×10^7 CFU D39 (hereafter referred to simply as *Strep* unless otherwise specified) induced no signs of disease in the majority of mice, although a small proportion developed high clinical scores (Fig. 1D) and reached endpoint (Fig. 1C). In contrast, IAV and *Strep* coinfection hugely increased weight loss (Fig. 1A) and gave high clinical scores (Fig. 1D), with almost all mice reaching endpoint (Fig. 1C). As these pathogen doses caused almost complete mortality during coinfection, they are hereafter referred to as "high dose".

Assessment of lung infiltrate by histology showed little cell recruitment during *Strep* infection, and a moderate infiltrate during IAV, but large regions of overwhelming infiltrate and few unobstructed airspaces during coinfection (Fig 1E) (experimental assistance provided by Histology Section, NIMR). The strong synergy observed during coinfection and the relatively mild to moderate effects of single infections made this model highly suitable for the study of synergy.

Figure 1

A

Influenza A
X31 (IAV) or
mock i.n.

S. pneumoniae
D39 (Strep) or
mock i.n.

C57BL/6
mice

0 dpi
(Days
post
infection)

5 dpi

B Weights

○ *Strep*
□ IAV
▲ IAV + *Strep*] ****

% of starting weight

Days post infection

C Survival

○ *Strep*
□ IAV
▲ IAV + *Strep*] ****

% survival

Days post infection

D Clinical score

○ *Strep*
□ IAV
▲ IAV + *Strep*] ****

Clinical score

Days post infection

E

Naive — Strep

IAV — IAV + *Strep*

7 dpi

200 µm (×4)
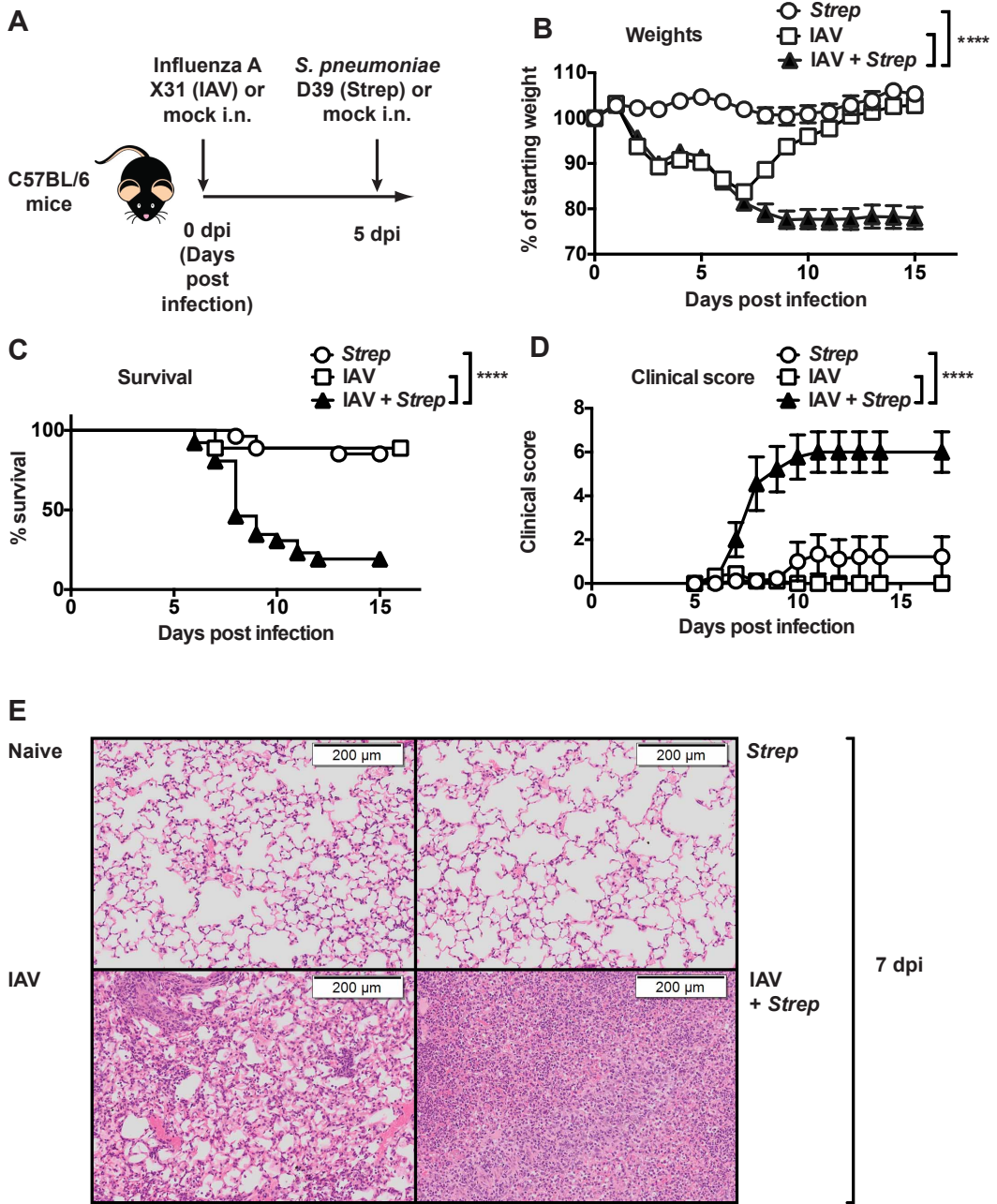
Figure 1: *Influenza A predisposes mice to S. pneumoniae coinfection.*
(A) Infection scheme. (B) Weight loss and (C) mortality following infection of C57BL/6 mice
with 8×10^3 $TCID_{50}$ IAV X31, 2×10^7 CFU *S. pneumoniae* D39 or mock (PBS) (data shown is
pooled from 4 independent experiments, n=6-9. On weight loss plot mice reaching endpoint
retained thereafter at final weight. Dosing hereafter referred to as "high dose"). (D) Clinical
scores (n=9, representative of 2 independent experiments, mice reaching endpoint retained
thereafter at final clinical score). (E) H+E staining of lung tissue sections (n=3) at 7dpi. Data
displayed as arithmetic means ±SEM (weights and clinical scores) or percent survival
(mortality). Significance assessed by 2-way ANOVA (weights and clinical scores) or Log-
rank (Mantel-Cox) test (mortality). ****p<0.0001.

Coinfection leads to bacterial but not viral outgrowth

I then profiled pathogen loads during coinfection to elucidate the causes of mortality. Consistent with its low virulence, *Strep* was not detected at any point in the first three days following secondary infection. However, coinfection led to high bacterial loads in the lung (Fig. 2A), and systemic spread by 7 to 8dpi to the spleen (Fig. 2B) and brain (Fig. 2C). Viral clearance was not affected by coinfection, with viral load as assessed by RNA in the lung falling from 6dpi to 8dpi (Fig. 2D) and live virus being below the limit of detection by 8dpi - the point where typically coinfection induced mortality begins to occur (Fig. 2E). IAV does not typically colonize other tissues; however, this may have been altered by coinfection. To assess this, spleen viral RNA was quantified relative to control infected and uninfected lungs. Viral RNA was not detected (i.e. the level observed was equivalent to an uninfected lung) in the spleen during IAV or coinfection (Fig. 2F). Therefore bacterial, but not viral, outgrowth occurs during coinfection and is likely to be a driver of mortality.

Figure 2
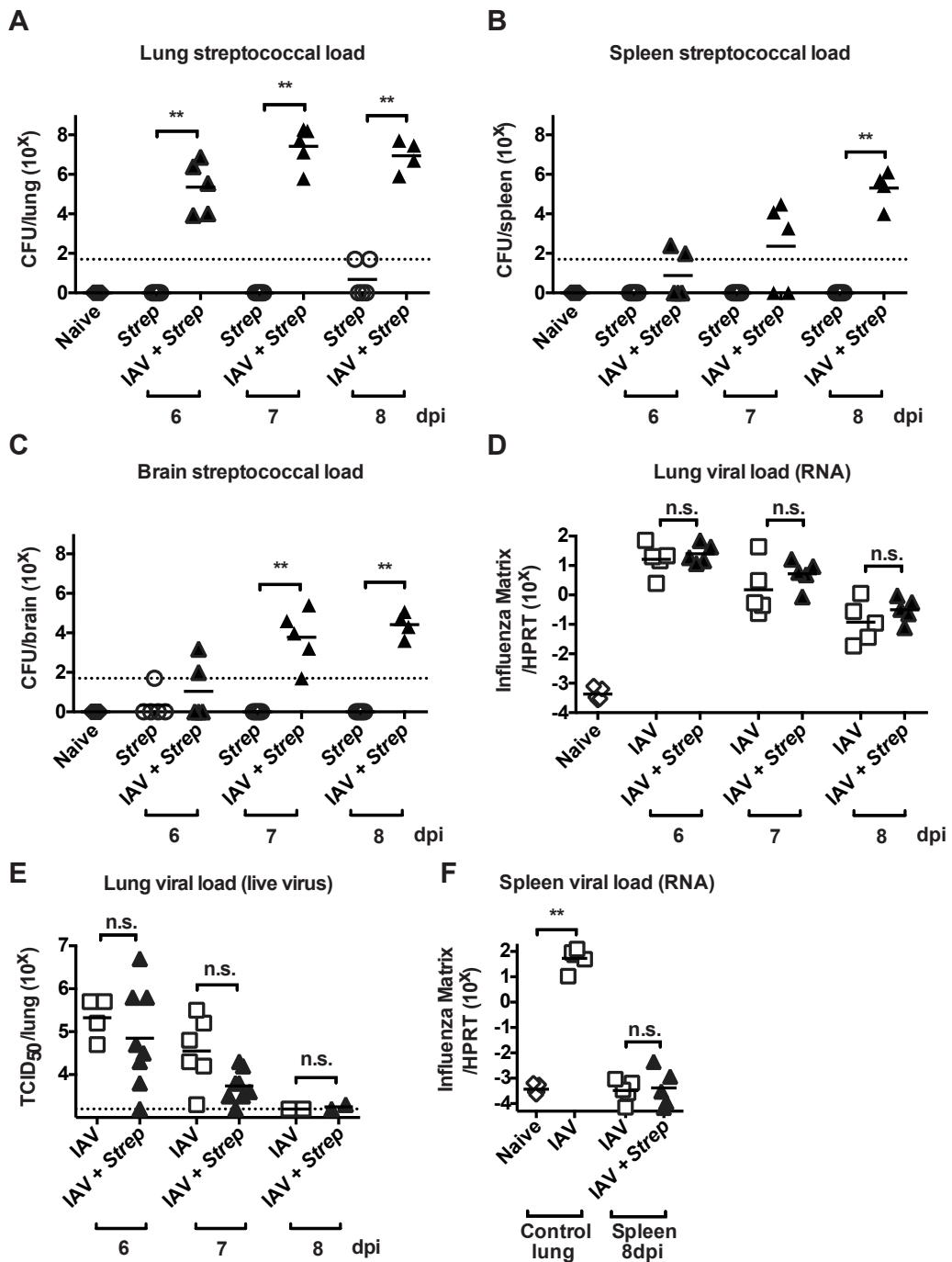
Figure 2: *Coinfection causes bacterial but not viral outgrowth.*
(A) Streptococcal load in the lung, (B) spleen and (C) brain during high dose coinfection (n=4-5, representative of 2 independent experiments, dashed line indicates detection limit). (D) Quantitative PCR for influenza matrix RNA in the lung (n=5). (E) Live virus assayed by tissue culture in the lung (n=2-5) (data shown is pooled from 2 independent experiments, dashed line indicates detection limit). (F) Quantitative PCR for influenza matrix in the spleen at 8dpi. Lungs used as positive (6dpi IAV) and negative (naive) controls (n=5). Data displayed as geometric means. Significance assessed by Mann-Whitney test. n.s. = not significant, **p<0.01

Bacterial outgrowth occurs following an initial decrease from inoculum

High streptococcal loads in the lung indicate two possibilities: that the inoculum of bacteria largely survives and persists, or largely dies, but a small proportion colonizes and subsequently outgrows. Profiling of airway bacterial loads at 5dpi + 4hrs to 5dpi + 16hrs (i.e. 4 to 16 hours after secondary infection) showed that most inoculum (amount given shown as a datapoint on the figure) dies in the first 4 hours following infection, although slightly more survives in coinfected mice. In coinfected mice, the remaining small proportion of inoculum rapidly grows, leading to high bacterial loads by 5dpi + 16hrs (Fig. 3A).

To ensure that low levels of bacteria at 5dpi + 4hrs relative to the inoculum is due to bacterial death rather than a technical issue - i.e. to ensure that the bacterial inoculum reaches the lung - I assessed bacterial 16s rRNA levels in the lung at 5dpi + 4 and 5dpi + 48 hours during coinfection. Bacterial 16s rRNA can be used to detect both live bacteria and total streptococcal material, and therefore by assessing this at 4 hours after inoculation, both live and recently deceased bacterial inoculum will be detected. 16s rRNA profiling showed high levels of streptococcal material (relative to uninfected baseline background signal) at 4 hours in both *Strep* infected and coinfected mice, indicating successful inoculation; however, high levels of streptococcal material only persist to 48 hours in coinfected mice where bacterial replication occurs (Fig. 3B). Therefore the initial bacterial inoculum reaches the lung, but largely does not survive. In coinfection a small surviving proportion is able to colonize and outgrow.

Figure 3

A

Airway early streptococcal load

B

Lung streptococcal RNA
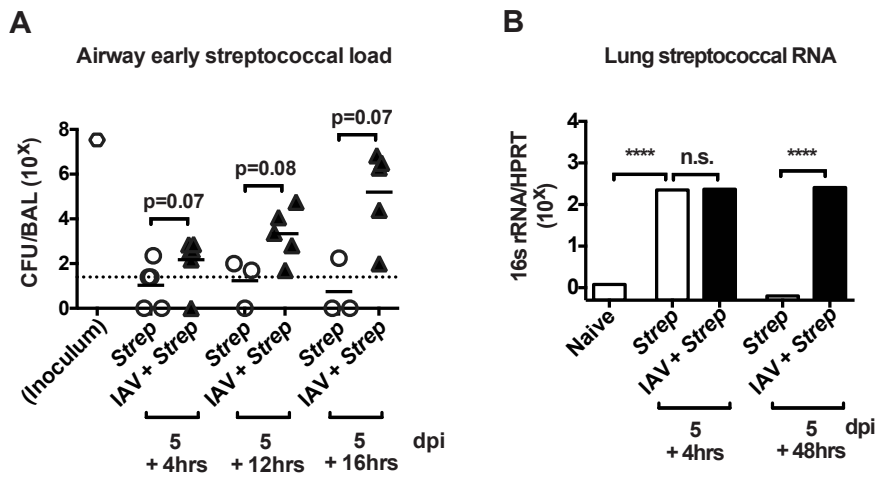
Figure 3. *Rapid death of the majority of bacterial inoculum is followed by outgrowth during coinfection.*
(A) Streptococcal load in the airways early during high dose coinfection (n=3-6). Bacterial inoculum given per mouse shown on the left. (B) Quantitative PCR for streptococcal 16s rRNA early and late during high dose coinfection (n=10). Data shown as geometric means. Significance assessed by Mann-Whitney test. n.s. = not significant. ****p<0.0001.

Live bacteria are required for mortality

Having observed bacterial outgrowth, I then attempted to determine whether bacterial outgrowth was required for mortality. Mice were coinfected either with live bacteria, heat-killed bacteria, or Pam3CSK4, a TLR2 agonist that mimics lipopeptides found in gram-positive bacterial cell walls (Manukyan *et al*., 2005). Only live bacteria caused synergy in weight loss (Fig. 4A) and mortality (Fig. 4B) with influenza. This shows live bacteria are required for synergistic mortality, and a non-replicating bacterial stimulus alone is not sufficient to induce immunopathology. This further supports the hypothesis that bacterial outgrowth is the primary driver of mortality. Immunopathology, which may still contribute to mortality, may be a downstream consequence of bacterial outgrowth.

Figure 4

A
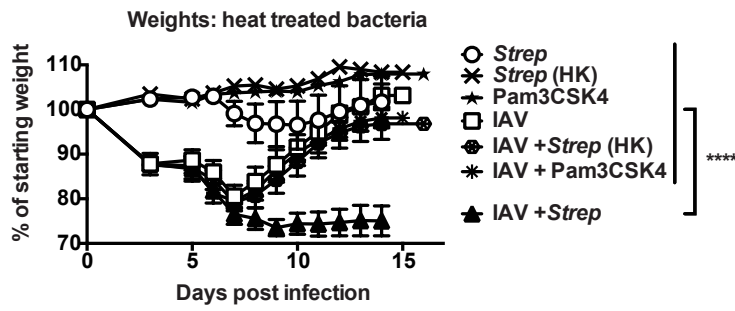
B
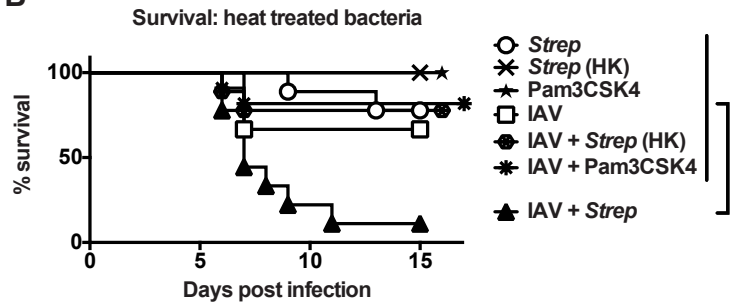
Figure 4. *Live bacteria are required for mortality during coinfection.*
(A) Weight loss and (B) mortality following high dose coinfection with live bacteria, heat-killed bacteria, or 15μg Pam3CSK4 (n=9, representative of 2 independent experiments, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final data point extended). Data displayed as arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by 2-way-ANOVA (weights) or Log-rank (Mantel-Cox) test (mortality). *p<0.05, ****p<0.0001.

3.4. Conclusions and Discussion

I established a mouse model of IAV-*Strep* coinfection with mild to moderate single infections, which, as will be shown in later chapters, allowed study of synergy rather than strong effects of the single infections. Consistent with other studies (Goulding *et al*., 2011), coinfection is characterised by bacterial but not viral outgrowth.

These results expanded upon previous studies by confirming that bacterial inoculum, although reaching the lung, largely does not survive; however, a small proportion colonizes and leads to bacterial outgrowth. This is in contrast to a study showing stable levels of *Strep* in the lung from 0-6hrs following inoculation, followed by outgrowth in coinfected mice (Li *et al*., 2012). However, in this study *Strep* infection alone is more virulent, and is detected in the lung as late as 60 hours following secondary infection. This may account for this discrepancy.

Live bacterial replication is required for mortality, suggesting bacterial outgrowth is the primary driver of mortality. Observation of bacterial outgrowth and its role as the primary driver is consistent with the implications of previous antibiotic and immunosuppression intervention studies outlined previously (Damjanovic *et al*., 2013). Although neither the implications of antibiotic versus immunosuppressive treatment or the approach used here of infecting with non-replicating bacterial-like stimuli directly confirm that bacterial outgrowth rather than immunopathology is the primary driver of mortality, the combination of

observations is highly suggestive that this is the case. It is still possible that a strong immune response to high bacterial loads and resulting immunopathology is the ultimate cause of death; however, bacterial outgrowth is required for this to occur.

A critique of the experiment showing that non-replicating bacterial stimuli such as heat-killed bacteria and TLR2 agonist cannot induce synergistic mortality through immunopathology is that these may represent an insufficient stimulus relative to live bacteria, as live, replicating bacteria may provide more constant immune stimulation. Furthermore, heat treatment may destroy some of the immune stimulating properties of bacteria, and TLR2 agonist represents only one signal, while live *Strep* is detected by multiple mechanisms (Koppe *et al*., 2012). However, if these critiques were correct some trend towards increased mortality would be expected even with an insufficient heat-killed bacterial or TLR2 agonist stimulus. As no trend is detected at all, it suggests lack of synergy is due to the fundamental requirement for live bacteria to drive mortality. One possible strategy to address these critiques is to treat mice with multiple bacteria like-stimuli, such as combined TLR2 and TLR4 stimulation, or repeated TLR2 stimulation.

It is somewhat unclear whether persistent high bacterial loads in the lung itself or dissemination to other organs, or a combination of both factors, is the driver of mortality. This is difficult to experimentally test; hypothetically coinfection with a mutant strain of *Strep* that can colonize the lung but is deficient in epithelial translocation, and therefore does not disseminate, could be a method to test this.

Chapter 4. The immune response in coinfection

4.1. Background

Having established a model and determined that bacterial outgrowth is the primary driver of mortality during coinfection, I then proceeded to profile the immune response. Results are somewhat contradictory on the immune response to coinfection.

Many studies observe a strong cytokine response in coinfection, with increases in neutrophil numbers and proinflammatory cytokines such as TNF-α, IL-6, MIP-2 and RANTES (LeVine *et al*., 2001, Seki *et al*., 2004, Shahangian *et al*., 2009). However, as previously described, other studies suggest the immune response to coinfection is impaired. Reduced IL-17 production relative to single *Strep* infection has been reported (Li *et al*., 2013); although it must be noted that this study also shows increases in several other proinflammatory cytokines such as TNF-α and G-CSF. One possibility is that immune profiling at different timepoints may give different results, and it has been hypothesised that although bacterial outgrowth during coinfection elicits a strong immune response, the early response when bacterial colonization occurs is defective. One study supports this by showing early TNF-α and IL-1β production during coinfection is reduced (Sun *et al*., 2008).

Several prior studies do not include all single infection controls, making it difficult to assess which aspects of the immune response are induced by which

infection. For example, one study shows greater neutrophil recruitment to the lung in IFNαβR$^{-/-}$ mice compared to wild-type mice during coinfection, but does not compare this to the number of neutrophils induced by *Strep* alone (Shahangian *et al.*, 2009). It is therefore hard to determine whether neutrophil recruitment in wild-type coinfected mice could be considered "impaired".

4.2. Hypotheses and Aims

I therefore decided to profile the immune response during coinfection, and look for evidence of immune impairment. I decided to use broad profiling using flow cytometry and multiplex to test the numbers of many immune cells and multiple cytokine levels during coinfection. I proposed to investigate the immune response both early and late during coinfection to determine whether at any time point immune impairment was evident. I also ensured that all appropriate single infection controls were included.

Based on the previous literature, I hypothesised that:

- Coinfection would induce a strong, overall proinflammatory immune response.
- Some aspects of the immune response would be impaired in coinfection relative to *Strep* single infection.

4.3. Results

Coinfection induces a strong neutrophil response

Flow cytometry was performed on lung at 7dpi to assess the cellular response to bacterial outgrowth. A small number of neutrophils were recruited by both *Strep* and IAV infection. However, coinfection led to a very large neutrophil response (Fig. 5A). Inflammatory monocytes are induced by IAV, and to a slightly greater extent during coinfection, but synergy is not a striking as for the neutrophil response (Fig. 5B). A trend was observed for reduced alveolar macrophages during influenza infection and coinfection although this change was not significant (Fig. 5C). Typical FACS gating strategies are shown in Appendix 1 A (myeloid) and B (lymphoid).

Influenza-induced recruitment of CD4 (Fig. 5D), CD8 (Fig. 5E) and NK (Fig. 5F) cells was unchanged by coinfection. These cells were not recruited during *Strep* infection. No significant change in B cell numbers was observed during any infection profiled (Fig. 5G). Therefore coinfection induces a strong neutrophil response and gave no clear sign of immune impairment in terms of cell recruitment relative to the modest response induced by mild *Strep* infection alone.

Figure 5
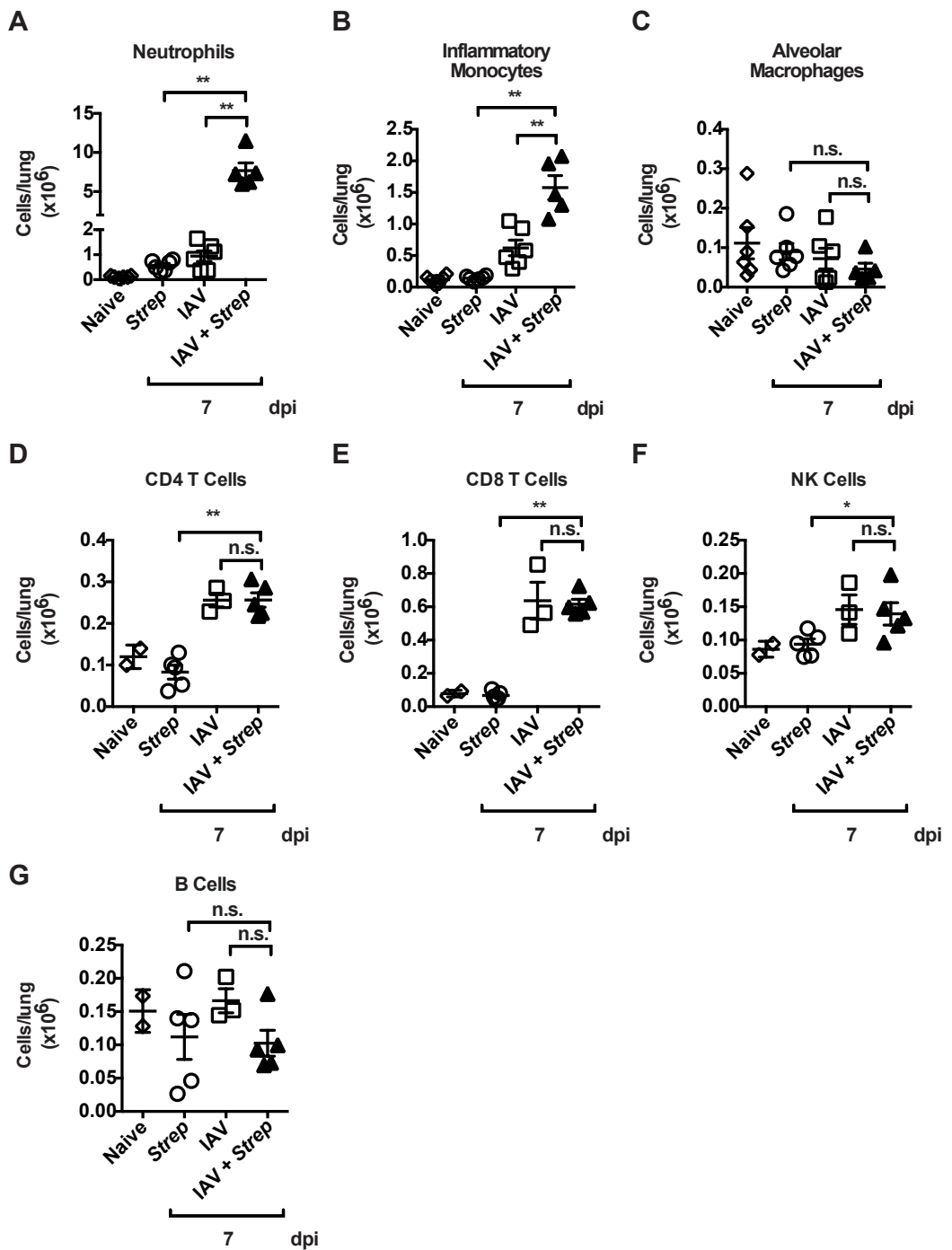
Figure 5. *Coinfection induces a strong neutrophil response.*

Flow cytometry quantification of (A) Neutrophils (Ly6G+CD11b+) (B) Inflammatory monocytes (Ly6C+CD11b+F4/80+) (C) Alveolar macrophages (CD11c+F4/80+) (A-C data pooled from 2 independent experiments, n=2-3), (D) CD4 T cells (CD3+CD4+) (E) CD8 T cells (CD3+CD8+) (F) Natural killer cells (CD3-CD4-CD8-NKp46+) and (G) B cells (CD3-CD19+) (D-G n=2-5) in the lung during high dose coinfection. Data displayed as arithmetic means ± SEM. Significance assessed by Mann-Whitney test. n.s. = not significant. *p<0.05. **p<0.01.

Coinfection induces a strong proinflammatory cytokine response

Having profiled the cellular response, cytokine concentrations at 7dpi in the airways (BAL fluid) were then assessed. Broad profiling of a range of cytokines was performed by multiplex (select cytokines shown in Fig. 6A, table of all shown in top section of table in Fig. 6B). The concentration of IL-22 and type I IFNs was also assessed by ELISA (bottom section of Fig. 6B). *Strep* modestly induced a small number of cytokines relative to uninfected mice, including G-CSF and IL-6. IAV induced a broader range of cytokines at higher levels, including IFN-γ and IL-10. However, coinfection hugely increased the majority of cytokines tested relative to both single infections. Of particular note, TNF-α, of which only very low levels were induced by both *Strep* and IAV, was greatly upregulated by coinfection. Chemokines associated with neutrophil recruitment such as KC and MIP-2, and with inflammatory monocyte recruitment such as MCP-1, were also greatly increased. Crucially, almost every cytokine tested was increased in coinfection relative to either single infection, and therefore there is no clear immune impairment in the cytokine response (note – some cytokines assessed by multiplex, such as LIX, show somewhat high background levels in uninfected mice; this is likely an artefact of the assay, as the detection limits for different cytokines vary). The only cytokine where a very slight decrease was observed was IL-22, which was reduced during both influenza and coinfection from already low levels in *Strep* and uninfected mice. Therefore coinfection induces a strong and seemingly unimpaired cytokine response.

Figure 6

A
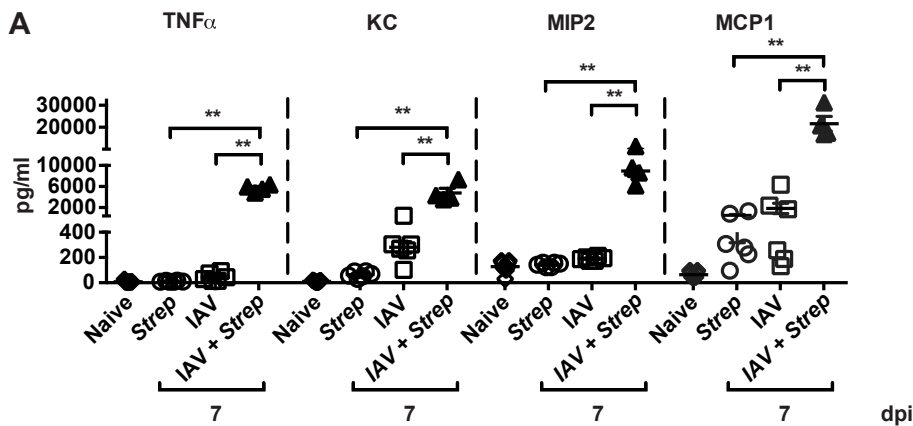
B

	Cytokine at 7dpi (pg/ml)				Fold change vs. single infections
	Naïve	**Strep**	**IAV**	**IAV + Strep**	
TNFα	8	11	45	5682	256
MIP1α	61	321	1338	87599	134
RANTES	12	10	16	1517	119
GCSF	31	1002	4864	183797	83
IL10	2	4	137	1858	80
IL6	5	101	9325	69182	71
MIP1β	101	228	1139	30002	59
IP10	10	2006	10511	253229	55
MIP2	128	143	190	8962	54
IFNγ	26	26	3591	14232	47
KC	10	65	281	4778	36
MIG	26	671	10622	75288	28
MCP1	63	506	1828	21613	22
LIF	2	3	311	431	13*
MCSF	11	15	48	349	13*,†
Eotaxin	14	18	565	830	8 *
VEGF	15	65	86	426	6*,†
IL1α	137	130	36	366	5 *,†
IL15	16	14	7	48	5*,†
IL1β	84	96	90	441	5 *,†
IL17	2	3	16	34	5 *
LIX	222	176	174	717	4
IL5	4	9	429	225	4 *
GMCSF	32	41	37	75	2
IL12 (p40)	21	50	22	57	2 †
IFNα	1	7	12	115	13 *
IFNβ	0	1	6	29	11
IL22	24	52	18	26	0.8 *

The "*,†" annotation applies to the bracketed group spanning from TNFα through MCP1.

IL2, IL3, IL4, IL7, IL12p70, IL13 all excluded as means all <20pg/ml

Figure 6. *Coinfection induces a strong proinflammatory cytokine response.*
Multiplex and ELISA quantification of airway cytokines during high dose coinfection - select cytokines shown in (A) and table of all shown in (B). Multiplex is top section of table, lower section is ELISAs (Multiplex: n=2-3, samples pooled from 3 independent experiments; IL-22 ELISA: n=3-8, samples pooled from 3 independent experiments; type I IFN ELISA: n=3-8; samples pooled from 2 independent experiments). Data shown as arithmetic means ±SEM (graph) or arithmetic means only (table). Cytokines in table ranked by fold change relative to single infections, shown in the right hand column. This is calculated by taking the geometric mean of fold change IAV + *Strep* vs. IAV and fold change of IAV + *Strep* vs. *Strep*. Significance assessed by Mann-Whitney test. **p<0.01 (graph). *p<0.05 IAV+*Strep* vs. *Strep*; †p<0.05 IAV + *Strep* vs IAV (table).

IAV does not impair the early cellular response to Strep

There is a strong immune response and no clear impairment in cell recruitment or cytokine production at 7dpi, showing bacterial outgrowth induces a robust response. However, it is possible that the immune response is impaired at an earlier phase in secondary infection, allowing bacterial colonization. Therefore the cellular response in the airways from 5dpi + 4hrs to 5dpi + 16hrs hours during coinfection (i.e. 4 to 16 hours following secondary infection) was profiled by flow cytometry.

Strep induced a small neutrophil response from 5dpi + 12 hours onwards (Fig. 7A), consistent with the small neutrophil infiltrate observed in the lung at 7dpi; no neutrophils were detected earlier at 5dpi + 4hrs. IAV maintains a consistent level of neutrophils, higher than induced by *Strep*, throughout the period observed. The number of neutrophils during coinfection is equivalent to IAV at 5dpi + 4hrs and begins to rise substantially at 5dpi + 12hrs, with high levels at 5dpi + 16hrs - consistent with the high levels in the lung at 7dpi. Therefore the number of neutrophils present during coinfection is higher than *Strep* at early time points during secondary infection, and therefore neutrophil recruitment cannot be considered to be impaired.

As at 7dpi in the lung, inflammatory monocytes are not induced by *Strep* but are recruited by both IAV and coinfection (Fig. 7B). Alveolar macrophage numbers are somewhat variable in all infection settings, and no clear trend is discernible; however, at no point are the number present in coinfected mice significantly reduced relative to *Strep* infection (Fig. 7C). As observed at 7dpi, the developing

CD8 T cell response to IAV is unaffected by coinfection (Fig. 7D). Therefore overall coinfection induces a strong neutrophil response, which develops as bacterial loads increase, and at no time point was a reduction in cell numbers during coinfection detected.

Figure 7
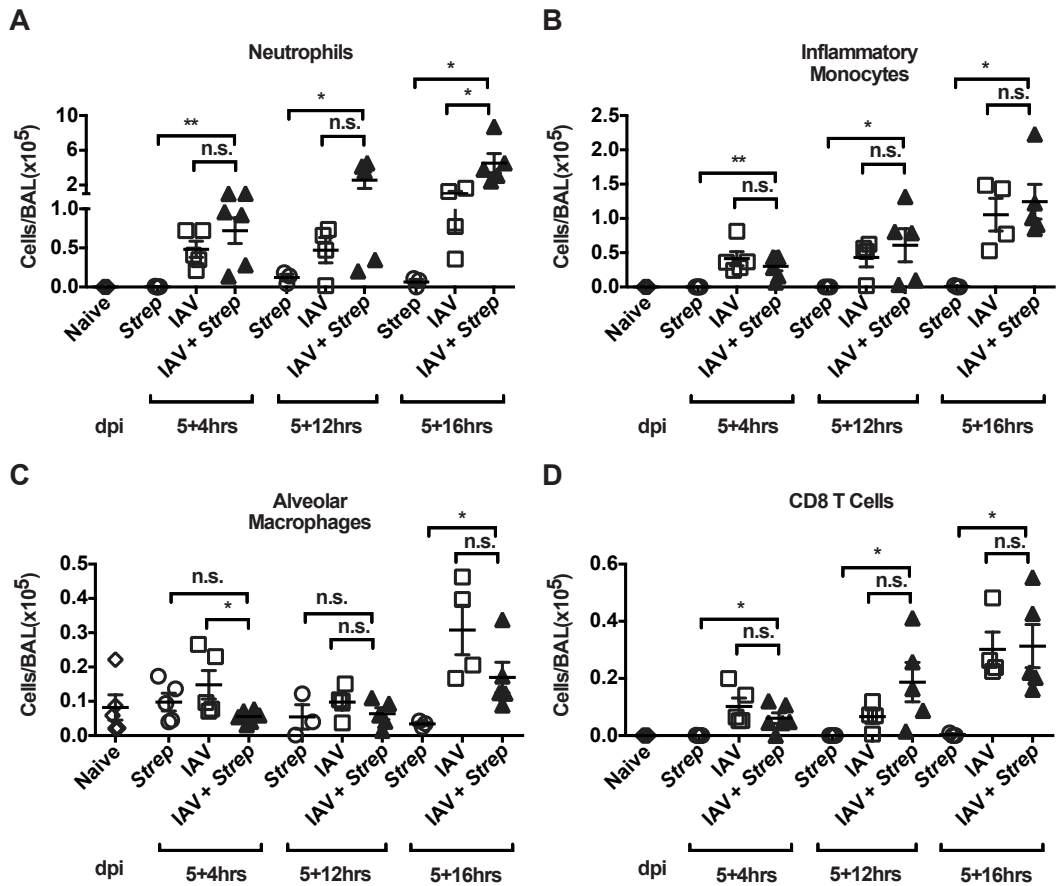
Figure 7. *Cell recruitment to the airways is not impaired early during coinfection.*
Flow cytometry quantification of (A) Neutrophils (Ly6G+CD11b+) (B) Inflammatory Monocytes
(Ly6C+CD11b+F4/80+) (C) Alveolar Macrophages (CD11c+F4/80+) (D) CD8 T Cells (CD3+CD8+) in
the airways early during high dose coinfection (n=3-6). Data displayed as arithmetic means
±SEM. Significance assessed by Mann-Whitney test. n.s. = not significant. *p<0.05.**p<0.01.

*IAV does not impair the early cytokine response to Strep*

Although a strong cytokine response was observed at 7dpi, it is possible this could be impaired early following secondary infection. Cytokine levels were therefore profiled in the airways at 5dpi + 4hrs. Broad profiling of a range of cytokines was performed by multiplex (select cytokines shown in Fig. 8A, table of all shown in top section of table in Fig. 8B). Concentrations of IL-22 and type I IFNs were also assessed by ELISA (bottom section of Fig. 8B).

The cytokine response to *Strep* was greater than observed later at 7dpi, with increases in proinflammatory cytokines and neutrophil-recruiting chemokines such as TNF-α, MIP-2 and KC, possibly reflecting the recent bacterial stimulus. During IAV, a similar cytokine profile to 7dpi was evident. TNF-α, MIP-2 and KC are less induced by IAV than *Strep* infection at this point. The strong synergy in cytokine production during coinfection observed at 7dpi was much less evident at this time point. Relative to *Strep* infection, several cytokines such as TNF-α and MIP-1α were moderately increased in coinfection, although much less so than at 7dpi, while others, such as KC, GM-CSF and MIP2 showed a very modest, non-significant decrease. Somewhat refuting the concept of immune impairment, no cytokine observed was significantly reduced relative to either single infection during coinfection. The only exceptions to this were an extremely slight decrease during coinfection in IL-22 relative to *Strep* infection and uninfected mice, and a small decrease in IL-5 relative to IAV infection; however, IL-5 is not induced by *Strep* and therefore is unlikely to be a crucial factor in the antibacterial immune response.

Broad cytokine profiling by multiplex and ELISA was also performed on two further time points – 5dpi + 12hrs and 5dpi + 16hrs (see Appendix 2A and B for tables) with no reduction in any cytokine during coinfection relative to single infections evident. Therefore cytokine production does not appear to be impaired early during secondary infection.

Figure 8

A
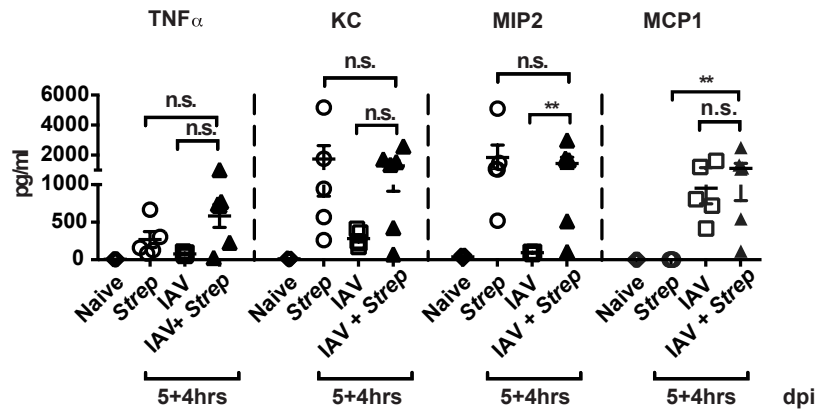
B

	Cytokine at 5dpi + 4hrs (pg/ml)				Fold change vs. single infections
	Naïve	***Strep***	**IAV**	**IAV +*Strep***	
MIG	4	3	7786	5522	38 *
MCP1	1	1	953	1119	30 *
IP10	2	16	3182	3406	15 *
IFNγ	4	9	3084	2284	13 *
IL17	0	0	11	30	13 *
TNFα	6	268	76	583	4
LIF	1	5	142	104	4 *
IL10	1	2	57	44	4 *
MIP2	38	1842	93	1442	3 †
RANTES	4	6	30	45	3 *
MIP1β	64	648	953	2531	3
IL4	1	1	84	35	3 *
VEGF	10	16	11	42	3
IL5	13	13	766	307	3 *,†
Eotaxin	2	2	124	51	3 *
GCSF	1	629	1757	2901	3
IL6	5	936	6109	6104	3 *
IL1β	28	58	57	123	2
LIX	76	93	139	229	2
IL12 (p40)	6	6	20	22	2
KC	12	1740	281	1285	2
MIP1α	45	392	1124	1210	2
GMCSF	44	374	84	248	1.4
IL1α	33	71	20	51	1.4
IL15	29	23	51	47	1.4 *
IL12 (p70)	12	16	17	21	1.3
IL9	71	125	87	79	0.8
IFNβ	3	3	8	14	3
IFNα	4	3	73	46	3 *
IL22	24	29	21	14	0.6 *

IL2, IL3, IL7, IL13 and MCSF excluded as all means <20pg/ml

Figure 8. *Cytokine production is not impaired early during coinfection.*
Multiplex and ELISA quantification of airway cytokines during high dose coinfection select cytokines shown in (A) and table of all shown in (B). Multiplex is top section of table, lower section is ELISAs (Multiplex: n=3-6, ELISAs: n=3-6). Data shown as arithmetic means ±SEM (graph) or arithmetic means only (table). Cytokines in table ranked by fold change relative to single infections, shown in the right hand column. This is calculated by taking the geometric mean of fold change IAV+*Strep* vs. IAV and fold change of IAV+*Strep* vs. *Strep*. Significance assessed by Mann-Whitney test. **p<0.01 (graph). *p<0.05 IAV+*Strep* vs. *Strep*; †p<0.05 IAV + *Strep* vs IAV (table)

4.4. Conclusions and Discussion

In summary, coinfection induced a large neutrophil and proinflammatory cytokine response. This response increased in similar time frame to previously shown bacterial outgrowth and was strongly developed by 7dpi. In contrast, *Strep* alone induced a much more modest, transient proinflammatory response at 5dpi + 4hrs, with recruitment of small number of neutrophils by 5dpi + 12hrs. As low virulence *Strep* is largely cleared from the lung by 5dpi + 4hrs, it is likely the small cytokine and downstream neutrophil response is a by-product of bacterial stimulus, and is not required for clearance. This suggests, although does not confirm, that the immune response is only required for bacterial clearance in the context of coinfection; this is further investigated in later chapters.

No aspect of the immune response during coinfection was reduced relative to *Strep* infection including, in contrast to prior studies (Sun *et al.*, 2008; Li *et al*, 2012), IL-17 and TNF-α. However there was a very slight decrease in IL-22 from very low baseline levels. This is of some interest and will be discussed in chapter 9. Overall no clear impairment in either cell recruitment or cytokine production was observed.

As a relatively comprehensive timepoint range was profiled it is unlikely that this accounts for the lack of immune impairment detected as compared to previous studies. Most likely differences are due to the virulence of the *Strep* strain used. It could be argued that use of non-colonizing, low virulence *Strep* is not that suitable for investigating immune impairment in coinfection, as it induces a very modest

immune response, leaving little scope for reduction. However, as low virulence *Strep* is capable of outgrowth and causing mortality following influenza, it is still highly relevant for studying mechanisms of coinfection susceptibility. The virulence of *Strep* used may accentuate or reduce the relative importance of influenza-mediated immune impairment as a mechanism of coinfection. A possible experiment to assess whether influenza impairs cellular and cytokine responses independent of the virulence of the *Strep* strain used is influenza followed by TLR2 stimulation.

As expected, IFN-α is present during influenza infected and coinfected mice at 5dpi + 4hrs. However, it only persists and is somewhat increased in coinfected mice at 7dpi. It is probable the increase in IFN-α in coinfected mice at 7dpi is a side effect of general strong inflammation; it is more likely that interferon induced during the viral infection (i.e. that detected at 5dpi + 4hrs) influences the downstream immune response. The presence of type I IFNs is of interest due to the reported inhibition of MIP2 and KC production by type I IFNs, and the resulting resistance of IFNαβR$^{-/-}$ mice to coinfection (Shahangian *et al*., 2009). However, in this setting KC and MIP2 production are greatly increased in coinfection relative to *Strep* alone at 7dpi, and showed only marginal, non-significant reductions at 5dpi + 4hrs. Furthermore, despite the reported antagonism between type I IFN and IL-1β in other experimental systems (Guarda *et al*., 2011), prior influenza infection and the presence of IFN-α does not appear to inhibit IL-1β production during coinfection. Furthermore, IL-1β is not significantly induced by low virulence *Strep* alone, suggesting it is not required for bacterial control in this context. Therefore, although IFN-α is present during

both viral infection and coinfection, it does not appear to inhibit the downstream immune response in this model.

Although quite broad profiling has been performed, it is difficult to conclusively rule out immune impairment as a factor during coinfection, as other aspects of the immune response not assessed here may be impaired. One aspect that did show a moderate reduction - consistent with other studies (Ghoneim *et al.*, 2013) – was the number of alveolar macrophages during coinfection at 7dpi. However trends at earlier timepoints were somewhat unclear, and the reduction relative to *Strep* infection was at no timepoint significant, suggesting that insufficient alveolar macrophage numbers is not the primary cause of coinfection.

It is possible that reduced alveolar macrophage function may be a factor in coinfection susceptibility (Goulding *et al.*, 2011), as here only alveolar macrophage numbers are assessed (neutrophil function will be assessed in a later chapter). Furthermore there are other cells implicated in coinfection susceptibility that have not been profiled such as γδ T cells (Li *et al.*, 2012; Cao *et al.*, 2014), which are outside the scope of this study. However, despite these caveats, there is a strong immune response induced by coinfection, with no clear impairment in any factor assessed here.

There is no change in the antiviral aspects of the immune response such as CD8 T cells numbers during coinfection, consistent with intact viral control previously observed, although it must be noted total CD8 T cell numbers does not indicate that these cells have equivalent influenza-infected cell specificity or cytotoxic

activity. The presence of cytokines induced in the antiviral response to IAV could also be a factor in coinfection susceptibility – for example, the reported detrimental role of IFN-γ (Sun *et al*., 2008), particularly as this is increased during coinfection.

IL-10, a cytokine with many anti-inflammatory roles that has been reported to contribute to coinfection susceptibility (van der Sluijs *et al.*, 2004), is also modestly induced by IAV and greatly increased during coinfection. However, despite its presence and well-characterised anti-inflammatory role, many proinflammatory cytokines and a strong neutrophil response are induced, suggesting IL-10 is not blocking the immune response. Furthermore, the role of IL-10 as a factor contributing to coinfection susceptibility has been disputed, as the original result showing anti-IL-10 ameliorates coinfection (van der Sluijs *et al*., 2004) could not be reproduced in a subsequent study with IL-10$^{-/-}$ mice (Sun *et al*., 2008).

Although the immune response to coinfection appears to be strong and unimpaired, it is possible that this response is excessive and harmful, promoting immunopathology. Previously bacterial outgrowth was identified as required to drive mortality, but whether the ultimate cause of death is high bacterial loads or an excessive immunopathological response to outgrowth is not clear. To address this, in later chapters, some aspects of the immune response such as neutrophils and TNF-α are depleted, and found to be protective, suggesting this response is not excessive. However, the possibility remains that other factors of the strong immune response to coinfection induced contribute to eventual mortality.

Chapter 5. Neutrophils in coinfection

5.1. Background

Having observed a strong proinflammatory immune response to coinfection and somewhat countered the concept of immune impairment, I decided to investigate whether this response was protective or pathogenic. I initially focused on neutrophils due to their striking recruitment in large numbers to the lung. Although previous studies have attempted to assess the role of neutrophils in coinfection, no study has specifically depleted them in a context where both a positive and negative effect of intervention could be detected. In addition, previous results are somewhat variable, which may reflect different disease settings in various models.

Some studies deplete neutrophils in coinfection with anti-Gr-1, which is poorly specific (Daley *et al*., 2008), making these results difficult to interpret. One study reported that anti-Gr-1 treatment increased bacterial loads 24 hours following secondary infection in a moderate to severe coinfection (Sun *et al*., 2008). Another severe coinfection study also used anti-Gr-1, which exacerbates of bacterial loads if secondary infection is performed at 3dpi, but not 6dpi (McNamee *et al*., 2006). This study highlights the difficulties in using more virulent *Strep* strains to investigate coinfection, as in this study anti-Gr-1 also exacerbates bacterial loads in single infection. The authors attribute lack of effect of anti-Gr-1 treatment during coinfection at 6dpi to neutrophil impairment, arguing that depletion of already impaired neutrophils has no effect. An

alternative interpretation is that anti-Gr-1 exacerbates disease in both *Strep* and coinfection, and as bacterial loads are already very high following coinfection at 6dpi, there is little capacity for further exacerbation. Overall, anti-Gr-1 treatment appears to increase bacterial loads when they are not already extremely high due to severe coinfection, although these results are hard to interpret due to poor specificity.

Use of a severe coinfection setting, and thus an inability to exacerbate already high bacterial loads, may also apply in another study using more specific anti-Ly6G to deplete neutrophils. In this severe coinfection study it was hypothesised that neutrophils were harmful, causing immunopathology. Depletion of neutrophils failed to ameliorate severe pathology and did not affect very high bacterial loads (Damjanovic *et al.*, 2013). Although this suggests neutrophils have no role, there is no capacity to detect a harmful effect of neutrophil depletion in this setting.

In contrast to studies showing no effect of neutrophil depletion, another study suggests neutrophils are protective: IFN$\alpha\beta$R$^{-/-}$ mice are better protected from a moderate to severe coinfection, which is attributed to production of more of the neutrophil chemoattractants KC and MIP2, and greater neutrophil recruitment (Shahangian *et al.*, 2009). Addition of KC and MIP2 to wild-type mice confers resistance, while blockade of CXCR2, the common receptor for KC and MIP2, renders IFN$\alpha\beta$R$^{-/-}$ mice susceptible. This indirectly implies, although does not directly confirm, that neutrophils are protective in coinfection.

Other studies have reported neutrophils are functionally impaired during coinfection, although this is somewhat unclear. It has been reported that neutrophils from IAV infected mice have reduced ROS production and phagocytic capacity. (McNamee *et al*., 2006). Another study reported reduced levels of total myeloperoxidase and myeloperoxidase per neutrophil during coinfection, but no impairment in ROS production (LeVine *et al*., 2001). In contrast, another study reports an increase in total myeloperoxidase levels, but also reports a very large increase in the number of neutrophils, so it is also possible myeloperoxidase per neutrophil is decreased (this is not assessed). This study also reports a marginal reduction in neutrophil phagocytic capacity (Damjanovic *et al*., 2013). Overall these results suggest neutrophils show some functional impairment during coinfection, although these effects are somewhat marginal and variable in different contexts.

There are various factors that may account for the discrepancy between these studies. The first study outlined (McNamee *et al.*, 2006) uses LPS aerozolisation to increase the number of neutrophils available for assays, which may be a confounding factor. This may explain why impaired ROS production was not detected in the second study described (LeVine *et al*., 2001). Severity of coinfection may account for differences in reported myeloperoxidase production; the study reporting increased total myeloperoxidase (Damjanovic *et al*., 2013) shows more severe outgrowth than that reporting reduced total myeloperoxidase (LeVine *et al*., 2001), which may different amounts of neutrophil degranulation in response to more or less bacterial stimulus.

5.2. Hypotheses and Aims

Therefore I decided to attempt to clarify the role of neutrophils in coinfection. I aimed to:

- Test whether neutrophils are functionally impaired during coinfection.

- Optimise the coinfection model to give a context where both a positive and detrimental effect of depleting neutrophils could be observed. This is to avoid the disadvantages of only using a severe context, where only a very strong positive effect of neutrophil depletion could be observed.

- Specifically deplete neutrophils in this context and assess whether they are on balance protective or harmful.

- If possible to optimise a setting where neutrophil depletion does not affect single infections, to allow study of their role in coinfection alone.

As prior studies have suggested inconclusively that neutrophils are somewhat impaired during coinfection, and others imply but do not confirm they are protective, I hypothesised:

- Neutrophils may exhibit functional impairment during coinfection.

- Neutrophil depletion would have no effect during low virulence single *Strep* or IAV infection.

- Depletion of neutrophils would be detrimental during coinfection.

- Depletion of neutrophils in coinfection would exacerbate bacterial outgrowth.

5.3. Results

Collagenase D digestion gives highly pure mouse lung neutrophils

To test neutrophil function during coinfection, mouse neutrophils were purified from coinfected mice at 6dpi. To achieve this a positive separation of Ly6G$^+$ cells on a Magnetic Activated Cell Sorting (MACS) column was utilised. Several steps had to be optimised before high purity neutrophils were derived (I am grateful for the help of Dr. C. De Santo (WIMM, University of Oxford) and Dr. V. Papayannopoulos, (Division of Molecular Immunology, NIMR) with neutrophil purification). Initially, different enzyme mixes for digestion of the lung tissue prior to separation were tested. Collagenase D digest of manually macerated tissue resulted in the highest purity neutrophils after one round of MACS separation - approximately 30% of total material was comprised of neutrophils (Fig. 9A, neutrophils defined as proportion of ungated material Ly6G$^+$CD11b$^+$), while over 80% of live cells were neutrophils (Fig 9B, neutrophils defined as proportion of Death Stain$^-$FSC$^{>\text{very low}}$SSC$^{>\text{very low}}$ material Ly6G$^+$CD11b$^+$). This suggested good purification; however, the discrepancy between the proportion of neutrophils in the total material and the live cell gate suggested the main contaminant was a large population of dead cells.

To remove dead cells, a second run through a MACS column and, where very high purity was required, a separation over a Percoll gradient, was performed. This removed much of the dead cell contamination, increasing the proportion of neutrophils in the total preparation (Fig. 9C), and ensuring neutrophils made up

the huge majority of live cells (Fig. 9D). To improve the speed of processing replacing manual maceration of the lung tissue with a gentleMACS homogeniser was tested; however, this reduced the proportion of neutrophils in both total material (Fig. 9E) and the live cell gate (Fig. 9F) and therefore was not used in subsequent purifications.

Therefore the final optimised purification protocol (shown in Fig. 9G) was a manual homogenisation of tissue, followed by digestion of the lung with collagenase D digest mix, then sequential positive selection for Ly6G$^+$ cells on two MACS columns. Where further removal of dead cells was desirable, neutrophils were also further purified over a Percoll gradient (whether this step was performed is specified in subsequent figure legends).
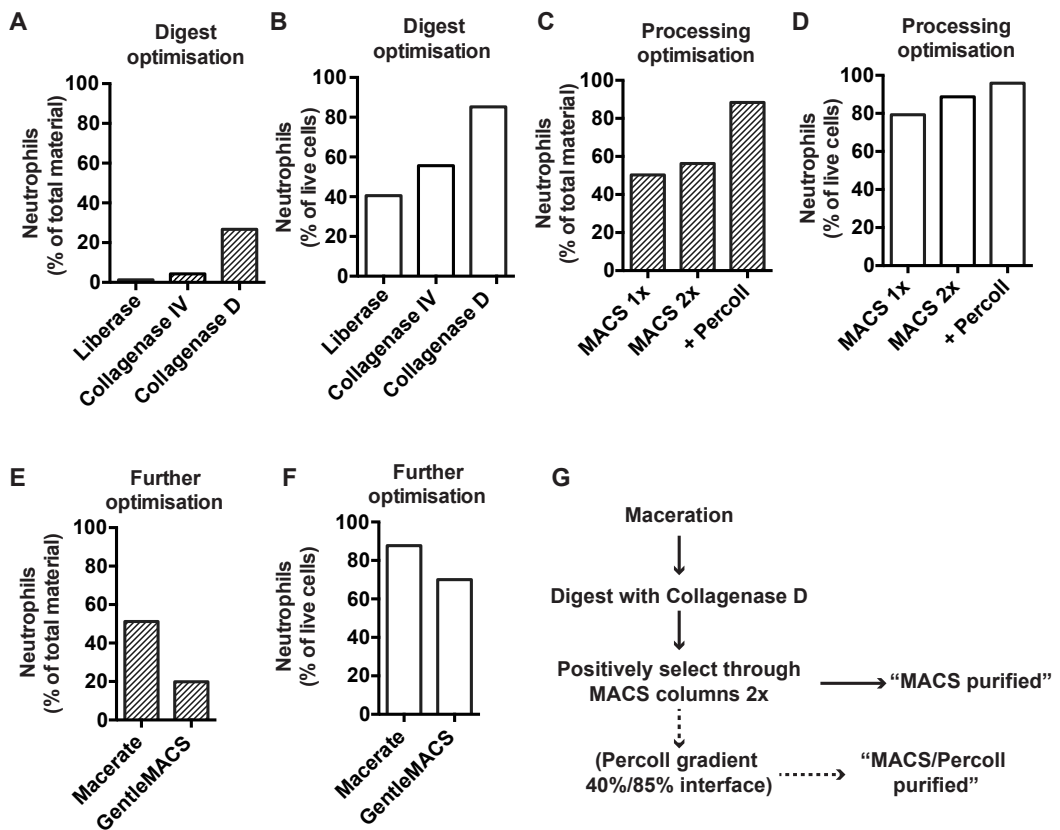
Figure 9

Figure 9. *Optimisation of neutrophil purification.*

% Neutrophils (Ly6G+CD11b+) derived from 6dpi IAV infected mouse lungs after different purification regimes: Neutrophils obtained through one round of MACS separation following lung digestion with different enzyme mixes containing Liberase, Collagenase IV or Collagenase D - total material shown in (A) and live cells only in (B). Neutrophils obtained following Collagenase D digestion with one or two rounds of MACS separation, followed by a 40/85% Percoll gradient, taking cells at the interface - total material shown in (C) and live cells only in (D). Neutrophils obtained with Collagenase D digestion and two rounds of MACS separation following homogenisation by maceration or using a gentleMACS tissue disassociator - total material shown in (E) and live cells only in (F). Each experiment represents neutrophils pooled from the lungs of 2-6 mice. Data is shown as % Neutrophils of either total material (ungated) or live cells (Death stain-FSC>very low SSC>very low). (G) Scheme of optimised purification protocol.

Using the established purification protocol, neutrophils were purified from mouse lungs at 6dpi during coinfection or *Strep* infection and their capacity to respond to restimulation was assessed. Neutrophil ROS production has been extensively studied using phorbol myristate acetate (PMA) stimulation (Nauseef *et al*., 1991). Coinfected neutrophils had equivalent ROS production to those from *Strep* infected mice upon restimulation with the PMA analogue phorbol 12,13-dibutyrate (PDBu) (Fig. 10A). *C. albicans* has been shown to induce neutrophil NET formation (Urban *et al*., 2006). Similar levels of NET formation upon restimulation with *C. albicans* were observed (Fig. 10B) (experiment performed in collaboration with Ms. Nora Branzk, Division of Molecular Immunology, NIMR; *C. albicans* kindly provided by Dr. J. Duarte, Division of Molecular Immunology, NIMR).

I then assessed capacity of purified neutrophils to produce cytokines. Production of TNF-α and KC by purified mouse bone marrow neutrophils has previously been described (Zhang *et al*., 2009). TNF-α and KC production by mouse lung neutrophils was similar or greater in those from coinfected mice (Fig. 10C) upon stimulation with the TLR2 agonist Pam3CSK4, the TLR4 agonist LPS, or PDBu. The ability to produce similar levels of reactive oxygen species and cytokines, and an equivalent capacity to form NETs, suggests that neutrophil function on a per-cell basis is not impaired during coinfection.

In addition to profiling the response of restimulated neutrophils *in vitro*, *in vivo* function was tested by assessing myeloperoxidase levels in the BAL fluid of coinfected and *Strep* infected mice at 6dpi. Myeloperoxidase levels were moderately higher in the BAL fluid of coinfected mice (Fig. 10D). This suggests neutrophil degranulation is not impaired in the lung during coinfection *in vivo*.
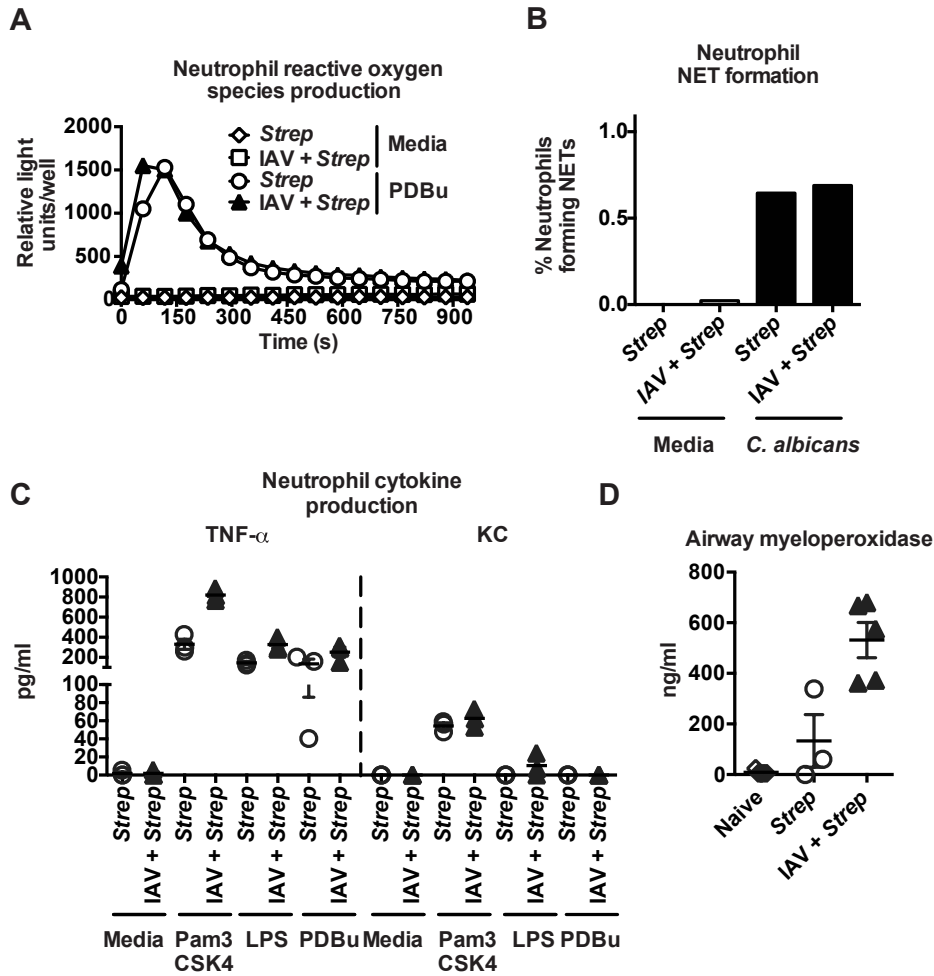
Figure 10

Figure 10. *Lung neutrophils are not functionally impaired during coinfection.*
(A) ROS production by luminol assay of PDBu (50nM) stimulated MACS purified lung neutrophils from high dose *Strep* or coinfected mice at 6dpi (neutrophils from 9 mice pooled into 3 separate replicates/group, with technical triplicates performed in the assay/condition; representative of 2 independent experiments). (B) Percentage of NET forming cells assessed by microscopy of *C. albicans* stimulated MACS/Percoll purified neutrophils (neutrophils from 3 mice pooled/group). (C) ELISA quantification of TNF-α and KC produced by Pam3CSK4 (1μg/ml), LPS (1μg/ml) or PDBu (50nM) stimulated MACS purified neutrophils (neutrophils from 3 mice pooled/group, with technical triplicates performed in the assay/condition). (D) ELISA quantification of myeloperoxidase in the airways at 6dpi (n=3-5). Data displayed as percentage of neutrophils (NET formation) or arithmetic means ±SEM (ROS, cytokine production, airway MPO).

Low dose coinfection gives moderate mortality

Having confirmed there is a strong neutrophil response during coinfection and that on a per-cell basis they do not appear functionally impaired, I then attempted to directly assess *in vivo* the role of neutrophils in coinfection. However, to achieve this, the current "high dose" coinfection setting, where almost all mice reach endpoint - although highly suitable for profiling synergistic effects in coinfection - was not appropriate for interventions, as only a strong positive effect of neutrophil depletion would be detected. Therefore both IAV and *Strep* doses were titrated down to give a "low dose" setting where approximately 50% of mice reached endpoint during coinfection (Fig. 11A, titration of single infections also shown in Fig. 11B).

Low dose coinfection indicates bacterial outgrowth correlates with mortality

Although optimised for the purpose of assessing interventions, low dose coinfection setting was also useful to further confirm bacterial outgrowth as the driver of mortality during coinfection. As not all mice reach endpoint at a low dose, mice can be classified by disease status as "endpoint" or "recovering", and therefore it was possible to determine whether bacterial outgrowth correlates with mortality.

Therefore in a low dose coinfection, mice reaching endpoint (this occurred from 8-10 dpi) were harvested. Mice showing weight gain - classified as "recovering" were concurrently harvested, as weight gain is typically an indicator of recovery.

For comparison, a high dose coinfection, where all mice reach endpoint, was also performed. There was a very strong correlation between reaching endpoint and high streptococcal loads in the lung (Fig. 11C, left panel shows all high dose mice versus all low dose mice, right panel shows low dose mice separated by disease status), spleen (Fig. 11D) and brain (Fig. 11E). Recovering mice had little, if any, bacteria present. All high dose coinfected mice reached endpoint and exhibited high bacterial loads. Therefore low dose coinfection, as well as enabling a greater range of inventions to be investigated, demonstrates clearly that bacterial outgrowth correlates very closely with mortality.
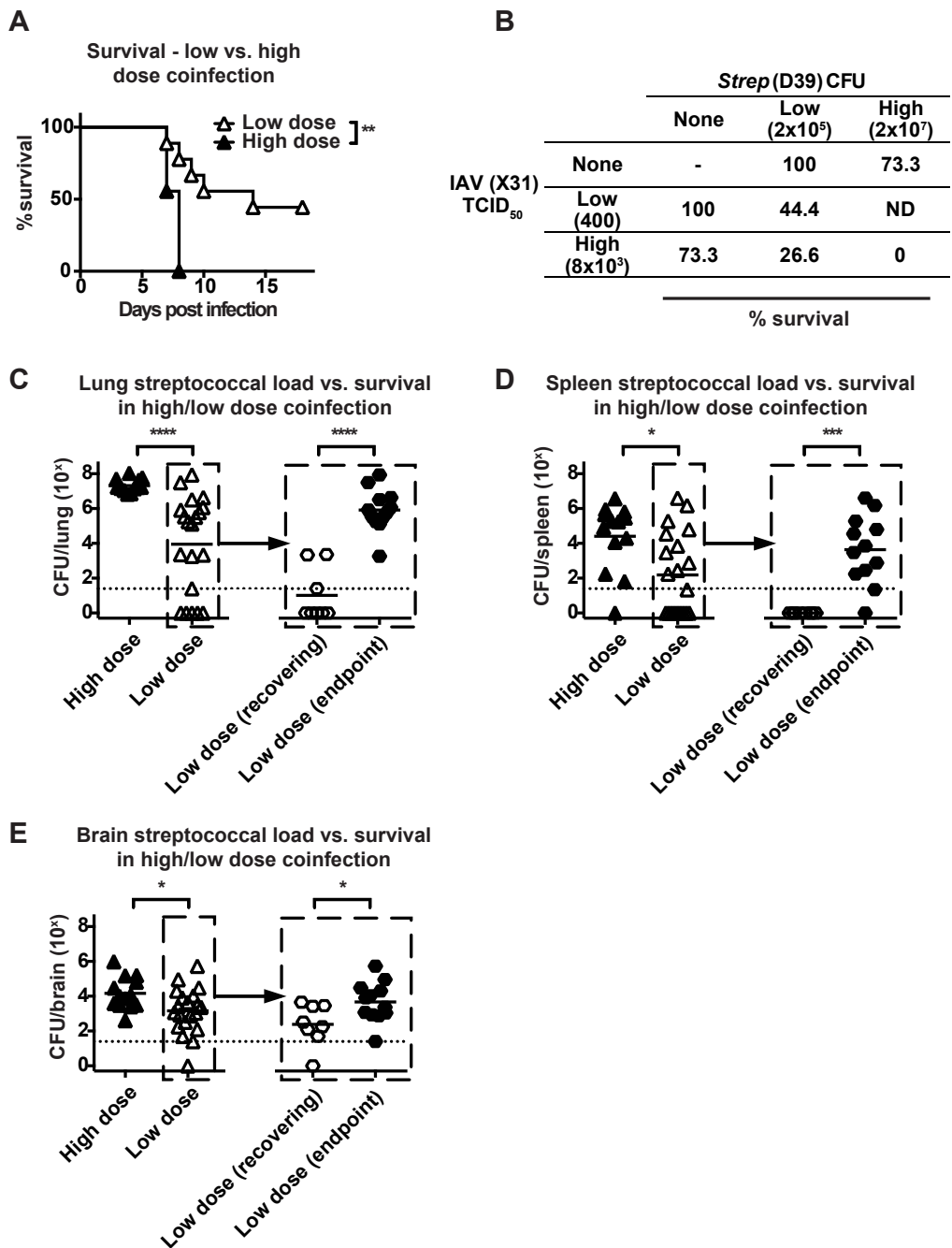
Figure 11

A

Survival - low vs. high dose coinfection

B

		Strep (D39) CFU		
		None	Low (2x10^5)	High (2x10^7)
IAV (X31) TCID$_{50}$	None	-	100	73.3
	Low (400)	100	44.4	ND
	High (8x10^3)	73.3	26.6	0

% survival

C

Lung streptococcal load vs. survival in high/low dose coinfection

D

Spleen streptococcal load vs. survival in high/low dose coinfection

E

Brain streptococcal load vs. survival in high/low dose coinfection

Figure 11. *Bacterial outgrowth correlates with mortality in a low dose coinfection.*
(A) Mortality following coinfection with high (8x10^3 TCID$_{50}$ IAV, 2x10^7 CFU *Strep*) or low (400 TCID$_{50}$ IAV, 2x10^5 CFU *Strep*) doses (n=9, representative of 3 independent experiments). All infections are shown in table (B). (C) Comparison of streptococcal load in the lung, (D) spleen and (E) brain in mice harvested upon reaching endpoint or concurrently harvested recovering (gaining weight) mice, during low and high dose coinfection from 8-10 dpi. All mice at high dose reached endpoint; all low dose mice are grouped (left panels) and then separated into recovering and endpoint groups (right panels) (n=13-21). Data displayed as percent survival (mortality) or geometric means (bacterial loads). Significance assessed by Mann-Whitney test (bacterial loads) or Log-rank (Mantel-Cox) test (mortality). n.s. = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ND=not done.

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Having established a suitable low dose coinfection setting for interventions, neutrophils were depleted by treating with anti-Ly6G prior to secondary infection (every 24 hours from 4-12dpi). Antibody treatment was performed one day prior to secondary infection to ensure neutrophils were depleted upon bacterial inoculation. However, in order to focus on their effect in coinfection, neutrophils were not depleted during the early influenza phase of infection (i.e. from 0-3dpi). The efficacy of anti-Ly6G treatment was verified through neutrophil quantification by flow cytometry in coinfected lungs at 6 and 7dpi (Fig. 12A). In this quantification it was not possible to identify neutrophils as previously done (for example, in Fig. 5A) based on their expression of Ly6G, as the same antibody clone is used for depletion and staining. Therefore neutrophils were identified on the basis of other marker characteristics as granular ($SSC^{>low}$) cells expressing CD11b and with intermediate (i.e. lower than inflammatory monocytes) or low expression of Ly6C. They were negative for the markers of other cell types MHC Class II, F4/80 and CD11c (for flow cytometry gating strategy, see Appendix 3).

Specific neutrophil depletion increased weight loss (Fig. 12B) and mortality (Fig. 12C) during low dose coinfection, with no effect during single IAV or *Strep* infections. Neutrophil depletion also led to increased streptococcal loads in the lung (Fig. 13A), spleen (Fig. 13B) and brain (Fig. 13C) at 8dpi. No effect on viral titres was observed (Fig. 13D). I also corroborated these findings by depleting with less specific anti-Gr-1 from one day prior to secondary infection (every 48 hours from 4-8dpi), which exacerbated weight loss (Fig. 14A) and mortality (Fig.

14B), with no substantial effects during single IAV or *Strep* infections. A small increase in weight loss in *Strep* infected mice did not translate into a significant difference in mortality. In summary neutrophil depletion during secondary infection is detrimental, confirming that the neutrophil response during coinfection is protective.
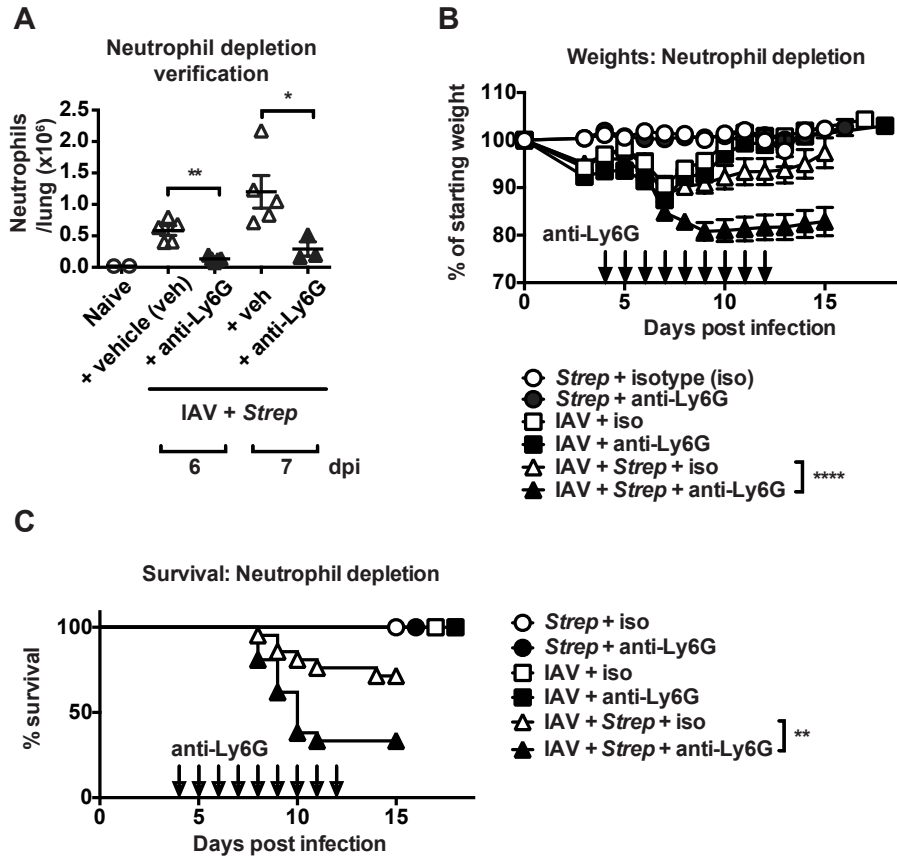
Figure 12

A

Neutrophil depletion verification

B

Weights: Neutrophil depletion

○ *Strep* + isotype (iso)
● *Strep* + anti-Ly6G
□ IAV + iso
■ IAV + anti-Ly6G
△ IAV + *Strep* + iso
▲ IAV + *Strep* + anti-Ly6G] ****

C

Survival: Neutrophil depletion

○ *Strep* + iso
● *Strep* + anti-Ly6G
□ IAV + iso
■ IAV + anti-Ly6G
△ IAV + *Strep* + iso
▲ IAV + *Strep* + anti-Ly6G] **

Figure 12. *Neutrophil depletion exacerbates low dose coinfection.*
(A) Flow cytometry quantification of lung neutrophils following vehicle (veh) or anti-Ly6G treatment during low dose coinfection. Neutrophils identified as CD11b+ SSC>low F4/80- CD11c- MHCII- Ly6Cint (n=3-5, representative of 2 independent experiments). (B) Weight loss and (C) mortality during low dose coinfection with isotype (iso) or anti-Ly6G treatment (n=9, data pooled from 2 independent experiments, arrows indiciate timing of antibody treatments, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final datapoint extended). Data is shown as arithmetic means ± SEM (depletion and weights) or % survival (mortality). Significance assessed by Mann-Whitney test (depletion), 2-way ANOVA (weights) or Log-Rank (Mantel-Cox) test (mortality). *p<0.05, **p<0.01, ****p<0.0001.
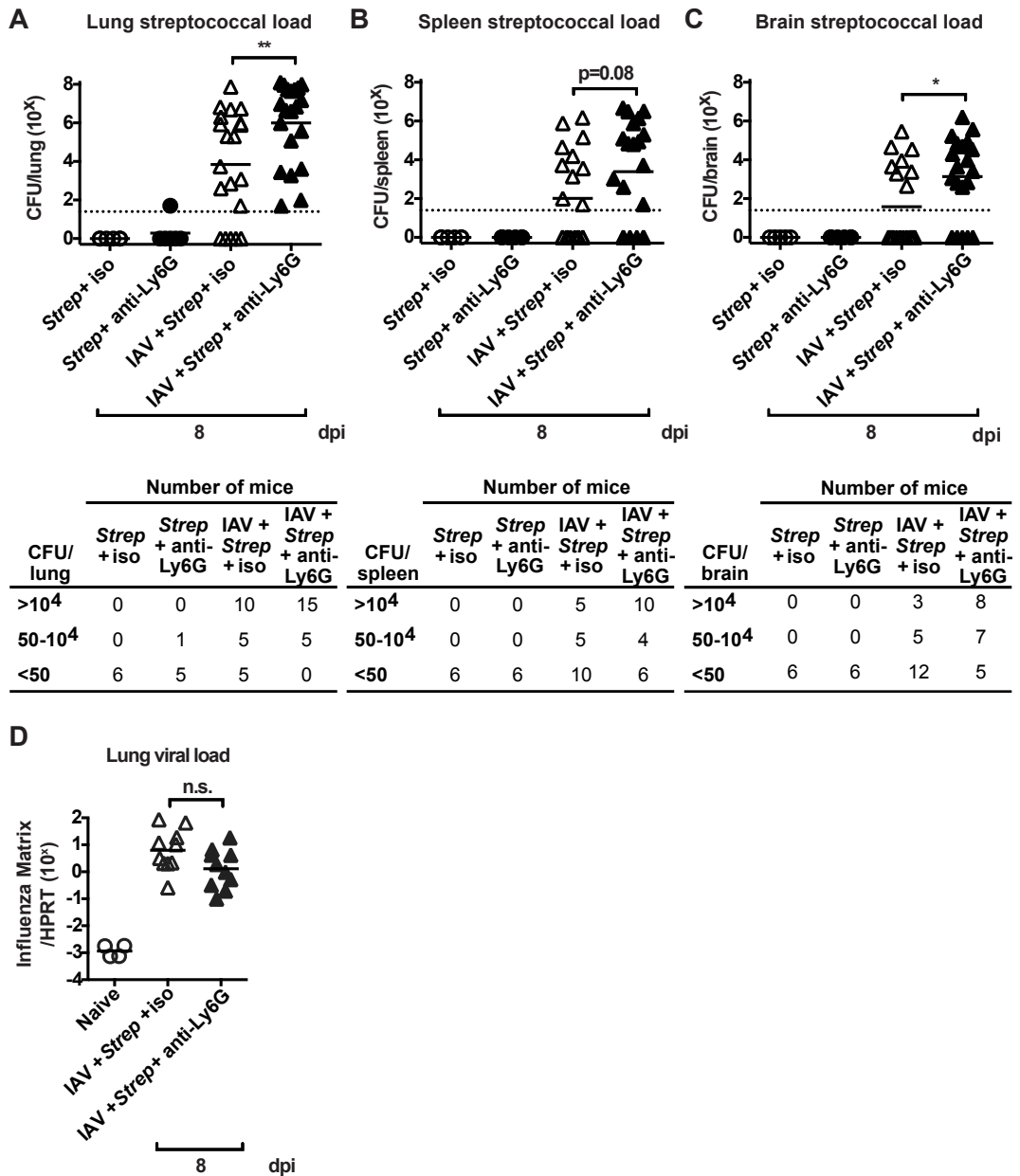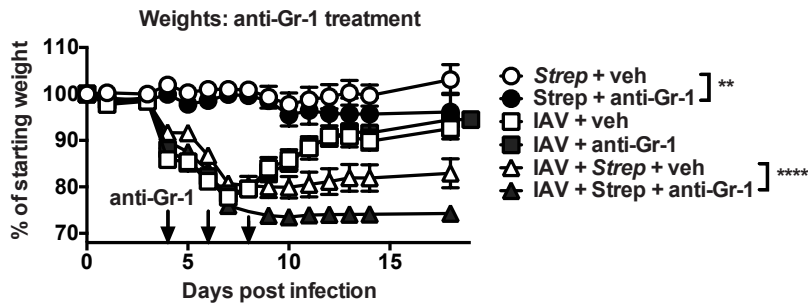
Figure 13

CFU/lung	Strep +iso	Strep + anti-Ly6G	IAV + Strep +iso	IAV + Strep + anti-Ly6G
>10^4	0	0	10	15
50-10^4	0	1	5	5
<50	6	5	5	0

CFU/spleen	Strep +iso	Strep + anti-Ly6G	IAV + Strep +iso	IAV + Strep + anti-Ly6G
>10^4	0	0	5	10
50-10^4	0	0	5	4
<50	6	6	10	6

CFU/brain	Strep +iso	Strep + anti-Ly6G	IAV + Strep +iso	IAV + Strep + anti-Ly6G
>10^4	0	0	3	8
50-10^4	0	0	5	7
<50	6	6	12	5

Figure 13. *Neutrophil depletion exacerbates bacterial outgrowth during low dose coinfection.*

(A) Streptococcal load during low dose coinfection in the lung, (B) spleen and (C) brain following treatment with anti-Ly6G or isotype control (data shown is pooled from 2 independent experiments, n=5-10). For clarity bacterial loads are also represented in a table immediately below. (D) Quantitative PCR for influenza viral matrix in the lung during low dose coinfection with anti-Ly6G or isotype control treatment (n=4-10). Data displayed as geometric means (graphs) or number of mice (tables). Significance assessed by Mann-Whitney test. n.s. = not significant, *p<0.05, **p<0.01
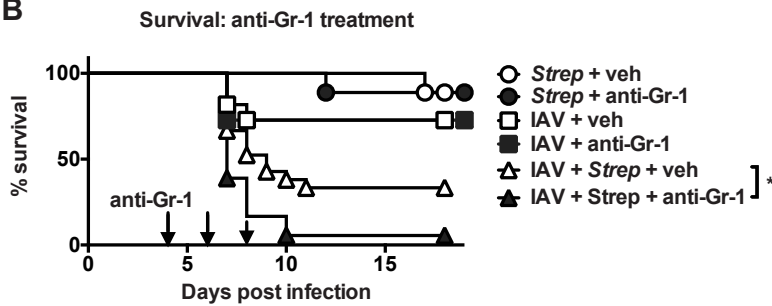
Figure 14

A

B

Figure 14. *Anti-Gr-1 treatment exacerbates low dose coinfection.*
(A) Weight loss and (B) mortality during low dose coinfection with vehicle or anti-Gr-1 treatment (data pooled from 2 independent experiments, n=5-12, arrows indiciate timing of antibody treatments, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final datapoint extended). Data shown as arithmetic means ± SEM (weights) or % survival (mortality). Significance assessed by Mann-Whitney test (weights) or Log-Rank (Mantel-Cox) test (mortality). *p<0.05, ****p<0.0001.

5.4. Conclusions and Discussion

It can therefore be concluded that the coinfection induces a strong, functional neutrophil response that is on balance protective, contributing to control of bacterial outgrowth. The protective role of neutrophils is consistent with the increased protection observed in granulophilic IFNαβR$^{-/-}$ mice (Shahangian *et al.*, 2009) and with previous studies showing exacerbated coinfection after poorly specific anti-Gr-1 treatment (McNamee *et al.*, 2006; Sun *et al.*, 2008). The use of low dose coinfection allows the detrimental effect of neutrophil depletion to be observed, which was not detected in a more severe context (Damjanovic *et al.*, 2013). Therefore the combination of specific depletion and low-dose coinfection allows the role of neutrophils to be clearly demonstrated for the first time.

The complex role of neutrophils in both *Strep* and IAV single infections has previously been outlined. Here neutrophil depletion has no effect during IAV when given at 4 - 12dpi. This is consistent with a previous study showing no effect of neutrophil depletion commencing several days into IAV infection (Tate *et al.*, 2011). It is likely, although the antibacterial role of neutrophils is well characterised, that neutrophil depletion does not exacerbate *Strep* single infection due to the low virulence, non-colonizing nature of *Strep* in this model (as shown in Fig. 3A), which is largely cleared prior to the induction of any neutrophil response (Fig. 7A).

By the readouts assessed, neutrophil function does not appear to be impaired by prior coinfection. This contradicts aspects of previous studies. One study

reporting deficiencies in ROS production and phagocytosis (McNamee *et al.*, 2006), as already outlined, is somewhat confounded by the use of LPS aerozolisation to induce greater neutrophil recruitment, and therefore is difficult to interpret. Another study reports no differences in ROS generation, consistent with the results shown here (LeVine *et al.*, 2001). However it also suggests myeloperoxidase per neutrophil in the lung is reduced. This is somewhat contradictory with another study that total myeloperoxidase in the lung is increased (Damjanovic *et al.*, 2013). It is possible the results shown here are consistent with both studies. I observed an increase in total myeloperoxidase in the airway (Fig. 10D) in coinfected mice relative to single *Strep* infection; however, it is notable that this increase is proportionally smaller than the rise in neutrophil numbers in the lung (Fig. 5A). Therefore it is possible that on a per-neutrophil basis myeloperoxidase release is reduced in coinfection, and this could be assessed by performing flow cytometry to enumerate neutrophils and quantifying myeloperoxidase by ELISA on the same airway samples. Nevertheless, as total myeloperoxidase is increased, it is unlikely that lack of myeloperoxidase production is a cause of coinfection susceptibility. Phagocytosis is not assessed here, although the reductions reported by a previous study are somewhat marginal (Damjanovic *et al.*, 2013).

Another criticism that could be raised of *in vitro* profiling of neutrophil function is that purification from the lung prior to restimulation is a biased selection of the neutrophil population. As neutrophils are terminally differentiated, short-lived effector cells (Nathan *et al.*, 2006), it is possible that the purification process selects against those that have already begun to perform their effector functions,

as they may be more susceptible to cell death during processing. Purification may select for recently recruited neutrophils, which may be less likely to be "impaired" due to less exposure to influenza and subsequent effectors in the lung (unless the effects of influenza on neutrophils are systemic). Therefore to complement the observation that neutrophils are not impaired upon purification and restimulation, readouts not requiring purification may be useful. Profiling of myeloperoxidase levels in the airway somewhat achieves this, although levels of other effectors such as elastase could also be assessed. Furthermore *in vivo* neutrophil cytokine production could be assessed by performing intracellular staining on whole lung cell populations.

In summary, low dose coinfection showed a strong correlation between bacterial outgrowth, spread to other organs, and poor disease outcome. Absence of bacterial outgrowth correlated with signs of recovery. This supports the earlier conclusions that bacterial outgrowth is the primary driver of mortality during coinfection. Neutrophils appear to be functional and on balance protective during coinfection, helping to counter bacterial outgrowth and dissemination.

Chapter 6. TNF-α in coinfection

6.1. Background

Another aspect of the strong proinflammatory response that was strikingly increased during coinfection is TNF-α. Interestingly, this was not present in either single infection. I therefore wanted to test whether this part of the response was protective or pathogenic. Furthermore, although it cannot be assumed to be a surrogate for all proinflammatory cytokines, investigating TNF-α does give information on whether the broader proinflammatory cytokine response observed in coinfection is protective. TNF-α is also highly relevant to the neutrophil response, promoting neutrophil recruitment to infected tissues (Borregaard, 2010), release of reactive oxygen species, and degranulation (Nathan *et al*., 2006).

Although in this model TNF-α was not observed in single infections, it has a well-characterised role in bacterial infections, and is protective during a virulent *Strep* infection (Hatta *et al*., 2010). It has also been identified as a possible source of immunopathology during severe IAV infection (Hussell *et al*., 2001), although reducing TNF signalling does not always show a beneficial effect (Salomon *et al*., 2007).

The role of TNF-α in IAV-*Strep* coinfection has not been studied; although several studies have observed a rise in TNF-α levels either 24 or 48 hours following coinfection (LeVine *et al*., 2001; Seki *et al*., 2004; Smith *et al*., 2007). Another study reported a decrease in TNF-α in coinfection relative to *Strep*

infection 4 hours following secondary infection (Sun *et al*., 2008). This discrepancy may be due to the different time points observed, although another study reported increases in TNF-α at both 4 and 24 hours following coinfection (Li *et al*., 2012). Furthermore, consistent with this, I observed increases in TNF-α at 4 and 24 hours following coinfection (Fig. 6A, Fig. 8A). Why one study observes a decrease in TNF-α in contrast to the majority of others is not clear, as studies reporting a rise in TNF-α during coinfection reflect inoculation with different amounts and strains of *Strep*, with a range of virulences.

A severe IAV-*H. influenzae* model (with 100% mortality) is the only coinfection study performing a direct intervention to assess the role of TNF-α (Lee *et al*., 2010). In this model both bacterial outgrowth and mortality are observed, but the drivers of mortality are not determined. The authors hypothesised TNF-α may be harmful, and TNFR1$^{-/-}$ mice would have reduced immunopathology and less severe disease. However, in the context of complete mortality, a protective effect was not observed.

6.2. Hypotheses and Aims

Having observed that neutrophils are protective during coinfection, but are not required during single *Strep* infection, I decided to test if the TNF-α cytokine response was also protective. TNF-α was a good target for investigation as it was highly upregulated versus both single infections, and its antibacterial role and ability to activate neutrophils are well defined.

Two loss of function approaches to test the role of TNF-α in coinfection were considered – use of TNFR1$^{-/-}$ mice, which are deficient in what are believed to be the primary aspects of TNF-α signalling (Bradley *et al*., 2008), and treatment with anti-TNF-α antibody, which has been shown to be effective *in vivo* previously (Hussell *et al*., 2001). I proposed to use a low dose coinfection setting so both positive and negative effects of interventions could be detected.

As I had already confirmed that the neutrophil response to coinfection was protective, and TNF-α promotes this response, I hypothesised that:

- TNF-α is protective during coinfection.

- TNFR1 deficiency would exacerbate disease during coinfection.

- TNFR1 deficiency would increase bacterial loads during coinfection.

- Blockade of TNF-α would exacerbate disease during coinfection.

- Blockade of TNF-α would increase bacterial loads during coinfection.

- Blockade of TNF-α or TNFR1 deficiency would have no effect on low virulence *Strep* or IAV infections.

6.3. Results

TNFR1$^{-/-}$ mice are more susceptible to IAV and coinfection

TNFR1$^{-/-}$ mice were infected with a low dose coinfection, and demonstrated increased weight loss (Fig. 15A) and mortality (Fig. 15B) relative to wild-type mice, suggesting TNF-α is protective during coinfection. However, TNFR1 deficiency also increased susceptibility to IAV and led to slight increase in weight loss during *Strep* infection. In this context it is difficult to assess the causes of increased susceptibility to coinfection, as it may be due to a worse response to the primary viral infection; therefore use of these mice was discontinued.
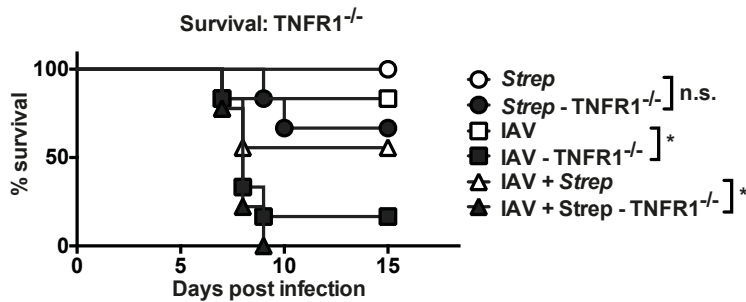
Figure 15

A
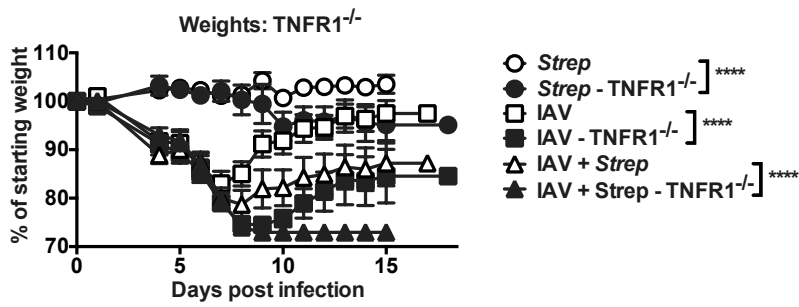
B

Figure 15. *TNFR1 deficiency exacerbates influenza and coinfection.*
(A) Weights and (B) mortality of wild-type (C57BL/6) or TNFR1$^{-/-}$ (B6) mice during low dose coinfection (n=9, representative of 2 independent experiments, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final data point extended). Data displayed as arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by 2-way ANOVA (weights) or Log-rank (Mantel-Cox) test (mortality). n.s. = not significant. *p<0.05. **p<0.01. ****p<0.0001.

Anti-TNF-α was used to blockade TNF-α signalling during a low dose coinfection. To assess the role of TNF-α during coinfection, without affecting the prior viral phase, treatment was given immediately prior to and during secondary infection at 5 and 7dpi. Anti-TNF-α treatment led to increased weight loss (Fig. 16A) and mortality (Fig. 16B) during coinfection. No effect on IAV or *Strep* infections was observed, suggesting that TNF-α is only required in the lung during coinfection, consistent with its absence in single infections. The more severe disease observed during single infections with TNFR1$^{-/-}$ mice is likely due to other confounding factors, rather than the absence of TNF-α in the lung.

Anti-TNF-α treatment also exacerbated bacterial loads in the lung (Fig. 17A), spleen (Fig. 17B) and brain (Fig. 17C). Consistent with the lack of effect on weights or survival during IAV infection, no effect on viral titres was observed (Fig. 17D). Therefore the high level of TNF-α produced during coinfection is on balance protective.
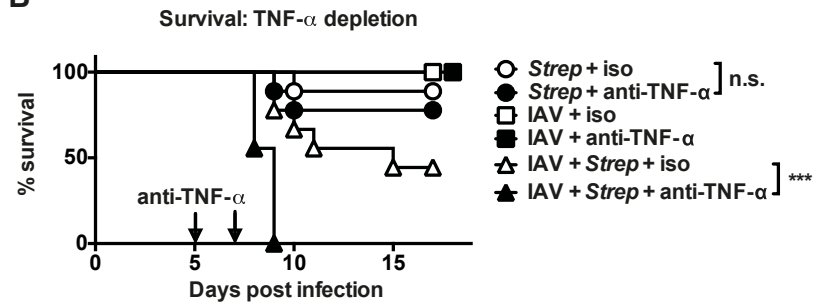
Figure 16

A
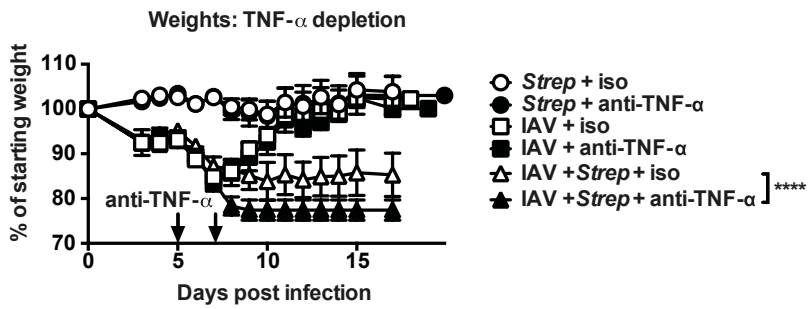
B

Figure 16. *TNF-α depletion exacerbates low dose coinfection.*
(A) Weights and (B) mortality during low dose coinfection following treatment with anti-TNF-α or isotype control (n=9, representative of 2 independent experiments. On weight loss plot mice reaching endpoint retained thereafter at final weight, arrows indicate timing of antibody treatments, in some groups to aid visualisation final data point extended). Data displayed as arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by 2-way ANOVA (weights) or Log-rank (Mantel-Cox) test (mortality). n.s. = not significant. ***p<0.001. ****p<0.0001.
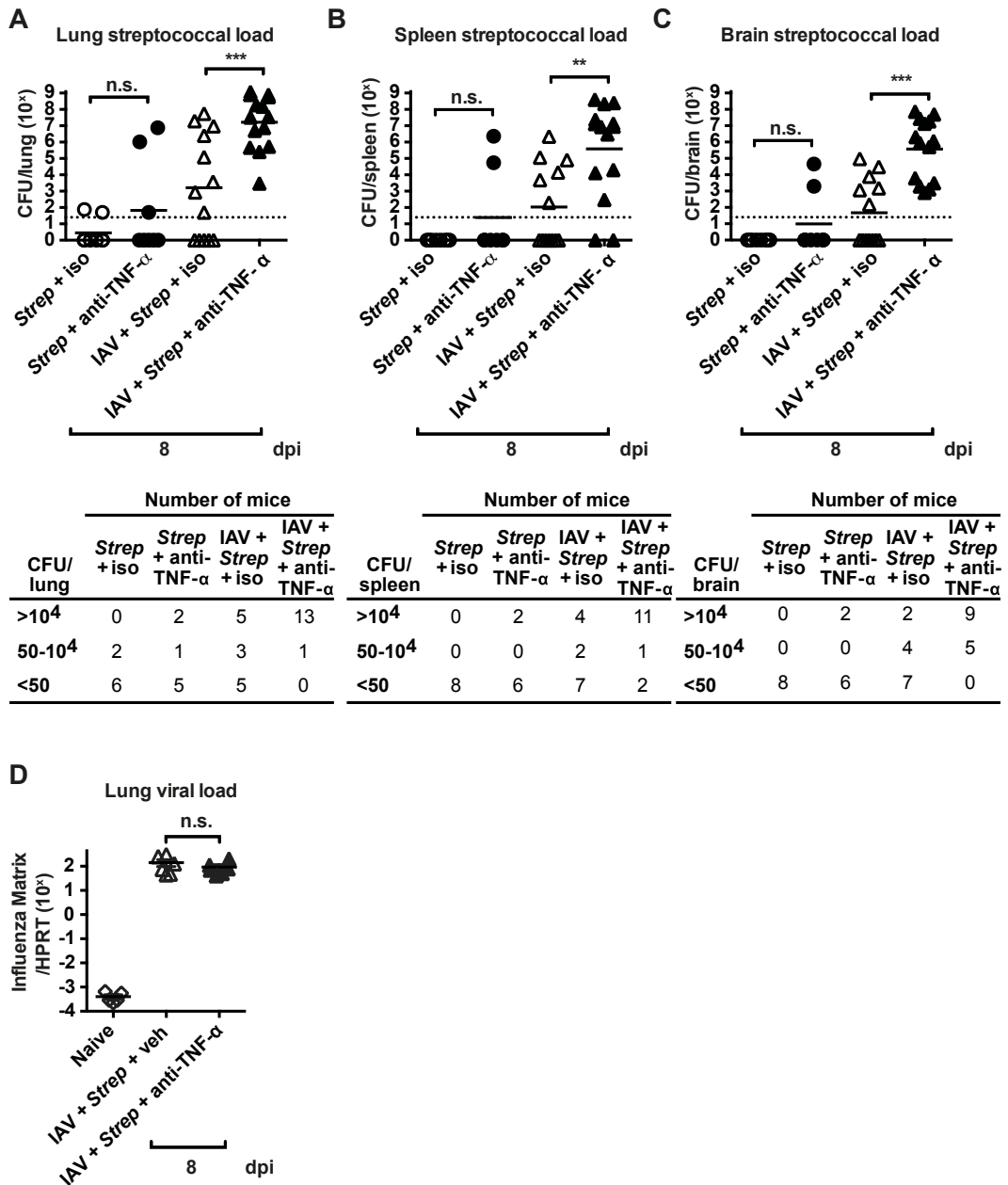
Figure 17

A — Lung streptococcal load

CFU/lung	Strep +iso	Strep + anti-TNF-α	IAV + Strep +iso	IAV + Strep + anti-TNF-α
>10^4	0	2	5	13
50-10^4	2	1	3	1
<50	6	5	5	0

B — Spleen streptococcal load

CFU/spleen	Strep +iso	Strep + anti-TNF-α	IAV + Strep +iso	IAV + Strep + anti-TNF-α
>10^4	0	2	4	11
50-10^4	0	0	2	1
<50	8	6	7	2

C — Brain streptococcal load

CFU/brain	Strep +iso	Strep + anti-TNF-α	IAV + Strep +iso	IAV + Strep + anti-TNF-α
>10^4	0	2	2	9
50-10^4	0	0	4	5
<50	8	6	7	0

D — Lung viral load

Figure 17. *TNF-α blockade exacerbates bacterial outgrowth during low dose coinfection.*
(A) Streptococcal load during low dose coinfection in the lung, (B) spleen and (C) brain following treatment with anti-TNF-α or isotype control (data shown is pooled from 2 independent experiments, n=3-9). For clarity bacterial loads are also represented in a table immediately below. (D) Quantitative PCR for influenza viral matrix in the lung during low dose coinfection with anti-TNF-α or isotype control treatment (n=5-6). Data displayed as geometric means (graphs) or number of mice (tables). Significance assessed by Mann-Whitney test. n.s. = not significant, **p<0.01, ***p<0.001.

6.4. Conclusions and Discussion

The harmful effect of anti-TNF-α treatment during low dose coinfection confirms the strong TNF-α response observed is on balance protective during coinfection. This is somewhat at odds with a previous study, which reports that treatment with linezolid - which substantially reduces IFN-γ and marginally reduces TNF-α - ameliorates coinfection (Breslow-Deckmann *et al*., 2013). However the authors primarily attribute the protective effect to reducing IFN-γ, which has already been reported to play a detrimental role during coinfection (Sun *et al*., 2008). Therefore it is difficult to draw clear conclusions of the role of TNF-α from linezolid treatment.

The increased susceptibility of TNFR1^{-/-} mice to IAV is of interest. Although TNFR1 signalling appears to be slightly detrimental due to TNF-α driven immunopathology in severe IAV (Salomon *et al*., 2007; Szretter *et al*., 2007; Belisle *et al*., 2010), its absence has not previously been shown to exacerbate disease in a mild to moderate IAV setting. As TNFR1^{-/-} mice have been reported to have a defect in antibody production (Le Hir *et al*., 1995), TNFR1 deficiency may lead to an impaired antibody response against influenza. This may not have been previously observed in severe influenza infections where immunopathology leads to rapid mortality, before a defect of antibody mediated viral control may be detectable.

However, it is notable that the absence of TNFR1^{-/-} does not reduce CD8 T cell numbers during severe or mild influenza (Szretter *et al*., 2007; Deberge *et al*.,

2014). Therefore viral control may still be partially intact in TNFR1 deficient mice, as there is some redundancy between CD8 and antibody responses (Brown *et al*, 2004). Whether the antibody response is defective in TNFR1 deficient mice, and the relative contribution of antibodies and CD8 T cells to viral control, may be worthy of further investigation, although it is not directly relevant to coinfection.

Although anti-TNF-α slightly prolongs survival in severe IAV models (Hussell *et al*., 2001; Peper *et al*., 2005), and may be modestly detrimental during infection with some *Strep* strains (Kirby *et al.*, 2005; Hatta *et al.*, 2010), in the mild to moderate single infections used here no effect was observed. This is particularly useful as it allowed investigation of the role of TNF-α in coinfection without confounding changes in susceptibility to single infections. It is likely that specific blockade of TNF-α in the lung during acute disease has substantially different effects to constitutive deficiency in TNF signalling throughout life, therefore it is unlikely that anti-TNF-α treatment, unlike TNFR1 deficiency, leads to impairment in antiviral antibody production. It should be noted that anti-TNF-α is not given during the early stage of influenza, when TNF-α may play a role; this may be an alternative explanation for the discrepancy with the susceptibility of TNFR1^{-/-} mice to influenza. To address this, anti-TNF-α treatment could be performed throughout influenza infection.

To summarize, upon bacterial colonization and outgrowth in the lung during coinfection, a strong immune response is mounted. The neutrophil and TNF-α components of this response are protective. The TNF-α and neutrophil response in

coinfection is of greater magnitude than the mild, transient and seemingly non-essential response to low virulence *Strep* infection, and therefore cannot be considered "impaired". This suggests that the fundamental issue during coinfection is the underlying factors that promote initial bacterial colonization in the lung, rather than subsequent immune impairment. In a coinfection setting where colonization occurs, a strong TNF-α and neutrophil response contributes to controlling bacterial outgrowth, but is frequently insufficient. Therefore in later chapters the underlying causes of initial bacterial colonization will be investigated.

Chapter 7. Inflammatory-monocyte mediated damage as a mechanism of coinfection

7.1. Background

As clear immune impairment was not detected during coinfection, it is possible early bacterial colonization may instead be due to lung damage. In addition to damage mediated directly by viral cytolysis, the immune response to influenza could be a source of damage. One of the effects of influenza is to induce inflammatory monocyte recruitment, which has been previously shown to induce damage in severe influenza (Lin *et al*., 2008). The role of influenza-induced inflammatory monocytes during coinfection has not yet been investigated.

Whether inflammatory monocytes are on balance beneficial or harmful during influenza is somewhat unclear, although their role in causing damage is well established. Inflammatory monocytes are difficult to deplete with a high specificity, and therefore the majority of studies assessing their role use CCR2$^{-/-}$ mice. CCR2$^{-/-}$ mice lack the crucial chemokine receptor required for inflammatory monocyte release from the bone marrow into the bloodstream, and therefore are deficient in peripheral inflammatory monocytes (Serbina *et al*., 2006). Most studies of CCR2$^{-/-}$ mice during influenza are in a context of severe infection with high mortality. An early study established that CCR2$^{-/-}$ mice have reduced early pathology but less subsequent T-cell recruitment (Dawson *et al*., 2000). Another study corroborated this, showing that inflammatory monocytes (in this case termed tipDCs) caused pathology but were required for full CD8 T-cell responses

(Aldridge *et al.*, 2009). CCR2$^{-/-}$ mice and those treated with a CCR2 small molecule inhibitor had reduced lung damage (Lin *et al.*, 2008; Lin *et al.*, 2010). Overall, inflammatory monocytes promote damage during (severe) influenza, but may be required for full adaptive responses. The role of inflammatory monocytes in *Strep* infections has not been extensively studied, although CCR2$^{-/-}$ mice show slightly higher bacterial loads during a virulent *Strep* infection (Davis *et al.*, 2011).

TRAIL, a pro-apoptotic ligand, has been identified as a source of inflammatory monocyte mediated damage during influenza. The results of different studies are somewhat contradictory and share similarities to results obtained with CCR2$^{-/-}$ mice; again, the main focus is severe influenza. TRAIL has been shown to signal to DR5 on epithelial cells during severe influenza and promote pathology; and anti-TRAIL treatment ameliorates this (Herold *et al.*, 2008; Davidson *et al.*, 2014). In contrast another study has shown that TRAIL$^{-/-}$ mice are more susceptible to severe influenza, which may be due to a defective CD8 response (Brincks *et al.*, 2008; Brincks *et al.*, 2011). The role of TRAIL in *Strep* infection has not been extensively studied, although TRAIL$^{-/-}$ mice are more susceptible to a virulent *Strep* infection (Steinwede *et al.*, 2012).

The role of inflammatory monocytes during coinfection has been briefly alluded to in a previous study, which was largely focused on IAV alone. This study, to demonstrate the safety of using a CCR2 small-molecule inhibitor to ameliorate influenza, also performs a secondary streptococcal coinfection (Lin *et al.*, 2011). In this very severe coinfection context there is an extremely marginal reduction in

very high bacterial loads. These results are difficult to interpret due to the severe coinfection context, the only partial block in inflammatory monocyte recruitment by the inhibitor, and effects of the inhibitor on other populations such as dendritic cells.

7.2. Hypotheses and Aims

Previous results in this thesis suggested that influenza allows bacterial colonization during coinfection, inducing a strong, unimpaired and on balance protective - but frequently insufficient - neutrophil and TNF-α response. This immune response was only required for protection in the context of coinfection. Single *Strep* infection induces only a modest immune response and depletion of neutrophils or TNF-α blockade during *Strep* infection has no effect. This implies immune impairment is not responsible for allowing bacterial colonization. An alternative cause of colonization may be reduced lung integrity.

As inflammatory monocytes have been shown to cause lung damage in severe influenza, I speculated that this may be a mechanism during moderate influenza to allow bacterial colonization. I also proposed that TRAIL may be a molecular mechanism for this. To investigate this I decided to assess the susceptibility of CCR2$^{-/-}$ and anti-TRAIL treated mice to low dose coinfection.

I hypothesised that:

- Influenza-induced inflammatory monocyte-mediated lung damage promotes early bacterial colonization during coinfection.

- CCR2$^{-/-}$ mice would therefore be less susceptible to coinfection.

- TRAIL is a molecular mechanism of inflammatory monocyte-mediated damage.

- Anti-TRAIL treated mice would therefore be less susceptible to coinfection.

- Damage at the point of bacterial inoculation would be the determinant of susceptibility. Therefore only anti-TRAIL treatment during the influenza phase of coinfection, prior to bacterial colonization, would be protective. Treatment during bacterial phase, following bacterial colonization, would be ineffective.

- Neither CCR2 deficiency or anti-TRAIL treatment would substantially affect my mild to moderate single IAV and *Strep* infections.

7.3. Results

CCR2$^{-/-}$ mice have reduced mortality and bacterial outgrowth during low dose coinfection

Low dose coinfection was performed on CCR2$^{-/-}$ and wild-type mice and weights (Fig. 18A) and mortality (Fig. 18B) observed. No major changes during single *Strep* and IAV infections were observed, although there was a slight protective effect on weights during *Strep* infection; however, this was not significant in terms of mortality. In contrast, during coinfection CCR2$^{-/-}$ mice showed significantly less weight loss and mortality. Consistent with reduced mortality, CCR2$^{-/-}$ mice had dramatically reduced streptococcal loads during low dose coinfection in the lung (Fig. 19A), spleen (Fig. 19B) and brain (Fig. 19C). No effect on viral titres was observed (Fig. 19D).
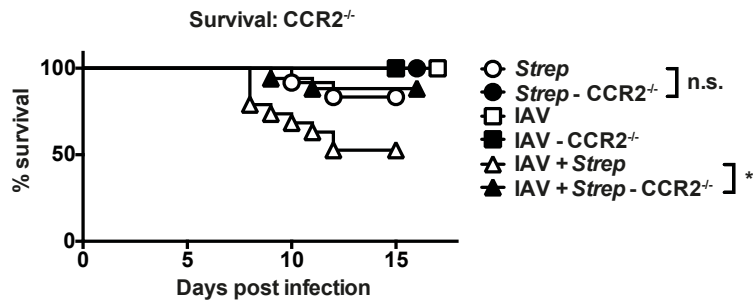
Figure 18

A
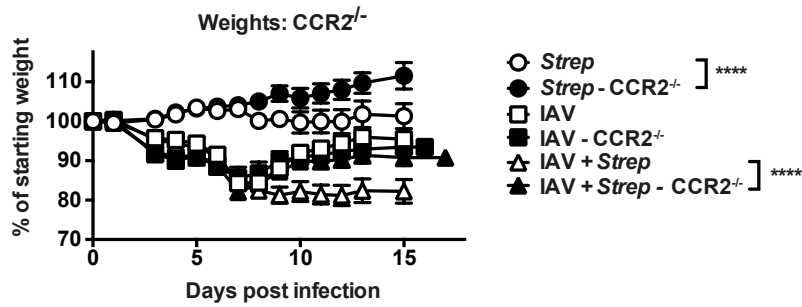
B

Figure 18. *CCR2 deficiency ameliorates low dose coinfection.*
(A) Weights and (B) mortality of wild type (C57BL/6) or CCR2$^{-/-}$ (B6) mice during low dose coinfection (n=6-9, data shown pooled from 2 independent experiments, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final data point extended). Data displayed as arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by 2-way ANOVA (weights) or Log-rank (Mantel-Cox) test (mortality). n.s. = not significant. *p<0.05. ****p<0.0001.

Figure 19

A Lung streptococcal load **B** Spleen streptococcal load **C** Brain streptococcal load

CFU/ lung	Strep	Strep -CCR2 -/-	IAV + Strep	IAV + Strep -CCR2 -/-
>10⁴	0	1	10	2
50-10⁴	0	2	3	5
<50	9	7	5	9

CFU/ spleen	Strep	Strep -CCR2 -/-	IAV + Strep	IAV + Strep -CCR2 -/-
>10⁴	0	0	4	0
50-10⁴	0	1	4	3
<50	9	9	10	13

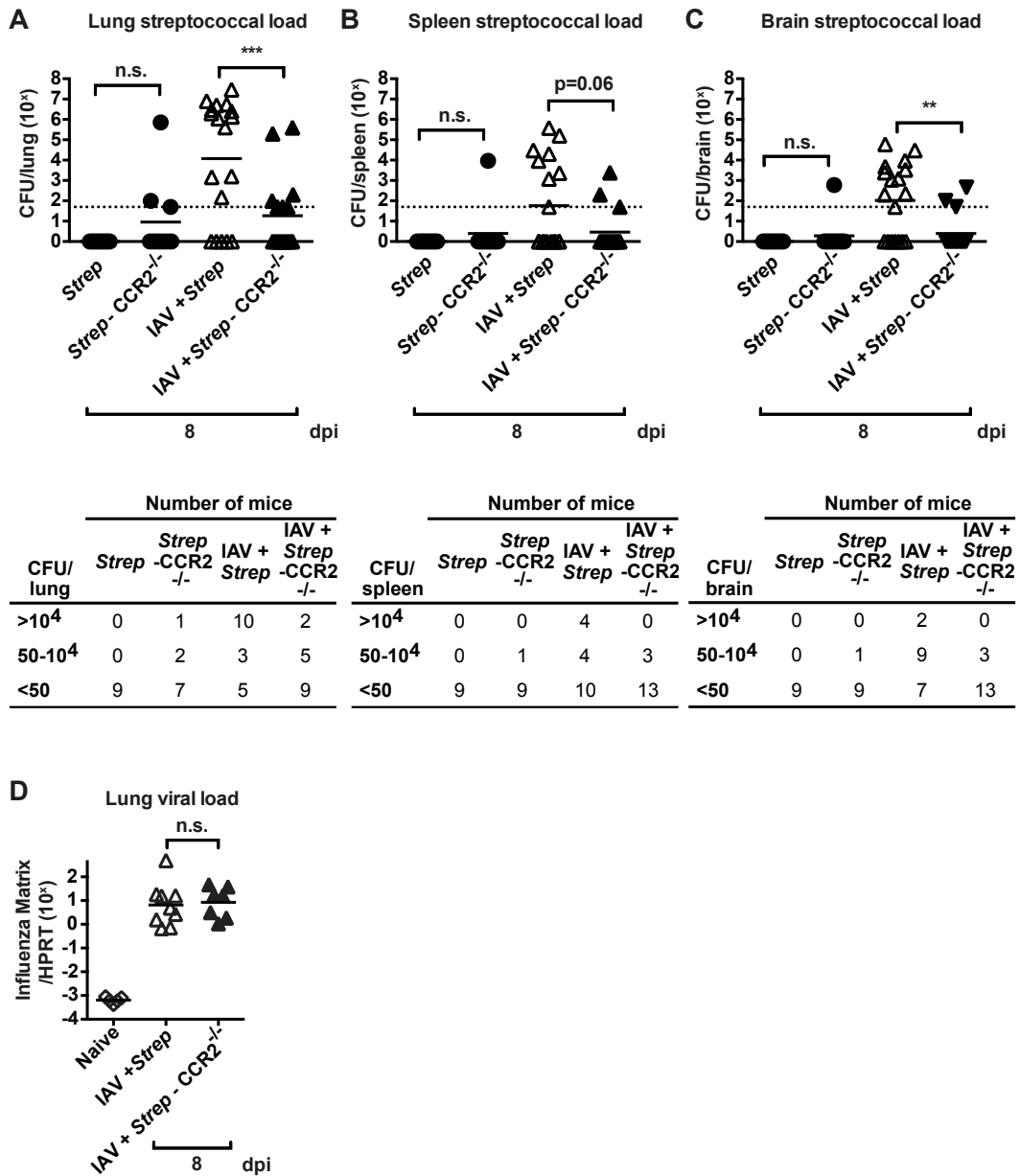| CFU/ brain	Strep	Strep -CCR2 -/-	IAV + Strep	IAV + Strep -CCR2 -/-
>10⁴	0	0	2	0
50-10⁴	0	1	9	3
<50	9	9	7	13

D Lung viral load

Figure 19. *CCR2 deficiency ameliorates bacterial outgrowth during low dose coinfection.* (A) Streptococcal load in the lung, spleen (B) and brain (C) of wild type (C57BL/6) or CCR2$^{-/-}$ (B6) mice during low dose coinfection (data shown is pooled from 2 independent experiments, n=4-9). For clarity bacterial loads are also represented in a table immediately below. (D) Quantitative PCR for influenza viral matrix in the lung during low dose coinfection (n=5-9) Data displayed as geometric means. Significance assessed by Mann-Whitney test. n.s. = not significant, **p<0.01, ***p<0.001.

Having confirmed CCR2$^{-/-}$ mice are less susceptible to coinfection, cell recruitment to the lung was then tested at the point of (5dpi) and during (7dpi) high dose coinfection. There were no significant changes in CCR2$^{-/-}$ mice in neutrophil (Fig. 20A), alveolar macrophage (Fig. 20C), CD3^{+} (Fig. 20D) or B cell levels (Fig. 20E) at 5 or 7dpi, although, consistent with greatly reduced bacterial colonization and outgrowth, there was a strong trend for reduced neutrophil recruitment at 7dpi. As expected, no inflammatory monocytes were present in CCR2$^{-/-}$ mice in any context at 5 or 7dpi (Fig. 20B).

Some modest differences in influenza-induced cytokine production in CCR2$^{-/-}$ mice were observed at the point of coinfection (5dpi), including significant increases in some cytokines such VEGF and MCSF were observed (see Appendix 4 for table). However, the fold changes observed were relatively small. Consistent with the trend for reduced neutrophils observed, proinflammatory cytokines such as MIP2 and TNF-α were reduced at 7dpi during high dose coinfection (see Appendix 4 for table). This confirms CCR2 deficiency removes inflammatory monocytes at the point of coinfection, without affecting other cell populations or causing major changes in the influenza-induced cytokine response. After bacterial superinfection, less bacterial outgrowth in CCR2 deficient mice leads to reduced neutrophil recruitment and proinflammatory cytokine production.
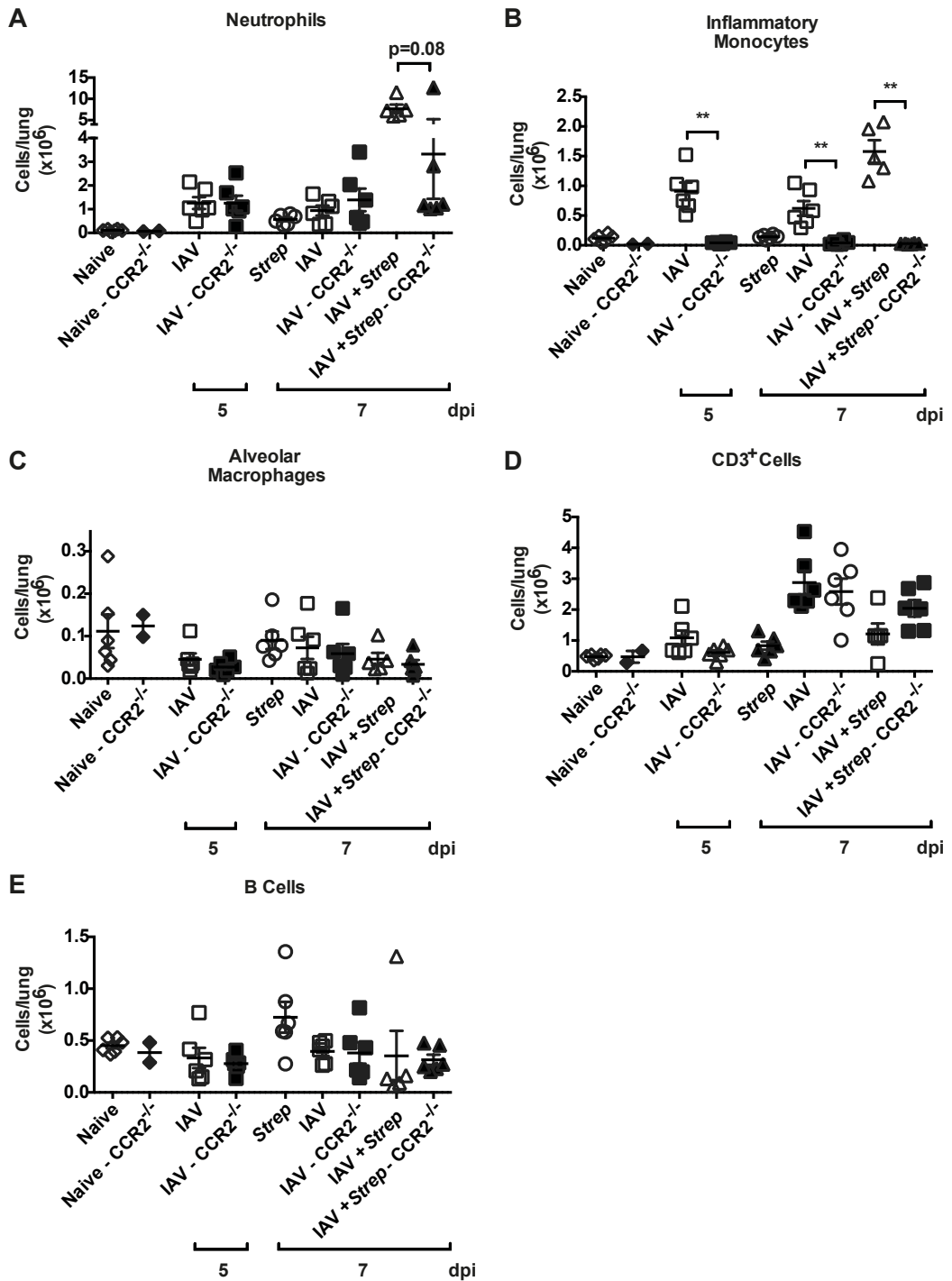
Figure 20

Figure 20. *CCR2$^{-/-}$ mice do not recruit inflammatory monocytes to the lung during influenza or coinfection.*
Flow cytometry quantification of (A) Neutrophils (Ly6G$^+$CD11b$^+$) (B) Inflammatory monocytes (Ly6C$^+$CD11b$^+$F4/80$^+$) (C) Alveolar macrophages (CD11c$^+$F4/80$^+$) (D) CD3$^+$ cells (CD3$^+$) and (E) B cells (CD3$^-$CD19$^+$) (pooled from 2 independent experiments, n=2-3) in the lung during high dose coinfection. Data displayed as arithmetic means ± SEM. Significance assessed by Mann-Whitney test. n.s. = not significant. **p<0.01.

CCR2$^{-/-}$ mice have reduced damage at the point of coinfection

Having confirmed that inflammatory monocytes are absent in CCR2$^{-/-}$ mice, the effect of this on lung damage was then assessed. Free lactate dehydrogenase (LDH) in the airway, which indicates cell lysis, and total protein levels, which indicate alveolar leakage, have previously been used as readouts for lung damage during influenza (Lin *et al*., 2008). CCR2$^{-/-}$ mice had reduced levels of airway protein (Fig. 21A) and LDH activity (Fig. 21B) at the point of coinfection (5dpi). Lung histology also showed reduced pathology (Fig. 21C) (experimental assistance provided by Histology Section, NIMR). Therefore CCR2$^{-/-}$ mice have reduced lung damage at the point of coinfection, consistent with their reduced susceptibility to bacterial colonization. As major changes in other immune cell populations and cytokine levels at the point of coinfection were not observed, it is likely that reduced damage in CCR2$^{-/-}$ mice is responsible for their reduced susceptibility to coinfection.

It is however notable that airway protein (Fig. 21D) and LDH activity (Fig. 21E) are not reduced in CCR2$^{-/-}$ mice during influenza or coinfection at 7dpi. This suggests that there is redundancy between inflammatory monocytes and other sources of damage later in influenza - this will be discussed later.
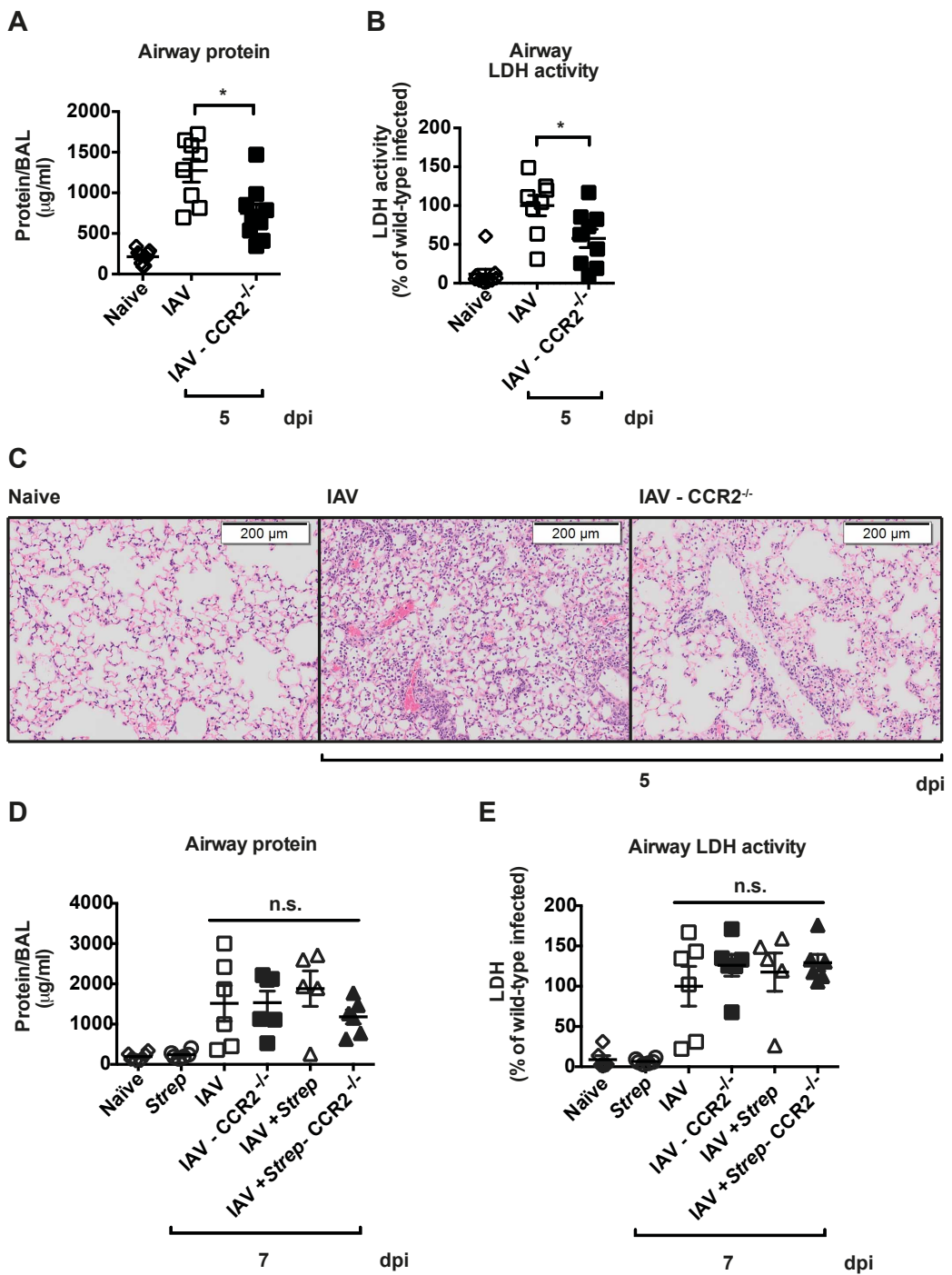
Figure 21

**Figure 21. *CCR2 deficiency reduces lung damage prior to coinfection.***
(A) Airway protein and (B) LDH activity during high dose influenza infection at 5dpi in wild type (C57BL/6) or CCR2^{-/-} (B6) mice assessed (n=2-3, data shown is pooled from 3 independent experiments, LDH activity shown relative to mean of 5dpi wild type IAV infected group, defined as 100%). (C) H+E staining of lung tissue sections at 5dpi (n=2-3). (D) Airway protein and (E) LDH activity during high dose coinfection at 7dpi in wild type (C57BL/6) or CCR2^{-/-} (B6) mice assessed (n=2-3, data shown is pooled from 2 independent experiments, LDH activity shown relative to mean of 7dpi wild type IAV infected group, defined as 100%). Data shown as arithmetic means ±SEM. *p<0.05.

TRAIL expressing inflammatory monocytes are present at the point of coinfection

Therefore CCR2$^{-/-}$ mice have reduced susceptibility to coinfection, and that this likely due to reduced damage and subsequent bacterial colonization at the point of coinfection. Inflammatory monocyte damage may be mediated through TRAIL. A substantial population of TRAIL expressing cells is recruited at the point of coinfection (5dpi) (Fig. 22A). These are largely inflammatory monocytes, and therefore are absent - greatly reducing the levels of total TRAIL expressing cells - in CCR2$^{-/-}$ mice. DR5, the receptor for TRAIL, is upregulated during influenza, and as expected this is not affected by CCR2 deficiency (Fig. 22B). Therefore reduced damage in CCR2$^{-/-}$ mice at 5dpi is likely due to less TRAIL ligand, as TRAIL-expressing inflammatory monocytes are absent. Note - FACS strategy for identification of TRAIL$^+$ cells is shown in Appendix 5A. Strategy for assessing DR5 expression on epithelial cells is shown in Appendix 5B.
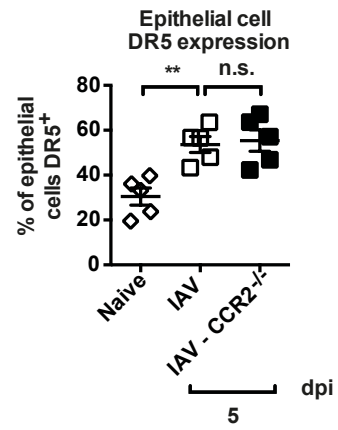
Figure 22

A
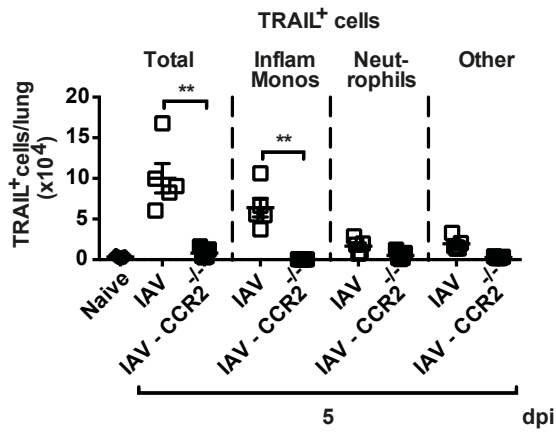
B

Figure 22. The majority of TRAIL expressing cells at the point of coinfection are CCR2-dependent inflammatory monocytes.

Flow cytometry quantification of (A) TRAIL$^+$ cells and (B) DR5$^+$ epithelial cells (E-cadherin$^+$Ep-Cam$^+$) at 5dpi during high dose influenza infection (n=5, data shown representative of 2 independent experiments). Data displayed as arithmetic means ± SEM. Significance assessed by Mann-Whitney test. n.s. = not significant. **p<0.01.

Anti-TRAIL treatment during influenza phase is protective during low dose coinfection

To confirm that inflammatory-monocyte mediated damage is harmful, the effect of TRAIL blockade was tested during low dose coinfection. Initially, to prevent all TRAIL-mediated damage, continuous treatment with anti-TRAIL was given during both influenza and bacterial phases of coinfection (from 1 to 9dpi). This ameliorated weight loss (Fig. 23A) and mortality (Fig. 23B), and had no effect in IAV or *Strep* single infections. This confirms TRAIL expressing-inflammatory monocyte mediated damage is harmful during coinfection, but did not confirm at when this harmful effect is mediated.

I hypothesised that the presence of influenza-induced damage at the point of bacterial inoculation is crucial for determining whether colonization will occur. This implied that only anti-TRAIL treatment starting during the influenza phase of infection would be effective. Therefore treatment with anti-TRAIL was given in the influenza phase ("early", 1 and 3dpi) or bacterial phase ("late", 6 and 8dpi) of low dose coinfection. Only early anti-TRAIL treatment ameliorated weight loss (Fig. 23C) and mortality (Fig. 23D). This confirms that the protective effect of blocking inflammatory-monocyte TRAIL is mediated during the influenza phase. Once bacteria have colonized due to inflammatory monocyte induced damage, anti-TRAIL treatment is ineffective. Confirming the role of TRAIL in promoting bacterial colonization, early anti-TRAIL treatment also significantly reduced lung bacterial load following high dose coinfection at 5dpi + 16hrs; a trend for reduction was also observed following low dose coinfection (Fig. 23E).
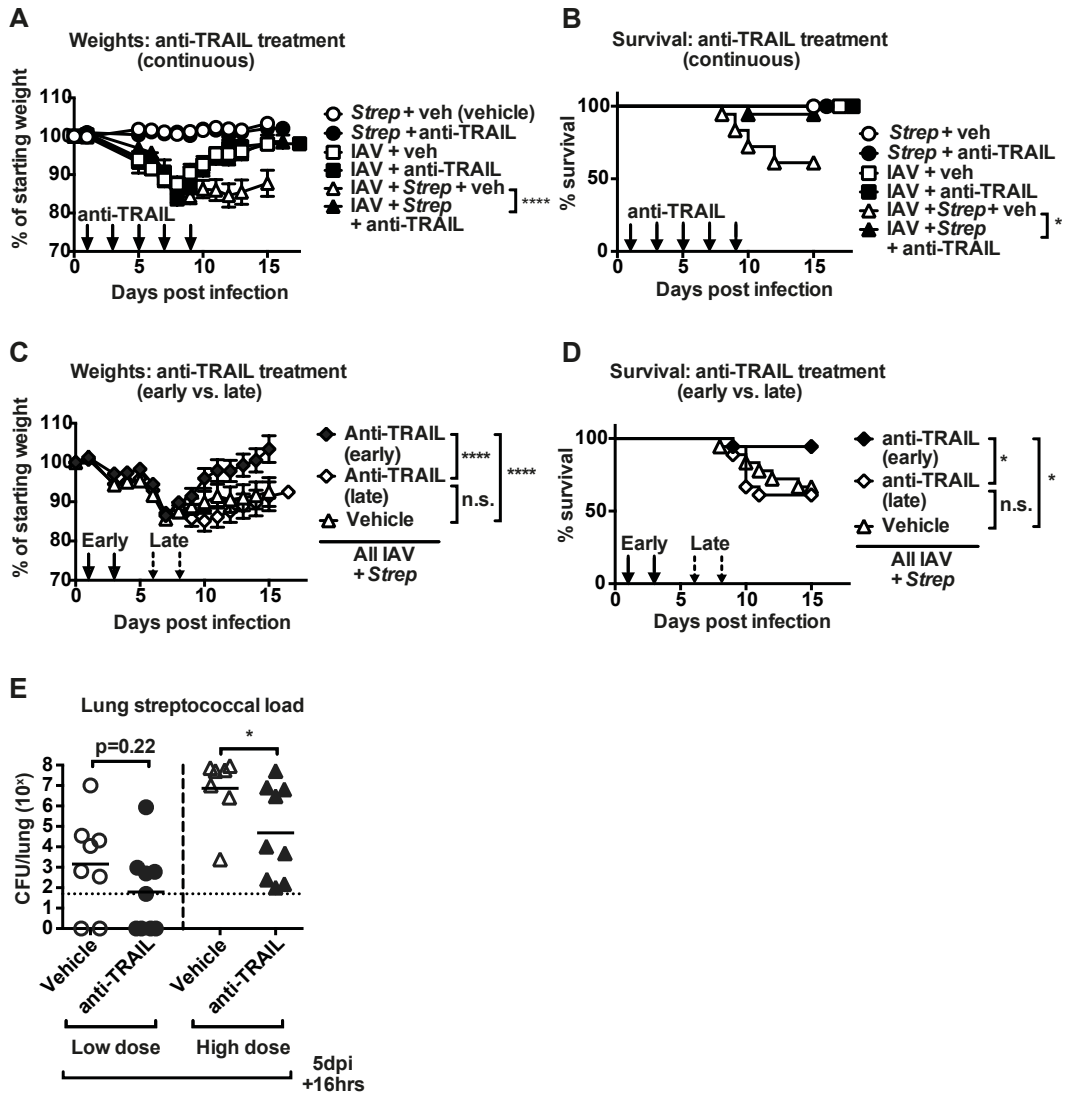
Figure 23

Figure 23. *Anti-TRAIL treatment during prior influenza ameliorates low dose coinfection.*
(A) Weights and (B) mortality during low dose coinfection with either anti-TRAIL treatment or vehicle control (PBS) every 48 hours from 1-9dpi ("continuous") (n=6-9, pooled from 2 independent experiments, arrows indicate timing of antibody treatment, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final data point extended). (C) Weights and (D) mortality during low dose coinfection with either anti-TRAIL treatment at 1 and 3 dpi ("early"), 6 and 8dpi ("late") or vehicle control (1,3,6 and 8dpi) (n=8-9, pooled from 2 independent experiments, solid arrows indicate early treatment, dashed arrows indicate late treatment, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final data point extended). (E) Lung streptococcal load during high or low dose coinfection at 5dpi+16hrs following treatment with anti-TRAIL or vehicle control at 1 and 3 dpi (n=7-9). Data shown as geometric mean (bacterial loads) or arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by Mann-Whitney test (bacterial loads), 2-way-ANOVA (weights) or Log-Rank (Mantel-Cox) test (mortality). n.s.=not significant. *p<0.05. ****p<0.0001

7.4. Conclusions and Discussion

From these results a mechanism for coinfection emerges where influenza-induced inflammatory monocytes, via TRAIL signalling to DR5 on epithelial cells, cause lung damage, which enables bacterial colonization and subsequent outgrowth. Prior data in this thesis suggests that this bacterial outgrowth induces a strong and unimpaired neutrophil and pro-inflammatory cytokine response, which is protective but frequently insufficient to prevent mortality.

A model of pre-colonization with *Strep* followed by influenza somewhat contrasts with the resistance of CCR2$^{-/-}$ mice to coinfection shown here. In this context these pathogens synergised to produce type I IFN, which reduced MCP-1 (a CCR2 ligand) production, reducing macrophage function and bacterial clearance (Nakamura et al., 2011). The protective role of MCP-1 in this study implies CCR2 deficiency would be harmful in coinfection. It is likely this discrepancy is due to different order of pathogen exposure between models; pre-existing pneumococcal carriage exacerbated by influenza differs greatly from the model investigated here of secondary bacterial infection during the acute phase of influenza.

The lack of effect of CCR2 deficiency or anti-TRAIL treatment during moderate influenza is not necessarily in contradiction with the varied literature regarding severe influenza. Consistent with severe influenza models (Lin *et al*., 2008), CCR2-dependent cells and TRAIL cause damage during the mild to moderate influenza used here; however, in this context this effect is likely too small for CCR2 deficiency or TRAIL blockade to substantially alter disease. Furthermore,

in severe influenza CCR2 and TRAIL appear to play both harmful and beneficial roles (Brincks *et al.*, 2008; Herold *et al.*, 2008; Aldridge *et al.*, 2009), which may in a moderate setting "cancel out" in terms of overall disease severity. Although TRAIL$^{-/-}$ mice have been reported to be more susceptible to a virulent *Strep* strain (Steinwede *et al.*, 2012), the lack of effect observed on *Strep* infection of anti-TRAIL treatment in the present model is likely due to the low virulence of the strain used.

Several other factors previously observed in other studies may indirectly corroborate the theory that influenza-induced inflammatory-monocyte mediated damage is responsible for bacterial colonization during coinfection. For example, the reported increased resistance of IFNαβR1$^{-/-}$ mice to coinfection (Shahangian *et al.*, 2009) may be due to reduced TRAIL-expressing inflammatory monocytes, as IFNαβR$^{-/-}$ mice have been shown to have reduced monocyte numbers in severe influenza infection (Seo *et al.*, 2011), and upregulation of TRAIL appears to be somewhat dependent on type I IFNs (Hogner *et al.*, 2013; Davidson *et al.*, 2014).

Furthermore, studies showing influenza strains encoding PB1-F2 - a pro-apoptotic viral peptide - lead to more severe coinfections (McAuley *et al.*, 2007; Alymova *et al.*, 2013) support lung damage as a mechanism of coinfection. These studies focus on direct viral rather than immune-mediated damage, which may be sufficient in a severe context to cause coinfection susceptibility. It could also be argued that more severe viral infections often induce a greater immune response, and therefore the source of damage in these systems is difficult to determine.

It is notable that, although inflammatory monocyte deficiency leads to reduced damage at the point of coinfection (5dpi), it does not affect damage later in influenza infection (7dpi). Other sources of damage, such as viral cytolysis or the cytotoxic CD8 T-cell response, rather than inflammatory monocytes, may be the major cause of damage at this point. If the model of damage allowing bacterial colonization is correct, CCR2$^{-/-}$ mice given secondary infection at 7dpi may be susceptible to coinfection; this may be a useful future experiment. In addition, blockade of other sources of damage, for example by depleting CD8 T cells, may merit investigation. Different sources of damage at different time points highlight that coinfection is a multifactorial process, and even if underlying lung damage is the primary mechanism, it may have multiple sources. As coinfection at 7dpi has been reported to be more severe than at other time points (McCullers *et al*., 2002), it is possible that this reflects multiple sources of damage at this point. Therefore although in this model - where coinfection is performed at 5dpi - early influenza-induced TRAIL-expressing inflammatory monocytes appears to be the primary cause of damage that allows subsequent bacterial colonization, other sources of damage must also be considered.

It is possible that CCR2 deficiency affects other cells types in influenza and coinfection in addition to inflammatory monocytes; one study reports moderately reduced levels of CD11c^{+}MHCII^{+} dendritic cells in CCR2$^{-/-}$ mice during influenza (Lin *et al*., 2008). Here changes are not observed in lymphocyte, neutrophil or alveolar macrophage numbers in CCR2$^{-/-}$ mice; however the dendritic cell numbers are not assessed. As inflammatory monocytes are almost completely abolished by CCR2 deficiency, in comparison to the modest reduction in dendritic

cells described in the study cited, absence of inflammatory monocytes can be considered the major effect of CCR2 deficiency. However, the possible reduction in dendritic cell numbers must be considered as another factor. Relating to this, another study also reports a drop in "tipDCs" in CCR2$^{-/-}$ mice during influenza (Aldridge *et al.*, 2009). However, tipDCs are classified as Ly6C^{+}CD11b^{+}, and therefore can be considered more equivalent to inflammatory monocytes than CD11c^{+} conventional dendritic cells (Serbina *et al.*, 2006).

Overall, several other studies indirectly corroborate a model where influenza-induced inflammatory-monocyte mediated damage causes susceptibility to coinfection. This model will be discussed further in chapter 10; however, chapters 8 and 9 will focus on other investigations into coinfection, including bacterial spread to the brain and other aspects of the immune response.

Chapter 8. Bacterial spread to the brain in coinfection

8.1. Background

A striking feature of coinfection is bacterial spread to the brain (Fig. 2C). Furthermore during low dose coinfection bacterial dissemination to the brain is common even among recovering mice (Fig. 11E), that otherwise show no spread to the spleen (Fig. 11D), and exhibit low bacterial loads in the lung (Fig. 11C). Bacterial spread to the brain therefore merited further investigation.

Meningitis is well-characterised complication of *Strep* infection (Koedel *et al*., 2002), and has been modelled in mice through direct intracisternal application of streptococci (Mook-Kanamori *et al*., 2012). This led to high bacterial loads, brain inflammation and pathology. It has been speculated that there is an association between viral infection and bacterial meningitis (Krasinski *et al*., 1987), although this finding has been disputed (Foy *et al*., 1988). A recent mouse model reported bacterial spread to many peripheral organs during IAV-*Strep* coinfection, including the brain, although they did not pursue these investigations further (Damjanovic *et al*., 2013).

8.2. Hypotheses and Aims

As only one study had previously tested whether *Strep* spreads to the brain during coinfection, I aimed to verify this and confirm that it was not an artefact. I also wanted to investigate whether spread caused inflammatory changes in the brain, as an indicator whether this area might merit further research.

I hypothesised that:

- Bacterial spread to the brain in coinfection was not an artefact of extraction technique, contamination from the blood, or intranasal infection.
- Bacterial spread to the brain induces inflammatory changes.

8.3. Results

Detection of bacteria in the brain is not due to contamination

To verify that bacterial spread to the brain was not an artefact of surgical contamination, mouse brains were extracted during a high dose coinfection. To exclude external contamination, brain tissue was washed serially in PBS prior to processing. To rule out contamination from the nasal cavity, the front of the brain - which may have come into contact with the nasal cavity during extraction - was excised. These mice were then compared to control mice, where whole brains were extracted conventionally without these measures. These alterations in

extraction protocol did not affect bacterial loads (Fig. 24A), confirming bacterial presence was not due to surgical contamination.

It was possible that bacteria spread directly from the nasal cavity to the brain following intranasal infection, rather than through dissemination from the lung. To test this, bacterial loads in the brain of high dose coinfected mice infected by the intranasal or intratracheal route were compared. Intratracheal inoculation bypasses the nasal cavity and directly infects the lungs. Intratracheal infection did not reduce, and indeed marginally increased streptococcal loads in the brain, confirming bacterial dissemination to the brain occurs via the lung (Fig. 24B).

As brains harvested for bacterial quantification were not perfused, it was necessary to confirm that the presence of streptococci in the brain was not due to bacteria from the blood. Brains and corresponding bloods were therefore harvested from high and low dose coinfected mice. In many cases, particularly during low dose coinfection, the bacterial load in the brain was higher than in a substantial (100μl) volume of blood (Fig. 24C). This strongly suggests bacterial presence in the brain is not due to blood contamination.
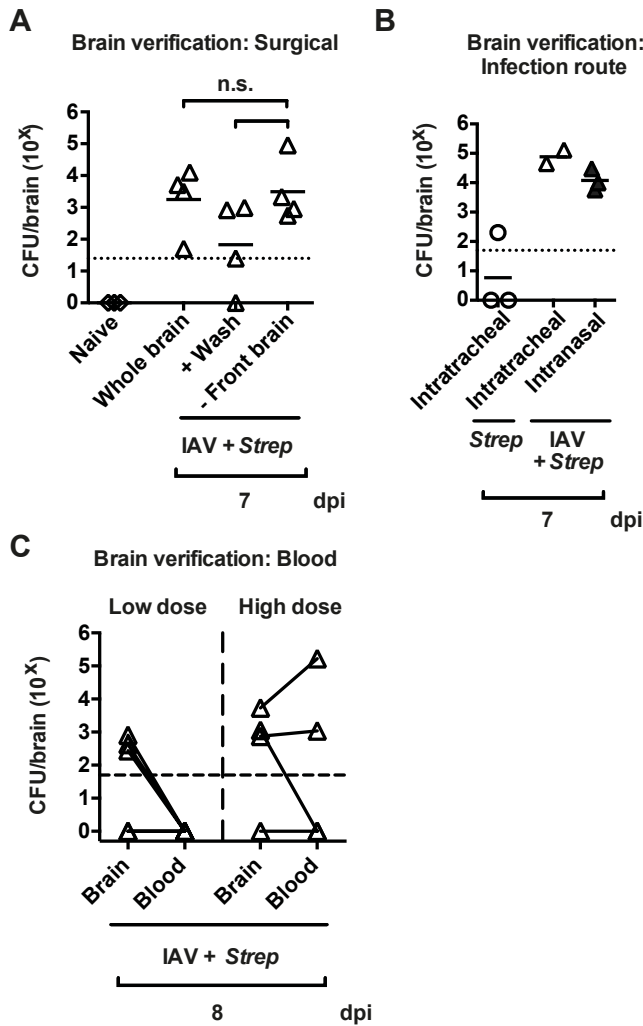
Figure 24

Figure 24. *Verification of streptococcal presence in the brain.*
(A) Streptococcal load in the brain during high dose coinfection at 7dpi; either extracted conventionally, with washing of the exterior in PBS, or with the front of the brain excised (n=3-4). (B) Streptococcal load in the brain during high dose coinfection at 7dpi with bacterial infection by the intranasal or intratracheal route (n=2-3). (C) Streptococcal load in whole brain and corresponding blood (100μl blood supernatant plated, brain and blood samples from same mice connected by line) during low or high dose coinfection at 8dpi (n=4-5). Data shown as geometric means. Significance assessed by Mann-Whitney test. n.s.=not significant.

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Bacterial spread to the brain leads to slight inflammation

As bacterial spread to the brain did not appear to be an artefact, whether this spread caused inflammation was then investigated. As an initial experiment, uninfected mice were compared with high dose coinfected mice, where the greatest bacterial spread to the brain is observed. Brains were extracted from naïve or high dose coinfected mice and cell numbers compared by flow cytometry. Coinfection led to a slight increase in neutrophils (Fig. 25A) and inflammatory monocytes (Fig. 25B), and caused a decrease in the number of (non-inflammatory monocyte) F4/80$^+$ cells (Fig. 25C), which may represent the resident microglia (Kettenmann *et al*., 2011). Very low levels of CD4 T cells were detected, which were unaffected by coinfection (Fig. 25D), while CD8 T cells were almost entirely absent from the brain in all settings (Fig. 25E). Therefore bacterial spread to the brain induces a modest neutrophil and inflammatory monocyte response, with a corresponding reduction in resident F4/80$^+$ cells.
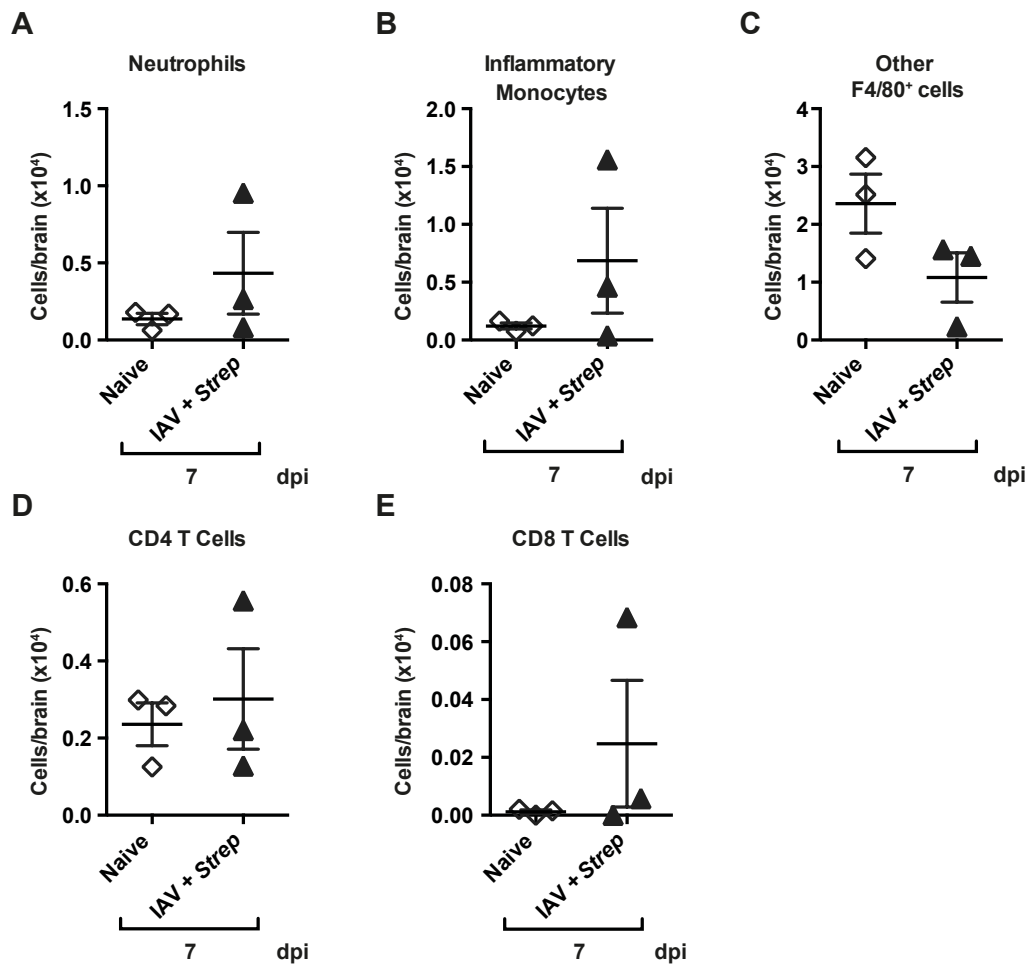
Figure 25

Figure 25. *Coinfection increases inflammatory cells and reduces F4/80⁺ cells in the brain.*

Flow cytometry quantification of (A) Neutrophils (Ly6G$^+$CD11b$^+$), (B) Inflammatory monocytes (Ly6C$^+$CD11b$^+$F4/80$^+$), (C) Non-inflammatory monocyte F4/80$^+$ cells (CD11b^{low}Ly6C-F4/80$^+$), (D) CD4 T cells (CD3$^+$CD4$^+$) and (E) CD8 T cells (CD3$^+$CD8$^+$) in the brain during coinfection (n=3). Data displayed as arithmetic means ± SEM.

8.4. Conclusions and Discussion

I have therefore demonstrated through several methods that bacterial spread to the brain during coinfection is not an artefact. Preliminary experiments suggest bacterial spread induces an inflammatory response.

It is notable that there is a modest, although not significant, decrease in bacterial loads detected in the brain when the exterior of the tissue is washed during harvesting (Fig. 24A). This implies some of the streptococci detected in the brain may be due to contamination from other tissues or the blood. As this result is not significant, this may simply be experimental variation, and therefore repeating this experiment, possibly with more mice, may be necessary. If this modest decrease represents a real result, some contamination from the blood may be an explanation, as some mice do show bacterial loads in the blood during high dose coinfection (Fig. 24C). Nevertheless, it is likely that a substantial proportion of the bacteria detected in the brain represents genuine infection of this tissue.

In this preliminary investigation single infection controls have not been performed for brain inflammatory cell quantification, and therefore another aspect of *Strep* or IAV single infections not related to coinfection may responsible for brain inflammation. *Strep* alone affecting brain inflammation is somewhat unlikely as no bacterial dissemination to the brain is observed during single infection (Fig. 2C). Nevertheless, a follow-up experiment should include all single infection controls.

Furthermore, as brain tissues are not perfused, it is possible the inflammatory response observed in the brain is a result of systemic changes in inflammatory cell proportions in the vasculature rather than infiltrate into the brain. Flow cytometry on perfused brain tissues, or comparison of changes in cell number during coinfection in the brain and blood may be useful future experiments to test this. Alternatively, histology of brain tissue to directly observe immune cells and bacteria may be useful.

As inflammation during bacterial meningitis has been correlated with severe disease (Kettenmann *et al*., 2002), bacterial spread to the brain may be one of the contributing factors to mortality during coinfection. However, it is notable that bacterial dissemination to the brain is also detected in recovering mice (Fig. 11E), although total loads are lower than in mice reaching endpoint. Therefore the absolute presence or absence of bacteria in the brain correlates poorly with disease, although the actual bacterial load correlates well. It is not clear whether bacteria persist in the brain in recovering mice, and this could be tested by harvesting at later time points. In summary, the presence of bacteria in the brain during coinfection appears to be a genuine phenomenon that moderately correlates with disease severity, and therefore merits further investigation.

Chapter 9. Other aspects of the immune response in coinfection

9.1. Background

In addition to studying the role of influenza-induced inflammatory monocyte mediated damage as an upstream cause of coinfection, and the downstream neutrophil and TNF-α response, I also investigated other aspects of the immune response in coinfection, although in a less comprehensive manner. Here studies relevant to the aspects investigated will be introduced.

IL-22 is an IL-10 family cytokine involved in antimicrobial immunity at the epithelial barrier. Expression of IL-22 is restricted to hematopoietic immune cells, while expression of its receptor is limited to non-hematopoetic organ surfaces (Sonnenberg *et al*., 2011). A recent study showed that IL-22 mice are moderately more susceptible to IAV-*Strep* coinfection (Ivanov *et al*., 2012). As IL-22 was marginally reduced during coinfection (Fig 6B), this may merit further investigation.

TLR2 is a pattern-recognition receptor involved in activating the immune system in response to recognition of bacterial lipopeptides in the cell walls of gram-positive bacteria (Yoshimura *et al*., 1999; Manukyan *et al*., 2005). It has been speculated that TLR2 may have a role in mediating the immune response to coinfection, although a previous study demonstrated no effect of TLR2 deficiency in a severe IAV-*Strep* coinfection (Dessing *et al*., 2007). In a recent IAV-*L.*

pneumophila coinfection model where tissue damage rather than bacterial outgrowth appears to be the primary driver of mortality, abolition of TLR2 and TLR4 signalling in conjunction with amphiregulin (an epidermal growth factor) treatment ameliorates coinfection (Jamieson *et al*., 2013). However the relevance of an *L. pneumophila* coinfection model to IAV-*Strep* coinfection, where bacterial outgrowth occurs, may be limited. Furthermore TLR2 deficiency only has an effect in the context of both TLR4 deficiency and amphiregulin treatment, and therefore its contribution to tissue damage is hard to assess. In summary, the role of TLR2 in a moderate IAV-*Strep* coinfection context has not yet been assessed.

As previously outlined, the role of type I IFNs in coinfection has been shown to be detrimental. IFNαβR$^{-/-}$ mice have improved survival in coinfection, due to greater neutrophil recruitment (Shahangian *et al*., 2009); however, this study does not assess whether neutrophil function is also increased. Type I IFN also induces IL-27, which leads to inhibition of IL-17 production by γδ T cells during coinfection (Cao *et al*., 2014). To recapitulate the negative effect of type I IFN, a recent study used Poly I:C, a TLR3 agonist that promotes a type I IFN response, to recapitulate the effects of prior influenza infection. This study reported that Poly I:C treatment increased bacterial loads during moderate to severe *Strep* infection (Tian *et al*., 2012). A similar study was performed using resiquimod (R848), a TLR7 agonist that can induce type I IFN and transient leukopenia; however, this did not cause susceptibility to subsequent *Strep* infection (Stegemann *et al*., 2009). In addition, Poly I:C treatment exacerbated *M. tuberculosis* infection through recruitment of a myeloid population that may be inflammatory monocytes (Antonelli *et al*., 2010). Therefore whether type I IFNs

affect neutrophil function, and whether susceptibility to secondary *Strep* infection can be caused by inducing a type 1 IFN response alone may be of interest.

The adaptive immune response to influenza may be a contributing factor to coinfection susceptibility. Production of IFN-γ by T cells during influenza can impair alveolar macrophage function during coinfection (Sun *et al*., 2008). As previously postulated, the CD8 T cell response to influenza may be responsible for the inflammatory-monocyte independent damage observed at 7dpi (Fig. 21D and E).

However, adaptive responses may also contribute to protection from *Strep* infection. Mice reach endpoint rapidly during coinfection, and it is unlikely an adaptive response to bacterial outgrowth is mounted during this timescale. However, it is possible that preformed natural antibodies may play a protective role, as has been described during *Strep* infection (Mold *et al*., 2002). A lethal IAV-*H. influenza* coinfection model directly modelled the role of the adaptive response in recombination-activating-gene (Rag) deficient mice, which lack T or B cells (Mombaerts *et al*., 1992); however, absence of the adaptive response did not ameliorate coinfection (Lee *et al*., 2010). Therefore the role of the adaptive response to IAV-*Strep* coinfection is a possible target for investigation.

9.2. Hypotheses and Aims

In this chapter I outline several experiments undertaken to investigate other aspects of the immune response, which were largely not comprehensively

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followed up. Generally this was due to negative results or corroboration of existing published data, which therefore did did not merit further investigation.

Several hypotheses were investigated in the course of these experiments:

- IL-22 is protective during coinfection.

- TLR2 plays a role in *Strep* recognition during coinfection.

- Neutrophil function during coinfection is not affected by type I interferon signalling.

- Poly I:C increases susceptibility to streptococcal infection.

- Adaptive immunity contributes to coinfection susceptibility.

9.3. Results

IL-22 is marginally protective during coinfection

As shown earlier in this thesis, the levels of IL-22 are marginally reduced from low levels by influenza infection and coinfection, both at 5dpi + 4hrs and 7dpi (in tables in Fig. 6B and 8B, shown graphically in Fig. 26A). To investigate whether the residual levels of IL-22 play a protective role, IL-22$^{-/-}$ mice were infected with a low dose coinfection. Total absence of IL-22 marginally but not significantly increased weight loss (Fig. 26B) and mortality (Fig. 26C). Therefore IL-22 is modestly reduced during coinfection, and total abolition of the remaining signalling is harmful, although this effect appears to be quite small.
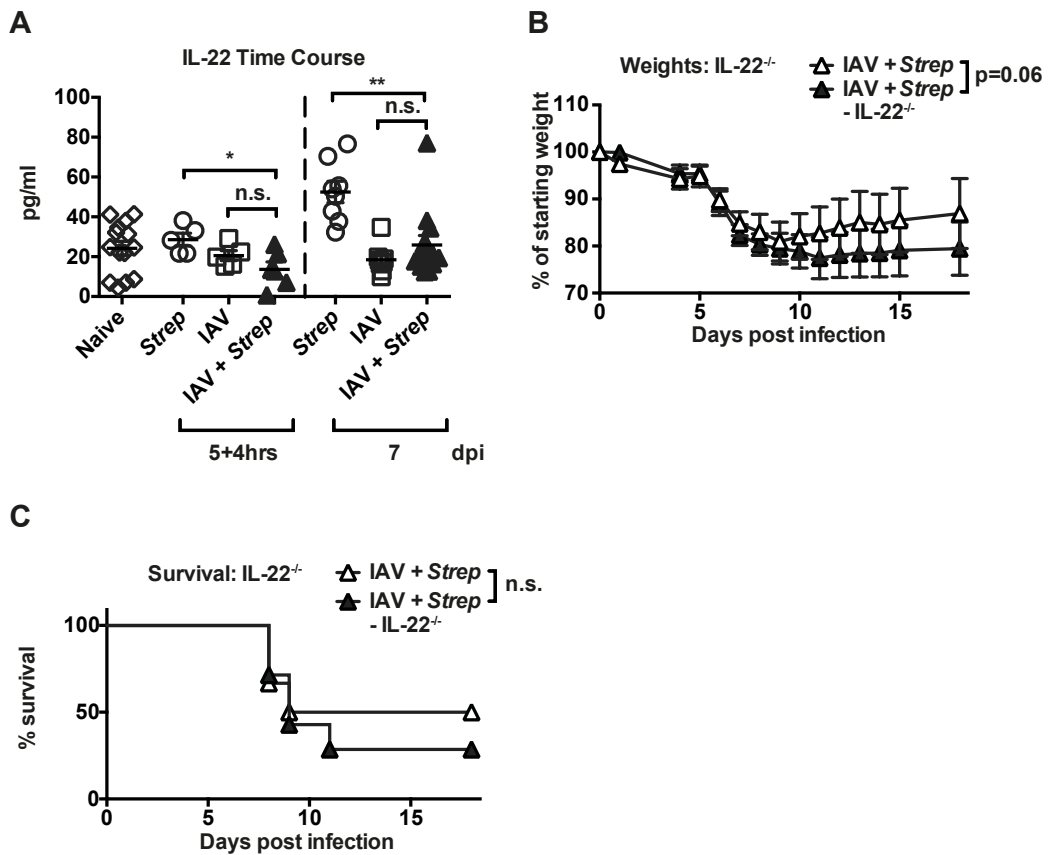
Figure 26

A

B

C

Figure 26. *IL-22 is reduced during coinfection; IL-22 deficiency slightly exacerbates low dose coinfection.*
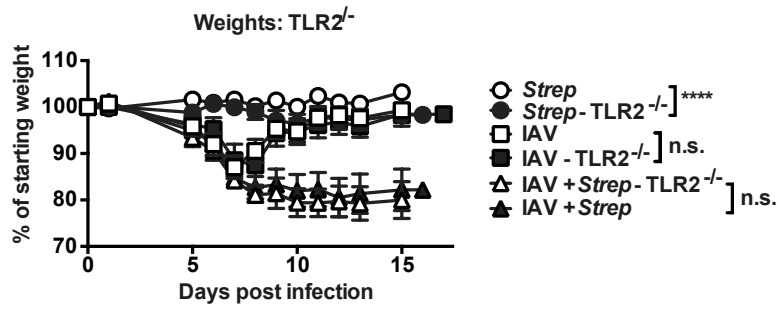
(A) ELISA quantification of IL-22 levels during high dose coinfection (n=3-8, data pooled from 3 independent experiments). (B) Weights and (C) mortality of wild-type (C57BL/6) and IL-22$^{-/-}$ (B6) mice during low dose coinfection (n=6-7, on weight loss plot mice reaching end-point retained thereafter at final weight). Data is shown as arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by Mann-Whitney test (IL-22 quantification), 2-way ANOVA (weights) or Log-Rank (Mantel-Cox) test (mortality). n.s.=not significant. *p<0.05, **p<0.01.

TLR2 does not play a role in coinfection

To assess the role of TLR2 in coinfection, the susceptibility of TLR2$^{-/-}$ mice to low dose coinfection was tested. TLR2$^{-/-}$ mice showed similar weight loss (Fig. 27A) and mortality (Fig. 27B) to wild-type mice during coinfection, although TLR2$^{-/-}$ mice did show slightly reduced weights following *Strep* infection. Therefore TLR2 plays no role in coinfection in this model.
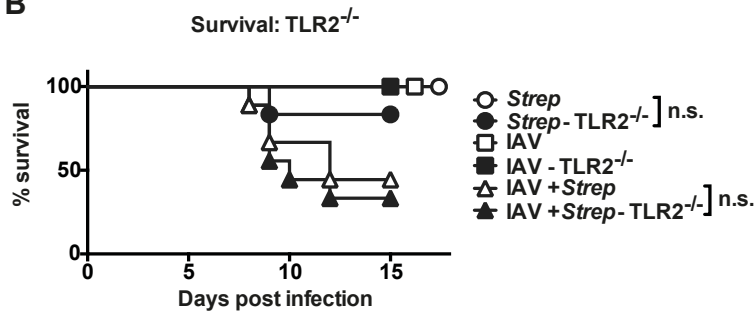
Figure 27

A

Weights: TLR2$^{-/-}$

% of starting weight

- ○ *Strep*
- ● *Strep* - TLR2 $^{-/-}$] ****
- □ IAV
- ■ IAV - TLR2$^{-/-}$] n.s.
- △ IAV +*Strep* - TLR2 $^{-/-}$] n.s.
- ▲ IAV +*Strep*

Days post infection

B

Survival: TLR2$^{-/-}$

% survival

- ○ *Strep*
- ● *Strep* - TLR2$^{-/-}$] n.s.
- □ IAV
- ■ IAV - TLR2$^{-/-}$
- △ IAV +*Strep*
- ▲ IAV +*Strep* - TLR2$^{-/-}$] n.s.

Days post infection

Figure 27. *TLR2 deficiency does not affect low dose coinfection.*
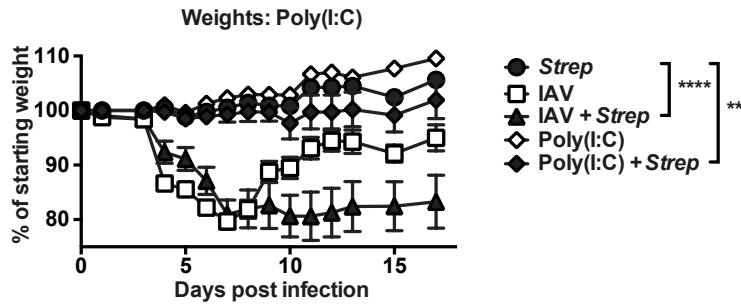(A) Weights and (B) mortality of wild-type (C57BL/6) and TLR2$^{-/-}$ (B6) during low dose coin-fection (n=6-9, on weight loss plot mice reaching endpoint retained thereafter at final weight, in some groups to aid visualisation final data point extended). Data shown as arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by 2-way-ANOVA (weights) or Log-Rank (Mantel-Cox) test (mortality). n.s. = not significant. ****p<0.0001.

Poly I:C treatment does not recapitulate the effect of influenza on secondary bacterial infection

Poly I:C has been shown to increase bacterial loads in a subsequent *Strep* infection (Tian *et al*., 2012). To attempt to reproduce this results the effects of prior influenza or intranasal Poly I:C treatment on secondary *Strep* infection were compared. Poly I:C treatment marginally exacerbated weight loss (Fig. 28A) and did not significantly affect mortality (Fig. 28B) during subsequent *Strep* infection, as opposed to the strong synergy observed between IAV and *Strep* infection. Therefore in a mild *Strep* infection, induction of a prior type I IFN response with Poly I:C is not sufficient to recapitulate the synergy seen during IAV-*Strep* coinfection.

Figure 28

A

B
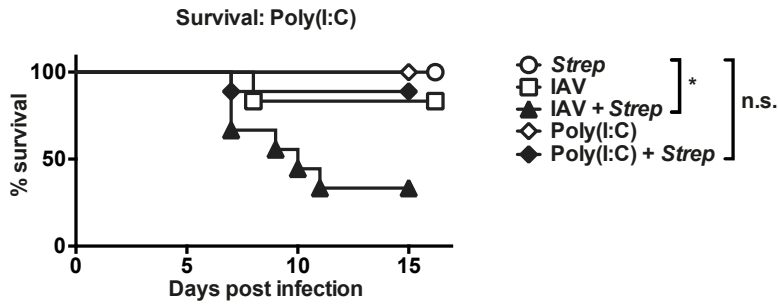
Figure 28. *Poly(I:C) treatment does not recapitulate coinfection.*
(A) Weights and (B) mortality during *Strep* infection preceeded by either treatment with 50μ g Poly(I:C) at 3 and 4 dpi or 1AV infection at 0 dpi (i.e. normal low dose coinfection) (n=6-9, on weight loss plot mice reaching endpoint retained thereafter at final weight). Data shown as arithmetic means ±SEM or percent survival. Signficance assessed by 2-way-ANOVA (weights)   or Mantel-Cox (Log-Rank) test. n.s.=not significant. *p<0.05, **p<0.01, ****p<0.0001.

Type I interferon signalling does not affect neutrophil function during coinfection

IFNαβR$^{-/-}$ mice are better protected from coinfection and recruit more neutrophils (Shahangian *et al*., 2009). As type I interferon has been reported to have detrimental effects on neutrophils (Navarini *et al.*, 2006), it is possible greater resistance is also due to increased neutrophil function. It has previously been verified in this thesis that type I IFNs are present during influenza and coinfection in wild-type mice (Fig. 6B and 8B).

To test whether this prior exposure to type I IFN *in vivo* affects neutrophil function, neutrophils extracted from coinfected IFNαβR$^{-/-}$ and wild-type lungs were functionally profiled. Wild-type and IFNαβR$^{-/-}$ neutrophils had similar ROS production (A) and cytokine production (Fig. 29B) upon restimulation. IFNαβR$^{-/-}$ neutrophils produced moderately less TNF-α following PDBu stimulation, and slightly more KC following Pam3CSK4, LPS and PDBu stimulation; however, these trends are quite marginal. This data suggests that prior type I interferon exposure does not substantially alter neutrophil function, although it may somewhat suppress KC production upon restimulation.

An alternative approach to testing whether type I IFN affects neutrophil function is to expose neutrophils that have not experienced interferon signalling to IFNα *in vitro*. Naïve neutrophils were used, as these cells would not have previously experience type I IFN *in vivo* (note – as few lung neutrophils are purified from naïve mice, only 5×10^4 neutrophils were used per replicate in this assay, as compared to 1×10^5 normally used to test cytokine secretion). Pretreatment of

naïve wild-type neutrophils in culture with increasing concentrations of IFNα did not affect cytokine (TNF-α) production upon stimulation with Pam3CSK4 (Fig. 29C). Therefore in an *in vitro* setting stimulation of type I IFN signalling does not appear to affect neutrophil function as assessed by cytokine production.
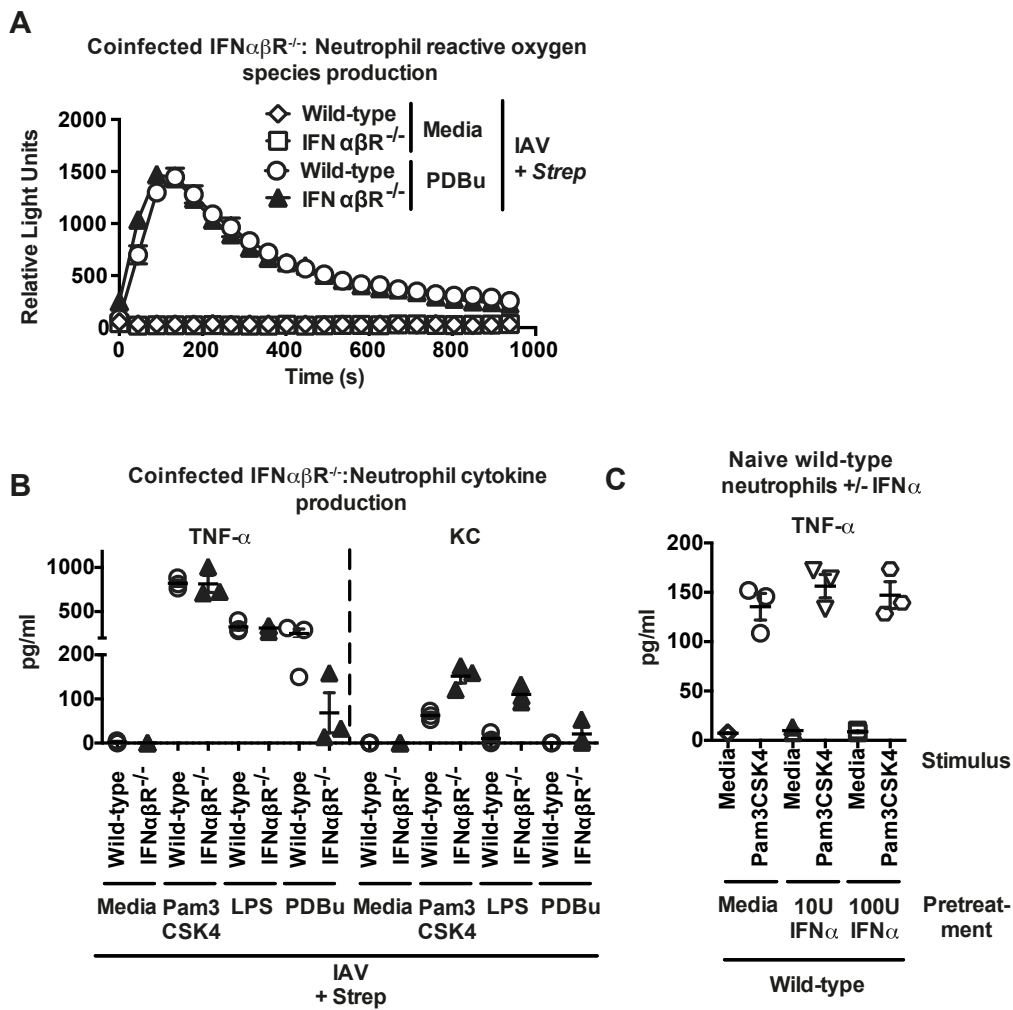
Figure 29

A

Coinfected IFNαβR⁻/⁻: Neutrophil reactive oxygen species production

B

Coinfected IFNαβR⁻/⁻:Neutrophil cytokine production

C

Naive wild-type neutrophils +/- IFNα

Figure 29. *Type I IFN does not affect neutrophil function.*
(A) ROS production by luminol assay of PDBu (50nM) stimulated MACS purified lung neutrophils from high dose coinfected wild-type (C57BL/6) or IFNαβR⁻/⁻ (B6) mice at 6dpi (neutrophils from 3 mice pooled into wild-type or IFNαβ⁻/⁻ group, with technical triplicates performed in the assay/condition). (B) ELISA quantification of TNF-α and KC produced by Pam3CSK4 (1μg/ml), LPS (1μg/ml) or PDBu (50nM) stimulated MACS purified neutrophils from high dose coinfected wild-type or IFNαβR⁻/⁻ (B6) mice at 6dpi (neutrophils from 3 mice pooled/group, with technical triplicates/condition). (C) ELISA quantification of TNF-α produced by Pam3CSK4 (1μg/ml) stimulated lung neutrophils from naive wild-type mice prestimulated with IFNα (10U/ml or 100U/ml) (neutrophils from 6 mice pooled, with technical triplicates/condition; note - in this assay, only 5x10⁴ neutrophils/replicate used). Data displayed as arithmetic means ±SEM.

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Rag1-/- mice display moderate synergy during coinfection and are susceptible to Strep infection

To investigate whether the adaptive immune response contributes to coinfection susceptibility, Rag1$^{-/-}$ mice, which lack peripheral T and B cells (Mombaerts *et al.*, 1992), were used. As the adaptive immune response is required for clearance of influenza (Brown *et al.*, 2004), Rag1$^{-/-}$ mice eventually succumb to influenza infection. Therefore in this context total mortality could not be used to test whether IAV-*Strep* coinfection was more severe than IAV infection alone. However, as IAV-*Strep* coinfection leads to rapid mortality in wild-type mice at a high dose, whether coinfected mice reach endpoint more rapidly than IAV infected mice could be assessed. To attempt to induce the strongest synergy and the most rapid mortality in coinfected mice, high dose coinfection was used.

High dose IAV-*Strep* coinfection caused moderately more rapid weight loss (Fig. 30A) and mortality (Fig. 30B) than IAV infection alone. Therefore synergy between IAV and *Strep* still occurs in the absence of adaptive immunity. However the modest nature of the synergy observed highlights the technical challenge in assessing the rapidity of mortality in a setting where all mice eventually reach endpoint due to influenza infection. Notably, a substantial proportion of Rag1$^{-/-}$ mice also eventually succumb to *Strep* infection, which is somewhat surprising given that *Strep* appears to be rapidly cleared in the first four hours following inoculation (Fig. 3A). These results are discussed further below.

Figure 30

A

Weights: Rag1$^{-/-}$

B

Survival: Rag1$^{-/-}$
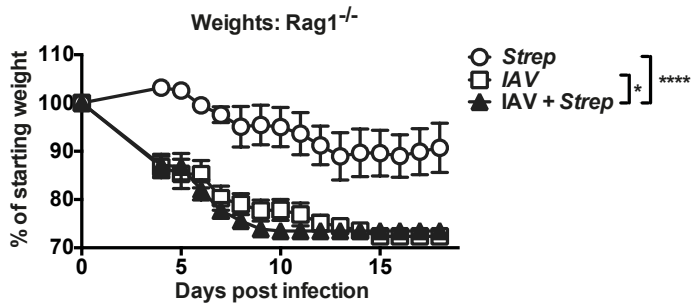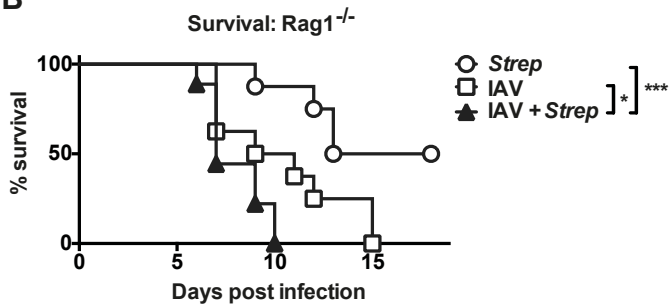
Figure 30. *Coinfection synergy is not dependent on adaptive immunity.*
(A) Weights and (B) mortality of wild type (C57BL/6) or Rag1$^{-/-}$ ^{MOM} (C57BL/6/J/CD45.1) mice during high dose coinfection (n=8-9, on weight loss plot mice reaching endpoint retained thereafter at final weight). Data displayed as arithmetic means ±SEM (weights) or percent survival (mortality). Significance assessed by 2-way ANOVA (weight time course) or Log-rank (Mantel-Cox) test (mortality). n.s. = not significant. *p<0.05. ***p<0.001. ****p<0.0001.

9.3. Conclusions and Discussion

Investigation of other aspects of the immune response has given much useful information regarding coinfection. The small protective role of IL-22 described in a previous study (Ivanov *et al*., 2012) has been corroborated. Furthermore it has been shown that IL-22 levels are slightly suppressed during moderate influenza infection. This is in contrast to the study cited above, which shows an induction in IL-22 early at several different timepoints during coinfection from 3 to 7dpi, during both moderate and severe influenza infections. The reasons for this discrepancy are not clear, but may reflect the different influenza strains used.

The suppression of IL-22 during coinfection may explain why further reduction in signalling to zero using IL-22 deficient mice only causes a modest increase in mortality. Whether the modest suppression of IL-22 plays a role in susceptibility is not clear. As taking a loss-of-function approach using IL-22 deficient mice may be limited here due to existing suppression, taking a gain-of-function approach by adding exogenous IL-22 during coinfection may be more informative. Exogenous IL-22 marginally reduced bacterial loads in IL-22$^{-/-}$ mice during coinfection (Ivanov *et al*., 2012), but addition of IL-22 to wild-type mice has not been previously been performed.

It has been shown that TLR2 does not have a role in severe coinfection (Dessing *et al*., 2007); however, this setting does not allow a negative effect of TLR2 deficiency to be detected. As TLR2 is involved in bacterial recognition, it was hypothesised that its absence might exacerbate coinfection, which would be

detectable in a low dose context. However, in a moderate, low dose coinfection context, TLR2 deficiency had no effect. This is consistent with the study cited above, and confirms that the lack of effect in this study this was not due to the severe coinfection used.

Another study also observes no effect of TLR2 deficiency in a severe coinfection (Karlstrom *et al.*, 2011). This study however does propose that bactericidal ampicillin treatment, although protective, is less so than bacteriostatic antibiotics as it leads to bacterial lysis. This study speculates that lysed bacteria activate TLR2 and cause subsequent immunopathology. Therefore in the context of antibiotic treatment, where immunopathology may replace bacterial outgrowth as the primary driver of mortality during coinfection, TLR2 may play a detrimental role, and this may be worthy of further investigation. Combination of this with addition of factors promoting tissue repair such as amphiregulin could also be considered, as this may ameliorate immunopathology further in this setting.

Prior type I interferon signalling did not appear to affect neutrophil function upon restimulation. In addition, Poly I:C did not recapitulate the effect of influenza upon secondary bacterial infection, in contrast to a recent study (Tian *et al.*, 2012). The lack of effect of Poly I:C prestimulation may be due to the different severities of *Strep* infections used. This result suggests that, although influenza-induced type I IFNs have been reported to play a detrimental role during coinfection, induction of a type I IFN response alone without other aspects of influenza such as lung damage is not sufficient to promote susceptibility to *Strep* infection. Alternatively - although equivalent to the amount used in the previous

study described - the amount of Poly I:C stimulation used here may be inadequate to recapitulate an equivalent type 1 IFN response to IAV. Furthermore although Poly I:C can induce a type I IFN response via TLR3, it does not mimic the multiple signalling pathways (such as the RIG-I pathway) stimulated by influenza (Iwasaki *et al.*, 2014), therefore crucial aspects that promote susceptibility to *Strep* may be absent. Possible further experiments could include quantification of type I IFN levels following Poly I:C stimulation, and to test the levels of damage-causing inflammatory monocytes, TRAIL-expression, and DR5 expression on epithelia induced by this.

More rapid mortality during coinfection than either IAV or *Strep* single infections was evident in Rag1$^{-/-}$ mice, which lack adaptive immunity (Mombaerts *et al.*, 1992). This suggests that the adaptive response to influenza is not required to cause coinfection susceptibility. The synergy observed however was modest, showing the difficulty in assessing rapidity of mortality in a context where all mice eventually succumb to influenza. A possible experiment that may address this is to perform secondary bacterial infection earlier in Rag1$^{-/-}$ mice, giving a longer period to observe coinfection synergy before mice succumb to viral infection. However, it has been reported that coinfection at earlier time points than 5dpi is less severe (McCullers *et al*, 2002) - possibly reflecting reduced lung damage - and therefore changing timepoint may make synergy even more difficult to detect.

Alternatively, other parameters than eventual mortality could be used to assess synergy in Rag1$^{-/-}$ mice - for example, cytokine levels in coinfection versus

influenza infection, or bacterial loads in *Strep* versus coinfection, assessed at the latest possible timepoint (such as 6dpi). However, as Rag1$^{-/-}$ mice, unlike wild-type mice, control both virus and bacterial single infections poorly, this is likely to elevate all readouts of disease such as bacterial load and cytokine induction. This suggests it may be difficult to detect synergy by any readout in the complete absence of adaptive immunity and viral control, and other approaches may be more suitable.

One approach that could be taken is specific depletion of some aspects of the adaptive response, such as depletion of CD8 T cells, in wild-type mice. As there is some redundancy in the adaptive response (Brown *et al.*, 2004) this may leave some viral control intact and give more scope to observe synergy. This may allow a more clear assessment than in Rag deficient mice of whether adaptive immune aspects are involved in promoting coinfection susceptibility.

It is possible that lung damage later in influenza infection is due to the adaptive response. I have previously shown in wild-type mice that lung damage in influenza infection at 5dpi, but not 7dpi, appears to be inflammatory monocyte-dependent (Fig. 21A-B; Fig. 21D-E). This suggests at later timepoints other factors, which may include the antiviral CD8 cytotoxic T cell response, cause damage. Therefore performing secondary infection at 5dpi may not be suitable for assessing the contribution of adaptive immunity to coinfection susceptibility. Although not possible with Rag1$^{-/-}$ mice, which begin to succumb to influenza infection from 7dpi onwards, it would be instructive to perform coinfection at

7dpi in wild-type mice and assess the role of adaptive immunity by other methods; for example, as suggested above, by depleting CD8 T cells.

Strikingly, a substantial proportion of *Strep* infected Rag1$^{-/-}$ mice also succumb to coinfection. This suggests a role for adaptive immunity in protection against *Strep*. However, somewhat paradoxically, *Strep* is almost completely cleared from the airways 4 hours following infection in wild-type mice (Fig. 3A), which strongly suggests an adaptive response - which typically takes several days to develop - is not required. A possibility is that the absence of preformed natural antibodies in Rag1$^{-/-}$ mice may cause susceptibility, as natural antibodies have been described to be protective against *Strep* infection (Mold *et al*., 2002). Opsonophagocytosis mediated by neutrophils or other phagocytes may also be impaired in the absence of natural antibodies, although I have previously shown that neutrophils are not required to clear low virulence single *Strep* infection. In summary, the role of adaptive immunity in coinfection remains unclear, and Rag1$^{-/-}$ mice may be a relatively unsuitable model to investigate this, due to their susceptibility to both *Strep* and IAV single infections. Other approaches - for example, antibody mediated depletion of adaptive immune cells during coinfection – could be considered.

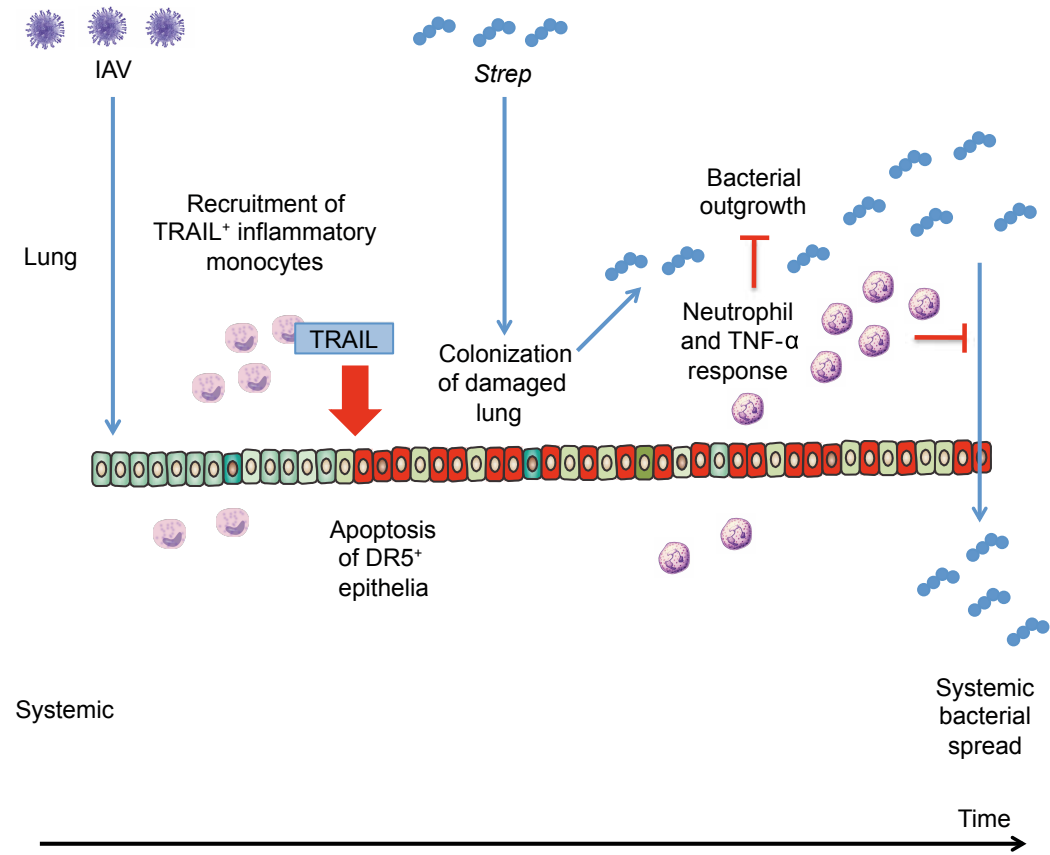# Chapter 10. Summary and General Discussion

10.1. Summary

In this thesis I have established and analysed mouse model of Influenza A-*Strep* coinfection. This has led to several conclusions (summarised diagrammatically in Fig. 31):

- Moderate severity influenza infection predisposes mice to secondary coinfection with a mild *Strep* strain, which in the absence of influenza colonizes poorly and rarely induces disease.

- Coinfection is characterised by bacterial, but not viral, outgrowth and spread.

- Bacterial outgrowth appears to be the primary driver of mortality during coinfection.

- Bacterial outgrowth during coinfection induces a strong proinflammatory response, including greatly elevated levels of neutrophils and TNF-α.

- The neutrophil and TNF-α response to coinfection is on balance protective.

- Neutrophils and TNF-α are not required for clearance of *Strep* alone, supporting the concept that in the absence of influenza low virulence *Strep* does not colonize.

- Influenza infection leads to recruitment of inflammatory monocytes, which induce lung damage by a TRAIL-dependent mechanism at the point of coinfection.

- Lung damage allows colonization by *Strep*, leading to bacterial outgrowth.

Highly specific and technical aspects of these conclusions relating to pertinent studies have already been discussed in prior chapters. Here I will discuss the merits and disadvantages of the scientific approach taken, and these conclusions in more general, conceptual terms.

Figure 31

IAV

Strep

Lung

Recruitment of
TRAIL⁺ inflammatory
monocytes

TRAIL

Colonization
of damaged
lung

Bacterial
outgrowth

Neutrophil
and TNF-α
response

Apoptosis
of DR5⁺
epithelia

Systemic

Systemic
bacterial
spread

Time

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10.2. General Discussion

10.2.1. Advantages and disadvantages of the Influenza A-*S. pneumoniae* mouse model

Mouse models

There are both advantages and disadvantages to using mouse models to investigate coinfection. Inbred mice are the most common models for both IAV and *Strep* infections (Chiavolini *et al*., 2008; Bouvier *et al*., 2010), and this, along with the low genetic variability of inbred mice, allows substantial consistency and comparison with other studies. However, IAV is not a natural mouse pathogen, and it has been argued that ferret models more accurately recapitulate human disease (Bouvier *et al*., 2010). However, mouse models have practical advantages over the use of ferrets, as much larger numbers of animals can be used for experimentation, and many more species-specific reagents and genetically modified strains are available.

Although rare in a laboratory setting, natural infections of mice with *Strep* have been reported (Baker *et al*., 1998), with transmission primarily from infected humans. Mouse models of *Strep* have similar advantages of mouse models to those investigating influenza. Therefore, the requirement for large numbers of animals, the range of reagents and genetically modified strains, and the abundance of existing information make mice a highly suitable model for studying coinfection.

Pathogen virulence and dosing

In this thesis mild to moderate virulence single *Strep* and IAV infections have been used to model coinfection. There are multiple advantages to this. Neutrophils and TNF-α have been reported to have roles during more severe single infections (Tate *et al*., 2009; Hussell *et al*., 2001). Use of moderate virulence strains reduces the likelihood of interventions such as neutrophil depletion perturbing single infections, and therefore allows focus on the role of neutrophils and TNF-α in coinfection.

Inflammatory monocytes have also been reported to have a detrimental effect during severe influenza infection (Herold *et al*., 2008). Use of moderate virulence influenza means alterations that affect the inflammatory-monocyte response and reduce lung damage, such as CCR2 deficiency or anti-TRAIL treatment, do not substantially alter the outcome of viral disease. This allows investigation of the role of inflammatory monocytes in promoting coinfection, where the positive effect of inflammatory monocyte deficiency can be detected.

In addition to using mild to moderate virulence strains of *Strep* and IAV, both "high" and "low" dose settings of these pathogens were used to investigate coinfection, giving different strength synergy. High dose induced almost 100% mortality during coinfection, while low dose caused approximately 50%. Many studies only use a setting that gives 100% mortality (for example, Damjanovic *et al*., 2013). Both settings have different advantages and drawbacks. High dose coinfection gives consistently severe disease and therefore is useful to profile

coinfection as results are highly homogenous. However, in a setting where all mice reach clinical endpoint, only strongly positive interventions can be detected. Therefore low dose coinfection - with variable disease and moderate mortality - was used to assess the effect of interventions, where both positive and negative effects of interventions could be detected. Low dose coinfection is less suitable for profiling disease due to the greater heterogeneity of coinfected mice, as demonstrated by the large spread in bacterial loads (for example, in Fig. 11C).

It could be argued that a low dose setting should be used for all experiments, as is likely more similar to physiological pathogen doses. Furthermore, it cannot be assumed that conclusions drawn profiling in a high dose setting always apply at a lower dose. However, performing profiling experiments at a low dose would, due to huge diversity in bacterial outgrowth and disease outcome, greatly increase the number of mice required to draw a clear conclusion, and therefore is deeply impractical. Furthermore, bacterial outgrowth appears to be the cause of mortality at both low and high dose, suggesting mechanisms that promote coinfection are similar between doses, and only differ in intensity.

Difficulty of correlating readouts with mouse fate

Where possible in this thesis the effects of coinfection were profiled *in vivo* in the lung or other organs. This is useful as the lung is the primary disease site and gives direct readouts on changes occurring during coinfection. However, to harvest the lung or other organs mice must be sacrificed at a defined timepoint. This does not allow monitoring of changes in a single mouse over time. It also

prevents directly relating readouts with mouse fate, as once mice are sacrificed, subsequent disease development cannot be monitored.

In some instances lung and other organs were harvested at endpoint, rather than a defined timepoint (for example, showing bacterial outgrowth correlated with mortality in Figure 11). This allowed direct correlation between disease severity and subsequent readouts. However, this is not practical for all experiments, as mice harvested at variable timepoints cannot always be directly compared. It may be useful to develop readouts that do not require sacrifice, such as cytokine levels or bacterial loads in the blood that can be assessed serially in the same mouse. Serial *in vivo* imaging of fluorescent bacteria in mice may also be useful, as has been performed in a previous coinfection study (Diavatopoulous et *al.,* 2010). These readouts could be predictive and correlated with eventual mouse fate.

Defining the sequence of events in coinfection

An issue in IAV-*Strep* coinfection models is that it is difficult to separate out upstream causes and downstream consequences of bacterial outgrowth. Here these factors were separated out in two ways. Firstly, the immune response was profiled after coinfection at both early (5dpi + 4hrs) and late (5dpi + 48 hrs) timepoints. Therefore the early response to bacterial colonization and the downstream response to bacterial outgrowth can be temporally separated.

Secondly, a temporal component was given to interventions during IAV-*Strep* coinfection. Neutrophils and TNF-α were depleted during coinfection but not

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during prior influenza infection, allowing assessment of their downstream role in bacterial phase. CCR2 mice were used to investigate upstream causes of coinfection. By definition CCR2$^{-/-}$ mice lack inflammatory monocytes in both viral and bacterial phases of infection. Therefore from these mice alone it was not possible to tell when the harmful effects of inflammatory monocytes were mediated, although reduced lung damage at 5dpi in CCR2$^{-/-}$ mice was highly suggestive. Use of anti-TRAIL to block inflammatory monocyte mediated damage allowed more clear temporal assessment. Anti-TRAIL treatment was protective if given during viral phase of infection. This intervention alone does not confirm when the harmful effect of TRAIL is mediated, as anti-TRAIL antibodies may persist into the bacterial phase of infection. However, anti-TRAIL treatment is ineffective if given in bacterial phase only, indicating the beneficial effect of TRAIL blockade is prior to this. Therefore, the harmful effect of TRAIL-expressing inflammatory monocytes is mediated in viral phase. Overall these interventions enabled separation of the upstream role of inflammatory monocytes in promoting bacterial colonization from the downstream protective role of neutrophils and TNF-α.

10.2.2. Bacterial outgrowth during coinfection

The conclusion that bacterial outgrowth occurs in coinfection is consistent with many other mouse models (LeVine *et al.*, 2001, van der Sluijs *et al.*, 2004; Sun *et al.*, 2008). Furthermore increased bacterial loads relative to single infection are also observed in IAV-*S. aureus* models, although these do not consistently show clear outgrowth over time (Kudva *et al*, 2010, Small *et al.*, 2011). Increased

bacterial loads are also observed during a systemic *L. monocytogenes* infection (Jamieson *et al.*, 2010)*,* and an IAV-*B. pertussis* model observes delayed bacterial clearance (Zavitz *et al.*, 2010). In addition, in all cases and interventions investigated here, bacterial outgrowth and mortality strongly correlate. The diversity of bacterial species for which prior influenza promotes outgrowth suggests two possibilities: that influenza causes a universal single problem that leads to susceptibility to multiple species, or it promotes a variety of problems, with specific disease mechanisms in each setting. As this thesis focuses on IAV-*Strep* coinfection, it is not possible to give a definitive answer to this proposition; the range of mechanisms suggested in different coinfection models suggests the latter, but influenza-mediated damage is likely to relatively non-specifically promote colonization.

In contrast to the results shown here and other IAV-*Strep* coinfection models, a study modelling IAV-*L. pneumophila* coinfection does not observe changes in bacterial load, and instead attributes mortality to immune-mediated tissue damage (Jamieson *et al.*, 2013). In this study the authors state bacterial outgrowth can complicate analysis of the causes of morbidity and mortality, and they therefore sought a model where this did not occur. This study is therefore useful in highlighting how tissue damage can contribute to mortality during coinfection; however, the lack of bacterial outgrowth may not reflect the events in physiological coinfections.

10.2.3. Immune impairment during coinfection

A striking feature of the model of IAV-*Strep* coinfection described here is that no clear immune impairment is observed. No cytokine (with the exception of a marginal reduction in IL-22) or cell type profiled is significantly reduced in coinfection; in contrast, a strong immune response is observed. Furthermore, depletion of neutrophils and TNF-α does not affect low virulence single *Strep* infection, suggesting that these responses are not required at all in the absence of prior influenza. Therefore in this context impairment in neutrophils and TNF-α cannot be the cause of coinfection susceptibility. In contrast, several previous studies have suggested the immune response is impaired during coinfection. There are several possible reasons for this discrepancy.

Firstly, the immune profiling performed here, while broad, is by no means absolutely comprehensive, and there are aspects of the immune response reported to be impaired by other studies, such as γδ T cell (Li *et al.,* 2012) and alveolar macrophage function (Goulding *et al*., 2011), that are outside the scope of this study. It is possible that these are contributing factors to susceptibility in this model. However, it must be noted that alveolar macrophage numbers show a trend towards reduction in both coinfection susceptible (wild-type) and resistant (CCR2$^{-/-}$) mice, suggesting their role is not crucial here.

Furthermore several studies reporting immune impairment do not include comparisons of the response between coinfection and single infections. This makes it difficult to assess the extent of immune impairment. For example, a gain-

of-function study shows IFNαβR$^{-/-}$ mice recruit more neutrophils than wild type and are less susceptible to coinfection. This implies that neutrophil recruitment is deficient during coinfection in wild-type mice (Shahangian *et al*., 2009). However, neutrophils recruited during *Strep* alone are not quantified. Therefore in this context it is not possible to assess whether neutrophil recruitment during coinfection can be considered impaired relative to *Strep* infection alone.

Some studies reporting immune impairment have not been reproduced. For example, while one study showed anti-IL-10 ameliorates coinfection (van der Sluijs *et al*., 2004), this effect was not reproduced in IL-10$^{-/-}$ mice (Sun *et al*., 2008). This may reflect differences in mouse and streptococcal strains, as well as timing differences between models, which further underlines the difficulties involved in comparing mouse models of IAV-*Strep* coinfection.

A criticism of some coinfection studies is that they focus on a single aspect of the immune response which is reduced during coinfection, while overlooking a simultaneous very strong proinflammatory response. For example, one study attributes susceptibility to reduced IL-17 production, while simultaneously observing increased IL-1β, IL-6, TNF-α, KC, MIP-2 and G-CSF (Li *et al*, 2012). Therefore whether the overall immune response can be considered impaired in this context is not clear.

Finally, I use low virulence *Strep* and IAV infections, which themselves only induce modest immune responses. As low virulence *Strep* largely does not colonize, it is likely that the immune response (with the possible exception of pre-

existing natural antibodies) is not required for bacterial clearance. However previous studies have shown aspects of the immune response are required in more virulent *Strep* single infections. Therefore a more virulent *Strep* infection may induce a stronger immune response than observed here, giving more scope for immune impairment in coinfection. In conclusion there are many reasons why some studies observe immune impairment and others, including this thesis, do not, and its relative importance during IAV-*Strep* coinfection remains to be determined. It is possible that in coinfection models with a more virulent *Strep*, influenza mediated immune impairment is a major factor; however, in low virulence *Strep* models such as in this thesis, immune impairment does not appear to contribute to coinfection.

10.2.4. Lung damage and integrity during coinfection

In this thesis I propose a mechanism for bacterial colonization where influenza-induced inflammatory monocytes cause lung damage. Most previous studies focusing on lung damage have used severe influenza strains and focused on direct viral damage. Here a moderate influenza strain is used, and the proposed mechanism focuses on damage mediated by the immune response to the virus, rather than direct viral damage. Nevertheless, these other studies corroborate the concept that the extent of lung damage is strongly correlated to the severity of secondary streptococcal infection. However, their focus on a different source of damage - directly viral mediated - highlights how multiple factors contribute to coinfection susceptibility.

Studies showing direct viral damage can increase the severity of coinfection focus on expression of PB1-F2, a pro-apoptotic peptide. Influenza strains expressing this lead to worse damage and more severe secondary bacterial coinfections (McAuley *et al*., 2007). This has been shown to be dependent on specific PB1-F2 sequences (Alymova *et al*., 2013), and different PB1-F2 expressing variants have different severity coinfections (Weeks-Gorosope *et al*., 2012). Furthermore addition of C-terminal peptide from PB1-F2 alone is sufficient to predispose to secondary *Strep* infection (McAuley *et al*., 2007). This suggests that in severe influenza infection, viral damage alone can promote coinfection susceptibility. The relative contributions of viral and immune-mediated damage in promoting coinfection susceptibility remain to be determined, and may differ greatly depending on the severity of the viral strain used.

Other studies also suggest that influenza reduces lung integrity through other mechanisms than damage. This suggests that there may be other factors promoting coinfection in addition to viral or inflammatory-monocyte mediated damage. Reduced mechanical clearance by the cilia of the bacteria is observed in an *ex vivo* trachea model (Pittet *et al*., 2010). *In vivo* models have also suggested that influenza increases adhesion of streptococci to epithelial cells (Plotkowski *et al*., 1986), and that this is partly viral neuraminidase dependent (McCullers *et al*., 2004). In summary, the results in this thesis showing that inflammatory monocyte mediated damage can lead to bacterial colonization during coinfection are consistent with other studies on lung integrity prior to coinfection, but suggest a new immune-mediated mechanism for this in addition to direct viral damage.

10.2.5. The multifactorial nature of coinfection

Several key points have been outlined in this discussion. Firstly, influenza causes susceptibility to coinfection by a range of bacterial species, typically leading to bacterial outgrowth. Multiple mechanisms have been proposed for this, which largely fall into the categories of immune impairment or lung damage – whether directly viral mediated, or, as is shown here, immune-mediated. Although immune impairment is not observed in this thesis, there are a range of conceptual and technical reasons which may account for this discrepancy. Studies that show increasing lung damage leads to more severe coinfection indirectly corroborate the findings shown here.

Influenza appears to promote a variety of mechanisms that allow secondary bacterial coinfection by a range of species. These may be detectable in different experimental contexts, suggesting coinfection is a highly multifactorial issue. Therefore it is likely that the mechanism proposed here - while evidently a crucial component of susceptibility - is not the sole cause of coinfection, and that many other factors also play a role.

10.3. Possible further investigations

Testing immune impairment in other aspects of the downstream response to coinfection

There are several possible follow-up experiments that could further elucidate mechanisms of coinfection. More in depth investigation into the downstream immune response to bacterial outgrowth during coinfection could be carried out. Immune cell function or numbers that have not profiled in this thesis could be investigated. Flow cytometry could be used to enumerate $\gamma\delta$ T cells, and intracellular staining could be used to assess $\gamma\delta$ T cell cytokine production. Furthermore, alveolar macrophage function could be profiled through different approaches. Purification of alveolar macrophages and *in vitro* restimulation may be suitable, or use of *in vivo* readouts such as uptake of fluorescently labelled bacteria by alveolar macrophages in the lung.

The strong immune response in coinfection appears to correlate with bacterial outgrowth. Therefore it could be argued that this represents a greater stimulus to the immune system, and thus it is not possible in the context of bacterial outgrowth to determine whether influenza "impairs" the immune response – i.e. influenza may reduce the immune response per bacterium, but this cannot be detected due to greater bacterial outgrowth. It would be technically difficult to assess this experimentally. One approach would be to observe the immune response to secondary infection with a non-replicating bacterial stimulus such as Pam3CSK4, where the secondary stimulus is of a consistent strength. However, as

non-replicating stimuli do not replicate coinfection synergy (Fig. 4B) this may not be a suitable model system.

Assessing neutrophil phagocytosis during coinfection

Neutrophil phagocytosis has previously been reported to be impaired during coinfection (Damjanovic *et al.*, 2013). In this thesis phagocytosis of streptococci by neutrophils was not assessed. Although neutrophils in coinfection appeared functional by all readouts tested, it is possible that phagocytosis is impaired. Different approaches could be taken: purified neutrophils could be incubated with streptococci (as well as appropriate opsonisation factors) *in vitro* and uptake assessed by gentamicin protection assay. Alternatively, use of fluorescent bacteria would allow *in vitro* and *in vivo* uptake to be assessed by flow cytometry.

Further investigations into the role of TNF-α in coinfection

Although it is clearly demonstrated in this thesis that TNF-α is protective during coinfection, how this is mediated remains unclear. Several aspects of the role of TNF-α during coinfection could therefore be further investigated. The source of TNF-α could be determined by intracellular staining of different lung cell populations. Furthermore, as both neutrophils and inflammatory monocytes have been reported as TNF-α producers (Zhang *et al.*, 2009), the levels of TNF-α in neutrophil depleted or $CCR2^{-/-}$ mice could be profiled. Also, the downstream targets of TNF-α signalling that promote its protective effects could be investigated; as TNF-α promotes neutrophil recruitment to peripheral tissues

(Nathan, 2006; Bradley, 2008) it may be that its primary protective effect is enhancing neutrophil recruitment. Flow cytometry to quantify neutrophils and other cells in coinfected lungs following anti-TNF-α treatment could test this.

Improving readouts for and further investigation into inflammatory-monocyte mediated lung damage

More investigation into inflammatory-monocyte mediated lung damage and its role in coinfection could be carried out. LDH activity and protein in the airways are somewhat blunt readouts, and more specific aspects of epithelial damage could be investigated, such as assessing the levels of cellular apoptosis. Furthermore, in addition to the previous loss-of-function approach taken to inflammatory-monocyte mediated damage using $CCR2^{-/-}$ mice and anti-TRAIL, a gain of function approach could be taken, supplementing $CCR2^{-/-}$ mice with inflammatory monocytes early during influenza.

It is possible inflammatory monocytes mediate damage through other mechanisms than TRAIL. Anti-TRAIL treatment reduces coinfection susceptibility to a similar extent as CCR2 deficiency, suggesting this is the main effector of inflammatory monocytes in this context. However, an alternative explanation is that anti-TRAIL treatment, by coating inflammatory monocytes with antibody, promotes phagocytic clearance of these cells. This may leads to partial depletion of monocytes, which would also reduce other monocyte effector mechanisms. Therefore whether anti-TRAIL treatment reduces inflammatory monocyte numbers at the point of coinfection should be tested by flow cytometry.

Furthermore, other inflammatory monocyte killing mechanisms could be investigated. Monocytes have been reported to mediate cell death through surface expression of Fas ligand (Imanishi *et al.*, 2002), which could be investigated by flow cytometry.

The specific mechanisms of how lung damage allows colonization at the level of an individual bacterium is not clear, and it would be useful to investigate this further. It is possible the death of epithelial cells exposes possible bacterial binding sites – for example, PAFR (van der Sluijs *et al.*, 2006). This could be possibly be investigated using immunohistochemistry staining for PAFR. Another possibility is that epithelial death reduces production of antibacterial compounds and mucus; these could be quantified in the lung. Furthermore, increased epithelial permeability may allow more bacterial dissemination. However, the greater bacterial loads in the lung observed than in other organs throughout coinfection suggest colonization in the lung itself is the crucial determinant of susceptibility.

Replicating influenza-mediated damage with a non-infectious stimulus

Although somewhat technically challenging, the importance of lung damage could be demonstrated directly by following a non-infectious but damaging stimulus with *Strep* infection. Different agents have previously been used in other settings to induce lung damage, such as bleomycin, which induces damage and fibrosis (Wilson *et al.*, 2010) or napthalene, which kills lung clara cells found in the bronchioles (Van Winkle *et al.*, 1995). Although these reagents have

drawbacks (for example, bleomycin is an antibiotic, and the damage caused may not be similar to that caused by viral infection), they could be used to model influenza-induced damage, and to determine if lung damage alone is sufficient to cause susceptibility to bacterial colonization. A previous study showing the C-terminal peptide of viral PB1-F2 alone can promote susceptibility to secondary *Strep* infection strongly suggests this is the case (McAuley *et al.*, 2007).

Investigating the link between type I IFNs and monocytes in coinfection

The link between type I IFNs and inflammatory monocytes may be highly relevant to coinfection. Other studies focusing on influenza-induced inflammatory-monocyte mediated lung damage have suggested it is partially type I IFN dependent (Hogner *et al.*, 2013; Davidson *et al.*, 2014), and an absence of type I IFNs reduces inflammatory monocyte recruitment during influenza (Seo *et al.*, 2011). This may be an additional factor contributing to the reported resistance of IFNαβR$^{-/-}$ mice to coinfection (Shahangian *et al.*, 2009).

It may be difficult to separate out the two proposed protective effects of IFNαβR deficiency – namely reduced inflammatory monocyte recruitment and TRAIL expression, and increased neutrophil recruitment. A method of investigating this could be to take a gain-of-function approach, supplementing IFNαβR$^{-/-}$ mice with inflammatory monocytes early during influenza, which may increase susceptibility to coinfection. Neutrophils could also be depleted in IFNαβR$^{-/-}$ mice to confirm directly their protective effects.

Investigating neutrophils as a source of early damage during influenza infection

It would also be useful to investigate whether other aspects of the immune response to influenza - in addition to inflammatory monocytes - contribute upstream to damage and bacterial colonization. Neutrophils have been described to cause lung damage during influenza (Narasaraju *et al*., 2011). Therefore neutrophils, while protective once bacterial colonization occurs, may contribute to damage early in influenza. It is possible that neutrophil depletion during the viral phase only would ameliorate coinfection. However this experiment would be technically challenging for two reasons: firstly, depleting antibody may persist into bacterial phase, which has been demonstrated here to exacerbate coinfection. Secondly, early neutrophil depletion may affect single IAV infection, as 1A8 treatment has been shown to exacerbate IAV (Tate *et al*., 2009). Despite this, comparing the effect of neutrophil depleting antibody given during viral and bacterial phases of coinfection may be of some interest.

Confirming how the protective effect of anti-TRAIL is mediated

One limitation of the results shown here is that it has not been explicitly confirmed that the protective effect of anti-TRAIL is mediated through blocking cell-surface TRAIL on inflammatory monocytes in the lung and preventing lung damage. The presence of TRAIL-expressing inflammatory monocytes and their absence in resistant CCR2$^{-/-}$ mice strongly suggest that the therapeutic effect of anti-TRAIL is through blocking damage mediated by these cells; however, this has not been confirmed. Various experiments to further investigate this could be

performed. Whether anti-TRAIL treatment reduces lung damage in influenza could be assessed by observing airway LDH and protein. Furthermore, if the main effect of anti-TRAIL is on inflammatory monocytes, it could be confirmed that anti-TRAIL treatment has no effect on lung damage in CCR2$^{-/-}$ mice.

Furthermore, TRAIL can be a soluble or cell-surface ligand (Benedict *et al.*, 2012), and soluble TRAIL has been shown to play a role in *Strep* infection (Steinwede *et al.*, 2012). Although in this thesis the majority of cell-surface TRAIL was detected on inflammatory monocytes, the levels of soluble TRAIL have not been assessed. Soluble TRAIL could be quantified by ELISA. If soluble TRAIL is detected the cellular source could be investigated; although it would be somewhat difficult to distinguish from cell-surface TRAIL by flow cytometry or qPCR.

Further study on bacterial spread to the brain in coinfection

Bacterial spread to the brain was investigated in a preliminary manner in this thesis. Bacterial spread induced inflammation in the brain; a more comprehensive flow cytometry analysis of this could be performed. Furthermore, bacterial invasion and inflammation in the brain may affect permeability. Brain permeability could be assessed by testing protein levels by BCA assay in perfused brain tissue, or by application of dyes in the blood and observation of colour changes in the brain. As bacteria spreads more consistently to the brain than other organs, this may merit more detailed investigation into streptococcal adhesion to the brain endothelium, and more research on the systemic effects of influenza.

Bacterial meningitis is frequently modelled in mice by somewhat unphysiological intracisternal application of bacteria (Chiavolini, 2008). As during IAV-*Strep* coinfection bacteria spread to the brain following intranasal infection, another use of the IAV-*Strep* coinfection model may a more physiological system to assess bacterial meningitis.

Investigating treatment options for coinfection

Different treatment options could be investigated in the IAV-*Strep* coinfection mouse model used here. Two approaches could be taken to ameliorating disease: controlling downstream bacterial outgrowth, or preventing bacterial colonization by reducing influenza-induced lung damage. Patients exhibiting severe symptoms are likely to have bacterial outgrowth, and therefore controlling this may represent a more suitable therapeutic strategy. However, in the instance of an influenza pandemic, ameliorating viral-induced lung damage may be a suitable prophylactic strategy.

The role of bacterial outgrowth as the primary driver of mortality suggests therapeutic antibiotic treatment would be highly effective during coinfection. Other studies have assessed the merits of different antibiotics in coinfection (Karlstrom *et al.*, 2009; Damjanovic *et al.*, 2013; Fukada *et al*., 2014). One study suggests bacteriostatic antibiotics are superior to bactericidal, as they do not cause rapid bacterial lysis and excessive inflammation (Karlstrom *et al*., 2009). Another method of controlling bacterial outgrowth may be to stimulate the neutrophil and

TNF-α response. Direct treatment with recombinant TNF-α or neutrophil recruiting chemokines such as MIP2 and KC may be protective. In a previous IAV-*Strep* mouse model, treatment with MIP2 and KC reduced bacterial loads (Shahangian *et al*., 2009).

To reduce viral-mediated damage two approaches could be taken. Direct antiviral treatment is likely to lead to reduced viral loads and possibly reduced damage. Consistent with this, neuraminidase inhibitors have been shown to be protective in coinfection (McCullers *et al*., 2003). Alternatively, an implication of the results here is that inhibition of the inflammatory monocyte response to influenza may be protective. Inhibition of monocyte-recruiting chemokines such as MCP-1 may be a treatment option; anti-MCP1 treatment has been shown to reduce inflammatory infiltrate during influenza (Narasaraju *et al*., 2010, Damjanovic *et al*., 2011).

Another strategy to counter lung damage may be to promote epithelial repair. In a coinfection study where bacterial outgrowth does not occur, and tissue damage is the driver of mortality, treatment with the epithelial growth factor family member amphiregulin treatment is beneficial (Jamieson *et al*., 2013). It is possible treatment with amphiregulin may be protective if given during the viral phase of coinfection to promote epithelial repair, countering inflammatory-monocyte mediated lung damage. However, its usefulness as a therapeutic option in the in the bacterial phase, where outgrowth is the driver of mortality, may be more limited. In conclusion the results in this thesis suggest several possible treatment strategies during coinfection.

10.4. Concluding remarks

In this thesis, I have attempted to investigate the role of the immune response in IAV-*Strep* coinfection. Susceptibility to coinfection is a multifactorial issue, and a new aspect of this has been uncovered. Influenza-induced inflammatory-monocyte mediated lung damage promotes susceptibility to secondary *Strep* colonization via a TRAIL-dependent mechanism. This leads to bacterial outgrowth, which is countered by a protective and unimpaired, but frequently insufficient, neutrophil and TNF-α response. This is a significant contribution to the understanding of IAV-*Strep* coinfection and will hopefully promote further research into this pressing public health problem.

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- Dr. Anne O'Garra and Dr. Xuemei Wu (NIMR) for providing CCR2$^{-/-}$ and IFNαβR$^{-/-}$ mice.

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Appendices

1A – Typical flow cytometry gating strategy for myeloid cells

1B – Typical flow cytometry gating strategy for lymphoid cells

2A – Cytokines in the airway 12hrs following coinfection

2B – Cytokines in the airway 16hrs following coinfection

3 – Flow cytometry gating strategy for identifying neutrophils without anti-Ly6G (1A8) staining

4 – Cytokines in the airway in $CCR2^{-/-}$ and wild-type mice at the point of and during coinfection

5A – Identification of $TRAIL^+$ cells

5B – Determination of DR5 expression on epithelial cells

Appendix 1 A
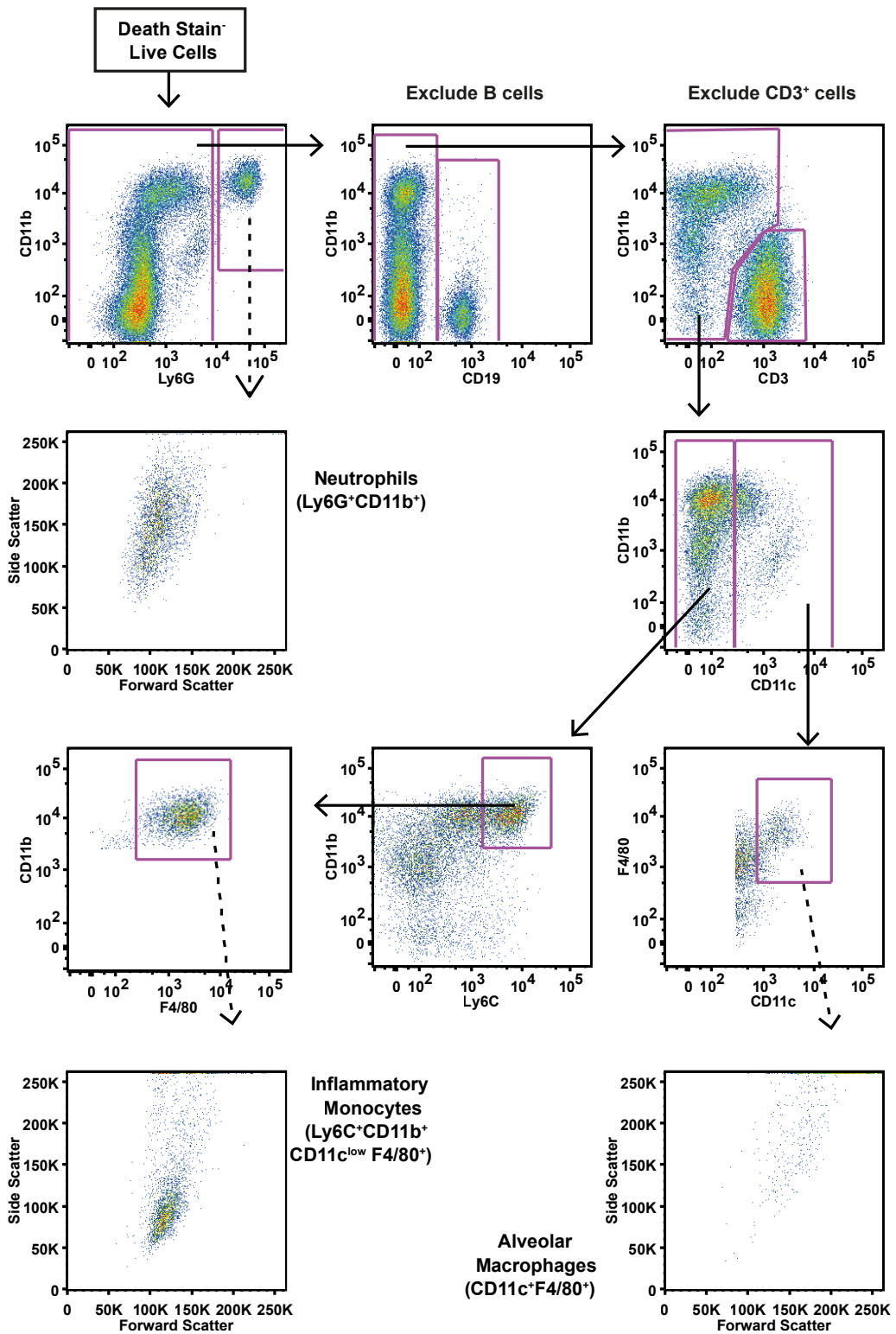
Appendix 1 A: *Typical flow cytometry gating strategy for myeloid cells.*
Typical flow cytometry gating strategy used for myeloid cells (neutrophils, inflammatory monocytes, alveolar macrophages) Representative 7dpi high dose IAV infected lung shown. Black arrows indicate progressionto the next gate. Dashed arrows indicate populations derived, for which their respective forward and side scatter is shown.
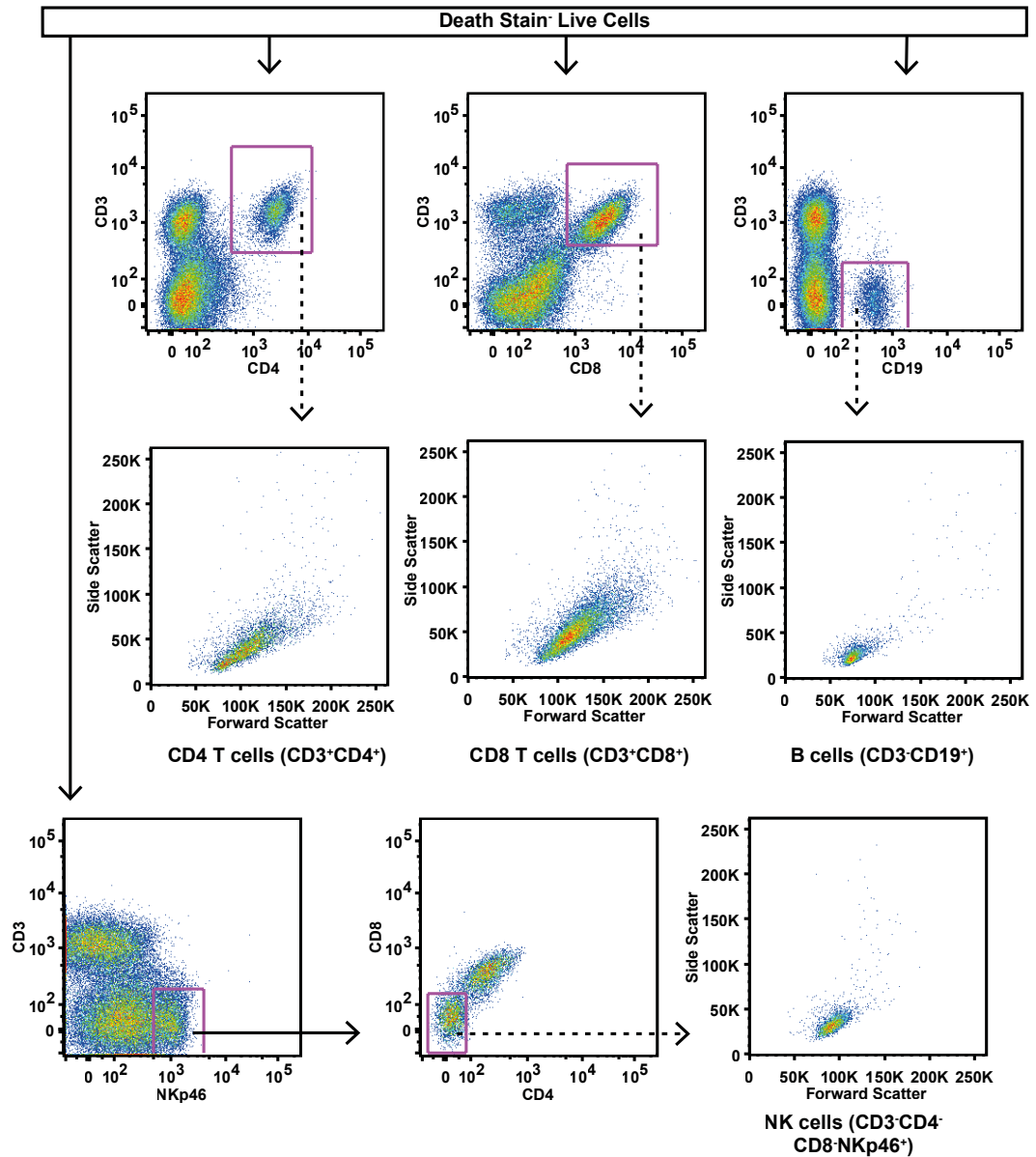
Appendix 1 B: *Typical flow cytometry gating strategy for lymphoid cells.*
Typical flow cytometry gating strategy used for lymphoid cells (CD4 and CD8 T cells, B cells, NK cells). Representative 7dpi high dose IAV infected lung shown. Black arrows indicate progression to the next gate. Dashed arrows indicate populations derived, for which their respective forward and side scatter is shown.

Appendix 2 A

|	Cytokine at 5dpi + 12hrs (pg/ml)				Fold change vs. single infections
	Naïve	*Strep*	IAV	IAV + *Strep*	
MCP1	1	1	821	867	25
IFNγ	4	17	2932	5048	23 *
IL10	1	1	102	101	9 *
Eotaxin	2	2	72	108	8
MIG	4	613	6526	10546	5.3 *
MIP1β	64	334	1142	3074	5 *
RANTES	4	7	28	55	4.0 *
IP10	2	792	2430	5529	4.0
IL5	13	39	376	408	3.4
TNF	6	122	95	350	3.2
MIP1α	45	657	774	2267	3.2
GCSF	1	1801	1381	4820	3.1
MIP2	38	161	150	395	2.5
LIF	1	27	112	131	2.4
IL6	5	1809	4654	5955	2.1
IL4	1	2	45	18	1.9
IL1α	33	38	21	50	1.8
IL17	0	25	10	23	1.5
KC	12	235	277	375	1.5
MCSF	1	30	12	26	1.4
IL1β	28	99	34	78	1.4
GMCSF	44	71	44	76	1.4
VEGF	10	95	13	48	1.4 †
IL15	29	25	38	34	1.1
IL9	71	43	77	58	1.0
LIX	76	379	121	146	0.7
IFNα	4	3	35	18	1.8
IL22	24	35	15	23	1.0

IL2, IL3, IL7, IL12p40, IL12 (p70), IL13, IFNβ excluded as means all < than 20pg/ml

Appendix 2 A. *Cytokines in the airway 12hrs following coinfection.*
Multiplex and ELISA quantification of airway cytokines at 5dpi + 12hrs during high dose coinfection (n=3-6). Multiplex is top section of table, lower section is ELISAs. Data shown as arithmetic means only.Cytokines in table ranked by fold change relative to single infections, shown in the right hand column. This is calculated by the taking the geometric mean of fold change IAV+*Strep* vs. IAV and fold change of IAV+*Strep* vs. *Strep.* Significance assessed by Mann-Whitney test. *p<0.05 IAV+*Strep* vs. *Strep*; †p<0.05 IAV + *Strep* vs IAV.

Appendix 2 B

	Cytokine at 5dpi + 16hrs (pg/ml)				Fold change vs. single infections
	Naïve	*Strep*	IAV	IAV + *Strep*	
IFNγ	4	18	7422	8203	22 *
MIP1β	64	242	2356	16960	22 *,†
TNFα	6	52	150	1553	18 *
MIP1α	45	457	1445	10028	12 *,†
MIP2	38	80	222	1439	11 *,†
LIX	76	18	96	443	10.7 *,†
IL10	1	3	357	298	10 *
RANTES	4	8	49	175	9 *,†
IL6	5	735	9553	21874	8 *
GCSF	1	1044	3689	15505	7.9 *
LIF	1	7	282	315	7.3 *
MIG	4	1159	16629	30895	7.0 *
MCP1	1	60	1463	2074	7.0 *
IP10	2	649	4712	10462	6.0 *
IL1β	28	66	44	311	5.8 †
Eotaxin	2	2	246	114	4.7 *
IL1α	33	36	17	96	3.9 *,†
IL9	71	63	114	322	3.8 *
KC	12	113	510	841	3.5 *
IL5	13	34	1104	666	3.4 *
IL12 (p70)	12	8	16	40	3.4 *,†
MCSF	1	18	15	50	3.1 †
IL15	29	33	41	99	2.7 *,†
GMCSF	44	103	70	217	2.6
IL4	1	2	60	27	2.2 *
IL17	0	16	18	24	1.4
VEGF	10	78	13	33	1.0
IFNβ	4	2	10	71	16.0 *
IFNα	4	2	49	34	3.6 *
IL22	24	52	19	28	0.9

IL2, IL3, IL7, IL12 (p40) and IL13 excluded as means all < than 20pg/ml

Appendix 2 B. *Cytokines in the airway 16hrs following coinfection.*
Multiplex and ELISA quantification of airway cytokines at 5dpi + 16hrs during high dose coinfection (n=3-6). Multiplex is top section of table, lower section is ELISAs. Data shown as arithmetic means. Cytokines in table ranked by fold change relative to single infections, shown in the right hand column. This is calculated by the taking the geometric mean of fold change IAV+*Strep* vs. IAV and fold change of IAV+*Strep* vs. *Strep*. Significance assessed by Mann-Whitney test. *p<0.05 IAV+*Strep* vs. *Strep*; †p<0.05 IAV + *Strep* vs IAV.
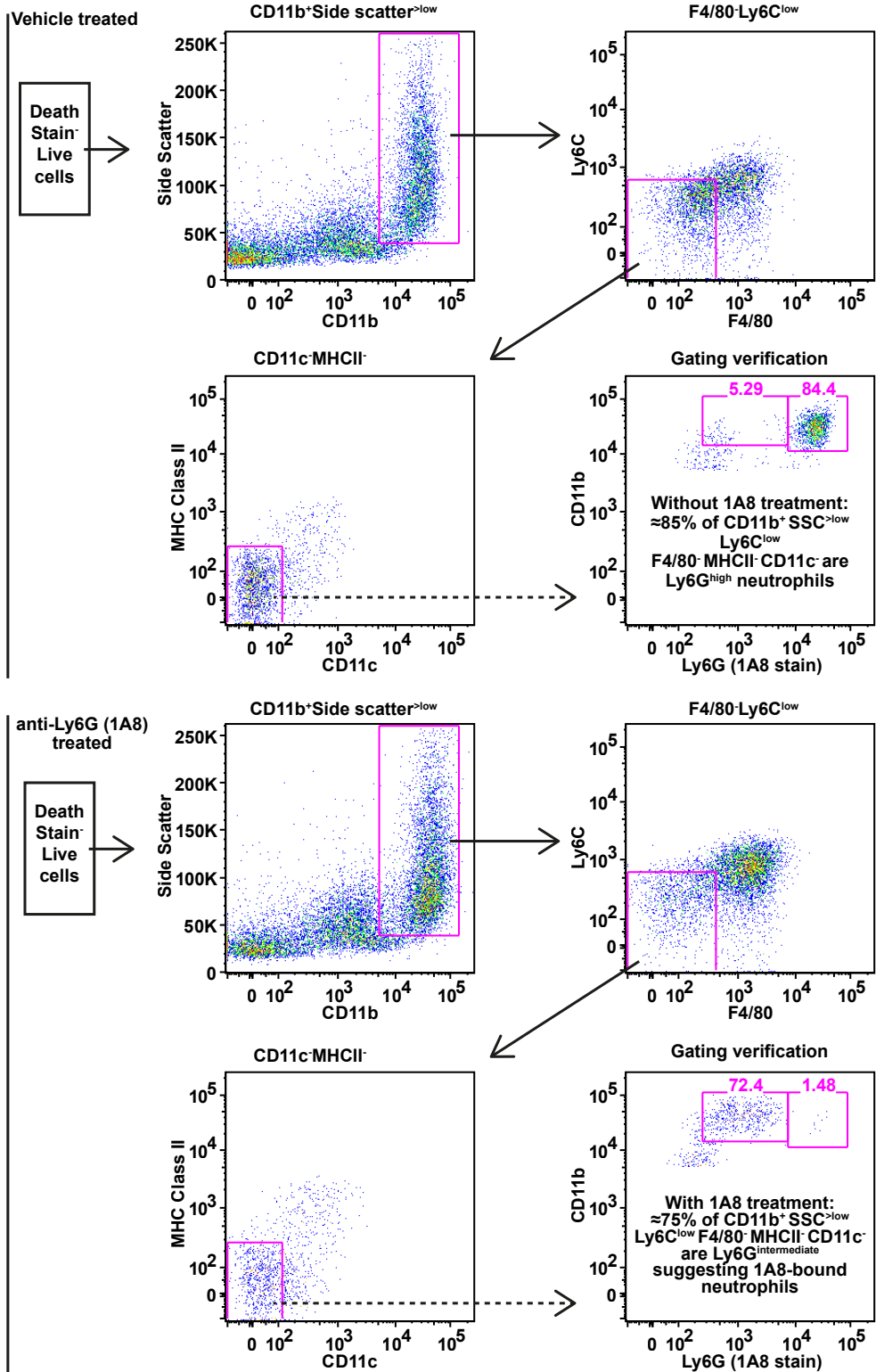
Appendix 3

Appendix 3: Flow cytometry gating strategy for identifying neutrophils without anti-Ly6G (1A8) stain:
Gating strategy used to identify neutrophils in 1A8 treated mice. Top section shows vehicle treated (i.e. no 1A8 treatment) representative 6dpi low dose coinfected mouse. Neutrophils identified as CD11b$^+$ SSC$^{>low}$ Ly6C^{low} F4/80$^-$ MHCII$^-$ CD11c$^-$ (1st three panels) and this strategy verified by comparing population derived to Ly6G staining (fourth panel). Bottom section shows 1A8 treated representative 6dpi low dose coinfected mouse. Neutrophils identified as above, and suppression of anti-Ly6G staining 1A8 treatment in residual population derived shown in fourth panel.

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Appendix 4

	Cytokine (pg/ml) at:						
	5dpi IAV		7dpi IAV		7dpi IAV + *Strep*		
	Wild-type	CCR2$^{-/-}$	Wild-type	CCR2$^{-/-}$	Wild-type	CCR2$^{-/-}$	Fold change CCR2$^{-/-}$ vs. wt 5dpi
VEGF	29	158	86	403	426	226	5.4 *
MCSF	61	257	48	512	349	892	4.2 *,†,‡
IL10	21	78	137	817	1858	741	3.6 *,†
MCP1	2076	7425	1828	7611	21613	10867	3.6 *,†
MIP1α	1107	2676	1338	3894	87599	10518	2.4 *,†,‡
MIP2	158	334	190	324	8962	614	2.1 *,†,‡
MIP1β	1514	2800	1139	4025	30002	9534	1.8 *,‡
IL1α	67	110	36	61	366	273	1.7 *
RANTES	32	53	16	59	1517	117	1.6 †
GCSF	4674	6799	4864	19130	183797	20696	1.5 †,‡
Eotaxin	58	60	565	381	830	186	1.0 ‡
IL1β	100	104	90	89	441	269	1.0
GMCSF	53	54	37	37	75	111	1.0
KC	484	462	281	335	4778	382	1.0 ‡
IP10	13204	12001	10511	11414	253229	22443	0.9 ‡
MIG	4636	4044	10622	15494	75288	41236	0.9
LIX	404	288	174	109	717	905	0.9
TNFα	110	75	45	85	5682	191	0.7 ‡
IFNγ	1613	985	3591	3315	14232	1556	0.7 ‡
IL15	19	11	7	8	48	18	0.6 ‡
IL6	15206	7701	9325	9414	69182	8430	0.6 ‡
IL4	55	27	11	13	9	7	0.5
LIF	217	98	311	249	431	257	0.5 *,‡
IL12 (p40)	61	26	22	25	57	83	0.5 *
IL5	674	212	429	296	225	203	0.4 *
IL17	13	4	16	9	34	8	0.3 ‡

IL2, IL3, IL7, IL12 (p70) and IL13 excluded as means all < than 20pg/ml

Appendix 4. *Cytokines in the airway in CCR2$^{-/-}$ and wild-type mice at the point of and during coinfection.*
Multiplex quantification of airway cytokines in wild-type (C57BL/6) or CCR2$^{-/-}$ (B6) at during high dose influenza infection or coinfection at 5 and 7dpi (n=5-6). Data shown as arithmetic means. Cytokines in table ranked in descending order of fold change of CCR2$^{-/-}$ IAV infected mice at 5dpi relative to wild-type. Significance assessed by Mann-Whitney test. *p<0.05 wild-type IAV 5dpi vs. CCR2$^{-/-}$ IAV 5dpi; †p<0.05 wild type IAV 7dpi vs CCR2$^{-/-}$ IAV 7dpi; ‡p<0.05 wild type IAV + *Strep* 7dpi vs CCR2$^{-/-}$ IAV + *Strep* 7dpi.

Appendix 5 A
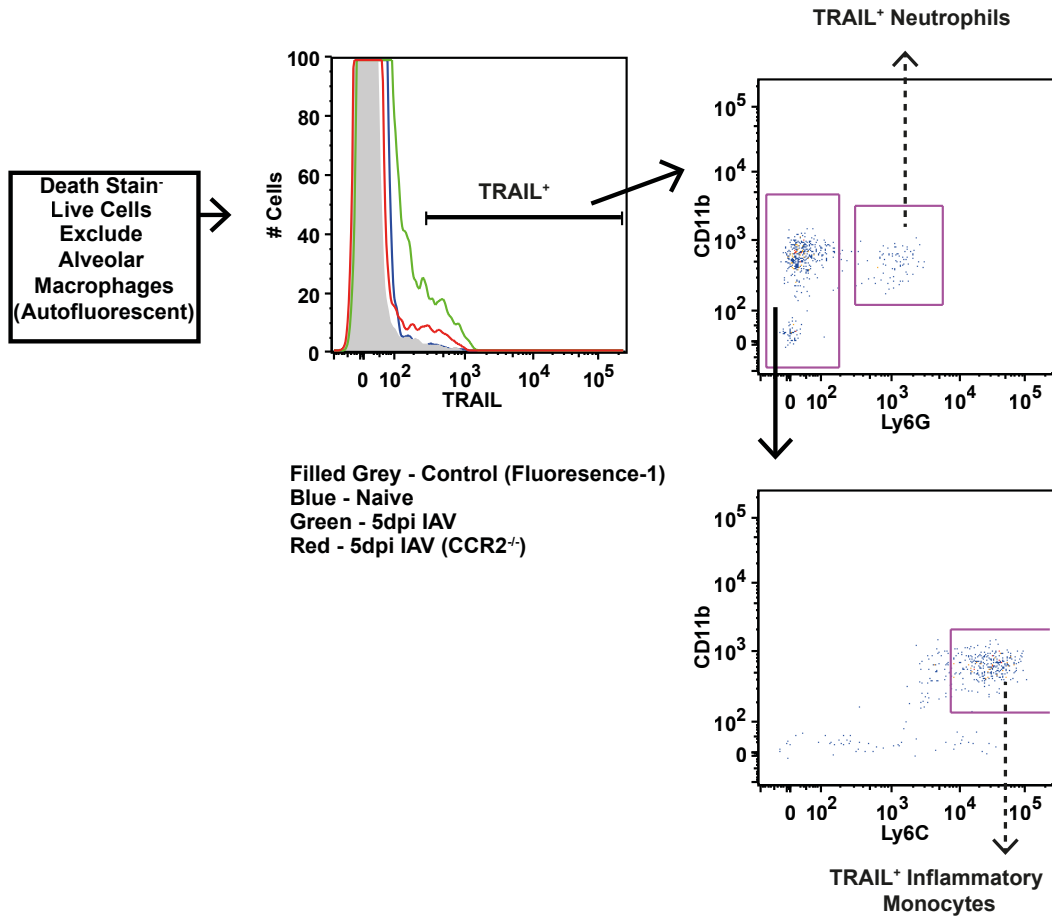
Appendix 5 A: *Identification of TRAIL+ cells.*

Gating strategy used to determine TRAIL+ cells and identify them as neutrophils (Ly6G+CD11b+) or inflammatory monocytes (Ly6G-CD11b+Ly6C+). TRAIL staining histogram shows representative samples - colours as indicated. Alveolar macrophages (here classed as CD11b-SSChigh cells) excluded due to autofluorescence confounding stain. Control is fluorescence-1 - a mix of all samples stained for all markers except TRAIL and used to determine background. Dot plots show representative 5dpi high dose IAV infected lung. Black arrows indicate progression to the next gate. Dashed arrows indicate populations identified.

Appendix 5 B

Filled Grey - Control (Fluorescence-1)
Blue - Naive
Green - 5dpi IAV
Red - 5dpi IAV (CCR2$^{-/-}$)
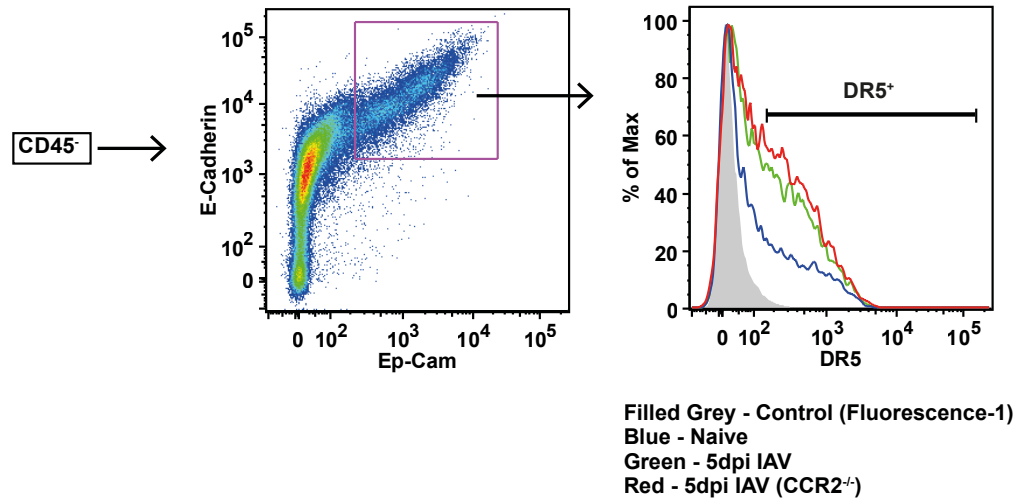
Appendix 5 B: *Determination of DR5 expression on epithelial cells.*
Gating strategy used to determine DR5$^+$ on epithelial cells. CD45$^-$ cells identified as epithelial by expression of Ep-Cam and E-cadherin. DR5 staining histogram shows representative samples - colours as indicated. Control is fluorescence-1 - a mix of all samples stained for all markers except DR5 and used to determine background. Dot plot shows representative 5dpi high dose IAV infected lung. Black arrows indicate progression to the next gate.

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