LUNG IMMUNOPATHOLOGY FOLLOWING INFLUENZA AND PNEUMOCOCCUS INFECTION: MECHANISMS OF DISEASE AND THERAPEUTIC APPROACHES

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ABSTRACT

Influenza is a highly contagious respiratory disease. Yearly epidemics and pandemics account for high morbidity and mortality worldwide. Lung immunopathology is a major factor causing death following influenza. In addition, secondary bacterial superinfections that occur after influenza further complicate the lung immunopathology and contribute to higher morbidity and mortality. The research presented in this thesis addressed important, understudied questions in the complicated field of tissue immunopathogenesis and host defense to influenza and pneumococcal infections. Firstly, in a model of acute respiratory influenza infection, we found that the classically proinflammatory cytokine TNF plays a dual and biphasic role at different times post-infection. While it does have pro-immune roles in the beginning stages, TNF acts as a negative type 1 immune regulator at later points of infection. TNF controls the level of immune activation and has a key role in preventing lung immunopathology and aberrant tissue remodeling. Secondly, to further investigate mechanisms of lung pathology, we elucidated the role of bacterial replication and over activated host immune responses during bacterial superinfection following influenza. In our model of pulmonary *Streptococcus pneumoniae* infection after influenza, we found that dual infected animals experience rapid weight loss and succumb to infection. Bacterial outgrowth, dysregulated cytokine and chemokine expression, and severe lung neutrophilia and immunopathology are linked to the poor clinical outcome. Combined treatment with both an antibiotic azithromycin and corticosteroid dexamethasone best improves clinical outcome, bacterial clearance, cellular and cytokine responses, and immunopathology. Thirdly, in our

continuing interest for improved therapies during pulmonary infections, we tested the transgenic expression of type I IFN as a treatment during *S. pneumoniae* infection. We found that IFN-α controls bacterial outgrowth and improves clinical outcome. Together, our findings provide novel insights into the mechanisms of lung immunopathology and treatment protocols for pulmonary influenza and pneumococcal infections.

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LIST OF ABBREVIATIONS

Ad	adenovirus
AEC	airway epithelial cell
Ag	antigen
AIM2	absent in melanoma 2
AM	alveolar macrophage
APC	antigen presenting cell
AZT	azithromycin
BAL	bronchoalveolar lavage
BALF	BAL fluid
CAP	community acquired pneumonia
CCR2	C-C chemokine receptor type 2
CD200	cluster of differentiation 200
CFU	colony forming unit
CPS	pneumococcal capsular polysaccharide
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DEX	dexamethasone
DR5	death receptor 5
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
GM-CSF	granulocyte-macrophage colony-stimulating factor
HA	haemagglutinin
HGF	hepatocyte growth factor
HIV/AIDS	human immunodeficiency virus infection / acquired immunodeficiency syndrome
IAV	influenza A virus
ICCS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.n.	intranasal
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IPD	invasive pneumococcal disease
IPF	idiopathic pulmonary fibrosis
IRAK-M	IL-1 receptor-associated kinase-M
i.t.	intratracheal
KC	keratinocyte chemoattractant
LCMV	lymphocytic choriomeningitis virus
LTA	lipoteichoic acid
M	matrix protein

MARCO	macrophage receptor with collagenous structure
MCP-1	monocyte chemotactic protein 1
MDR	multidrug resistant
MIP-2	macrophage inflammatory protein 2
MMP	metalloproteinase
MO	macrophage
MPO	myeloperoxidase
MS	multiple sclerosis
NA	neuraminidase
NETs	neutrophil extracellular traps
NK	natural killer
NLRP3	NOD-like receptor family, pyrin domain containing 3
NO	nitric oxide
NOS2	nitric oxide synthase 2
NP	nucleoprotein
PAFR	platelet activating factor receptor
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCV	pneumococcal CPS-protein conjugate vaccine
PF	pulmonary fibrosis
PFU	plaque forming unit
PLY	pneumolysin
PMN	polymorphonuclear cell
PRR	pattern recognition receptor
PspA	pneumococcal surface protein A
RA	rheumatoid arthritis
rINF	recombinant IFN
ROS	reactive oxygen species
SIGN-R1	SIGN-related 1
tipDC	TNF/iNOS-producing DC
TLR	toll-like receptor
TNF	tumor necrosis factor (alpha)
TRAIL	TNF-related apoptosis-inducing ligand
VCAM-1	vascular cell adhesion molecule 1
VLA-1	very late activation antigen 1

DECLARATION OF ACADEMIC ACHIEVEMENT

The three research articles included in the thesis are:

1. Negative regulation of lung inflammation and immunopathology by TNF-α during acute influenza infection (Chapter 2) by Daniela Damjanovic, Maziar Divangahi, Kapilan Kugathasan, Cherrie-Lee Small, Anna Zganiacz, Earl G. Brown, Cory M. Hogaboam, Jack Gauldie and Zhou Xing.

The experiments were designed by DD, MD and ZX. The experiments were executed mostly by DD and KK. Training was provided by CS and technical assistance by AZ. Materials were in part provided by EGB, CMH, and JG. The manuscript was written by DD. This work was performed from late 2007 to 2011.

2. Marked improvement of severe lung immunopathology by influenza-associated pneumococcal superinfection requires the control of both bacterial replication and host immune responses (Chapter 3) by Daniela Damjanovic, Rocky Lai, Mangalakumari Jeyanathan, Cory M. Hogaboam and Zhou Xing.

The experiments were designed by DD and ZX. The experiments were executed by DD, RL and MJ. Some materials were provided by CMH. The manuscript was written by DD. This work was performed from 2010 to 2012.

3. Transgenic expression of interferon alpha protects against acute pulmonary *Streptococcus pneumoniae* infection (Chapter 4) by Daniela Damjanovic, Amandeep Khera, Maria F. Medina, Jane Ennis, Jeffrey D. Turner, Jack Gauldie and Zhou Xing.

The experiments were designed by DD and ZX. The experiments were executed by DD and AK. The adenovirus was developed by JE, JDT, JG, and prepared by MFM. The manuscript was written by DD. This work was performed from 2012 to 2013.

CHAPTER 1: INTRODUCTION

1. Lung homeostasis

As we inhale 10,000 liters of air per day, the lung is exposed to many airborne particles and becomes extremely vulnerable to infection. As an exposed mucosal surface that acts as an interface between the body and the external atmosphere, the lung is constantly in direct contact with inhaled pathogens, toxic gases, and particulate matter. Still, the lung is generally maintained in a quiescent, non-inflamed state. Physical barriers and innate and adaptive immune mechanisms together protect the host from infection and keep the lower respiratory tract a sterile site (1). Such immune responses must be tightly regulated to prevent chronic airway inflammation and lung immunopathology. Otherwise, injury would impair gas exchange, the primary function of the lungs, a vital process that provides oxygen required for metabolic processes (2, 3). In fact, acute respiratory failure is the most common organ failure seen in the intensive care unit (4). In the United Kingdom, more people die from respiratory disease than from coronary heart disease or cancer, and in Canada more than 16% of deaths are attributed to respiratory diseases (5). Also, due to their potentially wide spread, inhaled respiratory pathogens are the most likely bioterrorism weapons (6). Therefore lung infection and disease remains a significant problem worldwide.

1.1 Innate immunity and lung homeostasis

Local immune cells in the bronchial mucosa of the conducting airways (Figure 1A) and in the alveoli of the lung parenchyma (Figure 1B) are involved in the innate immune responses initiated in the lungs (7). Ciliated epithelial cells and mucus trap inhaled particles and perform mucociliary clearance in the lower airways, moving the

entrapped foreign material to the oropharynx where it is either swallowed or expectorated (2, 7). Antimicrobial peptides including lysozyme, lactoferrin, α/β-defensins, cathelicidins, and surfactant proteins SP-A and SP-D that are secreted by airway epithelial cells (AECs), resident alveolar macrophages (AMs) and recruited leukocytes protect against pathogens (2). AECs, AMs, and resident dendritic cells (DCs) act as the first line of defense against inhaled microorganisms in the lung (2). AECs cover the whole mucosal surface in contact with the air and act as a barrier from environmental antigens. They also secrete a range of inflammatory mediators including surfactant proteins, mucins, complement, antimicrobial peptides, nitric oxide (NO), cytokines, chemokines, and growth factors (7-9). AMs are in close proximity or adherent to AECs and comprise more than 90% of all cells in the alveoli, while the rest are DCs and T cells (7, 10). They phagocytose inhaled antigens and actively suppress the induction of adaptive immunity to keep unnecessary inflammation from damaging the lung (7, 10-12). DCs are positioned within and directly beneath the surface epithelium and extend protrusions into the airway lumen, which can sample airway antigens and present them after moving to the draining lymph nodes, initiating the adaptive immune response (7, 10, 13). The rest of the parenchyma is composed of scattered macrophages, DCs, T cells, B cells and mast cells found in the interstitium beneath the alveoli (7). Antigen presenting cells (APCs) recognize conserved microbial components known as pathogen associated microbial patterns (PAMPs) via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), leading to production of proinflammatory cytokines (2). Neutrophils are subsequently recruited to the lungs and phagocytose and degrade foreign pathogens

(2, 8). Inflammation is resolved by the induction of neutrophil apoptosis and clearance by macrophages (2). Subepithelial fibroblasts found in lung connective tissue also contribute to lung homeostasis not only by performing tissue repair and generating extracellular matrix proteins, but also by releasing prostaglandins, IL-10, and TGF-β1 to regulate inflammation (10).

1.2 Post-infection return to homeostasis

Following infection with a virus such as influenza, transient lung damage and loss of epithelial integrity occur due to virus-induced cytotoxicity and apoptosis of AECs, as well as host responses that kill infected cells in order to clear the virus (3, 9, 10, 14). This includes actions by recruited natural killer (NK) cells, cytotoxic T lymphocytes (CTLs), apoptosis-inducing cytokines, and the activation of death receptor 5 (DR5) on the epithelium by TNF-related apoptosis-inducing ligand (TRAIL) on macrophages (10, 15). In addition, other cell types including macrophages, NK cells and fibroblasts can be infected and lysed by influenza, causing further lung injury (10, 14). Damaged epithelial cells are replenished following infection by macrophage-derived hepatocyte growth factor (HGF) release, fibroblasts and matrix metalloproteinases (MMPs), and type II pneumocytes that have stem-cell-like properties (3, 8-10, 16, 17). However, the lung does not return to the same basal state of homeostasis for some time following infection, as indicated most strikingly by the enhanced susceptibility to secondary bacterial superinfection that occurs following influenza, which will be further discussed below (9, 10, 18).

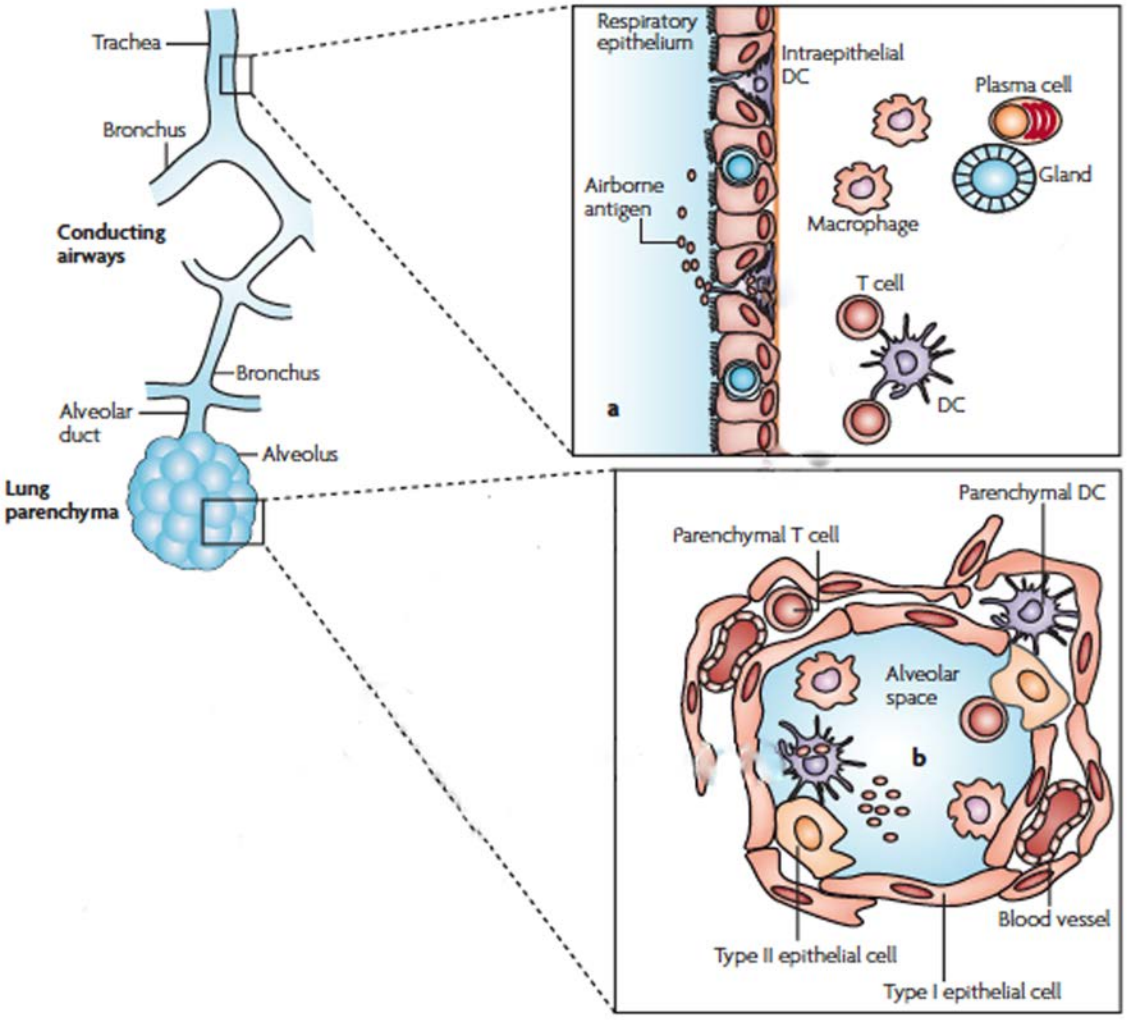

Figure 1. Local immune cells in the two lung compartments. The conducting airways **(A)** and the lung parenchyma **(B)** come into contact with airborne antigens. (Adapted from Holt *et al*. 2008).

2. Influenza

Acute pulmonary influenza infection is a very common and highly contagious respiratory disease. Annual worldwide influenza epidemics account for a significant number of deaths, particularly in high risk populations such as children, the elderly, and people with chronic illness. The influenza viruses are responsible for more than 100,000 hospitalizations and 20,000 deaths each year in the United States alone (19). Influenza viruses of varying pathogenicity not only cause annual epidemics, but also continue to cause unpredictable and perilous worldwide pandemics. These include the 1918 influenza pandemic that killed over 50 million people worldwide, the pandemic influenza strains such as the H5N1 influenza that re-emerged in 2003, and the pandemic influenza H1N1 of 2009 (20). The protective efficacy of the influenza vaccine is suboptimal and the social and economic impact of influenza infections remains high (19).

2.1 The virus

Influenza viruses are negative stranded, enveloped RNA viruses belonging to the family *Orthomyxoviridae* (Figure 2) (21, 22). There are three types of influenza virus (A, B and C) which are distinguished by antigenic differences in two of the internal proteins, nucleoprotein (NP) and matrix protein (M) (21). Influenza A viruses are the most pathogenic and clinically relevant, and are divided into subtype viruses based on antigenic differences in the surface molecules, haemagglutinin (HA) and neuraminidase (NA) (23). At present, there are two subtypes of influenza A (H1N1 and H3N2) as well as influenza B circulating in the community, and these viral strains compose the annual vaccine (21). The viruses preferentially replicate in the epithelial cell layers of the upper

respiratory tract, but monocytes, macrophages, DCs and NK cells can also be infected as they express the virus receptor, a sialic acid containing cell surface glycoprotein (24). Productive influenza A virus infection in epithelial cells leads to death of the host cells either by cytolytic or apoptotic mechanisms (24).

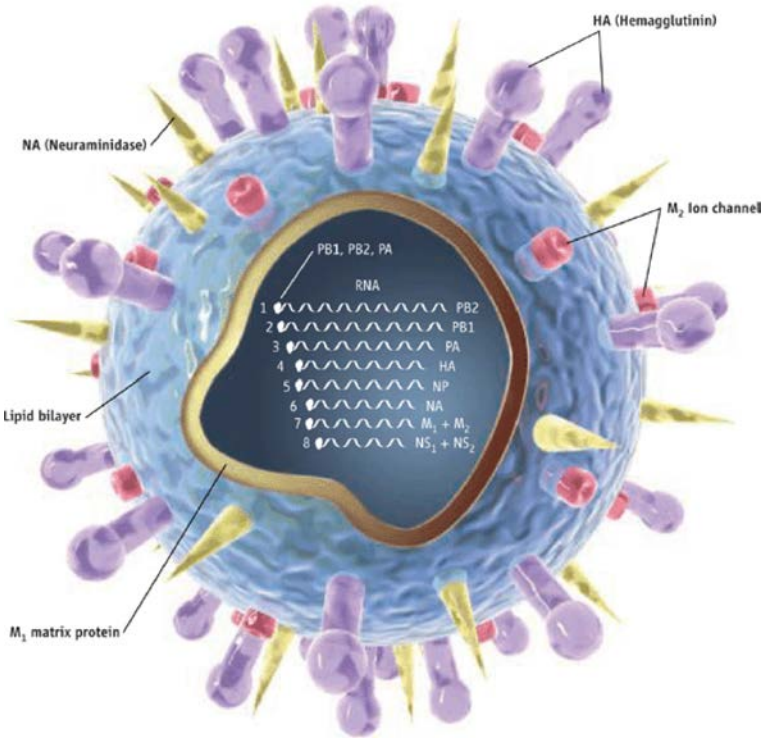

Figure 2. The influenza virus. (Adapted from Kaiser *et al*. 2006)

2.2 The immune response to influenza infection

Both innate and adaptive immune responses are key players in the host defense against influenza infection (Figure 3). Influenza virus infected epithelial cells and leukocytes respond to the infection by producing cytokines and chemokines. Among the chemokines released are IL-8, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-3, MIP-3α and IP-10 (25, 26). Type I interferons IFN-α/β are the key cytokines produced by infected cells, as they have direct antiviral activities and multiple immunomodulatory roles (26). Infected macrophages also efficiently produce TNF, IL-1β, IL-6, IL-18, and IFN-γ (25, 26). These cytokines have multiple roles such as inhibition of different steps of the viral cycle, recruitment of inflammatory cells and activation of their effector functions, and upregulation of MHC expression (26). Chemokines give the signal for the directed migration of neutrophils, monocytes and lymphocytes into the lung (26). Other players in innate immunity against influenza infection include inhibitory factors in the mucus of the respiratory tract, apoptosis-dependent phagocytosis of infected cells by macrophages, and NK cell production of IFN-γ and lysis of infected cells (23).

Infected APCs such as DCs and macrophages present viral antigens and activate antigen-specific CD4 and CD8 T cells (23). Cytolysis of influenza-infected cells is mediated by CTLs via two primary molecular mechanisms: the perforin/granzyme-mediated pathway of cytolysis, and the Fas-FasL pathway of induction of target cell apoptosis (21, 23). Thus, effective viral clearance from the lung is achieved within 7 to 10 days after primary infection (21, 23). Moreover, influenza infection induces a strong CD4 T helper response, which can direct CD8 responses by secreting cytokines including

IFN-γ, IL-2, and TNF and plays an important role in the full activation of macrophages, CD8 T cells and other immune cells (27). In addition, the CD4 T cell response can drive B cell production of antibodies which neutralize influenza viruses (27). CD4 T cells can also become killers of infected cells themselves (23, 27).

The humoral immune system produces antibodies against various influenza antigens, which are critical to immune protection from a secondary influenza virus exposure (21). HA-specific and NA-specific antibodies are the most important for neutralization of the virus and consequently for prevention of illness following a secondary infection (21). The peak antibody response lags behind that of T cells, usually occurring between 10 and 14 days post-infection (23). IgA, IgG, and to some extent IgM are actively secreted locally in the lung, whereas the main circulating anti-influenza antibody found in serum is IgG (21, 23).

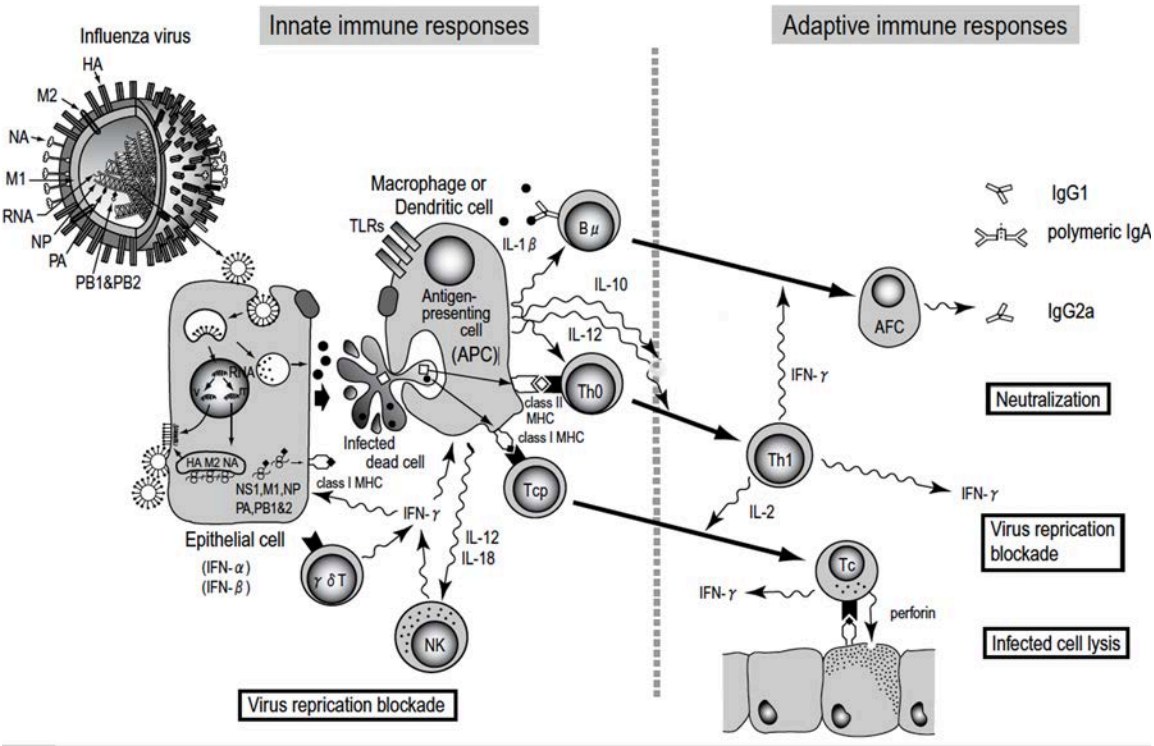

Figure 3. Defense mechanisms induced upon influenza infection. Both innate and adaptive immune responses are critical following influenza infection. (Adapted from Tamura *et al*. 2004)

3. Tumor necrosis factor (TNF)

Tumor necrosis factor has been classically referred to as TNF-α, due to the nomenclature of lymphotoxin as TNF-β. However, lymphotoxin is no longer referred to as TNF-β, and therefore TNF-α will be referred to simply as TNF in the thesis (other than the published manuscript in *Chapter 2*).

Members of the TNF superfamily are critically involved in the maintenance of homeostasis of the immune system, and have many pleiotropic effects. These include the induction of proliferation, survival, differentiation or apoptosis of responding cells (28). Monocytes and macrophages are the largest source of TNF, although T and B cells also produce significant amounts, as do many other cell types (29). TNFR1 (p55) is expressed by most cells in the body, and TNFR2 (p75) is mainly found on immune cells and endothelial cells (28). The two receptors are also functionally different and the details of their effects have not been fully elucidated. Some reports suggest that TNFR1 mediates apoptosis as it contains a death domain and that TNFR2 mediates proliferation, while others suggest that the two receptors work cooperatively (28, 30). Thus, a combination of the signaling pathways from the two receptors determines the diverse biological activities of TNF. It is the balance between the pro-apoptotic and anti-apoptotic signals that eventually determines cell fate in response to TNF (28).

TNF is classically known as an important proinflammatory cytokine that has a central role in instigating the inflammatory reactions of the innate immune system including induction of cytokine production, as well as activation and expression of adhesion molecules (30). However, there are contradicting results regarding the immune

role of TNF in different experimental models. Current knowledge of infections in TNF-deficient mice suggests that these mice appear to be susceptible to infections with mostly intracellular bacteria, parasites, and fungi, but not to viral infections (31). Moreover, novel studies question whether TNF always plays its classical immunostimulatory role and highlight the immunoregulatory effects of TNF.

3.1 Immunostimulatory roles of TNF

TNF contributes to host defense at the early stages of the immune response to influenza infection. TNF was found to enhance influenza virus induced expression of antiviral cytokines in human lung epithelial cells (32). TNF secreted from virus infected AMs induced monocyte chemoattractant CCL2 (MCP-1) release and VCAM-1 expression in infected epithelial cells, aiding in monocyte transepithelial migration into the alveolar space (33). VLA-1 and TNFR2 were found to synergize to protect effector CD8 T cells in the lung from apoptosis by delivery of a pro-survival, anti-apoptotic signal during acute influenza infection (34). TNF released by T cells mediated pathology via TNF mediated apoptosis of AECs, by induction of chemokine release from epithelial cells, and subsequent recruitment of macrophages (35, 36). Therefore TNF plays an immunostimulatory role during the early stages of influenza, and contributes to acute inflammatory pathology that is characteristic of the transient illness associated with pulmonary influenza infection. However, in line with the pleiotropic nature of TNF, immunoregulatory roles have also been identified.

3.2 Immunoregulatory roles of TNF

In support of its immune suppressive role in anti-infection host defense, neutralization of TNF has been found to worsen the severity of a number of autoimmune conditions in humans with multiple sclerosis (MS) and in experimental models of experimental autoimmune encephalomyelitis (EAE) and lupus (28, 37-40). Interestingly, a study found that treatment of RA patients with etanercept (a soluble TNF receptor) led to increased peripheral T cell reactivity both to microbial antigens such as influenza and to self-antigens such as collagen type II (41). Recent evidence has also implicated an anti-fibrotic activity of TNF in models of idiopathic pulmonary fibrosis (IPF), ureteral obstruction and scleroderma (42-44). Furthermore, studies have found a novel role for TNF as an immunoregulatory cytokine during intracellular infections. We have shown that TNF acts as a negative regulator of type 1 immune activation during intracellular bacterial infection. Its absence resulted in uncontrolled T cell proliferation and immune activation, massive lung immunopathology, respiratory failure and 100% mortality (45). In addition, a series of studies with lymphocytic choriomeningitis virus (LCMV) infection showed that the two TNFRs have overlapping roles in downregulating CD4 and CD8 T cell expansion, thus aiding in establishment of immune homeostasis (46, 47). TNF was also found to limit the duration of the effector phase of the CD8 T cell response by regulating apoptosis of effector cells *in vivo*, while not being required for LCMV clearance (48). Some evidence leads to a potential regulatory role of TNF in influenza infection as well, attributed to its role in apoptosis. For example, TNFR2-mediated editing of influenza-specific CD8 T cells was shown to limit the numbers of effectors that

have localized to the site of pathology in the lung (49). Furthermore, it was shown that murine bone marrow B lineage cells were severely depleted *in vivo* during influenza virus infection, and that TNF dependent apoptosis was required for this depletion (50). We have recently found that TNF plays an important immunoregulatory role during influenza immunopathogenesis, and that TNF deficiency leads to dysregulated immune responses, heightened lung immunopathology and development of fibrotic tissue remodeling (Chapter 2) (51). Thus during influenza infection, TNF plays a biphasic role and contributes to immune activation during the early immune response, but down regulates the immune response at later timepoints when the system must return to homeostasis.

4. *Streptococcus pneumoniae*

S. pneumoniae (the pneumococcus) is the most common bacterial respiratory tract pathogen and is the main cause of bacterial pneumonia, as well as a major cause of otitis media, bacterial meningitis and septicaemia (52-54). Pneumococcal septicaemia is a major cause of infant mortality in developing countries, causing 25% of all preventable deaths in children under the age of 5 and resulting in over 1.6 million child deaths per year (53, 55-57). Pneumococcal infection is also the leading cause of community-acquired pneumonia (CAP) and it is estimated that CAP alone accounts for 1 million doctor visits and 60,000 hospitalizations annually in Canada, costing $100 million (58). In addition, *S. pneumoniae* superinfection is the most common complication of influenza, which together form the leading cause of death from infectious diseases in Canada (58).

4.1 The bacterium

S. pneumoniae is an encapsulated, Gram-positive, facultatively anaerobic extracellular bacterium that resides on the mucosal surface of the upper respiratory tract (Figure 4A) (53, 59, 60). The bacterium colonizes the nasopharynx of 20-50% of children and 8-30% of adults, and while colonization is asymptomatic, the bacterium causes disease if it reaches the normally sterile parts of the lung (53, 59). Progress to invasive disease depends on the immune status of the host, on preceding viral infections such as influenza and on the pneumococcal serotype (61). There are over 92 known pneumococcal capsular serotypes that have structurally and antigenically different capsular polysaccharides (53). The capsule is covalently attached to the outer surface of the cell wall peptidoglycan and its thickness determines virulence (Figure 4B) (53, 62). It is strongly anti-phagocytic, as it can i) reduce the amount of deposited complement, ii) prevent either the Fc region of IgG or complement component iC3b from interacting with phagocyte receptors, and iii) reduce bacterial trapping in neutrophil extracellular traps (NETs) (53, 57, 63). On the other hand, protein virulence factors include pneumolysin (PLY, cytotoxic to mammalian cells as it oligomerizes in the membrane of target cells and forms transmembrane pores), pneumococcal surface protein A (PspA, interferes with the fixation of complement component C3), and the neuraminidases (NanA/B/C, aid colonization by revealing receptors for adherence) (53, 61, 63).

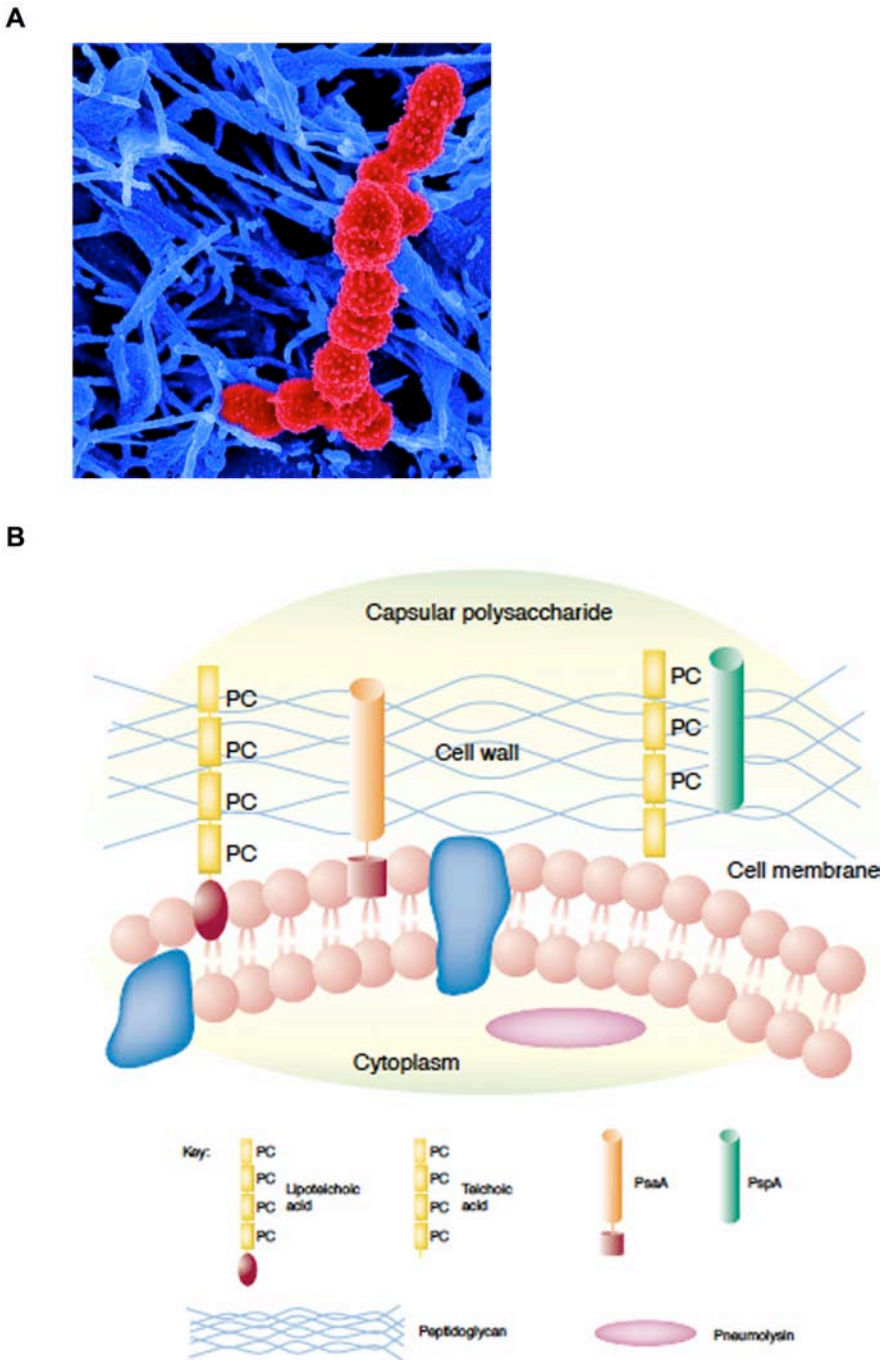

Figure 4. Features of *S. pneumoniae*. (A) Adherence of *S. pneumoniae* to the human epithelial cell line HEp-2 at 3h after infection as seen by high-resolution field emission scanning electron microscopy. (Adapted from Hammerschmidt, *et al*. 2005) **(B)** The structural features of *S. pneumoniae*, a plasma membrane, cell wall, and a polysaccharide capsule. Phosphorylcholine (PC). Pneumococcal surface adhesin A (PsaA). Pneumococcal surface protein A (PspA). (Adapted from Snapper, *et al*. 2001)

4.2 Prevention and treatment

Vaccines against *S. pneumoniae* have been successful at decreasing carriage rates and incidence of pneumococcal disease caused by vaccine serotypes, however they are still suboptimal. First, the pneumococcal capsular polysaccharide (CPS) vaccine provides strictly serotype-specific protection, covering 23 out of the more than 92 serotypes known (53). Also, being T cell independent antigens, polysaccharides are poorly immunogenic in young children which are the highest risk group, therefore conjugation to protein carriers has provided the pneumococcal CPS-protein conjugate vaccines (PCVs) (53, 64). The PCV13 (Prevnar 13®) contains 13 pneumococcal serotypes and provides coverage for over 85% of epidemiologically important serotypes in the world (65). However, protection is again serotype-specific and does not include many of the serotypes that cause disease in the developing world, and infection with non-vaccine pneumococcal strains is still of concern (64). In addition, PCVs are difficult and expensive to manufacture, need refrigeration and require multiple injections (53, 64). There is also evidence that while the carriage of vaccine serotypes has been reduced, non-vaccine serotypes have replaced them so that the serotype distribution has changed (a phenomenon known as serotype replacement), which may reduce the future efficacy of such vaccines (53, 64, 66). Therefore development of better vaccines is crucial, and vaccines against pneumococcal protein virulence factors are currently being investigated (53, 64, 67).

Antibiotic resistance is a major global concern in the treatment of pneumococcal infection and has increased in past three decades (54, 68-70). Almost half of the

pneumococcal isolates across the world from Spain, Eastern Europe, S. Africa, S. America, N. Guinea and S. Korea are penicillin resistant, and vancomycin resistant strains have also been reported (54). The situation is further complicated by resistance to other β-lactams, macrolide and fluoroquinolone antibiotics, and generation of multidrug resistant (MDR) clones (68-70).

4.3 The immune response to *S. pneumoniae* infection

As the pneumococcus enters the nasal cavity it is entrapped by mucus, however the capsule reduces mucus binding allowing the bacteria to reach the AECs (6, 53). Surfactant proteins (SP-A and SP-D) in the fluid lining the alveolar epithelium have been found to bind *S. pneumoniae* causing aggregation and enhancing mucociliary clearance and uptake by neutrophils (54, 63).

The bacteria then access various receptors on the surface of the epithelial cells, such as platelet-activating factor receptor (PAFR) and host cell glycoconjugates (glycoproteins and glycolipids) (53, 71). *S. pneumoniae* produces various enzymes, including NanA (a neuraminidase), BgaA (a β-galactosidase), and StrH (a β-N-acetylglucosaminidase) that cleave the terminal sugars on such glycoconjugates and thus reveal bacterial adhesion receptors (53). Various other bacterial adhesion molecules also bind to host structures including extracellular matrix components to aid in invasion across the epithelium (Figure 4A and Figure 5).

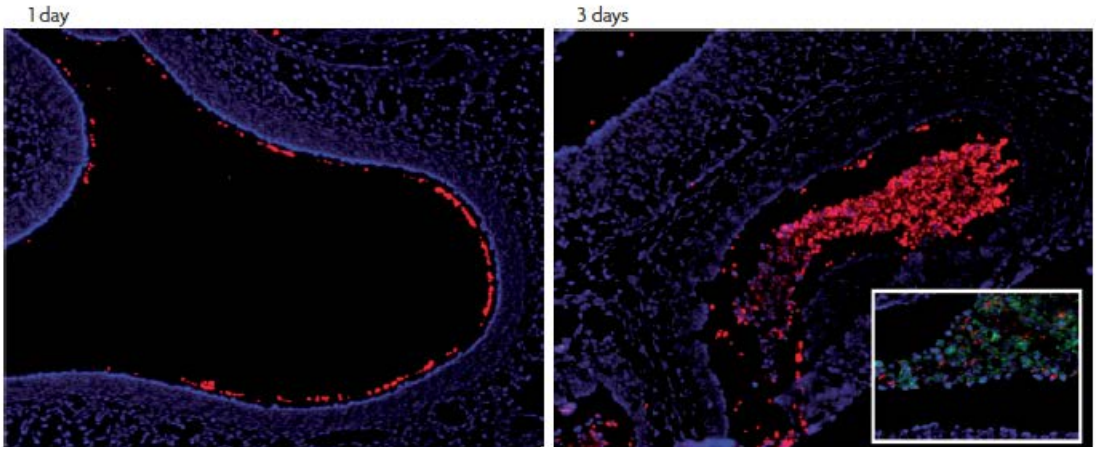

Figure 5. Nasal colonization by *S. pneumoniae*. The infection as it progresses from 1 to 3 days after infection (x40 magnification) in BALB/c mice with serotype 23F. Mouse tissue is stained blue (DAPI), bacteria are red (antisera), and neutrophils are green (anti-Ly6-G). (Adapted from Kadioglu *et al*. 2008).

When it successfully reaches the lower respiratory tract, host defense against *S. pneumoniae* involves complement-mediated phagocytosis, of which the classical pathway is dominant (Figure 6) (6, 54, 63). Natural antibodies and the acute phase proteins (serum amyloid protein and C-reactive protein) are involved in the activation of the classical pathway during pneumococcal infection (57). However, the capsule is a key factor in bacterial resistance as it acts to reduce the amount of complement deposited (53, 63).

Clearance of *S. pneumoniae* involves complement activation and AMs as the first line of defense, followed by neutrophils (Figure 6) (52-54, 72, 73). The cellular responses that lead to *S. pneumoniae* clearance begin with AMs which partially clear some of the bacteria from the airway lumen in the first 4 hours of infection, as NO levels transiently rise in the bronchoalveolar lavage (BAL) (74). In the next 20h as bacteria continue to replicate in the alveoli, neutrophils are recruited to the airway and the lung parenchyma

from the blood, and become the major cell type phagocytosing bacteria (74). Transient tissue injury is visible at 24-48h post-infection, and bacteria spread to the bloodstream and to other tissues (71, 74). By 72h post-infection, monocytes and lymphocytes are recruited to the BAL (74). Macrophages also have an important protective regulatory role that involves clearance of apoptotic neutrophils (72).

In order to clear the bacteria, the immune system must be able to recognize them. In the case of the pneumococcus, the polysaccharide capsule is recognized by the macrophage receptors MARCO (a scavenger receptor) and SIGN-R1 (a C-type lectin) (57, 61, 63). Also, various extra- and intracellular PRRs are involved in the recognition of *S. pneumoniae* by the innate immune system. Components of the pneumococcal cell wall, such as lipoteichoic acid (LTA) and lipoproteins are recognized by TLR2, pneumococcal DNA containing unmethylated CpG motifs within endosomes is recognized by TLR9, PLY activates TLR4, and NOD2 recognizes cell wall peptidoglycan (54, 57, 61, 63, 74). In addition, NLRP3 and the AIM2 inflammasomes are activated by PLY and bacterial DNA, respectively (61, 75). While the specific cytosolic PRR has not been identified, cytosolic recognition of pneumococcal DNA has recently been found to activate type I IFN signaling that may be protective against infection, although the mechanisms are unknown (61, 76-81). A variety of other cytokines including TNF, IL-1, IL-6, IL-8, IL-10, IL-12, IL-17 and IL-18 are released during the innate response to pneumococci (57, 74). TNF and IL-1 have been found to enhance cytokine expression, neutrophil recruitment, and bacterial killing (57, 74). Patients treated with anti-TNF therapies may

have increased risk of invasive pneumococcal disease, and deletion or neutralization of TNF was shown to be detrimental in experimental models (57, 74, 82-85).

Other cell types that are involved in the innate immune response to *S. pneumoniae* are NK cells and CD4 T cells. We have found that NK cell responses and their association with macrophage activation are required for host defense against another extracellular bacterium, *Staphylococcus aureus* (86). Involvement of NK cells in the host defense against *S. pneumoniae* has also recently been demonstrated (87). Also, as it was observed that HIV/AIDS patients who have lower CD4 T cell counts frequently develop severe pneumococcal infections, CD4 T cells have been implicated in host defense (53). CD4 T cells rapidly infiltrate into the lungs at early timepoints of infection, release cytokines and contribute to host resistance (54, 63). Pneumococcus-specific CD4 Th17 cells have also been found to confer protection after immunization with an inactivated and unencapsulated strain, and also in clearance of primary pneumococcal infection in mice (88, 89). Secretion of IL-17A from these cells may recruit neutrophils or macrophages to the site of colonization and help reduce the duration of carriage (64).

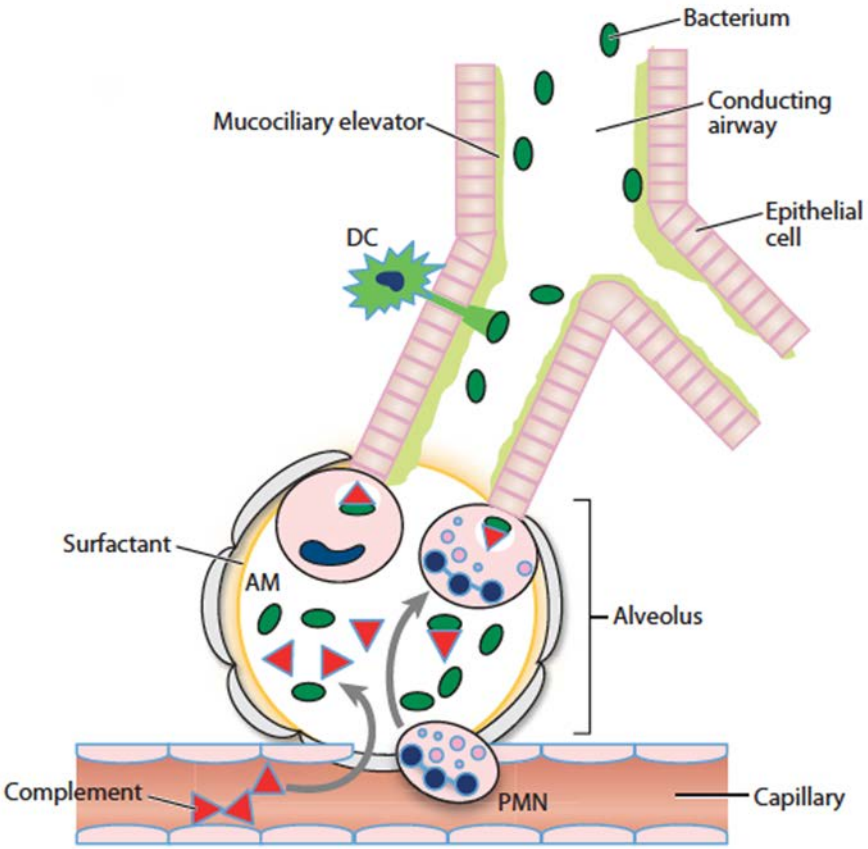

Figure 6. Innate immunity in host defense against the pneumococcus. In the naive lung exposed to bacteria, the innate defenses include the mucociliary elevator, surfactant, complement, AMs, neutrophils, and subepithelial DCs. (Adapted from Lipscomb *et al*. 2010)

5. Bacterial superinfection following influenza

Influenza infection increases the susceptibility to secondary bacterial superinfection (18, 59, 90). This occurs in the first two weeks and in some cases, increased susceptibility to bacteria is seen even several weeks after the resolution of the viral infection (Figure 7) (18, 73). In fact, during the 1918 pandemic where over 50 million people died, most of the deaths were attributed to secondary bacterial pneumonia (59, 91-93).

After the recognition that previous influenza infection causes increased susceptibility to *S. pneumoniae* infection, various mechanisms have been discovered that mediate this susceptibility. The effects of the influenza virus on the host include epithelial damage, changes in airway function, and upregulation and exposure of receptors for bacterial adhesion (59). Also, direct alteration of innate cell function, modification of the cytokine milieu, and long term modifications of the microenvironment after resolution of the viral infection are involved (73). Much literature has focused on neutrophil dysfunction (94-96). Cytokines are also implicated, including IFN-α/β which have been shown to inhibit the release of neutrophil-attracting chemokines KC and MIP2, leading to decreased bacterial clearance (97, 98). IFN-γ was found to cause inhibition of pulmonary antibacterial defense during recovery from influenza infection by suppressing MARCO expression on AMs (99). The suppressive effect of IL-10 on neutrophils has been shown to cause bacterial outgrowth during secondary pneumococcal pneumonia (96, 100, 101). Sustained desensitization of AMs to *S. pneumoniae* TLR ligands was also demonstrated months after resolution of respiratory influenza infection, and led to decreased neutrophil

recruitment (102). We have found that prior influenza infection leads to impaired NK cell responses in the lung and increased susceptibility to subsequent *S. aureus* infection (103). It has also been well characterized that influenza upregulates expression of PAFR in the lung, allowing pneumococcal strains that express its ligand phosphorylcholine on their surface to attach and invade more efficiently (Figure 8) (104-106). Furthermore, the influenza neuraminidase (NA) cleaves sialic acids from the host cell glycoproteins during budding, and in concert with pneumococcal neuraminidases exposes receptors for bacterial adherence (107-109). It has also recently been shown that influenza virus infection decreases tracheal mucociliary velocity and clearance of *S. pneumoniae* (110).

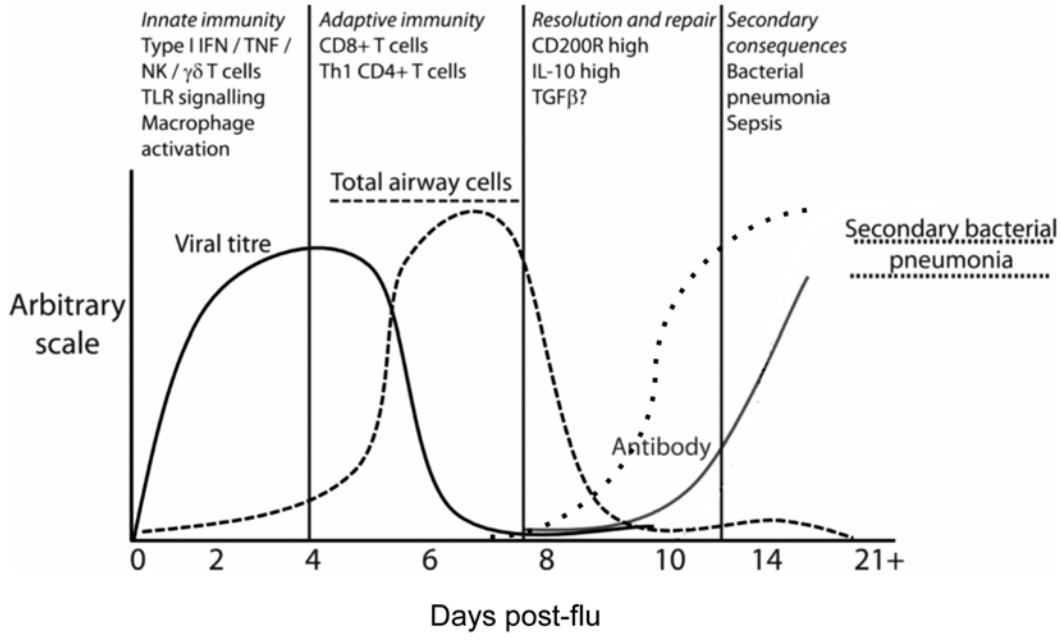

Figure 7. Bacterial superinfection following influenza. The sequence of events during influenza infection and the timing of secondary bacterial infection. (Adapted from Hussell *et al*. 2009)

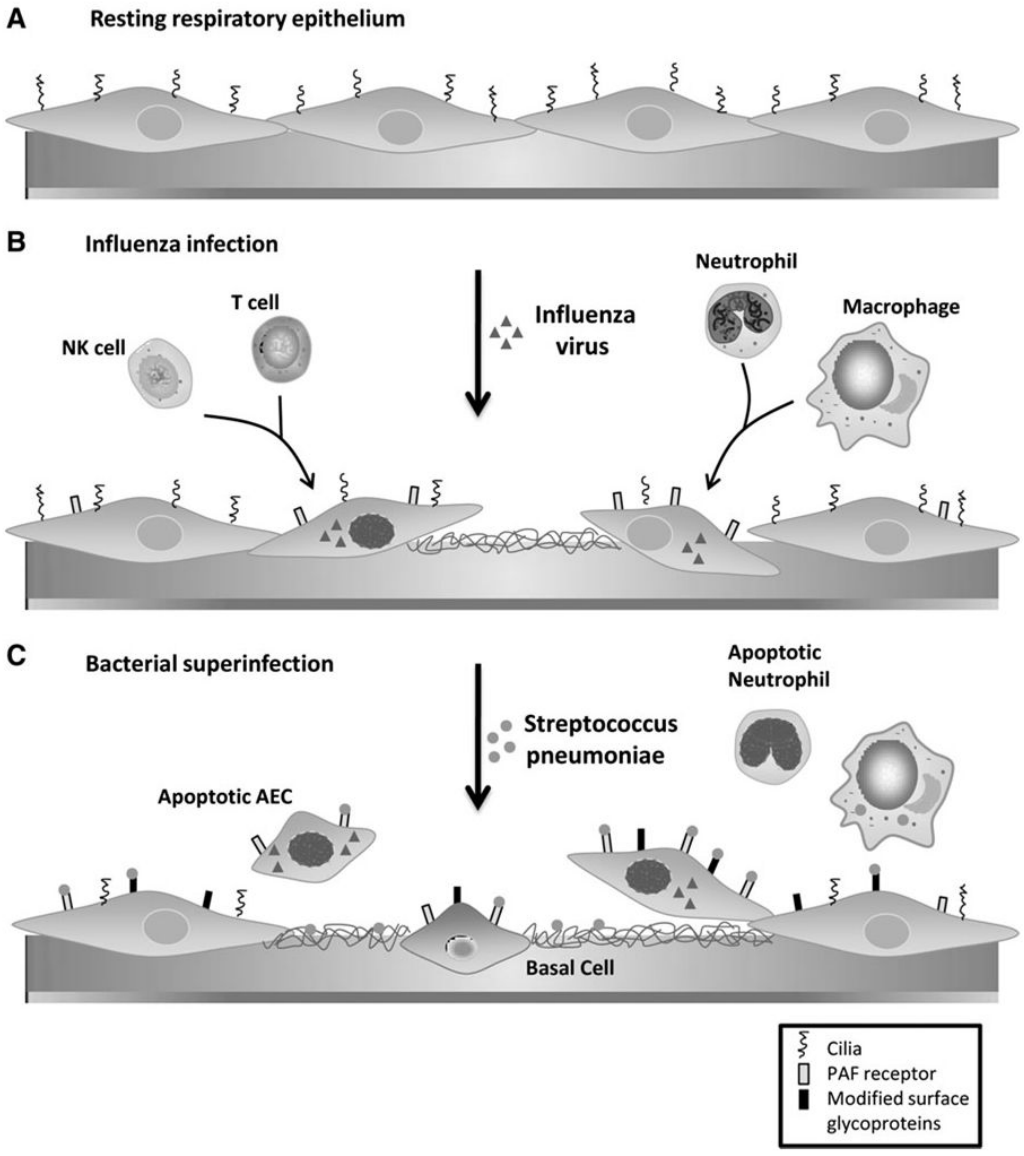

Figure 8. Effects of influenza on airway epithelium promoting bacterial adherence. **(A)** Airway AECs expressing cilia provide a protective barrier. **(B)** Influenza virus infects AECs, immune cells are recruited to the lung, PAFRs are upregulated, and cilia are lost. **(C)** Bacteria bind to PAFRs and other glycoproteins made accessible after influenza. (Adapted from Ballinger *et al*. 2010)

6. Lung immunopathology in influenza infection

A major cause of morbidity and mortality during influenza epidemics and pandemics is pulmonary immunopathology (14). During influenza infection cytotoxic mechanisms destroy infected cells in order to clear the virus and subsequent tissue remodeling restores the lung architecture. However, these host immune responses also have the potential to cause deleterious pulmonary immunopathology. Interestingly, the lung histopathology features seen in autopsies for different influenza strains are very similar, regardless of viral pathogenicity (111, 112). The main features seen are destruction and desquamation of the tracheal and bronchial epithelium, submucosal edema, vascular thrombosis, mixed inflammatory cell infiltration around the airways and throughout the lung parenchyma, alveolar damage and hyaline membrane formation, as well as sustained lung injury and chronic tissue remodeling that lead to squamous metaplasia, pneumocyte hyperplasia and interstitial fibrosis (20, 111-115). The mechanisms involved in immunopathogenesis during influenza infection are complex and involve various parts of the host immune response, including contributions of acute inflammatory responses, T cells, tissue remodeling processes, and bacterial superinfection (Figure 9) (14).

6.1 Acute inflammatory responses

Excessive acute inflammatory responses can lead to death following severe influenza infection, as large numbers of innate cells invade the lungs to fight the infection by killing infected cells and producing inflammatory cytokines and chemokines, culminating in life-threatening pulmonary immunopathology (14).

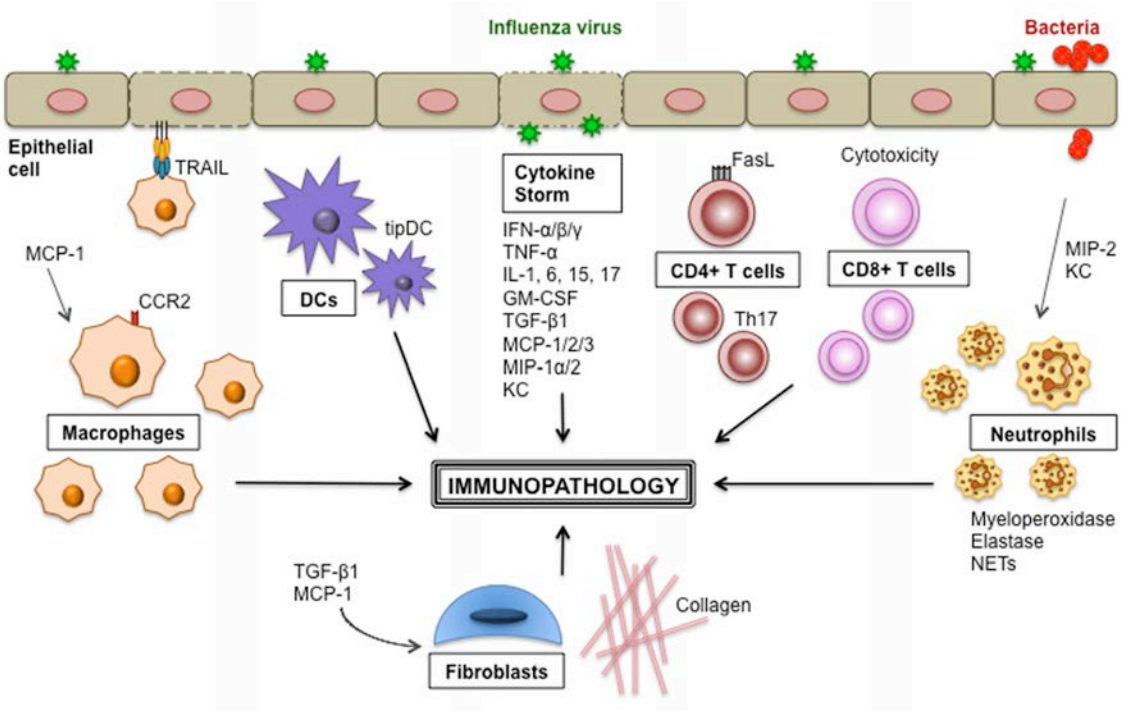

Figure 9. Influenza immunopathology caused by excessive host responses to infection. Excessive acute inflammation, T cell responses, lung remodeling processes, and bacterial superinfection all contribute to severe lung immunopathology following influenza infection. (Adapted from Damjanovic *et al*. 2012).

6.1.1 Macrophages

Macrophages help control viral load as part of the innate immune response, however, they can also become destructive and cause lung immunopathology following influenza infection. CC-chemokine receptor 2 (CCR2) expressing cells recruited by MCP-1 in the first few days following influenza include monocyte-derived DCs and exudate macrophages, and were found to be the predominant cause of immunopathology, morbidity and mortality in a mouse model of influenza infection (116). Thus, CCR2-deficient mice or pharmacologically blocked CCR2 resulted in significantly reduced

monocyte-derived cell numbers, markers of lung injury, weight loss, and mortality with no change in viral clearance (116, 117). These macrophages contributed to AEC apoptosis by releasing TRAIL, causing increased lung leakage and mortality (15). Among the mechanisms that regulate macrophage responses following infection is the negative regulator CD200 that is expressed on AECs and binds to CD200R expressed on macrophages (118). CD200 deficiency led to increased macrophage numbers, delayed resolution of lung inflammation and death, while CD200R agonists alleviated influenza-induced illness and prevented the excessive lung inflammation (118). Overall, it appears that macrophages are not absolutely required and play a redundant role in influenza infection by controlling viral replication until the adaptive immune response is mounted. Thus under normal circumstances a certain level of the macrophage response is helpful, however, if excessive influx of macrophages into the lung occurs, they become significant contributors to immunopathology (14).

6.1.2 Neutrophils and DCs

Neutrophils are rapidly recruited into the airway following influenza infection, and help control viral replication and clear dying cells (119). Neutrophil mechanisms that have been found to contribute to immunopathology include myeloperoxidase (MPO) activity and release of NETs, which were found to be entangled with alveoli and observed in areas of tissue injury (120, 121). DCs are important in host defense against influenza by inducing adaptive immune responses. Not much is known about their contribution to lung immunopathology, however the TNF/iNOS (inducible nitric oxide synthase)-producing DCs (tipDCs) are excessively recruited to the lungs during influenza infection

and are associated with weight loss and mortality (122).

6.1.3 Toll-like receptors

TLR activation is an important step in recognition of influenza virus by the innate immune system, however overstimulation of TLR receptors may lead to excessive acute inflammation (14). Deficiency in IL-1 receptor-associated kinase-M (IRAK-M), an inhibitor of MyD88-dependent TLR signaling, led to heightened lung immunopathology and mortality following influenza infection, correlated with excessive neutrophils, inflammatory cytokines and chemokines, and activated T cells (123). Similarly, dampening responses mediated by the TLRs 3, 7, and 9 has individually been shown to reduce lung immunopathology and improve survival during influenza (124, 125). Therefore, TLR signaling must be tightly controlled to prevent immunopathology during influenza.

6.1.4 Cytokines

Transient cytokine release after infection is an important step in the initiation of the immune response, however it must be properly regulated as both insufficient and excessive levels of certain cytokines can be detrimental (14). The 'cytokine storm' that can occur following influenza is a major cause of lethal immunopathology (14). Implicated cytokines and chemokines include IFN-γ, TNF, IL-1α, IL-6, IL-17, MIP-1α, MCP-1, KC, and TGF-β1 (51, 126, 127). Recent studies with granulocyte-macrophage colony-stimulating factor (GM-CSF) have illustrated the importance of having proper levels of cytokines during the immune response to influenza. GM-CSF was found to be required for reducing lung immunopathology and improving survival, however when

either deficient or overexpressed, the mice succumbed to infection associated with pneumonia and interstitial lung disease (128, 129).

6.2 T cells

Both CD8 and CD4 T cells are crucial during the adaptive immune response to influenza infection, in order for viral clearance to be achieved by 10 days post primary infection, and for proper antibody responses to be raised for defense against secondary infection (21, 23).

6.2.1 CD8 T cells

CD8 T cells and their cytolytic activity against influenza infected cells are critical for viral clearance, however improper CD8 T cell contraction, excessive cytolytic activity and proinflammatory cytokine production can cause lung immunopathology (14). It was found that deficiency in the inhibitory receptor NKG2A on CD8 T cells or its ligand Qa-1b on APCs led to greatly increased lung immunopathology associated with CTL activity (130). Similarly, mice lacking the negative regulator CD200 developed more severe disease associated with increased immunopathology, associated with CD8 T cell numbers (131). Therefore, CD8 T cells are needed as they are important to clear the virus, but proper regulation is required to prevent immunopathology during influenza.

6.2.2 CD4 T cells

CD4 T cells provide the necessary cytokine and co-stimulatory signals to activate B cell and antibody responses, and can directly target infected cells (14). However, immunopathogenic Th1 CD4 T cells have recently been found to cause lung damage in influenza models (132-134). We found that influenza-specific, Fas-L-expressing CD4 T

cells in the lung caused rapid and severe lung damage and respiratory failure, which were under control of the immunoadaptor DAP12 (134). Several reports have also shown that Th17 responses may not be necessary for influenza viral clearance, but rather contribute directly to tissue immunopathology (126, 135).

6.3 Tissue remodeling processes

Tissue remodeling is necessary for the lung to return to homeostasis after infection, however pulmonary fibrosis (PF) following influenza has been observed in humans (14). Animal models are scarce, however two studies have shown that following non-lethal infection with either H5N1 or H1N1 in mice, fibrosis did develop at later times post-infection (14-30 days) when the virus was cleared (51, 136). We found that the lung fibrosis and severe immunopathology in H1N1 infected TNF deficient mice was associated with excessive expression of fibrocyte-attracting MCP-1 and fibrogenic cytokine TGF-β1 (Chapter 2) (51). These results have implications in long-term clinical management of survivors of H5N1 infection or for patients on TNF inhibiting therapy infected with influenza (14).

6.4 Bacterial superinfection

Bacterial superinfections cause exacerbated lung immunopathology and are major contributors to morbidity and mortality of influenza patients. Autopsy lung samples show severe pathological changes that are mostly characterized by a massive infiltration of neutrophils (14). The precise mechanisms for heightened immunopathology remain poorly understood, but seem to be closely correlated with uncontrolled bacterial replication and the resulting overwhelming acute inflammatory responses in the lung (14).

In a mouse model of influenza and *S. aureus* infection, we found that the worsened lung immunopathology was associated with persisting tissue neutrophilia, neutrophil apoptosis and excessive mononuclear cell infiltration, and was associated with bacterial outgrowth due to suppressed macrophage and NK cell responses (137). In addition, it was recently found that use of β-lactam antibiotics worsened patient outcome and in mouse models caused worsened immunopathology, as the products of lysed bacteria increased inflammation and neutrophil influx into the lung (138-140). We recently also found that the use of a bacteriostatic macrolide antibiotic together with a corticosteroid provided the best clinical outcome and improvement of lung immunopathology in an influenza and *S. pneumoniae* mouse model. Uncontrolled bacterial outgrowth, dysregulated cytokine and chemokine responses, and excessive neutrophil infiltration in the lungs were found to be the main contributors to deleterious lung immunopathology and death (Chapter 3).

7. Thesis hypotheses and objectives

The scope of my PhD thesis was to study host immune responses during viral and/or bacterial infection, which were introduced in *Chapter 1*. In particular, we focused on investigating the consequences on lung tissue immunopathology of dysregulated host immune responses and on discovering possible treatments for such pathology. First, several novel studies of viral and bacterial infections suggested that the classically proinflammatory cytokine TNF performed an important immunoregulatory role in immune activation, depending on the antigen or pathogen studied. We hypothesized that

TNF is not required for protection from respiratory influenza infection, but is rather an indispensable immune suppressive cytokine required for controlling the level of immune activation and tissue immunopathology. As shown in *Chapter 2*, we developed a model of acute influenza infection established in wild type and TNF-deficient mice and evaluated viral clearance, inflammatory responses, and lung immunopathology. Next, we continued our work in the area of lung immunopathogenesis by using a dual infection model. Secondary bacterial superinfections occur following influenza and further complicate the lung immunopathology. We hypothesized that the mechanisms of severe immunopathology in dual infections are multifactorial, involving both the uncontrolled bacterial outgrowth and exacerbated host immune responses, and that effective intervention strategies need to control both factors. As shown in *Chapter 3*, we established a murine model of acute respiratory influenza infection and *S. pneumoniae* superinfection. We investigated the mechanisms of severe lung immunopathology and intervention strategies for effective improvement of clinical outcomes and pathology using antibiotic and corticosteroid treatment. Finally, to continue our interest in therapeutic interventions, we focused on primary pneumococcal infection. It has recently been shown that classically anti-viral type I IFNs are induced by *S. pneumoniae*, and we hypothesized that such responses may be protective during pneumococcal infection. As seen in *Chapter 4*, we tested the protective efficacy of transgenic expression of IFN-α in a murine model of primary *S. pneumoniae* infection. The major findings and implications of these studies are summarized in the discussion in *Chapter 5*.

CHAPTER 2: Negative regulation of lung inflammation and immunopathology by TNF-α during acute influenza infection

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Negative regulation of lung inflammation and immunopathology by TNF-α during acute influenza infection

In this publication, we used a murine model of acute respiratory influenza infection to investigate the immunoregulatory role of TNF. For the first time, our study demonstrated that TNF is not required for effective influenza viral clearance in the lung, but is critically required for controlling the extent of inflammatory immune responses and lung immunopathology. Thus we discovered that the classically proinflammatory cytokine TNF in fact plays a dual and biphasic role at different times post-infection. While it does have pro-immune roles in the beginning stages of infection, TNF acts as a negative immune regulator at later times following infection.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

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Immunopathology and Infectious Diseases

Negative Regulation of Lung Inflammation and Immunopathology by TNF-α during Acute Influenza Infection

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Lung immunopathology is the main cause of influenza-mediated morbidity and death, and much of its molecular mechanisms remain unclear. Whereas tumor necrosis factor-α (TNF-α) is traditionally considered a proinflammatory cytokine, its role in influenza immunopathology is unresolved. We have investigated this issue by using a model of acute H1N1 influenza infection established in wild-type and TNF-α–deficient mice and evaluated lung viral clearance, inflammatory responses, and immunopathology. Whereas TNF-α was up-regulated in the lung after influenza infection, it was not required for normal influenza viral clearance. However, TNF-α deficiency led not only to a greater extent of illness but also to heightened lung immunopathology and tissue remodeling. The severe lung immunopathology was associated with increased inflammatory cell infiltration, anti-influenza adaptive immune responses, and expression of cytokines such as monocyte chemoattractant protein-1 (MCP-1) and fibrotic growth factor, TGF-β1. Thus, *in vivo* neutralization of MCP-1 markedly attenuated lung immunopathology and blunted TGF-β1 production following influenza infection in these hosts. On the other hand, *in vivo* transgenic expression of MCP-1 worsened lung immunopathology following influenza infection in wild-type hosts. Thus, TNF-α is dispensable for influenza clearance; however, different from the traditional belief, this cytokine is critically required for negatively regulating the extent of lung immunopathology during acute influenza infection. ***(Am J Pathol 2011, 179:2963–2976; DOI: 10.1016/j.ajpath.2011.09.003)***

Pulmonary influenza is a common and highly contagious respiratory infectious disease. Annual influenza epidemics account for significant morbidity and mortality worldwide. The 1918 influenza pandemic killed 50 million people worldwide, and emerging strains such as the novel swine-origin influenza A (H1N1) virus pose a continuing threat to the world.[1,2] It is now well established that lung immunopathology is one of the main causes of influenza-related morbidity and mortality.[3–11]

Influenza viruses are negative-stranded, enveloped RNA viruses belonging to the family *Orthomyxoviridae* that preferentially infect and replicate in bronchial epithelial cells.[7] Both innate and adaptive immune responses are key players in the host defense against influenza.[8,9,12–14] Part of such host immune responses involves the production of antiviral and inflammatory cytokines and chemokines from infected airway epithelial cells and leukocytes, including interferon (IFN)-α/β, TNF-α, IFN-γ, keratinocyte-derived chemokine (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1)α/β, MIP-3α, interferon-γ inducible protein 10 (IP-10), and regulated on activation normal T-cell expressed and secreted (RANTES).[9,15–17] Cytotoxic CD8 T cells play an important role in influenza viral clearance by lysing virus-infected cells, and effective viral clearance from the lung is usually achieved within 7 to 10 days after primary infection.[8,12,13] A strong CD4 T-helper response is believed to contribute to the generation of robust hu-

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moral responses, important for neutralization of the virus.[8,12,13] Although these host immune responses are critical to influenza viral clearance, they often cause undesired lung immunopathology, severe cases of which may also develop irreversible tissue injury and fibrotic remodeling.[7–10,18–20] Much of the immunoregulatory mechanisms of influenza immunopathology and tissue injury still remain to be understood.

Members of the TNF superfamily are critically involved in the maintenance of homeostasis of the immune system and have many pleiotropic effects such as proliferation, survival, differentiation, or apoptosis of responding cells.[21,22] TNF-α is traditionally considered as a proinflammatory and proimmune cytokine.[21,22] Indeed, there is evidence to suggest its proimmune role at several early points of host defense against influenza infection.[23–27] In this regard, the limited data also suggest that production of TNF-α by influenza-specific CD8 T cells may contribute to lung immunopathology.[28,29] The immune-activating role of TNF-α was also shown in the models of other viral infections.[30,31] However, recent emerging evidence has suggested an immune regulatory nature of TNF-α. It has been shown that TNF-α is required for controlling the level of Th1 cell activation and immunopathology following pulmonary mycobacterial infection.[32] Furthermore, TNF-α was found to negatively regulate CD4 and CD8 T-cell responses to lymphocytic choriomeningitis virus (LCMV) infection.[33–35] In support of its immune regulatory role in anti-infection host defense, neutralization of TNF-α has also been found to worsen the severity of a number of autoimmune conditions in humans with multiple sclerosis and in models of experimental autoimmune encephalomyelitis and lupus.[21,36–39] Of interest, treatment of rheumatoid arthritis patients with etanercept, a soluble TNF-α receptor, led to increased peripheral T-cell reactivity both to microbial antigens such as influenza and to tissue antigens such as collagen type II.[40] Recent evidence has also implicated an antifibrotic activity of TNF-α in models of idiopathic pulmonary fibrosis, ureteral obstruction, and scleroderma.[41–43] However, whether TNF-α plays an immunoregulatory role in host defense and immunopathology during influenza infection still remains poorly understood.

In our current study, we have used a murine model of acute respiratory influenza A infection to investigate the immunoregulatory role of TNF-α in viral clearance, host immune responses, and lung immunopathology. For the first time, our study demonstrates that TNF-α is not required for effective influenza viral clearance in the lung, but it is critically required for controlling the extent of lung immunopathology. Such heightened immunopathology in the animals lacking TNF-α is linked to markedly increased inflammatory cellular infiltration and cytokine responses. Thus, selective inhibition of up-regulated chemokine MCP-1 in these animals dampens lung immunopathology. On the other hand, overexpression of MCP-1 in the lung of infected wild-type animals worsens immunopathology. These findings suggest that different from the conventional belief, TNF-α is a critical negative regulator of inflammatory responses and immunopathology in the lung during acute influenza infection.

Materials and Methods

Mice

Male 7- to 10-week-old C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). TNF-α knockout (*TNF*$^{-/-}$) breeding pairs on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred in the barrier at the McMaster University Central Animal Facility (Hamilton, ON, Canada). Mice were housed in specific pathogen-free–level facilities. For all experiments, mice were euthanized by exsanguination of the abdominal artery under anesthesia. All experiments were conducted in accordance with the animal research ethics board of McMaster University.

Pulmonary Influenza Infection

The virus strain used in the studies was a mouse-adapted strain of influenza A/FM/1/47 (H1N1)[44], prepared and used as previously described.[45] Mice anesthetized by inhalation of isoflurane were infected with 2×10^4 plaque forming units of influenza virus in 20 μL of PBS, intranasally. Body weight was measured at various times after infection.

Viral Titer Determination

At 3, 7, and 14 days after infection, lungs were homogenized in PBS and centrifuged for 10 minutes at 800 $\times$ *g* at 4°C. Serial dilutions of the supernatants were applied to a confluent monolayer of Madin-Darby canine kidney (MDCK) cells, as previously described.[46] Briefly, an enriched agarose medium (1% agarose, 2$\times$ F-11 minimal essential medium, 2% L-glutamine, 2% penicillin-streptomycin, and 5 mg/mL trypsin) was added to the infected cells after 30 minutes at 37°C, and then incubated at 37°C for 2 days. The cells were fixed with Carnoy's fixative (25% acetic acid and 75% methanol), and plaques counted.

Pulmonary Histopathological and Morphometric Analysis

Lungs were inflated with 10% formalin and then kept in 5 mL of 10% formalin for at least 72 hours. Tissue sections were stained with hematoxylin and eosin (H&E) for conventional histopathological examination or with Masson's trichrome for collagen deposition. Immunohistochemistry for the myofibroblast marker α-smooth muscle actin (α-SMA) was performed on deparaffinized lung tissue sections using the ARK (Animal Research Kit) Peroxidase kit, according to the manufacturer's protocol (Dako, Glostrup, Denmark).[47] Blinded lung histopathology scoring was performed by using a semiquantitative method ranging from 0 to 5, with 5 being the most extensive pathology. Morphometric analysis was performed by blinded observers to quantify the extent of immunopathological and fibrotic responses with the Northern Eclipse Image Analysis Software (Empix Imaging, Mississauga, ON, Canada).

Bronchoalveolar Lavage and Differential Cell Counting

Airway luminal cells were collected by bronchoalveolar lavage (BAL) of the lungs by using a standard procedure previously described.[32,48] Lungs were lavaged twice with 250 μL and 200 μL of PBS and then centrifuged. BAL fluids were stored at −20°C until antibody and cytokine measurement. Cell pellets were combined with cells from lavages of 900 μL of PBS in total, and resuspended in 800 μL of complete RPMI medium (RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine). Differential cell counts were determined using cytocentrifuged specimens prepared from 10^5 cells stained with a modified Wright-Giemsa stain solution (Polysciences, Warrington, PA). The cells were classified according to standard morphological criteria as alveolar macrophages, polymorphonuclear cells, and lymphocytes.

Lung Cell Isolation

Mononuclear cells were isolated from lung tissue, as previously described.[45] Briefly, the lungs were cut into small pieces and incubated in 10 mL of 150 U/mL collagenase type I (Sigma-Aldrich, St. Louis, MO) for 1 hour at 37°C, with agitation. The lung pieces were then crushed through 40-μm basket filters, red blood cells lysed with ACK lysis buffer [0.15 mol/L NH_4Cl, 1 mol/L $KHCO_3$, 0.1 mmol/L Na_2EDTA (pH 7.4)], and the remaining lung cells resuspended in complete RPMI.

Flow Cytometric Analysis

Fluorescence-activated cell sorting and intracellular cytokine staining were performed on BAL and lung cells. For intracellular cytokine staining, BD GolgiPlug (protein transport inhibitor containing Brefeldin A) (BD Biosciences, San Jose, CA) was added to *ex vivo* cultures stimulated with the nucleoprotein (NP)$^{366-374}$ or NS2$^{114-121}$ influenza peptides for 5 to 6 hours. Cells were subsequently blocked for 15 minutes with the CD16/CD32 Fc block antibody, and then stained with the extracellular antibodies Pacific Blue anti-CD3, APC-Alexa Fluor 750 anti-CD4, and PECy7 anti-CD8 (BD Biosciences). Cells were then fixed and permeabilized according to the manufacturer's protocol using the BD Cytofix/Cytoperm solutions (BD Biosciences), and subsequently stained with the intracellular antibody APC anti–IFN-γ (BD Biosciences). Alternatively, unstimulated cells were first stained with PE-DbNP366 tetramers (MHC I; specific to the influenza NP^{366} epitope) (Baylor College of Medicine, Houston, TX) and then with the extracellular antibodies mentioned above. For antigen-presenting cell staining, the extracellular antibodies APC anti-CD11c and PECy7 anti-CD11b (BD Biosciences) were used. Stained cells were run on the LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Influenza-Specific Antibody ELISA

Levels of anti-influenza antibodies in the BAL fluid and blood serum were measured by ELISA using a modified protocol.[49] Ninety-six-well ELISA plates (Nalge Nunc International, Rochester, NY) were coated with 2.5 μg per well of influenza-infected MDCK cell lysate and incubated at 4°C overnight. Wells were then blocked with Blocking buffer (1% bovine serum albumin in PBS) for 1 hour at 37°C. Samples diluted in dilution buffer (0.3% bovine serum albumin in PBS) were added to the wells and incubated for 1 hour at 37°C. Goat anti-mouse IgG1-biotin or IgG2a-biotin (Southern Biotechnology Associates, Birmingham, AL) or IgA-biotin conjugates (Sigma-Aldrich) were added and incubated for 1 hour at 37°C, followed by streptavidin conjugated alkaline phosphatase (R&D Systems, Minneapolis, MN) for 1 hour at 37°C. As a substrate, a p-nitrophenyl phosphate tablet (Sigma-Aldrich) was dissolved in 5 mL of 10% diethanolamine [50 g diethanolamine, 0.1 g NaN_3, 0.05 g $MgCl_2.6H_2O$ in 500 mL distilled water (pH 9.8)], 50 μL were added to each well, and incubated at room temperature for 15 to 30 minutes in the dark. The reaction was then stopped with 25 μL of 2N NaOH per well, and the colorimetric change was evaluated with a microplate reader at 405 nm. The titer was calculated as: antibody titer = (sample dilution factor × OD_{405} reading)/0.05, where OD_{405} is the optical density at 405 nm.

Cytokine and Chemokine Quantification

Levels of cytokines and chemokines in BAL fluid and lung homogenates were quantified using DuoSet ELISA Development Kits (R&D Systems) or using Luminex multianalyte technology (Luminex Molecular Diagnostics, Toronto, ON, Canada), according to the manufacturer's protocols.

In Vivo *Cytokine/Chemokine Neutralization and Gene Transfer*

For MCP-1 neutralization experiments, rabbit anti-murine MCP-1 serum or normal rabbit serum as control (200 μL/injection) was injected intraperitoneally into influenza-infected *TNF*$^{-/-}$ mice at 2, 5, and 9 days after infection. For MCP-1 overexpression experiments, a replication-deficient adenoviral gene transfer vector expressing murine MCP-1 (AdMCP-1) or an empty adenoviral vector (AdDl) as a control were used.[50] Wild-type (WT) mice were infected intranasally with 2×10^8 plaque forming units of AdMCP-1 or AdDl, and were infected with influenza the next day. For TNF-α neutralization experiments, rabbit anti-murine TNF-α serum or normal rabbit serum as control (200 μL/injection) was injected intraperitoneally into influenza-infected WT mice on days −1, 2, 5, 8, and 12 after infection.

Statistical Analysis

To determine whether the differences among infection groups were significant, Student's *t*-test was used for two-sample comparisons. For comparison between more than two groups, one-way analysis of variance and Tukey's

post hoc test were performed with Prism (GraphPad Software, Inc., La Jolla, CA). A P value of <0.05 was regarded as statistically significant.

Results

TNF-α Is Increased in the Lung but Is Not Required for Viral Clearance during Primary Influenza Infection

Following respiratory infection with a nonlethal dose of influenza A virus, TNF-α was up-regulated between days 3 and 14 in the lungs of wild-type C57BL/6 mice (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). To determine the role of TNF-α in influenza viral clearance, WT and TNF-α–deficient ($TNF^{-/-}$) mice were infected with influenza virus, and body weight changes were monitored as a measure of sickness and disease recovery. For WT mice, body weight was stable during the period of acute infection (1 to 7 days after infection) and then began to steadily increase from day 7 onward (Figure 1A). On the other hand, $TNF^{-/-}$ mice lost weight between days 5 and 10 (Figure 1A). After day 10, although $TNF^{-/-}$ mice began to regain weight, their body weight status remained well below that in WT controls until day 30 after infection (Figure 1A). These data suggest that the infected animals lacking TNF-α suffered a certain degree of illness from an otherwise nonlethal, small dose of influenza infection.

To examine whether the greater illness seen in infected $TNF^{-/-}$ mice was due to dysregulated influenza viral clearance, viral titers in the lungs were examined by a plaque formation assay. We detected a high, but comparable, level of influenza viral titers in the lung of WT and $TNF^{-/-}$ mice at 3 days after infection (Figure 1B). By day 7, the viral titers drastically declined in the lungs of both WT and $TNF^{-/-}$ mice, and by day 14, the infection was completely cleared (Figure 1B). These results suggest that $TNF^{-/-}$ mice were able to clear influenza virus from the lungs equally well as WT controls and that the illness seen with these mice was not due to delayed or uncontrolled influenza viral infection in the lung.

Sustained Lung Immunopathology in TNF-α–Deficient Hosts after Influenza Infection

To understand the cause of illness in $TNF^{-/-}$ hosts, we assessed histopathology in the lung. At 3 days after infection, when viruses could be easily detected in the lungs (Figure 1B), peribronchial inflammation characterized by a mononuclear cell infiltrate in the interstitial space surrounding the airways and blood vessels was observed in both strains of mice, whereas a greater extent of inflammatory infiltration in the bronchial epithelium was seen in $TNF^{-/-}$ lungs (data not shown). By 7 days after infection, when few viruses could be detected in the lungs of WT and $TNF^{-/-}$ mice (Figure 1B), WT mouse lungs had only mild remaining inflammatory infiltration in the peribronchial region, but in contrast, the lungs of the $TNF^{-/-}$ counterparts displayed severe mononuclear infiltration in the peribronchial and perivascular regions and bronchial hyperplasia (Figure 2, A and B; Table 1). After the infection was completely cleared in the lung from 14 days on (Figure 1B), the lungs of WT mice appeared largely clear and returned to normal at days 14, 21, and 30 (Figure 2, C, E, and G; Table 1). In contrast, $TNF^{-/-}$ lungs showed worsened inflammatory infiltration and extensive bronchial epithelium injury and sloughing (Figure 2D; Table 1). By day 21, immunopathology in $TNF^{-/-}$ lungs continued to worsen, showing bronchial and alveolar structural injury, hemorrhage, metaplasia, and numerous dense cellular aggregates characteristic of fibrotic tissue remodeling (Figure 2F; Table 1). At day 30 after infection, although $TNF^{-/-}$ lungs had reduced inflammatory infiltration, there still existed significant bronchial epithelial hyperplasia and fibrotic cellular aggregates (Figure 2H; Table 1).

To further dissect the fibrotic tissue remodeling in the lung of $TNF^{-/-}$ mice, we examined lung samples col-

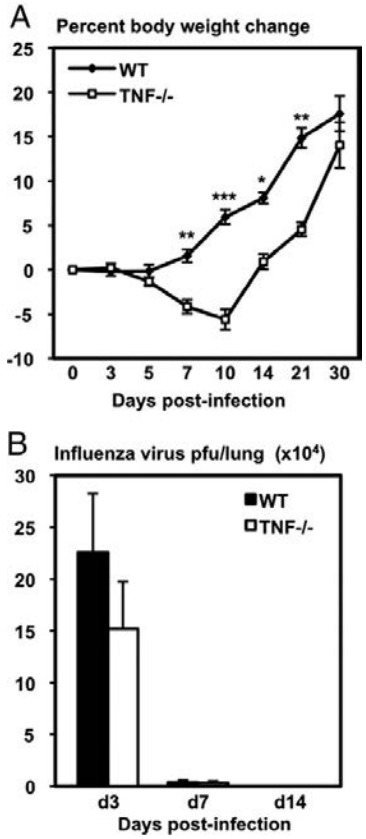

Figure 1. TNF-α is not required for influenza viral clearance in the lung. WT and $TNF^{-/-}$ mice were intranasally infected with influenza virus. **A:** Average percent body weight change of WT (n = 28/time point) and $TNF^{-/-}$ (n = 85/time point) mice was monitored. Results are from multiple independent experiments. **B:** At day 3, 7, and 14 after infection, the viral titer in the lung was evaluated by plaque forming assay (n = 4 to 8/strain/time point from two independent experiments). Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to WT controls.

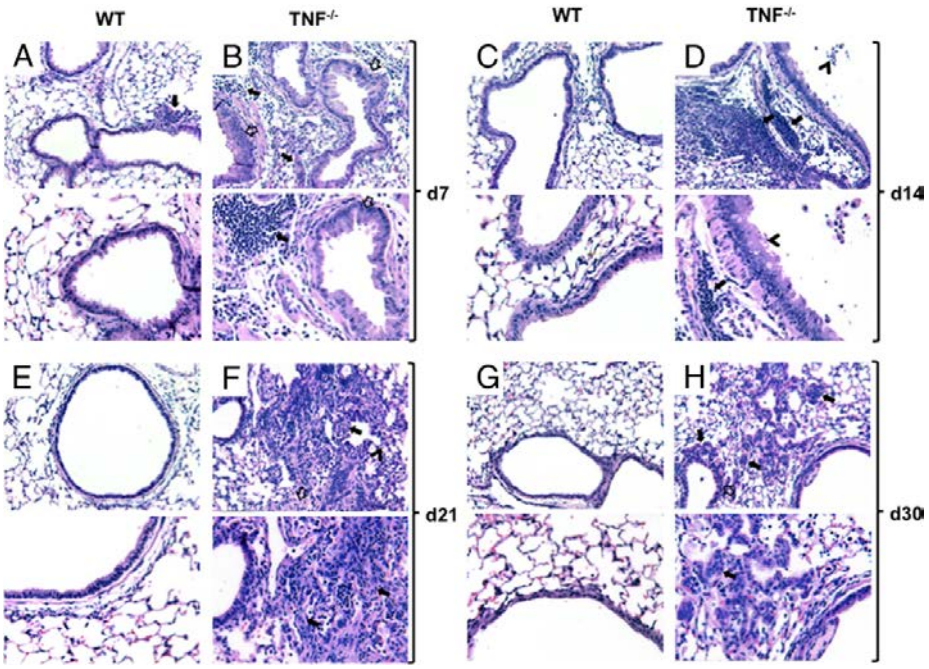

Figure 2. Immunopathological changes in the lungs following influenza infection. Mice were sacrificed 7, 14, 21, and 30 days after influenza infection, and lung tissue sections were stained with H&E. Representative slides are shown at ×5 (**top**) and ×10 (**bottom**) original magnification. **A**, **C**, **E**, and **G:** WT mice exhibited transient lung inflammation. **B**, **D**, **F**, and **H:** *TNF*$^{-/-}$ mice showed persistent lung immunopathology and injury. **A**, **B**, and **D:** Mononuclear infiltration (**closed arrow**). **B** and **H:** Bronchial hyperplasia (**open arrow**). **D:** Bronchial epithelium injury and sloughing (**arrowhead**). **F:** Structural injury (**arrowhead**), metaplasia (**open arrow**). **F** and **H:** Fibrotic cellular aggregates (**closed arrow**).

lected at days 14 and 21, using Masson's trichrome staining and immunohistochemical staining for α-SMA. α-SMA is highly expressed by collagen-releasing myofibroblasts, the key cellular component of fibrosis.[51,52] There was a lack of any signs of pulmonary fibrotic reaction in influenza-infected WT lungs at any time point after influenza infection (Figure 3, A, B, C, and D; Table 1). However, infected *TNF*$^{-/-}$ lungs had overt signs of fibrotic tissue remodeling at days 14 and 21 (Table 1), including increased extracellular collagen deposition in lung parenchyma (Figure 3, A and C), the appearance of myofibroblasts (Figure 3, B and D), and the formation of fibrotic foci with abundant collagen deposition and myofibroblast accumulation (Figure 3E). Furthermore, by using the Sirius Red colorimetric assay (Sircol collagen assay), we measured the total collagen content and found that at day 21 after infection, *TNF*$^{-/-}$ mouse lungs had more collagen than WT lungs (data not shown). The above results together indicate that the illness observed in influenza-infected TNF-α–deficient hosts was due to sustained severe lung immunopathology and tissue remodeling.

Markedly Increased Inflammatory Cellular Responses in the Lung of TNF-α–Deficient Hosts after Influenza Infection

To begin examining the mechanisms of pronounced immunopathology and tissue remodeling in the lung of in-

Table 1. Assessment of Histopathological Changes in the Lung

	Day 7		Day 14		Day 21		Day 30	
	WT	*TNF*$^{-/-}$	WT	*TNF*$^{-/-}$	WT	*TNF*$^{-/-}$	WT	*TNF*$^{-/-}$
Inflammation	+	++	+/−	+++	−	+++	−	++
Injury	+	++	+	+++	+/−	++++	−	+++
Remodeling	−	−	−	++	−	+++	−	+++

Lung inflammation (mononuclear cell infiltration), lung injury (degeneration of airway epithelium, necrosis, bronchoepithelial hyperplasia, metaplasia in the lung parenchyma, hemorrhaging), and tissue remodeling (pulmonary fibrosis: myofibroblasts, collagen deposition, scarring, fibrotic foci) were assessed.

Results are representative of n = 8 to 12/strain/time point from two to three independent experiments.

−, absent; +, minimal; ++, slight; +++, moderate; ++++, marked; +++++, severe.

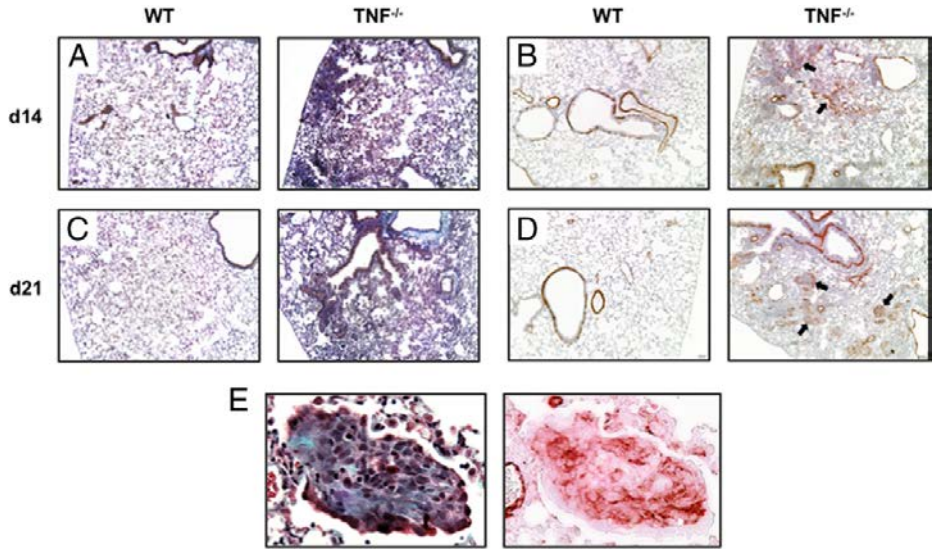

Figure 3. Tissue fibrotic remodeling in the lungs of *TNF*$^{-/-}$ mice following influenza infection. WT and *TNF*$^{-/-}$ mice were sacrificed 14 and 21 days after influenza infection. **A** and **C:** Lung tissue sections were stained with Masson's trichrome that stains collagen fibers blue. **B** and **D:** Lung sections were immunohistochemically stained using a monoclonal antibody against α-SMA found on myofibroblasts in fibrotic foci, which stains brown-red (**arrows**). Representative images are shown at ×5 original magnification. **E:** Representative fibrotic foci stained with Masson's trichrome (**left**) or for α-SMA in *TNF*$^{-/-}$ (**right**) lungs at 21 days after infection are shown at ×40 original magnification.

fluenza-infected *TNF*$^{-/-}$ mice, we first evaluated the types of inflammatory cell infiltrates in the respiratory tract. To this end, BAL was performed on infected WT and *TNF*$^{-/-}$ mice, and leukocytes were differentiated on cytospins. In the lung of WT mice, the number of inflammatory cells, including alveolar macrophages, neutrophils, and lymphocytes, steadily declined from day 7 until day 30, the last time point examined (Figure 4, A, B, and C), which coincided well with a marked drop in viral titers by day 7 and a complete viral clearance by day 14 (Figure 1B). On the contrary, compared to WT controls *TNF*$^{-/-}$ mouse lungs had significantly greater numbers of alveolar macrophages, neutrophils, and lymphocytes at all time points examined (Figure 4, A, B, and C). Of note, whereas the overall numbers of macrophages and neutrophils decreased over time in the lung of *TNF*$^{-/-}$ mice, the number of lymphocytes was somewhat sustained between days 14 and 30 (Figure 4, A, B, and C). These data suggest that severe immunopathology and tissue remodeling in infected *TNF*$^{-/-}$ hosts are linked to an exaggerated inflammatory cellular response in the lung.

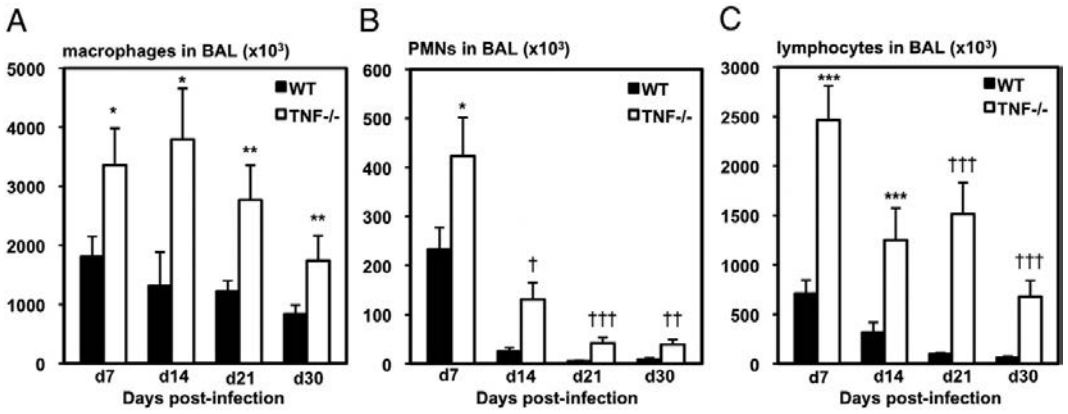

Figure 4. Inflammatory cellular infiltration in the lungs following influenza infection. WT and *TNF*$^{-/-}$ mice were sacrificed 7, 14, 21, and 30 days after influenza infection, and differential cell counting was performed on cytospins of bronchoalveolar lavages. Alveolar macrophages (**A**), polymorphonuclear cells (PMNs) (**B**), and lymphocytes (**C**) were enumerated. Results are expressed as mean ± SEM (n = 8 to 12/strain/time point from two to three independent experiments). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{\dagger}P < 0.0005$, $^{\dagger\dagger}P < 0.00005$, and $^{\dagger\dagger\dagger}P < 0.0000005$ as compared to WT.

Markedly Increased Influenza-Specific Adaptive Immune Responses in the Lung of TNF-α–Deficient Hosts after Influenza Infection

Antigen-specific CD8 T-cell responses play an important role in anti-influenza host defense.[8,12,13] Thus, by using influenza NP366-374 (H-2Db) tetramer immunostaining and intracellular cytokine staining techniques, we further examined the properties of markedly increased total lymphocytes seen in the lung of infected *TNF*$^{-/-}$ mice (Figure 4C). *TNF*$^{-/-}$ mice had more total CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ T cells than WT mice, both in the BAL (airway lumen) and lung interstitium at all time points after infection (days 7 to 30) (data not shown). On tetramer immunostaining, there were much greater frequencies and numbers of NP-specific tetramer$^+$CD8$^+$ T cells in the BAL and lung of *TNF*$^{-/-}$ mice over the entire course of infection examined (days 7 to 30) compared to WT mice (Figure 5, A and B). Furthermore, on NP peptide stimulation *ex vivo*, there were also significantly higher frequencies and numbers of IFN-γ$^+$CD8$^+$ T cells in the BAL and lung of *TNF*$^{-/-}$ mice at various time points after infection, compared to WT (Figure 5, C and D). Similar results were obtained with NS2$^{114-121}$ peptide stimulation (data not shown). These results indicate that TNF-α deficiency does not impede adaptive T-cell responses to primary influenza infection.

As B-cell–mediated antibody responses also play a role in anti-influenza host defense,[8,13,14] and given the observed heightened influenza antigen–specific T-cell responses, we next examined the level of influenza-specific antibody responses, both in the BAL (airway lumen) and the peripheral blood, by ELISA. We found that the levels of influenza-specific IgA were slightly higher at 14

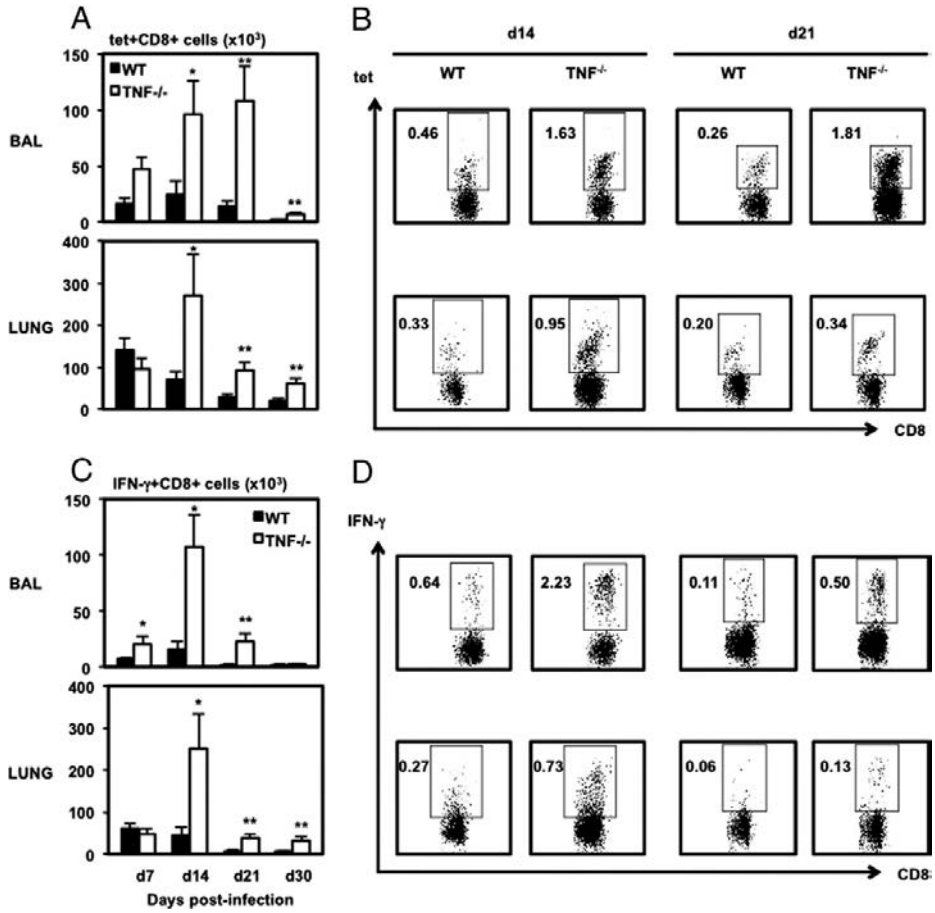

Figure 5. Flu antigen–specific CD8 T cells in the lungs following influenza infection. WT and *TNF*$^{-/-}$ mice were sacrificed 7, 14, 21, and 30 days after influenza infection. **A** and **B:** Cells from the BAL and lung were stained with DbNP366 tetramers and analyzed by flow cytometry for NP366-specific tet$^+$CD8$^+$ T cells. **C** and **D:** Cells from the BAL and lung were stimulated with the NP366-374 influenza peptide, stained for intracellular IFN-γ, and analyzed by flow cytometry for antigen-specific IFN-γ$^+$CD8$^+$ T cells. The charts represent total numbers of tet$^+$CD8$^+$ or IFN-γ$^+$CD8$^+$ T cells in the BAL or lung at each time point. The dot plots show representative populations from the BAL and lung at 14 and 21 days after infection (gates show frequency of total cells). Data are expressed as mean ± SEM (n = 8 to 12/strain/time point from two to three independent experiments). *$P < 0.05$, **$P < 0.01$ as compared to WT controls.

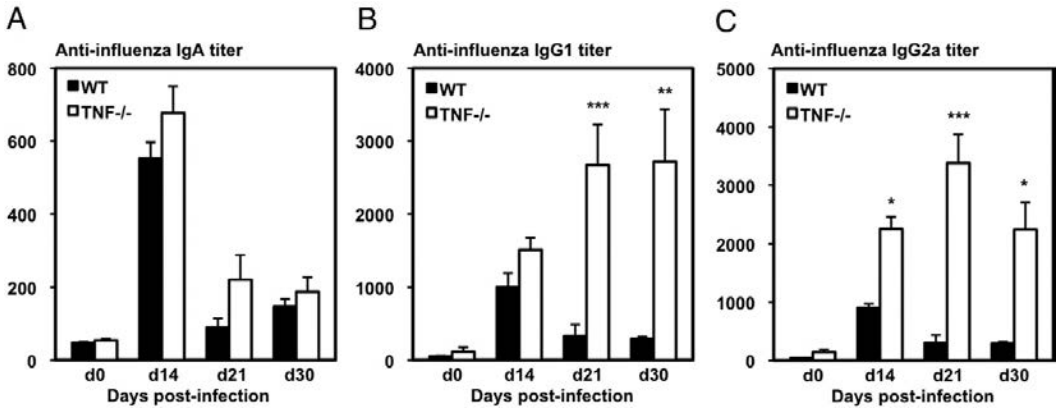

Figure 6. Flu antigen–specific antibody responses in the lungs following influenza infection. WT and *TNF*$^{-/-}$ mice were sacrificed before infection (d0), as well as 14, 21, and 30 days after influenza infection. IgA (**A**), IgG1 (**B**), and IgG2a (**C**) levels in the BAL fluids were measured by ELISA. Data are expressed as mean ± SEM (n = 8 to 10/strain/time point from two independent experiments). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to WT controls.

and 21 days after infection in the BAL of *TNF*$^{-/-}$ mice than in WT mice (Figure 6A). However, the levels of influenza-specific IgG1 and IgG2a were generally much higher at days 14, 21, and 30 after infection in the BAL of *TNF*$^{-/-}$ mice than in WT mice (Figure 6, B and C). Of note, the titers of IgG1 and IgG2a in the lung of WT mice declined sharply after day 14, whereas they continued to rise and remained high in the lung of *TNF*$^{-/-}$ mice after day 14 (Figure 6, B and C). In comparison, both infected WT and *TNF*$^{-/-}$ mice had, by and large, comparable circulating levels of influenza-specific IgG1 and IgG2a (data not shown). These results show that consistent with heightened anti-influenza T-cell responses seen in the lung of influenza-infected *TNF*$^{-/-}$ hosts, there were also heightened humoral immune responses.

Dysregulated Proinflammatory Cytokine and Chemokine Responses in the Lung of Influenza-Infected TNF-α–Deficient Hosts

To investigate the potential molecular mechanisms of severe inflammatory cellular responses and immunopathology in the lung of influenza-infected *TNF*$^{-/-}$ mice, we examined the level of proinflammatory cytokines in the lung. Although IFN-γ levels were similar initially in the BAL of both WT and *TNF*$^{-/-}$ mice at day 3 after infection, they were significantly higher in the lung of *TNF*$^{-/-}$ mice both at days 7 and 14 (Table 2). We also elected to examine the levels of IL-17, which is known to play an important role in triggering both CXC and CC chemokine responses in the lung.[53] There were relatively small, detectable levels of IL-17 in the lung of WT mice at days 3 and 7, which became undetectable after day 7 (Table 2). In contrast, the levels of IL-17 were much more elevated in the lung of *TNF*$^{-/-}$ mice by day 3 and remained sustained between days 7 and 21 after infection (Table 2). In contrast to heightened IFN-γ and IL-17 in the lung of *TNF*$^{-/-}$ hosts, the lung levels of IL-1β were comparable between WT and *TNF*$^{-/-}$ mice (Table 2). We also measured the levels of anti-inflammatory cytokine IL-10 and found comparably low levels of this cytokine in both strains of mice (Table 2).

Given significantly higher levels of IL-17 responses in the lung of infected *TNF*$^{-/-}$ mice beginning from day 3 after infection, we next examined the levels of chemokines including MCP-1 (CCL2), IP-10 (CXCL10), and KC (CXCL1) in the lung. We found that these chemokines were highly inducible in the lung of both strains of mice on influenza infection and peaked between days 3 and 7 (Figure 7, A, B, and C). However, the levels of MCP-1 and IP-10 were significantly higher around 7 days after infec-

Table 2. Cytokine and Chemokine Expression in the Lung during Influenza Infection (pg/mL)

	Day 3		Day 7		Day 14		Day 21	
	WT	*TNF*$^{-/-}$	WT	*TNF*$^{-/-}$	WT	*TNF*$^{-/-}$	WT	*TNF*$^{-/-}$
IFN-γ *	58.1 ± 0.3	58.4 ± 0.6	51.4 ± 1.1	180 ± 81	56.8 ± 0.9	116.0 ± .7	40.3 ± 5.5	43.6 ± 5.6
IL-17	2.1 ± 2.1	41.5 ± 12.6	20.2 ± 5.2	41.2 ± 18.6	0	5.4 ± 5.4	0	20.6 ± 12.5
IL-1β	ND	ND	20.1 ± 2.4	24.5 ± 2.4	17.9 ± 0.9	15.0 ± 0.8	21.7 ± 0.5	18.6 ± 1.7
IL-10	ND	ND	7.6 ± 1.9	12.8 ± 1.4	7.2 ± 0.2	6.3 ± 0.4	9.9 ± 1.0	13.8 ± 3.1

Results are expressed as mean ± SEM (n = 4/strain/timepoint from one to two independent experiments).
*ELISA (all others Luminex).
ND, not determined.

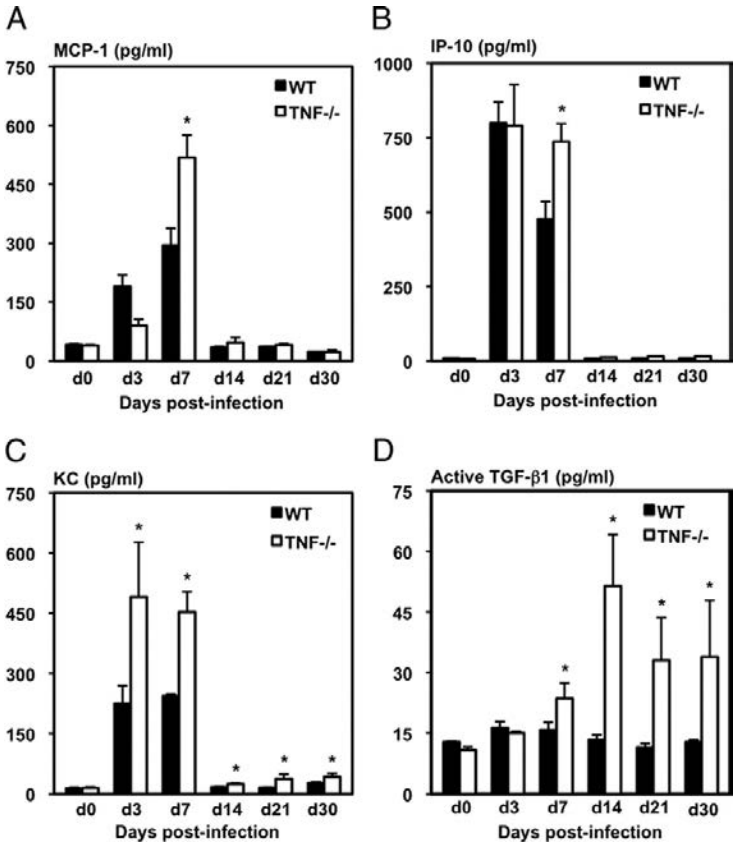

Figure 7. Levels of chemokines and fibrogenic growth factor in the lungs following influenza infection. WT and *TNF*$^{-/-}$ mice were sacrificed at different time points after influenza infection. Levels of MCP-1 (**A**), IP-10 (**B**), and KC (**C**) were measured in the BAL fluid by Luminex. **D:** Active TGF-β1 was measured by ELISA. Data are expressed as mean ± SEM (n = 4 to 8/strain/time point from two independent experiments). *$P <$ 0.05 as compared to WT controls.

tion in the lung of *TNF*$^{-/-}$ mice than in WT controls (Figure 7, A and B), whereas the levels of KC were significantly higher at all time points examined in the lung of infected *TNF*$^{-/-}$ mice, with the largest differences seen at 3 and 7 days after infection (Figure 7C). Of note, the peak response of CXC neutrophil chemokine KC preceded that of MCP-1, a chemokine for monocytes/macrophages, T cells, and fibrocytes.[54] Thus, earlier peak KC responses correlate with an early and transient nature of the neutrophilic response seen in the lung of influenza-infected *TNF*$^{-/-}$ hosts (Figure 4B). On the other hand, delayed peak MCP-1 responses correlate with relatively sustained mononuclear cellular responses (Figure 4, A and C; Table 1) and immunopathology (Figure 2), and the subsequent development of fibrotic remodeling (Figures 2 and 3; Table 1) in the lung of influenza-infected *TNF*$^{-/-}$ hosts.

On the basis of the documented association of MCP-1 to, and the critical role of, TGF-β1 in tissue fibrogenesis,[51,52,54–60] we examined the level of profibrogenic growth factor TGF-β1. Indeed, we found that the levels of active TGF-β1 in the lung of influenza-infected *TNF*$^{-/-}$ mice were significantly higher than in WT control mice (Figure 7D), and the peak of MCP-1 responses (day 7) preceded the peak of TGF-β1 responses seen at day 14 in the lung of influenza *TNF*$^{-/-}$ mice (Figure 7A). Of importance, the elevated active TGF-β1 responses were sustained between days 14 and 30 in the lung (Figure 7D) and correlated well with the development of fibrotic remodeling seen in the lung of influenza-infected *TNF*$^{-/-}$ mice (Figures 2 and 3; Table 1). The above data together thus indicate a temporal relationship between dysregulated MCP-1 responses and fibrotic tissue remodeling in the lung of *TNF*$^{-/-}$ hosts following acute influenza infection.

Attenuation of Lung Immunopathology in Influenza-Infected TNF-α–Deficient Hosts by MCP-1 Depletion

Compelling evidence suggests that MCP-1 is not only a chemokine for mononuclear cells, but of importance, it is also a profibrotic cytokine capable of direct up-regulation of TGF-β1 expression[55–61] and recruitment and induction of collagen synthesis by fibrocytes.[51,62–68] Thus, given the dysregulated MCP-1 and its association with heightened TGF-β1 production seen in influenza-infected *TNF*$^{-/-}$ lungs (Figure 7), we further investigated the role of MCP-1 in heightened immunopathology and fibrotic

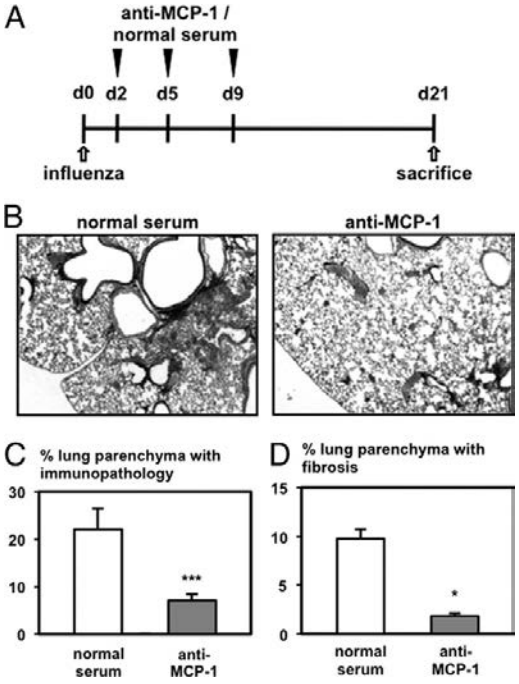

Figure 8. Modulation of lung immunopathology in the lungs of infected $TNF^{-/-}$ mice by MCP-1 depletion. $TNF^{-/-}$ mice were infected with influenza. **A:** On days 2, 5, and 9 after infection, anti–MCP-1 serum or normal control rabbit serum was injected intraperitoneally, and mice were sacrificed on day 21. **B:** Lung tissue sections were stained with H&E, and representative images are shown at ×5 original magnification. **C:** Morphometric quantification of lung immunopathology performed on H&E-stained sections. **D:** Morphometric quantification of lung fibrotic remodeling performed on Masson's trichrome–stained sections. Results are expressed as mean ± SEM (n = 8 to 13/group from two independent experiments). $^{*}P < 0.05$, $^{***}P < 0.001$ compared to normal serum–treated controls.

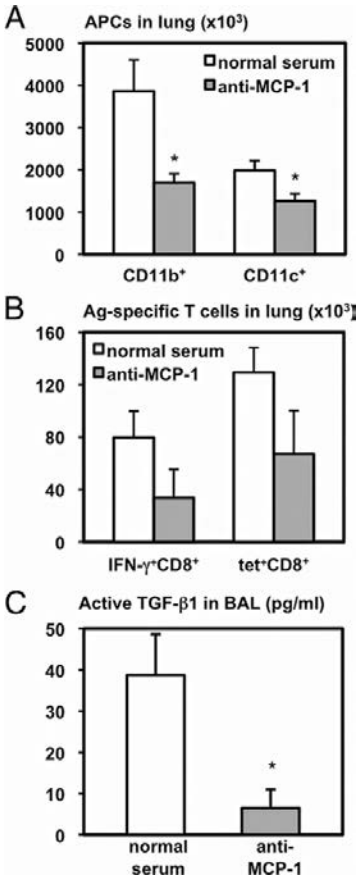

Figure 9. Modulation of immune cellular responses and active TGF-β1 production in the lungs of infected $TNF^{-/-}$ mice by MCP-1 depletion. $TNF^{-/-}$ mice were infected with influenza, and MCP-1 was depleted as in Figure 8A. At day 21 after infection, macrophages/dendritic cells (**A**) and influenza-specific $CD8^+$ T cells (**B**) were enumerated in the lungs by flow cytometry. **C:** BAL fluids were analyzed for active TGF-β1 by ELISA. Results are expressed as mean ± SEM (n = 4/group). $^{*}P < 0.05$ compared to normal serum–treated controls.

reaction in the lung of $TNF^{-/-}$ mice by means of MCP-1 depletion. To this end, MCP-1 anti-serum (anti–MCP-1) or the control serum was administered intraperitoneally to $TNF^{-/-}$ mice on days 2, 5, and 9 following influenza infection, and the mice were sacrificed at day 21 after infection for morphometric quantification of lung immunopathology and fibrotic remodeling (Figure 8A). MCP-1 depletion did not affect the rate of influenza viral clearance (data not shown). However, compared to the control group, depletion of MCP-1 resulted in significantly reduced inflammation and immunopathology as assessed on H&E-stained tissue sections (Figure 8, B and C). Furthermore, it also significantly reduced the area of fibrotic reaction in the lung as assessed on Masson's trichrome–stained lung sections for collagen deposition (Figure 8D). Such potent attenuating effect by MCP-1 depletion on lung immunopathology in infected $TNF^{-/-}$ animals contrasts with the modest effect exerted by CD8 T-cell depletion (data not shown).

We further examined the potential mechanisms by which MCP-1 depletion reduced lung immunopathology and fibrotic reactions in influenza-infected $TNF^{-/-}$ mice. We found that MCP-1 significantly reduced the number of $CD11b^+$ and $CD11c^+$ macrophages/dendritic cells in the lung (Figure 9A). It also decreased the number of influenza antigen–specific CD8 T cells (Figure 9B). There was also a significantly reduced level of fibrogenic growth factor TGF-β1 in the lung by MCP-1 depletion (Figure 9C). The above data together suggest that MCP-1 plays an important role in the development of severe immunopathology in the lung of influenza-infected $TNF^{-/-}$ hosts.

Worsening of Lung Immunopathology in Influenza-Infected Wild-Type Mice by Transgenic Expression of MCP-1

To further understand the role of MCP-1 in immunopathology, we examined whether transient overexpression of MCP-1 in the lung of influenza-infected WT mice would worsen lung immunopathology. Thus, a recombinant rep-

lication-defective adenoviral vector expressing murine MCP-1 gene (*AdMCP-1*) was delivered intranasally to the lungs of WT mice 1 day before influenza infection (Figure 10A). The control WT mice received AddI. The mice were sacrificed for analysis at day 21 after infection. MCP-1 gene transfer to the lung led to much greater lung immunopathology compared to influenza-infected control mice (Figure 10B). This was accompanied by significantly increased numbers of influenza NP–specific IFN-γ^+CD8^+ and tetramer$^+CD8^+$ T cells (Figure 10C). These results further support our conclusion that MCP-1 is one of the key cytokines involved in influenza-associated lung immunopathology.

Enhanced Lung Immunopathology in Influenza-Infected Wild-Type Mice Depleted of TNF-α

We have shown that following influenza infection, TNF-α was up-regulated on days 3, 7, and 14 in the lungs of WT mice (see Supplemental Figure S1 at *http://ajp.amjpathol.org*) and have identified the critical regulatory role of TNF-α in influenza immunopathology and immune responses by using mice genetically deficient for TNF-α. To verify the phenotype seen in influenza-infected TNF-α–deficient mice, we examined the effects of TNF-α depletion in WT mice following influenza infection. To this end, TNF-α anti-serum (anti–TNF-α) or control serum was administered intraperitoneally to WT mice repeatedly on days −1, 2, 5, 8, 12, and 15 following influenza infection, and the mice were sacrificed at day 21 (see Supplemental Figure S2A at *http://ajp.amjpathol.org*). Compared to the control group, depletion of TNF-α in WT mice indeed resulted in markedly increased immunopathology in H&E-stained sections (see Supplemental Figure S2B at *http://ajp.amjpathol.org*) and fibrotic remodeling by Masson's trichrome staining (data not shown). Also, significantly increased numbers of influenza antigen–specific CD8 T cells were seen in the lung (see Supplemental Figure S2, C and D, at *http://ajp.amjpathol.org*). These results further support the critical role of TNF-α in controlling the level of immune activation and lung immunopathology following influenza infection.

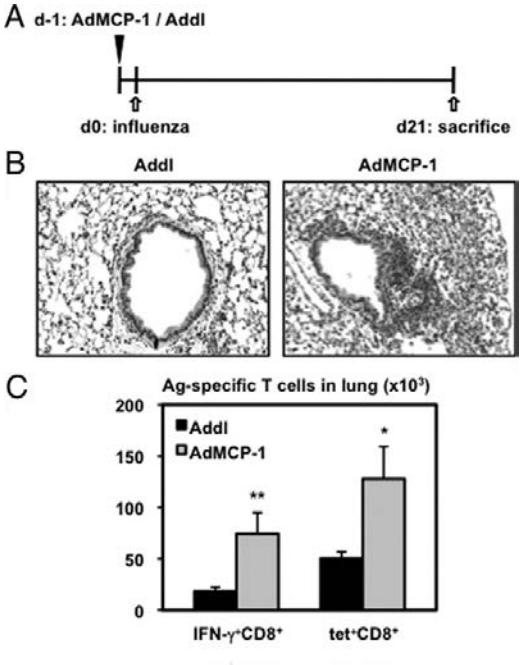

Figure 10. Enhancement of lung immunopathology in the lungs of infected WT mice by transgenic MCP-1 overexpression. **A:** WT mice were infected intranasally with AddI or AdMCP-1, the next day infected with influenza, and sacrificed on day 21. **B:** Lung tissue sections were stained with H&E, and representative images are shown at ×5 original magnification. **C:** Influenza-specific $CD8^+$ T cells were enumerated in the lungs with flow cytometry. Results are expressed as mean ± SEM (n = 4 to 5/group). $^*P < 0.05$, $^{**}P < 0.005$ compared to AddI-treated control mice.

Discussion

Severe lung immunopathology is known to be one of the main causes of influenza-related morbidity and mortality.[3–11] TNF-α, a classical proinflammatory cytokine, has recently been shown to play anti-inflammatory and anti-fibrotic roles in various models.[32–43] However, whether TNF-α plays an immunoregulatory role during influenza infection remains unclear. Using a mouse model of respiratory influenza infection, we investigated this issue. We show that TNF-α is not required for influenza viral clearance, but it is required for controlling the level of anti-influenza inflammatory and immune responses. Thus, dysregulated anti-influenza inflammatory responses resulting from the genetic deficiency or antibody-mediated depletion of TNF-α lead to illness associated with severe immunopathology and tissue fibrotic remodeling in the lung. We further identify that the dysregulated MCP-1 response represents an important molecular mechanism in heightened immunopathology. Our study thus establishes that although it is dispensable in influenza viral clearance, TNF-α is critically required for controlling immunopathology and aberrant tissue repair in the course of acute influenza infection.

Transient lung inflammation was observed in WT lungs between 3 and 14 days following infection, which decreased thereafter as lungs returned to normal by 21 days. In sharp contrast, the lung immunopathology was much more severe and sustained in *TNF*$^{-/-}$ lungs throughout the infection. At day 7, there was increased cellular infiltration, degeneration, and exfoliation of the airway epithelium and edema in *TNF*$^{-/-}$ lungs. After the virus was completely cleared at 14 days, *TNF*$^{-/-}$ lungs showed more extensive necrotizing injury, edema, and cellular infiltration into the airways. By day 21 after infection, when WT lungs returned back to normal, *TNF*$^{-/-}$ lungs continued to worsen, showing a loss of clear alveolar structure, hemorrhaging, and epithelial metaplasia, which sustained even out to 30 days after infection. Associated with the heightened immunopathology observed in the lung of influenza-infected *TNF*$^{-/-}$ hosts is

fibrotic tissue remodeling. The fibrotic lesion seen in these mice began from 14 days after infection and is characterized by the appearance of fibrotic foci containing α-SMA–expressing myofibroblasts and collagen deposition. The antifibrotic role of TNF-α was also previously seen in models of idiopathic pulmonary fibrosis, ureteral obstruction, and scleroderma.[41–43] It is noteworthy that much of the previous evidence to suggest a proinflammatory or proimmune role of TNF-α in anti-influenza host defense is drawn from *in vitro* studies[23,24,29] or deduced from the historical belief of its proinflammatory nature. To our knowledge, there have been only few direct *in vivo* investigations on the role of TNF-α in influenza immunopathology. One study shows the involvement of CD8 T-cell–derived TNF-α in causing bronchial epithelial injury.[28] The opposing conclusions regarding the role of TNF-α in influenza immunopathology drawn from this study and our current study are likely because the former used an adoptive transgenic T-cell transfer approach and a transgenic mouse model in which the bronchial epithelial cells overexpress hemagglutinin.[28] Two other studies have shown a decrease in disease severity in TNFR1 knockout mice infected with highly virulent H5N1 avian or a reconstructed 1918 pandemic influenza virus. In agreement with our study, these studies report the lack of involvement of TNFR1 in influenza viral clearance.[26,27] However, infected TNFR1-deficient mice had a significant delay in weight loss compared to WT from day 3 to 8 after infection, although they ultimately succumbed.[26,27] On one hand, these findings lend further support to the current belief that TNF-α is a proimmune or proinflammatory cytokine, particularly in the early response (first 7 days of infection) and that a "cytokine storm" may contribute to disease severity.[23–25] On the other hand, these findings differ from our current results with respect to the role of TNF-α in influenza disease severity and immunopathology, which is likely due to the choice of model influenza viruses of different pathogenicity. In support, use of a less virulent strain of H5N1 in the same study did not lead to significant differences in weight loss or disease outcome.[26] The choice of a mouse-adapted strain of influenza virus with relatively low virulence in our current study was to allow us to study the role of TNF-α at later phases of influenza, when the immune system is required to return to homeostasis. Thus, although the earlier studies suggest a proinflammatory role of TNF-α in anti-influenza host defense, our current investigation suggests that proinflammatory molecules other than TNF-α induced by influenza could replace the proinflammatory activities by TNF-α in the early course of influenza infection, which explains the unimpaired influenza viral clearance in *TNF*$^{-/-}$ hosts that we and others have observed. Our observation that TNF-α deficiency leads to severe immunopathology and tissue remodeling strongly suggests that the immunoregulatory function of this cytokine is indispensable, particularly when influenza viral clearance is near completion and lung homeostasis needs to be restored at later phases of infection. On the basis of previous and our current findings, we argue that TNF-α plays a differential and biphasic role during the course of primary influenza infection. In the early phase of infection, it is proinflammatory and may contribute to disease severity, particularly in cases of highly pathogenic influenza, but it is immunoregulatory in later phases of infection after viral clearance. Overall, our study thus lends strong support to the recent emerging conviction that TNF-α is a double-edged sword, and besides its proinflammatory properties, it plays an important immunoregulatory function in the host response to a number of intracellular infections and autoimmunogens.[21,22,32–40]

In our current study, we have found TNF-α regulation of MCP-1 responses to be an important molecular mechanism by which TNF-α negatively controls the level of influenza immunopathology. MCP-1 has previously been reported to be a major profibrotic chemokine involved in various fibrotic processes, including recruitment of fibrocytes, induction of collagen synthesis, and up-regulation of TGF-β1 expression in various models.[50,51,55–61,63–68] Indeed, we observed that MCP-1 responses preceded the responses of fibrogenic growth factor TGF-β1,[51,52] which were significantly higher in the lung of infected *TNF*$^{-/-}$ lungs and were well correlated with fibrotic tissue remodeling seen in *TNF*$^{-/-}$ lungs at later time points during influenza infection. Thus, depletion of MCP-1 in influenza-infected *TNF*$^{-/-}$ hosts markedly reduced lung immunopathology and tissue remodeling, and such MCP-1 depletion–improved welfare was associated with diminished production of bioactive TGF-β1. On the other hand, by using a transient gene transfer approach, we observed that transgenic MCP-1 overexpression in infected WT mouse lungs markedly increased influenza-associated immunopathology and tissue injury. Our observations together suggest an influenza–TNF-α–MCP-1–TGF-β1–immunopathology axis. It is likely that in addition to its fibrogenic property, MCP-1 also contributes to tissue immunopathology via its robust effects on inflammatory cells including macrophages and monocytes. Excessive accumulation of these inflammatory cells in the lung contributes to severe immunopathology and tissue injury that precipitates tissue remodeling.[8,19,51,52,56,69]

The dysregulated anti-influenza T-cell responses that we observed in infected *TNF*$^{-/-}$ or TNF-depleted hosts are likely due in part to reduced T-cell contraction following viral clearance. These results are consistent with studies of intracellular mycobacterial and LCMV infections. Indeed, it has been previously shown that during mycobacterial infection, TNF-α deficiency results in uncontrolled T-cell proliferation and immune activation, and massive lung immunopathology.[32] Furthermore, lack of TNF-α leads to dysregulated CD4 and CD8 T-cell expansion and a prolonged T-cell effector phase following LCMV infection.[33–35] In addition, Turner and colleagues have suggested a role of TNFR2 in editing CD8 T cells following PR8 infection.[70] Thus, it is likely that heightened helper T cells, as well as decreased B-cell apoptosis[71] resulting from TNF-α deficiency, leads to the increased anti-influenza humoral responses that we have also observed in our current study. Of interest, we found that depletion of CD8 T cells in influenza-infected *TNF*$^{-/-}$ animals around the time of viral clearance had only a modest improving effect on lung immunopathology (data not shown). First, this suggests a complex role played by

anti-influenza T cells and the difficulty of determining the right timing for T-cell depletion, as these cells are critical to viral clearance. Second, the much-heightened lung immunopathology in influenza-infected $TNF^{-/-}$ animals may involve multiple inflammatory cell subsets including macrophages and monocytes. This contention is strongly supported by our findings that compared to T-cell depletion, MCP-1 depletion proved to be a much more effective way to attenuate the severe immunopathology seen in the lung of infected $TNF^{-/-}$ hosts.

In conclusion, our results indicate that TNF-α is dispensable for influenza viral clearance. However, this cytokine is critically required for controlling the extent of influenza immunopathology and tissue injury. Thus, lack of TNF-α results in dysregulated inflammatory responses and tissue immunopathology. As TNF-α depletion has been widely implemented as a means to counter inflammatory conditions in humans,[21,36–40] our results caution the clinical use of anti–TNF-α therapeutics, which may potentially worsen influenza-associated immunopathology in the lung, particularly during flu seasons or epidemics.

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Supplementary Figures

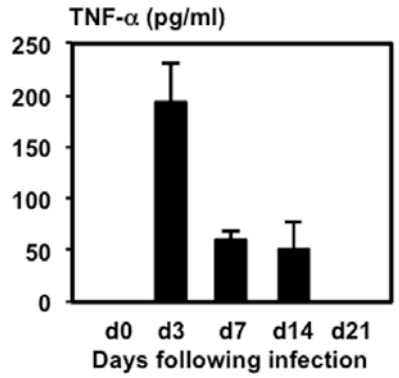

Figure S1. TNF-α upregulation in the lung following influenza infection. WT mice were infected intranasally with influenza. TNF-α levels were measured in lung tissue homogenates by ELISA. Results are expressed as Mean ± SEM (n=4-5/timepoint from one experiment).

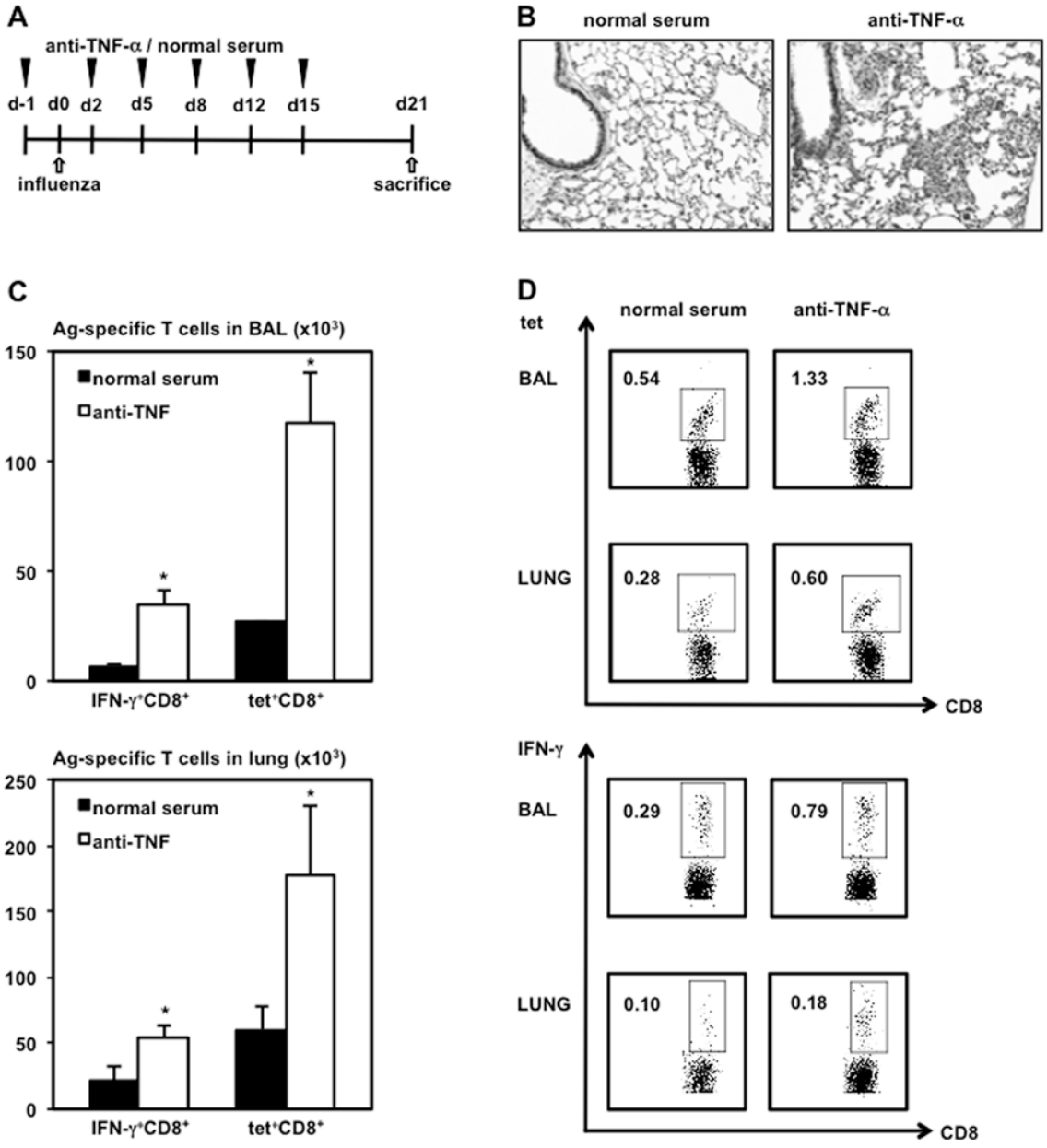

Figure S2. Enhancement of lung immunopathology and influenza-specific T cell numbers in WT mice after TNF-α depletion. **A:** WT mice were infected with influenza and TNF-α was depleted on days -1, 2, 5, 8, 12 and 15 after infection using intraperitoneally injected anti-TNF-α rabbit serum (control: normal rabbit serum), and mice were sacrificed on day 21. **B:** Lung tissue sections were stained with H&E, and representative slides are shown at 5x magnification. **C, D:** Influenza-specific CD8+ T cells were analyzed in the lungs with flow cytometry. Results are expressed as Mean ± SEM (n=4/group from one experiment). *, $p < 0.05$; compared to normal serum mice.

CHAPTER 3: Marked improvement of severe lung immunopathology by influenza-associated pneumococcal superinfection requires the control of both bacterial replication and host immune responses

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Marked improvement of severe lung immunopathology by influenza-associated pneumococcal superinfection requires the control of both bacterial replication and host immune responses

For this study, we established a murine model of acute respiratory influenza infection and *Streptococcus pneumoniae* superinfection to investigate the mechanisms of severe lung immunopathology and possible intervention strategies. We were interested in elucidating the role of bacterial replication and over activated host immune responses during bacterial superinfection following influenza. Our study identified uncontrolled bacterial outgrowth, dysregulated cytokine and chemokine responses, and excessive neutrophil infiltration in the lungs to be the main contributors to deleterious lung immunopathology and death. The use of a bacteriostatic antibiotic alone effectively controlled bacterial replication and improved clinical outcome, but failed to significantly reduce lung immunopathology. Importantly, the use of antibiotic and corticosteroid in combination led to the best clinical outcome with markedly improved survival, bacterial clearance and lung immunopathology.

Data not shown in the manuscript are included in *Appendix I*, with corresponding pages.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

Marked improvement of severe lung immunopathology by influenza-associated pneumococcal superinfection requires the control of both bacterial replication and host immune responses

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ABSTRACT

Bacterial superinfection and associated lung immunopathology are major contributors to hospitalizations and mortality following influenza. However, the underlying mechanisms and effective intervention strategies remain poorly defined. Using a model of influenza and pneumococcal superinfection, we found that dual infected animals experienced rapid weight loss and succumbed to infection. Bacterial outgrowth, dysregulated cytokines including KC and MIP-2, and severe lung neutrophilia and immunopathology were linked to the poor clinical outcome. *In vivo* neutralization of highly induced MIP-2 did not affect clinical outcome, bacterial loads or lung immunopathology. On the other hand, *in vivo* neutrophil depletion did not alter the clinical outcome and bacterial burden, while it moderately improved lung immunopathology. Treatment with a bacteriostatic antibiotic azithromycin alone significantly improved clinical outcome and bacterial clearance but failed to reduce lung immunopathology. In comparison, treatment with a global inflammation inhibitor dexamethasone alone failed to alter clinical outcome, bacterial infection and immunopathology despite its moderate reducing effects on neutrophilic and cytokine responses. In contrast, combined treatment with both azithromycin and dexamethasone best improved clinical outcome, bacterial clearance, lung cellular and cytokine responses, and immunopathology. Our study suggests that marked improvement of clinical outcome and lung immunopathology caused by bacterial superinfection requires the control of both bacterial infection and aberrant host immune responses. Our findings hold important implications in clinical management for influenza-associated bacterial superinfections.

INTRODUCTION

Influenza remains one of the leading causes of deaths from respiratory infections. Influenza predisposes to secondary bacterial superinfection that often occurs during the recovery phase from influenza and accounts for significant hospitalizations and mortality. For instance, during the Spanish flu pandemic of 1918 an estimated 50 to 100 million people died and the secondary bacterial pneumonia was the most common cause of death (1-4). Although the virus that causes seasonal influenza is less virulent and lethal, most hospitalizations and mortality are due to bacterial superinfections or exacerbations of underlying disease (1). With the looming threat of a highly pathogenic influenza virus pandemic and increasing bacterial antibiotic resistance, it is imperative that pandemic preparedness includes not only influenza vaccines and antivirals, but also well-developed protocols to fight bacterial superinfections (5, 6).

Seasonal influenza infection alone typically causes a transient illness in otherwise healthy individuals, and viral clearance is achieved by 7 to 10 days post-infection (7). Bacterial superinfections often occur within the first two weeks of influenza infection, and are caused primarily by *Streptococcus pneumoniae*, *Staphylococcus aureus*, or *Haemophilus influenzae* (6, 8). *S. pneumoniae* is the most common bacterial respiratory tract pathogen and is the fifth leading cause of death worldwide (9). Host defense against the bacterium involves complement-mediated phagocytosis by alveolar macrophages and neutrophils, followed by clearance of apoptotic neutrophils by macrophages (9-11). A number of mechanisms have been identified to mediate influenza-increased susceptibility to bacterial superinfection, including airway epithelial damage, upregulation and

exposure of adhesion receptors, changes in airway function, modification of the cytokine milieu, and impaired innate immune cell function (1, 2, 8, 12).

In addition to impaired bacterial clearance, deleterious lung immunopathology is a prominent feature of influenza-associated bacterial superinfection, characterized by massive neutrophilic infiltration and suppurative bronchopneumonia (6, 13-16). However, the mechanisms underlying such excessive immunopathology have remained poorly understood. Mouse models of influenza and bacterial superinfection have been used to recapitulate the clinical histopathological findings. Mounting evidence suggests a role of the ongoing inflammatory responses to influenza and uncontrolled bacterial replication resulting in excessive neutrophilic infiltration and tissue injury (6, 17-19). However, important questions still remain about whether the poor clinical outcome following bacterial superinfection is due to lung immunopathology or uncontrolled bacterial replication or both, and whether these two events can be uncoupled and effectively controlled by using various intervention strategies (6).

During influenza epidemics and pandemics the use of antibiotic is the mainstay of treatment against bacterial superinfections (12). However, in order to attenuate detrimental immunopathology treatment protocols must be carefully selected. For instance, recent clinical and experimental data suggest that treatment with β-lactam bacteriolytic antibiotics may worsen the outcome of hospitalized patients or lung immunopathology (20-22). The use of bacteriostatic macrolide antibiotics that inhibit bacterial protein synthesis may improve lung immunopathology and control bacterial infection, while other strategies aimed to restore impaired innate immunity may in fact

exacerbate immunopathology (6, 21). On the other hand, while corticosteroids are used in severe cases of influenza infection or community-acquired pneumonia (CAP), there is still no consensus on their benefit particularly in cases of influenza-associated bacterial superinfection (23-29).

In our current study, we have established a murine model of acute respiratory influenza infection and *Streptococcus pneumoniae* superinfection to investigate the mechanisms of severe lung immunopathology and the intervention strategies for effective improvement of clinical outcomes and lung immunopathology. Our study identifies uncontrolled bacterial outgrowth, dysregulated cytokine and chemokine responses, and excessive neutrophil infiltration in the lungs to be the main contributors to deleterious lung immunopathology and death. While the use of a bacteriostatic macrolide antibiotic alone effectively controlled bacterial replication and improved clinical outcome, it failed to significantly reduce lung immunopathology. On the other hand, the use of dexamethasone alone had no impact on bacterial clearance and only a minimum beneficial effect on immunopathology. Strikingly, the use of antibiotic and dexamethasone in combination led to the best clinical outcome with markedly improved survival, bacterial clearance and lung immunopathology. Therefore, our study reveals that the mechanisms of severe lung immunopathology by influenza-associated bacterial superinfection are multifactorial and are in part independent of bacterial burden. Effective intervention strategies need to involve the effective control of both bacterial infection and aberrant host immune responses.

MATERIALS AND METHODS

Mice

Female 7-10 week old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in specific pathogen-free level facilities at the McMaster University Central Animal Facility (Hamilton, Ontario, Canada). For all experiments, mice were euthanized by exsanguination of the abdominal artery under anesthesia. All experiments were conducted in accordance with the animal research ethics board of McMaster University.

Infectious agents

A mouse-adapted strain of influenza A/FM/1/47 (H1N1) was prepared and used as previously described (7, 30). A clinical isolate *Streptococcus pneumoniae* serotype 3 (ATCC 6303) was prepared by plating frozen stock on blood-agar plates and incubating overnight, then culturing the resultant colonies for ~5h at 37°C in 5% CO_2 in Todd Hewitt broth to midlogarithmic phase. The bacteria were harvested and resuspended in PBS. The infectious dose was verified by plating 10-fold dilutions on blood-agar plates.

Heterologous infection model

Mice anesthetized by inhalation of isoflurane were infected intranasally (i.n.) with 10^5 plaque forming units (pfu) of influenza virus in 20 μl of PBS (or PBS only as control). After 7 days, mice were infected intratracheally (i.t.) with 10^3 colony forming units (cfu) of *S. pneumoniae* in 40 μl of PBS (or PBS only as control). Mice were monitored for changes in body weight and other disease symptoms following influenza infection and then at 20, 48, 60, and 72 hours (h) after *S. pneumoniae* infection. Moribund mice were

terminated (usually by 60 hours post-*Strep*) and endpoint was defined as 20% loss of the initial body weight (prior to influenza infection), which occurred between 60h and 72h post-*Strep* for all dual infected mice.

Determination of influenza and streptococcus load in tissue

Influenza levels in the lung were quantified at 7 days post-flu by plaque forming assay using Madin-Darby canine kidney cells, as previously described (7). Levels of streptococcus in the lung, spleen, heart, liver, kidney, and brain were determined by homogenizing the tissues in PBS, plating serial dilutions on blood-agar plates, and incubating overnight at 37°C, 5% CO_2. Colonies were counted and calculated as cfu per whole organ.

Pulmonary histopathologic analysis

Lungs were inflated with 10% formalin and kept in 5 ml of 10% formalin for at least 72h. Tissue sections were stained with hematoxylin and eosin (H&E) for histologic examination. Blinded lung histopathology scoring was performed by using a semiquantitative method ranging from 1 to 10, with 10 being the most extensive pathology.

Bronchoalveolar lavage and lung cell isolation

Airway luminal cells were collected by bronchoalveolar lavage (BAL) of the lungs by using a standard procedure previously described (7). BAL fluids were stored at -20°C until cytokine measurement. Cell pellets were resuspended in complete RPMI (cRPMI) medium (RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine). Mononuclear cells were isolated from lung tissue, as previously described

(7). Briefly, the lungs were cut into small pieces and incubated in 10 ml of 150 U/ml collagenase type I (Sigma-Aldrich, St. Louis, MO) for 1h at 37°C, with agitation. The lung pieces were then crushed through 40-μm basket filters, red blood cells lysed with ACK lysis buffer (0.15M NH_4Cl, 1M $KHCO_3$, 0.1mM Na_2EDTA, pH 7.4), and the remaining cells resuspended in cRPMI.

Flow cytometric analysis

Flow cytometry was performed on BAL and lung cells. Cells were incubated for 15 min. with the CD16/CD32 Fc block antibody, and then stained with extracellular antibodies (BD Biosciences unless otherwise stated) for antigen presenting cells (APC-Cy7-anti-CD45, Alexa Fluor 700-anti-MHCII [eBioscience], Pacific Orange-anti-Gr1 [Invitrogen], APC-anti-CD11c, PECy7-anti-CD11b), NK cells (V450-anti-CD3, PE-anti-NK1.1), and T cells (V450-anti-CD3, APC-Cy7-anti-CD4, PECy7-anti-CD8), in separate wells for each population. Stained cells were run on the LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Myeloperoxidase assay

Myeloperoxidase (MPO) was measured in total lung tissue as previously described (19, 31). Briefly, lungs were homogenized in 1 ml PBS, the pellets were resuspended in Hexadecyltrimethylammonium bromide (HTAB) buffer (Sigma-Aldrich, Oakville, Ontario, Canada) to a concentration of 100 mg/ml, homogenized again, and the supernatant was frozen at -80°C until the assay (performed no more than 7 days later). 10 μl of supernatant were added to 200 μl of the reaction mixture (16.7 mg o-dianisidine [Sigma-Aldrich], 90 μl dH_2O, 10 μl of 50 mM dibasic potassium-phosphate buffer [EMD

Chemicals], and 50 μl of 1% H_2O_2) in a 96-well flat-bottom plate. Absorbance was measured at 450 nm in a spectrophotometer at one minute intervals. MPO activity is expressed as units per gram of lung tissue, where 1 U MPO is defined as the amount of enzyme capable of degrading 1 μmol H_2O_2/min at room temperature.

Cytokine and chemokine quantification

Levels of cytokines and chemokines in BAL fluid and lung homogenates were quantified using Luminex multianalyte technology (Luminex Molecular Diagnostics, Toronto, ON, Canada), according to the manufacturer's protocols.

***In vivo* MIP-2 chemokine neutralization and neutrophil depletion**

For MIP-2 neutralization, 200 μg of rabbit anti-murine MIP-2 serum or normal rabbit serum as control were injected intraperitoneally (i.p.) into dual infected mice (7 days after influenza) at 10h before-*Strep*, as well as at 20h and 40h post-*Strep*. Mice were sacrificed at 60h post-*Strep*. For neutrophil depletion, anti-Ly6G (1A8) antibody (UCSF Monoclonal Antibody Core, San Francisco, CA, USA) or total rat IgG as control were given to dual infected mice as follows: at 8h before-*Strep* 200 μg i.p., at 16h post-*Strep* 200 μg i.p. + 100 μg i.n., and at 40h post-*Strep* 350 μg i.p. Mice were sacrificed at 60h post-*Strep*.

Antibiotic and corticosteroid treatment

Dual infected mice were injected with 4 mg/kg of the corticosteroid dexamethasone (DEX) (PCCA, Houston, Texas, USA) i.p. at 15h and 29h post-*Strep*. Other mice were injected with 10 mg/kg of the antibiotic azithromycin (AZT) (Sandoz Canada Inc., Quebec, Canada) i.p. at 30h and with 5 mg/kg at 44h post-*Strep*. Some mice were treated

with both DEX and AZT at the indicated timepoints, while control mice were given only PBS at those timepoints.

Statistical analysis

To determine significance between infection groups, Student's *t* test was used for two-sample comparisons. Otherwise, one-way ANOVA and Tukey's post hoc test were performed with Prism (GraphPad Software, Inc., La Jolla, CA, USA). A p value of < 0.05 was regarded as statistically significant.

RESULTS

Influenza infection increases susceptibility to pneumococcal superinfection

To determine the effect of influenza infection on subsequent bacterial superinfection, mice were infected intranasally with a nonlethal dose of influenza virus. By 7 days post-infection, the virus was almost completely cleared from the lungs (data not shown). At this point, mice were infected intratracheally with a very small dose (10^3 cfu) of *S. pneumoniae* and sacrificed at various time points after infection (Fig. 1A). Dual infected mice are designated as 'Flu/Strep', while the mice that were infected only with influenza are 'Flu' and mice only infected with *S. pneumoniae* are 'Strep'. Body weight changes and survival were monitored as a measure of overall illness or clinical outcome. Flu mice experienced no weight loss, and all groups of mice gained approximately 5% of initial body weight over the course of 7 days by the timepoint of streptococcus infection at 0 hours (Fig. 1B). Over the next 60 hours, Flu mice continued to gain weight and Strep mice lost minimal weight (< 5%), while Flu/Strep mice on average had almost reached

endpoint (20% weight loss) (Fig. 1B). Some Flu/Strep mice did reach endpoint by 60h post-*Strep* (28% lethality), and 100% lethality was seen by 72h (Fig. 1C).

Consistent with the illness and lethality, the Flu/Strep mice had significantly higher lung bacterial loads than the Strep mice at all timepoints (Fig. 1D). The bacterial load in the lungs of Strep mice decreased between 48h and 60h post-infection (Fig. 1D) and was cleared by 5 days (data not shown), while the Flu/Strep mice had consistently increasing bacterial loads up to endpoint at 60h (Fig. 1D). Bacterial dissemination past the lung was not observed in Strep mice, while Flu/Strep mice had increasing levels of bacterial burden in the spleen from 20h to 60h post-*Strep* (Fig. 1D), and marked systemic dissemination to other organs including the heart, liver, kidney, and brain at 60h (Fig. 1E). These results show that infection with a nonlethal dose of influenza increases susceptibility to streptococcus infection, causing an otherwise small inoculum of *S. pneumoniae* to lead to bacterial outgrowth, wide systemic dissemination, and 100% lethality within 3 days post-infection.

In order to determine if the influenza-increased susceptibility to bacterial superinfection is sustained longer than 7 days, mice were infected with *S. pneumoniae* 11 days or 21 days following influenza and sacrificed at 60h post-*Strep*. After infection at 11 days post-influenza, Flu/Strep mice still suffered significantly higher bacterial loads in the lung and systemic dissemination to the spleen compared to Strep mice, while there was no significant difference at 21 days post-influenza (data not shown). This suggests that the effects of influenza on susceptibility to superinfection are sustained out at least to 11 days following the initial influenza infection.

Severe lung immunopathology by pneumococcal superinfection

To further investigate the cause of illness and death in Flu/Strep mice, we examined histopathological changes in the lung. At 9 to 10 days following influenza infection when the virus is cleared (data not shown), the Flu mice had minimal residual inflammation in the lung (Table 1). After low-dose *S. pneumoniae* infection the Strep mice displayed minimal lung inflammation, with only slight neutrophil infiltration seen at 48h and 60h post-infection (Fig. 2 and Table 1). In contrast, *S. pneumoniae* superinfection in Flu/Strep mice caused severe lung immunopathology and tissue injury, which increased from 20h post-infection to endpoint at 60h (Fig. 2, Table 1). Immunopathology was associated with excessive neutrophilia, including the appearance of apoptotic and necrotic neutrophils, particularly at 60h (Fig. 2C). Further evidence of lung injury included large areas of consolidation spreading to whole lung lobes by 60h post-*Strep*, degeneration and sloughing of airway epithelium, hemorrhaging, metaplasia, and plugged airways filled with neutrophils, cell debris, and mucus (Fig. 2). Pleuritis was observed on the lung surfaces, including excessive amounts of mucus and lung fluid exudate that filled the chest cavity (Fig. 2). These findings further support the role of influenza in predisposing to subsequent bacterial superinfection that leads to severe lung immunopathology underlying illness and death.

Excessive neutrophil infiltration in the lungs following pneumococcal superinfection

Given severe tissue neutrophilia and immunopathology seen in the lung of Flu/Strep hosts, we further dissected neutrophilic responses by quantifying neutrophils both in the bronchoalveolar lavage (BAL) fluids and lung tissue at various timepoints by flow cytometry. Excessive neutrophil influx was observed in the BAL and lung tissue of Flu/Strep mice between 12h and 60h post-*Strep*, and was significantly higher than in the other groups (Fig. 3A). There were no significant differences in macrophage and dendritic cell numbers between the groups, and T cell numbers were slightly lower in the Flu/Strep mice than in Flu mice at 60h (data not shown).

Myeloperoxidase (MPO) activity was also measured as an indication of neutrophil anti-bacterial functionality in lung homogenates at 48h and 60h post-*Strep*. At the 48h timepoint, while there were 8-fold more neutrophils in the lungs of Flu/Strep mice compared to Strep mice (Fig. 3A), the MPO activity was only 5-fold higher (Fig. 3B). Furthermore, at 60h post-infection MPO activity was the same between the two groups (Fig. 3B) even when neutrophil numbers remained significantly (4-fold) higher in the Flu/Strep mice (Fig. 3A), suggesting that neutrophil anti-bacterial activity on a per cell basis was impaired in the dual-infected mice. These results suggest that the overwhelming tissue neutrophilia and the resulting lung immunopathology may have contributed to illness and death of dual-infected mice. In addition, impaired neutrophil anti-bacterial functionality in previous influenza-imprinted lungs could be the cause for impaired clearance of pneumococci from the lungs, leading to its systemic dissemination.

Aberrant cytokine and chemokine responses following pneumococcal superinfection

To investigate the potential molecular mechanisms leading to overwhelming tissue neutrophilia and immunopathology of Flu/Strep mice, we examined cytokine responses in the lung. Cytokine and chemokine levels were assessed in lung homogenates and BAL fluids at 12h, 20h, and 48h following *S. pneumoniae* infection. Flu infected mice had low, naïve levels of all analytes measured in the lung, while Strep mice had slight increases in some analytes at 48h post-infection (Fig. 4). In contrast, the levels of most cytokines and chemokines measured including IFN-γ, TNF, KC and MIP-2 were much higher between 20h and 48h in the lungs of Flu/Strep mice (Fig. 4). This corresponded with the sharp increase in wide systemic bacterial dissemination (Fig. 1D, E) and the rapid weight loss observed (Fig. 1B). Other cytokines and chemokines IFN-β, IL-1β, GM-CSF, IL-17, IL-10, MCP-1, MIP-1α, MIP-1β, IP-10, and RANTES were also significantly increased while IL-12 and IL-15 did not increase in Flu/Strep mice (data not shown). Corresponding increases in the same analytes were observed in the BAL fluids (data not shown). The levels of the neutrophil attracting chemokines KC and MIP-2 were induced to much higher levels (up to 50,000 pg/ml concentrations) than any other increased analytes measured, in accordance with overwhelming neutrophil influx into the lungs (Fig. 3). This data suggests that overwhelming cytokine and chemokine responses culminating in a 'cytokine storm' in the lungs of dual infected animals contributed to the influenza-induced susceptibility to lung neutrophilia, severe immunopathology, and fatal clinical outcome.

Effects of MIP-2 neutralization and neutrophil depletion on lung immunopathology and clinical outcome of dual infected hosts

Chemokine overexpression (Fig. 4) and excessive neutrophil influx into the lungs (Fig. 3) were characteristic of dual infected mice, and correlated with severe lung immunopathology (Fig. 2) and fatal clinical outcome (Fig. 1C). We therefore set out to investigate whether *in vivo* neutralization of the neutrophil chemokine MIP-2 which was the most highly induced analyte measured (Fig. 4) would significantly dampen severe lung immunopathology and improve the clinical outcome in Flu/Strep animals. Dual infected mice were treated with three doses of anti-MIP-2 serum beginning at 10h before *S. pneumoniae* infection and sacrificed at 60h. MIP-2 neutralization did not lead to an improvement in weight loss or bacterial burden compared to control mice (data not shown). Furthermore, histopathological assessment showed no changes in lung immunopathology following MIP-2 neutralization compared to the control animals (Table 2). These results suggest that neutralization of one selected chemokine may be insufficient to improve lung immunopathology and clinical outcome, as other functionally redundant chemokines such as KC were also highly induced in Flu/Strep hosts. Based on this consideration, in separate experiments we went on to *in vivo* deplete neutrophils by using three repeated doses of anti-Ly6G antibody beginning at 8h before *S. pneumoniae* infection and the mice were sacrificed at 60h. While similar to MIP-2 depletion, neutrophil depletion did not lead to an improvement in body weight losses or altered bacterial burden (data not shown), it led to somewhat improved lung immunopathology, though moderate lung inflammation and injury associated with residual neutrophils were

still observed in the lung parenchyma (Table 2). These results suggest that firstly, excessive neutrophil infiltration contributes to severe lung immunopathology and injury in Flu/Strep animals. Secondly, neutrophils have impaired anti-bacterial activities due to discordant MPO activity (Fig. 3B) and the observed unaltered bacterial burden in the lungs of neutrophil-depleted Flu/Strep animals.

Marked improvement of lung immunopathology and clinical outcome by combined antibiotic and corticosteroid treatment in dual infected hosts

Our data so far has shown that neutrophil depletion led to only partially improved lung immunopathology, but had no altering effects on bacterial burden and clinical performance of Flu/Strep animals (Table 2 and data not shown). This observation suggests that lung immunopathology and bacterial clearance may be independently regulated on one hand, and that a significant improvement in clinical outcome of these animals may require the stringent control of both bacterial infection and lung immunopathology on the other hand. This consideration led us to next examine antibiotic and/or corticosteroid treatment options in dual infected mice. To this end, Flu/Strep mice were treated with a bacteriostatic, macrolide antibiotic azithromycin (AZT) and/or the global inflammatory/immune inhibitor corticosteroid dexamethasone (DEX) from 15-44h post-*Strep* and sacrificed at 60h (Fig. 5A). Untreated (PBS) mice expectedly lost almost 20% of body weight and some reached the endpoint by 60h, and the DEX treated mice had the similar poor clinical outcome (Fig. 5B). In comparison, AZT alone treated mice stopped losing weight soon after the first dose was given, and regained weight to almost pre-infection levels by 60h, while the mice treated with both DEX and AZT had the best

clinical outcome with the most rapid recovery in their body weight (Fig. 5B). The weight changes were significantly different between the PBS and DEX groups versus the AZT and DEX/AZT groups at the later timepoints, and the difference in body weight recovery rates was also significantly different between the AZT and DEX/AZT groups at 44h (Fig. 5B). This data shows that clinical outcome is most efficiently improved by treatment with both a bacteriostatic antibiotic azithromycin and a global inflammatory/immune inhibitor dexamethasone.

To dissect the mechanisms for improved clinical outcome by AZT or DEX/AZT treatment, we first examined the bacterial burden in the lung and spleen. In accordance with improved clinical outcome, mice treated with DEX/AZT or AZT had most significantly decreased bacterial burden in the lungs and led to no systemic bacterial dissemination to the spleen, while DEX treated mice had the same levels of bacteria as untreated control mice (Fig. 6A). We further examined the cellular and cytokine responses in the lung of these animals. Neutrophil influx into both the BAL and lung tissue was significantly lower in AZT and DEX/AZT mice, and slightly lower in DEX mice when compared to PBS mice (Fig 6B). On the other hand, levels of the cytokines and chemokines IFN-γ, TNF, MIP-1α, KC, MIP-2 and MCP-1 (Fig. 6C) were significantly higher in the untreated PBS group compared to all other groups. Of all groups, the combined DEX and AZT treatment resulted in the greatest overall level of reduction in cytokine and chemokine responses (Fig. 6C), in keeping with the most improved clinical outcome (Fig. 5B). In comparison, AZT treatment alone only moderately reduced TNF, MIP-1α and MCP-1 while it markedly reduced IFN-γ, KC and

MIP-2 (Fig. 6C). DEX treatment alone only moderately, but significantly, reduced almost all cytokines examined (Fig. 6C), in contrast to its negligible modulating effect on clinical outcome (Fig. 5B) and bacterial burden (Fig. 6A).

Upon histopathological examination, we found that corresponding with the best clinical outcome (Fig. 5B), the most reduced bacterial burden (Fig. 6A), and the most reduced neutrophilic (Fig. 6B) and cytokine/chemokine responses (Fig. 6C) achieved by the combined DEX and AZT treatment, the apparent and dramatic improvement in lung immunopathology was observed only in DEX/AZT animals (Fig. 7A-D and Table 3). Despite its significant improving effects on clinical outcome and bacterial burden (Fig. 5, 6), AZT treatment alone only modestly improved lung immunopathology (Fig 7B and Table 3). A significant level of immunopathology in AZT animals persisted up to 144h, well beyond the point of complete bacterial clearance (88h) (data not shown). On the other hand, in accordance with unaltered clinical outcome and bacterial burden, and moderately reduced cytokine responses (Fig. 5, 6), DEX alone hardly improved lung immunopathology (Fig. 7C). Overall, the above results indicate that firstly, multiple cellular and molecular mechanisms are involved in increased susceptibility of influenza infected hosts to pneumococcal superinfection and that targeting a single pathway will be ineffective. Secondly, the significant improvement in lung immunopathology and clinical outcome in Flu/Strep hosts requires the effective control of both bacterial infection and host immune responses. Thirdly, bacterial clearance and lung immunopathology can be two uncoupled, independently regulated processes, and thus clinically effective control of

bacterial infection alone may not lead to markedly improved lung immunopathology in dual infected hosts.

DISCUSSION

Secondary bacterial superinfections following influenza are major contributors to hospitalizations and mortality. With increasing bacterial antibiotic resistance and a continuous threat of an influenza virus pandemic, well-developed protocols to fight such heterologous infections must be in place (1, 4-6). Importantly, the severely exacerbated lung immunopathology associated with bacterial superinfections must be addressed during treatment (6, 13-16, 32). Also, the underlying mechanisms of such excessive immunopathology need to be further investigated (6, 8, 17-19). Using a mouse model of acute respiratory influenza infection and *Streptococcus pneumoniae* superinfection, we have investigated these questions. We show that uncontrolled bacterial outgrowth and systemic dissemination, as well as dysregulated host immune responses including enhanced cytokine and chemokine responses and excessive neutrophil infiltration are the main contributors to severe lung immunopathology and death. We provide novel information that targeting a single immune cell type or molecular pathway is ineffective. We have identified that the most effective treatment protocol for pneumococcal superinfection following influenza requires both bacteriostatic antibiotic and corticosteroid treatment, and that mere antibiotic control of bacterial infection is ineffective in improving lung immunopathology.

Our model of dual infection, where mice were infected with a nonlethal dose of influenza followed 7 days later with a very small dose of *S. pneumoniae* recapitulated the well-established clinical observation that influenza infection markedly increases susceptibility to pneumococcal superinfection. The Flu/Strep mice lost over 20% of body weight and reached 100% mortality by 72h. Consistent with their severe illness and lethality, Flu/Strep mice had significantly higher lung bacterial loads than Strep mice, as well as dissemination to other organs including the spleen, heart, liver, kidney, and brain. Our further investigation into the potential mechanisms leading to bacterial outgrowth and lethality in Flu/Strep mice showed that cytokine and chemokine responses following streptococcus superinfection were severely dysregulated. In fact, most cytokines and chemokines measured were markedly increased in the Flu/Strep lungs compared to the Strep and Flu alone groups. This corresponded with the observed wide systemic bacterial dissemination and rapid weight losses. Enhanced cytokine and chemokine expression has been previously reported in most models of bacterial superinfection following influenza, whereas some studies have also reported influenza-induced defects in cytokine and chemokine expression (33-36). The occurrence of ongoing inflammatory responses to influenza and a 'cytokine storm' has been suggested to be one of the mechanisms for exacerbated lung immunopathology after bacterial superinfection, and our model supports this notion (1, 6). In addition, enhanced chemokine responses have been shown to lead to excessive inflammatory cell infiltration, particularly that of neutrophils (17-19, 33, 37, 38). In our model, excessive neutrophil influx was observed in Flu/Strep mice corresponding to highly expressed KC and MIP-2 chemokines, which were the two most

induced analytes measured. Also, in accordance with previous observations, neutrophil functionality measured by myeloperoxidase activity was impaired in the Flu/Strep mice (19, 33, 36, 39). Thus the overwhelming number of neutrophils in the lung, some of which were not functional at clearing the bacteria, contributed to lung immunopathology and ill-controlled bacterial infection.

In our study, we addressed the largely understudied mechanisms of increased immunopathology following bacterial superinfection. As was previously observed in other models, bacterial superinfection following influenza caused severe lung immunopathology and tissue injury, with excessive neutrophilia, large areas of consolidation, degeneration of airway epithelium, hemorrhaging, and plugged airways (8, 19, 37). However, questions still remain about whether the poor clinical outcome following superinfection is due to lung immunopathology or uncontrolled bacterial replication and dissemination, or both (6). Uncoupling these factors or processes proved to be difficult in the past. We found that neutralization of the neutrophil chemokine MIP-2 did not impact bacterial burden, and did not improve clinical outcome or affect lung immunopathology. These results suggest that the highly induced chemokines including KC played a compensatory role in recruiting neutrophils into the lung, and together with the ‘cytokine storm’ and the bacterial outgrowth, caused lethality in the Flu/Strep mice. We then depleted neutrophils and found that this did not change bacterial loads either, further showing that the excessive neutrophil numbers in the lung are not helpful in clearing the infection, especially as these cells were shown to be partially functionally impaired. However, neutrophil depletion did cause modestly to moderately improved

immunopathology illustrating that lung immunopathology is dependent in part on neutrophil influx. As clinical outcome was not improved in neutrophil-depleted hosts, bacterial outgrowth and systemic dissemination appeared to be the important factors for mortality. Therefore, we next investigated a treatment protocol involving antibiotic use, in order to clear the bacteria beginning at 30h post-*Strep* when marked lung immunopathology is clearly observed in Flu/Strep mice. We used a non-lytic, bacteriostatic, macrolide antibiotic azithromycin, as studies have shown that treatment with bactericidal β-lactam antibiotics can worsen clinical outcome and pathology (6, 20-22). All AZT treated mice eventually recovered, and expectedly had significantly decreased bacterial burden in the lungs with no systemic bacterial dissemination, but they demonstrated only moderately reduced cytokine responses and neutrophil influx in the lungs. Thus, marked lung immunopathology was still observed in AZT treated mice and sustained even to later timepoints after bacteria were fully cleared. This is an important observation and it, for the first time, uncouples bacterial infection and lung immunopathology in Flu/Strep animals. As unresolved lung pathology can cause chronic obstructive pulmonary disease, after the infectious agents are cleared, we modified the treatment protocol to include the corticosteroid dexamethasone. While corticosteroids are used in influenza infection and community-acquired pneumonia, there has been no consensus on their benefit particularly in cases of secondary bacterial superinfection, and use of corticosteroids in animal models of dual infection has been scarcely attempted (23-29, 40). In our study, we show that while DEX treatment alone did not improve survival, DEX/AZT treated mice had the quickest recovery, showing that the clinical outcome is

most efficiently improved by treatment with both azithromycin and dexamethasone. Furthermore, DEX/AZT mice had most significantly decreased bacterial burden in the lungs with no systemic dissemination, no excessive neutrophil influx into the lungs, and the lowest cytokine and chemokine expression of all the groups. Importantly, vast improvement of lung immunopathology was seen only in DEX/AZT treated mice when compared to the AZT or DEX alone treated animals.

In conclusion, our results indicate that both the clinical outcome and severe lung immunopathology are most efficiently improved only by treatment with both antibiotic and corticosteroid, and antibiotic treatment alone is insufficient. In other words, marked improvement of severe lung immunopathology and illness caused by influenza-associated pneumococcal superinfection cannot be accomplished unless both bacterial infection and host immune responses are under effective control. Our findings hold important implications in future clinical practice. During influenza epidemics and pandemics, well-developed protocols must be in place to fight bacterial superinfections, which are the largest contributors to hospitalizations and mortality of otherwise healthy patients. While antibiotic treatment will remain essential in clinical practice, concurrent use of corticosteroids is imperative in preventing complicating lung immunopathology and injury.

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Table 1. Semi-quantification of histopathological changes in the lung.

	Flu			Strep			Flu/Strep		
	20h	**48h**	**60h**	**20h**	**48h**	**60h**	**20h**	**48h**	**60h**
Neutrophilia	ND	ND	-	+	+	+	++	++++	+++++
Inflammation	ND	ND	+	-	-/+	+	++	++++	+++++
Injury	ND	ND	+	-	-/+	-/+	++	++++	+++++

Tissue neutrophilia, overall lung inflammation (mononuclear cell infiltration), and *lung injury* (consolidation, degeneration of airway epithelium, necrosis, hemorrhaging, metaplasia) were assessed. Results are representative of 3-5 experiments, n=5-10 mice/group/timepoint. −, absent; +, minimal; ++, slight; +++, moderate; ++++, marked; +++++, severe

Table 2. Assessment of histopathological changes in the lung after immunotherapy.

	Control	**α-MIP-2**	**α-Ly6G**
Neutrophilia	+++++	+++++	++
Inflammation	+++++	+++++	+++
Injury	+++++	+++++	+++

Tissue neutrophilia, overall lung inflammation, and *lung injury* were assessed in control or treated Flu/Strep mice at 60h post-*Strep*. Results are representative of 1-2 experiments, n=7-14 mice per group. −, absent; +, minimal; ++, slight; +++, moderate; ++++, marked; +++++, severe

Table 3. Semi-quantification of histopathological changes in the lung following AZT and DEX.

	PBS	**AZT**	**DEX**	**DEX / AZT**
Neutrophilia	+++++	+++	+++++	+
Inflammation	+++++	++++	+++++	+
Injury	+++++	++++	+++++	+

Tissue neutrophilia, overall lung inflammation, and *lung injury* were assessed in control (PBS) or treated Flu/Strep mice at 60h post-*Strep*. Results are representative of 2-3 experiments, n=12 mice per group. −, absent; +, minimal; ++, slight; +++, moderate; ++++, marked; +++++, severe

Figures

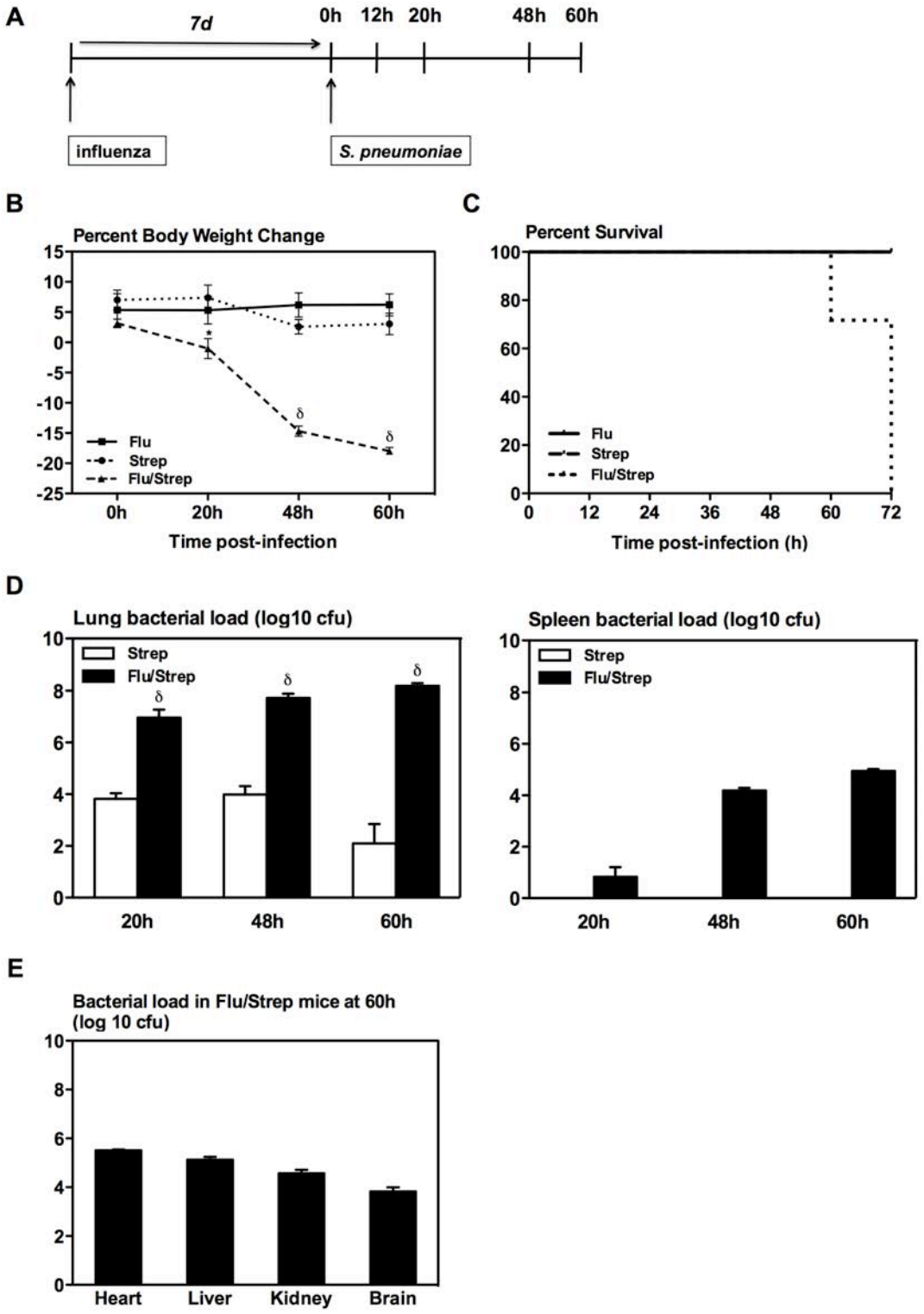
A
7d
0h 12h 20h 48h 60h
influenza
S. pneumoniae
B
Percent Body Weight Change
Flu
Strep
Flu/Strep
Time post-infection
C
Percent Survival
Flu
Strep
Flu/Strep
Time post-infection (h)
D
Lung bacterial load (log10 cfu)
Strep
Flu/Strep
20h 48h 60h
Spleen bacterial load (log10 cfu)
Strep
Flu/Strep
20h 48h 60h
E
Bacterial load in Flu/Strep mice at 60h
(log 10 cfu)
Heart Liver Kidney Brain

Figure 1. Influenza infection causes increased susceptibility to pneumococcal superinfection. ***A***, Experimental schema. Female C57BL/6 mice were infected with 10^5 pfu of influenza virus and 7 days later with 10^3 cfu of *S. pneumoniae*. ***B***, Average percent body weight change. ***C***, Percent survival. Results are from multiple experiments, n=10-44/group/timepoint. ***D***, Lung and spleen bacterial load in Strep and Flu/Strep mice was measured with a CFU assay at 20h, 48h, and 60h post-*Strep* infection. Results are from 2-4 independent experiments, n=10-34 /group/timepoint. ***E***, Heart, liver, kidney, and brain bacterial load in Flu/Strep mice was measured at 60h post-*Strep*. Results are from 1-2 experiments, n=6-10. Data are expressed as Mean ± SEM. *, $p < 0.05$; δ, $p < 0.0001$.

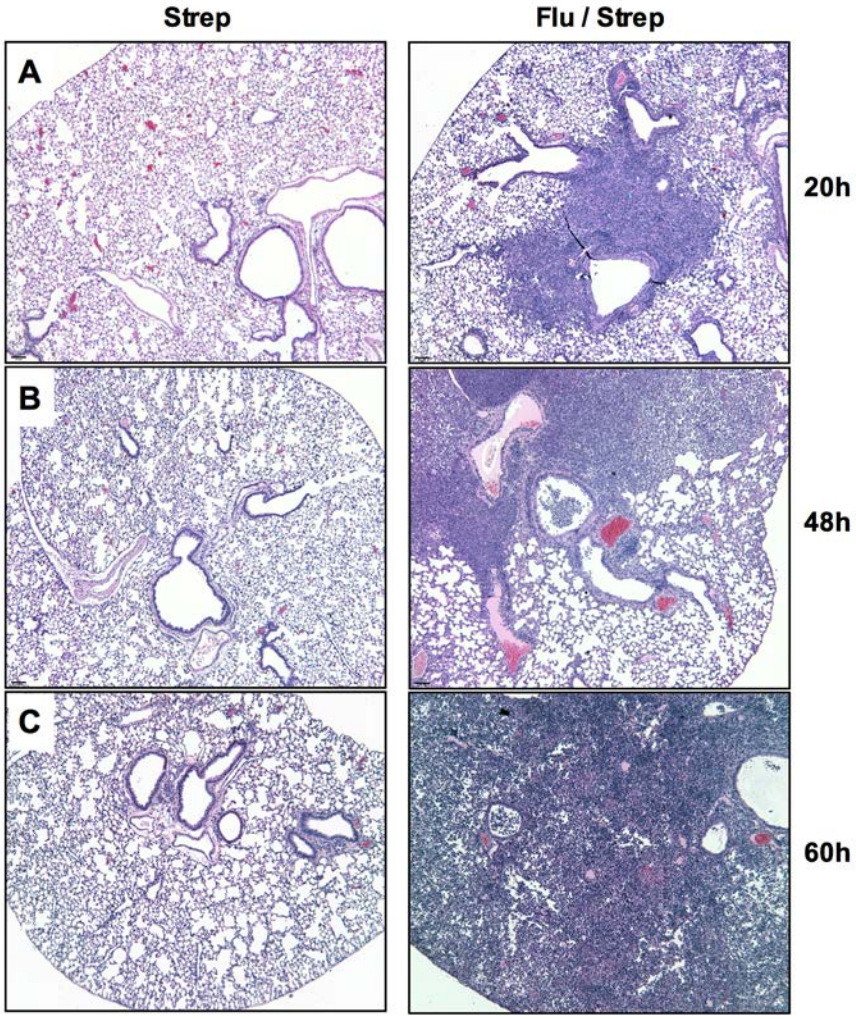

Figure 2. Severe lung immunopathology following pneumococcal superinfection in influenza-infected hosts. Histopathological changes were examined by H&E staining in the lungs of Strep and Flu/Strep mice at ***A,*** 20h, ***B***, 48h, and ***C***, 60h post-*Strep* infection. Results are representative of 3-5 experiments, n=5-10 /group/timepoint.

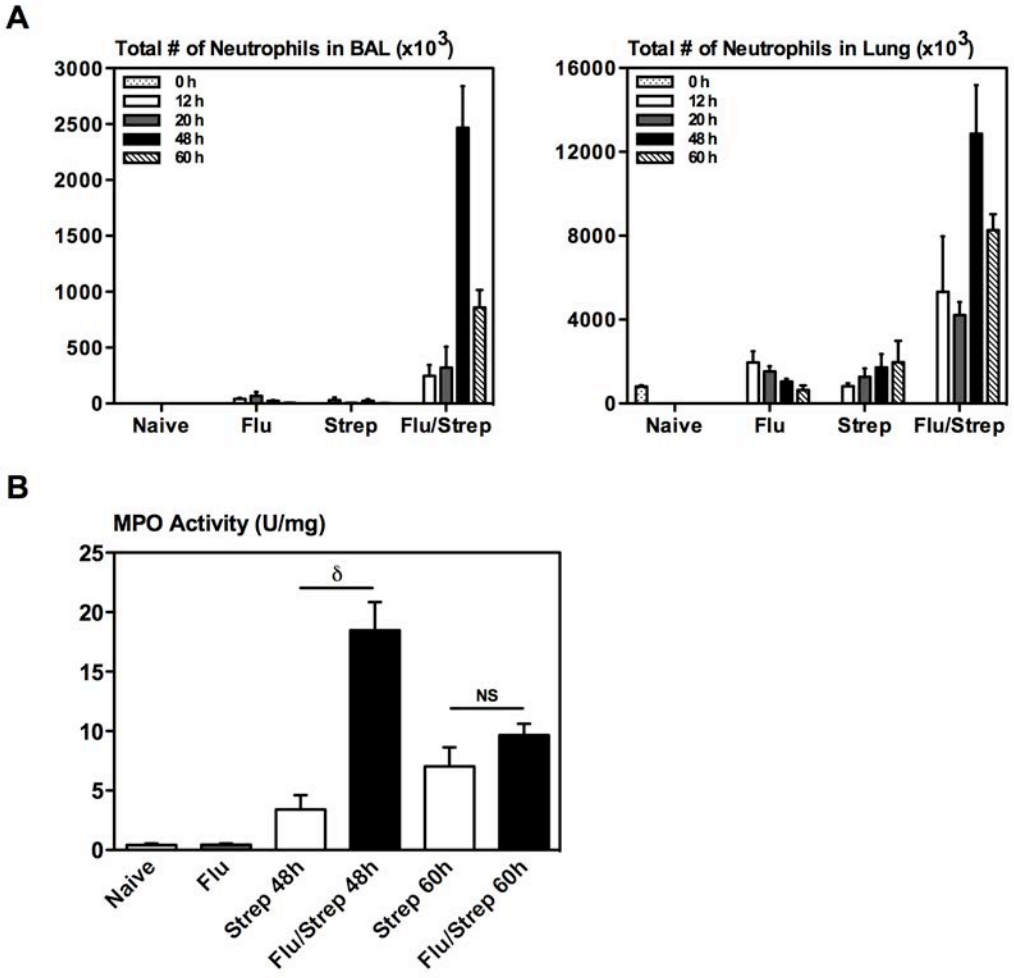

Figure 3. Excessive neutrophil infiltration in the lungs following pneumococcal superinfection in influenza-infected hosts. ***A***, Neutrophil (CD11b+Gr1+) influx into the BAL and lung was assessed by flow cytometry in naïve, Flu, Strep, and Flu/Strep mice at various timepoints post-*Strep* infection. Results are total neutrophil numbers in the tissue compartment, from 4 experiments, n=4-10 /group/timepoint. Data are expressed as Mean ± SEM. ***B***, Neutrophil activity was measured with a MPO assay of lung homogenates at 48h and 60h post-*Strep*. Results are from 2 experiments, n=6 /group/timepoint. Data are expressed as Mean ± SEM. δ, $p < 0.0001$.

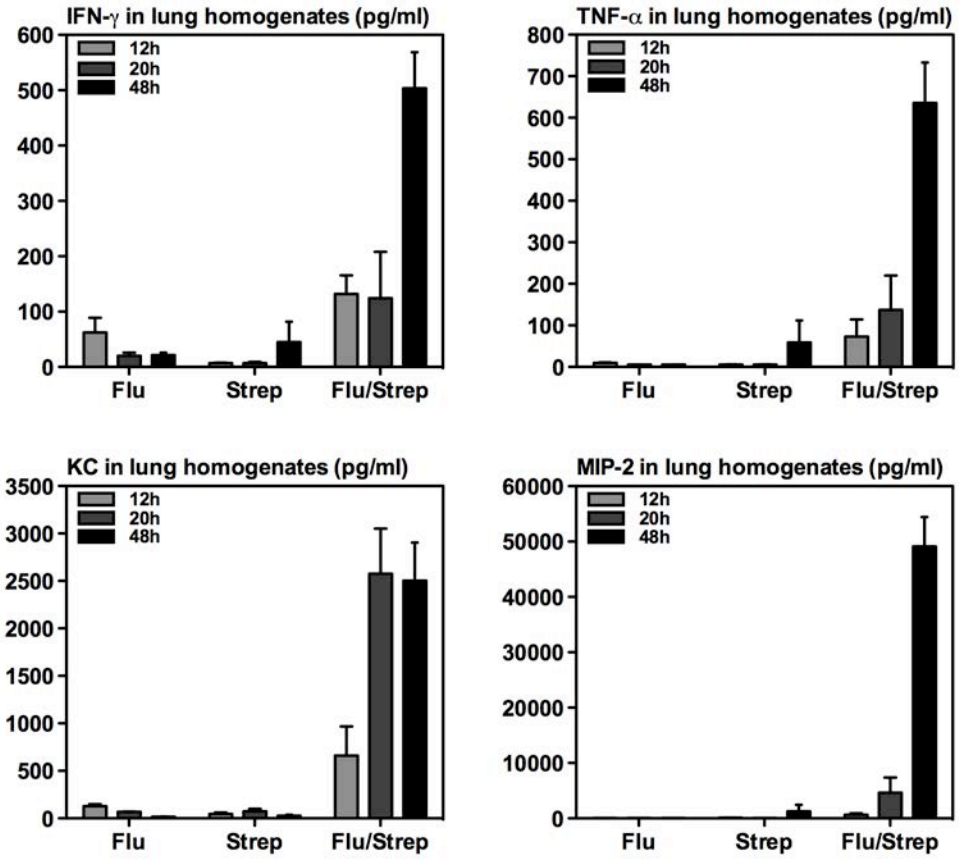

Figure 4. Dysregulated cytokine and chemokine responses in the lungs following pneumococcal superinfection in influenza-infected hosts. Cytokine and chemokine levels in lung homogenates were measured in Flu, Strep and Flu/Strep mice at 12h, 20h, and 48h post-*Strep* infection. Results are from 3 experiments, n=3-4 /group/timepoint. Data are expressed as Mean ± SEM.

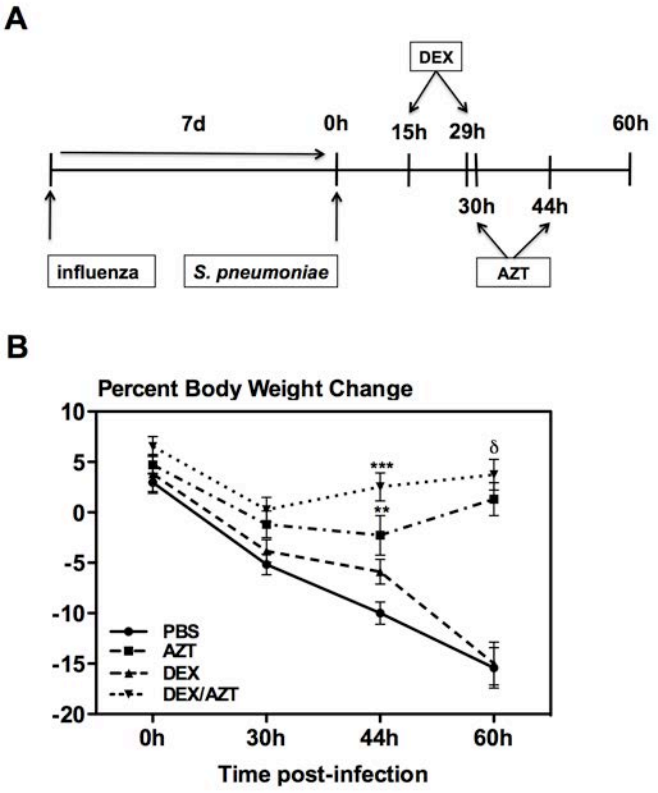

Figure 5. Combined antibiotic and corticosteroid treatment improves clinical outcome of dual infected mice. ***A***, Experimental schema. Mice were infected with 10^5 pfu of influenza virus and 7 days later with 10^3 cfu of *S. pneumoniae*. Dexamethasone (DEX) was given at 15h and 29h, azithromycin (AZT) was given at 30h and 44h post-*Strep* and mice were sacrificed at 60h. ***B***, Average percent body weight changes. Results are from 2-3 experiments, n=14-21 per group. Data are expressed as Mean ± SEM. **, $p < 0.01$; ***, $p < 0.001$; δ, $p < 0.0001$ as compared to the PBS group.

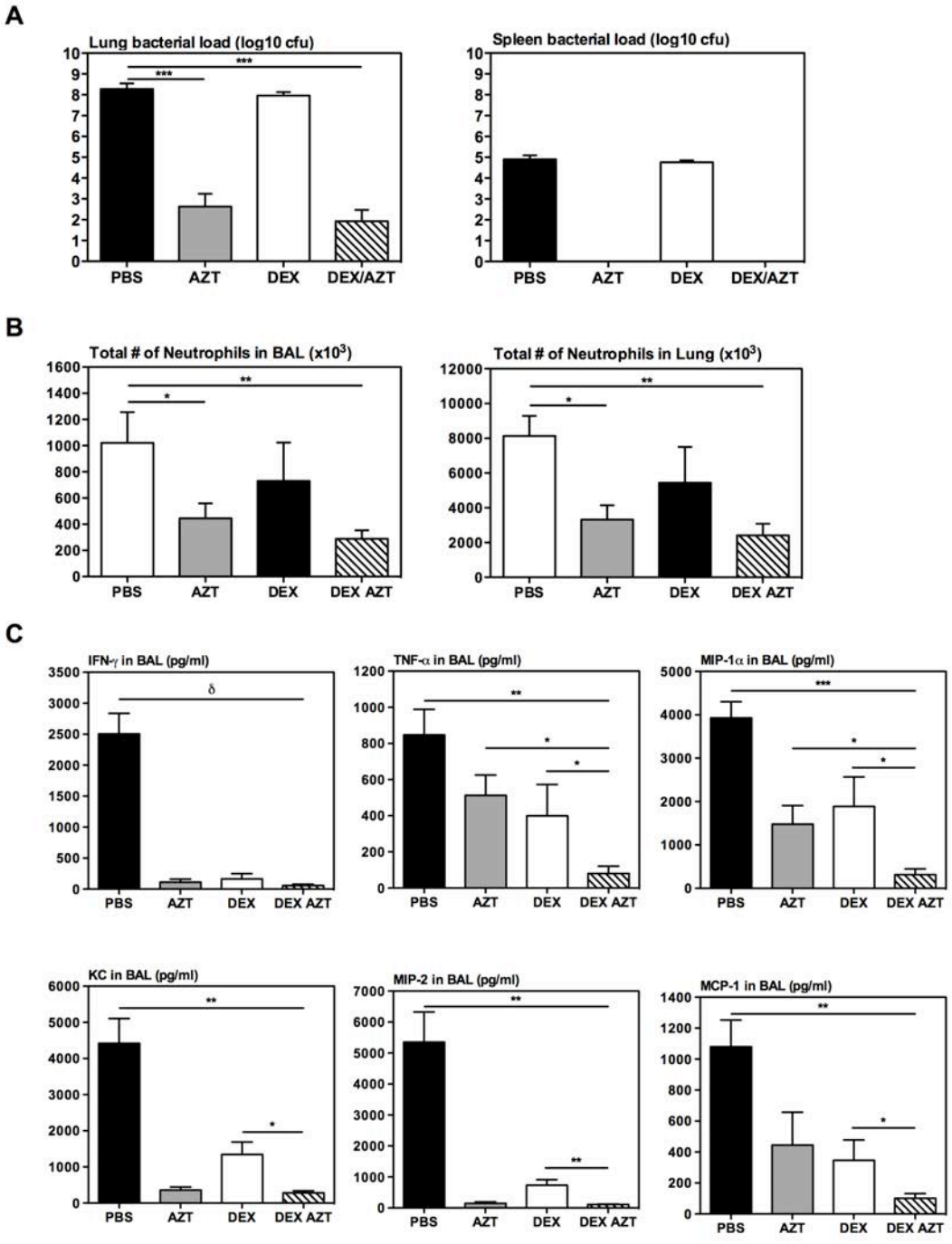

Figure 6. Combined antibiotic and corticosteroid treatment markedly decreases bacterial load, neutrophil influx, and cytokine responses in the lungs of dual infected mice. ***A***, Lung and spleen bacterial load was measured with a CFU assay. ***B***, Total neutrophil numbers in the BAL and lungs were assessed by flow cytometry. ***C***, Cytokine and chemokine levels in BAL fluids were measured. Results are from 2-3 experiments, n=11-15 per group. Mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; δ, $p < 0.0001$.

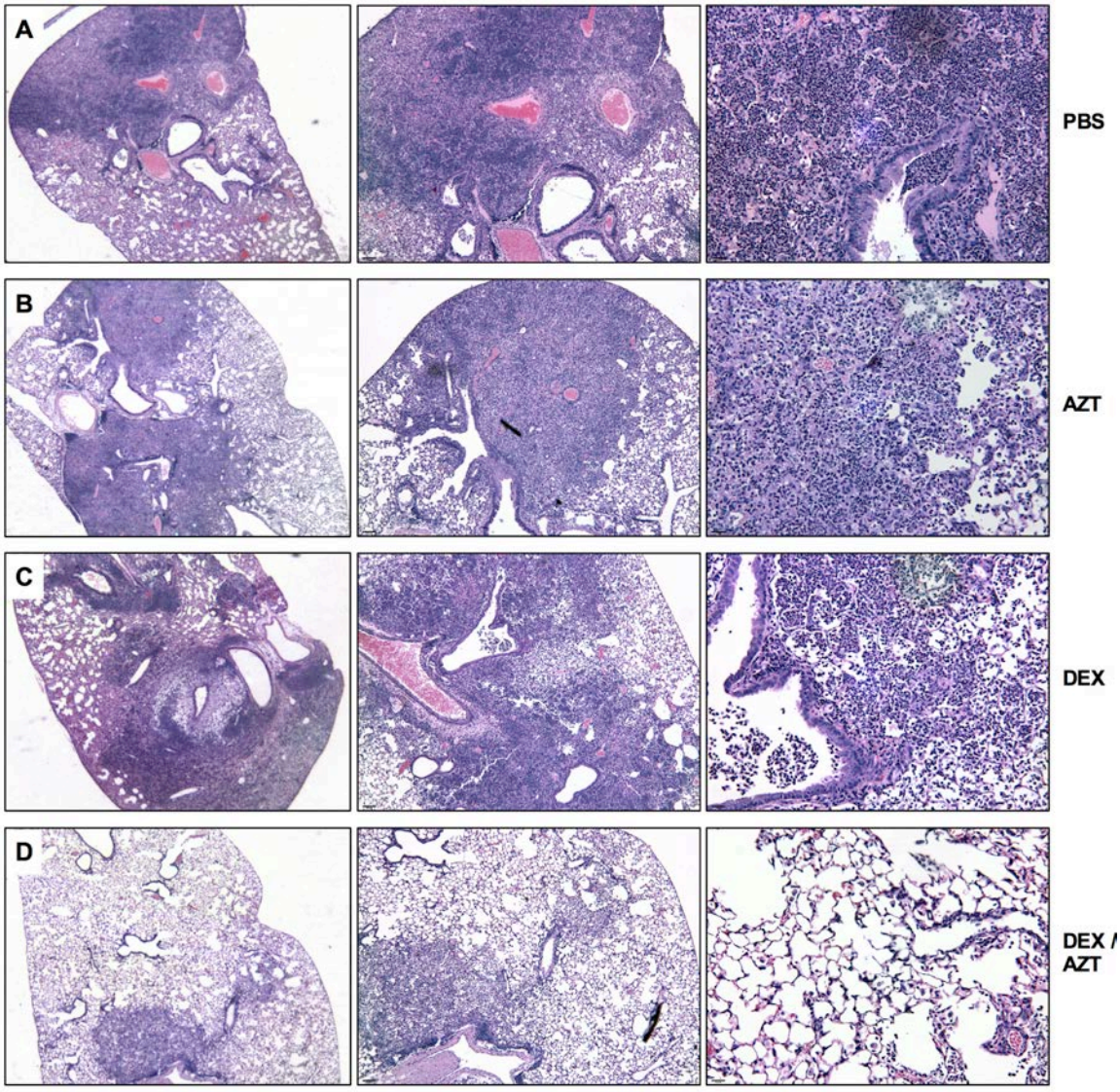

Figure 7. Combined antibiotic and corticosteroid treatment markedly improves lung immunopathology of dual infected mice. Histopathological changes were examined in the lungs of ***A,*** PBS, ***B***, AZT, ***C***, DEX, and ***D***, DEX/AZT treated mice. Magnifications 1.6x (left column), 5x (middle), 20x (right). Results are from 2-3 experiments, n=12 per group.

CHAPTER 4: Transgenic expression of interferon alpha protects against acute pulmonary *Streptococcus pneumoniae* infection

**Daniela Damjanovic, Amandeep Khera, Maria F. Medina,
Jane Ennis, Jeffrey D. Turner, Jack Gauldie and Zhou Xing**

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Transgenic expression of interferon alpha protects against acute pulmonary *Streptococcus pneumoniae* infection

For this study, in our continuing interest for improved therapies during pulmonary infections, we evaluated the transgenic expression of type I IFN as a treatment during *S. pneumoniae* infection. Strikingly, a single intranasal dose of AdIFN-α led to control of bacterial replication, lung inflammation, and improvement in clinical outcome. Our study demonstrated the protective role of type I IFN during *S. pneumoniae* infection, and for the first time established the successful use of adenovirus-delivered IFN-α for pulmonary pneumococcal infection.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

Transgenic expression of interferon alpha protects against acute pulmonary *Streptococcus pneumoniae* infection

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Running title: Control of pneumococcal replication by IFN-alpha

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ABSTRACT

Streptococcus pneumoniae remains a major human pathogen worldwide. Pneumococcal infections are the leading cause of community-acquired pneumonia (CAP), invasive pneumococcal disease (IPD), complications during influenza epidemics, and bacterial infections in children. During a *S. pneumoniae* pandemic with an antibiotic resistant or non-vaccine strain, readily available fast-acting drugs would be crucial. The type I interferon alpha (IFN-α) has recently been shown to be induced by *S. pneumoniae* and some data suggests that it may be protective. However, such potential protective effects of IFN-α in pneumococcal infections have been largely understudied and the mechanisms of protection are entirely unknown. A novel broad-spectrum anti-viral drug, the IFN-α-expressing adenovirus 5 vector AdIFN-α was for the first time evaluated as a treatment during a bacterial infection, in a *S. pneumoniae* mouse model. AdIFN-α was administered as a single intranasal treatment given at 48h prior to infection. We found that *S. pneumoniae* infection in untreated animals caused excessive lung inflammation and severe weight loss, associated with rapid bacterial outgrowth in the lung and systemic dissemination to the spleen. In contrast, transgenic expression of IFN-α led to rapid and effective control of bacterial replication and lung inflammation, and to an improvement in clinical outcome. Our study shows that the classically anti-viral cytokine IFN-α is protective against extracellular bacterial infection with *S. pneumoniae* and our findings hold important implications for the use of AdIFN-α to combat pneumococcal infections.

INTRODUCTION

Streptococcus pneumoniae remains a major human pathogen worldwide. Pneumococcal infection is the leading cause of community-acquired pneumonia (CAP) and can lead to invasive pneumococcal disease (IPD) that includes bacteremia, septicemia, pneumonia, and meningitis (1, 2). It is estimated that CAP alone accounts for 1 million doctor visits and 60,000 hospitalizations annually in Canada, costing about $100 million (3). The incidence of IPD in adults remains high, while the highest incidence is in the elderly and in children less than 5 years old, particularly in Aboriginal children living in northern Canada (1, 4). Worldwide, *S. pneumoniae* continues to be the most significant bacterial pathogen in children, causing approximately 25% of all preventable deaths in children under 5 and 1.6 million infant deaths per year in developing countries (2, 5, 6). In addition, *S. pneumoniae* superinfection is the most common complication of influenza, which together form the leading cause of death from infectious diseases in Canada (3). Alarmingly, *S. pneumoniae* outbreaks have occurred across Canada in the last 10 years (7). Treatment is complicated by the continuous emergence of antibiotic resistant *S. pneumoniae* serotypes, vaccines that provide narrow serotype-specific protection, and evidence of changing serotype distribution to non-vaccine serotypes in invasive infections of vaccinated children (2, 8, 9). The threat of a pneumococcal outbreak remains a reality worldwide and development of novel therapeutic agents is critical.

A replication-deficient adenovirus 5 (Ad5) vector containing the human consensus interferon alpha (IFN-α) gene, known as DEF201, has been developed. Used prophylactically or therapeutically it has provided protection from lethality in animal

models of Ebola virus, yellow fever virus, arenavirus, and phlebovirus infection after a single intranasal dose (10-13). The mouse IFN-α construct, mDEF201, has been shown to be protective from lethal SARS virus, Western equine encephalitis virus, Venezuelan equine encephalitis virus, and vaccinia virus infection (14-17). While IFN-α is classically known to be anti-viral, *S. pneumoniae* has recently been found to activate type I IFN signaling which may be protective against infection, although the mechanisms of protection are unknown (18-21). Exploitation of type I IFNs for protection against pneumococcal infection has scarcely been attempted. Recombinant IFN-α/β (rIFN) is a possible delivery mechanism for IFNs (22). However, as the half-life of rIFN is very short, such treatment would require frequent administrations. Adenoviral delivery on the other hand provides steady-state expression of IFN after a single dose, and has not been investigated in *S. pneumoniae* infection to date (17).

In our current study, transgenic expression of IFN-α was evaluated for treatment of *S. pneumoniae* respiratory infection in mice. In our model, *S. pneumoniae* infection led to bacterial outgrowth in the lungs, systemic dissemination to the spleen, excessive lung inflammation and severe weight loss in the first 72h post-infection in control animals. Strikingly, a single intranasal dose of AdIFN-α administered 48h before *S. pneumoniae* infection led to control of bacterial replication, lung inflammation, and improvement in clinical outcome. Therefore, our study demonstrates the protective role of type I IFN during *S. pneumoniae* infection, and for the first time establishes the successful use of adenovirus-delivered IFN-α for pulmonary pneumococcal infection.

MATERIALS AND METHODS

Mice

Female 7-10 week old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in specific pathogen-free level facilities at the McMaster University Central Animal Facility (Hamilton, Ontario, Canada). For all experiments, mice were euthanized by exsanguination of the abdominal artery under anesthesia. All experiments were conducted in accordance with the animal research ethics board of McMaster University.

Infections

A replication-deficient adenoviral gene transfer vector (Ad5) expressing murine IFN-α (AdIFN-α) was obtained from Defyrus Inc. (Toronto, ON, Canada; construct mDEF201 prepared at the Robert Fitzhenry Vector Laboratory, McMaster University, Hamilton, Canada), and an empty adenoviral vector (Addl) was used as a control. Mice anesthetized by inhalation of isoflurane were infected intranasally (i.n.) with 10^7 plaque forming units (pfu) of AdIFN-α in a volume of 20 μl. *S. pneumoniae* serotype 3 (ATCC 6303) was prepared by plating frozen stock on blood-agar plates and incubating overnight, then culturing the resultant colonies for ~5h at 37°C in 5% CO_2 in Todd Hewitt broth to midlogarithmic phase. The bacteria were harvested and resuspended in PBS. Anesthetized mice were infected intratracheally (i.t.) with 10^4 colony forming units (cfu) of *S. pneumoniae* in a volume of 40 μl, 48h after the Ad administration.

Determination of streptococcus load in tissue

Levels of streptococcus in the lung and spleen were determined by homogenizing the tissues in PBS, plating serial dilutions on blood-agar plates, and incubating overnight at 37°C, 5% CO_2. Colonies were counted and calculated as cfu per whole organ.

Bronchoalveolar lavage and lung cell isolation

Airway luminal cells were collected by bronchoalveolar lavage (BAL) of the lungs and mononuclear cells were isolated from lung tissue, using standard procedures previously described (23). Briefly, the lungs were cut into small pieces and incubated in 10 ml of 150 U/ml collagenase type I (Sigma-Aldrich, St. Louis, MO) for 1h at 37°C, the lung pieces crushed through 40-μm basket filters, red blood cells lysed with ACK lysis buffer, and cell pellets resuspended in complete RPMI medium (RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine).

Flow cytometric analysis

Flow cytometry was performed on BAL and lung cells. Cells were incubated for 15 min. with the CD16/CD32 Fc block antibody, and then stained with extracellular antibodies (BD Biosciences unless otherwise stated) for antigen presenting cells (APC-Cy7-anti-CD45, Alexa Fluor 700-anti-MHCII [eBioscience], Pacific Orange-anti-Gr1 [Invitrogen], APC-anti-CD11c, PECy7-anti-CD11b), NK cells (V450-anti-CD3, PE-anti-NK1.1), and T cells (V450-anti-CD3, APC-Cy7-anti-CD4). Stained cells were analyzed on the LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Neutrophils (CD45+CD11b+Gr1+), macrophages (CD45+Gr1-AF+CD11c+), natural killer (NK) cells (CD3-NK1.1+) and CD4+ T cells (CD3+CD4+)

were assessed as total number of cells per tissue compartment by multiplying the 'frequency of total' of the population by total cell number in the tissue.

Cytokine and chemokine quantification

Levels of cytokines and chemokines in BAL fluid (BALF) were quantified using Luminex multianalyte technology (Luminex Molecular Diagnostics, Toronto, ON, Canada), according to the manufacturer's protocols. Levels of IFN-α were measured in BALF, lung homogenates, serum, and culture supernatants using the VeriKine™ Mouse Interferon Alpha ELISA Kit (PBL Interferon Source, NJ, USA).

Killing assay

A standard bacterial phagocytosis and killing assay was used to evaluate the effect of pre-treatment of macrophages with AdIFN-α (24). Naïve C57BL/6 mice were used to obtain the cells, by BAL for alveolar macrophages (AMs) and by mononuclear cell isolation followed by purification of CD11c+ cells and CD11b+ cells (which were then pooled) by magnetic cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), from lung tissue for lung macrophages (MOs). In separate experiments, $5x10^5$ AMs were used per well and $2x10^5$ lung MOs were used per well, in 24-well plates. Cells were infected with AdIFN-α or Addl at MOI=100 in serum-free cRPMI for 3h at 37°C (during this time cells adhered to the bottom of the wells), washed, resuspended in Pen/Strep-free cRPMI and left overnight at 37°C. *S. pneumoniae* was freshly grown to midlogarithmic phase and then opsonized by adding 10% naive C57BL/6 serum to bacteria for 30 min at 37°C. Bacteria were then washed twice with PBS to remove excess serum and added to the macrophage culture (bacteria to

macrophage ratio 10:1). After incubation for 1-1.5h, phagocytosis was stopped by treating with Pen/Strep-containing cRPMI for 30 min at room temperature to kill any extracellular bacteria. Some wells were then lysed to determine cfu at the 1-1.5h timepoint, while other wells were washed with PBS, resuspended with Pen/Strep-free cRPMI and returned to 37°C for an additional 1-2h. To determine cfu, cells were washed with PBS, lysed with 1 ml distilled autoclaved water for 30 min at room temperature, diluted in PBS, plated in 10-fold serial dilutions on blood-agar plates, and incubated overnight. Colonies were counted the next day and determined as intracellular CFU.

Histopathology

Lungs were inflated with 10% formalin and kept in 5 ml of 10% formalin for at least 72h. Tissue sections were stained with hematoxylin and eosin (H&E) for histologic examination.

Statistical analysis

To determine if the differences between Addl and AdIFN-α groups were significant, Student's *t* test was used for two-sample comparisons with Prism (GraphPad Software, Inc., La Jolla, CA, USA). A p value of < 0.05 was regarded as statistically significant.

RESULTS

IFN-α expression controls *S. pneumoniae* replication and improves clinical outcome

To determine the effect of IFN-α expression on subsequent bacterial infection, mice were infected intranasally with AdIFN-α or Addl control, and 48h later infected intratracheally with *S. pneumoniae* (Fig. 1A). At the time of bacterial infection IFN-α was expressed locally in the airway lumen and lung, and released into the serum of AdIFN-α mice, while Addl mice had very low baseline levels of IFN-α (Fig. S1). After *S. pneumoniae* infection, IFN-α expression was further induced in the AdIFN-α mice (Fig. S2). Body weight changes were monitored as a measure of overall illness and clinical outcome. Control (Addl infected) mice began to lose weight soon after the infection and lost 8.5% of initial body weight by 48h, significantly more than AdIFN-α mice that lost 5.0% (Fig. 1B). By 72h post-infection Addl mice continued to lose weight to 11.3%, while AdIFN-α mice began to recover, and were at a weight loss of 4.5% at 72h (Fig. 1B). As further indication of illness, lung histopathology was examined at 48h, and the lungs of AdIFN-α mice had visibly less inflammation and areas of injury, corresponding to the time when they began to recover in weight (Fig. 1C).

Consistent with the illness and lung inflammation, the Addl mice had significantly higher lung and spleen bacterial loads than the AdIFN-α mice at 48h and 72h post-infection (Fig. 2). The bacterial load in the lungs and spleens of AdIFN-α mice decreased between 48h and 72h post-infection, while the Addl mice had consistently high bacterial loads with no change between 48h and 72h (Fig. 2). These findings show that local and

systemic expression of IFN-α during *S. pneumoniae* infection helps control *S. pneumoniae* replication and outgrowth, in turn improving lung inflammation and clinical outcome.

IFN-α expression leads to rapid induction of cytokine and chemokine responses and cellular infiltration in the lungs

To investigate the mechanisms of improved bacterial control mediated by IFN-α, we measured cytokine responses in the lung. Cytokine and chemokine levels were assessed in bronchoalveolar lavage (BAL) fluids at 20h following *S. pneumoniae* infection. AdIFN-α mice had markedly higher levels of most analytes measured in the lung, and significantly higher levels of MIP-2, IP-10, MCP-1, MIG, and IL-1β compared to Addl mice (Fig. 3).

Given the increased chemokine expression in AdIFN-α mice, we next analyzed the cellular components in the lung at 20h post-infection using flow cytometry. Total cell numbers were almost 3-fold higher in the BAL of AdIFN-α mice (Table 1), and neutrophil influx was markedly increased compared to Addl mice (Fig. 4A). Higher cell numbers and neutrophil influx in AdIFN-α BAL were also observed at 48h (Table 1 and data not shown). Neutrophil numbers in the lung parenchyma were similar between the two groups, as were macrophages in the BAL and lung (Fig. 4A, B). However, AdIFN-α lungs had noticeably higher numbers of lung NK cells and CD4+ T cells compared to Addl lungs (Fig. 4C). The increased numbers of neutrophils, NK cells, and CD4+ T cells in the lungs of AdIFN-α mice corresponded with the better control of bacterial replication (Fig. 2), as these cell types have been shown to be involved in the clearance of *S.*

pneumoniae (9, 25). These results show that transgenic expression of IFN-α during *S. pneumoniae* infection leads to rapid induction of cytokine responses and cellular infiltration into the lungs at early timepoints post-infection, leading to better control of bacterial replication and improved clinical outcome.

Excessive lung inflammation in the lungs of control mice following *S. pneumoniae* infection

To further investigate the potential mechanisms leading to immunopathology and poor clinical outcome of control mice, we examined responses at the later timepoint of 72h post-infection, when Addl mice had 11.3% of body weight loss (Fig. 1B) and uncontrolled bacterial replication (Fig. 2). At this time, Addl mice had significantly higher and dysregulated expression of most analytes measured in the BAL, including KC, MIP-2, IP-10, MCP-1, MIG, MIP-1α, TNF, IL-1β, and IFN-γ (Fig. 5). Correspondingly, Addl mice also had excessive influx of neutrophils in the BAL and lung when compared to AdIFN-α mice, as well as 12-fold more total cells in the airway (Fig. 6 and Table 1). This data suggests that at this later timepoint, as the AdIFN-α mice were clearing the bacteria and recovering, their anti-bacterial immune responses were decreasing. On the other hand, the control mice were unable to control bacterial outgrowth and consequently lung inflammation was increasing.

Improved phagocytic killing of *S. pneumoniae* by AdIFN-α infected lung macrophages

As prophylactic treatment with AdIFN-α led to rapid induction of immune responses in the lungs (Fig. 3 and 4), we further investigated the mechanisms of improved

bacterial control (Fig. 2) by assessing the functionality of macrophages. Freshly isolated naïve macrophages were infected with AdIFN-α or AddI and a killing assay was performed. After initial phagocytosis, levels of IFN-α were measured in culture supernatants and the cytokine was highly induced (Fig. S3). Both the alveolar macrophages (Fig. 7A) and purified lung macrophages (Fig. 7B) that were infected with AdIFN-α displayed more effective *S. pneumoniae* killing in the first hour after phagocytosing the bacteria compared to AddI infected cells. The assay was performed for an additional hour with lung macrophages, and showed that the AdIFN-α macrophages continued to better control bacterial growth, even when the bacteria were overcoming the anti-microbial control (Fig. 7B). These experiments suggest that the mechanisms of improved bacterial control mediated by IFN-α expression include improved phagocytic killing by airway and lung macrophages.

DISCUSSION

Infections with *Streptococcus pneumoniae* continue to have significant clinical, social and economic impacts in Canada and worldwide. As a leading cause of CAP and IPD, as the most significant bacterial pathogen in children and the most common complication of influenza, *S. pneumoniae* contributes to significant morbidity and mortality worldwide (1, 2, 5, 6). Treatment is complicated by the continuous emergence of antibiotic resistant strains and suboptimal vaccines, making the threat of *S. pneumoniae* outbreaks a reality (2, 8, 9). A novel broad-spectrum drug that has shown promise in providing protection from a variety of viruses, the IFN-α expressing adenovirus, would

be an ideal candidate during a *S. pneumoniae* outbreak (10-17). Indeed, it has been shown that *S. pneumoniae* activates type I IFN signaling, however the protective effects of IFN-α have not been confirmed and the mechanisms of protection are unknown (18-21). Using a mouse model of acute respiratory *S. pneumoniae* infection, we have investigated these questions. We show that *S. pneumoniae* infection that leads to bacterial outgrowth in the lung and systemic dissemination, excessive lung inflammation and severe weight loss in control animals was ameliorated by transgenic expression of IFN-α. We provide novel information that a single intranasal dose of AdIFN-α administered prior to *S. pneumoniae* infection led to control of bacterial replication, lung inflammation, and improvement in clinical outcome. We have thus for the first time identified successful use of adenovirus-delivered IFN-α for pulmonary extracellular bacterial infection.

In our model of pulmonary pneumococcal infection, mice were infected with *S. pneumoniae* 48h after receiving one intranasal dose of AdIFN-α or Addl control. At this time IFN-α was expressed locally in the airway lumen and lung, and was also released into the serum. Measured at 20h after *S. pneumoniae* infection in the airway, IFN-α expression was induced 2-fold more in AdIFN-α mice. Therefore the adenovirus vector provided sustained and inducible expression of IFN-α in treated mice. It has previously been reported that intramuscular injection of AdIFN-α (mDEF201) rapidly led to high levels of IFN-α in the serum (within 3-5h) that were undetectable by 7d, however the protective effects of the interferon lasted, so that animals challenged with Western equine encephalitis virus at 7d after AdIFN-α treatment were 100% protected (15). In addition,

intranasal delivery of AdIFN-α was previously observed to be protective when given 14d and 56d prior to infection with SARS and vaccinia virus, respectively (14, 17). Thus a single dose of AdIFN-α induces a long-term antiviral state that activates as of yet unknown immune mechanisms.

We found that in our model of extracellular bacterial infection, transgenic expression of IFN-α controls *S. pneumoniae* replication and improves clinical outcome. While control (Addl) mice began to lose weight soon after the infection and lost more than 11% by 72h, AdIFN-α mice transiently lost 5% and began to recover from 48-72h post-infection. In agreement with the improved clinical outcome, AdIFN-α mice had significantly less lung inflammation, as well as significantly lower lung and spleen bacterial loads than control mice up to 72h post-infection. Later timepoints post-infection are currently being investigated to examine if such improvement is sustained. Our results are in agreement with a 1986 murine study where either recombinant IFN-α or IFN-β markedly improved survival in streptococcal infection when given intraperitoneally 5h before infection (63-72% survival compared to 0% survival in controls) (22). In the same study, anti-IFN-α/β serum significantly increased mortality (20% survival compared to 95% survival in controls) (22). Similarly, in a colonization model study, IFN-α/β receptor null mice had significantly increased nasal colonization with *S. pneumoniae* compared with wild-type mice (21). However in these studies the mechanisms were not addressed.

In our study, we also investigated the unknown mechanisms of improved bacterial control in AdIFN-α treated animals. We observed that cytokine and chemokine levels in the airway of AdIFN-α mice 20h after *S. pneumoniae* infection were markedly higher

compared to controls. Significantly increased levels of key chemokines correlated with the observed higher influx of immune cells into the airway and lung, including neutrophils, NK cells, and CD4+ T cells. Recently, NK cells and CD4+ T cells have been shown to have important roles in early host defense against extracellular bacteria (2, 9, 25, 26). Importantly, higher neutrophil numbers in the airway are indicative of the improved bacterial control, as neutrophils are the key phagocytes involved in *S. pneumoniae* clearance (2). As macrophages are also known to be major cells involved in the first line of defense at the site of infection, and were not found to be increased in number in AdIFN-α mice, we further investigated their functionality as a potential mechanism of improved bacterial control in the treated animals. Both alveolar macrophages and purified lung macrophages displayed more efficient *S. pneumoniae* killing and control of bacterial outgrowth when previously infected with AdIFN-α as opposed to Addl. Our study therefore sheds light onto the mechanisms mediated by IFN-α to provide improved bacterial control in *S. pneumoniae* infection. Further investigation is essential, as questions remain about the role of IFN-α on activation of other important cell types such as neutrophils, NK cells, and CD4+ T cells. Also, it would be interesting to examine the effects of IFN-α on epithelial integrity, as it has been observed that type I IFNs can inhibit invasion by bacterial species in the intestine (20). Other treatment protocols, including therapeutic use of AdIFN-α, delivered post-infection are imperative and will be explored.

In conclusion, our results indicate that the classically anti-viral cytokine IFN-α is protective against extracellular bacterial infection with *S. pneumoniae*. Furthermore, we

identify for the first time that a single intranasal dose of AdIFN-α led to rapid and effective control of bacterial replication and lung inflammation, and consequently to an improvement in clinical outcome. Our findings hold important implications for transgenic expression of IFN-α in future clinical practice, in particular during outbreaks where a rapid therapy delivered directly to the site of infection, the lung, would be invaluable.

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Table 1. Total cell numbers in BAL following *S. pneumoniae* infection ($x10^6$).

20h		48h		72h	
AddI	**AdIFNα**	**AddI**	**AdIFNα**	**AddI**	**AdIFNα**
0.35 ± 0.03	0.92 ± 0.26	0.21 ± 0.02	0.44 ± 0.07	3.2 ± 1.15	0.26 ± 0.02

Results are expressed as Mean ± SEM (n=3-5/group/timepoint from one experiment/timepoint).

Figures

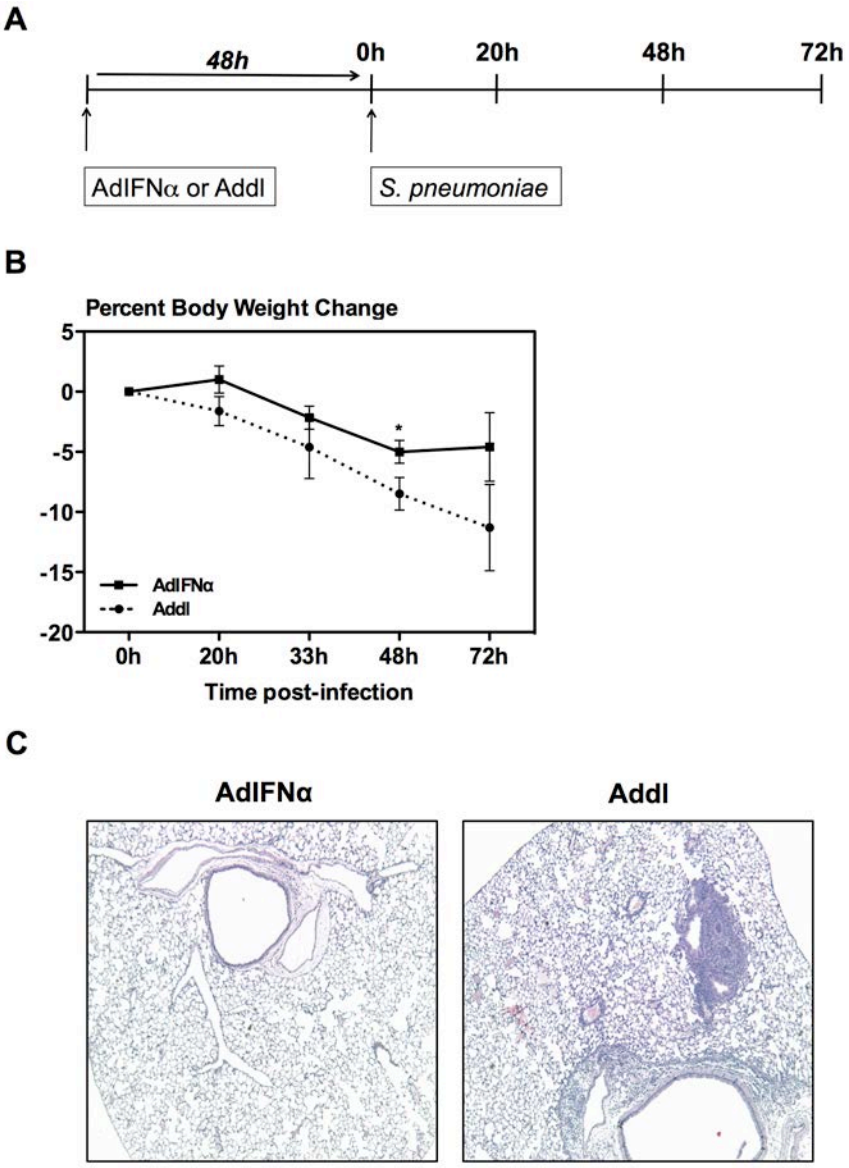

Figure 1. Transgenic expression of IFN-α improves clinical outcome following pneumococcal infection. ***A***, Experimental schema. Female C57BL/6 mice were infected with 10^7 pfu of AdIFN-α or AddI and 48h later with 10^4 cfu of *S. pneumoniae*. ***B***, Average percent body weight change was monitored as an indicator of illness. Results are from four independent experiments, n=5-8/group/timepoint. Data are expressed as Mean ± SEM. *, $p < 0.05$. ***C***, Histopathological changes were examined by H&E staining in the lungs of AdIFN-α or AddI treated mice at 48h post-*Strep* infection. Results are representative of one experiment, n=3/group, magnification 5x.

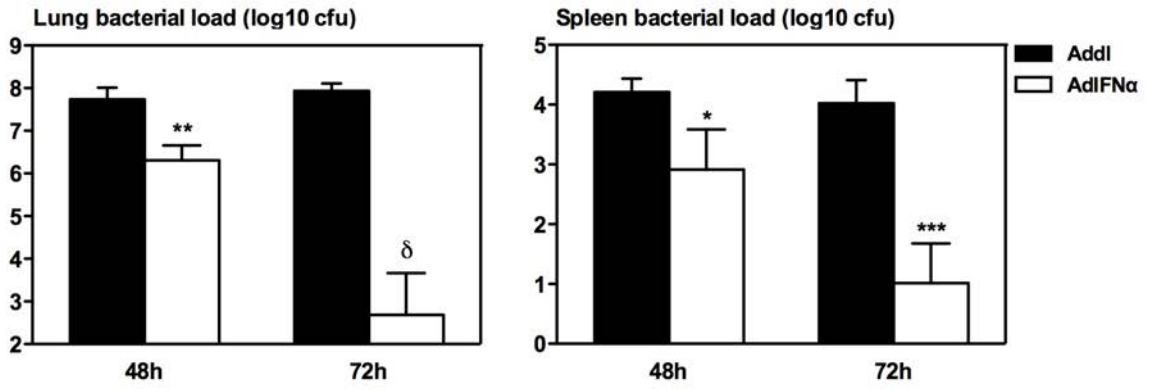

Figure 2. Transgenic expression of IFN-α controls *S. pneumoniae* replication. Lung and spleen bacterial load was measured in AdIFN-α or AddI treated mice using a CFU assay at 48h and 72h post-*Strep* infection. Results are from three independent experiments, n=5-8/group/timepoint. Data are expressed as Mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$, δ, $p < 0.0005$.

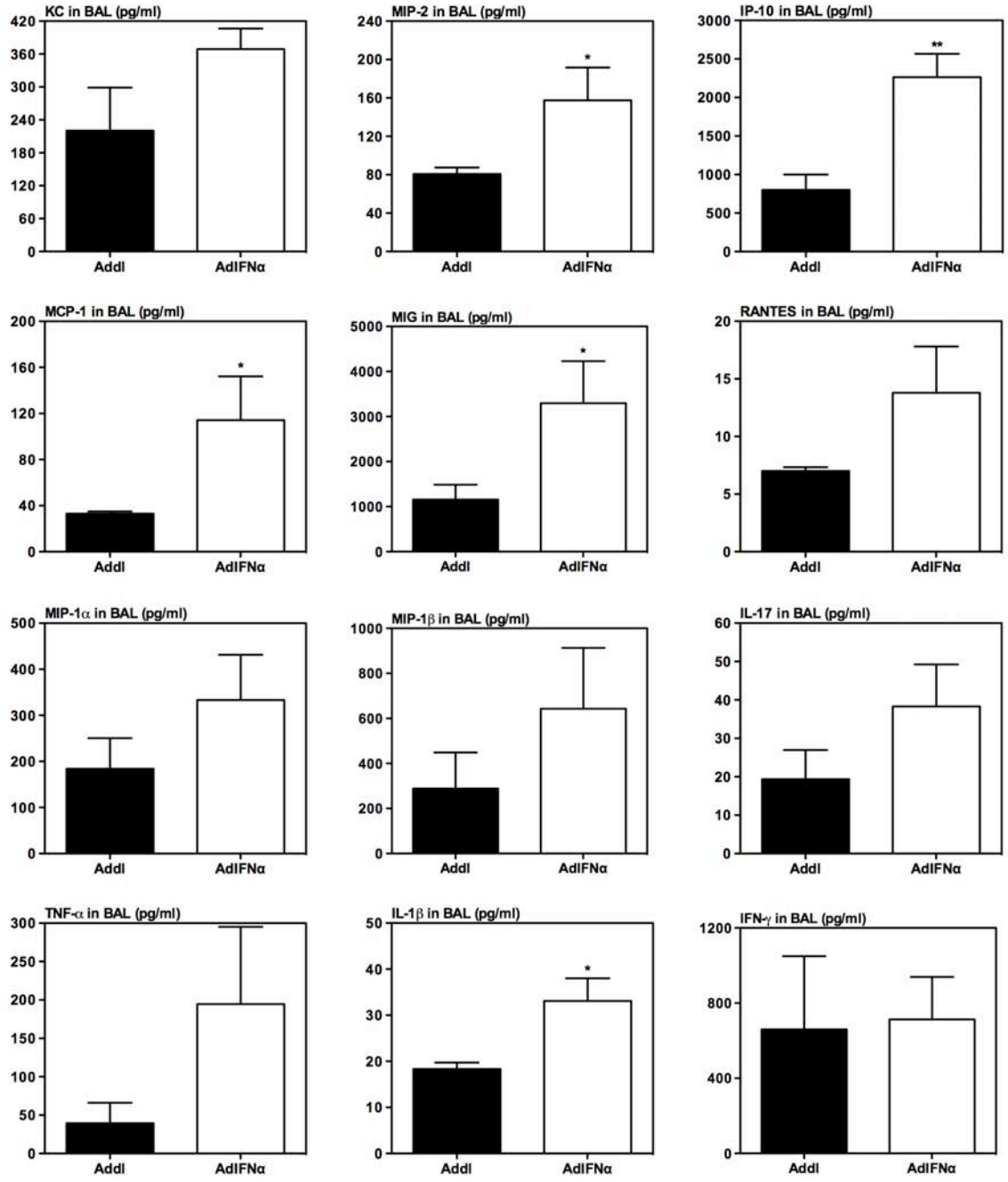

Figure 3. Transgenic expression of IFN-α leads to rapid induction of cytokine and chemokine responses in the lungs of *S. pneumoniae* infected mice. Cytokine and chemokine levels in BALF were measured in AdIFN-α or AdIl treated mice at 20h post-*Strep* infection. Results are from one experiment, n=5/group. Data are expressed as Mean ± SEM. *, $p < 0.05$; **, $p < 0.005$.

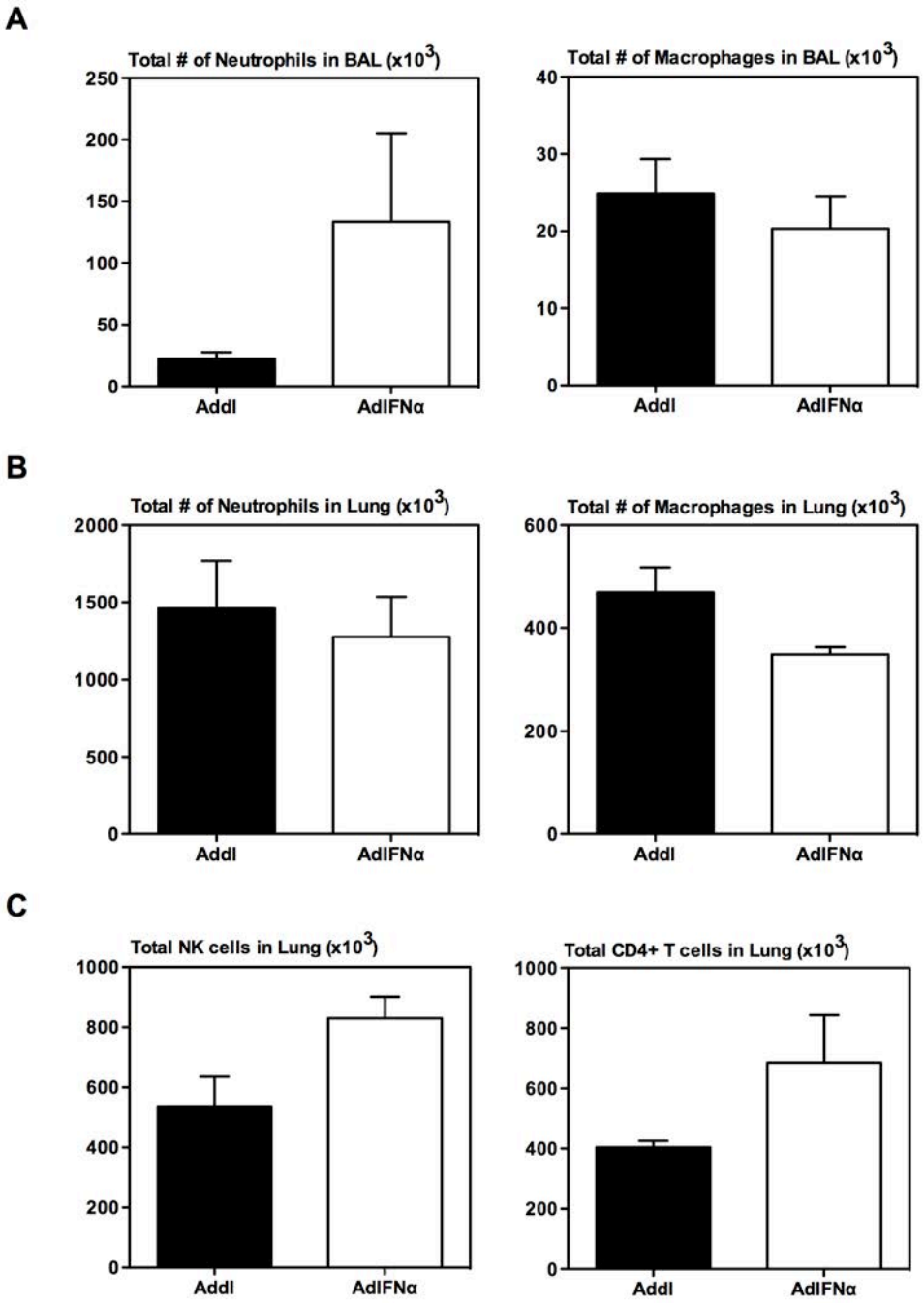

Figure 4. Rapid cellular infiltration in the lungs of AdIFN-α treated mice at 20h post-*Strep* infection. ***A***, Neutrophil and macrophage numbers in the BAL. ***B***, Neutrophil and macrophage numbers in the lung. ***C***, NK cell and CD4+ T cell numbers in the lung. Cells were assessed by flow cytometry in AdIFN-α or Addl treated mice at 20h post-*Strep* infection. Results are total cell numbers in the tissue compartment from one experiment, n=5/group. Data are expressed as Mean ± SEM.

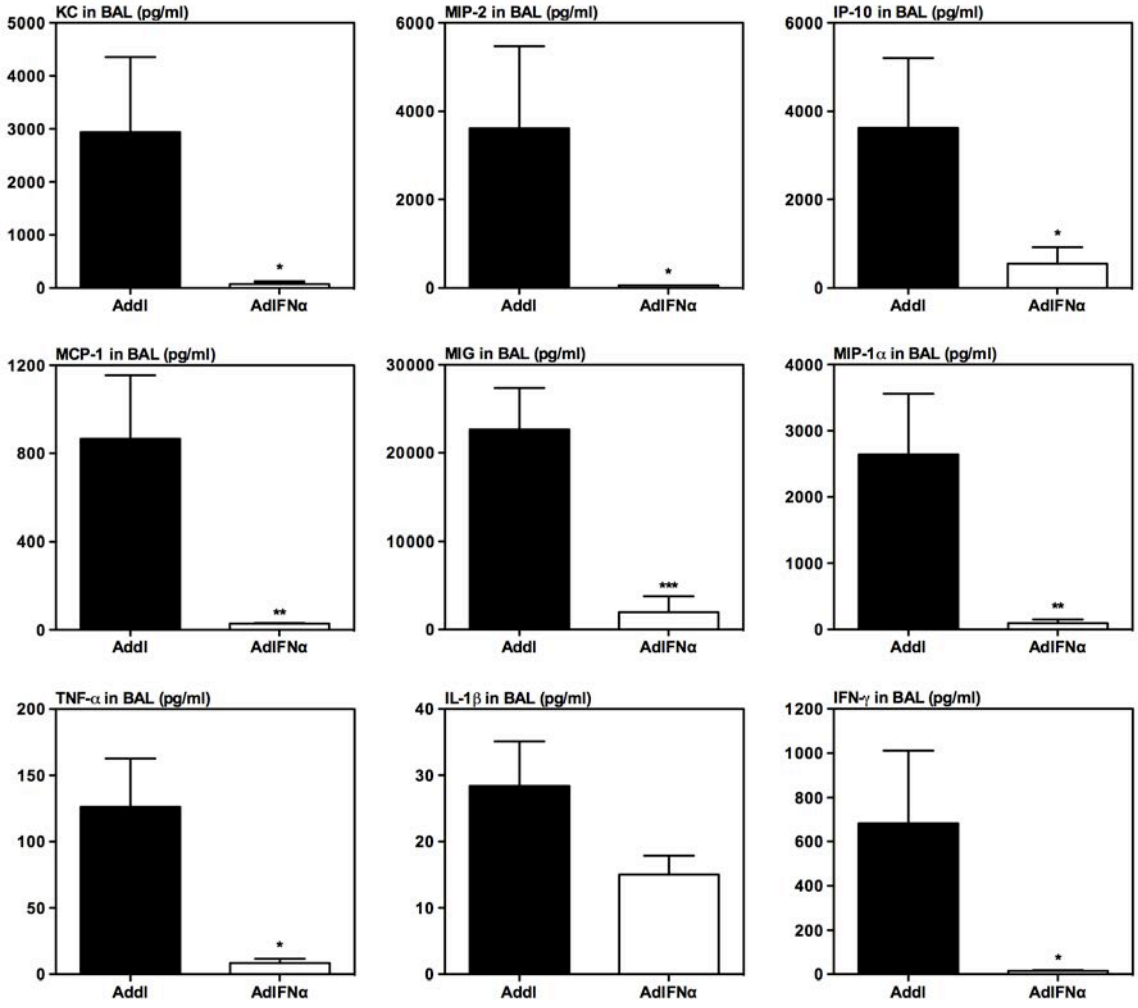

Figure 5. Dysregulated cytokine and chemokine responses in the lungs of AddI treated mice at 72h post-*Strep* infection. Cytokine and chemokine levels in BALF were measured in AdIFN-α or AddI treated mice at 72h post-*Strep* infection. Results are from one experiment, n=5/group. Data are expressed as Mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.005$.

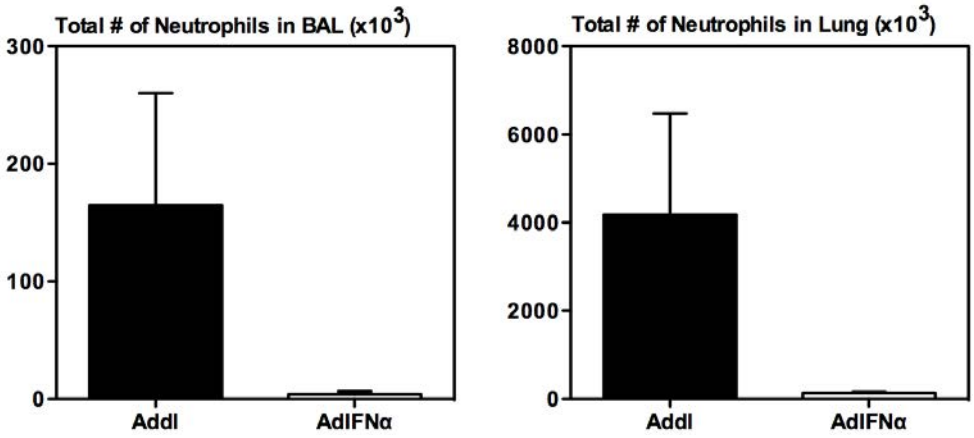

Figure 6. Excessive neutrophil infiltration in the lungs of AddI treated mice at 72h post-*Strep* infection. Neutrophil numbers in the BAL and lung were assessed by flow cytometry in AdIFN-α or AddI treated mice at 72h post-*Strep* infection. Results are total cell numbers in the tissue compartment from one experiment, n=5/group. Data are expressed as Mean ± SEM.

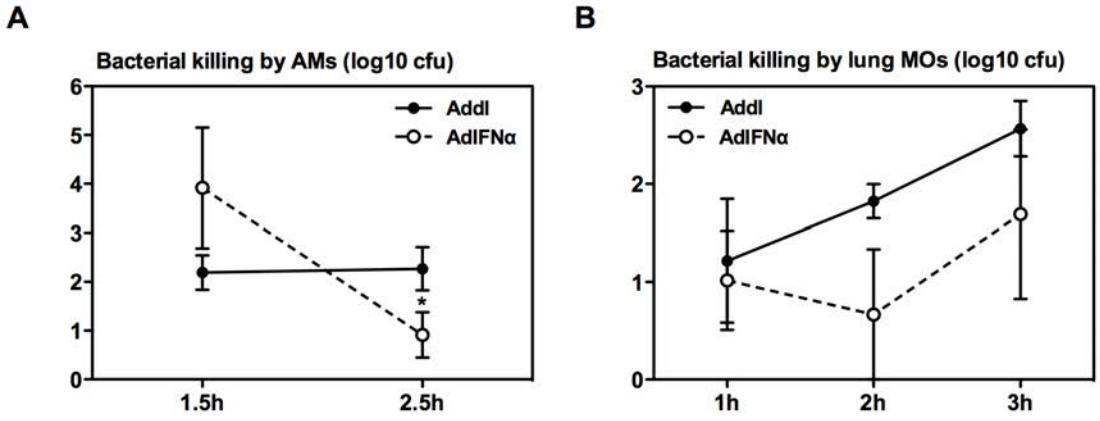

Figure 7. AdIFN-α infection improves *S. pneumoniae* killing ability of lung macrophages. Alveolar macrophages (***A***) and purified lung macrophages (***B***) were infected with AdIFN-α or AddI for 3h, and the following day *S. pneumoniae* was added to the cells. Phagocytosis was allowed to occur for 1-1.5h and then bacterial killing was allowed for another 1-2h. Cells were lysed and the live bacteria were enumerated by a CFU assay. Data are expressed as Mean ± SEM of triplicate wells/group/timepoint, representative of two independent experiments. *, $p < 0.05$.

Supplementary Figures

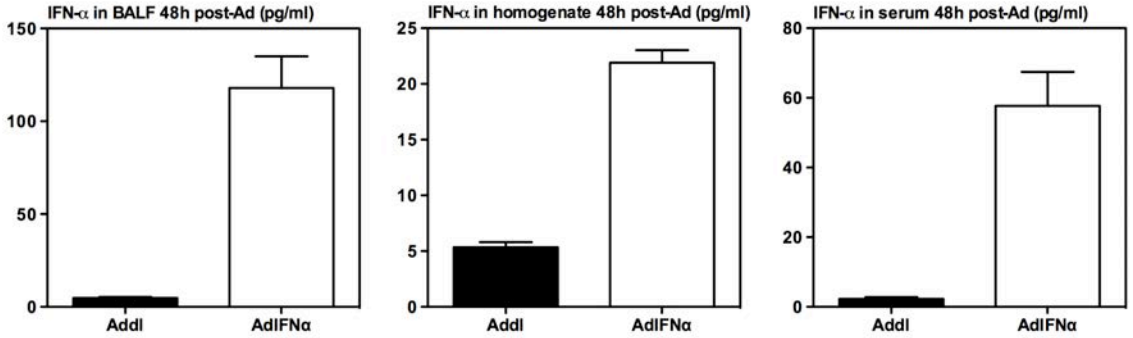

Figure S1. Levels of IFN-α following *in vivo* infection with AdIFN-α. Female C57BL/6 mice were infected with 10^7 pfu of AdIFN-α or AddI, and IFN-α was measured in the BALF, lung homogenate, and serum 48h later by ELISA. Results are from one experiment, n=3/group. Data are expressed as Mean ± SEM.

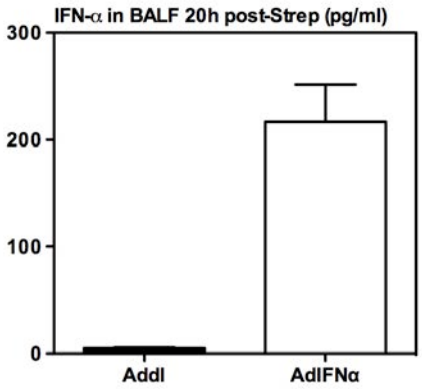

Figure S2. Levels of IFN-α following AdIFN-α pre-treatment and subsequent *S. pneumoniae* infection. Mice were infected with 10^7 pfu of AdIFN-α or AddI, and 48h later with 10^4 cfu of *S. pneumoniae*. IFN-α was measured in the BALF at 20h post-*Strep*. Results are from one experiment, n=3/group. Data are expressed as Mean ± SEM.

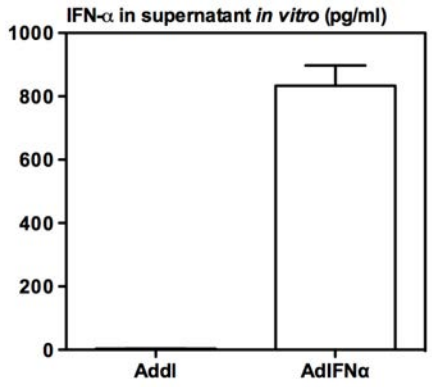

Figure S3. Levels of IFN-α following *in vitro* infection of macrophages by AdIFN-α. Alveolar macrophages and purified lung macrophages were infected with AdIFN-α or AddI for 3h, and the following day *S. pneumoniae* was added to the cells for 1-1.5h. IFN-α was measured in these supernatants. Data are expressed as Mean ± SEM of triplicate wells/group, representative of two independent experiments.

CHAPTER 5: DISCUSSION

1. Summary of major findings

Lung immunopathology is a major contributing factor to morbidity and mortality of influenza infection. Secondary bacterial superinfections that occur following influenza further complicate the lung immunopathology and result in increased hospitalizations and deaths. The research presented in this thesis addressed important, understudied questions in the complicated field of tissue immunopathogenesis, as well as potential treatment protocols for influenza and pneumococcal infections. In *Chapter 2*, we used a murine model of acute respiratory influenza infection to investigate the immunoregulatory role of TNF. For the first time, our study demonstrated that TNF is not required for effective influenza viral clearance in the lung, but is critically required for controlling the extent of inflammatory immune responses and lung immunopathology. In *Chapter 3*, we next established a murine model of acute respiratory influenza infection and *Streptococcus pneumoniae* superinfection to investigate the mechanisms of severe lung immunopathology and possible intervention strategies. Our study identified uncontrolled bacterial outgrowth, dysregulated cytokine and chemokine responses, and excessive neutrophil infiltration in the lungs to be the main contributors to deleterious lung immunopathology and death. The use of a bacteriostatic antibiotic alone effectively controlled bacterial replication and improved clinical outcome, but failed to significantly reduce lung immunopathology. Importantly, the use of antibiotic and corticosteroid in combination led to the best clinical outcome with markedly improved survival, bacterial clearance and lung immunopathology. In *Chapter 4*, we continued our investigations into possible therapeutics and evaluated transgenic expression of IFN-α for treatment of *S.*

pneumoniae respiratory infection. Strikingly, a single intranasal dose of AdIFN-α led to control of bacterial replication, lung inflammation, and improvement in clinical outcome. Our study demonstrated the protective role of type I IFN during *S. pneumoniae* infection, and for the first time established the successful use of adenovirus-delivered IFN-α for pulmonary pneumococcal infection.

In summary, the major findings presented in my thesis are: (i) TNF is not required for effective influenza clearance, (ii) TNF is a critical negative regulator of inflammatory responses and immunopathology during influenza, (iii) the mechanisms of severe lung immunopathology by influenza-associated bacterial superinfection are multifactorial and are in part independent of bacterial burden, (iv) overactive immune responses during bacterial superinfection contribute to deleterious lung immunopathology and death, (v) effective intervention strategies during superinfection should involve the effective control of both bacterial infection and aberrant host immune responses, (vi) IFN-α plays a protective role during *S. pneumoniae* infection by controlling bacterial replication, and (vii) transgenic expression of IFN-α is an effective treatment of respiratory pneumococcal infection. These major findings will be further discussed below.

2. The role of TNF in lung immunopathogenesis during influenza infection

Pulmonary influenza continues to be a common respiratory infectious disease worldwide. It is now well established that lung immunopathology is one of the main causes of influenza related morbidity and mortality (141-146). Recent studies using experimental animal models have significantly enhanced our understanding of the

complex mechanisms involved in the immunopathogenesis during influenza infection (as reviewed in *Appendix I*) (14). This involves acute inflammatory responses including the role of cytokines and chemokines. Current studies together suggest that carefully selected targeting of certain cytokine pathways may improve influenza immunopathology without negatively affecting viral clearance, which would be the ideal treatment. However, cytokine biology is complex, as most cytokines are required at proper levels and at specific times in the immune response, and any deviation from this well controlled expression can cause complications such as extensive lung immunopathology. Also, proper expression of immunoregulatory cytokines is essential in the return to homeostasis during the resolution phase of influenza infection.

We have shown that TNF plays an important immunoregulatory role during influenza immunopathogenesis. This finding adds to recent studies that have discovered immune suppressive roles for the classically proinflammatory cytokine (37-45, 48). We found that TNF is not required for influenza clearance and thus plays a redundant role in the early times of the infection, as immune responses are effectively initiated to clear the virus in TNF deficient hosts. However these same responses that successfully clear the virus are excessive and prolonged, culminating in lung immunopathology. Therefore we have shown that TNF plays a differential and biphasic role during the course of primary influenza infection. In the early phase of infection it is proinflammatory, and may in fact contribute to the 'cytokine storm' and immunopathology, seen particularly in cases of highly pathogenic influenza. However, TNF is immunoregulatory in later phases of infection after viral clearance. This role is in agreement with the pleiotropic nature of

TNF and with its role in apoptosis, which is reflected in the reduced T cell contraction following viral clearance in our model (28, 30). Recent evidence has also shown an anti-fibrotic activity of TNF in various models, including idiopathic pulmonary fibrosis (IPF) (42-44). Interestingly, we have also found that TNF is anti-fibrotic during influenza infection, through suppression of profibrotic chemokine MCP-1 expression. Thus we identified that the dysregulated MCP-1 and in turn TGF-β1 responses represent an important molecular mechanism in abberant tissue repair and heightened immunopathology in influenza.

TNF depletion has been widely implemented as a means to counter inflammatory conditions in humans (37-41). Our results caution the clinical use of such therapeutics as they may worsen influenza-associated immunopathology during flu seasons or epidemics. Interestingly, a study found that treatment of RA patients with etanercept (a soluble TNF receptor) led to increased peripheral T cell reactivity to microbial antigens that included influenza (41). Several studies have also indicated that TNF blockade with infliximab (a monoclonal antibody) increases T cell reactivity (147, 148). As such, it has been suggested that in autoimmune diseases in which TNF blockade is therapeutic (RA, Crohn's disease, psoriasis) the overproduction of TNF is part of the innate immune response and released by macrophages, rather than by T cells such as in diseases where neutralization of TNF worsens disease severity (MS, diabetes, mouse models of EAE and lupus) (28, 30, 37). However, to date there is little evidence showing that patients treated with TNF inhibitors exhibit more frequent or severe influenza infections. Clinical trials of the safety and immunogenicity of influenza vaccination in patients with RA have shown

that the vaccine induces an adequate humoral response and does not induce clinical exacerbation of RA (149-151). There is a lack of reports of influenza illness itself in RA patients being treated with TNF inhibitors (152). However, as mentioned above, TNF deficiency may have stimulating effects on T cell activity against microbial antigens and may assist in the generation of CTLs to clear the infection, as was shown in our model. Thus an adequate humoral response following immunization and influenza viral clearance following infection may occur in patients under TNF deficiency, which is in agreement with our model. However, the question still remains about the fate of such patients at later times post-infection after the virus is cleared which is when TNF acts as a negative regulator preventing lung immunopathology. Also, RA patients are concurrently on glucocorticoid immunosuppressive therapy used to control the disease, which may mask the effect of TNF deficiency seen in our model, where TNF deficient mice had excessive inflammatory responses including cytokine and chemokine expression that led to fibrosis and immunopathology. Nevertheless, influenza patients on TNF inhibiting medication should be more closely monitored, including at later stages after the virus has been cleared and the lung architecture is returning to homeostasis, in order to prevent complicating lung disease that may occur under TNF deficiency.

3. Control of lung immunopathology in pneumococcal superinfection

Secondary bacterial superinfections following influenza are major contributors to hospitalizations and mortality, and lung immunopathology is severely exacerbated in dual infections (14, 91, 112, 115, 153, 154). The underlying mechanisms of such excessive

immunopathology need to be further investigated, and such lung injury must be addressed during treatment.

We have shown that uncontrolled bacterial outgrowth and systemic dissemination, as well as dysregulated host immune responses including enhanced cytokine and chemokine responses and excessive neutrophil infiltration are the main contributors to severe lung immunopathology and death. We observed that antibiotic control of bacterial infection alone in Flu/Strep animals is ineffective in improving lung immunopathology. Importantly, this observation showed that bacterial replication and lung immunopathology are not so closely interdependent as was previously believed, and can indeed be uncoupled. As unresolved lung pathology can cause chronic pulmonary disease even after the infectious agents are cleared, we recognized that it was imperative to create a treatment protocol to target the persisting immunopathology. While corticosteroids are used in influenza infection and CAP, there is no consensus on their benefit particularly in cases of secondary bacterial superinfection (155-162). By using dexamethasone concurrently with azithromycin, we found that the most effective treatment protocol for pneumococcal superinfection following influenza requires both bacteriostatic antibiotic and corticosteroid treatment. This therapeutic protocol most efficiently improved not only the lung immunopathology of the treated animals, but also the clinical outcome.

A number of mechanisms have been identified which mediate the well known increased susceptibility to bacterial superinfection following influenza, including changes in airway function, modification of the cytokine milieu, and impairment of innate immune cell function (59, 73, 137, 163). While our project did not directly address the

mechanisms of increased susceptibility to superinfection, some preliminary investigation on lung surfactants tackled this subject. Pulmonary surfactant covers the airway epithelial cells (AECs) and is well known to reduce alveolar surface tension and thus prevent collapse of the alveoli on expiration (164, 165). Surfactant proteins SP-A and SP-D are oligomeric collectins that bind microbes and their debris, allergens, and other foreign bodies that invade the lungs and opsonize them for phagocytic clearance by alveolar macrophages (164, 166, 167). Surfactant proteins have also been found to have other anti-inflammatory properties and to increase phagocytosis of apoptotic epithelial cells and neutrophils and thus facilitate the resolution of inflammation (164, 166, 167). These functions are mainly mediated by SP-A and SP-D (168). These surfactant proteins have been found to bind to *S. pneumoniae*, causing their aggregation and enhancing their uptake by phagocytes (54, 165, 169-173). The aggregation of pneumococci also immobilizes the pathogen in the lung, limits the spread of bacteria through the lung tissue, and enhances mucociliary clearance (54). SP-D deficiency is associated with increased colonization, infection, bacteraemia, and lung inflammation in mice (171). It has also been suggested that following influenza infection, disruption of surfactant and increased mucinous secretions contribute to obstruction of the airways and create a culture medium for bacteria (59). However, it is currently not known whether altered expression of surfactant proteins after influenza contributes to increased susceptibility to bacterial superinfection. To address this question, we analyzed SP-A levels in our dual infection model (*Appendix II*). SP-A levels were increased at 7 days after influenza infection in Flu mice when compared to naïve mice, suggesting that decreased surfactant expression is not

a mechanism contributing to the initial increased susceptibility to bacterial superinfection. SP-A levels increased following *S. pneumoniae* infection in Strep mice, in agreement with the role of surfactants in pneumococcal clearance. On the other hand, while SP-A levels were induced in Flu/Strep mice at 12h post-*Strep*, by 48h SP-A levels were markedly decreased. This was consistent with the illness and lethality of Flu/Strep mice seen in our model, which corresponded with significantly higher lung bacterial loads and systemic dissemination when compared to Strep mice between 20-60h post-*Strep*. This data suggests that decreased surfactant levels may contribute to bacterial outgrowth at later times following bacterial superinfection, when control of bacterial replication and dissemination is lost.

4. Control of pneumococcal replication by IFN-α

Streptococcus pneumoniae contributes to significant morbidity and mortality worldwide and treatment is complicated by the continuous emergence of antibiotic resistant strains and suboptimal vaccines (53, 66, 174). It is currently recognized that the bacterium activates type I IFN signaling, which is typically associated with antiviral infections (76-79). However it is unclear whether type I IFNs are protective against pneumococcal infection, and the mechanisms of protection are unknown.

We have shown that transgenic expression of IFN-α ameliorates the bacterial outgrowth, excessive lung inflammation and severe weight loss observed in control animals following *S. pneumoniae* infection. We have observed that a single intranasal dose of AdIFN-α administered prior to *S. pneumoniae* infection is protective, and have

for the first time identified successful use of adenovirus-delivered IFN-α for pulmonary extracellular bacterial infection. This protocol allows for the expression of IFN-α locally in the lung and systemically in the serum at the time of *S. pneumoniae* infection. There is evidence that the AdIFN-α vector induces a long-term antiviral state that activates unknown immune mechanisms (175, 176). As we have now shown that IFN-α is protective against pneumococci, it is of interest to investigate long-term effects of the transgenic expression in our model, which would hold important implications for prophylactic treatment during outbreaks. Also, other treatment protocols including therapeutic use of AdIFN-α, delivered after *S. pneumoniae* infection are important to investigate. Moreover, with our well-established model of influenza and *S. aureus* infection, the specificity of IFN-α mediated protection can be tested with a different extracellular pathogen (103). We have also illustrated that at early timepoints post-infection, AdIFN-α leads to enhanced innate immune responses in the airway, including cytokine and chemokine expression, influx of neutrophils, NK cells, and CD4 T cells, and macrophage phagocytic killing. These results shed light onto the mechanisms of IFN-α associated protection from pneumococcal infection. Thus the intriguing results in this study are promising and hold possibilities for various further investigations, which have significant implications in treatment of pneumococcal infections.

5. Concluding Remarks and Future Directions

Influenza and *Streptococcus pneumoniae* remain major global pathogens that contribute to significant morbidity and mortality, whether infecting alone or in concert.

The pathogens themselves cause overwhelming harm to the delicate tissues of the lung, and the elicited immune responses often contribute to lung immunopathology. Therefore, it is apparent that current research must focus on further understanding the host immune responses to these pathogens, the mechanisms of lung immunopathogenesis following infection, and on discovery of novel therapeutics. The research presented in this thesis addressed important, understudied questions this field.

Firstly, we found that the classically proinflammatory cytokine TNF is a negative type 1 immune regulator that controls the level of immune activation, lung immunopathology, and aberrant tissue remodeling during influenza infection. Further investigations to be done with our model include: (i) the role of TNF in T cell, B cell, and fibroblast apoptosis, proliferation, and activation, (ii) specific roles of the two TNF receptors, (iii) molecular mechanisms of the influenza—TNF—MCP-1—TGF-β1 axis (gene expression regulation), and (iv) cellular sources involved in TNF, MCP-1, and TGF-β1 release. Also, improved reporting of influenza illnesses in patients receiving TNF inhibitors will allow us to determine to what extent such patients need to be monitored after being infected with influenza.

Secondly, we observed that in animals superinfected with *S. pneumoniae* following influenza, concurrent treatment with both an antibiotic and corticosteroid best improves clinical outcome, bacterial clearance, cellular and cytokine responses, and immunopathology. These findings hold important implications in future clinical practice. Further investigations to be done in the field include: (i) other novel mechanisms of increased susceptibility and immunopathology, (ii) further uncoupling of the contribution

of bacterial replication and immune responses to immunopathology, (iii) use of other treatment protocols (antibiotics, timing, dose), and (iv) aged or infant mouse models which are more reflective of the most susceptible human populations. While much is known about the various mechanisms of increased susceptibility to bacterial infection following influenza, much remains to be discovered in these complex processes. We demonstrated that decreased surfactant levels may contribute to bacterial outgrowth at later times following bacterial superinfection. These observations need to be further investigated, as well as possible surfactant replacement therapy for pneumococcal infections.

Thirdly, we discovered that transgenic expression of IFN-α using a single dose of an adenovirus vector protects against acute pulmonary pneumococcal infection. These results yield many possibilities for further research into the development of improved therapeutics for pneumococcal infections. Further investigations to be done with our model include: (i) mechanisms of the effect of IFN-α on bacterial control (cellular activity, epithelial invasion), (ii) effect of prolonged IFN-α expression, (iii) other protocols such as AdIFN-α administration at different time points before or after *S. pneumoniae* infection, (iv) protection from high dose challenge with *S. pneumoniae*, (v) administration route (intramuscular), and (vi) confirmation with our *Staphylococcus aureus* model. Therefore, together our findings provide important insights into the mechanisms of lung immunopathogenesis, as well as novel treatment protocols and therapeutics for pulmonary influenza and pneumococcal infections.

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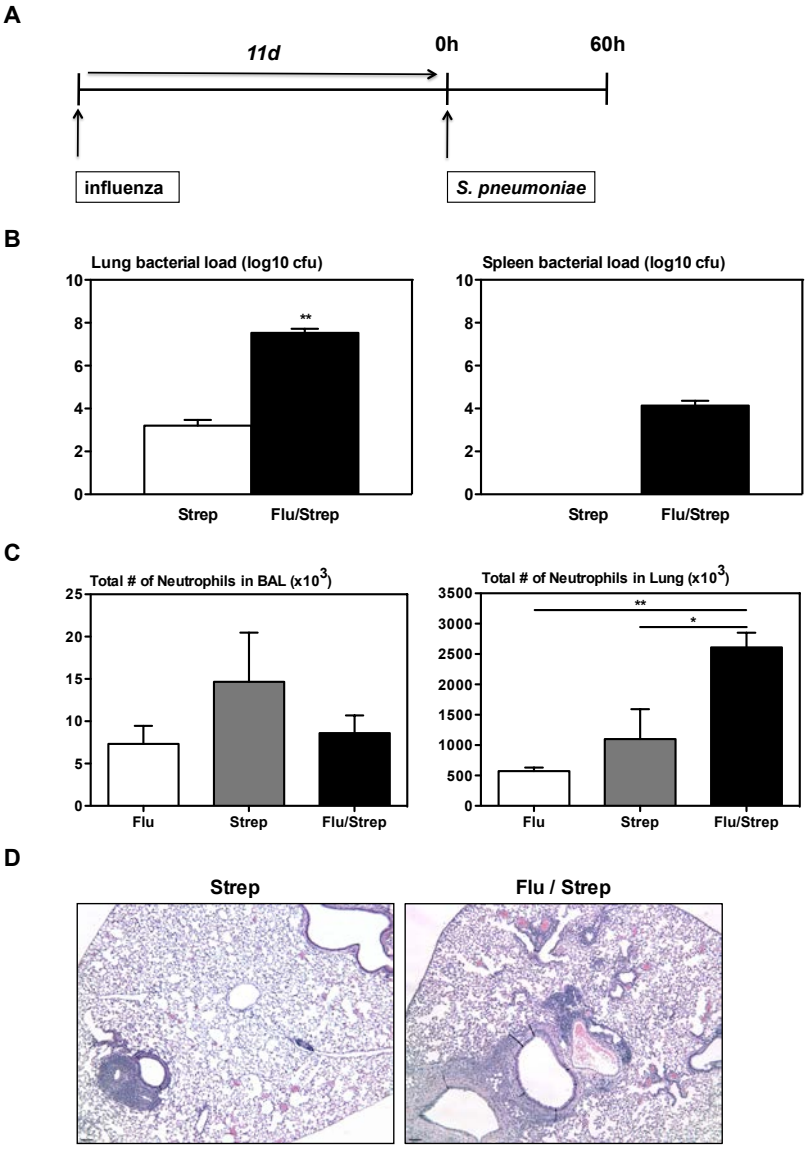
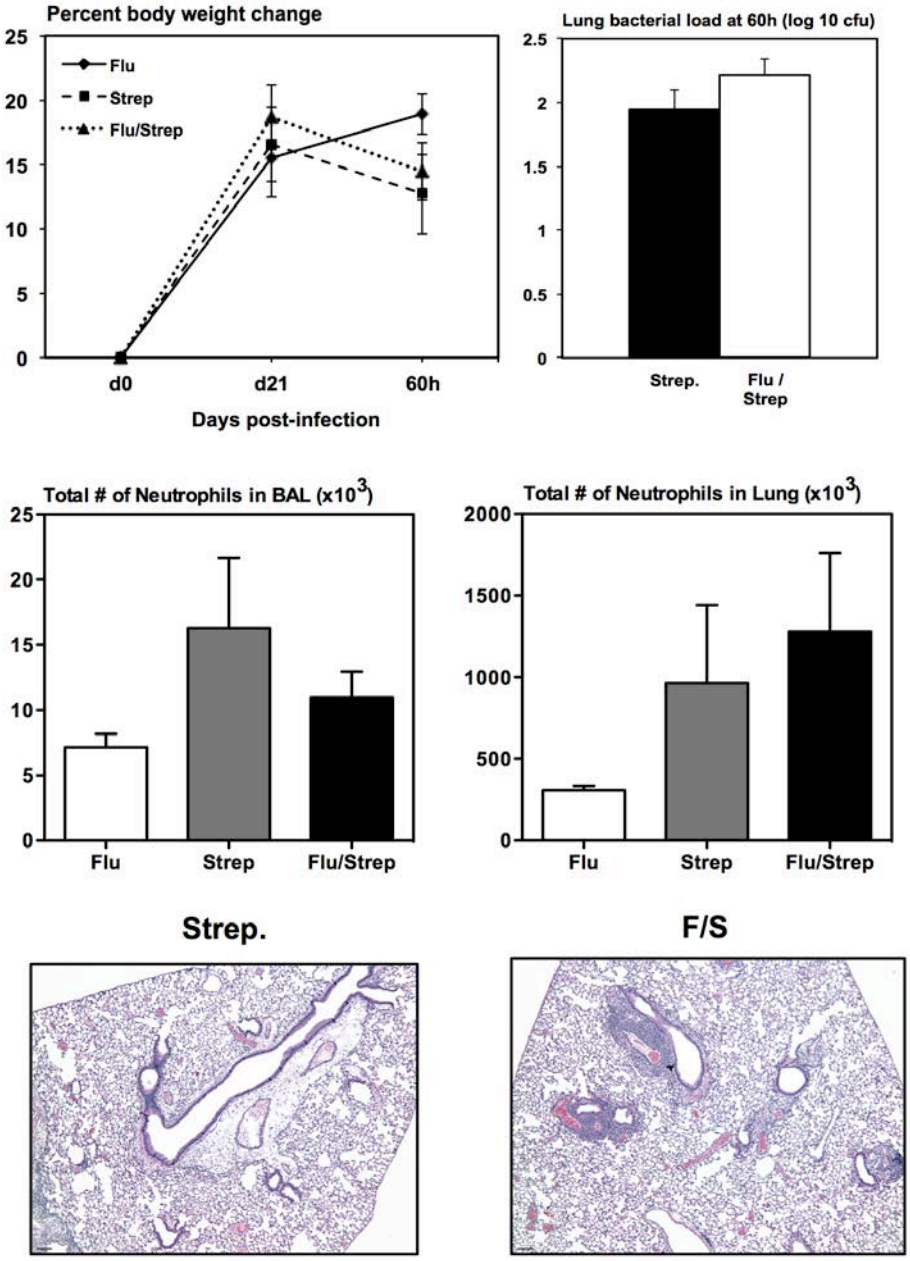
APPENDIX I

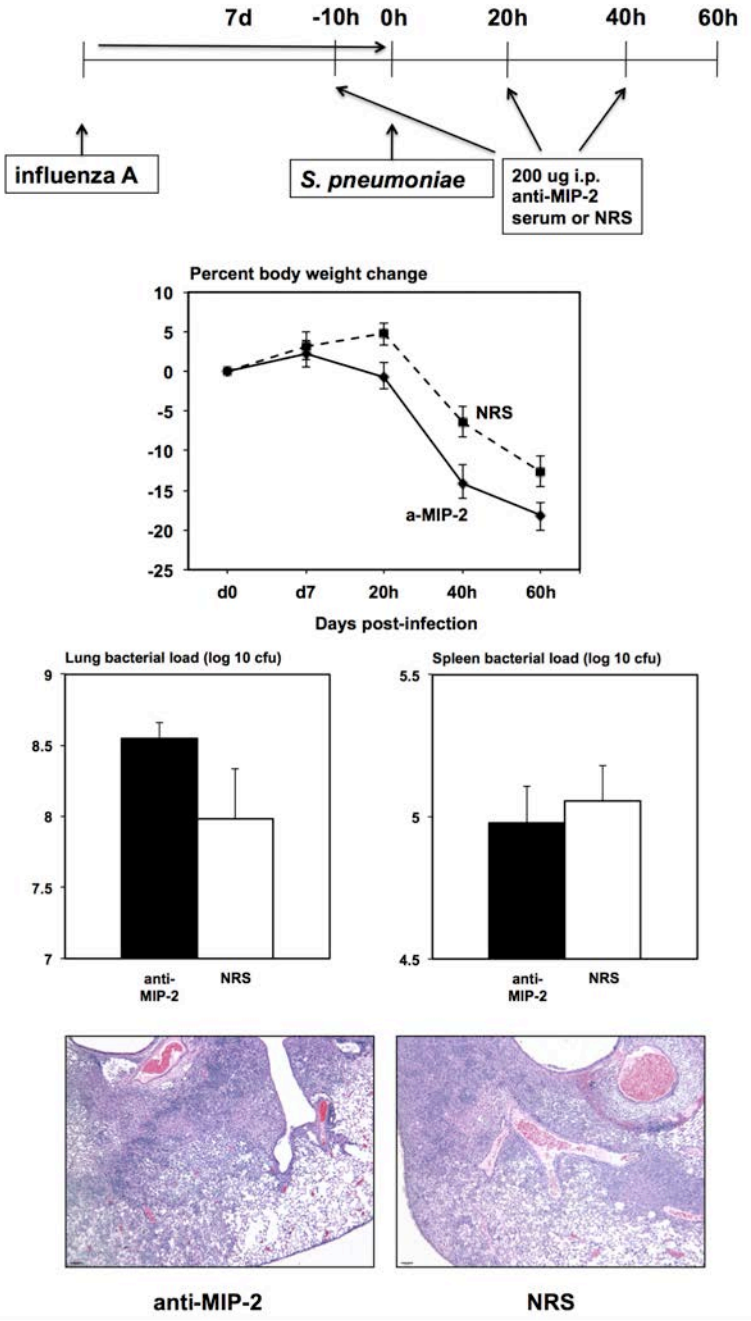
Data not shown for Chapter 3



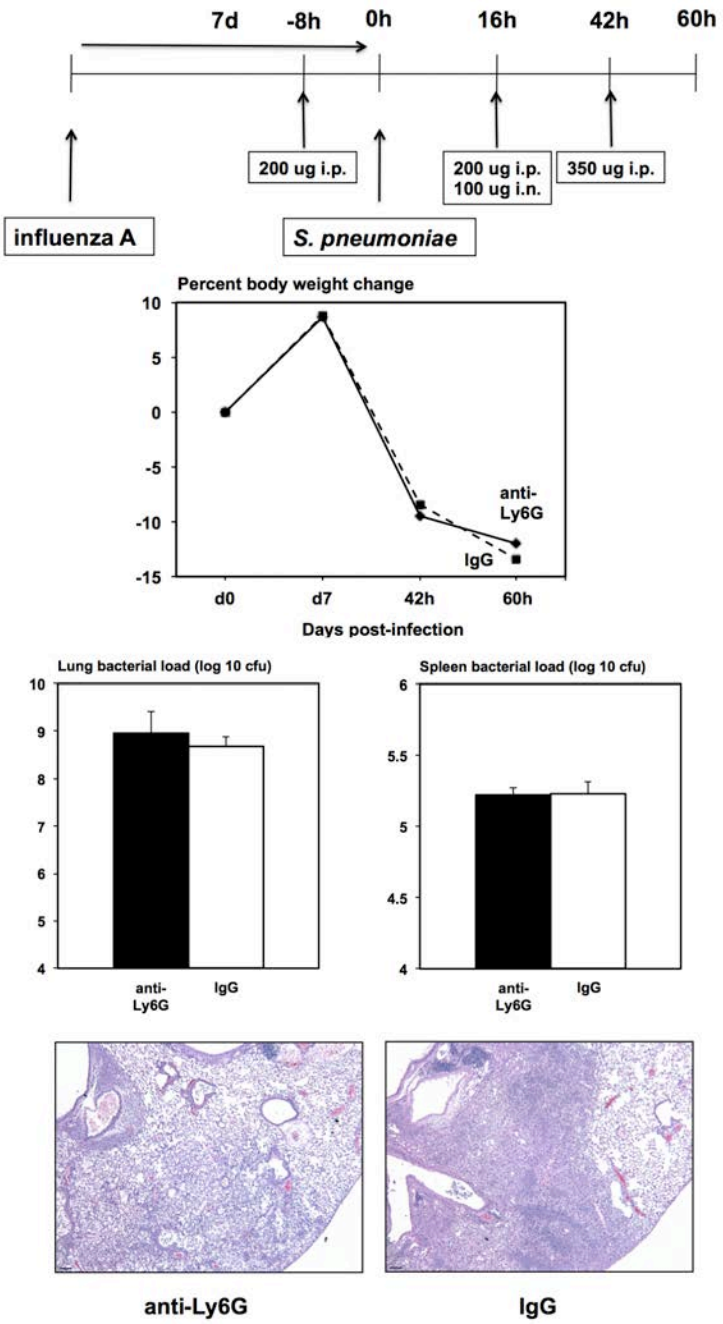
Increased susceptibility and enhanced lung immunopathology are still observed in mice infected with *S. pneumoniae* 11 days post-flu (pg. 64). WT mice were infected with 10^5 pfu of influenza (Flu) or 1,000 cfu *S. pneumoniae* (Strep) or dual infected with flu and 11 days later with *S. pneumoniae* (Flu/Strep) (***A***). Lung and spleen bacterial load (***B***), neutrophil influx into the BAL and lung (***C***), and lung histopathology (***D***) were assessed at 60h post-Strep. Results are expressed as mean ± SEM and representative of 6-10 mice/group from two experiments.



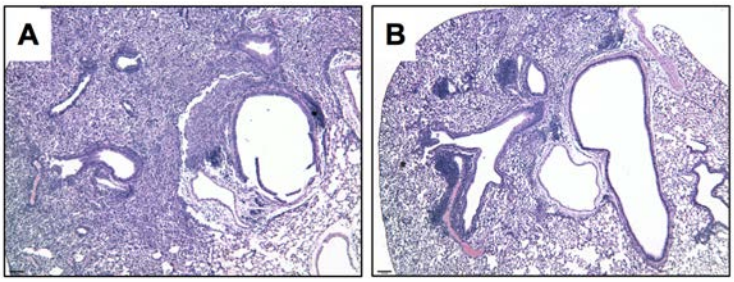
No increased susceptibility and lung immunopathology in mice infected with *S. pneumoniae* 21 days post-flu (pg. 64). WT mice were infected with 10^5 pfu of influenza (Flu) or 1,000 cfu *S. pneumoniae* (Strep) or dual infected with flu and 21 days later with *S. pneumoniae* (Flu/Strep). Body weight changes, lung bacterial load, neutrophil influx into the BAL and lung, and lung histopathology were assessed at 60h post-Strep. Results are expressed as mean ± SEM and representative of 3-6 mice/group from one experiment.



MIP-2 depletion does not improve the clinical outcome, bacterial load, or immunopathology in dual infected mice (pg. 68). WT mice were infected with 10^5 pfu of influenza and 7 days later with 1,000 cfu *S. pneumoniae*. Anti-MIP-2 rabbit serum or normal rabbit serum (NRS) were administered at the regimen outlined above, and mice were sacrificed at 60h. Body weight changes, bacterial load, and lung histopathology of the two groups were monitored. Results are expressed as mean ± SEM of 7 mice/group from one experiment.



Neutrophil depletion moderately improves lung immunopathology in dual infected mice (pg. 68). Mice were infected as before. Anti-Ly6G antibody or total rat IgG were administered at the regimen outlined above, and mice were sacrificed at 60h. Body weight changes, bacterial load, and lung histopathology of the two groups were monitored. Results are expressed as mean ± SEM of 7 mice/group from two experiments.



Lung immunopathology is sustained in AZT alone treated dual infected mice (pg. 71). Mice were infected with 10^5 pfu of influenza virus and 7 days later with 10^3 cfu of *S. pneumoniae*. Azithromycin (AZT) was given at 30h and 44h post-*Strep* and mice were sacrificed at 88h (***A***) and 144h (***B***) post-*Strep*.

APPENDIX II

Immunopathology in influenza virus infection: Uncoupling the friend from foe

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REVIEW

Immunopathology in influenza virus infection: Uncoupling the friend from foe

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KEYWORDS
Influenza;
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Mechanisms

Abstract Influenza epidemics and pandemics cause significant morbidity and mortality worldwide associated with severe immunopathology in the lung, and the mechanisms of such immunopathogenesis still remain poorly understood. While human studies help to understand influenza immunopathology, they provide only limited mechanistic information. On the other hand, recent studies using experimental animal models have significantly enhanced our understanding of the complex mechanisms involved in the immunopathogenesis during primary influenza or influenza-associated bacterial superinfection. This includes the involvement of acute inflammatory responses (macrophages, neutrophils, dendritic cells, toll-like receptors, cytokines, chemokines), CD4 and CD8 T cells, tissue remodeling processes, and contribution of bacterial superinfection. In particular, progress has been made in uncoupling the mechanisms that are involved in both anti-viral host defense and in immunopathogenesis from those that solely contribute to lung immunopathology. Uncoupling such events will facilitate the discovery of new intervention strategies to treat pulmonary immunopathology associated with influenza infection.

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1. Introduction

Influenza is a common and highly contagious respiratory infectious disease. Influenza viruses of varying pathogenicity not only cause annual epidemics but also continue to cause unpredictable and perilous worldwide pandemics. Epidemics cause hospitalizations and significant mortality in immune compromised individuals, as well as the very young and the elderly. Pandemics have historically shown to be much harder to control and have resulted in more deaths [1,2]. These include the 1918 influenza pandemic that killed approximately 50 million people worldwide, the pandemic influenza strains such as the H5N1 influenza that re-emerged in 2003, and the pandemic influenza H1N1 of 2009. A major cause of morbidity and mortality during influenza epidemics and pandemics is pulmonary immunopathology [1,3]. Upon influenza infection, cytotoxic mechanisms destroy infected cells in order to clear the virus and subsequent tissue remodeling restores the lung architecture. While such events are required for resolution of the infection, they also have the potential to cause deleterious pulmonary immunopathology [4,5].

Histological and pathological indicators suggest that influenza induces much more immunological activity than is necessary to clear the virus and it is the 'cytokine storm' and the ensuing viral pneumonia that cause most of the damage in the lung [1,3,6,7]. In most influenza infections the severe complications are restricted to the lung. Viremia is rare and is not a significant contributor to mortality [2]. However, infection with the highly pathogenic avian influenza H5N1 causes a fast-progressing pneumonia that is complicated by viremia and extra-pulmonary symptoms contributing to multiorgan failure [7]. Interestingly, autopsy examinations have shown very similar lung histopathology for different influenza strains, regardless of viral pathogenicity [1,8]. Observed pathological changes include destruction and desquamation of the tracheal and bronchial epithelium, submucosal edema, vascular thrombosis, mixed inflammatory cell infiltration around the airways and throughout the lung parenchyma, alveolar damage and hyaline membrane formation [1,3,6,8,9]. Therefore, lethal infections are not localized only to the larger airways like non-lethal infections with low virulence viruses are, but extend to the alveoli and lung parenchyma, infecting pneumocytes and other cell types including macrophages [2,8]. In many cases, sustained lung injury and chronic tissue remodeling occur and lead to squamous metaplasia, pneumocyte hyperplasia and interstitial fibrosis [1,3,7]. Secondary bacterial superinfections following influenza further complicate the picture and have been found to be more deadly than the primary viral infection itself. Bacterial superinfections often occur within the first two weeks of influenza infection [10]. In humans, superinfections induce a massive neutrophilic infiltration and thus worsen tissue injury and pneumonia in the lung, causing production of pus and suppurative bronchopneumonia [1,2,6,8]. Overall, while human studies are invaluable, they are limited to late-stage disease with a lethal outcome and understandably provide very limited mechanistic insights to influenza immunopathogenesis. In this regard, the use of animal models is instrumental.

The most commonly used species in experimental models of influenza are laboratory mice, domestic ferrets, and cynomolgus macaques [1,6,7]. The mouse remains the most favorable model animal as mice are inexpensive, their genetics are well-characterized, inbred strains and knock-outs are available, important reagents are readily available, and they display most of the histopathological features seen in human influenza [6,11,12]. However, the data from animal models is complicated by the use of vastly different doses and influenza viral strains of varying virulence, which makes it difficult to resolve the exact cause of lung immunopathology — the virus or the resulting host immune responses or both. In this regard, some of the most recent studies have begun to uncouple such mechanisms. This review will examine and discuss the latest information on influenza immunopathogenesis from mouse models of primary influenza infection and influenza-associated bacterial superinfection. The primary focus will be on the role of immune cells rather than humoral aspects of the immunity as little information is available on the immunopathogenic role of antibody responses.

2. Acute Inflammatory Responses and Their Role in Immunopathology

Human autopsy studies have pointed to the contribution of excessive acute inflammatory responses to death following severe influenza infection, including influx of innate cells into the lungs and overproduction of cytokines and chemokines that culminate in life-threatening pulmonary immunopathology. Data from experimental animal models have added much insight into the contribution of various mechanisms of acute

inflammation to influenza immunopathology. Recent advances include findings on the roles of macrophages, neutrophils and dendritic cells (DCs), toll-like receptors (TLRs), and cytokines.

2.1. Macrophages

Macrophages accumulate in the lungs upon influenza infection, release proinflammatory cytokines, and help control viral load as part of the innate immune response [13]. However, accumulating evidence shows that macrophages can also become destructive and cause lung immunopathology following influenza infection.

The majority of inflammatory cells present in the lungs by 5 days post-infection were found to arise from CCR2 (CC-chemokine receptor 2) expressing monocytes recruited by MCP-1/CCL2 (monocyte chemoattractant protein-1) produced by infected airway epithelial cells [14]. These included monocyte-derived DCs and exudate macrophages that are the source of TNF-α and NO synthase 2 (NOS2) in infected lungs and were found to be the predominant cause of immunopathology, morbidity and mortality [14]. Infected CCR2-deficient mice had significantly reduced numbers of monocyte-derived inflammatory cells, markers of lung injury, weight loss, and mortality with no change in viral clearance or dissemination [14]. Likewise, pharmacologically blocking CCR2 with a small molecule inhibitor reduced weight loss and hypothermia, lung injury, and mortality [15]. Again, viral clearance was not affected in these mice and there was even an increase in antigen-specific cytotoxic T cell activity in the lungs of treated mice [15]. In further support for a detrimental role for MCP-1 signaling, we have recently found that neutralization of MCP-1 in TNF-α-deficient mice markedly attenuated influenza immunopathology [16]. Neutralization of MCP-1 was associated with reduced numbers of macrophages and DCs, while also not affecting viral clearance [16]. In addition, we observed that transgenic expression of MCP-1 worsened lung immunopathology in WT hosts [16]. However, Tate et al. reported contrasting results when comparing selective depletion of airway macrophages during infection with a low virulence H3N2 influenza virus that led to exacerbated virus replication, dysregulated cytokine and chemokine production and severe viral pneumonia, and a highly virulent PR8 influenza virus where viral clearance nor disease severity were affected after macrophage depletion [17]. Such comparisons suggest that the role of macrophages in host defense and immunopathology may differ based on influenza virus subtype or pathogenicity. However, due to the wide range of virus subtypes and doses used in most studies, a conclusion remains elusive.

Other studies have further investigated the specific mechanisms by which macrophages may contribute to lung immunopathology during influenza infection. Herold et al. found that exudate macrophages recruited into the lung contributed to alveolar epithelial cell (AEC) damage and apoptosis [18]. These macrophages released TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) that via binding to DR5 (death receptor 5) on epithelial cells led to their apoptosis, causing increased lung leakage and mortality [18]. In contrast, another study found that defective macrophage recruitment caused by MCP-1 neutralization led to enhanced AEC damage and apoptosis without affecting viral clearance [19]. The lungs had decreased levels of hepatocyte growth factor (HGF), a mitogen for alveolar epithelium suggesting that macrophage-released HGF plays a role in the regeneration of alveolar epithelium or tissue repair upon influenza infection [19]. Therefore the role of macrophages in AEC regeneration or apoptosis remains controversial.

Studies examining the negative regulators of macrophages during influenza infection have also reported that increased macrophage numbers and activity in the lung can lead to delayed resolution of lung inflammation and death, even in the context of lower viral titers. Among the mechanisms that regulate immunopathogenic responses following influenza infection are regulatory T cells (Tregs), found to limit pathology by reducing the accumulation and altering the distribution of macrophages in the lungs to more confined areas, which was also associated with a delay in MCP-1 induction [20]. In addition, the negative regulator CD200 is expressed on airway epithelial cells and binds to CD200R expressed on macrophages [21]. Mice lacking CD200 had increased macrophage numbers and activity which led to delayed resolution of lung inflammation and death, even in the context of lower viral titers [21]. CD200R agonists alleviated influenza-induced illness and prevented the excessive lung inflammation [21].

These findings together illustrate a complex role of lung macrophages in influenza viral clearance and immunopathology. They suggest that a certain level of the macrophage response is important for viral clearance and lung repair. However, when infection causes excessive influx of macrophages into the lung, these cells become significant contributors to immunopathology. It still remains to be elucidated whether the relative contribution of lung macrophages to influenza immunopathology can be determined by the subtype or pathogenicity of virus. Further comparisons of different influenza viruses need to be performed, and may prove vital in future epidemic and pandemic preparedness. Importantly, the above studies show that reduction of macrophage accumulation following influenza infection can be beneficial by reducing immunopathology without sacrificing viral clearance. In particular, mounting evidence points to the CCR2 chemokine pathway in uncoupling anti-viral host defense from immunopathology. These findings hold important implications in design of potential CCR2 blockade-based therapeutics for influenza epidemics and pandemics. Thus if macrophages are inconsequential to viral clearance and only act to control viral replication until the adaptive immune response is mounted, a role that is also fulfilled by other cells of the innate immune system including natural killer cells and neutrophils, then depleting macrophages to prevent them from contributing to lung immunopathology could be a sound therapeutic strategy.

2.2. Neutrophils and DCs

Like monocytes, neutrophils are rapidly recruited to the airway following influenza infection, and play a role in host defense by controlling viral replication and clearing dying cells [22]. Neutrophil depletion in influenza infected mice has been shown to cause rapid weight loss, enhanced virus replication, exacerbated pulmonary inflammation and respiratory

dysfunction [23]. On the other hand, mounting evidence also reveals an important contribution of neutrophils to the excessive acute inflammatory responses that cause immunopathology in influenza infections. Narasaraju et al. found that neutrophil depletion did not change viral clearance and only caused mild lung pathology [24]. When macrophages were depleted, the resulting lung immunopathology was associated with excessive neutrophilic infiltration [24]. Neutrophil mechanisms that contributed to the immunopathology included myeloperoxidase activity and release of neutrophil extracellular traps (NETs) that were found to be entangled with alveoli and observed in areas of tissue injury and endothelial damage [24]. In a model of influenza infection under PAFR (platelet activating factor receptor) deficiency, reduced tissue injury and mortality were associated with decreased neutrophil recruitment, suggesting that neutrophils contributed to lung injury in WT hosts [25]. Moreover, at 8 days post-infection with a low dose influenza virus, titers were significantly lower in PAFR-deficient mice compared to WT [25]. Thus, enhanced viral clearance was coupled with reduced tissue neutrophilia and lung immunopathology in this study.

Dendritic cells, which are important in host defense against influenza by inducing adaptive immune responses, have also recently been identified to contribute to influenza immunopathology. A particular dendritic cell subset, the tipDCs (TNF-α/inducible nitric oxide synthase (iNOS)-producing DCs), is excessively recruited to the lungs during influenza infection [26]. While these cells were essential to mounting a proper anti-viral CD4 and CD8 T cell response, partially compromising their recruitment reduced weight loss and mortality with no effect on viral clearance [26].

Therefore, other than macrophages, the above evidence points to the immunopathogenic dangers of excessive neutrophil and DC influx into the lung following influenza infection. Again, partially reducing influx of these cell types seems to be inconsequential to viral clearance, which makes them possible targets for immunotherapy during influenza outbreaks. However, studies of neutrophils and DCs in immunopathology are still limited, and more work needs to be done to better understand their roles in viral clearance and immunopathology.

2.3. Toll-like Receptors

TLR stimulation is an important step in recognition of influenza virus by the innate immune system. Thus, in theory increased stimulation of these receptors may be protective against influenza immunopathology and mortality, and agonists have recently been explored in vaccine design. Prestimulation of TLR2 and TLR4 with a synthetic mycoplasmal lipoprotein (FSL-1) or lipopolysaccharide (LPS), during influenza infection was found to enhance viral clearance and survival [27]. Furthermore, we observed that TLR4 stimulation with a fimbriae H protein (FimH) reduced pulmonary immunopathology, morbidity, and mortality in influenza infection, associated with decreased viral titers [28].

Overstimulation of TLR receptors, however, can be detrimental as it may lead to excessive acute inflammation. As such, deficiency in IRAK-M (IL-1 receptor-associated kinase-M), an inhibitor of MyD88-dependent TLR signaling, led to heightened viral loads, lung immunopathology and mortality following influenza infection [29]. Immunopathology correlated with enhanced early influx of neutrophils and release of elastase and myeloperoxidase, increased inflammatory cytokines and chemokines, and greater numbers of activated T cells [29]. These results further demonstrate how the downstream effects of TLR overstimulation can cause excessive and detrimental acute inflammation. In such cases, dampening TLR signaling may be beneficial. For instance, down-regulating TLR7/9-mediated innate immune responses by using a microsatellite DNA mimicking oligodeoxynucleotide (MS ODN) led to improved survival and reduced lung immunopathology associated with reduced neutrophil recruitment and TNF-α expression in the lungs, without impacting viral clearance [30]. Furthermore, the TLR3 pathway was found to be detrimental in influenza-induced acute pneumonia, as TLR3 deficiency led to significantly reduced lung inflammation and immunopathology and improved survival, although this improvement occurred at the expense of higher viral titers [31].

While the above studies with TLR 2/4 indicate that prestimulation of TLRs may be beneficial during influenza infection, such strategies remain controversial due to contrasting findings with TLR3/7/9 and IRAKM which indicate that TLR signaling must be tightly controlled to prevent immunopathology. Whether differences in the results are due to the specific TLRs studied is unclear, as is the requirement for different TLR signaling for viral clearance. Thus, much more investigation of TLR pathways is required, as ideally the potential immunointervention strategies would need to improve survival and lung immunopathology without affecting viral clearance.

2.4. Cytokines

The 'cytokine storm' that occurs in the first few days following influenza infection is a major cause of lethal immunopathology. A surging response of proinflammatory cytokines and chemokines reflects an overzealous attempt by the host to control the infection. Recent experimental studies have revealed the importance of proper regulation of cytokine expression, as both insufficient and excessive levels of certain cytokines can be detrimental.

Influenza infection was found by Perrone et al. to cause rapid expression of high levels of proinflammatory cytokines and chemokines in the lungs, including IFN-γ, IL-1α, IL-6, MIP-1α, MCP-1, and KC [32]. The excessive cytokine response played a role in the observed acute lung inflammation including severe lung consolidation and destruction of tissue architecture [32]. In the first day of influenza virus infection, lung levels of IL-17 and IL-17-responsive cytokines and chemokines were found to be elevated and correlated with severe acute lung injury [33]. IL-17 deficiency or depletion ameliorated lung inflammation and immunopathology and improved survival while it did not negatively affect viral clearance [33]. On the other hand, another study found that during influenza infection mice deficient in TNF-α, IL-6, or MCP-1, or mice treated with glucocorticoids for suppression of cytokines were inconsequential to the survival rates [34]. These data together suggest that carefully selected targeting of certain cytokine pathways such as IL-17 may improve influenza immunopathology without negatively affecting

viral clearance, whereas targeting other cytokines or globally suppressing cytokine expression may be futile or even deleterious. In fact, we have recently investigated the role of TNF-α in influenza host defense and immunopathology. We found that TNF-α was not required for influenza clearance, but was critically required for controlling the levels of anti-viral inflammatory and immunopathogenic responses [16]. Thus, even after viral clearance TNF-α deficient mice suffered a greater extent of illness, continued to have heightened lung immunopathology and developed fibrotic tissue remodeling (Fig. 1, top) [16]. These immunopathological changes were associated with dysregulated responses of cytokines, particularly MCP-1 and fibrogenic cytokine TGF-β1 [16]. Neutralization of MCP-1 markedly attenuated immunopathology and fibrotic remodeling in the lungs of TNF-α deficient mice [16]. These results suggest that TNF-α plays an important immune regulatory role during influenza immunopathogenesis.

Recent findings have provided more insight into the role of GM-CSF (granulocyte-macrophage growth factor) in influenza infection, and further illustrated the importance of cytokine production at proper levels. Transgenic expression of GM-CSF in lung epithelium or intranasal treatment with GM-CSF were found to improve survival after influenza infection, which correlated with quick resolution of inflammation that led to reduced viral load, weight loss and lung immunopathology [35]. Another recent study showed that GM-CSF enhanced resistance to influenza infection, as GM-CSF deficient mice had shorter survival times than WT counterparts [36]. However, the transgenic mice with high levels of GM-CSF also eventually succumbed to infection that was associated with pneumonia and development of secondary interstitial lung disease [36]. Interestingly, conditional expression of optimal GM-CSF levels at the time of infection, using a doxycycline inducible mouse model, resulted in improved lung immunopathology and survival [36].

The above evidence collectively suggests that transiently increased expression of cytokines and chemokines at proper levels protects the host from influenza by enhancing host anti-viral defense and effectively controlling viral replication. However, excessive or prolonged proinflammatory cytokine expression can lead to undesired lung immunopathology and aberrant tissue remodeling. Moreover, proper

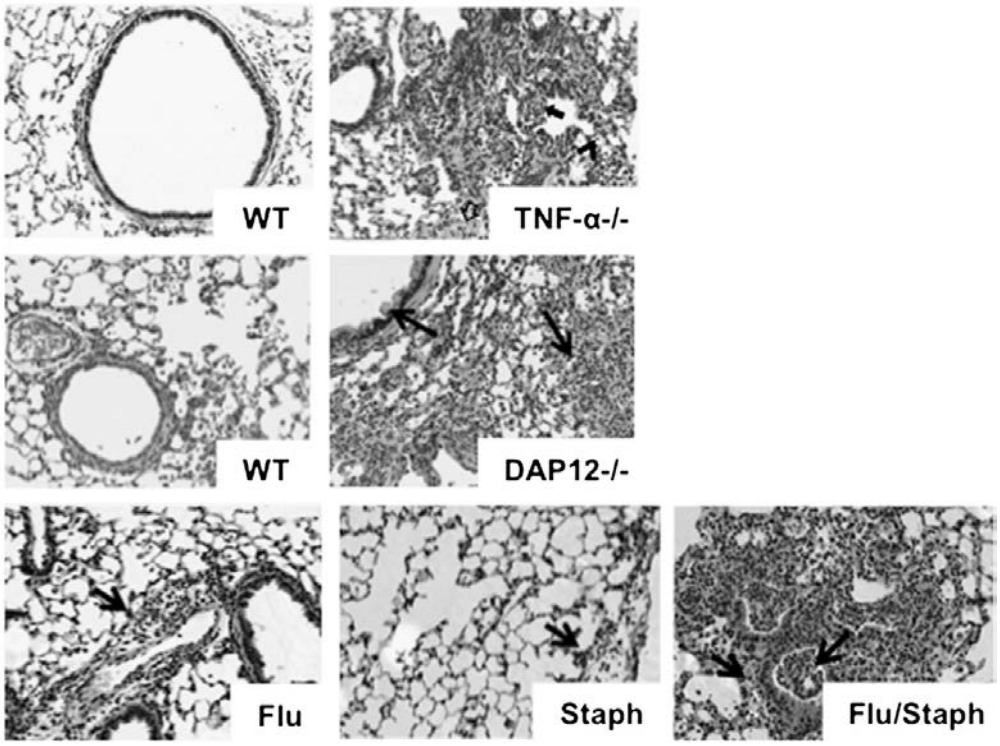

Figure 1 Increased influenza-associated lung immunopathology by TNF-α deficiency, DAP12 deficiency or *S. aureus* bacterial superinfection. Wild type (WT) or gene-deficient mice were intranasally infected with a mouse-adapted H1N1 influenza virus, sacrificed, and lung tissue sections were stained with H&E. Top panels: WT lungs returned to normal by 21 days post-infection, while TNF-α−/− lungs had persistent lung immunopathology and injury. Middle panels: WT lungs showed low residual lung inflammation by 9 days post-infection, while DAP-12−/− lungs displayed exaggerated lung immunopathology and injury. Bottom panels: WT mice were infected only with influenza (7d) or *S. aureus* (1d), or infected with influenza (7d) and then superinfected with *S. aureus* (1d). Flu lungs had residual mononuclear infiltrates, Staph lungs showed predominantly neutrophilic infiltration, and Flu/Staph lungs displayed mixed neutrophilic and mononuclear infiltration, epithelial sloughing, and formation of intrabronchial inflammatory plugs [10,16,42].

expression of immunoregulatory cytokines such as TNF-α is also essential in the return to homeostasis during the resolution phase of influenza infection.

3. CD4 T Cells and Immunopathology

CD4 T cells play a vital role in influenza host defense by not only providing the necessary cytokine and co-stimulatory molecules required to activate protective B cell and antibody responses, but also through direct targeting of virally infected cells [37]. In support of this notion, deficiency in CD4 T cells leads to decreased antibody titers and impaired viral clearance [38,39]. However, many of the effector functions of CD4 T cells that are essential for host anti-viral defense also have the potential to cause serious immunopathology.

CD4 T cells are able to directly recognize and target influenza infected cells and actively eliminate the viral infection, even in the absence of CD8 T cell or B cell responses [40,41]. Interestingly, the CD4 T cell response was found to be more diverse than the CD8 T cell response, suggesting that CD4 T cells have a much greater potential to react against self antigens and cause tissue destruction. In spite of the obvious potential for CD4 T cells to cause lung immunopathology, there is little evidence to directly support this notion. Following influenza infection, Th1 cells secrete IFN-γ. However, we found that in vivo neutralization of excess IFN-γ did not reduce severe immunopathology and mortality caused by dysregulated Th1 cells in influenza infected DAP12-deficient mice [42]. These findings imply that cytokine production alone by CD4 T cells is not likely the mainstay mechanism by which CD4 T cells cause lung immunopathology.

CD4 T cells lyse virally infected cells through perforin/granzyme and Fas-L, and their expression during influenza is protective and essential for viral clearance [43]. These mechanisms have also been implicated in tissue immunopathology in a variety of diseases including viral hepatitis and pulmonary fibrosis [44–46]. However, the role of CD4 T cells in influenza immunopathology remained elusive until recently. Teijaro et al. found that a memory CD4 T cell response to a secondary influenza exposure caused lung immunopathology [47]. Of note, co-stimulation modulation via blocking CD28 signaling in CD4 T cells, reduced lung immunopathology while still maintaining proper viral clearance in this model of secondary influenza challenge [47]. We also recently investigated the regulatory mechanisms of immunopathogenic CD4 T cells during influenza infection and found that the transmembrane immunoadaptor DAP12 was critically required to regulate the level of immunopathogenic CD4 T cell responses [42,48]. DAP12 deficiency led to remarkably increased influenza-specific, Fas-L-expressing CD4 T cells in the lung which caused rapid and severe damage to the airway epithelium, bronchial inflammatory plug formation, and ultimately respiratory failure (Fig. 1, middle) [42]. Blocking Fas-L by using a neutralizing antibody prevented lethal lung immunopathology and improved survival [42]. These findings for the first time demonstrate that CD4 T cells can directly contribute to severe immunopathology and tissue destruction in a cytokine-independent manner during primary influenza infection.

In addition to Th1 CD4 T cells, Th17 responses have been observed in cases of severe, life-threatening influenza suggesting that they may play a role in tissue immunopathology [33,49,50]. There are several reports to support the idea that Th17 responses do not directly contribute to anti-influenza host defense, but rather contribute directly to tissue immunopathology. Indeed, it has been shown that IL-17 depletion does not alter the ability to clear lethal viral infection but it improves survival by ameliorating influenza immunopathology [33,51]. Such evidence seems to support the selective targeting of Th17 cells or IL-17 as an attractive immunointervention strategy for alleviating influenza immunopathology, as it will not affect viral clearance. However, Wang et al. recently showed that IL-17 plays a critical regulatory role in preventing lung immunopathology through the modulation of B cell recruitment to the infected lung [52]. These apparently contradictory findings illustrate the complex nature of the host response to primary influenza infection and highlight an urgent need for further in-depth investigation of the role of CD4 T cells in influenza immunopathology.

4. CD8 T Cells and Immunopathology

CD8 T cells and their cytolytic activity against influenza infected cells have long been known to be critical for viral clearance, as part of the adaptive immune response [5,53,54]. Studies continue to support this point. Lin et al. showed that during severe influenza infection the co-stimulatory receptor 4-1BB on T cells is induced, binding to its ligand 4-1BBL on APCs [55]. It was found that 4-1BBL deficiency led to decreased CD8 T cell accumulation in the lungs, decreased viral clearance, impaired lung function and increased mortality [55]. Interestingly, while a low dose of intranasally delivered 4-1BBL improved survival, a high dose exacerbated disease due to increased immunopathology [55]. In addition to their host defense activity, recent evidence also suggests an immune regulatory role of effector CD8 T cells during influenza. It was found that during acute influenza infection, the anti-inflammatory cytokine IL-10 was produced in the lungs by recruited effector CD8 T cells [56]. This IL-10 was shown to have a protective effect, as blocking of the IL-10 receptor resulted in lethal lung immunopathology without affecting viral clearance [56]. The critical immune regulatory role of IL-10 during influenza infection is reminiscent to our finding of a similar role by TNF-α required to control lung inflammation, immunopathology and fibrotic tissue remodeling during influenza infection [16].

However, as for effecter CD4 T cells, improper CD8 T cell contraction following viral clearance, exuberant cytolytic activity and proinflammatory cytokine production can cause lung immunopathology. For instance, the inhibitory natural killer cell receptor NKG2A is induced on CD8 T cells upon influenza infection and binds to its ligand Qa-1b on APCs [57]. Qa-1b deficiency led to greatly increased lung immunopathology associated with CD8 T cell cytolytic activity, as the influenza virus was effectively cleared [57]. Also, when NKG2A ligation was blocked in vivo, lung injury was amplified in transgenic mice expressing hemagglutinin (HA) in the lung upon adoptive transfer of HA-specific CD8 T cells [57]. Furthermore, Nakamura et al. found that IL-15 deficiency led to improved survival during influenza infection, associated with decreased numbers of antigen-specific CD8

T cells in the lung, while viral clearance was unchanged [58]. While this piece of evidence suggests that IL-15 could be a therapeutic target for taming influenza immunopathology, this cytokine is known to be critical to maintaining memory CD8 T cells [59]. Other studies have also linked CD8 T cells with influenza-mediated immunopathology. Mice lacking the negative regulator CD200 developed more severe disease associated with increased immunopathology upon influenza infection, without impacting viral clearance [60]. Depletion of CD4 or CD8 T cells in these mice led to improved animal welfare despite dramatically increased viral load [60]. This is an interesting study which is in agreement with our recent finding that depletion of immunopathogenic CD4 T cells in influenza infected DAP12 deficient mice markedly improves survival, even though viral clearance is delayed [42].

The above results together suggest that like their CD4 counterparts, effector CD8 T cells are essential for viral clearance and immune regulation. However, dysregulated CD8 T cell cytolytic activity and proinflammatory cytokine production are significant contributors to immunopathology. Such findings have important implications in the way we understand the complex scenarios at play during influenza infections. It is important to continue to identify the immunological pathways that are not vital for influenza viral clearance, but that are critical contributors to undesired lung immunopathology. However, in cases of severe influenza infection the top priority is to control devastating immunopathology and tissue injury for host survival, even if such control measures may hinder or delay viral clearance.

5. Tissue Remodeling and Immunopathology

Tissue remodeling is a necessary process for the lung to return to homeostasis after transient lung immunopathology following influenza. However, excessive remodeling has been shown to cause pulmonary fibrosis (PF) following influenza infection in humans [1,3,7]. However, fibrosis in animal models of influenza has been very much understudied, in part due to the acute nature of the infection. Qiao et al. have reported that following infection with a non-lethal dose of H5N1 influenza, acute lung immunopathological changes including typical diffuse pneumonia with inflammatory cellular infiltration, edema, and hemorrhaging were observed [61]. However, chronic immunopathological changes around 30 days post-infection were also seen, including severe interstitial and intra-alveolar fibrosis with thickened alveolar walls, collapsed alveoli and large fibrotic areas [61]. The development of PF was associated with significantly elevated hydroxyproline levels, collagen deposition in lungs, and increased dry lung-to-body weight ratio in infected mice [61]. In this regard, we have recently found that infection of TNF-α deficient mice with a non-lethal dose of mouse-adapted H1N1 influenza led to severe lung immunopathology and fibrosis characterized by myofibroblast accumulation, increased collagen deposition, and appearance of fibrotic foci in the lung parenchyma [16]. The fibrotic tissue remodeling was observed beginning at 14 days post-infection after viral clearance was completed, and was associated with excessive expression of fibrocyte-attracting MCP-1 and fibrogenic cytokine TGF-β1 [16]. In turn, neutralization of MCP-1 markedly decreased TGF-β1 expression and attenuated lung immunopathology and fibrosis [16].

Thus it appears that non-lethal influenza infections may evolve into a phase of fibrotic tissue remodeling long after viral clearance has occurred, particularly in the immune-dysregulated hosts. Such findings call for caution in long-term clinical management of survivors of H5N1 infection or for patients on anti-TNF-α therapy, including those infected with only the seasonal low virulence H1N1. Clearly, further investigation is still needed to understand the immune regulatory mechanisms of chronic tissue remodeling in non-lethal influenza infections.

6. Immunopathology Associated with Bacterial Superinfection

Seasonal influenza infection alone typically causes a debilitating yet transient illness in otherwise healthy individuals. Since the 1918 pandemic, studies have shown exacerbated lung immunopathology associated with secondary bacterial superinfections that typically occur in the first two weeks following influenza infection [1,2,6,8,62–64]. Bacterial superinfections are therefore major contributors to hospitalizations and deaths of influenza patients. In the majority of clinical cases, autopsy lung samples during influenza pandemics show severe pathological changes that include massive infiltration of neutrophils [63,65,66]. With new methods of identifying respiratory pathogens, such pathological evidence is indicative of secondary bacterial pneumonia caused primarily by *Streptococcus pneumoniae*, *Staphylococcus aureus*, or *Haemophilus influenzae* in such patients [63,65,67,68]. However, bacterial superinfections were largely underinvestigated in animal models until the last few years. Several mouse models of heterologous influenza and bacterial superinfection developed by us and others can recapitulate the clinical histopathological findings [10,69–73]. Despite the varying underlying mechanisms identified to date, there is a consistent finding that previous influenza infection markedly increases the susceptibility to subsequent bacterial superinfection. While the precise mechanisms for heightened immunopathology seen in influenza/bacterium infected lungs still remain poorly understood, they seem to be closely correlated with uncontrolled bacterial replication and overwhelming acute inflammatory responses in the lung.

One possible mechanism is that the ongoing or unresolved inflammatory responses to influenza may promote subsequent responses to bacterial superinfection. Massive 'cytokine storms' of TNF-α, IL-1β, IL-6, KC/IL-18, MIP-2, MIP-1α and the resulting inflammatory cell infiltration have been observed in a number of influenza/bacterial superinfection animal models, as well as in lungs of patients with acute respiratory distress syndrome [69,72–76]. Of note, in these studies such responses preceded peak bacterial burden, suggesting that the host inflammatory response is not necessarily only driven by uncontrolled bacterial burden. McAuley et al. showed that mice sequentially infected with a strain of influenza virus that expresses a proapoptotic viral accessory protein PB1-F2 and then with *S. pneumoniae*, had an influx of inflammatory cell infiltrates consisting of macrophages, neutrophils and T cells which occluded the airspace, and led to severe immunopathology and tissue injury [74].

Such severe immunopathology was not seen in mice infected with an isogenic mutant strain of influenza virus engineered to have reduced PB1-F2 expression [74]. Similar findings were also reported using *S. aureus* or an influenza virus strain expressing PB1-F2 with coding changes identical to the 1918 influenza strain [72,74]. How exactly these various strains of influenza virus lead to differential immunopathological outcome upon bacterial superinfection, still remains unclear. Nonetheless, these studies do suggest that the degree of virus virulence may mediate the immunopathology associated with subsequent bacterial superinfection.

Another mechanism involves the influenza-imprinted innate immune microenvironment that plays into the immune response to subsequent bacterial superinfection. For instance, type I IFNs and IFN-γ are produced at different times during influenza infection and can aid in ameliorating the severity of lung immunopathology caused by influenza infection by aiding viral clearance. However, these cytokines were found in some studies of heterologous influenza/bacterial superinfection to inhibit Th17 cells and chemokine expression, thus leading to depressed alveolar macrophage and neutrophil function, bacterial outgrowth and decreased survival [77–80]. The current theory supported by the evidence from us and others, is that innate cells become desensitized to avoid excessive lung immunopathology during a primary influenza infection, while leaving the host vulnerable to a secondary bacterial superinfection, which in turn worsens immunopathology [10,75,81]. Indeed, Didierlaurent et al. showed that over time, NF-κB activation in alveolar macrophages is impaired causing these cells to have sustained desensitization to TLR2, 4 and 5 agonists [81]. Such TLR desensitization led to attenuated neutrophilic infiltration and increased bacterial burden in the lung [81]. Also, it appears that uncontrolled bacterial infection and impaired function of macrophages and neutrophils can lead to continuous recruitment of neutrophils, which then contribute to worsened immunopathology without helping to clear the bacteria. In this regard, a recent study showed that depletion of neutrophil chemokine MIP-2 markedly improved immunopathology and clinical outcome without impacting bacterial burden during influenza and *Bordetella parapertussis* superinfection [69]. However, our recent unpublished results from a model of influenza and *S. pneumoniae* superinfection show that depletion of neutrophils does not improve the clinical outcome and only modestly improves immunopathology.

In addition, we have recently identified another innate cell, the NK cell, to be suppressed in a previously influenza infected lung environment leading to increased *S. aureus* bacterial load and severe immunopathology (Fig. 1, bottom) [10]. The immunopathology was associated with persisting tissue neutrophilia, neutrophil apoptosis and excessive mononuclear cell infiltration [10]. These findings thus suggest that NK cells are likely initially suppressed to limit influenza-associated immunopathology, but are critically required for host defense against the subsequent bacterial superinfection [82]. Therefore, suppressed NK cell responses indirectly account for exacerbated immunopathology during *S. aureus* superinfection. Whether the impaired NK cell response by previous influenza infection also plays an important role in *S. pneumoniae* superinfection remains unclear. Together, these findings support the notion that exacerbated immunopathology seen during influenza/bacterial superinfection results from impaired innate immunity and uncontrolled bacterial growth.

During several of the influenza epidemics and pandemics that stemmed after the 1918 pandemic, antibiotic use has played a major part in treatment against bacterial superinfections. Thus, controlling superinfection represents perhaps the most effective way to attenuate detrimental immunopathology in influenza infected hosts. However, treatment with β-lactam antibiotics worsened the outcome of hospitalized patients [83]. Furthermore, microscopic examination of lungs from influenza infected mice treated with or without ampicillin for pneumococcal pneumonia showed similar pathological features, including extensive epithelial bronchial and coagulative necrosis, edema, neutrophil infiltration within alveolar spaces and exudates along the pleura [84,85]. Investigators suggested that ampicillin lysed the bacteria, and subsequent TLR2 recognition of bacterial cell wall components triggered massive neutrophil accumulation resulting in immunopathology [85]. By contrast, these investigators showed that by using a number of bacteriostatic macrolide antibiotics which inhibit bacterial protein synthesis, the bacteria were also cleared but neutrophil influx was lowered resulting in decreased immunopathology [85]. Therefore, in the event of another influenza pandemic, care should be given upon administering β-lactam antibiotics.

Apparently much still remains to be understood about the mechanisms underlying the excessive immunopathology upon bacterial superinfection. Also, little attention has been paid to potentially uncouple the host anti-bacterial mechanisms from those involved in immunopathology. At this point, it also remains unclear whether the poor clinical outcome in dual-infected hosts is due to lung immunopathology or uncontrolled bacterial replication and systemic dissemination or both. As previous influenza infection predisposes to bacterial superinfection, the priority for regulating lung immunopathology is to control bacterial infection by using non-lytic antibiotics. The strategies aimed to restore impaired innate immunity have to be cautiously developed and applied since overwhelming tissue inflammatory cellular responses are always associated with bacterial superinfection. Untimely restoration of any of these cellular responses may further worsen immunopathology.

7. Conclusions

Lung immunopathology observed during primary influenza or during influenza/bacterial superinfection is clearly one of the main causes of influenza-related morbidity and mortality. The current challenge in the research field is to delineate the mechanisms shared by both anti-viral/anti-bacterial host defense and tissue immunopathogenesis, and those that are not required for viral or bacterial clearance but are major culprits in immunopathology. The latter would be the ideal targets for future immunotherapies. Recent experimental studies have identified the involvement of a number of immunopathogenic mechanisms including excessive acute inflammatory responses, CD4 and CD8 T cells, lung remodeling and bacterial superinfection, many of which overlap with the mechanisms required for viral or bacterial clearance (Table 1, Fig. 2). Unfortunately, the precise role of each of these mechanisms has been clouded by several factors including the use of differing influenza strains and

Table 1 The roles of various immune factors in influenza-induced lung immunopathology.

Immune factor	Role	Mechanisms	References
Acute inflammation	Protective	Macrophage and neutrophil influx to lung CD200 IRAK-M TLR2, TLR4 GM-CSF	[17,23,27,28,35,36]
	Detrimental	Excessive neutrophils and macrophages CCR2+ moDCs and exMACS NET formation tipDCs TRAIL PAFR TLR3, TLR7, TLR9 IRAK-M deficiency Excessive proinflammatory cytokines IL-17 TNF-α deficiency	[14–16,18–21,24–26,29–33]
CD4 T cells	Protective	Perforin/granzyme Th17	[43,52]
	Detrimental	Excessive CD4 T cells Fas-L Th17	[42,47–51]
CD8 T cells	Protective	4-1BBL IL-10	[55,56]
	Detrimental	Excessive CD8 T cells Cytolytic activity CD200 deficiency NKG2A inhibition IL-15	[57,58,60]
Remodeling	Detrimental	Myofibroblasts Hydroxyproline Collagen TGF-β1 MCP-1	[16,61]
Superinfection	Detrimental	Excessive neutrophils Excessive proinflammatory cytokines IFN-α/β IFN-γ MIP-2 TLR desensitization TLR2 signaling Neutrophil, macrophage and NK cell impairment	[69,72,74,77–82,84,85]

doses. Aside from the daunting task ahead, emerging evidence from models of primary influenza infection suggest that CCR2 expressing macrophages and IL-17 may be promising therapeutic targets. In addition, carefully tailored GM-CSF or IL-10 delivery, and CD200 activation may also be therapeutically beneficial. However, compared to the work carried out in models of primary influenza infection, little is known about whether it is possible to uncouple some of the anti-bacterial host defense mechanisms with those solely responsible for deleterious immunopathology during bacterial superinfection. Furthermore, mounting evidence also implies that there are severe cases of influenza infection where the top priority is to control devastating immunopathology and tissue injury to improve host survival. In such cases, the consequently increased viral or bacterial loads would have to be controlled by host defense and anti-viral/anti-bacterial treatments.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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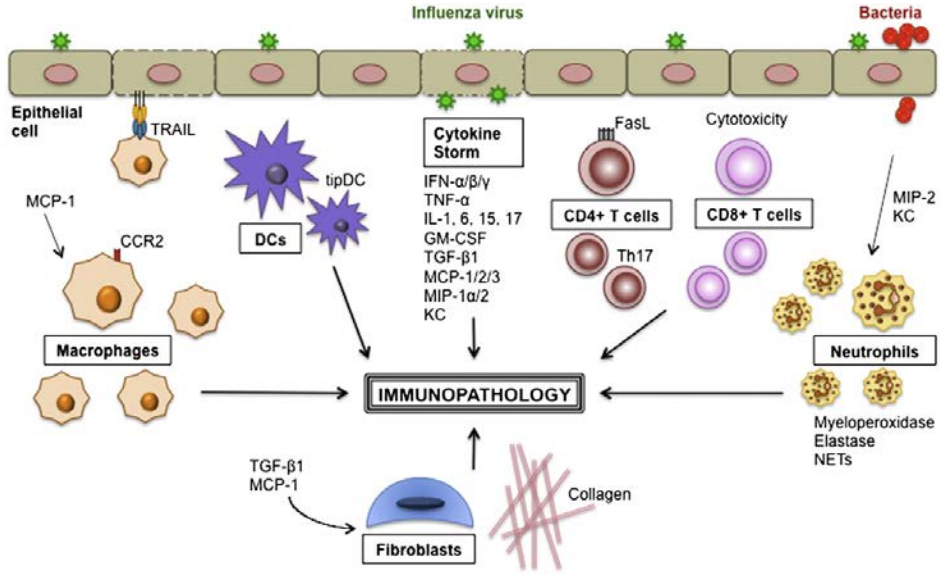

Figure 2 Influenza immunopathology is caused by excessive host responses to infection. Excessive acute inflammatory responses (macrophages, neutrophils, DCs, and the 'cytokine storm'), CD4 and CD8 T cells, lung remodeling, and bacterial superinfection all contribute to severe lung immunopathology following influenza infection.

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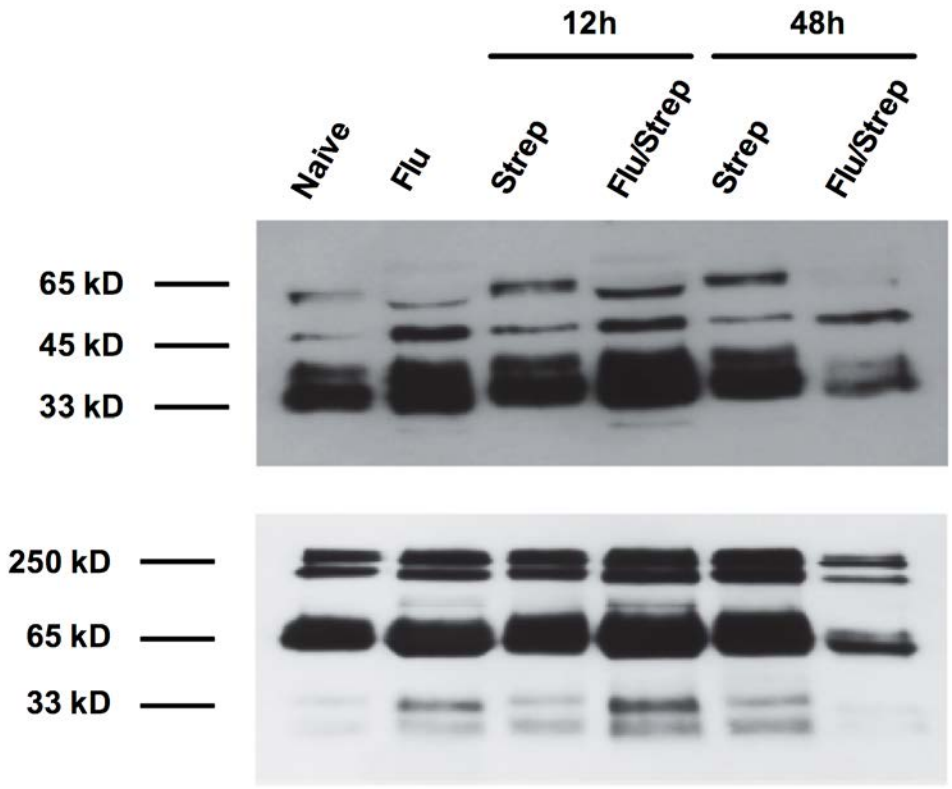
APPENDIX III



Surfactant protein A (SP-A) expression in pneumococcal superinfection following influenza. Mice were infected with influenza and 7 days later with *S. pneumoniae* (as in *Chapter 3*). BAL fluids were analyzed by reducing (top) and non-reducing (bottom) Western blots. Under reducing conditions, the band size of monomeric SP-A is 32 kDa, glycosylated SP-A is ~ 35 kDa, and non-reducible dimers are 64 and 70 kDa. In non-reducing conditions, SP-A multimers are visible. Data was generated via collaboration with Dr. Nades Palaniyar and generated by Pascal Djiadeu (Physiology & Experimental Medicine, The Hospital For Sick Children, University of Toronto).