Mechanisms of Increased Susceptibility to Influenza Infection in Mouse Models of Chronic Lung Diseases

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Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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The works presented in this thesis have been done in collaboration with other researchers. I have included the Statement of Collaboration which clearly outlines the extent of the collaborations.

Irwan Hanish bin Warsanah
Statement of collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included below a statement clearly outlining the extent of collaboration, with whom and under what auspices.

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List of figures

74  Figure 1. Antigenic drift and antigenic shift in influenza viruses........................................23
75  Figure 2. Structure of influenza virus. ..................................................................................27
76  Figure 3. Influenza virus replication cycle in host epithelial cells.................................29
77  Figure 4. Viral detection by RIG-I leads to the production of Type I and III IFNs.........34
78  Figure 5. Pathogenesis of COPD. .........................................................................................42
79  Figure 6. PI3K catalytic and regulatory subunits...............................................................49
80  Figure 7. Influenza virus utilises PI3K signalling pathways to increase infection........53
81  Figure 8. Pathophysiological features of asthma...............................................................60
82  Figure 9. IL-13 and its receptors.......................................................................................69
83  Figure 10. Mice with experimental COPD are predisposed to more severe influenza virus infection .................................................................................................................91
84  Figure 11. Experimental COPD predisposes to exaggerated airway inflammation during influenza virus infection..................................................................................................................95
85  Figure 12. Influenza virus infection in experimental COPD leads to further impairment of lung function............................................................................................................................98
86  Figure 13. Influenza virus infection in experimental COPD had no effect on emphysema-like alveolar enlargement..............................................................100
87  Figure 14. Mice with experimental COPD have impaired antiviral IFN responses during influenza virus infection..................................................................................................................103
88  Figure 15. Experimental COPD leads to impaired antiviral cytokine and chemokine responses but increased pro-inflammatory cytokine responses during influenza virus infection..............................................................................................................106
Figure 16. PI3K activity is increased during influenza virus infection in experimental COPD, and its inhibition enhances antiviral responses and suppresses viral titres. 

Figure 17. Inhibition of PI3K in experimental COPD leads to increased inflammatory cell responses and improved lung function during influenza infection. 

Figure 18. Administration of rIL-13 to naïve mice promotes more severe influenza virus infection. 

Figure 19. Administration of rIL-13 impairs antiviral responses to influenza virus infection. 

Figure 20. Administration of rIL-13 increases airway inflammatory cell influx in response to influenza virus infection. 

Figure 21. Administration of rIL-13 promotes increased AHR during influenza virus infection. 

Figure 22. Administration of rIL-13 leads to more severe histopathology during influenza virus infection. 

Figure 23. Administration of rIL-13 leads to increased MSCs during influenza virus infection. 

Figure 24. Administration of rIL-13 leads to increased levels of IL-13 and IL-13Rα1 during influenza virus infection. 

Figure 25. AAD promotes more severe influenza virus infection. 

Figure 26. AAD impairs antiviral IFN responses to influenza virus infection. 

Figure 27. Influenza virus infection leads to exaggerated airway inflammatory cell influx in AAD. 

Figure 28. Influenza virus infection leads to increased AHR in AAD. 

Figure 29. Influenza virus infection leads to more severe histopathology in AAD. 

Figure 30. Influenza virus infection leads to increased numbers of MSCs in AAD.
Figure 31. Influenza virus infection in AAD increased IL-13Rα1. ........................................149
Figure 32. Inhibition of IL-13 in AAD leads to reduced influenza virus infection. ..... 152
Figure 33. Inhibition of IL-13 in AAD leads to improved antiviral responses to influenza virus infection.................................................................................................................. 154
Figure 34. Inhibition of IL-13 in AAD leads to decreased eosinophil infiltration during influenza virus infection.................................................................................................................. 157
Figure 35. Inhibition of IL-13 in AAD leads to improved lung function during influenza virus infection.................................................................................................................. 159
Figure 36. Inhibition of IL-13 in AAD lead to increased histopathology during influenza virus infection.......................................................................................................................... 161
Figure 37. Inhibition of IL-13 in AAD leads to decreased numbers of MSCs during influenza virus infection.................................................................................................................. 163
Figure 38. Inhibition of IL-13 in AAD lead to reduced levels of IL-13 protein and IL-13Rα1 mRNA expression following influenza infection.................................................................................. 166
Figure 39. Interaction of influenza virus infection with experimental COPD............ 169
Figure 40. Interaction of influenza virus infection with AAD................................. 172
Figure 41. Influenza virus infection is more severe in experimental COPD. ......... 175
Figure 42. Antiviral responses to influenza infection are reduced in COPD......... 181
Figure 43. Inhibition of exaggerated PI3K activity during influenza virus infection in experimental COPD improves infection outcomes.......................................................................................... 185
Figure 44. Influenza virus infection in either combination with rIL-13 administration or in AAD........................................................................................................................................ 190
Figure 45. Influenza virus infection in AAD is suppressed by IL-13 inhibition. ....... 191
List of tables

Table 1. GOLD classification of COPD severity. .......................................................... 40
Table 2. Histopathological scoring system for mouse lungs. .................................... 82
List of abbreviations

AAD: Allergic airway disease

AHR: Airway hyperresponsiveness

Akt-in: Akt inhibitor NH(2)-AVTDHPRLWAEKFCOOH

APC: Antigen presenting cell

BALF: Bronchoalveolar lavage fluid

CD: Cluster of Differentiation

cDNA: Complementary DNA

CARD: Caspase-recruitment domain

CLDSI: Chronic lung disease severity index

COPD: Chronic obstructive pulmonary disease

DC: Dendritic cell

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Dpi: Days post infection

ELISA: Enzyme linked immunosorbent assay

FcεRI: Fc epsilon receptor I

FEV₁: Forced expiratory volume in 1 second
FVC: Forced vital capacity

GOLD: Global Initiative for Chronic Obstructive Lung Disease

GPCR: G protein-coupled receptor

GTP: Guanosine triphosphate

H$_2$O$_2$: Hydrogen peroxide

H&E: Haematoxylin and eosin

HDM: House dust mite

HEK293: Human embryonic kidney 293

HO-1: Heme oxygenase 1

HPRT: Hypoxanthine-guanine phosphoribosyltransferase

Ig: Immunoglobulin

IFN: Interferon

pfu: Plaque forming unit

IKKi: IκB kinase-i

IL: Interleukin

IL-13Rα1: Interleukin-13 receptor alpha 1

ILC: Innate lymphoid cell

ILC2: Group 2 innate lymphoid cell
i.n: Intranasal
i.p: Intraperitoneal
IP-10: IFN-γ-induced protein-10
IPS-1: IFN-β promoter stimulator 1
JAK: Janus kinase
KC: Keratinocyte-derived chemokine
MDA-5: Melanoma Differentiation-Associated protein-5
MDCK: Madin-Darby Canine Kidney
MHC: Major histocompatibility complex
MIP-1α: Macrophage inflammatory protein-1α
mRNA: Messenger ribonucleic acid
miRNA: MicroRNA
MSC: Mucus secreting cell
NF-κB: Nuclear factor kappa light chain enhancer of activated B cells
NK cell: Natural killer cell
NKT cell: Natural killer T cell
Nrf2: Nuclear factor (erythroid-derived 2)-like 2
NO: Nitric oxide
O$_2^-$: Superoxide

Ova: Ovalbumin

PAS: Periodic acid–Schiff

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate-buffered saline

PC: Physical containment

PIP$_2$: Phosphatidylinositol 4,5–bisphosphate

PIP$_3$: Phosphatidylinositol 3,4,5–triphosphate

PIV-3: Parainfluenza virus type 3

PKB: Protein Kinase B

PRR: Pattern recognition receptor

PVDF: Polyvinylidene difluoride

qPCR: Quantitative real-time Polymerase Chain Reaction

RIG-I: Retinoic acid-inducible gene-I

ROS: Reactive oxidant species

rIL-13: Recombinant Interleukin-13

RIPA: radio-immunoprecipitation assay

RLR: RIG-I-like receptor
RNA: Ribonucleic acid

RSV: Respiratory syncytial virus

RV: Rhinovirus

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SPF: Specific pathogen free

SH: Src Homology

STAT6: Signal transducer and activator of transcription 6

TANK: TRAF family member-associated NF-κB activator

TBK1: TANK binding kinase-1

Th: T helper lymphocyte

TLC: Total lung capacity

TLR: Toll-like receptor

TNF: Tumour necrosis factor

TRAF: TNF receptor associated factor

VEGF: Vascular endothelial growth factor

VPg: Virion protein genome linked protein
# Table of contents

243
244 Acknowledgements ........................................................................................................... 2
245 Statement of originality ........................................................................................................ 3
246 Statement of collaboration .................................................................................................... 4
247 List of figures ........................................................................................................................ 5
248 List of tables .......................................................................................................................... 8
249 List of abbreviations ............................................................................................................. 9
250 Table of contents .................................................................................................................... 14
251 Synopsis ............................................................................................................................... 20
252 CHAPTER 1: INTRODUCTION .......................................................................................... 22
253 1.1 Influenza .......................................................................................................................... 22
254 1.1.1 Epidemiology of influenza .......................................................................................... 22
255 1.1.2 Pathogenesis of influenza ......................................................................................... 26
256 1.1.3 Host immune responses to influenza virus infection ............................................... 31
257 1.2 Influenza infection in COPD .......................................................................................... 37
258 1.2.1 Epidemiology of COPD ............................................................................................. 37
259 1.2.2 Pathogenesis of COPD ............................................................................................. 39
260 1.2.3 Influenza virus infection in COPD and the role of PI3K ............................................. 45
261 1.3 Influenza infection in asthma ......................................................................................... 57
262 1.3.1 Epidemiology of asthma ........................................................................................... 57
1.3.2 Pathophysiology of asthma ................................................................. 59
1.3.3 Immunology of asthma ....................................................................... 61
1.3.4 Influenza infection in asthma and the role of IL-13 .......................... 63
1.4 Hypotheses and aims ........................................................................... 73

CHAPTER 2: MATERIALS AND METHODS .................................................. 75
2.1 Mice ...................................................................................................... 75
2.2 Influenza virus infection ........................................................................ 75
2.3 Induction of cigarette smoke-induced experimental COPD .................. 75
2.4 PI3K inhibition ..................................................................................... 76
2.5 Induction of AAD .................................................................................. 76
2.6 Administration of rIL-13 ..................................................................... 77
2.7 Neutralisation of IL-13 ......................................................................... 77
2.8 Bronchoalveolar lavage fluid (BALF) .................................................... 78
2.9 Plaque assay ......................................................................................... 79
2.10 Lung function analysis in experimental COPD ...................................... 79
2.11 Assessment of AHR in AAD ............................................................... 80
2.12 Lung histology .................................................................................... 80
2.13 Cytokine concentrations in BALF ....................................................... 83
2.14 Immunoblotting .................................................................................. 83
2.15 Total RNA extraction .......................................................................... 84
2.16 Reverse transcription .......................................................................... 85
2.14 Quantitative real-time Polymerase Chain Reaction (qPCR) ........................................86
2.15 Statistical analyses ........................................................................................................87
2.16 Study approvals ..............................................................................................................88

CHAPTER 3: RESULTS .................................................................................................................89

3.1 Influenza virus infection in COPD ......................................................................................89
3.1.1 Experimental COPD predisposes to more severe influenza virus infection...89
3.1.2 Experimental COPD predisposes to exaggerated airway inflammation
following influenza virus infection.........................................................................................93
3.1.3 Influenza virus infection in experimental COPD alters lung function ...........96
3.1.4 Antiviral IFN responses to influenza virus infection are impaired in
experimental COPD........................................................................................................102
3.1.5 Inflammatory cytokine production is increased in the lung during influenza
virus infection in experimental COPD...........................................................................105

3.2 Experimental COPD increases PI3K activity in the lung and promotes more
severe influenza infection and exacerbation of COPD.................................................107

3.3 Influenza infection and IL-13 ..............................................................................................114
3.3.1 Administration of rIL-13 to naïve mice promotes more severe influenza virus
infection ..............................................................................................................................114
3.3.2 Administration of rIL-13 impairs antiviral responses to influenza virus infection ................................................................. 116
3.3.3 Administration of rIL-13 increases airway inflammatory cell responses to influenza virus infection .............................................. 118
3.3.4 Administration of rIL-13 promotes increased AHR during influenza virus infection ........................................................................ 120
3.3.5 Administration of rIL-13 leads to more severe histopathology and increased MSCs following influenza infection ................................. 122
3.3.6 Administration of rIL-13 leads to increased levels of IL-13 and IL-13Rα1 during influenza virus infection ................................................ 127
3.4 Influenza virus infection in AAD ........................................................................................................................................ 129
3.4.1 AAD promotes more severe influenza virus infection ................... 129
3.4.2 AAD impairs antiviral responses to influenza virus infection ........... 133
3.4.3 Influenza virus infection leads to exaggerated airway inflammatory cell influx in AAD ............................................................................ 135
3.4.4 Influenza virus infection leads to increased AHR in AAD ................. 138
3.4.5 Influenza virus infection leads to more severe histopathology and increased MSCs in AAD ............................................................. 142
3.4.6 Influenza virus infection in AAD increases IL-13Rα1 ...................... 147
3.5 Inhibition of IL-13 in AAD during influenza infection protects against virus infection and prevents associated exacerbation of AAD ........................................... 150
3.5.1 Inhibition of IL-13 in AAD leads to reduced influenza virus infection ..... 150
3.5.2 Inhibition of IL-13 in AAD leads to improved antiviral responses to influenza virus infection

3.5.3 Inhibition of IL-13 in AAD leads to decreased eosinophil infiltration during influenza virus infection

3.5.4 Inhibition of IL-13 in AAD leads to improved lung function during influenza infection

3.5.5 Inhibition of IL-13 in AAD leads to more severe histopathology but decreased MSCs during influenza virus infection

3.5.6 Inhibition of IL-13 in AAD leads to reduced levels of IL-13 and IL-13Rα1 during influenza virus infection

CHAPTER 4: DISCUSSION

4.1 Overall Findings and Significance of Research

4.2 Influenza infection virus in COPD

4.2.1 Influenza infection is more severe in experimental COPD

4.2.2 Antiviral responses to influenza virus infection are reduced in COPD

4.2.3 Inhibition of exaggerated PI3K activity during influenza virus infection in COPD improves infection outcomes

4.3 Influenza virus infection in AAD

4.3.1 IL-13 plays an important role in promoting more severe influenza virus infection in AAD

4.3.2 AAD impairs antiviral responses to influenza virus infection, which can be improved by inhibition of IL-13
4.3.3 IL-13 plays an important role in pulmonary inflammation during influenza virus infection in AAD ......................................................... 195

4.3.4 IL-13 plays an important role in increased AHR during influenza virus infection in AAD ................................................................. 197

4.3.5 Influenza virus infection leads to increased numbers of MSCs in AAD, which is reduced by inhibition of IL-13 .............................................. 199

4.3.6 IL-13 signalling is important in influencing the disease outcomes during influenza virus infection in AAD ............................................. 200

4.4 Future Directions ........................................................................ 201

4.4.1 Future directions in the study of influenza virus infection in COPD ...... 201

4.4.2 Future directions in the study of influenza virus infection in AAD ....... 202

4.5 Conclusions ................................................................................ 205

References ....................................................................................... 206
Synopsis

Influenza infections are of major importance as they have a significant impact on the health of individuals and impart substantial socio-economic ramifications on society. Prevention and treatment of influenza infections are complicated by frequent genetic mutations of the influenza virus. Patients with underlying chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) and asthma are more susceptible to influenza infection, and infection with influenza exacerbates these diseases. Therefore, elucidation of the mechanisms underpinning increased susceptibility to influenza in these patients is vital. Here, we established an experimental mouse model of COPD and utilised an existing ovalbumin-induced allergic airways disease (AAD) model to investigate the effects of influenza infection in COPD and asthma, respectively. Influenza infection in experimental COPD resulted in increased viral titre, exaggerated airway inflammation and further impaired lung function. These effects were accompanied by decreased neutrophil influx into the airways, reduced antiviral interferon responses, and the suppression of a range of cytokines and chemokines, including interferon (IFN)-\(\gamma\), tumour necrosis factor (TNF)-\(\alpha\), IFN-\(\gamma\)-induced protein (IP)-10, macrophage inflammatory protein (MIP)-1\(\alpha\), keratinocyte-derived chemokine (KC, or IL-8 in humans) and interleukin (IL)-10, as well as increased IL-6. This increased susceptibility was mediated by an increase in phosphoinositide 3-kinase (PI3K) protein expression. The inhibition of PI3K effectively reduced viral titre, enhanced antiviral IFNs and improved lung function.

Influenza infection in recombinant IL-13-treated or ovalbumin (Ova)-induced AAD models led to increased viral titre, impaired antiviral responses and increased
airway hyper-responsiveness (AHR). It also resulted in exaggerated airway inflammation, more severe histopathology, increased mucus secreting cell numbers and increased IL-13. Importantly, we also showed that inhibition of IL-13 by administration of anti-IL-13 (αIL-13) monoclonal antibody during influenza infection reduced viral titre, AHR, eosinophil infiltration and MSCs, which were associated with improved antiviral IFN responses.

In summary, these data highlight the important roles of PI3K and IL-13 in the increased susceptibility to influenza infection in experimental models of COPD and asthma, respectively. Such findings offer evidence for new and promising avenues for influenza disease management in these chronic lung diseases. In fact, both PI3K inhibitors and anti-IL-13 antibodies have already entered clinical trials and may be utilised as novel therapeutic approaches for influenza infections in the future.
CHAPTER 1: INTRODUCTION

1.1 Influenza

1.1.1 Epidemiology of influenza

Influenza is one of the most globally important respiratory viral infections in the world. It was responsible for three global pandemics in the 20th century alone (1). For example, the Spanish Flu pandemic in 1918 killed more than 40 million people (2). In addition, seasonal influenza infection causes 250,000 to 500,000 deaths every year worldwide (3).

Influenza is able to evade the host immune system through two phenomena referred to as “antigenic drift” and “antigenic shift” (Figure 1). The antigenic glycoprotein called haemagglutinin (HA) that exists on the surface of influenza virus is the primary target for the host immune system to neutralise the infection. However, in “antigenic drift”, cumulative minor modifications occur in HA that allow the antigenic site to “drift” in configuration until it is no longer recognised by antiviral recognition molecules previously developed by the host (4). The segmented nature of the influenza virus genome also allows for more dramatic changes to occur in its antigenic sites, this is known as “antigenic shift”. This typically occurs during viral replication within host cells infected with multiple viruses from different species that enables recombination of different viruses. Modifications of this nature are more significant as they may alter the genomic profile of the virus into a totally new configuration. Indeed, recent analysis of the 2009 H1N1 influenza virus shows clear associations with influenza A viruses that
have been isolated from different geographical locations: namely Asia, Europe and North America (5).

Figure 1. Antigenic drift and antigenic shift in influenza viruses.

Cumulative modifications that occur over time to influenza virus RNAs cause a “drift” in antigenic site configuration. During the viral replication process in host cells infected with influenza viruses from different species, the segmented nature of the genome allows for a more radical “shift” in the antigenic profile. Both antigenic drift and
antigenic shift render the new influenza virus strain no longer susceptible to the host immune system. Figure was created by the author and incorporates images (graphical representations of viruses) adapted from The Acute Respiratory Infections Atlas, World Lung Foundation (6).
In addition to the significant rate of morbidity and mortality, influenza infections also result in a substantial economic burden. The economic loss in the United States alone caused by this disease can reach up to USD $187 per capita (approximately AUD $202) (7). Without effective medical intervention this may result in a total cost of up to USD $167 billion per year (approximately AUD $178 billion) (8). Beyond these quantifiable markers, the emotional impacts influenza has on families and communities following severe infections or deaths are no less significant. All of these factors have contributed to placing this disease as a high priority in global healthcare and medical research.
1.1.2 Pathogenesis of influenza

Influenza A viruses are members of the family Orthomyxoviridae, and comprised of eight single-stranded, negative sense RNA segments that make up the viral genome (Figure 2). These RNA segments are conserved within ribonucleoprotein complexes that are contained within the viral membrane (9). These RNA segments encode multiple viral proteins: haemagglutinin (HA), which contains specific locations of influenza antigenic sites on the outer surface of the membrane (10); neuraminidase (NA), which cleaves sialic acid groups from glycoproteins and is currently a primary target for antiviral drugs (such as neuraminidase inhibitors zanamivir and oseltamivir) (11, 12); polymerase acidic (PA) and polymerase basic (PB1, PB2, PB1-F2) protein subunits, which are essential catalysts for polymerisation processes during replication (13); nucleoprotein (NP), which encapsulates the negative strand of viral RNA (14); matrix proteins M1 and M2, which are gated ion channels located along the transmembrane region that are highly selective for protons and responsible for proton conductance (a vital chemical condition during viral replication process) (15, 16); as well as NS1 and NS2, the non-structural proteins that are also known as nuclear export protein (NEP), which mediate the export of ribonucleoprotein complexes from the nucleus (17-19).
Figure 2. Structure of influenza virus.

The typical structure of influenza virus consists of eight negative sense single-stranded RNA segments within ribonucleoprotein complexes. These RNAs can express up to eleven types of proteins including haemagglutinin (HA); neuraminidase (NA); polymerase proteins PA, PB1, PB2, PB1-F2; nucleoprotein (NP); matrix proteins M1 and M2; and non-structural proteins NS1 and NS2 (also known as nuclear export protein, NEP). These RNA segments are enveloped within the viral membrane. The M2 ion channel is located along the transmembrane region, whereas NA and HA are distributed on the outer surface of the membrane structure. Figure was adapted from Centers for Disease Control and Prevention website (20) and labels were added by the author. **Light green: ribonucleoprotein complexes. Brown: viral membrane. Purple: M2 ion channel. Red: neuraminidase (NA). Blue: haemagglutinin (HA).**
There are two other genera in the Orthomyxoviridae family: Influenza B and C viruses (21). Nevertheless, Influenza A viruses are the main focus of contemporary studies, primarily because of their roles in major influenza pandemics, including the most recent H1N1 outbreak in 2009 (5). Typically, patients infected with influenza present with a fever that is accompanied by cough, sore throat, and muscle pain. This may be followed by chest discomfort during respiration and lower respiratory tract complications that result from infections, such as bronchiolitis, bronchitis and bronchopneumonia (22, 23). In humans, influenza infection is initiated at the epithelial lining of the respiratory mucosa, where the virus adheres to the sialic acid-terminated glycans on these cells (Figure 3). This allows for the internalisation of the virus into host cells. The HA proteins then undergo changes in their conformation that is induced by the low pH environment in the endosome. These changes release multiple complexes of viral ribonucleoproteins into the cytoplasm, which then translocate into the nucleus (24, 25). Inside the nucleus, viral messenger RNAs (mRNAs) and complementary RNAs (cRNAs) are synthesised from viral ribonucleoprotein templates. Transcription of viral mRNAs with positive polarity is achieved by the utilisation of 5’-methylated cap structures belonged to the host cell (26). This is then delivered to the cytoplasmic region of the host cell and translated into viral proteins. Concurrently, cRNAs remain in the nucleus and are utilised as templates for creating new viral negative sense RNAs. These daughter RNAs then are associated back with M1 proteins before being exported into the cytoplasmic region. Viral components including RNA segments, HA, NA and M2 move towards the apex of host membrane, where eventually the NA cleaves the host sialic acid residues, liberating newly formed virions from the infected cell (27).
Figure 3. Influenza virus replication cycle in host epithelial cells.

Influenza virus adheres to the epithelial lining of the respiratory mucosa, which leads to the internalisation of the virus into host cells. Viral proteins then undergo changes in their conformation that is induced by the low pH environment in the endosome. Multiple complexes of viral ribonucleoproteins (RNPs) are then released into the cytoplasm and translocate into the nucleus. Inside the nucleus, mRNAs and cRNAs are synthesised. The mRNAs are delivered to the cytoplasmic region of the host cell and translated into viral proteins. The cRNAs are utilised as templates for creating new viral negative sense RNAs that become part of RNPs of the progeny virus. All viral components are then mobilised towards the apex of the host membrane, eventually
liberating newly formed virions from the infected cell. \textit{RNPs}: ribonucleoproteins.

\textit{mRNA}: messenger RNAs. \textit{cRNA}: complementary RNA
1.1.3 Host immune responses to influenza virus infection

1.1.3.1 Innate immune response

During the initial stage following influenza viral infection, viral cRNA and mRNA are released as part of the replication process (28). These components are vital pathogen-associated molecular patterns (PAMPs) and recognised by the pattern recognition receptors (PRRs) on the surface of host cells. A cascade of signals is initiated downstream of these PRRs, ultimately resulting in the release of numerous chemokines and cytokines. These signals are critical in activating the innate arm of the immune system by directing the surrounding leukocytes to the site of infection and initiating preliminary host defences against influenza virus.

The primary PRR for influenza viruses is the cytoplasmic retinoic acid-inducible gene-I (RIG-I) (29-31). Toll-like receptors (TLRs), such as TLR3 and TLR7; and another RIG-I-like receptor (RLR) named Melanoma Differentiation-Associated protein-5 (MDA-5) have also been reported as important viral PRRs (32). Nevertheless, compared to RIG-I, their contribution in the context of influenza infection is varied and restricted. Because of its location on the cell surface, the capacity of TLR3 to serve as a defensive sensor against influenza viruses is diminished once the virus has successfully invaded the cytoplasm (33). TLR7, although capable of inducing HA-specific antibodies important in vaccination, is dispensable for protection against influenza A virus infection (34). MDA-5 recognises different ligands to RIG-I, such as those on Picornaviruses, which do not have the uncapped 5′-triphosphate end (instead, the ligands possess virion protein genome linked protein (VPg), which is covalently
associated to the 5’-ends of their genome) (32, 35). Therefore, compared to RIG-I, the role of these receptors during influenza virus infection is limited. Nevertheless, this might not negate their role completely, as these receptors may be part of collaborative elements that work with RIG-I in the protection against influenza virus infection (36).

Detection of influenza virus by RIG-I initiates its conformational changes and a cascade of downstream signals, which leads to the production of IFN-α/β (type I IFNs) and IFN-λ1/2/3 (type III IFNs, Figure 4) (29, 37). The RIG-I structure contains two caspase-recruitment domains (CARDs) as well as a DExD/H-box helicase domain (30). These domains play a vital interfacing role during the interaction between RIG-I and the target viral RNA (30). RIG-I recognises the uncapped 5’-triphosphate end of viral RNA, which is distinctly different from host RNA (31, 38). Binding of influenza viral RNA to RIG-I results in structural changes in the receptor, leading to exposure of the CARD domain. This subsequently allows this domain to interact with the IFN-β promoter stimulator 1 (IPS-1, also known as VISA, Cardif or MAVS) (39).

Downstream of this signalling pathway, tumour necrosis factor (TNF) receptor associated factor (TRAF) family member-associated NF-κB activator (TANK) binding kinase-1 (TBK1) and IκB kinase-i (IKKi) are activated as a result of the interaction between IPS-1 with TRAF-3 (40). The TBK1/IKKi complex then phosphorylates interferon regulatory factor-7 (IRF-7) and IRF-3, which promotes the production of IFN-α/β and IFN-λ1/2/3(41). These IFNs subsequently induce the transcription of over 300 IFN-stimulated genes that are capable of disrupting the influenza infection cycle via cleavage of viral RNA and inhibition of subsequent protein synthesis (42).

The host also generates IFN-γ (a type II IFN), in order to begin the initiation of adaptive immune response against influenza virus infection (43). These antiviral
responses are reinforced by the release of other cytokines, such as TNF-α, and chemokines, including IFN-γ-induced protein (IP)-10 (CXCL10), macrophage inflammatory protein, (MIP)-1α (CCL3), as well as IL-8 (CXCL8, Keratinocyte-derived chemokine (KC) is the murine homolog) (44, 45). IP-10 is a chemokine produced by multiple cell types such as neutrophils and airway epithelial cells in response to IFN-γ (46-48). It has been shown to contribute to T cell proliferation or differentiation, and neutrophil recruitment (45, 49). For example, in response to the oxidative stress factor trioxygen (O2[μ-O]), specific antibody inhibition of IP-10 in vivo led to a more than 70% decrease in neutrophil recruitment (49). MIP-1α is produced by macrophages, T- and B-cells, neutrophils, dendritic cells (DCs), mast cells and natural killer (NK) cells following infection by viruses, including influenza virus (50-52). It has been associated with generation and differentiation of primed cluster of differentiation (CD)8+ T cells into effector cells which is important in reducing viral infection (44, 53, 54). For example, following in vivo infection with mouse hepatitis virus, genetic deletion of MIP-1α resulted in reduction in the number of CD8+ T cells and associated with delayed viral clearance (54). IL-8 is produced by several cell types including epithelial, endothelial, and airway smooth muscle cells (55, 56). IL-8 is also associated with neutrophil chemotaxis. In an in vitro study, neutrophil chemotaxis by IL-8 was demonstrated to be mediated by Janus kinase 3 (JAK3), where specific inhibition of JAK3 by apigenin (4′,5,7-trihydroxyflavone, a plant derived pharmaceutical compound) resulted in disruption of chemotaxis activity (57).
Figure 4. Viral detection by RIG-I leads to the production of Type I and III IFNs.

Retinoic acid-inducible gene-I (RIG-I) detects of influenza virus and initiates a cascade of downstream signals. The DExD/H-box RNA helicase domain of RIG-I interacts with target viral RNA. This leads to conformational alterations that expose the caspase-recruitment domains (CARDs), which bind with IFN-β promoter stimulator 1 (IPS-1). Interaction between IPS-1 and tumour necrosis factor (TNF) receptor associated factor-3 (TRAF-3) activates TRAF family member-associated NF-κB activator (TANK)-binding kinase-1 (TBK1) and IκB kinase-i (IKKi). The TBK1/IKKi complex then phosphorylates interferon regulatory factor-7 (IRF-7) and IRF-3. IRF-7 and IRF-3 then mobilise into the nucleus and induce the production of type I IFNs (IFN-α/β) and type III IFNs (IFN-λ1/2/3). RIG-I: Retinoic acid-inducible gene-I. CARDs: caspase-
recruitment domains. IPS-1: IFN-β promoter stimulator-1. TRAF-3: tumour necrosis factor (TNF) receptor associated factor-3. TBK-1: TRAF family member-associated NF-κB activator (TANK) binding kinase-1. IKKi: IκB kinase-i. IRF-7 and -3: interferon regulatory factor-7 and -3.
In healthy lungs, alveolar macrophages along with epithelial cells and DCs act as surveillance cells, continuously scanning for foreign proteins and microorganisms through their PRRs (58). Activation and terminal differentiation of DCs is significantly enhanced by IFN-β, which assists the cells in executing their role as antigen presenting cells (APCs) (59). Upon recognition, DCs integrate influenza viral antigens as part of their surface structure, prior to maturation and migration to lymph nodes. Once they arrive at lymph nodes, the DCs present the antigen to T cells with receptors uniquely specific to that antigen. This, along with accessory signals delivered through co-stimulatory molecules, initiates T cell priming. During this process, influenza-specific CD8+ T cells undergo significant proliferation in the lymph nodes, and are subsequently redeployed to the site of infection, for example, the lung (60).

In the lung, influenza virus antigens, in association with major histocompatibility complex (MHC) class 1 molecules, are presented on the surface of infected airway epithelial cells. They are utilised as recognition elements by CD8+ T cells that are recruited from lymph nodes. This allows the CD8+ T cells to bind to infected epithelial cells, consequently deliver cytotoxic factors such as granzymes and perforins into the target epithelial cells, and ultimately destroy those cells (61). B cells have also been reported to be an important part of the adaptive immune response against influenza virus, and their absence significantly increases susceptibility to lethal influenza A infection (62). This is achieved by the production of influenza virus-specific IgM, which is an important intermediate factor that affects virus clearance (62).
1.2 Influenza infection in COPD

1.2.1 Epidemiology of COPD

In 2014, Global Initiative for Chronic Obstructive Lung Disease (GOLD) defined COPD as:

“A common preventable and treatable disease [that] is characterised by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients” (63).

COPD is one of the most important causes of morbidity and mortality worldwide that affects more than 64 million people globally (64, 65). In the 2014 Australian Institute of Health and Welfare report, COPD was identified as a significant underlying cause for 5,767 deaths among patients aged 55 and over in the country for the year 2011. This represents 102 per 100,000 people and 4.4% of mortality cases in that age group (66).

The most significant risk factor for COPD is active smoking of tobacco products and wood smoke, and eradication of smoking would substantially reduce the occurrence of COPD (67). Although not to the same extent, other factors have also been associated with the aetiology of COPD. Exposure to significant amounts of air pollution and living in close proximity to busy roads has been reported to have a strong connection with shortened life expectancy associated with COPD (68-71). Physical inactivity and occupational exposure to airborne particulates (such as dust and fumes) are also
attributable as causative factors of COPD (72, 73). Studying the prevalence of COPD in humans presents an important challenge diagnosis in patients is dependent on the definition used in particular cases. Estimation of incidences or cases appears to offer significant variability among doctors’ diagnoses, respiratory symptoms-based diagnoses and diagnoses indicated by spirometric criteria (74). For epidemiological studies, although high variation in estimations still exist, the use of spirometry as pulmonary function tests has been widely accepted for diagnosing COPD (75).

COPD also causes substantial economic burden. In Europe alone, the annual cost of this disease can reach up to €38.7 billion (approximately AUD$ 58 billion) (76). This is primarily from hospitalisation costs, including the cost for drug distribution, inpatient care, ambulatory care, as well as lost productivity by work absenteeism and early retirement (76-78). However, these approximations are likely an underestimate of the real impact of COPD on individuals as they exclude indirect financial burdens of the family members who care for COPD sufferers (75-79).
1.2.2 Pathogenesis of COPD

Typical characterised symptoms of COPD often include chronic airway inflammation, emphysema, and airflow limitation, which is predominantly caused by structural damage of bronchiolar walls (64). Indeed, a diagnosis of COPD often relies heavily on spirometric indicators in order to detect airflow obstructions during respiration (78). This is usually followed by other tests such as blood panels and X-rays to exclude other conditions and determine appropriate treatment and management strategies (80). In COPD, the severity of airflow limitation is classified based on FEV\textsubscript{1} (forced expiratory volume in 1 second) and FEV\textsubscript{1}/FVC (forced expiratory volume in 1 second/forced vital capacity) parameters. In benchmark GOLD classifications, patients with FEV\textsubscript{1}/FVC value lower than 70 and FEV\textsubscript{1} higher than 80% predicted value are categorised as “mild” or GOLD 1. Reduction in this value increases the level of GOLD category: GOLD 2, with FEV\textsubscript{1} predicted between 50-79%, indicating “moderate” severity; GOLD 3, with FEV\textsubscript{1} predicted between 30-49%, representing “severe” COPD; and GOLD 4, which is the highest classification, with FEV\textsubscript{1} predicted lower than 30%, or FEV\textsubscript{1} predicted lower than 50% with chronic respiratory failure present, indicating a “very severe” COPD (Table 1)(81).
### Table 1. GOLD classification of COPD severity (81).

In GOLD classification of COPD severity, symptoms are assessed and categorised based on the measured value of FEV$_1$ and FEV$_1$/FVC parameters. When patients have FEV$_1$/FVC value lower than 70, reduction in FEV$_1$ over predicted value increases the level of GOLD category. **FEV$_1$: forced expiratory volume in 1 second. FEV$_1$/FVC: forced expiratory volume in 1 second/forced vital capacity.**

<table>
<thead>
<tr>
<th>GOLD classification</th>
<th>Criteria (where FEV$_1$/FVC &lt; 0.70)</th>
<th>COPD Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLD 1</td>
<td>FEV$_1$ ≥ 80% normal</td>
<td>Mild</td>
</tr>
<tr>
<td>GOLD 2</td>
<td>FEV$_1$ 50-79% normal</td>
<td>Moderate</td>
</tr>
<tr>
<td>GOLD 3</td>
<td>FEV$_1$ 30-49% normal</td>
<td>Severe</td>
</tr>
<tr>
<td>GOLD 4</td>
<td>FEV$_1$ &lt; 30% normal, or FEV$_1$ &lt; 50% normal with chronic respiratory failure present</td>
<td>Very Severe</td>
</tr>
</tbody>
</table>
This airflow limitation and the underlying emphysematous changes in the lung are usually the result of interactions of toxicants and host response against them (Figure 5). Inhalation of tobacco products, despite not being the only cause of COPD, is the most significant contributor of these toxicants. In addition to nicotine and carcinogenic compounds, cigarette smoke also contains reactive oxygen species, such as nitric oxide (NO) and superoxide (O$_2^-$), which are highly toxic to the lung (82, 83). Inhalation of these toxicants, and the resulting oxidative stress, lead to inflammation in the lung, which in turn further generates more reactive oxygen species (84, 85).
Figure 5. Pathogenesis of COPD.

Inhalation of toxicants in cigarette smoke leads to inflammation and generation of reactive oxygen species. These provide an environment where antiproteases are more susceptible to oxidation, which reduces their antiproteolytic function. At the same time, proteolytic activities increase, resulting from elevated level of proteases. This imbalance, where deficiency of antiproteases is combined with excess proteases, along with cellular apoptosis, results in emphysematous destruction in the lung. Figure was created by the author and incorporates an image (graphical representation of normal versus emphysematous alveoli) adapted from *Molecular Pathogenesis of Emphysema* by Laimute Taraseviciene-Stewart and Norbert F. Voelkel (86). ROS: reactive oxidant species.
At the same time, the reactive oxygen species generated by compounds in the cigarette smoke also provide an environment where antiproteases are more susceptible to oxidation, compromising their normal antiproteolytic function. An example of this is the methionyl residue at the active site of α-1 protease inhibitor (an antiprotease, also referred to as α1-antitrypsin), which can be rendered ineffective because of the oxidation process caused by the encounter with reactive oxygen species (87, 88). This reduction of antiproteolytic effectiveness leads to the inability of the host to naturally balance the rate of cellular proteolysis and antiproteolysis. This imbalance is amplified by the increased proteolytic activities that result from elevated levels of proteases (such as neutrophil elastase, which contributes to elastin degradation in extracellular matrix proteolysis) that are generated during inflammation (89, 90). This loss of equilibrium, where deficiency of antiproteases is combined with increased proteases, results in excess proteolytic activity and consequently the emphysematous destruction in alveoli (91-95).

In addition, apoptosis may contribute to loss of alveolar epithelial and endothelial cells (96-98). This is demonstrated in vivo where loss of vascular endothelial growth factor (VEGF) function (achieved by the administration of VEGF receptor blocker SU5416) leads to apoptosis of alveolar septae (97, 98). Moreover, increased epithelial and endothelial alveolar septal deaths are observed in the lungs of emphysema patients (99). Similarly, apoptosis of epithelial or endothelial cells also occurs in mouse models of emphysema (100, 101). Indeed, experimental ablation of genetic factor Nrf2 (Nuclear factor [erythroid-derived 2]-like 2; a transcription factor that is typically involved in regulations of antioxidant genes) leads to increased number of apoptotic alveolar septal cells (100). Similarly, another study showed that direct intratracheal
instillation of apoptosis-associated effector molecules, such as ceramide, induces alveolar airspace enlargement (101).
1.2.3 Influenza virus infection in COPD and the role of PI3K

1.2.3.1 Epidemiology of influenza virus infection in COPD

Respiratory viral infections, including influenza, are significant causes of COPD exacerbations and death (102, 103). For example, during winter respiratory virus seasons; COPD patients, irrespective of age, are at higher risk of more frequent hospitalisations (103). These patients are also associated with worsened health-related quality of life, as measured by chronic lung disease severity index (CLDSI) (103).

Moreover, strong links between COPD and prevalence of respiratory infections have also been shown, in which viruses are highlighted to be among the most significant triggers of exacerbations (104-106). Up to 64% of COPD exacerbations have been found to be associated with respiratory viral infection (107-110). In many of these cases, multiple viruses can be detected, including influenza virus (both influenza A and influenza B virus strains), respiratory syncytial virus (RSV), adenovirus, coronavirus, and parainfluenza viruses (110). In one clinical study, viral infections were reported in a third of patients who sought treatment within ten days of the onset of COPD exacerbations (105). Interestingly, influenza virus was identified in 29% of those cases, and was the most prevalent virus detected; followed by RSV at 24% and parainfluenza virus type 3 (PIV-3) at 21% (105). In another study, the spectra of respiratory infections among hospitalised patients who had COPD (among other underlying respiratory illnesses) were described. In this study, influenza virus was detected in 36% of patients who presented with COPD exacerbations (108).
There is often a high variability between studies in reporting the proportion of COPD exacerbations that are specifically linked to isolation of influenza virus (103, 111). This variability is influenced by differences in the number of COPD patients who had received influenza vaccination (103, 111). In population samples that do not receive influenza vaccination, there is an increased rate of hospitalisation for exacerbations of COPD during influenza season compared to the rest of the year (112, 113). In regard to pandemic infection, investigators have predicted the shifting of pandemic H1N1 influenza infection bias from school-age children towards older age groups (114). This is potentially in line with increased risk of those with COPD, who are typically older individuals, to pandemic influenza (105, 114, 115).

At present, preparative strategies implemented to address potential influenza pandemics are mainly focused on vaccination and accumulation of antiviral drugs. Annual vaccinations lower the risk of influenza virus infection, including in patients with COPD (115). However, the ability of viral antigens to change frequently prevents lasting protection of vaccination against infection, meaning that therapeutic interventions still have a significant role to play. Neuraminidase inhibitors such as zanamivir and oseltamivir were used in many cases of influenza infections during the 2009 pandemic (116). M2 inhibitors, which include drugs such as amantadine and rimantadine, are used as alternative options especially in USA and UK, although in many cases there are high-levels of resistance by influenza viruses due to antigenic mutations (117-119).

A number of mechanisms have been implicated in virus-induced COPD exacerbations. For example, COPD exacerbations are linked to increased oxidative stress (84, 120). Infections by respiratory viruses, such as rhinovirus and influenza virus
induce increased oxidative stress in the lung (121-123). Infection of respiratory epithelial cells by rhinovirus increases the concentration of reactive oxygen species including hydrogen peroxide (H$_2$O$_2$) *in vitro* (121). *In vitro* infection of rhinovirus and *in vivo* infection of influenza virus also stimulate the activation of oxidative stress-response factors such as nuclear factor kappa light chain enhancer of activated B cells (NF-κB) (121, 123). Both *in vitro* and *in vivo* infections with influenza virus induce the expression of oxidative stress-response and antioxidant enzyme-producing genes: including heme oxygenase 1 (HO-1), manganese superoxide dismutase, and glutathione peroxidase (122, 123).

In addition to these factors, another mechanism has also been strongly associated with influenza infection in COPD: the PI3K signalling pathway (124, 125). The following sections will further explore what is currently known of the role that PI3K may play during influenza infection in COPD.
1.2.3.2 PI3K

Phosphatidylinositol-4,5-bisphosphate 3-kinase, also known as phosphoinositide 3-kinase (PI3K), is an enzyme that consists of eight catalytic subunits (126). These subunits have been divided into three classes according to the differences in the primary domain structures, sequence alignment, function and lipid substrate specificity (Figure 6) (126-129).
Figure 6. PI3K catalytic and regulatory subunits

PI3K consists of eight catalytic and eight regulatory subunits. These subunits are divided into three classes according to the differences in the primary domain structures, sequence alignment, function and lipid substrate specificity.
Class I PI3K structurally consists of multiple variants of catalytic subunits of p110 and regulatory subunits (127). In vertebrates, three of the four variants of the catalytic subunits, namely p110α, p110β and p110δ are categorised as Class IA PI3K (127, 128). Another variant in this subfamily, p110γ is separated from the other three variants into a second category, Class IB PI3K (127, 128). Both Class 1A and 1B prefer phosphatidylinositol 4,5-bisphosphate (PIP₂, a minor phospholipid component of cell membranes) as their primary catalytic substrate (130). PIP₂ is then converted to phosphatidylinositol (3,4,5) -triphosphate (PIP₃) (130). All variants of p110 subunits also contain a Ras-binding domain, which may interact with guanosine triphosphate (GTP)-Ras signal transduction protein to amplify the overall PI3K activity at the plasma membrane (131). Class IA and IB PI3K differ from each other in the utilisation of two distinct regulatory subunits associated with their catalytic activities. Class IA PI3K associates with a p85 regulatory subunit which contains Src Homology (SH) 2 protein domain (132). The p85 subunit also has five known variants; p85α, p55α, p50α, p85β, and p85γ (127, 128, 133). Class IB PI3K utilises p101 and p84 regulatory subunits (134). p110α and p110β are ubiquitously expressed in most cell types whilst p110γ and p110δ are largely limited to immune cells (128).

Class II PI3K is composed of three isoforms C2α, C2β, and C2γ. In vitro studies have demonstrated that Class II PI3K isoforms can phosphorylate either phosphatidylinositol 4-phosphate (also known as PI4P) or phosphatidylinositol, with a preference towards the latter (128, 135). Activation of Class II PI3Ks can be triggered by G protein-coupled receptors (GPCRs), tumour necrosis factor (TNF) family receptors, epidermal growth factor receptors and insulin receptors (128, 135). While the exact mechanism of interaction between these isoforms and receptors remains to be
verified, it may involve coupling with phospholipids, clathrin, and calcium and translocation of the Class II PI3Ks to the plasma membrane (128, 135).

Class III PI3K has a single member, Vps34, and is the oldest class of PI3K discovered (127). The primary substrate for Vps34-mediated catalysis is a minor amphiphilic component of eukaryotic cellular membranes, phosphatidylinositol, which is converted to phosphatidylinositol 3-phosphate (PI3P). PI3P is the most abundant form of phosphorylated phosphatidylinositol and a vital binding site of proteins that contain FYVE zinc finger domain and PX structural domain (136). Vps34 is typically part of two complexes with Vps15 and Vps30 (137). Vps15 and Vps30 play supporting roles as catalytic activity regulators, transporters to lipid membranes and adapter proteins for Vps34 (138). Vps34 itself has been shown to be important in protein sorting, endocytosis, phagocytosis and autophagy in various cell types (137-140). However, its role in cells and immune responses to influenza virus is poorly understood. While genetic deletion of Vps34 leads to embryonic death in mice, the use of conditional knockouts may contribute in understanding the role of Vps34 in selected cell types, including those that are vital in immune system (141-144).
1.2.3.3 The role of PI3K during influenza infection

Influenza virus has the ability to modulate existing cell signalling pathways in order to suppress antiviral responses and increase the efficiency of viral infection (145, 146). This process may include exploiting the signalling pathways that are originally activated as defence mechanisms against the infection itself (145, 146). One of the important intracellular pathways that is activated during influenza infection is the PI3K signalling pathway (Figure 7) (147-150). PI3K was initially thought to act in an antiviral fashion. In an *in vitro* study using Human Embryonic Kidney 293 (HEK293) cells, pre-treatment with the specific PI3K inhibitor LY294002 resulted in the failure of mRNA induction of p56 (an antiviral IFN-inducible protein) following dsRNA treatment that mimics viral infection (151). However, in contrast to that initial characterisation, viral infections were later demonstrated to promote PI3K activation (147-149). For example, infection of lung epithelial cells with different strains of influenza virus: A/Puerto-Rico/8/34 (H1N1), A/FPV/Bratislava/79 (H7N7), and WSN/HK (H1N2); resulted in increased phosphorylation of Akt (also known as Protein Kinase B, or PKB), a marker of PI3K pathway activation (147).
Figure 7. Influenza virus utilises PI3K signalling pathways to increase infection

Following influenza virus infection, NS1 protein binds to the SH3 and C-terminal SH2 domains of the PI3K regulatory subunit, p85. This activates the conversion of PIP2 to PIP3 and phosphorylation of Akt (also known as Protein Kinase B, PKB). This is then utilised by influenza virus for intracellular entry that subsequently increases viral replication.
Early activation of the PI3K signalling pathway in influenza infection typically occurs transiently and regulates the initial phase of viral entry into the host cells (147, 152). Following specific inhibition of PI3K by wortmannin in adenocarcinomic human alveolar basal epithelial (A549) cells that were infected with influenza virus, analysis via immunofluorescence microscopy showed the accumulation of viral particles on the cellular surface that had failed to enter the target cells (147). Similarly, in another study, treatment with a specific Akt inhibitor peptide, Akt inhibitor NH(2)-AVTDHPDLWAWEF-COOH (Akt-in, encompassing the betaA strand of human TCL1) in A549 cells infected with influenza virus, also resulted in the suppression of viral entry and replication (152, 153).

PI3K also plays an important role in the endosomal transport during virus uptake localising on cellular membranes. A study demonstrated that during receptor-mediated endocytosis, PI3K inhibition by wortmannin in A549 cells resulted in the absence of co-localisation of influenza viral nucleoprotein with Cluster of Differentiation 63 (CD63, a late endosomal antigen marker) (154). This co-localisation of viral nucleoprotein and CD63 is an indicator of virus trafficking towards the endosome (154). Therefore, the absence of this co-localisation following PI3K inhibition indicates a failure in the trafficking of viruses towards the endosome in the target cells during the early phase of influenza infection (147).

During the later stage of infection (at approximately 6 hours after the infection in in vitro settings), a more sustained activation of the PI3K pathway can be detected (147, 148). In contrast to the early activation that occurs in infection by both influenza A and B viruses, the later stage of PI3K activation can only be observed in infection by type A, but not type B, influenza viruses (155). This sustained PI3K activation requires
induction by influenza virus structural protein, NS1. During influenza infection in vitro, activation of PI3K signalling, which is indicated by the phosphorylation of Akt, cannot be induced in the absence of NS1 (147, 148). Furthermore, NS1 alone, without the requirement for additional virus-derived factors, is sufficient to induce PI3K signalling in 1321N1 (a human astrocytoma cell line) cells (148). Importantly, the effect that NS1 expression has on the induction of PI3K signalling is negated when the cells are treated with the PI3K inhibitor, LY294002 (148).

The induction of PI3K signalling by NS1 is achieved through direct binding of the viral protein with the SH3 and C-terminal SH2 domains of the PI3K regulatory subunit, p85 (148, 149). More specifically, NS1 binding occurs more efficiently with the p85β subunit than the related p85α subunit (148, 149). In addition, the binding between NS1 and the p85β can be disrupted (for example, by amino acid substitutions), which consequently leads to the failure of activation of the PI3K signalling pathway (148).

In the presence of NS1, PI3K activity becomes advantageous for influenza virus in its replication process. On the other hand, mutations of the influenza NS1 protein hinder the viruses’ ability to utilise the PI3K signalling for efficient infection. For example, in vitro infection with influenza A/Udorn/72 virus, which contains phenylalanine for Tyr-89 (Y89F) mutation in NS1 expression (which renders the NS1 less effective), is unable to induce Akt phosphorylation (148). Furthermore, this Y89F mutation leads to smaller plaque formations and lower viral titres following infection with the virus, in comparison to infection with wild type influenza virus that has intact NS1 (148).
Similarly, in another study, A549 cells were infected with a variant strain of A/Puerto Rico/8/34 influenza virus (149). This virus contains NS1 that expresses multiple-site mutations (a total of six site substitutions: one substitution of phenylalanine for Tyr-89 and five substitutions of alanine for proline) (149). In this experiment, the variant virus was unable to activate PI3K signalling pathway, due to the failure of binding between SH-binding motifs on the mutated NS1 with SH domains of p85 on the PI3K, leading to attenuated infection and reduced viral titre (149).

Importantly, the PI3K signalling pathway has also been reported to be hyper-activated in bronchial epithelial cells and leukocytes in the lung of patients with COPD (124, 125). In light of the strong associations between influenza virus infection and COPD (as discussed previously in section 1.2.3.1: Influenza infection in COPD), as well as between PI3K signalling pathways and infection (as indicated by multiple studies mentioned in the current section), we hypothesised that the activation of PI3K signalling pathway may be a mechanism that promotes increased susceptibility of COPD patients to influenza infection (see section “1.4: Hypotheses and aims” for further descriptions). Furthermore, current prevention and treatment strategies for influenza virus infections have significant limitations, such as antigenic drift and shift and resistance to neuraminidase inhibitors (156-159). Thus, elucidation of the mechanisms underlying the susceptibility to influenza in COPD will potentially open new avenues for novel treatment strategies.
1.3 Influenza infection in asthma

1.3.1 Epidemiology of asthma

Globally, around 300 million people have asthma (160). In the past several years, there have been reports suggesting a plateauing or even a declining trend of asthma prevalence (161-163). Increased public awareness and better asthma management strategies have contributed to this improvement (164). Despite this, asthma remains a significant health issue, which has a correspondingly large economic burden. Annual expenditure on asthma in the United States is between USD $3.6 and $8 billion (approximately AUD $3.8 and $8.5 billion, respectively), with half of these costs due to hospitalisations (165). In Australia, an estimated 12% of the population is affected with asthma leading to an estimated 54,000 annual hospitalisations annually (166, 167). The corresponding annual health expenditure associated with asthma in Australia is AUD $693 million, which comprises approximately 1.4% of the national healthcare budget (166). The economic burden of asthma is expected to rise in the next decade as 100 million additional asthma patients are predicted to be diagnosed by 2025 (168).

The high prevalence of asthma, particularly in economically developed societies, such as the United States, the United Kingdom and Australia; has been linked to sanitary measures taken to improve overall health. The “hygiene hypothesis” postulates a strong correlation between decreased infections in early childhood and increased cases of allergy and asthma (169, 170). In immunologically naïve young individuals, the immune system is dominated by T helper cell type (Th) 2 phenotype. A balance between the counteracting Th1 and Th2 phenotypes is later achieved by natural exposure to immune stimulating infections, which enhance responses in a Th1-mediated
manner. Contact with older siblings and other children in day-care facilities may expose young children to microbial infection and leads to reductions in allergic sensitisation (169-171). Childhood exposure to infectious agents is greatly reduced in Western societies where there is better infection control, frequent prescriptions of oral antibiotics, improved sanitation, higher rates of immunisation and smaller family sizes (172, 173). These factors significantly impede the development of a Th1-Th2 balance and increase susceptibility to allergic asthma that results from a dominant Th2 phenotype.

As the importance and burden of asthma on respiratory health continue to be major and increasing, appropriate strategies are urgently required to manage the disease at both the individual and societal levels. Accordingly, development of the most effective strategies necessitates increasing our understanding of asthma pathophysiology and immunology.
1.3.2 Pathophysiology of asthma

Asthma is a complex allergic airways disease (AAD) that manifests as recurrences of wheezing, coughing, breathlessness and chest tightness (174, 175). These symptoms are often elicited by the combined effects of airway inflammation and hyperplasia and metaplasia of mucus secreting cells (MSCs, Figure 8) (174-176). These are usually accompanied by airway hyper-responsiveness (AHR), which refers to airway smooth muscle contraction following excessive sensitivity to non-specific stimuli. Despite the strong association with airway inflammation, the severity of hypersensitivity and AHR in asthma is not necessarily dependent on the level of airway inflammation (177-179). Patients may present with inflammation in atopic rhinitis without the presence of AHR (180). On the other hand, AHR can be stimulated, for example by hypertonic saline aerosol, in the absence of airway inflammation (181). Because of such disassociations between the level of airway inflammation and AHR, assessment of AHR (or other lung function criteria, such as FEV$_1$) may be a more reliable measurement of asthma severity. In the long term, airway remodelling may occur as a result of chronic inflammation and excessive cellular growth in the airway, leading to overall declines in lung function (182, 183). This airway remodelling is typically caused by hyperplasia, metaplasia and/or hypertrophy of epithelial cells, MSCs, and smooth muscle cells, angiogenesis, sub-epithelial basement membrane thickening and collagen deposition (184, 185). In humans, pulmonary inflammation, increased mucus production and AHR may eventually cause serious obstructions in airflow, leading to respiratory distress, or even death due to asphyxiation (108, 186-191).
Figure 8. Pathophysiological features of asthma

Schematic representations of the airways in (A) healthy individuals, (B) asthmatic patients, and (C) asthmatic patients during an asthma attack. In asthma, exposure to allergens leads to inflamed and thickened airway walls and hypersecretion of mucus inside the airway lumen. Hyper-responsiveness of the airway smooth muscles towards the allergens also leads to bronchoconstriction. These features collectively may result in serious airflow obstruction, leading to symptoms ranging from minor respiratory distress to asphyxiation and death. Figure was created by the author and incorporates images (graphical representations of airways) adapted from Turning Discovery into Health – Asthma, National Institute of Health (192).
1.3.3 Immunology of asthma

Immunological reactions in asthma generally occur following exposure to allergens. Allergens are typically innocuous antigens in the environment, such as plant pollens, animal and fungal antigens, house dust mites (HDM), as well as air pollutants. In asthmatics they trigger hypersensitivity as well as causing the production of immunoglobulin (Ig)E antibodies in the circulation (193-196). IgE antibodies have long been recognised as significant inducers of the immune response to allergens (194, 197).

Prior to the initiation of IgE synthesis, antigens must first encounter and be processed by DCs which act as APCs in the airway (197). These DCs then carry the processed antigens into mediastinal lymph nodes, which are a cluster of draining lymph nodes situated adjacent to the lung (197). In these lymph nodes, the processed antigens are presented to T and B cells (194). Activation of B cells is achieved following two signalling events. First, the binding of IL-4 or IL-13 to receptors on B cells and second, the structural interaction between CD40 (cell surface molecule on B cells) and CD40-L (surface ligand molecule on T cells) (194, 197, 198).

B cell activation in turn leads to the production of more cytokines, promoting switching of B cell antibody release from IgG to IgE and therefore subsequent production of IgE (199). IgE antibodies then encounter receptors known as Fc epsilon receptor I (FcεRI) (194). FcεRI are high-affinity IgE receptors, which are present on cell surface of mast cells, basophils and eosinophils (194). Mast cells may also express the surface molecule CD40 and produce IL-13, providing a feedback loop to amplify the IgE synthesis (194, 197). Formation of the cross-linking IgE-FcεRI complex leads to mast cell production of mediators, such as histamine and prostaglandins (197, 200). These mediators play a role in vascular leakage and the subsequent pulmonary
infiltration of inflammatory cells and oedema (197). Various interleukins are also generated during these responses, leading to the recruitment of macrophages, basophils, Th2 cells and potentially natural killer T (NKT) cells in chronic asthma (201-203). In particular, differentiated Th2 cells produce Th2-associated cytokines, including IL-4, IL-5 and IL-13 (194, 197). This leads to the suppression of Th1 cells, eosinophil activation and infiltration into the airways, hyperplasia and metaplasia of MSCs, and AHR (204-207).
1.3.4 Influenza infection in asthma and the role of IL-13

1.3.4.1 Epidemiology of influenza virus infection in asthma

Respiratory virus infections are the most common cause of asthma exacerbations in both adult and paediatric populations (191, 208, 209). There are fewer reports of asthma exacerbations in adults compared to those of children (208). Nevertheless, these reports still provide useful insights regarding the prevalence of viral infection in adult cases of asthma exacerbations, which have been estimated to be up to 78% (209-213). Most recently, investigators utilised a multiplex real-time PCR system to examine nasopharyngeal swab and sputum samples in order to rapidly estimate the causes of asthma exacerbations (209). In this study, viral infection was detected in over 35% of patients who presented with acute exacerbation of bronchial asthma, with an additional 11% who were diagnosed with viral and bacterial co-infections (209).

In children, the prevalence is significantly higher, where respiratory viral infection is detected in up to 95% of paediatric patients with acute wheezing episodes (214-224). These studies also cover both community and hospital settings, and are inclusive of the very early presentation of the disease in infants. For example, one study evaluated the presence of viral infections in the first episode of acute wheezing in hospitalised infants (under 12 months old) (223). In this study, real-time PCR detection was used to examine the presence of infectious agents in nasopharyngeal swabs collected from the patients. Viral infections were detected in 89.4% of patients, including 17.6% who were co-infected with more than one virus (223).
In those studies, rhinoviruses were most frequently detected in both adult and paediatric populations. However, other viruses were also shown to be significantly involved, including influenza virus. In adults, influenza viruses were identified in approximately 20% to 25% of patients who were admitted to hospitals for acute asthma exacerbations (108, 186, 212, 225). Methods of diagnosis also play an important role in determining the rate of detection. For example, one of these studies implemented the combination of induced sputum and PCR as a detection method, which was compared with detection using serology and immunofluorescent antigen testing (225). The induced sputum and PCR combination lead to better detection of influenza virus in exacerbating asthma (24%) compared to the serology and immunofluorescent antigen testing (18.3%) (225).

In children, influenza virus infection in asthma exacerbations are reported to be at lower prevalence, ranging from 0% up to 7% (215, 216, 220, 221, 223, 226, 227). Nonetheless, health care utilisation rates for influenza patients are higher in asthmatic compared to non-asthmatic children (228). For example, annual outpatient visit rates were reported to be higher in children with asthma compared to healthy children in different age groups: 6 to 23 months of age (316 vs 152 cases per 1000 children), and 24 to 59 months of age (188 vs 102 cases per 1000 children) (228).

Moreover, individuals with asthma (both adults and children) are more susceptible to influenza virus infection and have higher risk of medical complications in the event of an influenza pandemic (229-231). In one study, the clinical spectrum of illness and risk factors were analysed among hospitalised patients during the peak of the 2009 H1N1 influenza pandemic (229). More than 72% of the patients presented with at least one underlying medical condition. Importantly, asthma was the most common
underlying medical condition associated with the pandemic, both in adults (27%) and children (29%) (229).
1.3.4.2 Susceptibility to influenza virus infection in asthmatics

A number of factors have been suggested to contribute to the increased susceptibility of asthmatic patients to respiratory viral infections. For example, compromised epithelial barrier in the lungs of asthmatics may increase their susceptibility to viral infections. In a recent study, epithelial cells from bronchial biopsy specimens were obtained from paediatric patients with asthma (232). These samples were then evaluated in terms of the induction of antiviral response following rhinovirus type 16 infection (232). Along with impaired antiviral responses, samples from patients with asthma were found to have greater epithelial damage compared to those from healthy controls (232). This observation may also be important in the context of other viral infections, including influenza. Similar to rhinovirus, influenza virus also inflicts epithelial damage, either directly by cytolytic effects of the virus, or indirectly by the host immune responses generated against the infection (for example, by cellular apoptosis induced during viral replication to limit the spread of infection) (233-235).

Epithelial damage that occurs during viral infection in asthma may also induce the release of mediators, including alarmins IL-25 (236), IL-33 (237), and TSLP (238). The presence of these alarmins may skew the immune environment in terms of the type of cells that undergo activation and maturation processes. For example, exposure of resident antigen presenting cells (such as DCs) to IL-33 and TSLP may promote the differentiation of naive T cells to Th2 cells (239, 240). In addition, IL-25 and IL-33 may induce the activation and maturation of group 2 (or type 2) innate lymphoid (ILC2) cells (241). The combination of increased numbers of Th2 cells and ILC2s provide a microenvironment of elevated Th2-associated cytokines, including IL-4, IL-5, IL-9, and
most importantly, IL-13 (242-244). The following sections will further explore the role that IL-13 may play during influenza virus infection in asthma.

1143
1.3.4.3 IL-13

IL-13 was initially described as an inhibitor of inflammatory cytokine production (245-247). However the diverse functions of this Th2 cell-derived cytokine have been identified over the years. Along with Th2 cells, there is an increase number of other cell types that have been shown to produce IL-13, including mast cells, DCs, macrophages, epithelial cells and ILC2 cells (242-244). In contrast to early assumptions of functional redundancies between IL-13 and IL-4, studies utilising IL-4 and signal transducer and activator of transcription 6 (STAT6) deficient mice have demonstrated the independent role of IL-13 in the host immune response (248-250). IL-13 triggers signalling of the janus kinase (JAK)/STAT6 pathway through its binding with the multi-subunit IL-13 receptor, which is comprised of IL-13Rα1 and IL-4Rα (Figure 9) (249, 251, 252). This results in the phosphorylation and dimerisation of STAT6, which then translocates into the nucleus. In the nucleus, genes related to proteins involved in the development of AAD are transcribed, including those that encode IgE, eotaxin and vascular cell adhesion molecules (253-255).
Figure 9. IL-13 and its receptors

IL-13 binds to the multi-subunit IL-13 receptor, which comprises of IL-13Rα1 and IL-4Rα. This binding initiates signalling of the janus kinase (JAK)/STAT6 pathway, triggering phosphorylation and dimerisation of STAT6, which then translocates into the nucleus. In the nucleus, genes encoding IgE, eotaxin and vascular cell adhesion molecules are transcribed, leading to the development of airway inflammation, mucus production and AHR. Alternatively, IL-13 may also bind to IL-13Rα2, which may function as a decoy receptor or as an active receptor in IL-13-mediated pathways that are yet to be elucidated. This eventually also leads to airway inflammation, mucus production and AHR.
Beside IL-13Rα1, IL-13 may also bind to another receptor, IL-13Rα2. However, this receptor is thought to function as a decoy IL-13 receptor, based on the absence of signalling motifs that are vital for downstream signal induction (256, 257). Nevertheless, memIL-13Rα2, which is a membrane form of IL-13Rα2, has been shown to play a role in the development of allergic asthma (258). Following HDM challenge, the absence of memIL-13Rα2 in mice resulted in the attenuation airway inflammation, mucus production and AHR (258). Moreover, in IL-13Rα2-deficient mice, over-expression of memIL-13Rα2 was shown to restore airway inflammation, mucus production and AHR to levels in wild type controls (258). Therefore, IL-13Rα2 may play a role in either the decoy function to the IL-13 receptor or as an active receptor in IL-13-mediated pathways, depending on the disease context.
1.3.4.4 The role of IL-13 during influenza virus infection in asthma

Patients with asthma have increased levels of IL-13 in the lungs, which plays a vital role in promoting the development of goblet cell formation, increasing mucus secretion and profibrotic repair of airway epithelium and AHR (259-262). Inhibition of aberrant IL-13 signalling may reduce allergic responses in asthmatic patients (263, 264). Similar observations have been made in *in vivo* models, where the absence of IL-13 signalling decreases AAD-associated features, such as mucus hypersecretion and AHR (260, 262, 265). In addition, over-expression of IL-13 has been linked with asthma-associated abnormalities, including pulmonary allergic inflammation and mucus hypersecretion (266).

Respiratory viral infections, including influenza, have been shown to trigger AHR independently of the adaptive arm of the immune system, including Th2 cells. Instead, it requires “natural helper” ILCs (instead of the adaptive T- or B-cells) and utilises a pathway involving IL-13 (267). The role of IL-13 during viral infection is also vital in the context of pre-existing allergic conditions such as asthma. IL-13 has also been associated with decreased production of IFN-γ and IFN-λ, which are important factors in antiviral immune responses (259, 268-270). The relationship between IFN-γ and IL-13 in pulmonary inflammation is complex and their interaction is generally viewed in the larger context of balancing the number of Th1 and Th2 cells in the immune system (269). In the case of IFN-λ, inverse correlations between this type-III IFN and IL-13 have previously been observed. Production of IL-13 has been shown to be diminished in the presence of IFN-λ (271). This down-regulatory effect of IFN-λ is selective and has a more potent effect on IL-13 compared to other Th2-related cytokines, such as IL-5 (272).
In this study, we explored the potential role of IL-13 in promoting increased susceptibility to influenza in AAD. At present, prophylactic and treatment strategies for combating influenza infections and their efficacy in asthma patients have important limitations, and are circumvented by genetic mutations that lead to antigenic drift and the emergence of resistant strains to drug treatments, such as neuraminidase inhibitors (116, 156, 159, 273, 274). Enhancing our understanding of the mechanisms that promote influenza viral infection in asthma may provide insights in improving and augmenting our repertoire in disease management, both in those with underlying asthma as well as in healthy individuals.
1.4 Hypotheses and aims

Patients with COPD and asthma are more susceptible to influenza viral infection (104, 105, 108, 208). However, the mechanisms that underpin this association are largely unknown. The studies outlined hereafter were designed to elucidate the immunological mechanisms that lead to this increased susceptibility. In order to investigate these mechanisms in vivo, murine models of influenza virus infection in COPD and AAD were used. These murine models have been developed by our laboratory and shown to exhibit features associated with COPD (275) and AAD (276, 277). We hypothesised that cigarette smoke-induced experimental COPD would lead to increased susceptibility to influenza infection. We proposed that PI3K plays a vital role in the mechanism underpinning this susceptibility. We also proposed that inhibition of PI3K would lead to improvements in the outcomes of infection, including reduced viral titre, enhanced antiviral IFN responses and improved lung function. Similarly, we hypothesised that AAD would increase the susceptibility to influenza virus infection and, subsequently, the infection would exacerbate the underlying AAD. We proposed that IL-13 responses play an important role in mediating these effects. We also proposed that inhibition of IL-13 would improve infection outcomes, including reduced viral titre, improved antiviral IFN responses, reduced mucus hypersecretion and AHR.

Numerous studies were performed in order to address these hypotheses. These studies specifically aimed to:

- Characterise the effects of cigarette smoke-induced experimental COPD on the severity of influenza virus infection in terms of viral titre, inflammatory, antiviral and cytokine responses and lung function.
• Investigate the role of PI3K and characterise the effects of cigarette smoke-induced experimental COPD on influenza virus infection following the inhibition of PI3K.

• Characterise the effects of Ova-induced AAD on influenza virus infection in terms of viral titre, inflammatory, antiviral and cytokine responses, and AHR.

• Investigate the role of IL-13 and characterise the effects of both recombinant IL-13 administration in naive mice, as well as the effects of IL-13 inhibition in AAD mice following influenza virus infection.
CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

Six to eight-week old specific pathogen-free (SPF) female BALB/c mice were used in all the experiments. Animals were obtained from The University of Newcastle Animal Services Unit, or purchased from Animal Resources Centre (Perth, Australia) or Australian Bio Resources (Moss Vale, Australia). They were given access to food and water ad libitum. Animals were housed in individually ventilated cages in a specific pathogen-free facility with controlled environment of 12 hours light and dark cycles.

2.2 Influenza virus infection

On the last day of smoke-exposure or Ova challenge, groups of mice were anaesthetised with isoflurane and infected intranasally (i.n.) with 7.5 plaque forming unit (PFU) of the mouse-adapted H1N1 influenza virus A/PR/8/34 (WHO Collaborating Centre for Reference and Research of Influenza, Victoria, Australia) in 50µl of media vehicle (UltraMDCK, Lonza, NJ, USA). Controls were sham-inoculated with media. Mice were sacrificed at 3, 7 or 10 days post infection (dpi).

2.3 Induction of cigarette smoke-induced experimental COPD

Mice were exposed to the smoke from 12 x 3R4F reference cigarettes (University of Kentucky, USA) twice/day, five times/week, for eight weeks using an in-
house custom-designed and purpose-built specialised nose-only, directed flow
inhalation and smoke-exposure system contained in a laminar flow and smoke-
extraction unit (CH Technologies, NJ, USA) (275). Non-smoking control mice were
exposed to normal air for the same period of time (i.e. 8 weeks). The dose of cigarette
smoke was determined by performing acute (4 days) dose-response experiments using
different levels of smoke exposure (275).

2.4 PI3K inhibition

Mice were treated with the Class I pan PI3K inhibitor LY294002 (Selleck
Chemicals, TX, USA) at 1 mg/kg in 3% dimethyl sulfoxide (DMSO) vehicle (Sigma
Aldrich, Castle Hill, Australia), three times a week by i.n. administration under
isoflurane anaesthesia for the last two weeks of smoke exposure and throughout the
infection. Control mice were vehicle-treated with DMSO (278).

2.5 Induction of AAD

Mice were sensitised with 50 μg Ova (Sigma Aldrich) and 1 mg Rehydrogel
(Reheis) in 200 μl sterile phosphate buffered saline (PBS) by intraperitoneal (i.p.)
injection (276, 277). Mice were then challenged with Ova (10 μg in 50 μl sterile PBS)
under isoflurane anaesthesia at 12-15 days later by i.n. administration. Control mice
received PBS sensitisation and Ova challenges (276, 277).
2.6 Administration of rIL-13

Mice were treated with 100 ng of murine rIL-13 (R&D systems, Gymea, Australia) in 30 μl sterile PBS i.n., once every 24 hours, from the day before influenza virus infection (-1 dpi) through to 2 dpi. Mice were sacrificed at 3 dpi. Controls were vehicle-treated with PBS (279).

2.7 Neutralisation of IL-13

Rabbit anti-murine IL-13 antibodies were kindly prepared and supplied by Professor Nick Lukacs, the University of Michigan, USA (280). These antibodies were produced by multiple-site immunisation of New Zealand White rabbits with murine rIL-13 (R&D Systems) and titred by direct ELISA. The antibodies were specifically verified by the failure to cross-react to these murine (m) and human (h) proteins: mIL-3, mIL-1α, mTNF, hTNF, mIL-4, hIL-13, mIL-10, mIL-12, mMIP-1α, hMIP-1α, hMIP-1β, mMIP-1β, IL-6, mMCP-1, hMCP-1, hIL-8, and hRANTES. The in vivo half-life of the antibody was 30 hours. Mice were treated i.p. with 0.3 ml of anti-IL-13 antibody, 1 hour before influenza virus infection and at 2 dpi. Mice were sacrificed at 3 dpi. Controls were treated with isotype control antibody.
2.8 Bronchoalveolar lavage fluid (BALF)

The right lobes of the lung were tied off using a thread in order to block the bronchus (these lobes were reserved for protein and RNA extractions). BALF extraction was performed on the left lobe of the lung using 2 x 0.7 ml Hanks Buffered Saline Solution (HBSS) (Gibco, Invitrogen, Mount Waverly, Australia). 0.5 ml of the BALF was reserved for plaque assay, while the remainder was used for cytospin preparation.

BALF was centrifuged at 300 x g for 5 minutes at 4 °C. BALF supernatants were collected and stored at -20 °C for cytokine analysis. The remaining pellets were resuspended in 500 µl of red blood cell lysis buffer for approximately 5 minutes on ice. Cells were then washed with HBSS and centrifuged at 300 x g for 5 minutes at 4 °C. Supernatants were discarded and pellets were resuspended in 160 µl of HBSS. The total numbers of cells were enumerated using a hemocytometer. Cell suspensions were cytocentrifuged (300 x g, 10 min; Thermo Fisher Scientific, Norwood, Australia) and air-dried overnight.

Dried cytospin slides were stained with May Grunwald-Giemsa stain (Giemsa was diluted 1:25 with Giemsa buffer, Australian Chemical Reagents, Australian Scientific, Kotara, Australia). Slides were immersed in May-Grunwald stain for 5 minutes to allow staining of the nuclei. Slides were then washed in distilled water for 1 minute to remove excess stain. Following that slides were immersed in Giemsa stain for 20 minutes to allow staining of eosinophilic granulocytes. Slides were again washed in distilled water twice for 5 minutes. Slides were left to dry overnight and cover-slipped using Normount mounting medium (Thermo Fisher Scientific). The numbers of total
leukocytes, macrophages, lymphocytes and neutrophils were identified by morphology and enumerated with a total of 250 cells counted using light microscopy (281-284).

### 2.9 Plaque assay

Madin-Darby Canine Kidney (MDCK) cells were grown until approximately 70% confluence was achieved. The cells were then washed with Dulbecco’s Phosphate Buffered Saline (DPBS; Sigma Aldrich) three times. The cells were submerged in Leibovitz’s L-15 (L-15) medium (Invitrogen) supplemented with 2- [4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid (HEPES; Invitrogen) and N-p-tosyl-L-phenylalanine chloromethyl ketone treated trypsin (trypsin-TPCK; Invitrogen). BALF samples were serially diluted in L-15 medium, which had been supplemented with HEPES, and were added to the cells. After 60 minutes of incubation at 37 °C (with 5% CO₂), the inoculum was removed. A thin overlay of 1.8% agarose in L-15 medium containing trypsin-TPCK (Invitrogen) was placed on to the cell monolayers. After 48 hours of incubation at 37 °C (with 5% CO₂), plaques were stained with 0.1% crystal violet and counted (285, 286).

### 2.10 Lung function analysis in experimental COPD

Lung function parameters for experimental COPD model were assessed using forced oscillation (FlexiVent, Scireq Scientific Respiratory Equipment, Montreal, Canada) and forced manoeuvre (Buxco Electronics, NC, USA) techniques (275). Transpulmonary resistance and dynamic compliance were assessed using the snapshot
perturbation function, and tissue damping with the forced oscillation perturbation. For all perturbations, a minimum ventilation period of 20 seconds was allowed between each perturbation. A coefficient of determination (COD) of 0.95 was the minimum value allowable for each measurement. Total lung capacity (TLC) was determined using the quasistatic pressure-volume loop. Each manoeuvre was performed a minimum of three times, and the average determined.

2.11 Assessment of AHR in AAD

Assessment of AHR in AAD was done by whole body invasive plethysmography (Buxco Electronics) (276, 287). Mice were anaesthetised and tracheas were cannulated and attached to a ventilator. Dynamic compliance and transpulmonary resistance were measured by analysis of pressure and flow waveforms following challenge with increasing doses of aerosolised methacholine (Sigma Aldrich).

2.12 Lung histology

To assess alveolar diameter and destructive index, the left lobe was perfused with 0.9% saline using a 19-gauge needle after allowing the euthanised mice to bleed out by severing the aorta in the lower abdominal cavity. Buffered formalin (0.5 ml, 10%, Sigma Aldrich) was injected into the trachea, before being tied off. Lungs were embedded in paraffin, sectioned (4-6 μm) and stained with haematoxylin and eosin (H&E). For histopathological scoring, lungs were stained with H&E and scored according to a set of custom-designed criteria (Table 1) (288). For the assessment of the numbers of MSCs, lungs were stained with periodic acid Schiff (PAS) and enumeration
was determined in airways as previously described (277, 289, 290).
### Table 2. Histopathological scoring system for mouse lungs (288).

<table>
<thead>
<tr>
<th>Score 1: Airways inflammation Score (/4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = Lack of inflammatory cells around airways - Absent</td>
</tr>
<tr>
<td>1 = Some airways have small numbers of cells - Mild</td>
</tr>
<tr>
<td>2 = Some airways have significant inflammation - Moderate</td>
</tr>
<tr>
<td>3 = Majority of airways have some inflammation - Marked</td>
</tr>
<tr>
<td>4 = Majority of airways are significantly inflamed – Severe</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score 2: Vascular inflammation Score (/4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = Lack of inflammatory cells around vessels – Absent</td>
</tr>
<tr>
<td>1 = Some vessels have small numbers of cells - Mild</td>
</tr>
<tr>
<td>2 = Some vessels have significant inflammation - Moderate</td>
</tr>
<tr>
<td>3 = Majority of vessels have some inflammation - Marked</td>
</tr>
<tr>
<td>4 = Majority of vessels are significantly inflamed – Severe</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score 3: Parenchymal inflammation (at 10X magnification) Score (/5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 = &lt;1% affected</td>
</tr>
<tr>
<td>1 = 1-9% affected</td>
</tr>
<tr>
<td>2 = 10-29% affected</td>
</tr>
<tr>
<td>3 = 30-49% affected</td>
</tr>
<tr>
<td>4 = 50-69% affected</td>
</tr>
<tr>
<td>5 = &gt;70% affected</td>
</tr>
</tbody>
</table>

**Total score = Score 1 + Score 2 + Score 3 = /13**
2.13 Cytokine concentrations in BALF

Protein concentrations in BALF supernatants were determined for IFN-γ, IFN-λ3, TNF-α (eBioscience, CA, USA), KC, IL-5, IL-6, and IL-13 (Biolegend, CA, USA) using mouse ELISA kits; and IP-10, IL-10, and MIP-1α using a FlowCytomix assay kit (eBioscience), according to the manufacturers’ instructions (276, 291-293).

2.14 Immunoblotting

Protein expression of RIG-I and IFN-β were assessed by immunoblotting. Lung tissues were submerged into 1 ml of radio-immunoprecipitation assay (RIPA) buffer (Sigma Aldrich) in a 5 ml tube and were homogenised at 4 °C. The resulting homogenates were incubated for 5 minutes at 4 °C. The homogenates were then transferred into 1.5 ml microcentrifuge tubes and centrifuged at 8000 xg for 10 minutes at 4 °C. The supernatants were collected, sonicated, and re-centrifuged at 8000 xg for 10 minutes at 4 °C. The supernatants containing protein fractions were collected. The amount of proteins in each sample was determined using BCA Protein Assay Kit (Thermo Fisher Scientific) according to manufacturer’s instructions. Samples containing proteins (20-40 μg) were mixed with an equal volume of 2X sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (125mM Tris.Cl pH 6.8, 4.1% (w/v) SDS, 0.001% (w/v) bromophenol blue, 20% (v/v) glycerol and 300mM β-mercaptoethanol). These samples were then boiled at 95 °C for 5 minutes to reduce the proteins in the samples and loaded into the wells of electrophoresis gel.
Gels were electrophoresed in SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS) at 180V for 60 minutes. The gels were then equilibrated in Towbin transfer buffer (192 mM Glycine, 25 mM Tris at pH 8.3, 20% (v/v) Methanol) for approximately 30 seconds. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Hybond, CA, USA) at 30 V for 60 minutes while sandwiched between Whatman filter papers (Whatman, Mount Eliza, Australia) and sponges, which were pre-soaked with Towbin transfer buffer. The PVDF membranes were probed with primary antibodies specific to RIG-I or IFN-β and incubated overnight at 4 °C on an orbital shaker. The PVDF membranes were washed three times for approximately 10 minutes each time with TBS-T (120 mM NaCl, 10 mM Tris at pH 8.0, 0.05% (v/v) Tween 20). The PVDF membranes were then incubated with peroxidase-labelled secondary antibodies in TBS-T for 1 hour at room temperature. The membrane was visualised by chemiluminescence assay (Bio-Rad ChemiDoc MP System, Bio-Rad, Regents Park, Australia). The densitometries on the blots were analysed using Image J software (National Institutes of Health, USA).

2.15 Total RNA extraction

Total RNA was extracted from whole lung by guanidinium thiocyanate phenol chloroform (TRIzol) extraction (294). Lung tissues harvested from mice were stored in RNA Stabilisation Reagent, RNAlater (Qiagen, Chadstone Centre, Australia) prior to extraction. Tissues were transferred into 5 ml tubes containing 1 ml of TRIzol solution (Ambion, Thermo Fisher Scientific), carefully ensuring minimal RNAlater carry over. Tissues were then homogenised at 4 °C, transferred to 1.5 ml microcentrifuge tubes and centrifuged at 12 000 xg for 10 minutes at 4 °C. The clear homogenates were then
transferred into fresh microcentrifuge tubes and supplemented with 250 µl chloroform to separate RNAs from proteins. The microcentrifuge tubes were then pulse-vortexed for approximately 5 seconds, until the solutions were homogenous. The homogenous solutions were incubated at room temperature for 10 minutes before being centrifuged at 12 000 xg for 15 minutes at 4 °C.

The resulting aqueous phase was then transferred into fresh 1.5 ml microcentrifuge tubes and supplemented with 500 µl of cold isopropyl alcohol to precipitate the RNA. The solutions were again pulse-vortexed, incubated at room temperature for 10 minutes, and centrifuged at 12 000 xg for 10 minutes at 4 °C. Supernatants were discarded and the RNA pellets were washed with 1 ml of 75% (v/v) ethanol. The solutions were then pulse-vortexed to dislodge the pellets and to wash out contaminants that were trapped underneath. The solutions were centrifuged at 7500 xg for 5 minutes at 4 °C. A second wash was performed to remove any carbohydrate/phenol-based contaminants. Pellets were then allowed to air dry for 15 minutes at 4 °C. Pellets were eventually resuspended with 75-100 µl nuclease free water (Ambion, Thermo Fisher Scientific). The concentration of mRNA was measured using a NanoDrop Spectrophotometer (ND-1000, v3.8.0 Bio Lab, NanoDrop Technologies, DE, USA).

2.16 Reverse transcription

1000 ng of RNA in 8 µl of nuclease free water (Ambion) was prepared to generate complementary DNA (cDNA). mRNA samples were mixed with 1 µl of 10X reaction buffer (Bioline, Alexandria, Australia) and 1 µl of amplification grade DNAse I
(Sigma Aldrich) and were incubated for 15 minutes at room temperature. 1 µl of DNase Stop Solution (Sigma Aldrich) was added into the mixture, and the samples were heated at 65 °C for 10 minutes to inactivate and denature the DNAse I. The samples were mixed with 2 µl of 50 ng/ml random hexamer primers (Invitrogen) and 1 µl of 2.5mM dNTPs (Invitrogen), and incubated at 65 °C for 5 minutes. The samples were allowed to cool to 25 °C, before 4 µl of 5X reaction buffer, 1 µl of DTT (Bioline), 1 µl of nuclease free water, and 1 µl of Bioscript (Bioline) was added. The samples were then heated at 25 °C for 20 minutes, 42 °C for 50 minutes and 70 °C for 15 minutes. The samples were finally resuspended with 500 µl of nuclease free water.

2.14 Quantitative real-time Polymerase Chain Reaction (qPCR)

qPCR was performed to determine the relative abundance of cDNA of specific genes in samples in comparison with the reference gene HPRT. The cycles were done using Mastercycler Eppendorf RealPlex 2 System (Eppendorf South Pacific, North Ryde, Australia) or ViIA 7 Real-Time PCR System (Life Technologies, Thermo Fisher Scientific). 2 µl of sample cDNA was added to a mixture containing 3 µl SYBR Green and ROX as a passive reference dye (SYBR Green ERTM reagent system, Invitrogen), 0.5 µl (10 µM) of each forward and reverse primers (Integrated DNA Technologies, Baulkham Hills, Australia) and 4 µl of nuclease free water (Ambion), to make a total of 10 µl reaction volume. Primers that were used were HPRT (Forward: 5' - AGG CCA GAC TTT GTT GGA TTT GAA - 3'; Reverse: 5' - CAA CTT GCG CTC ATC TTA GGC TTT - 3'), RIG-I (Ddx58) (Forward: 5' – ACA AAC CAC AAC CTG TTC CTG
ACA - 3'; Reverse: 5' – TGG CGC AGA ATA TCT TTG CTT TCT - 3'), and IL-13Rα1
(Forward: 5' - CAC AGT CAG AGT AAG GTG GA - 3'; Reverse: 5' - ATG GTG TAG AAG GTG GA - 3').

Cycling conditions used for Mastercycler Eppendorf RealPlex 2 System were:
holding stages at 50 °C for 2 minutes and 95 °C for 2 minutes; cycling stages at 95 °C for 15 seconds and 60 °C (variable) for 30 seconds (cycling stages were repeated for 40 cycles); melt curve/dissociation stages at 95 °C for 15 seconds, 60 °C (variable) for 15 seconds, 95 °C for 8 minutes and 95 °C for 15 seconds. Cycling conditions used for ViiA 7 Real-Time PCR System were: holding stage at 95 °C for 30 seconds; cycling stages at 95 °C for 15 seconds and 60 °C (variable) for 30 seconds (cycling stages were repeated for 40 cycles); melt curve/dissociation stages at 95 °C for 15 seconds, 60 °C (variable) for 15 seconds and 95 °C for 15 seconds. The threshold value (Ct value) for each sample was measured as the number of cycles needed for the specific fluorescent signals to cross “threshold”, which is a value that is set above the background levels of fluorescence (background “noise”). The Ct value from each gene was normalised against the constant housekeeping gene: the HPRT gene.

2.15 Statistical analyses

Data were expressed as mean ± standard error of mean (SEM) (n≥6) from two or more independent experiments when normally distributed. Non-normally distributed data were analysed using non-parametric equivalents and summarised using the median and inter-quartile range. Comparisons between two groups were made using a two-tailed Mann-Whitney Test. Multiple comparisons were made using one-way ANOVA with Tukey’s post-test, or Kruskal-Wallis with Dunn’s post-test, where non-parametric
analyses were appropriate. Analyses were performed using GraphPad Prism Software version 6 (GraphPad Software, CA, USA). A p-value of < 0.05 was considered significant. To provide clarity of the presentation of the figures, we only display statistical comparisons between a group and its relevant controls. For example, in figures containing Control, Smk, Vir and Smk+Vir groups; we compare Smk+Vir with its controls (Smk and Vir), and Smk and Vir with their control (Control).

2.16 Study approvals

All animals and the protocols used in the experiments were approved and performed in accordance with guidelines by the Animal Care and Ethics Committee, The University of Newcastle.
CHAPTER 3: RESULTS

3.1 Influenza virus infection in COPD

3.1.1 Experimental COPD predisposes to more severe influenza virus infection

To investigate whether experimental COPD increases susceptibility to influenza virus infection, we exposed BALB/c mice to cigarette smoke for eight weeks. On the last day of smoke exposure, we infected the mice i.n. with 7.5 pfu of mouse-adapted H1N1 influenza A/PR/8/34 strain (Smk+Vir). Mice were sacrificed at 3, 7 and 10 dpi (Figure 10A). Controls were sham-inoculated (with media) and smoke-exposed (Smk), or were infected (Vir) or sham-inoculated (Control) and exposed to normal air. Viral titre and changes in weight gain were assessed.

Infected normal air-exposed mice (Vir) had a productive infection, characterised by detectable virus in the lungs at 3 dpi, which peaked at 7 dpi and resolved by 10 dpi (Figure 10B). No significant change in viral titre was observed in infected smoke-exposed mice (Smk+Vir) to that of infected normal air-exposed mice at 3 dpi. However, at 7 dpi, viral titre was significantly increased in infected smoke-exposed mice compared to infected normal air-exposed controls (Figure 10B).

In the absence of influenza infection, non-infected smoke-exposed (Smk) mice had reduced weight gain compared to non-infected normal air-exposed (Control) controls (Figure 10C). Similarly, influenza virus infection led to reduced weight gain from 4 dpi in infected normal air-exposed mice compared to non-infected normal air-
exposed controls. Cigarette smoke exposure prior to infection did not result in further reductions in weight gain in infected smoke-exposed mice compared to infected normal air-exposed controls.

Collectively, these observations indicate that influenza virus infection in experimental COPD leads to increased viral titre.
Figure 10. Mice with experimental COPD are predisposed to more severe influenza virus infection.

(A) BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Control) for eight weeks, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Smk+Vir) or media (Control, Smk) on the last day of smoke exposure and sacrificed at 3, 7 and 10
dpi. (B) Viral titres in bronchoalveolar lavage fluid (BALF). (C) Body weight. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * \( P \leq 0.05 \) versus Control, Smk and Vir control groups, respectively.
3.1.2 Experimental COPD predisposes to exaggerated airway inflammation following influenza virus infection

Next, we examined the impact of cigarette smoke-induced experimental COPD on the influx of inflammatory cells into the airways during influenza virus infection. This was assessed through quantification of the influx of leukocytes, macrophages, lymphocytes, neutrophils and eosinophils in BALF.

Non-infected smoke-exposed mice (Smk) had increased numbers of total leukocytes 3 days after the last smoking exposure (the same time point as 3 dpi for other infected groups), compared to non-infected normal air-exposed controls (Control, Figure 11A). This consisted of increased numbers of macrophages, lymphocytes and neutrophils compared to non-infected normal air-exposed controls (Figure 11B-D). Similarly, at 7 dpi, non-infected smoke-exposed mice also had increased numbers of total leukocytes, macrophages, lymphocytes, and neutrophils compared to normal air-exposed controls (Figure 11A-D). By 10 dpi, influx of all inflammatory cells had returned to baseline levels (Figure 11A-D).

During influenza virus infection, at 3, 7 and 10 dpi, infected normal air-exposed mice (Vir) had increased numbers of total leukocytes, macrophages and neutrophils, but not lymphocytes, compared to non-infected normal air-exposed controls (Figure 11A-D).

Infected smoke-exposed mice (Smk+Vir) had no changes in the numbers of leukocytes, macrophages and lymphocytes, but had reduced numbers of neutrophils, compared to infected normal air-exposed controls at 3 dpi (Vir, Figure 11A-D).
However, at 7 dpi, infected smoke-exposed mice had increased numbers of total leukocytes, macrophages and lymphocytes, but reduced numbers of neutrophils, compared to infected normal air-exposed controls (Figure 11A-D). At 10 dpi, infected smoke-exposed mice had increased numbers of total leukocytes, macrophages and lymphocytes, but reduced number of neutrophils, compared to infected normal air-exposed controls (Figure 11A-D). Eosinophils were not detected in any samples.

Taken together, the results show that cigarette smoke-induced COPD predisposes to exaggerated airway inflammation during influenza virus infection.
Figure 11. Experimental COPD predisposes to exaggerated airway inflammation during influenza virus infection.

BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Control) for eight weeks, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Smk+Vir) or media (Control, Smk) on the last day of smoke exposure. Inflammatory cells in BALF were assessed at 3, 7 and 10 dpi. Numbers of (A) total leukocytes, (B) macrophages, (C) lymphocytes, and (D) neutrophils. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P ≤ 0.05 versus Control, Smk and Vir control groups, respectively.
Influenza virus infection in experimental COPD alters lung function.

Influenza virus infection in COPD patients is associated with reduced lung function (295). Therefore, we investigated whether infection with influenza virus in experimental COPD alters lung function. We also examined whether the alterations are associated with changes in emphysema-like alveolar enlargement.

Non-infected smoke-exposed mice (Smk) had reduced transpulmonary resistance and increased dynamic compliance, but no changes in tissue damping or total lung capacity, compared to non-infected normal air-exposed controls (Control), at 3 and 7 days following the last smoke exposure (Figure 12A-H). Infected normal air-exposed mice (Vir) had no alterations in transpulmonary resistance, dynamic compliance, tissue damping or total lung capacity compared to non-infected normal air-exposed controls, at 3 dpi (Figure 12A-D). At 7 dpi, infected normal air-exposed mice had reduced transpulmonary resistance, but no changes in dynamic compliance, tissue damping or total lung capacity, compared to non-infected normal air-exposed controls (Figure 12E-H). Infected smoke-exposed mice (Smk+Vir) had no changes in transpulmonary resistance, dynamic compliance, tissue damping or total lung capacity compared to infected normal air-exposed group at 3 dpi (Figure 12A-D).

However, at the peak of infection (7 dpi), infected smoke-exposed mice had further impairment of lung function, characterised by increased in transpulmonary resistance, tissue damping and total lung capacity, as well as decreased dynamic compliance compared to infected normal air-exposed controls (Figure 12E-H).
The impairment of lung function at 7 dpi was not directly associated with changes in alveolar diameter as determined by mean linear intercept and destructive index. Non-infected smoke-exposed mice had increased alveolar diameter compared to non-infected normal air-exposed controls (Figure 13A-B). Infected smoke-exposed mice had increased alveolar diameter compared to infected normal air-exposed group. However, this increase was similar to that in non-infected smoke-exposed mice.

Overall, these results indicate that influenza virus infection in experimental COPD leads to further impairment that is not associated with infection-induced changes in alveolar diameter.
Figure 12. Influenza virus infection in experimental COPD leads to further impairment of lung function.
BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Control) for eight weeks, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Smk+Vir) or media (Control, Smk) on the last day of smoke exposure. Lung function was assessed at 3 dpi (A) transpulmonary resistance, (B) dynamic compliance, (C) tissue damping, (D) total lung capacity; and at 7 dpi (E) transpulmonary resistance, (F) dynamic compliance, (G) tissue damping, (H) total lung capacity. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Smk and Vir control groups, respectively.
Figure 13. Influenza virus infection in experimental COPD had no effect on emphysema-like alveolar enlargement.

BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Control) for eight weeks, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Smk+Vir) or media.
(Control, Smk) on the last day of smoke exposure. Mean alveolar diameter was assessed at 7 dpi. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * $P \leq 0.05$ versus Control, Smk and Vir control groups, respectively.
3.1.4 Antiviral IFN responses to influenza virus infection are impaired in experimental COPD

We then examined whether the levels of antiviral responses to influenza virus infection were reduced in experimental COPD, which may contribute to increased viral titre. At 3 dpi, infected normal air-exposed mice (Vir) had elevated levels of RIG-I, IFN-β and IFN-λ3 proteins compared to non-infected normal air-exposed controls (Control, Figure 14A-C). However, these levels were reduced in infected smoke-exposed mice (Smk+Vir) compared to infected normal air-exposed controls.

At 7 dpi, the level of IFN-β and IFN-λ3, but not RIG-I, was increased in infected normal air-exposed mice compared to non-infected normal air-exposed controls (Figure 14D-F). IFN-β, but not RIG-I and IFN-λ3, were again reduced in infected smoke-exposed mice compared to infected normal air-exposed controls.

These results suggest that experimental COPD leads to reduced innate antiviral responses, particularly IFN-β, during influenza virus infection. This reduction is associated with increased viral titre during the peak of infection (Figure 10B).
Figure 14. Mice with experimental COPD have impaired antiviral IFN responses during influenza virus infection.

BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Control) for eight weeks, and inoculated with influenza virus (Vir, Smk+Vir) or media (Control, Smk) on the last day of smoke exposure. Antiviral responses were assessed at 3 dpi (A) RIG-I (B) IFN-β (C) IFN-λ3, and at 7 dpi (D) RIG-I (E) IFN-β (F) IFN-λ3. RIG-I, IFN-β were assessed in lung homogenates, and IFN-λ3 in BALF. Densitometry results were calculated as RIG-I/β-actin and IFN-β/β-actin ratios, and expressed as fold change from Control. Data are presented as mean ± SEM (n≥6) and are representative of two or more
independent experiments. #, + and * \( P \leq 0.05 \) versus Control, Smk and Vir control groups, respectively.
Inflammatory cytokine production is increased in the lung during influenza virus infection in experimental COPD

Given that the increased viral titre and altered lung function were only observed at 7 dpi, we selected this time point to assess cytokine and chemokine responses. Infected normal air-exposed mice (Vir) had increased levels of IFN-γ, TNF-α, IP-10, MIP-1α, KC and IL-10, but not IL-6 compared to non-infected normal air-exposed controls (Control, Figure 15A-G). In contrast, infected smoke-exposed mice (Smk+Vir) did not mount the same level of cytokine or chemokine responses. Infected smoke-exposed mice generated decreased levels of IFN-γ, TNF-α, IP-10, MIP-1α, KC and IL-10, and increased in IL-6 compared to infected normal air-exposed controls (Figure 15A-G).

Collectively these results show that cigarette smoke-induced COPD leads to a reduction in appropriate cytokine and chemokine responses to influenza virus infection. Furthermore, anti-inflammatory IL-10 levels were suppressed and pro-inflammatory IL-6 responses were elevated, which are potentially involved in promoting inflammation.
Figure 15. Experimental COPD leads to impaired antiviral cytokine and chemokine responses but increased pro-inflammatory cytokine responses during influenza virus infection.

BALB/c mice were exposed to cigarette smoke (Smk) or normal air (Control) for eight weeks, and inoculated with influenza virus (Vir, Smk+Vir) or media (Control, Smk) on the last day of smoke exposure. Cytokine levels were assessed in BALF at 7dpi. (A) IFN-γ, (B) TNF-α, (C) IP-10, (D) MIP-1α, (E) KC, (F) IL-10, and (G) IL-6. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Smk and Vir control groups, respectively.
3.2 Experimental COPD increases PI3K activity in the lung and promotes more severe influenza infection and exacerbation of COPD

3.2.1 PI3K activity is increased during influenza virus infection in experimental COPD, and its inhibition enhances antiviral responses and decreased viral titre

PI3K has been shown to be important for influenza viral entry into cells, and can be further activated by the virus during infection (147). Thus, we investigated the role of PI3K as a potential mechanism of increased influenza virus infection in experimental COPD. We determined whether the inhibition of PI3K affected antiviral and cytokine responses, as well as the level of viral titre in experimental COPD model during influenza virus infection. To achieve this, the pan-PI3K inhibitor LY294002 was administered for the final two weeks of smoke exposure and for the duration of the infection (Figure 16A). The effects of PI3K inhibition on antiviral and cytokine responses, as well as viral titre, were assessed at 7 dpi.

Inhibition of PI3K resulted in reduced pAkt levels in both infected normal air-exposed (Vir) and infected smoke-exposed (Smk+Vir) groups, indicating that the activation of the PI3K signalling pathway was reduced (Figure 16B). In infected normal air-exposed mice, inhibition of PI3K did not alter the level of IFN-β. However, in infected smoke-exposed mice, inhibition of PI3K led to increased IFN-β. RIG-I and IFN-λ were not assessed as their levels were not reduced at this time point (7 dpi) (Figure 14 D and F).
In infected normal air-exposed mice, inhibition of PI3K resulted in reduced IFN-γ and TNF-α, increased IP-10, MIP-1α and IL-10, but not KC and IL-6 \((\text{Figure 16C-I})\). In contrast, in infected smoke-exposed mice, inhibition of PI3K led to increased IFN-γ, IP-10 and MIP-1α and IL-10, reduced IL-6, but not TNF-α or KC. Thus, following the inhibition of PI3K in infected smoke-exposed mice, the pattern of cytokine responses to influenza was largely returned to those observed in infected normal air-exposed controls.

In infected normal air-exposed mice, inhibition of PI3K did not lead to changes in viral titre \((\text{Figure 16J})\). However, inhibition of PI3K in infected smoke-exposed mice resulted in reduced viral titre.

Taken together, inhibition of PI3K in experimental COPD improves antiviral and cytokine responses to influenza virus infection. This improvement was associated with reduced viral titre.
Figure 16. PI3K activity is increased during influenza virus infection in experimental COPD, and its inhibition enhances antiviral responses and suppresses viral titres.

(A) BALB/c mice were exposed to cigarette smoke or normal air for eight weeks and infected with influenza virus (Vir, Smk+Vir) on the last day of smoke exposure. Mice were treated with LY294002 or vehicle three times per week for the last two weeks of smoke exposure and throughout the infection. PI3K activation, antiviral responses and infection were assessed at 7dpi. (B) pAkt (S473) and IFN-β expression in lung homogenates. (C) IFN-γ, (D) TNF-α, (E) IP-10, (F) MIP-1α, (G) KC, (H) IL-10, (I) IL-6 and (J) Viral titres in BALF. Densitometry results were calculated as pAkt/β-actin and IFN-β/β-actin ratios, respectively, and are expressed as fold change from vehicle-treated Vir groups. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Smk and Vir control groups, respectively.
3.2.2 Inhibition of PI3K during influenza virus infection in experimental COPD enhances antiviral inflammatory cell responses and improves lung function

Given our observations that the inhibition of PI3K led to improved immune responses and reduced viral titre in experimental COPD during influenza infection, we examined whether the same treatment also improves the airway inflammatory cell responses and lung function.

In infected normal air-exposed mice (Vir), inhibition of PI3K did not alter the numbers of total leukocytes, macrophages, lymphocytes or neutrophils (Figure 17A-D). Interestingly, in infected smoke-exposed mice (Smk+Vir), inhibition of PI3K resulted in increased numbers of total leukocytes and neutrophils, but not macrophages or lymphocytes.

In infected normal air-exposed mice, PI3K inhibition did not affect transpulmonary resistance, but did reduce total lung capacity (Figure 17E and F). In contrast, in infected smoke-exposed mice, inhibition of PI3K decreased both transpulmonary resistance and total lung capacity, which indicates an improvement in lung function.

These data, taken together with previous observations, suggest that influenza virus infection in COPD, increased the activation of PI3K and this is associated with impaired antiviral and cytokine responses, lung function, and increased viral infection. Furthermore, they indicate that suppression of PI3K partially, but significantly, reverses these alterations and thus improve infection outcomes.
Figure 17. Inhibition of PI3K in experimental COPD leads to increased inflammatory cell responses and improved lung function during influenza infection.
BALB/c mice were exposed to cigarette smoke or normal air for eight weeks and infected with influenza virus (Vir, Smk+Vir) on the last day of smoke exposure. Mice were treated with LY294002 or vehicle three times per week for the last two weeks of smoke exposure and throughout the infection. Numbers of inflammatory cells in BALF and lung function were assessed at 7dpi. (A) Leukocytes. (B) Macrophages. (C) Lymphocytes. (D) Neutrophils. (E) Transpulmonary resistance. (F) Total lung capacity.

Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Smk and Vir control groups, respectively.
3.3 Influenza infection and IL-13

3.3.1 Administration of rIL-13 to naïve mice promotes more severe influenza virus infection

To explore the potential role of IL-13 during influenza virus infection, we investigated whether infection in a background of increased pulmonary IL-13 is more severe. To achieve this, mice were administered with rIL-13 intranasally from the day before influenza virus infection (-1 dpi) through to 2 dpi. As in previous experiments, we infected the mice intranasally with 7.5 pfu of mouse-adapted H1N1 influenza A/PR/8/34 strain (rIL13+Vir). Mice were then sacrificed at 3 dpi (Figure 18A). Controls were sham-inoculated (with media) and rIL-13-treated (rIL13), infected and PBS-treated (Vir), or sham-inoculated and PBS-treated (Control).

To examine the effects rIL-13 on influenza virus infection, we first measured viral titre in the BALF. Influenza virus was detected in both infected PBS-treated and infected rIL-13-treated mice (Figure 18B). Infected rIL-13-treated mice had increased viral titre compared to infected PBS-treated controls. This demonstrates that IL-13 promotes more severe influenza virus infection.
Figure 18. Administration of rIL-13 to naïve mice promotes more severe influenza virus infection.

(A) BALB/c mice were administered with rIL-13 (rIL13) or PBS (Control), and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, rIL13+Vir) or media (Control, rIL13). Mice were sacrificed at 3 dpi. (B) Viral titres in BALF. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, rIL13 and Vir control groups, respectively.
3.3.2 Administration of rIL-13 impairs antiviral responses to influenza virus infection

Next, we determined whether the increase in viral titre following rIL-13 administration was due to altered antiviral responses. Infected PBS-treated mice (Vir), but non-infected rIL-13-treated (rIL13) had increased levels of RIG-I, IFN-β, IFN-γ and IFN-λ3, compared to non-infected PBS-treated controls (Control, Figure 19A-D). In infected rIL-13-treated mice (rIL13+Vir), the levels of both IFN-β and IFN-λ3 protein were reduced compared to infected PBS-treated mice. There was no change in RIG-I mRNA or IFN-γ protein, compared to infected PBS-treated controls.

Taken together, these results indicate that IL-13 impairs antiviral type I and III IFN responses to influenza virus infection.
Figure 19. Administration of rIL-13 impairs antiviral responses to influenza virus infection.

BALB/c mice were administered with rIL-13 (rIL13) or PBS (Control), and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, rIL13+Vir) or media (Control, rIL13). Mice were sacrificed and antiviral responses were assessed at 3 dpi. (A) RIG-I mRNA expression. (B) IFN-β protein expression densitometry, calculated as IFN-β/β-actin ratios, and expressed as fold change from Control. (C) IFN-γ and (D) IFN-λ3 protein expression assessed by ELISA in BALF. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, rIL13 and Vir control groups, respectively.
3.3.3 Administration of rIL-13 increases airway inflammatory cell responses to influenza virus infection.

To examine the effects of rIL-13 on the influx of inflammatory cells into the airways following influenza virus infection, we quantified the number of leukocytes including macrophages, lymphocytes, neutrophils and eosinophils in BALF.

In non-infected rIL-13-treated mice (rIL13), there were no differences in the numbers of total leukocytes, macrophages, lymphocytes and neutrophils compared to non-infected PBS-treated controls (Control, Figure 20A-D). However, we did observe an increase in the number of eosinophils in non-infected rIL-13-treated mice (Figure 20E). In infected PBS-treated mice (Vir), there was an increase in the number of total leukocytes, lymphocytes and neutrophils, but not macrophages or eosinophils, compared to non-infected PBS-treated controls (Figure 20A-D).

In infected rIL-13-treated mice (rIL13+Vir), there was an increase in the number of total leukocytes, macrophages, lymphocytes and neutrophils, but not eosinophils, compared to non-infected rIL-13-treated mice. There were also increases in the number of total leukocytes, macrophages, neutrophils, and eosinophils, but not lymphocytes, compared to infected PBS-treated controls.

Taken together, these results indicate that IL-13 promotes increased airway inflammatory cell influx during influenza virus infection.
Figure 20. Administration of rIL-13 increases airway inflammatory cell influx in response to influenza virus infection.

BALB/c mice were administered with rIL-13 (rIL13) or PBS (Control), and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, rIL13+Vir) or media (Control, rIL13). Mice were sacrificed at 3 dpi and inflammatory cells in BALF were assessed. Numbers of (A) leukocytes, (B) macrophages, (C) lymphocytes, (D) neutrophils, and (E) eosinophils. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, rIL13 and Vir control groups, respectively.
3.3.4 Administration of rIL-13 promotes increased AHR during influenza virus infection

We next determined whether the presence of elevated IL-13 increased influenza virus-induced AHR.

Infected PBS-treated (Vir), but not non-infected rIL-13-treated (rIL13) mice, had increased transpulmonary resistance and decreased dynamic compliance, compared to non-infected PBS-treated controls (Control, Figure 2A-D). Infected rIL-13-treated group (rIL13+Vir) had further increased transpulmonary resistance and decreased dynamic compliance, compared to infected PBS-treated controls.

These results indicate that IL-13 promotes increased AHR during influenza virus infection.
Figure 21. Administration of rIL-13 promotes increased AHR during influenza virus infection.

BALB/c mice were administered with rIL-13 (rIL13) or PBS (Control), and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, rIL13+Vir) or media (Control, rIL13). Mice were sacrificed and AHR were assessed at 3 dpi. (A) Transpulmonary resistance (% change from saline). (B) Transpulmonary resistance (unmanipulated). (C) Dynamic compliance (% change from saline). (D) Dynamic compliance (unmanipulated). Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, rIL13 and Vir control groups, respectively.
3.3.5 Administration of rIL-13 leads to more severe histopathology and increased MSCs following influenza infection

Next we assessed the effects of rIL-13 administration on influenza virus infection-induced pulmonary histopathology and MSC numbers.

Both non-infected rIL-13-treated (rIL13) and infected PBS-treated (Vir) mice had higher histopathological scores, compared to non-infected PBS-treated controls (Control, Figure 22). Significantly, the combination of rIL-13 and influenza virus infection (rIL13+Vir) led to a more severe histopathology compared to infected PBS-treated controls.

There was an increase in the number of MSCs in the airways of non-infected rIL-13-treated mice but not in infected PBS-treated mice, compared to non-infected PBS-treated controls (Figure 23). Infected rIL-13-treated mice had increased MSCs compared to infected PBS-treated mice, but numbers were lower compared to non-infected rIL-13-treated mice.

These results indicate that an environment with elevated levels of IL-13 leads to increased histopathology and MSC numbers during influenza virus infection.
Figure 22. Administration of rIL-13 leads to more severe histopathology during influenza virus infection.

BALB/c mice were administered with rIL-13 (rIL13) or PBS (Control), and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, rIL13+Vir) or media (Control, rIL13). Mice were sacrificed at 3 dpi. Histopathological score of lung tissue sections was
assessed. Data are presented as mean ± SEM (n≥6) and are representative of two or
more independent experiments. #, + and *P≤0.05 versus Control, rIL13 and Vir control
groups, respectively.
**Figure 23.** Administration of rIL-13 leads to increased MSCs during influenza virus infection.

BALB/c mice were administered with rIL-13 (rIL13) or PBS (Control), and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, rIL13+Vir) or media (Control, rIL13). Mice were sacrificed at 3 dpi. Number of mucus secreting cells per 100 µm of basal membrane (BM) was assessed. Data are presented as mean ± SEM (n≥6) and are
representative of two or more independent experiments. $^\#$, + and $^*P\leq0.05$ versus Control, rIL13 and Vir control groups, respectively.
3.3.6 Administration of rIL-13 leads to increased levels of IL-13 and IL-13Rα1 during influenza virus infection

Next, we examined the level of IL-13 following the administration of rIL-13 in mice during influenza virus infection. In non-infected rIL-13-treated mice (rIL13), IL-13 was increased in the lung compared to non-infected PBS-treated controls (Control, Figure 18A). Moreover, influenza virus infection in the presence of rIL-13 (rIL13+Vir) further increased the level of IL-13 in the lung compared to infected PBS-treated controls.

We also measured the effect of rIL-13 on the levels of the IL-13 receptor IL-13Rα1 and the Th2-associated cytokine IL-5 in the lung. In infected PBS-treated mice, but not non-infected rIL-13-treated mice, IL-13Rα1 mRNA was increased compared to non-infected PBS-treated controls (Figure 18B). In infected rIL-13-treated mice, IL-13Rα1 mRNA was increased compared to infected PBS-treated controls.

These results suggest that IL-13 feeds back to increase IL-13 responses, by increasing both IL-13 and IL-13Rα1.
Figure 24. Administration of rIL-13 leads to increased levels of IL-13 and IL-13Rα1 during influenza virus infection.

BALB/c mice were administered with rIL-13 (rIL13) or PBS (Control), and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, rIL13+Vir) or media (Control, rIL13). Mice were sacrificed at 3 dpi. (A) IL-13 protein and (B) IL-13Rα1 mRNA were assessed in BALF. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, rIL13 and Vir control groups, respectively.
3.4 Influenza virus infection in AAD

3.4.1 AAD promotes more severe influenza virus infection

To investigate whether AAD predisposes to more severe influenza virus infection, specific pathogen-free BALB/c mice were sensitised i.p. to Ova, and subsequently challenged i.n. with Ova 12-15 days after sensitisation. On the last day of Ova challenge, mice were infected i.n. with 7.5 pfu of mouse-adapted H1N1 influenza A/PR/8/34 strain (Ova+Vir). Controls were sham-inoculated (with media) and Ova-sensitised (Ova), or infected and PBS-sensitised (Vir), or sham-inoculated and PBS-sensitised (Control). Mice were sacrificed at 3, 7 and 10 dpi (Figure 25A).

In infected non-allergic mice (Vir), influenza virus was detected in the lungs at 3 dpi, viral titre peaked at 7 dpi and infection resolved by 10 dpi (Figure 25B). A significant increase in viral titre was observed in infected allergic mice (Ova+Vir) at 3 and 7 dpi compared to infected non-allergic controls.

In the absence of infection, no difference in weight loss was observed between non-infected non-allergic (Control) and non-infected allergic (Ova) mice (Figure 25C). Infected non-allergic mice had increased weight loss starting from 3 dpi compared to non-infected non-allergic controls. Similarly, infected allergic mice had increased weight loss from 3 dpi compared to non-infected allergic controls. However, no difference in weight loss was observed between infected allergic mice and infected non-allergic controls.

Collectively, these results indicate that AAD leads to increased viral titre in the lung during influenza virus infection. Although weight loss is not affected by AAD,
influenza virus infection is associated with marked increase in weight loss in all infected groups.
Figure 25. AAD promotes more severe influenza virus infection.

(A) BALB/c mice were sensitised with Ova (Ova) or PBS (Control), challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Ova+Vir) or media.
(Control, Ova) on the last day of Ova challenge. Mice were sacrificed at 3, 7 and 10 dpi.

(B) Viral titres in BALF. (C) Body weight. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Ova and Vir control groups, respectively.
3.4.2 AAD impairs antiviral responses to influenza virus infection

To investigate the factors that contribute to increased viral titre, we next examined whether AAD impairs antiviral responses following influenza virus infection at 3 and 7 dpi.

At both 3 and 7 dpi, infected non-allergic (Vir), but not non-infected allergic (Ova) mice, had increased expression of RIG-I mRNA and levels of, IFN-β, IFN-γ and IFN-λ3 proteins, compared to non-infected non-allergic controls (Control, Figure 26A-D). At 3 dpi, in infected allergic mice (Ova+Vir), RIG-I mRNA expression and IFN-γ protein levels were unchanged, but the levels of IFN-β and IFN-λ3 proteins were reduced compared to infected non-allergic controls. At 7 dpi, in infected allergic mice, IFN-γ was reduced, but there was no change in RIG-I, IFN-β, and IFN-λ3.

Taken together, these results indicate that increased influenza virus infection in AAD is associated with impaired early antiviral responses.
**Figure 26. AAD impairs antiviral IFN responses to influenza virus infection.**

BALB/c mice were sensitised with Ova (Ova) or PBS (Control), challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Ova+Vir) or media (Control, Ova) on the last day of Ova challenge. Antiviral factors were assessed at 3 and 7 dpi. (A) RIG-I mRNA expression. (B) IFN-β protein expression densitometry, calculated as IFN-β/β-actin ratios, and expressed as fold change from Control. (C) IFN-γ and (D) IFN-λ3 proteins expression were assessed by ELISA in BALF. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Ova and Vir control groups, respectively.
3.4.3 Influenza virus infection leads to exaggerated airway inflammatory cell influx in AAD

To investigate the effects of AAD on the influx of inflammatory cells into the airways following influenza virus infection, we quantified the number of total leukocytes, macrophages, lymphocytes, neutrophils and eosinophils in BALF.

At 3 dpi, in non-infected allergic mice (Ova), there was an increase in the numbers of total leukocytes, macrophages and eosinophils, but not lymphocytes or neutrophils, compared to non-infected non-allergic controls (Control, Figure 27A-E).

At 7 dpi there was no change in the numbers of all cell types compared to non-infected non-allergic controls. At both 3 and 7 dpi, infected non-allergic mice (Vir) had increased numbers of total leukocytes, macrophages, lymphocytes and neutrophils, but not eosinophils, compared to non-infected non-allergic controls.

At 3 dpi, infected allergic mice (Ova+Vir) had exaggerated airway inflammation, indicated by increased numbers of total leukocytes, macrophages and eosinophils, but not neutrophils, and reduced numbers of lymphocytes compared to infected non-allergic controls (Figure 27A-E). However, by 7 dpi, infected allergic mice no longer had increased numbers of these cells, but had increased numbers of lymphocytes and decreased numbers of neutrophils compared to infected non-allergic controls.

Collectively, these results show that influenza virus infection leads to an overall increased airway inflammation following AAD, which is greatest at 3 dpi.
Figure 27. Influenza virus infection leads to exaggerated airway inflammatory cell influx in AAD.

BALB/c mice were sensitised with Ova (Ova) or PBS (Control), challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Ova+Vir) or media (Control, Ova) on the last day of Ova challenge. Inflammatory cells in BALF were assessed at 3 and 7 dpi. Numbers of (A) leukocytes, (B) macrophages, (C) lymphocytes,
(D) neutrophils, and (E) eosinophils. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Ova and Vir control groups, respectively.
Next, we determined whether influenza virus infection increased the severity of AHR in AAD. AHR is identified by increased resistance and decreased compliance in response to methacholine.

At 3 dpi, infected non-allergic mice (Vir), but not non-infected allergic mice (Ova), had increased transpulmonary resistance compared to non-infected non-allergic controls (Control, Figure 28A and B). Importantly, infected allergic mice (Ova+Vir) had further increases in transpulmonary resistance compared to infected non-allergic mice.

Both non-infected allergic and infected non-allergic mice also had decreased dynamic compliance, compared to non-infected non-allergic controls (Figure 28C and D). However, in line with the resistance data, infected allergic mice had decreased dynamic compliance compared to infected non-allergic mice.

At 7 dpi, infected non-allergic mice, but not non-infected allergic mice, had increased transpulmonary resistance compared to non-infected non-allergic controls (Figure 28E and F). However, infected allergic mice did not show any changes in transpulmonary resistance compared to infected non-allergic mice.

In both infected non-allergic and non-infected allergic mice, dynamic compliance was reduced compared to non-infected non-allergic control group (Figure 28G and H). However, infected allergic mice did not show any changes in dynamic compliance compared to infected non-allergic mice.
Overall, these results show that influenza virus infection in AAD leads to increased AHR specifically at 3 dpi.
Figure 28. Influenza virus infection leads to increased AHR in AAD.

BALB/c mice were sensitised with Ova (Ova) or PBS (Control), challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Ova+Vir) or media (Control, Ova) on the last day of Ova challenge. AHR was assessed at 3 dpi, (A) transpulmonary resistance (% change from saline), (B) transpulmonary resistance (unmanipulated), (C) dynamic compliance (% change from saline), (D) dynamic compliance (unmanipulated); and at 7 dpi, (E) transpulmonary resistance (% change from saline), (F) transpulmonary resistance (unmanipulated), (G) dynamic compliance (% change from saline), (H) dynamic compliance (unmanipulated). Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Ova and Vir control groups, respectively.
3.4.5 Influenza virus infection leads to more severe histopathology and increased MSCs in AAD

We then focused our study on the 3 dpi time point, when increased viral titre, airway inflammation and AHR were observed. Specifically, we measured histopathological score, a scoring system in which pulmonary inflammation is its major determinant, as well as the number of MSCs (288).

Both non-infected allergic (Ova) and infected non-allergic (Vir) mice had higher histopathological scores compared to non-infected non-allergic (Control) controls (Figure 29). Significantly, in infected allergic mice (Ova+Vir), the combination of AAD and influenza virus infection resulted in more severe histopathology compared to both non-infected allergic and infected non-allergic controls.

Non-infected allergic and infected allergic, but not non-infected non-allergic or infected non-allergic groups, had detectable MSCs within their airways (Figure 30). Importantly, infected allergic mice had significantly higher numbers of MSCs compared to their non-infected allergic controls.

Taken together, these results show that influenza virus infection leads to worsened pulmonary histopathology and increased MSCs in AAD.
Influenza virus infection leads to more severe histopathology in AAD.

BALB/c mice were sensitised with Ova (Ova) or PBS (Control), challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Ova+Vir) or media (Control, Ova) on the last day of Ova challenge. Histopathological score of lung tissue sections was assessed at 3 dpi. Data are presented as mean ± SEM (n≥6) and are...
representative of two or more independent experiments. #, + and * $P \leq 0.05$ versus Control, Ova and Vir control groups, respectively.
Figure 30. Influenza virus infection leads to increased numbers of MSCs in AAD.

BALB/c mice were sensitised with Ova (Ova) or PBS (Control), challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Ova+Vir) or media (Control, Ova) on the last day of Ova challenge. Numbers of mucus secreting cells per 100µm of basal membrane (BM) were assessed at 3 dpi. Data are presented as mean ±
SEM (n≥6) and are representative of two or more independent experiments. #, + and *

P≤0.05 versus Control, Ova and Vir control groups, respectively.
3.4.6 Influenza virus infection in AAD increases IL-13Rα1

Our observations indicate that influenza virus infection in AAD leads to more severe infection and AAD including enhanced pulmonary inflammation, and increased AHR, histopathology and the numbers of MSCs. To determine whether these observations correspond with increased level of IL-13, we assessed the levels of IL-13, its receptor IL-13Rα1, and a closely related Th2-cytokine IL-5.

In non-infected allergic mice (Ova), IL-13 protein levels were increased compared to the non-infected non-allergic controls (Control, Figure 31A). However, influenza virus infection resulted in no increase in IL-13 protein in infected non-allergic mice (Vir). In contrast, infected allergic mice (Ova+Vir) had increased IL-13 protein levels compared to infected non-allergic, but not non-infected allergic, controls.

IL-13Rα1 receptor mRNA expression was increased in non-infected allergic mice, compared to the non-infected non-allergic controls (Figure 31B). Influenza virus infection also resulted in increased IL-13Rα1 receptor expression in infected non-allergic mice, compared to non-infected non-allergic controls. Infected allergic mice had further increases in IL-13 receptor expression compared to non-infected allergic, but not infected non-allergic, controls.

Increased IL-5 protein levels were observed in non-infected allergic mice compared to non-infected non-allergic controls (Figure 31C). Influenza virus infection did not increase IL-5 protein levels in infected non-allergic mice compared to non-infected non-allergic controls. However, infected allergic mice had increased IL-5 protein levels compared to infected non-allergic, but not non-infected allergic controls.
These results indicate that influenza virus infection in AAD does not increase the level of IL-13 and IL-5, above that in non-infected allergic controls. However, infection did increase the expression of IL-13 receptor, IL-13Rα1, which may increase IL-13 signalling.
**Figure 31. Influenza virus infection in AAD increased IL-13Rα1.**

BALB/c mice were sensitised with Ova (Ova) or PBS (Control), challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu, Vir, Ova+Vir) or media (Control, Ova) on the last day of Ova challenge. (A) IL-13 protein (B) IL-13Rα1 mRNA and (C) IL-5 protein levels were assessed at 3 dpi. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Control, Ova and Vir control groups, respectively.
3.5 Inhibition of IL-13 in AAD during influenza infection protects against virus infection and prevents associated exacerbation of AAD

3.5.1 Inhibition of IL-13 in AAD leads to reduced influenza virus infection

Our prior observations indicate that IL-13 plays a critical role in driving increased susceptibility to influenza virus infection and in infection-induced exacerbation of AAD. Therefore, we next assessed whether inhibition of IL-13 with a specific monoclonal antibody could protect against infection and reduce the severity of AAD during influenza virus infection.

Mice were sensitised i.p. to Ova, and subsequently challenged i.n. with Ova 12-15 days after sensitisation. On the last day of Ova challenge, mice were infected i.n. with 7.5 pfu of mouse-adapted H1N1 influenza A/PR/8/34 strain. Mice were treated i.p. with anti-IL13 on the day of infection and at 2 dpi (Ova+αIL13+Vir). Isotype controls (Ova+Vir and Vir groups) were treated with isotype antibody. Non-allergic controls (Vir and αIL13+Vir groups) were sensitised with PBS. All mice were sacrificed at 3 dpi (Figure 32A).

To assess the effects of IL-13 inhibition in AAD on infection, we measured viral titres in BALF. Isotype-treated infected allergic mice (Ova+Vir) had increased viral titre compared to isotype-treated infected non-allergic controls (Vir, Figure 32B). Most importantly, anti-IL13-treated infected allergic mice (Ova+αIL13+Vir) had reduced viral titre compared to isotype-treated infected allergic controls (Ova+Vir).
These results demonstrate that treatment with anti-IL13 in AAD suppresses influenza virus infection \textit{in vivo}. 
Figure 32. Inhibition of IL-13 in AAD leads to reduced influenza virus infection.

(A) BALB/c mice were sensitised with Ova or PBS, challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu). Mice were treated with anti-IL13 antibody (αIL13+Vir, Ova+αIL13+Vir) or isotype antibody (Vir, Ova+Vir) and were sacrificed at 3 dpi. (B) Viral titres in BALF. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Vir, αIL13+Vir and Ova+Vir control groups, respectively.
3.5.2 Inhibition of IL-13 in AAD leads to improved antiviral responses to influenza virus infection

Given that anti-IL-13 treatment suppressed influenza virus infection we next determined if this was associated with improved antiviral IFN responses. Isotype-treated infected allergic mice (Ova+Vir) had decreased levels of IFN-β compared to isotype-treated infected non-allergic controls (Vir, Figure 33A). However, inhibition of IL-13 in infected allergic mice (Ova+αIL13+Vir) increased IFN-β compared to isotype-treated infected allergic controls.

Isotype-treated infected allergic mice had similar levels of IFN-γ compared to isotype-treated infected non-allergic controls (Figure 33B). Inhibition of IL-13 in infected allergic mice increased IFN-γ compared to isotype-treated infected allergic controls.

Isotype-treated infected allergic mice had decreased levels of IFN-λ3 compared to isotype-treated infected non-allergic controls (Figure 33C). Significantly, in anti-IL13-treated infected allergic mice, inhibition of IL-13 resulted in increased IFN-λ3 compared to isotype-treated infected allergic controls.

Collectively, these results indicate that inhibition of IL-13 during AAD and influenza virus infection resulted in improved antiviral responses that suppressed infection.
**Figure 33.** Inhibition of IL-13 in AAD leads to improved antiviral responses to influenza virus infection.

BALB/c mice were sensitised with Ova or PBS, challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu). Mice were treated with anti-IL13 antibody (αIL13+Vir, Ova+αIL13+Vir) or isotype antibody (Vir, Ova+Vir) and were sacrificed at 3 dpi. (A) IFN-β protein expression densitometry, calculated as IFN-β/β-actin ratios, and expressed as fold change from Control. (B) IFN-γ and (C) IFN-λ3 proteins expressions were assessed by ELISA in BALF. Data are presented as mean ± SEM.
(n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Vir, αIL13+Vir and Ova+Vir control groups, respectively.
3.5.3 Inhibition of IL-13 in AAD leads to decreased eosinophil infiltration during influenza virus infection

Next, to investigate the effect of IL-13 inhibition on pulmonary influx of inflammatory cells, we quantified the number of leukocytes in BALF.

Isotype-treated infected allergic mice (Ova+Vir) had increased numbers of total leukocytes, macrophages and eosinophils, but not neutrophils, and reduced numbers of lymphocytes compared to isotype-treated infected non-allergic controls (Vir, Figure 34A-E).

Inhibition of IL-13 in infected allergic mice (Ova+αIL13+Vir) led to decreased numbers of eosinophils, but not total leukocytes, macrophages, lymphocytes and neutrophils compared to isotype-treated infected non-allergic controls (Figure 34A-E).

Overall, these results indicate that inhibition of IL-13 in AAD during influenza virus infection significantly reduced the influx of eosinophils into the airways, but not other types of inflammatory cells.
Figure 34. Inhibition of IL-13 in AAD leads to decreased eosinophil infiltration during influenza virus infection.

BALB/c mice were sensitised with Ova or PBS, challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu). Mice were treated with anti-IL13 antibody (αIL13+Vir, Ova+αIL13+Vir) or isotype antibody (Vir, Ova+Vir) and were sacrificed at 3 dpi. Inflammatory cells in BALF were assessed. Numbers of (A) leukocytes, (B) macrophages, (C) lymphocytes, (D) neutrophils, and (E) eosinophils. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Vir, αIL13+Vir and Ova+Vir control groups, respectively.
3.5.4 Inhibition of IL-13 in AAD leads to improved lung function during influenza infection

We next sought to determine if inhibition of IL-13 in AAD suppressed influenza virus-induced AHR.

Isotype-treated infected allergic mice (Ova+Vir), had increased transpulmonary resistance and decreased dynamic compliance compared to isotype-treated infected non-allergic controls (Vir, Figure 35A-D). In contrast, anti-IL13-treated infected allergic mice (Ova+αIL13+Vir) had decreased transpulmonary resistance, but had no changes in dynamic compliance, compared to isotype-treated infected allergic controls.

These results indicate that inhibition of IL-13 in AAD suppressed AHR during influenza virus infection.
Figure 35. Inhibition of IL-13 in AAD lead to improved lung function during influenza virus infection.

BALB/c mice were sensitised with Ova or PBS, challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu). Mice were treated with anti-IL13 antibody (αIL13+Vir, Ova+αIL13+Vir) or isotype antibody (Vir, Ova+Vir) and were sacrificed at 3 dpi. (A) Transpulmonary resistance (% change from saline). (B) Transpulmonary resistance (unmanipulated). (C) Dynamic compliance (% change from saline). (D) Dynamic compliance (unmanipulated). Data are presented as means ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Vir, αIL13+Vir and Ova+Vir control groups, respectively.
3.5.5 Inhibition of IL-13 in AAD leads to more severe histopathology but decreased MSCs during influenza virus infection

Next we determined the effect of IL-13 inhibition on pulmonary histopathology and MSC numbers.

Isotype-treated infected allergic mice (Ova+Vir) had increased histopathological score compared to isotype-treated infected non-allergic controls (Vir, Figure 36). Inhibition of IL-13 in infected allergic mice (Ova+αIL13+Vir) resulted in further increases in histopathological score compared to isotype-treated infected allergic mice.

MSCs were detected in the airways of isotype-treated infected allergic mice, but not in isotype-treated infected non-allergic groups (Figure 37). Inhibition of IL-13 in infected allergic mice led to decreased numbers of MSCs compared to isotype-treated infected allergic controls.

These results show that inhibition of IL-13 in AAD leads to increased histopathology but decreased numbers of MSCs during influenza virus infection.
Figure 36. Inhibition of IL-13 in AAD lead to increased histopathology during influenza virus infection.

BALB/c mice were sensitised with Ova or PBS, challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu). Mice were treated with anti-IL13 antibody (αIL13+Vir, Ova+αIL13+Vir) or isotype antibody (Vir, Ova+Vir) and were sacrificed at
3 dpi. Histopathological score of lung tissue sections was assessed. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. 

#, + and * P≤0.05 versus Vir, αIL13+Vir and Ova+Vir control groups, respectively.
Figure 37. Inhibition of IL-13 in AAD leads to decreased numbers of MSCs during influenza virus infection.

BALB/c mice were sensitised with Ova or PBS, challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu). Mice were treated with anti-IL13 antibody (αIL13+Vir, Ova+αIL13+Vir) or isotype antibody (Vir, Ova+Vir) and were sacrificed at
3 dpi. The numbers of mucus secreting cells per 100µm of basal membrane (BM) was assessed. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Vir, αIL13+Vir and Ova+Vir control groups, respectively.
3.5.6 Inhibition of IL-13 in AAD leads to reduced levels of IL-13 and IL-13Rα1 during influenza virus infection

Next, we examined the level of IL-13 following the anti-IL13 treatment in mice during influenza virus infection in AAD. Isotype-treated infected allergic mice (Ova+Vir) had increased IL-13 compared to isotype-treated infected non-allergic controls (Vir, Figure 38A). Importantly, anti-IL13-treated infected allergic mice (Ova+αIL13+Vir) had reduced IL-13 compared to isotype-treated infected allergic controls.

We also measured the effects of IL-13 inhibition on IL-13Rα1 receptor mRNA and IL-5 protein. Isotype-treated infected allergic mice had no change in IL-13Rα1 mRNA expression compared to isotype-treated infected non-allergic controls (Figure 38B). However, anti-IL13-treated infected allergic mice had reduced IL-13Rα1 mRNA compared to isotype-treated infected allergic mice controls.

These results show that inhibition of IL-13 in AAD during influenza virus infection not only reduces the IL-13 protein levels, but also the expression of its receptor, IL-13Rα1.
Figure 38. Inhibition of IL-13 in AAD lead to reduced levels of IL-13 protein and IL-13Rα1 mRNA expression following influenza infection.

BALB/c mice were sensitised with Ova or PBS, challenged with Ova, and inoculated with A/PR/8/34 influenza virus (7.5 pfu). Mice were treated with anti-IL13 antibody (αIL13+Vir, Ova+αIL13+Vir) or isotype antibody (Vir, Ova+Vir) and were sacrificed at 3 dpi. (A) IL-13 protein, (B) IL-13Rα1 mRNA and (C) IL-5 protein were assessed in BALF. Data are presented as mean ± SEM (n≥6) and are representative of two or more independent experiments. #, + and * P≤0.05 versus Vir, αIL13+Vir and Ova+Vir control groups, respectively.
CHAPTER 4: DISCUSSION

4.1 Overall Findings and Significance of Research

The severity of influenza virus infection varies among different cohorts of people in the population. In particular, patients with underlying chronic airways diseases, such as COPD and asthma, are more susceptible to influenza virus infection and consistently present with worse disease outcomes (104, 189, 208, 228, 231, 295, 296). In the current in vivo mouse studies, we utilised a cigarette smoke-induced model of experimental COPD and an Ova-induced model of AAD to elucidate the interactions of influenza virus infection with COPD and asthma, respectively.

We have made important observations that further our understanding of the mechanisms that underpin the association between influenza virus infection and chronic airways diseases, specifically COPD and asthma. In the first part of the study, we successfully demonstrated that influenza virus infection in experimental COPD led to increased viral titre, exaggerated airway inflammation and impaired lung function. These effects were accompanied by decreased neutrophil influx into the lung and reduced antiviral IFN responses, as well as the suppression of a range of cytokines and chemokines. Most significantly, we made a vital observation that this increased susceptibility to influenza virus infection is mediated by an increase in PI3K pathway activity. The inhibition of PI3K effectively reduced viral titre, enhanced antiviral IFNs and improved lung function (Figure 39).
Influenza virus infection in COPD led to increased PI3K, which impaired antiviral responses during infection. This was associated with more severe infection outcomes, including increased viral titre, exaggerated airway inflammation (but reduced neutrophils) and impaired lung function. Inhibition of PI3K improved antiviral responses, which consequently reduced viral titre, increased neutrophil infiltration into the lung and improved lung function.
In the second set of studies, we characterised the effects of influenza virus infection in AAD and the specific role of IL-13 in these events. These were delineated through three progressive sets of experiments. First, mice were infected with influenza virus following the administration of rIL-13, which increased the levels of IL-13 during the infection. In the second stage, mice were infected with virus in Ova-induced AAD. In the final stage, we inhibited IL-13 during Ova-induced AAD in the presence of influenza virus infection via administration of an anti-IL13 monoclonal antibody.

Following analysis of these studies we made important observations which demonstrate that the administration of rIL-13 during infection lead to higher viral titres, impaired antiviral responses, exaggerated airway inflammation, more severe histopathology, and increased IL-13Rα1 mRNA expression, mucus hypersecretion, and AHR. We also demonstrated that in AAD, influenza virus infection led to similar outcomes namely increased viral titre, impaired antiviral responses, exaggerated airway inflammation, more severe histopathology, and increased IL-13Rα1 mRNA expression, mucus hypersecretion, and AHR. Most importantly, in AAD, specific inhibition of IL-13 with anti-IL13 monoclonal antibody treatment during infection improved many of these outcomes including reduced viral titre, improved antiviral responses, reduced eosinophil infiltration (albeit the level of overall inflammation and histopathology were not significantly decreased), reduced IL-13 and IL-13Rα1, MSC numbers, and AHR. Collectively, these studies provide strong evidence of the importance of IL-13 in promoting influenza virus infection in AAD, and its potential as a therapeutic target in these situations (Figure 40).
Figure 40. Interaction of influenza virus infection with AAD.

Administration of rIL-13 or AAD promoted more severe influenza virus infection, which occurred through impaired antiviral responses, and lead to increased viral titres and exaggerated airway inflammation. In turn, increased influenza virus infection lead to more severe histopathology and increased mucus hypersecretion and AHR in AAD. Importantly, inhibition of IL-13 during influenza virus infection in AAD improved many of these outcomes, including reduced viral titre, increased antiviral responses, and reduced eosinophil infiltration, mucus secretion and AHR.
In completing both arms of this research, we have demonstrated the important and novel roles of PI3K and IL-13 signalling in increased influenza virus infection in COPD and asthma, respectively. These studies provide strong evidence of their roles as potential target for influenza management and therapy, particularly in patients with COPD and asthma. These observations have been used as the basis for numerous ongoing studies in our laboratory. The results of our in vivo study are progressing into clinical studies by our physician collaborators. In addition, ongoing studies are being pursued which further explore the questions that arise from our observations (see section 4.4: Future Directions).

Thus, findings from our studies have both contributed to the body of knowledge and become important resources for subsequent research in this area.
4.2 Influenza infection virus in COPD

Influenza virus infection in experimental COPD resulted in more severe infection outcomes. This included increased viral titre, exaggerated airway inflammation and altered lung function. These effects were accompanied by decreased neutrophil influx in the airways, reduced antiviral responses such as IFN-β, as well as key antiviral cytokines and chemokines. We demonstrated that the activity of the PI3K/pAkt pathway was increased and mediated the suppression of antiviral responses in smoke-exposed mice. Most importantly we showed that inhibition of PI3K led to improvements in infection outcomes, including reduced viral titre, increased levels of IFN-β, and other antiviral cytokine and chemokine responses as well as elevated numbers of neutrophil infiltration in the lungs, and improved lung function.

In the next sections, important observations will be discussed and the potential role of PI3K pathway in providing a novel therapeutic approach for the prevention and treatment of influenza infection in COPD will be highlighted.

4.2.1 Influenza infection is more severe in experimental COPD

In the current study, we have demonstrated that influenza virus infection is more severe in COPD, leading to increased viral titre, exaggerated airway inflammation and impaired lung function (Figure 41).
Figure 41. Influenza virus infection is more severe in experimental COPD.

Influenza viral infection in experimental COPD leads to more severe outcomes which includes increased viral titre, exaggerated airway inflammation and impaired lung function.
Some mice were exposed to tightly controlled doses of cigarette smoke. This exposure was conducted in puffs followed by pauses for 2 hours per day and for the duration of 8 weeks. The exposure are also specifically directed to the nose and airways of the animals (275). This exposure is in a concentration and volume to weight equivalent of human smokers, reflecting the exposure in a pack-a-day human smoker who takes approximately 6 minutes to smoke a cigarette (297). Our protocol is in contrast to existing models that typically involved whole-body exposure of mice to cigarette smoke (298). Using our direct-exposure strategy, we successfully established a model that requires only 8 weeks of smoking duration to induce hallmark features of COPD in mice, including tissue destruction, increased inflammatory cells influx into the lung and reduced lung function (275). This time frame is significantly shorter compared to 6 months usually taken by the whole-body exposure approach (298).

Following influenza virus infection, there was no difference in viral titre between infected smoke-exposed and normal air-exposed mice at 3 dpi. However, at 7 dpi, infection of smoke-exposed mice resulted in a higher viral titre. This is consistent with another in vivo study that used acute smoke exposure (4 days) where increased viral titre was observed following infection with H3N1 (299). We observed no reduction in weight gain in infected smoke-exposed mice compared to infected normal air-exposed group. However, this should not be inferred as an absence of physical symptoms in the mice following combined exposures of both cigarette smoke and infection. Rather, further weight loss is unlikely to manifest since the infection by itself has already led to significant reduction in weight gain (in infected air-exposed mice, compared to non-infected air-exposed controls).
COPD patients suffer influenza virus-induced exacerbations of their disease which involve exaggerated airway inflammation and reductions in lung function (295). However, there are few human studies that have directly examined inflammatory cell profiles in COPD patients during influenza infection. This is due to the difficulties in sample collection in those who undergo exacerbations. Therefore, our study using mouse models provides valuable insights into the inflammatory processes involved.

We show that influenza virus infection of smoke-exposed mice led to exaggerated airway inflammation involving the influx of macrophages and lymphocytes. Importantly, we also observed a reduction in the numbers of neutrophils. This may be associated with neutrophil death, as influenza viruses have been shown to infect and accelerate the apoptosis of neutrophils (300). By contrast, another study has shown increased numbers of neutrophils in mice acutely exposed to cigarette smoke that were infected with influenza virus (299). These differences are likely due to the reduced duration (4 days) of smoke exposure, which is not sufficient to induce chronic reductions in immunity, or the different influenza strain (H3N1, strain Mem71) used in that study (299). The role of neutrophils in the immune response to influenza is incompletely understood. Neutrophil influx has been implicated in increasing the pathology of infection by contributing to pulmonary oedema and epithelial cell apoptosis in murine models of influenza (301). Conversely, neutrophils have also been reported to inhibit influenza virus replication and cooperate with antibody responses to eliminate the virus in mice (302, 303). Other studies have shown that the depletion of neutrophils in vivo leads to increased peribronchiolar inflammation during influenza virus infection (304). Reduction in adhesion molecule expression may also contribute to lower numbers of neutrophils in the lung. Analysis of human sputum samples following
rhinovirus infection indicates a reduction in neutrophil adhesion molecule expressions, including CD11a, CD11b, CD62L and CD162 in COPD patients (305). Similar analyses from patients with influenza infection have not yet been reported and will be informative when combined with our observations. Thus, in the context of our study, the reduction in neutrophils may have resulted from increased apoptosis and reduced neutrophil adhesion molecule expressions.

Reduced lung function is observed in COPD patients with influenza virus infection but the precise abnormalities are difficult to examine during exacerbations in humans (295). In our study, infection of smoke-exposed mice resulted in reduced lung function that was characterised by increased transpulmonary resistance, tissue damping and total lung capacity and lower dynamic compliance. COPD patients also have increased lung resistance, which is associated with narrowing of small airways (306). Decreased lung compliance is linked with the narrowing of the airway which results from bronchial compression that is caused by the formation of perivascular fluid cuffs (307). Increased tissue damping has been reported to correspond with proliferation of airway smooth muscle in other murine chronic airway disease models such as asthma (308), or with increased stiffening and remodelling of the parenchyma and airway walls (309). Increased total lung capacity indicates hyperinflation during breathing, and is associated with COPD as shown by computed tomography (310). Thus, our model recapitulates clinical observations of impaired lung function in influenza in COPD and contributes to our understanding of the physiological mechanisms of these changes.

Infection had no effect on emphysema-like alveolar enlargement even at the peak of infection (7 dpi). Nevertheless, it is likely that longer periods after infection are needed to observe the effects of influenza on this most chronic feature of the disease.
4.2.2 Antiviral responses to influenza virus infection are reduced in COPD

To account for the increased susceptibility to influenza infection in COPD that we observed at 7 dpi, we also demonstrated that in experimental COPD, there is a deficiency in antiviral responses, including IFN-β and key cytokines and chemokines; including decreased in the levels IFN-γ, TNF-α, IP-10, MIP-1α, KC and IL-10, and increased IL-6 (Figure 42).
Figure 42. Antiviral responses to influenza infection are reduced in COPD.

Influenza virus infection in COPD led to reduced antiviral IFN, cytokine and chemokine responses, and thus resulted in exaggerated airway inflammation, increased viral titre and impaired lung function.
RIG-I and IFN-λ3 production were decreased at 3 dpi, although not at 7 dpi. RIG-I has been demonstrated to be vital for viral detection and eradication (30), and can be suppressed by exposure to cigarette smoke extract *in vitro* (311). Following endocytosis of influenza virus into host cells, RIG-I recognizes influenza viral RNA and, through phosphorylation of IRF3, induces IFN responses, including IFN-β. These IFN responses consequently amplify antiviral responses by inducing the expression of over 300 ISGs, which include positive feedback regulators (such as RIG-I itself), and antiviral proteins such as protein kinase R (29, 42, 312). Mice deficient in these factors have reduced ability to clear influenza virus (39, 313). Other studies also indicate that mice lacking functional IFN-λ receptors were also more susceptible to influenza (314). The direct effect of smoking on IFN-λ has not been studied extensively. However, it has been shown to be downregulated in other chronic lung conditions such as asthma (314). Thus, chronic smoke-exposure may suppress critical antiviral responses that promote increased susceptibility to influenza.

Other antiviral cytokines and chemokines were also suppressed during cigarette smoke-exposure and influenza virus infection. IFN-γ is critical for immunity against influenza as it inhibits replication and is immunostimulatory, and its levels are reduced in smoking subjects (315). In our study, we show that the levels of IFN-γ were decreased in infected smoke-exposed mice. These mice also had reduced levels of IP-10, a factor produced by neutrophils in response to IFN-γ and has been shown to generate antiviral activity (48, 316). Infected smoke-exposed mice also had decreased levels of TNF-α, MIP-1α and KC, which are neutrophil chemoattractants (317-319). Thus, decreases in cytokines and chemokines that were associated with reduced neutrophil recruitment in smoke-exposed mice may also contribute to increased
susceptibility to infection. By contrast, reduced levels of the anti-inflammatory cytokine IL-10, and elevations in the pro-inflammatory cytokine IL-6, were accompanied by increased influx of other leukocytes in the lung (319, 320). These changes likely induce a pro-inflammatory environment that contributes to exacerbations of COPD.

4.2.3 Inhibition of exaggerated PI3K activity during influenza virus infection in COPD improves infection outcomes

Influenza virus activates PI3K signalling pathways which increases viral titre by promoting viral endocytosis and suppressing antiviral responses (147, 148, 321). They also play a role in impairing the phosphorylation and dimerisation of factors, such as IRF3, that are important in the induction of IFN-β (147, 151). In our study, we identified links between elevated PI3K signalling, increased susceptibility to influenza virus infection and reduced antiviral responses in experimental COPD. We demonstrated heightened PI3K/pAKT pathway activity following influenza virus infection in experimental COPD, and that inhibition of PI3K led to significant improvements in infection outcomes (Figure 43).
Figure 43. Inhibition of exaggerated PI3K activity during influenza virus infection in experimental COPD improves infection outcomes.

Inhibition of PI3K during influenza virus infection in COPD partially restores antiviral responses, leading to improvements in many of the infection outcomes, including increased neutrophil influx into the airways, decreased viral titre and improved lung function.
We utilised the PI3K inhibitor, LY294002 to assess the role of the PI3K pathway in increasing the susceptibility of mice with experimental COPD to influenza virus infection and its potential as a therapeutic target. However, along with its ability to inhibit PI3K, LY294002 has also been reported to affect signalling functions of other molecules. For example, LY294002 directly inhibits the mammalian target of rapamycin (mTOR) \textit{in vitro} (322). mTOR has been shown \textit{in vivo} to modulate antibody responses against influenza virus infection where its inhibition during immunisation with H3N2 subtype lead to cross-protective immunity to secondary lethal infection against other subtypes, including H5N1, H7N9 and H1N1 (323). LY294002 also blocks casein kinase 2 (CK2) function \textit{in vitro} (324). CK2 activity is increased during influenza virus replication, and its inhibition \textit{in vitro} resulted in decreased virus shedding (325). LY294002 also inhibits glycogen synthase kinase (GSK)3β expression \textit{in vitro} (326). Recently, GSK-3β has been demonstrated \textit{in vitro} to be involved in influenza virus-induced vascular hyperpermeability by mediating the degradation of β-catenin, a key regulatory component of the vascular endothelial-cadherin cell adhesion complex (327). Although LY294002 has not been shown to directly increase the survival of neutrophils, indirectly, its contribution to neutrophil survival has been demonstrated, albeit in a limited and context-specific fashion. For example, hypoxia-mediated neutrophil survival is not affected by LY294002 inhibition \textit{in vitro} (328). However, LY294002 inhibition has been shown \textit{in vitro} to support the phosphorylation of Bad (an apoptosis-promoting BH3 domain-containing protein), which contributes to the survival of neutrophils (329). LY294002 inhibition may also play a role in altering the adhesion properties of neutrophils. For example, inhibition by LY294002 prevented the platelet-endothelial cell adhesion molecule (PECAM)-1, which is important in the adhesion of human neutrophils to the EA.hy926 endothelial cell line (330).
In our studies, we first showed that this pathway was elevated in smoke-exposed mice, and this was associated with more severe infection, reduced antiviral responses and impaired lung function. Then we demonstrated that inhibition of PI3K in infected smoke-exposed mice increased the expression of IFN-β, enhanced the influx of neutrophils, suppressed infection and improved lung function. This indicates that PI3K inhibitors may be effective preventions and treatments for influenza, particularly in COPD. Moreover, we demonstrated a role for PI3K in suppressing antiviral cytokine and chemokine production. Elevated PI3K/pAkt activity was associated with reductions in IFN-γ, IL-10, IP-10 and MIP-1α and increase in pro-inflammatory IL-6. Inhibition of PI3K led to increased IFN-γ, IL-10, IP-10 and MIP-1α and reduced IL-6 in infected smoke-exposed mice. These observations strengthen and provide a mechanistic link between increased PI3K activity and associated reductions in neutrophils and antiviral responses in COPD that can be reversed by PI3K inhibition. This occurred without an increase in overall inflammation (and with a decrease in IL-6) in smoke-exposed mice. Delineating the full implication of these multiple interactions between individual cytokines requires further study.

Current prevention and treatment strategies for influenza are limited and new therapies are urgently required (159, 331). In the absence of COPD, different individuals have differing susceptibility to influenza (332). Some otherwise healthy adults were affected by the recent swine flu, and children, the elderly, asthmatics and pregnant women were at increased risk (333, 334). Such individuals are known to have reduced antiviral responses, and there is the potential that they may also have elevated PI3K activity. Furthermore, influenza strains that are pathogenic in humans have non-structural protein (NS)1 that upregulates PI3K activation and suppresses IFN signalling
cascades and antiviral responses (147, 148, 286). Therefore, targeting PI3K pathways and enhancing antiviral responses may have substantial benefit in the general population beyond those with COPD, particularly during seasonal and pandemic outbreaks.
**4.3 Influenza virus infection in AAD**

In the second part of our studies, we demonstrated that influenza virus infection following rIL-13 administration or in AAD resulted in more severe infection and allergic airway inflammation and reduced lung function. These include increased viral titre, impaired antiviral responses such as IFN-β and IFN-λ, exaggerated inflammation in the airways, and increased severity in pulmonary histopathology, numbers of MSCs and AHR (Figure 44). Furthermore, and importantly, inhibition of IL-13 by anti-IL13 antibodies during influenza virus infection in AAD improved many of the infection and allergic outcomes including reduced viral titre, increased antiviral responses, and reduced AHR. Moreover, although the levels of overall airway inflammation and histopathology did not decrease, eosinophil influx and mucus secretion in the lungs were reduced significantly (Figure 45).

In the next section, important observations and the significant impacts of both experimental supplementation and inhibition of IL-13 will be discussed. These highlight the crucial role of IL-13 responses and signalling in influenza virus infection in AAD and thus potentially provide a novel therapeutic approach in managing influenza in asthma.
**Figure 44. Influenza virus infection in either combination with rIL-13 administration or in AAD.**

During experimentally-induced increased of IL-13 via rIL-13 administration or Ova-induction of AAD, influenza virus infection resulted in increased levels of IL-13 signalling and impaired antiviral responses, including IFN-β and IFN-λ. Consequently, this leads to more severe infection and allergic outcomes including increased viral titre, exaggerated inflammation in the airways, and increased pulmonary histopathology, mucus secretion and AHR.
Figure 45. Influenza virus infection in AAD is suppressed by IL-13 inhibition.

Inhibition of IL-13 during influenza virus infection in AAD improved antiviral responses including IFN-β and IFN-λ, leading to reductions in viral titre, eosinophil infiltration into the airways, mucus secretion and AHR.
4.3.1 IL-13 plays an important role in promoting more severe influenza virus infection in AAD

In the present study, increased influenza viral titres were detected in mice previously treated with rIL13. Interestingly, in AAD, we also detected similar increases in viral titre in infected allergic mice compared to infected non-allergic controls. Furthermore, and importantly, we demonstrated that inhibition of IL-13 in AAD effectively reduced viral titre during infection. Our observations indicate that hosts with underlying AAD are vulnerable to more severe influenza virus infection. Increased influenza viral titres have previously been shown in mice exposed to other irritants such as diesel exhaust particles (a potent oxidant air pollutant) (335). Our findings are also consistent with clinical observations that show similar increases in the susceptibility of asthmatic patients to viral infections in general, and influenza in particular (208, 228-230, 296).

In terms of weight loss, we detected no significant difference between infected allergic mice and infected non-allergic controls. This may not necessarily indicate an absence of additional disease impacts in infected allergic mice. Rather, this disconnect suggests that there is a threshold of viral load that is required before excessive weight loss occurs during an infection. Therefore, in the context of our current study, the usefulness of changes in body weight is limited in evaluating the symptomatic difference between infected mice.

We could find no literature on the direct functions of IL-13 in affecting viral replication in asthmatic patients. It is more likely that rather than directly affecting the
influenza viral titre on its own, IL-13 provides an antagonistic environment towards antiviral factors in AAD, leading to significant impairment in antiviral responses.

4.3.2 AAD impairs antiviral responses to influenza virus infection, which can be improved by inhibition of IL-13

We have shown that administration of rIL-13 led to reduced antiviral responses, specifically IFN-β and IFN-λ3 during influenza virus infection. Similarly, during influenza virus infection in AAD, mice also had decreased levels of IFN-β and IFN-λ3 compared to infected non-allergic controls. Most importantly, specific inhibition of IL-13 during influenza virus infection in AAD improved antiviral responses, including increasing the levels of IFN-β and IFN-λ3.

Our current understanding of the mechanisms underpinning the increased susceptibility to influenza virus infection in AAD is limited. Studies with other viruses have suggested that impairment of antiviral responses to infection may significantly contribute to increased susceptibility. Infection with rhinovirus in primary bronchial epithelial cells (pBECs) obtained from asthmatic patients leads to reduced production of IFN-β (336). A subsequent similar in vitro study reported that the level of IFN-λ1 was also decreased in asthmatic pBECs that were infected with rhinovirus (337). In this context, our findings further this understanding by showing that during influenza virus infection, mice with AAD are also incapable of generating sufficient antiviral responses. This may lead to failure in inhibiting viral titre via early apoptosis of infected host cells, comparable to what occurs during rhinovirus infections in asthmatic patients (336, 337).
Multiple other studies have demonstrated inverse relationships between the levels of IL-13 and IFN-β (338-340). IFN-β has been shown to inhibit IL-13 production and secretion by T cells (338). It also has the capacity to block IL-13 signalling events by inhibiting the activation of IL-13-response elements of STAT6 (339). Another way of achieving that signal blockage is through the induction of IL-13 decoy receptor (IL-13Rα2) expression, which also leads to suppression of IL-13 responsiveness (340).

Similarly, inverse associations between IL-13 and IFN-λ have also been reported (268, 271, 272). IFN-λ1 has been shown to inhibit the production and secretion of IL-13, and diminish the level of IL-13 in Th2-promoting conditions (272). IFN-λ1 also diminishes IL-13 levels in naïve T cells following exposure to allogeneic myeloid DCs (271). On the other hand, IL-13 can suppress IFN-λ1 expression stimulated by synthetic mimic of viral dsRNA in airway epithelial cells and alveolar macrophages (268). IL-13 has also been demonstrated to suppress IFN-λ3 induced by intratracheal instillation of dsRNA in the lung (268).

Therefore, our observations further our understanding by demonstrating that in the context of AAD, IL-13 responses and signalling may play a pivotal role in the immunomodulation of antiviral responses during influenza virus infection, thus increasing the level of viral titre during the course of infection.
4.3.3 IL-13 plays an important role in pulmonary inflammation during influenza virus infection in AAD

Exaggerated pulmonary influx of inflammatory cells and increased histopathology in the lungs was observed during influenza virus infection following both administration of rIL-13, and the induction of AAD. Interestingly, specific inhibition of IL-13 in AAD during influenza virus infection did not alleviate this inflammation.

Our observations highlight the multifactorial nature of pulmonary inflammation during influenza virus infection in AAD. Our findings are consistent with observations that typify infection-induced exacerbations in asthmatic patients, which involve exaggerated lung inflammation that worsens their symptoms (341, 342). Previous studies have shown the important role of IL-13 during inflammation, including in murine and primate models (266, 267, 343, 344). For example, mice deficient in IL-13 did not develop airway inflammation following H3N1 infection (267).

In the context of influenza virus infection in AAD, our findings suggest that while IL-13 signalling may contribute significantly to the increased inflammation, the absence of IL-13 signalling *per se* is insufficient to impede the overall inflammatory process. Nevertheless, IL-13 signalling may play a more specific role during this inflammatory process such as promoting eosinophilic influx into the lungs. Indeed, we detected an increased pulmonary influx of eosinophils following influenza virus infection in AAD mice, and most importantly, demonstrated that inhibition of IL-13 in this environment resulted in decreased numbers of eosinophils. IL-13 has been shown to
promote migration of eosinophils into the lung (345, 346). Another in vivo study also showed that increased IL-13 was associated with increased numbers of eosinophils in influenza virus infection in AAD mice following instillation of another inhaled irritant (i.e. diesel exhaust particles) (347).

In patients, asthma may present with low numbers of sputum eosinophilic counts that are consistent with those that do not have asthma (348, 349). However, the level of eosinophil infiltration into the lungs may play a larger role in the treatment of exacerbations. Therapeutic strategies directed at reducing the induced sputum eosinophil count has been demonstrated to reduce asthma exacerbations and eliminate the necessity for additional anti-inflammatory treatments (350). In virus-induced asthma attacks, the number of eosinophils may be partly responsible for the difference in degrees of severity among different types of viral infections (351). Importantly, in agreement with our findings, sputum eosinophilia in asthmatic patients has been shown to correlate positively with the level of IL-13 expression during severe episodes of asthma (351).

Thus, in the context of the multifactorial nature of inflammatory processes during influenza virus infection in asthma, our data add important evidence to support the vital roles of IL-13 responses and signalling, specifically in the pulmonary influx of eosinophils during the infection.
4.3.4 IL-13 plays an important role in increased AHR during influenza virus infection in AAD

In our study, we also detected a decline in lung function in influenza virus infection following administration of rIL-13, which is indicated by increased transpulmonary resistance and decreased dynamic compliance (combination of both signifies increased AHR). Interestingly, influenza virus infection in AAD also led to a similar decline. Importantly, we demonstrated that in AAD, inhibition of IL-13 reduced AHR during influenza virus infection.

Respiratory viral infections in asthmatic patients have been strongly linked with induction of exacerbations that require urgent medical attention to prevent hospitalisation or death (108, 186-191). These exacerbations normally include the decline in normal respiratory airflow and manifest in symptoms such as chest tightness, wheezing and shortness of breath (188, 191, 352).

In our *in vivo* model, we detected decreased dynamic compliance but not increased transpulmonary resistance in non-infected allergic mice following AAD induction in the absence of influenza virus infection. These may be due to the subsiding of the effects of AAD which typically wanes over time in non-infected allergic mice. Nevertheless, and importantly, the combination of AAD and influenza virus infection still triggered increased transpulmonary resistance and decreased dynamic compliance in infected allergic mice. This suggests that the presence of responses to both AAD and infection at the same time may trigger a potent synergism leading to a serious increase in AHR.
IL-13 has been strongly implicated in AHR both in asthma and in respiratory viral infection. Inhibition of normal IL-13 signalling has been shown to reduce allergic airway responses in patients with asthma (264). Similarly, the absence of IL-13 or its signalling (either through genetic deletion of IL-13 or blocking of its critical signalling elements, such as STAT6) has been demonstrated to decrease AHR (260, 262, 265, 353, 354). Conversely, genetic overexpression of IL-13 has been linked with increased AAD-related features, including pulmonary AHR (266). Respiratory viral infections, such as RSV, may result in increased AHR, which can be suppressed by inhibition of IL-13 (280).

Infection with influenza can also induce IL-13-dependent AHR (355). AHR was induced following H3N1 infection, but was attenuated in IL-13 deficient mice. Importantly, adoptive transfer of ILCs from wild-type mice into IL-13 deficient mice effectively restored influenza-induced AHR, highlighting ILCs as a critical source of IL-13 in the innate immune system (267). AHR induced by Sendai virus has also been shown to be dependent on NKT cells-activated IL-13 that was produced from macrophages (356). The importance of ILCs and NKT cells in our influenza model of AAD is the subject of ongoing investigations. The role of IL-13 in asthmatic patients has also been identified in recent clinical trials, where improvement in lung function was observed following treatment with anti-IL-13 monoclonal antibodies such as lebrikizumab (263), tralokinumab (357) and dupilumab (358). Our observations indicate the possibility of using similar anti-IL-13 therapeutic strategies to alleviate the decline in lung function in asthmatic patients contracted with influenza.

Our findings, along with studies, further our understanding and highlight the important role of IL-13 signalling in increased influenza virus infection in AAD.
4.3.5 Influenza virus infection leads to increased numbers of MSCs in AAD, which is reduced by inhibition of IL-13

Finally, our studies show that rIL-13 administration and similarly, AAD, led to increased numbers of MSCs. In AAD, there was a further increase in the numbers of MSCs during influenza virus infection. Moreover, we demonstrated that inhibition of IL-13 reduced the numbers of MSCs in AAD during infection.

Abnormal metaplasia and hyperplasia of epithelial cells into MSCs has been linked with asthma exacerbations, and can lead to excessive sputum production and compromised pulmonary gas exchange (174-176, 359). Our results indicate that simultaneous occurrence of AAD and influenza virus infection may contribute to such increased in mucus hypersecretion. Other viral infections such as rhinovirus have also been implicated in causing mucus hypersecretion (360, 361). With regard to influenza virus, a slight increase in mucus secretion has been considered a part of normal mucociliary clearance against infection (362). IL-13 has been shown to potently induce mucus hypersecretion in asthma (266, 363).

Therefore, our findings offer evidence that IL-13 may also be responsible for increased number of MSCs in the specific context of influenza virus infection in AAD.
4.3.6 IL-13 signalling is important in influencing the disease outcomes during influenza virus infection in AAD

Collectively, our findings demonstrate that mice with AAD are more susceptible to influenza virus infection, which leads to increased viral titre caused by compromised antiviral responses. This is associated with increases in airway inflammation, severity of pulmonary histopathology, mucus hypersecretion and AHR. Moreover, through administration of rIL-13 and specific inhibition of IL-13 using anti-IL-13 monoclonal antibodies, we illustrated that elevated levels of IL-13 in AAD may contribute significantly towards such susceptibility and that IL-13 targeting may be a viable therapeutic strategy to improve many of the disease outcomes.

Most importantly, we observed that although influenza virus infection did not further increase the levels of IL-13 to more than that of AAD, the infection induced an increased expression of IL-13 receptor, IL13Rα1. This signifies the importance of the degree of IL-13 signalling activation, along with the amount of IL-13 present, in increasing disease outcomes during influenza virus infection in AAD.
4.4 Future Directions

In achieving our aims in elucidating the mechanisms of increased susceptibility of influenza in COPD and AAD, our studies have uncovered findings that can be and have been used in several ongoing and future studies.

4.4.1 Future directions in the study of influenza virus infection in COPD

In the first part of our studies, we identified the important role of PI3K in increasing susceptibility to influenza virus infection in experimental COPD. We are furthering this study by exploring the degree of contributions of individual catalytic subunits of PI3K in this process. Class IA PI3K variants of the catalytic subunits, namely p110α, p110β and p110δ (127, 128), occur and may be involved, as our understanding of how these isoforms functions in influenza virus infection are unknown. We are also exploring whether combinations of these isoforms, rather than individual ones, are required for PI3K to functionally affect the increased susceptibility to influenza virus infection in COPD. This line of investigation will allow us to explore more specific targets that will be therapeutically useful for managing influenza in COPD. This is vital as increasing the specificity of pathway inhibition will significantly reduce the likelihood of off-target effects from occurring during therapy.

We are exploring the potential role of microRNAs (miRNAs), as upstream regulators of PI3K activity in influenza in COPD. One of the promising candidates for this study is miR-21, which has been shown to be a valuable predictive biomarker of COPD occurrence in heavy smokers (364). Despite this, the exact roles of miR-21 in
COPD, or in influenza infection, have not been demonstrated. Therefore, we are exploring the potential roles of miR-21 in regulating the expression of PI3K through its known association with phosphatase and tensin homologue (PTEN) (365-367), which negatively regulates Akt signalling (368). As demonstrated in the present study, this negative regulation of PI3K/Akt signalling can significantly improve the outcomes of influenza in COPD. This may provide alternative targets for managing this disease.

Our findings suggest that in COPD, the elevated PI3K activity contributes to the impairment of host antiviral responses. Therefore, we will explore the mechanisms of direct or indirect interactions between elevated PI3K signalling pathways and the suppression of IFNs against influenza virus infection in COPD. This may include delineation of possible epigenetic alterations in PI3K or IFN pathways. Novel findings from this study may further expand our understanding of how the underlying COPD increases susceptibility to influenza virus infection.

4.4.2 Future directions in the study of influenza virus infection in AAD

In the second part of our studies, we demonstrated a role for IL-13 in the increased susceptibility to influenza virus infection in AAD. To further these findings, we will identify the specific cellular sources of IL-13 production, in the context of influenza virus infection in AAD. In addition, we will identify whether the same cells also express IL-13 and IL-13Rα1, and whether the presence of influenza virus affects IL-13 mRNA stability. Likely cellular sources include innate immune cells, such as ILC2s and NKT cells, which have been shown to generate IL-13 either during asthma or
in viral infections (356, 369, 370). Identifying these cellular sources and quantifying the
level of IL-13 production will improve our understanding of the mechanisms
underpinning IL-13 responses during influenza virus infection in AAD.

In relation to that study, we will also explore the mechanisms of direct or
indirect interactions between the increased IL-13 in AAD and IFN production following
influenza virus infection. This will facilitate the delineation of the specific mechanisms
that contribute to the impairment of IFNs as an antiviral defence in the context of
influenza virus infection in AAD.

Macrophages can be divided into two major subsets. Classically activated or M1
macrophages promote Th1 responses and are associated with a pro-inflammatory
phenotype and elimination of pathogens (371-374), whilst alternatively activated or M2
macrophages promote Th2 responses and are linked to cell proliferation and tissue
repair (375-377). To further understand the changes in inflammatory cell profile in our
study, in the future we will characterize the polarity of macrophages and the associated
Th responses in the context of influenza infection in AAD.

Furthermore, as many viral-induced exacerbations of asthma occur in children
(189, 191, 231, 280), we will further our study from adult mice into neonatal and infant
AAD mouse models. This will further elucidate whether there are different mechanisms
of susceptibility that dominate at different ages.

Studies have also indicated a link between IL-13 and the induction and
activation of PI3K pathways (378-380). Increased goblet cell density in cultured human
bronchial epithelial cells was shown to be induced by IL-13, and this was sensitive to
inhibition of PI3K by LY29002 (378). In another study, using mouse epithelial cells,
Yan and colleagues demonstrated that LY29002 can block MUC5AC protein production induced by IL-13 (380). Therefore, in future studies we will explore this link further and identify its effects in the context of influenza virus infection in AAD. Specifically, we will investigate the effect of rIL-13 administration in combination with PI3K inhibitor treatment or in the absence of STAT6. This will potentially uncover common factors that significantly contribute to the increased susceptibility to influenza virus infection in both COPD and AAD, leading to therapeutic options that have broader effectiveness.
4.5 Conclusions

In conclusion, our studies have characterised the effects of influenza virus infection in experimental COPD and AAD. We discovered important roles for PI3K in increasing disease outcomes during influenza infection in COPD. We also established that increased influenza virus infection and exacerbation of AAD is, to a significant extent, due to elevated levels of IL-13 signalling. In both of these sets of studies, we demonstrated that inhibition of these factors (PI3K in COPD and IL-13 in AAD models) improve the outcomes in the respective disease settings. These studies have furthered our understanding of the pathogenesis of, and potential therapeutic approaches for, influenza virus infection in chronic lung diseases.


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