

Mechanisms and treatments for severe, steroid-resistant allergic airway disease and asthma

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Funding information

National Health and Medical Research Council (NHMRC); Hunter Medical Research Institute; Australian Research Council; NHMRC of Australia Principal Research Fellowship; Faculty of Health and Medicine, The University of Newcastle

Summary

Severe, steroid-resistant asthma is clinically and economically important since affected individuals do not respond to mainstay corticosteroid treatments for asthma. Patients with this disease experience more frequent exacerbations of asthma, are more likely to be hospitalized, and have a poorer quality of life. Effective therapies are urgently required, however, their development has been hampered by a lack of understanding of the pathological processes that underpin disease. A major obstacle to understanding the processes that drive severe, steroid-resistant asthma is that the several endotypes of the disease have been described that are characterized by different inflammatory and immunological phenotypes. This heterogeneity makes pinpointing processes that drive disease difficult in humans. Clinical studies strongly associate specific respiratory infections with severe, steroid-resistant asthma. In this review, we discuss key findings from our studies where we describe the development of representative experimental models to improve our understanding of the links between infection and severe, steroid-resistant forms of this disease. We also discuss their use in elucidating the mechanisms, and their potential for developing effective therapeutic strategies, for severe, steroid-resistant asthma. Finally, we highlight how the immune mechanisms and therapeutic targets we have identified may be applicable to obesity- or pollution-associated asthma.

KEYWORDS

infection, novel therapies, obesity, pollution, severe asthma, steroid resistance

1 | INTRODUCTION

1.1 | Effects of steroids in asthma management

Corticosteroid therapy is the mainstay treatment in the clinical management of asthma. Despite recent advances in the development and clinical application of disease-modifying biologics,^{1–3} treatment

regimens that incorporate anti-inflammatory corticosteroids are fundamental to successful asthma management and for controlling the frequency and extent of disease exacerbations.^{4,5} Glucocorticoids are a class of corticosteroids, which are steroid hormones that exert potent anti-inflammatory activity and are widely used to treat asthma patients.^{6,7} Inhaled corticosteroid (ICS) therapy uses synthetic, highly lipophilic glucocorticoids, that rapidly diffuse into airway cells upon inhalation and exert their effects by binding and activating cytosolic glucocorticoid receptors (GRs).^{7,8} GR ligation triggers the formation of GR homodimers that are transported into the nucleus by importin- α

and importin-13.^{6,7,9} In the nucleus, GR homodimers bind to glucocorticoid response elements (GREs) in promoter regions of responsive genes to induce or suppress gene transcription. Interaction with transcriptional co-activators, such as cyclic adenosine monophosphate response element (CREB)-binding protein (CBP), results in the acetylation of core histones associated with anti-inflammatory glucocorticoid response genes and allows RNA polymerase II-mediated gene transcription (also known as transactivation). Alternatively, activated GRs can interact with CBP proteins that are already complexed with promoter regions of pro-inflammatory genes, including nuclear factor (NF)- κ B and activator protein (AP)-1. This results in the suppression of pro-inflammatory gene transcription (also known as transrepression) by inhibiting histone acetylation and disallowing access to DNA for RNA polymerase II.^{6,7,10,11} Importantly, the anti-inflammatory activity of GRs is mediated, at least in part, by the recruitment of the enzyme histone deacetylase (HDAC)2, which is a key transcriptional co-repressor that deacetylates core histone proteins.^{12,13}

1.2 | Severe, steroid-resistant asthma

Although the majority of asthmatics respond well to steroid therapies that effectively control their symptoms, a subpopulation of 5%-25% of asthmatics, typically with more severe disease, have poor sensitivity to treatment even with high doses of steroids.^{5,14} This group, which fails to achieve control of their symptoms, has been described as having severe, steroid-resistant asthma.¹⁵⁻²⁰ Severe asthma is defined as asthma with poor symptom control, frequent severe exacerbations that require systemic corticosteroid treatment, and airflow limitation with minor improvements with bronchodilators, or controlled asthma that worsens on tapering of high dose or systemic steroids.²¹ Approximately 30% of severe asthmatics are steroid-refractory that, despite combinatorial treatment with high dose inhaled and systemic corticosteroids, still fail to achieve adequate symptom control.²¹ Steroid-resistant asthma was first described by Schwartz et al.²², who identified a small subpopulation of asthmatics with poorly controlled asthma that did not respond to treatment with high doses of orally administered steroid. Through refinement of the diagnostic criteria, steroid-resistant asthma, that may or may not be severe, is defined as an inability to achieve >15% improvement in forced expiratory volume in one-second (FEV₁) following a 14-day course of oral steroid therapy (prednisolone; 40 mg/day).^{15,21,23}

Severe, steroid-resistant asthma is currently the greatest unmet clinical need in asthma management and although patients with the disease make up 5%-25% of disease sufferers, they take up 50%-80% of resources and health care costs for asthma, which costs over \$8 billion/year in Europe, USA, and Australia combined.^{24,25} Thus, effective treatments for severe, steroid-resistant asthma are urgently required.

Severe, steroid-resistant asthma is commonly associated with non-eosinophilic endotypes of asthma, including neutrophilic asthma, with evidence of innate immune activation (such as toll-like receptor [TLR]2, 4, and nucleotide-binding oligomerization domain [NOD]-like receptor [NLR] family, pyrin domain-containing [NLRP]3, and interleukin [IL]-1 β , responses) and increased type 1 and type 17 responses in addition to

the classical type 2 responses that are associated with mild to moderate asthma.^{20,26-38} However, persistent eosinophilic inflammation, and increased type 2 responses have also been implicated in the pathogenesis of more severe disease.^{33,39-42} This heterogeneity of disease and likely involvement of different underlying immunological, inflammatory, and molecular mechanisms in different subtypes of severe, steroid-resistant asthma has hampered the development of effective therapies.

Multiple mechanisms have been implicated in promoting the pathogenesis of steroid resistance.^{27,43-45} Many studies have linked steroid insensitivity in asthma to defects in GR expression and activity, including reduced GR expression, reduced GR binding affinity to glucocorticoids and/or GREs, and elevated expression of pro-inflammatory transcription factors (Figure 1).^{20,23} Increased expression of IL-2, IL-4,

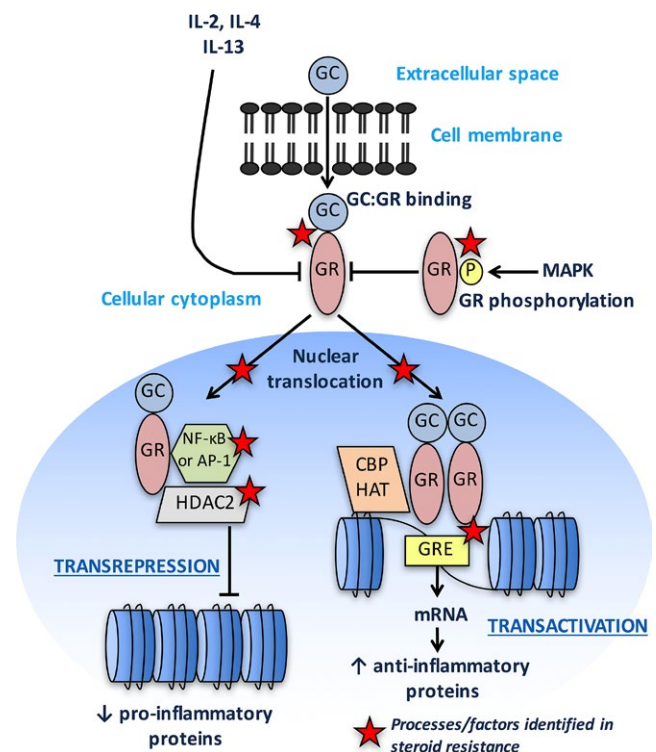


FIGURE 1 Glucocorticoid response and mechanisms of steroid resistance. Glucocorticoids (GC) cross the cell membrane and bind to glucocorticoid receptors (GR) in the cytoplasm. GC:GR complexes then translocate to the nucleus where they interact with CREB-binding protein (CBP) and histone acetyl transferase (HAT) in order to activate transcription from glucocorticoid response elements (GRE), which results in an increase in the expression of anti-inflammatory genes (transactivation). GC:GR complexes also inhibit inflammatory responses by interacting with transcription factors such as nuclear factor (NF)- κ B and activator protein (AP)-1 and through the recruitment and activation of histone deacetylase (HDAC)2, which results in the restriction of NF- κ B and AP-1-mediated pro-inflammatory gene transcription, respectively (transrepression). GC resistance can result from the increased presence of certain cytokines, decreased GR levels and GC:GR binding affinity, increased mitogen-activated protein kinase (MAPK), which phosphorylates and deactivates the GR, defective GC:GR nuclear translocation, and GR interactions with CBP/HAT and GRE, reduced HDAC2 activity, and increased activity of pro-inflammatory transcription factors

and IL-13 in the airways of asthmatics can induce local steroid insensitivity by reducing GR binding affinity in T cells.^{20,23} Additionally, Goleva et al.⁴⁶ found that silencing of the dominant negative GR- β isoform in bronchoalveolar lavage (BAL) macrophages from patients with steroid-resistant asthma improved the activity of the functional GR- α isoform. In another study, Irusen et al.⁴⁷ found that the activation of the p38 mitogen-activated protein kinase (MAPK) resulted in GR phosphorylation and impaired function. Some patients with steroid-resistant asthma also have defective nuclear translocation of GR that leads to reduced GR:GRE interactions.^{20,23} In the nucleus, excessive activation of NF- κ B and AP-1, as well as increased expression of c-Fos and c-Jun N-terminal kinase (JNK), can interfere with GR:GRE binding affinity.^{20,23,48,49}

Interestingly, many steroid-resistant asthma patients have normal nuclear translocation of GR and do not show reduced GR:GRE binding affinity.^{23,50} Thus, it is likely that steroid insensitivity in asthma is also induced by other mechanisms. Notably, reduced HDAC2 expression and activity is associated with steroid insensitivity and more severe disease in both asthma and chronic obstructive pulmonary disease (COPD).^{51–54} This suggests that deficiencies in transcriptional corepressor expression and activity may also play a role in the pathogenesis of steroid-resistant asthma.

Patients with steroid-resistant asthma also present with increased thickness of the airway epithelium, basement membrane, and airway remodeling, compared to steroid-sensitive asthmatics.^{55,56} Airway remodeling in asthma results from goblet cell metaplasia and/or hyperplasia and mucus hypersecretion, airway smooth muscle hyperplasia, angiogenesis, fibrosis, collagen accumulation and excess deposition of extracellular matrix proteins, including the recently identified fibulin-1.^{57–59} Mast cells and their mediators may contribute to these processes.⁶⁰ Angiogenesis is associated with reductions in anti-angiogenic factors such as tumstatin.^{61,62} Severe, steroid-resistant asthma patients have been shown to have a decreased response to bronchodilators compared to steroid-sensitive patients, which is indicative of fixed airflow obstruction and tissue remodeling.⁶³ The exact mechanisms that underpin the pathogenesis of lung remodeling in steroid-resistant asthma remain unclear, however, Goleva et al.⁶³ recently suggested that an imbalance of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP) may play a role with decreased TIMP-1 levels resulting in an increased MMP9/TIMP-1 ratio observed in BAL from steroid-resistant asthmatics, compared to steroid-sensitive controls. Furthermore, fibulin-1c is increased in airway smooth muscle cells in asthma and is necessary as a structural scaffold and plays a role in inflammation.^{57,58} These findings highlight a potential role for airway remodeling in the pathogenesis of severe, steroid-resistant asthma, and suggest that stressors that affect pulmonary development or homeostasis, and permanently alter lung structure, may compromise corticosteroid responsiveness.

The development of more effective therapies for severe, steroid-resistant asthma may be achieved by applying new strategies to increase our understanding of the mechanisms that underpin the pathogenesis of steroid insensitivity, and identifying, and therapeutically targeting, the factors that underpin disease. Increasing clinical

and experimental evidence highlights roles, particularly for infection, but also obesity/high fat diet or air pollution, in the development and exacerbation of asthma, particularly more severe and steroid-resistant forms of disease, in both children and adults. Here, we describe the experimental investigations that we have performed that explore the potential roles that infections and infection-induced responses play in the pathogenesis of disease, and highlight novel mechanisms and treatment strategies for severe, steroid-resistant asthma that we have identified. We also briefly outline the roles that obesity/high fat diet and air pollution play in asthma. We discuss how the mechanisms and treatments that we have identified from our infection-associated studies may be applicable for treating obesity- and/or pollution-associated disease.

2 | INFECTIONS AND SEVERE, STEROID-RESISTANT ALLERGIC AIRWAY DISEASE (AAD) AND ASTHMA

2.1 | Early life infections and the development of severe AAD and asthma

Increasing clinical and experimental evidence supports the role of early life infections in shaping the phenotype of immune responses to antigens in later life and in the development of asthma, particularly severe forms of the disease.^{27,64–68} Respiratory infections in early life may also induce irreparable damage to lung structure, resulting in reduced lung function in later life. In humans, the lungs are unique among organ systems in that they continue to undergo significant maturation during the first 2 years of life, with only approximately 10% of the adult pulmonary alveolar component present at birth.⁶⁹ Thus, deleterious alterations in pulmonary architecture caused by early life infection may be irreversible and lead to fixed alterations in lung function. Importantly, respiratory infections in early life have been shown to adversely affect lung function well into adulthood, even in the absence of asthma.⁷⁰

Several infections, particularly viral respiratory infections, are associated with the development of asthma.^{67,68,71} Interestingly, studies have shown that the offspring of parents who have asthma or allergy, are at increased risk of experiencing viral respiratory infections, and that these infections are more likely to provoke wheezing in infancy and lead to the development of asthma in later life.^{72–74} Indeed, asthmatic airway epithelial cells exhibit a heightened intrinsic capacity for IL-25 expression, and heightened type 2 responses, that promote aberrant inflammatory responses during rhinovirus infections.⁷⁵ Together, these findings suggest that atopy plays an important role in susceptibility to primary virus infection, and predisposition to, and virus-induced exacerbations of asthma.

Respiratory syncytial virus (RSV) infections in early life are strongly linked with the development of asthma.⁶⁷ Sigurs et al.⁷⁶ showed that family history of asthma in combination with severe RSV infection increases the likelihood of developing asthma in children at age 7. A recent study showed that RSV lower respiratory tract infection (LRTI) in infancy is associated with an increased risk of permanent

pulmonary obstruction in adulthood.⁷⁷ Another study showed that severe RSV infection-induced bronchiolitis in infancy is associated with increased risk of developing allergic asthma that persists into later life.⁷⁸ Indeed, a systematic review of 28 articles, that assessed estimates of asthma risk after RSV infection-associated hospitalization during infancy, found that infants that were hospitalized for RSV infection were more likely to develop asthma compared to those that were not hospitalized.⁷⁹ However, despite the number of studies showing associations of infant RSV infection with the development of asthma, the precise nature of the link between infection and the pathogenesis of disease is controversial and remains undefined. To address this, the links between RSV infection in infancy and the development of asthma have been explored experimentally.

Respiratory infection with RSV induced mucus hypersecretion and airway hyper-responsiveness (AHR) in murine models of AAD.^{80,81} Others showed that neonatal infection with RSV predisposes to the development of exaggerated airway eosinophil influx and AHR during re-infection, however, infection in later life was protective.⁸² These findings were extended using a natural mouse paramyxovirus (pneumonia virus of mice [PVM]) infection to model the natural host: pathogen interaction of RSV infections and asthma in mice. PVM infection resulted in allergic sensitization to ovalbumin (Ova), and subsequent Ova-induced AAD, in infected neonatal mice compared to uninfected controls.⁸³ PVM infection-induced AAD was characterized by elevated serum levels of allergen-specific IgE and expression of the type 2 cytokines IL-4, IL-5, and IL-13.⁸³ PVM infection-induced AHR even in the absence of AAD, which agrees with clinical findings that RSV infections in early life result in permanent lung obstruction. Type 2 cytokine-mediated signaling plays an important role, since infection-induced AAD was abrogated in IL-4 receptor alpha chain-deficient mice.⁸³ Conversely, natural killer (NK) cell-mediated type 1 responses protected against PVM-associated AAD.⁸⁴ These findings suggest that RSV infections in early life may augment type 2 responses in the lung that persist into later life and predispose to the development of AAD and asthma. Together, with clinical association studies, these findings indicate the potential for developing and testing RSV-targeted strategies for wheeze in infancy and the subsequent development of asthma, particularly in children with a family history of asthma and atopy.⁸⁵

In addition to the role of virus infections in early life, increasing evidence supports a role for *Chlamydia* respiratory infections in the development of asthma. *Chlamydia* respiratory infections are a primary cause of pneumonia in early life. *C. trachomatis* is regarded as one of the most common causes of neonatal pneumonia,^{86,87} while *C. pneumoniae* accounts for a significant proportion of CAP in children and young adolescents.⁸⁸ It has been reported that up to 20% of pregnant women have cervical *C. trachomatis* infections.^{89–91} However, due to the asymptomatic nature of *Chlamydia* infections, its prevalence among pregnant women may be substantially greater than reported. Significantly, numerous studies demonstrate that between 40% and 50% of pregnant women have antibodies against *C. trachomatis*.⁹² Transmission of *C. trachomatis* from mother to child occurs during passage through the infected cervix and it is estimated that approximately 100 000 neonates in the USA are exposed annually.^{91,93} Inclusion

conjunctivitis and a distinctive afebrile pneumonia often develop in the early stages of life following exposure to *C. trachomatis* during birth.⁹¹ Indeed, pneumonia develops in up to 20% of newborns born to infected women.^{91,92} Although the disease is usually mild and does not require hospitalization, infection with *C. trachomatis* accounts for one-third of all cases of pneumonia in children under 6 months of age that require hospitalization.⁹² Untreated infants are usually ill for several weeks, presenting with frequent cough and reduced weight gain. Significantly, children who developed pneumonia as a result of *C. trachomatis* infection in early life were more likely to progress to abnormal lung function and chronic cough in later life compared to age-matched, non-infected children.⁹⁴ It is possible that children may also have an asymptomatic infection that does not present as pneumonia, but results in altered immune programming and lung structure to promote the development of asthma in later life.

Chlamydia pneumoniae is also an important respiratory pathogen that is a common cause of community-acquired pneumonia (CAP) in children over the age of 5 years.^{88,95} Numerous recent clinical studies have demonstrated links between *C. pneumoniae* infection and the development of asthma in children. Teig et al.⁹⁶, investigated the presence of *C. pneumoniae* DNA in nasal swabs and induced sputum from 38 children with stable chronic lung disease, 26 of whom had asthma, compared to 42 healthy controls. They showed that approximately 24% of the children with lung disease, but none of the controls, were PCR-positive for *C. pneumoniae*, indicating that this bacterium is present in the airways of a substantial proportion of children with stable chronic lung disease. Webley et al.⁹⁷, investigated the frequency of viable *C. pneumoniae* infection in the blood and BAL fluid of pediatric patients with severe chronic respiratory syndromes, of which, 60% were asthmatic. Of the BALF samples collected from their patients, 54% were PCR-positive and 31% were culture-positive for *C. pneumoniae*. Importantly, 67% of asthmatic subjects were PCR-positive and 33% were culture-positive for *C. pneumoniae*. There was also evidence of systemic involvement where 34% of the whole cohort was culture-positive for *C. pneumoniae* in their blood. Interestingly, the authors also observed that *C. pneumoniae*-positive BALF cultures correlated with elevated serum levels of *Chlamydia*-specific IgE. *C. pneumoniae*-specific IgE has been detected at higher levels and more frequently in the BALF of pediatric patients who did not respond to corticosteroid treatment, indicating that *C. pneumoniae* infection may be associated with steroid-resistant asthma.^{98,99} These studies are of particular importance as they demonstrate that *C. pneumoniae* respiratory tract infection is associated with severe asthma in pediatric patients.^{96,97} Additionally, a recent study demonstrated that infants with diagnosed wheeze are more likely to develop asthma after infection with *C. pneumoniae* than non-infected, wheezing infants.¹⁰⁰ Collectively, these studies indicate that *Chlamydia* respiratory infections may promote the development, and increased severity, of asthma in children. *Chlamydia* resides low in the respiratory tract where it is difficult to sample and specific methods are needed for detection, which are not part of routine microbiology sampling.^{97,101,102} This suggests that it is a prevalent infection that could have insidious and underappreciated effects on the developing respiratory tract, and the links are greater than described above.

We have investigated the mechanisms of how early life *Chlamydia* respiratory infections are associated with the induction and increased severity of AAD in later life using mouse models.^{66,103–109} We developed neonatal, infant, and adult models of *Chlamydia* respiratory infection and combined these with an established model of Ova-induced AAD in order to investigate the impact of age of *Chlamydia* infection on AAD. Infections at all ages result in increased inflammation and reduced weight gain (neonates and infants) or weight loss (adults) with infections peaking at 10 days and clearing by 20 days postinfection. AAD was induced in infected mice 6 weeks postinfection. We showed that neonatal and infant, but not adult, infections result in mixed type 1/type 2 immunity with increased IL-13 and interferon (IFN)- γ responses that are associated with greatly increased mucus secreting cell (MSC, that is, goblet cell) hyperplasia and AHR in AAD in later life compared to uninfected, age-matched controls.^{103,104} These effects on respiratory structure and function were subsequently confirmed by Jupelli et al.¹¹⁰ Interestingly, we show that while eosinophilic inflammatory responses in AAD were reduced by neonatal infection, infant infection resulted in increased airway eosinophil numbers.^{103,104} Importantly, we show that early life infections also result in exaggerated AHR, and neonatal, but not infant or adult, infections result in impaired lung development with increases in alveolar diameter, in mice in the absence of AAD.^{103,104} We have recently found that the inflammation and AHR becomes resistant to steroid therapy. This suggests that early life infection has detrimental effects on lung function, perhaps through effects on lung development/structure, even in the absence of asthma.

Thus, we have used experimental models of disease to demonstrate how early life respiratory infections increase the severity of AAD in later life and show that this likely occurs through different mechanisms depending on the pathogen and age of infection. These findings support clinical links between early life infections with respiratory pathogens and the development of asthma, particularly more severe forms of disease. Further investigations into the mechanisms that underpin the effects of different infections, or age of infection, may identify new therapeutic targets to either protect against the development of asthma, particularly severe forms of disease, or effectively treat the condition. Our subsequent investigations that have examined potential mechanisms that underpin early life infection-induced severe disease in later life are discussed below.

2.2 | Infection-associated severe, steroid-resistant AAD and asthma

There is strong clinical evidence to suggest that respiratory infections play important roles in the pathogenesis of severe, steroid-resistant asthma and neutrophil-dominated endotypes of disease. The association between infection and severe, steroid-resistant asthma may explain the evidence for increased activation of innate immune responses, neutrophilic inflammation, and type 1 and type 17 responses observed in these endotypes of disease. Interestingly, recent studies investigating airway microbiomes in asthma have shown that bacterial burden (indicated by 16S expression) is higher in patients with low levels of expression of type 2 cytokines in the airways, and negatively

correlates with bronchial eosinophil numbers.^{111,112} Importantly, we also show decreased microbial diversity in patients with neutrophilic asthma.¹¹³

Chlamydia pneumoniae Cpn has been estimated to account for 5% of cases of bronchitis and sinusitis, and up to 22% of cases of CAP requiring hospitalization,^{114,115} making it the third most commonly diagnosed bacterial cause of CAP behind *Streptococcus pneumoniae* (50%–66% of all cases)^{116,117} and *Mycoplasma pneumoniae* (30% of all cases).¹¹⁸ Moreover, several studies have shown that *C. pneumoniae*-specific antibodies are found in 50%–80% of healthy young adults, suggesting that there is a high prevalence of asymptomatic infection within the healthy adult population.^{98,99,114,119} This further indicates that early life exposures may be prevalent and underappreciated. Importantly, increasing clinical evidence links *Chlamydia* respiratory infection with severe, steroid-resistant asthma. Exacerbating asthmatics with evidence of *Chlamydia* infection have increased neutrophil numbers in their airways.¹²⁰ Furthermore, asthmatics with evidence of *Chlamydia* infection are more likely to have disease that is resistant to steroid therapy and show clinical markers of severe asthma.^{121–123} Indeed, increased neutrophil numbers in a cohort of patients with severe, refractory asthma predicted the presence of *Chlamydia* infection.¹²¹ It is known that *Chlamydia* can infect and grow in immune cells, and so infection-associated inflammation may facilitate its spread.¹²⁴

Haemophilus influenzae is also commonly isolated from the airways of patients with neutrophilic asthma.^{38,125} Significantly, we showed that 60% of patients were culture-positive for *H. influenzae* in a cohort of stable asthmatics with high levels of potentially pathogenic bacteria in their airways and that this cohort was much more likely to have non-eosinophilic asthma and be on high doses of ICS.¹²⁵ Roles for potentially pathogenic bacteria, including *H. influenzae*, in severe, steroid-resistant asthma have also been reported by Green et al.¹²⁶, in a more recent study. In addition to bacterial infections, several viral infections are frequent exacerbators of asthma, namely influenza A, rhinovirus, and RSV infections,^{67,127–129} and steroid treatment has limited efficacy in controlling asthma that is associated with viral infections.^{67,129} However, while these clinical studies have highlighted links between infection and severe, steroid-resistant disease, they do not address the nature of the association, the mechanisms involved, or whether targeting infections, and/or infection-induced responses, in the lung may be effective for treatment.

We have developed experimental models of *Chlamydia*, *Haemophilus*, influenza, and RSV infections, and combined these with Ova-induced models of AAD that have the hallmark features of severe, steroid-resistant asthma. We are using them to investigate the clinical associations between infection and severe, steroid-resistant asthma, identify mechanisms and help inform novel therapies. In our early studies, we assessed the effects of ongoing *Chlamydia muridarum* and non-typeable *H. influenzae* infections during the induction of Ova-induced AAD in adult mice. We showed that the infections suppress type 2 and eosinophilic, but increase type 1 and/or type 17 and neutrophilic, inflammatory responses.^{130,131} This switch from type 2 immune-mediated eosinophilic AAD to neutrophilic AAD was dependent upon neutrophil-mediated responses induced during infection in *Chlamydia*-induced AAD and

infection-induced IL-17 responses in *Haemophilus*-induced AAD.^{130,131} We consider that severe asthma results when patients with asthma become infected and this drives the severe, steroid-resistant phenotype. Thus, we refined our model to induce AAD and then introduce infection. We know that AAD in mice wains over time and that a second set of Ova challenges is needed to recapitulate the AAD phenotype. We showed that when this occurs after an infection, AAD becomes severe and steroid-resistant compared to uninfected controls. This represents an allergen-induced exacerbation after infection in asthmatic patients and enables us to assess the impact of infection on AAD.^{132–134}

We used these models to show that inflammation is neutrophilic and that inflammation and AHR are resistant to intranasal treatment with the steroid, dexamethasone (DEX), in both *Chlamydia* and *Haemophilus*-associated AAD.^{132–135} Importantly, we also show that infection-induced, severe, steroid-resistant AAD is not only associated with increased neutrophilic inflammation and type 1 and/or type 17 immunity but also with increased TLR2, TLR4, IL-6, IL-1 β , caspase-1, and NLRP3 responses. All these responses have been associated with severe, steroid-resistant disease in humans as described above. We also show that, while these bacterial infections drive steroid-resistant, neutrophil-dominated disease, influenza and RSV infections result in the induction of severe, steroid-resistant asthma that is dominated by eosinophilic inflammation.¹³³ These were the first studies to demonstrate how *Chlamydia*, *Haemophilus*, influenza, and RSV infections may alter key immune and inflammatory responses in the adult asthmatic lung to drive the development of severe, steroid-resistant asthma. Our findings suggest that targeting infection and/or infection-induced responses in severe asthma may be effective therapeutic strategies. Indeed, we have also shown that the macrolide, clarithromycin, suppressed both *Chlamydia* and *Haemophilus*-induced, severe, steroid-resistant AAD by targeting the predominant immune responses that drive disease in either model, and that these immunomodulatory effects were independent of the antimicrobial effects of the drug.¹³² These studies provide valuable insights into the mechanisms of severe, steroid-resistant asthma and further strengthen the case for macrolide therapy, in the treatment of severe, steroid-resistant asthma in not only adults but also for children with infection-associated disease.^{101,136,137}

Our models of neutrophil and eosinophil-enriched, steroid-resistant AAD accurately represent different subsets of disease observed in humans and, therefore, provide a valuable platform for studying mechanisms of pathogenesis of severe, steroid-resistant asthma, and to test novel therapies that are applicable to multiple endotypes of disease. We now discuss some of the novel mechanisms and treatment strategies that we have identified using these models.

3 | NOVEL MECHANISMS OF SEVERE, STEROID-RESISTANT ASTHMA IDENTIFIED IN EXPERIMENTAL MODELS OF DISEASE

The significance of the experimental models that we have developed and used to examine the clinical associations between infection and

asthma extend beyond demonstrating roles for infection in the pathogenesis of severe, steroid-resistant asthma. They represent novel tools that can be used to gain valuable insights into the potential mechanisms that underpin the pathogenesis of disease. Indeed, our recently published studies used these experimental models to identify key roles for increased phosphoinositide-3-kinase (PI3K), TLR signaling and NLRP3 inflammasome responses, and altered microRNA (miRNA), cytokine and immune factor expression, in the pathogenesis of severe, steroid-resistant asthma (Figure 2). Notably, our studies provide compelling evidence that these processes may be involved in the pathogenesis of severe, steroid-resistant asthma and can be therapeutically targeted for the effective treatment of disease.

3.1 | PI3K

Several studies implicate PI3K signaling in the pathogenesis of asthma. In a previous study, Duan et al.¹³⁸, examined the role of PI3K in allergic airway inflammation in an Ova-induced model of mild to moderate

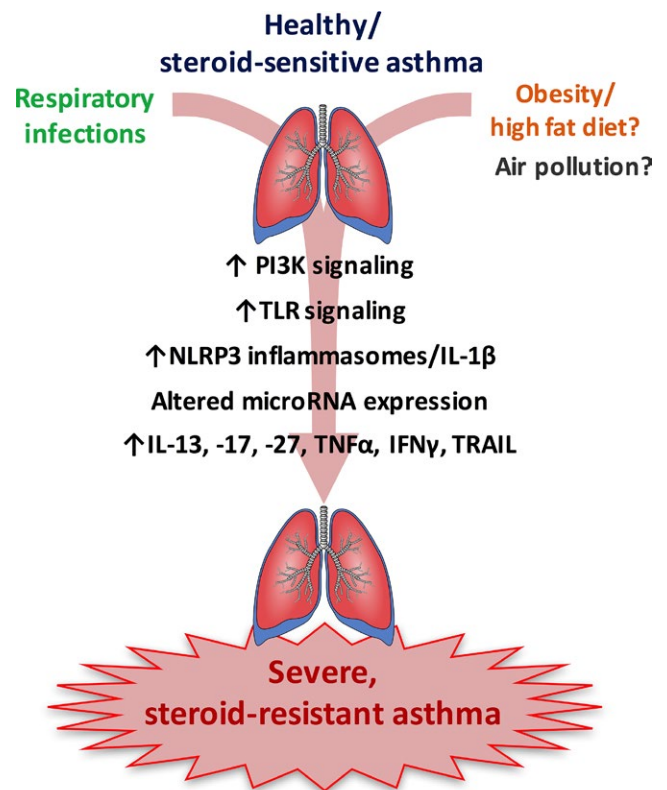


FIGURE 2 Mechanisms of severe, steroid-resistant asthma. Clinical and experimental studies demonstrate that respiratory infections in healthy individuals or individuals with steroid-sensitive asthma; increase phosphoinositide-3-kinase (PI3K) and TLR signaling and NLRP3 inflammasome-dependent, IL-1 β -mediated responses, alter microRNA (miR) expression profiles and increase the production of a number of cytokines and immune factors. These infection-induced changes in the lung play important roles in the development of severe, steroid-resistant asthma. Importantly, targeting these factors with PI3K inhibitors, MCC950, miR-specific antagonists, or cytokine-/immune factor-specific antibodies (Abs) may be effective for the treatment/prevention of disease

allergic asthma in mice. They showed that treatment with LY294002 (a non-selective pan-PI3K inhibitor), 2 hours before Ova-aerosol challenge of allergic mice, suppressed the levels of pAkt in the lungs in AAD compared to sham-treated, allergic controls. Furthermore, treatment with LY294002 decreased IL-5, IL-13, and eotaxin levels in the BALF, and suppressed eosinophilic airway inflammation, tissue eosinophilia, mucus production, and AHR, in AAD. In another study, Kwak et al.¹³⁹, demonstrated that the levels and activity of phosphatase and tensin homolog (PTEN) were decreased in mice with Ova-induced AAD. They also reported that the epithelial layers around the bronchioles in control mice had higher levels of PTEN, and that these levels were markedly decreased following Ova aerosol challenge. Treatment with wortmannin (another non-selective pan-PI3K inhibitor), LY294002, or adenovirus-mediated overexpression of PTEN, during the challenge phase suppressed Ova-induced increases in the levels of IL-4 and IL-5 in the BALF, and suppressed AHR in AAD. These results were supported by Ezeamuzie et al.¹⁴⁰, who showed a role for PI3K in allergen-induced increases of eosinophil peroxidase activity (assessed in BALF) and AHR in guinea pigs. Together, these studies show that targeting PI3K directly in the lungs with non-specific pharmacological inhibitors can suppress allergic inflammation. More recent studies have advanced these findings by investigating the roles of specific PI3K isoforms. In one study, Lee et al.¹⁴¹, treated Ova-sensitized mice with IC-87114 (a selective PI3K δ inhibitor) and demonstrated that PI3K δ activity promotes allergic airway inflammation (eosinophils, neutrophils, and lymphocytes) and AHR, as well as increases in the levels of total IgE in the serum, and Ova-specific IgE in the BALF. Additionally, two independent studies demonstrated that Ova-sensitized, PI3K γ -deficient mice had diminished allergen-induced eosinophilic airway inflammation and decreased airway remodeling.^{142,143} Collectively, these studies highlight the potential importance of the PI3K δ and PI3K γ isoforms in the pathogenesis of asthma. However, there is little direct evidence of the contribution of PI3K in the pathogenesis of severe, steroid-resistant asthma.

Significantly, several studies implicate PI3K signaling in the manifestation of steroid insensitivity, which may be relevant to severe asthma and its poor control. In a seminal study, Marwick et al.⁵³, demonstrated that the inhibition of PI3K δ restored steroid sensitivity in a mouse model of cigarette smoke-induced COPD. They showed that cigarette smoke exposure in mice reduced the activity of HDAC2 in the lung and also reduced glucocorticoid function. Significantly, PI3K δ kinase dead knock-in transgenic mice exhibited normal HDAC2 activity and glucocorticoid function as well as reduced tyrosine nitration of HDAC2. These results were supported by To et al.¹⁴⁴, who also examined the role of PI3K δ in the development of steroid insensitivity in COPD. The authors demonstrated that treatment of cigarette smoke-exposed mice with low-dose theophylline (another non-selective PI3K inhibitor) increased the anti-inflammatory effect of steroids (DEX) and restored HDAC2 activity. Similar effects were observed following treatment with IC-87114. We have also shown that PI3K signaling is exaggerated in COPD patients and in experimental COPD¹⁴⁵ that is resistant to steroid treatment.¹⁴⁶ Furthermore, theophylline treatment of peripheral blood mononuclear cells (PBMCs)

from patients with COPD restored steroid sensitivity as determined by decreased IC₅₀-DEX values. The authors also show that PBMCs pretreated with HDAC2 siRNA exhibited increased IC₅₀-DEX values. Taken together, these studies provide strong evidence for the role of PI3K signaling in promoting the development of steroid insensitivity. Furthermore, they highlight the potential importance of therapeutic strategies that target PI3K signaling in order to re-instate HDAC2 activity and sensitivity to steroids in the context of COPD. However, they do not address the role or potential for therapeutic targeting of aberrant PI3K signaling in severe, steroid-resistant asthma.

To assess this possibility, we investigated PI3K signaling pathways in our murine models of infection-induced, severe, steroid-resistant AAD.¹³³ We demonstrate that nuclear pAkt levels are increased in experimental severe, steroid-resistant AAD compared to steroid-sensitive AAD.¹³³ This increase in pAkt is indicative of exaggerated PI3K signaling. Significantly, this increase in pAkt in severe, steroid-resistant AAD was associated with reduced HDAC2 expression and nuclear protein levels compared to steroid-sensitive controls.¹³³ In order to demonstrate the functional relevance, and therapeutic targeting, of aberrant PI3K signaling in severe, steroid-resistant asthma, we treated mice intranasally with LY294002 with and without DEX treatment during infection-induced, severe, steroid-resistant AAD. LY294002 treatment suppressed aberrant pAkt nuclear levels and reversed the suppression of HDAC2 responses.¹³³ Significantly, we showed that treatment also restored steroid sensitivity to inflammation and AHR in severe, steroid-resistant AAD.¹³³ These findings implicate a potential role for aberrant PI3K signaling in severe, steroid-resistant asthma and highlight the therapeutic potential for PI3K inhibitors for the treatment of disease. Interestingly, we have also shown that enhanced PI3K signaling increases susceptibility to influenza virus infection in clinical and experimental COPD,¹⁴⁵ using mouse models that accurately recapitulate the hallmark features of that disease.¹⁴⁷ These findings suggest that targeting PI3K signaling may also be effective for treating infection in influenza-associated exacerbations of severe, steroid-resistant asthma.

3.2 | TLR signaling

Many studies have demonstrated important roles for TLR signaling in both sensitization and exacerbation in murine models of AAD, and in the development and exacerbation of asthma in humans.^{148–150} Importantly, as outlined earlier, clinical studies have shown that TLR responses may be altered in severe, steroid-resistant asthma, particularly in the context of infection-associated disease. We have performed several studies that have investigated the roles of TLR signaling in the pathogenesis of disease.

Using TLR2- and TLR4-deficient mice, we have shown that these TLRs play an important role in the induction of immune and inflammatory responses and clearance of infection during early life *Chlamydia* respiratory infection.¹⁰⁹ TLR2-deficient mice had increased IL-17 responses and neutrophil numbers in the lung, and reduced IFN- γ production, during infection compared to wildtype (WT) controls.¹⁰⁹ These events corresponded to more severe signs of disease and

increased chronic bacterial infection. Others have also shown that TLR2 ligation induces steroid resistance in lung epithelial cells.¹⁵¹ Interestingly, we also showed that TLR4-deficient mice had reduced IL-17 and IFN- γ responses, and were protected against infection-induced disease (although bacterial numbers were similar to WT controls).¹⁰⁹ These findings demonstrate important, albeit complex, roles for TLR signaling in early life *Chlamydia* respiratory infection and suggest that the interplay between different TLR signaling molecules may play an important role in determining the outcomes of early life infection-induced severe AAD in later life. Indeed, a deficiency in TLR7 signaling plays an important role in early life PVM infection-induced allergic sensitization and development of AAD.¹⁵²

We have also investigated the interactions between type 1 cytokine and TLR4 signaling in the development of steroid-resistant asthma.^{30,153} The adoptive transfer of Ova-specific IFN- γ -producing T-helper type (T_H)₁ cells into mice resulted in the macrophage-dependent induction of steroid-resistant AHR upon airway exposure to Ova and LPS, but not Ova or LPS alone.³⁰ The adoptive transfer of Ova-specific T_H2 cells induced AHR that was sensitive to steroid treatment. Using a combination of cytokine-depleting antibodies, and innate immune signaling molecule-deficient mice, we then extended these findings to show that both IL-27 and IFN- γ responses uniquely synergize with TLR4-induced, MyD88 signaling pathways to inhibit GR nuclear translocation in macrophages.^{30,153} These findings suggest that a combination of type 1 responses and TLR4 signaling can mediate the induction of macrophage-mediated, steroid-resistant AHR.⁴⁵ Since both increased type 1 signaling and TLR4 activation are potentially induced by pathogen exposure, these findings further support a role for respiratory infections in the pathogenesis of severe, steroid-resistant asthma.

Together, these findings demonstrate roles for altered TLR signaling in the pathogenesis of severe, steroid-resistant asthma, and that correcting aberrant or defective TLR signaling and/or the downstream effects of altered TLR signaling pathways may be effective treatments.

3.3 | NLRP3 inflammasome-mediated, IL-1 β responses

Several NLRs (NLRP1, NLRP3, and NLRC4) can assemble multiprotein complexes termed "inflammasomes" that serve as platforms for the recruitment and activation of inflammatory caspases such as Caspase-1.^{32,134,154–159} Active Caspase-1, in turn, activates the IL-1 family cytokines, IL-1 β and IL-18, and inactivates IL-33.^{154–157} Studies have also reported that the non-NLR protein absent in melanoma 2 (AIM2; also called PYHIN4) can assemble an inflammasome that recruits and activates Caspase-1.^{160–162} The NLRP3 inflammasome (also called NALP3, PYPAF1, or cryopyrin) is the best characterized, and has been widely implicated in a range of inflammatory diseases. This inflammasome is structurally comprised of NLRP3 and an apoptosis-associated speck-like protein containing a CARD (ASC) adapter protein and pro-Caspase-1.^{154,156,157,159} Importantly, inflammasomes require two separate events, assembly and activation, to exert their effector functions. Expression and assembly of inflammasome components

is induced by signal 1 from pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) and double-stranded (ds)DNA, that trigger TLR and NLR signaling.¹⁵⁶ Danger-associated molecular patterns such as extracellular ATP, potassium efflux, and monosodium urate crystals, provide a second signal and activate the assembled inflammasome.^{156,157} Upon activation, the NLRP3 inflammasome interacts with ASC, which results in autocatalysis of pro-Caspase-1 and activation of Caspase-1.^{156,163} This potentially induces inflammation through the cleavage of pro-IL-1 β and pro-IL-18 causing the release of active IL-1 β and IL-18.¹⁵⁶

There is mounting evidence that implicates the NLRP3 inflammasome in the pathogenesis of allergic asthma, however, its role remains controversial. The levels of ATP and P2X7 receptor (P2X7R) are increased in asthma, and the inhibition of ATP-mediated P2X7R signaling suppressed key disease features in experimental asthma.^{164,165} This suggests that ATP-mediated NLRP3 inflammasome activation is involved in asthma pathogenesis. Importantly, mouse models of asthma often involve sensitization to protein allergens (such as Ova) in the presence of the T_H2-inducing adjuvant aluminum hydroxide (alum), which is a known activator of the NLRP3 inflammasome.¹⁶⁶ A study by Eisenbarth et al.¹⁶⁷, used NLRP3-deficient mice to demonstrate that NLRP3 is required for adjuvanticity of alum in allergic antibody responses to antigen. Another study demonstrated the importance of NLRP3 in allergic airway inflammation using an adjuvant (alum)-free Ova model.¹⁶⁸ This study used mice deficient in NLRP3, IL-1 receptor (IL-1R)1, IL-1 β , or IL-1 α to show that NLRP3-mediated IL-1 β responses are required for Ova-induced allergic airway inflammation. Indeed, all of these factor-deficient mice exhibited marked decreases in the production of Ova-induced, T_H2-associated cytokines. In contrast, a different study by Kool et al.¹⁶⁹, demonstrated that uric acid potentially induces T_H2 lymphocyte immunity in an NLRP3-independent manner. They also showed that uric acid promoted T_H2 immunity through PI3K δ . These findings are supported by a different study performed by Allen et al.¹⁷⁰, which showed that WT and NLRP3-deficient mice exhibited no differences in the key features of acute or chronic Ova-induced allergic airway disease, including eosinophilic airway inflammation, mucus hypersecretion, and AHR. Taken together, the role of the NLRP3 inflammasome in the pathogenesis of allergic asthma is controversial and remains to be defined.

Most significantly, augmented NLRP3 inflammasome activation, and exaggerated production of IL-1 β , are strongly implicated in the pathogenesis of severe, steroid-resistant asthma.^{35,36,171–175} Simpson et al.³⁸, showed that neutrophilic asthmatics had increased expression of TLR2, TLR4, IL-1 β , and IL-8 and elevated levels of LPS in their sputum compared to other asthma phenotypes and healthy controls. These data are supported by a different study by Baines et al.³⁶, who performed gene expression profiling analyses on induced sputum from different subtypes of asthmatics and showed that the overexpression of factors involved in the IL-1 and tumor necrosis factor (TNF)- α /NF- κ B signaling pathways correlated with neutrophilic airway inflammation. Simpson et al., also showed that the sputum macrophages of neutrophilic asthmatics exhibited elevated expression of NLRP3, Caspase-1, Caspase-4, Caspase-5, and IL-1 β , compared to other

groups. They also showed that neutrophilic asthmatics have increased protein levels of IL-1 β in the sputum that correlated with IL-8 levels.³⁵ Furthermore, immunocytochemical analyses revealed that sputum neutrophils from patients with neutrophilic asthma, but not other asthma phenotypes, exhibited strong immunoreactivity for NLRP3 and Caspase-1. Collectively, these studies strongly implicate roles for innate immune activation and NLRP3 inflammasome signaling in the pathogenesis of severe asthma. Importantly, and as discussed earlier in detail, substantial clinical and experimental evidence links *Chlamydia* and *Haemophilus* respiratory infections with severe, steroid-resistant asthma. Infections with these bacteria, as well as a number of other infections, have been shown to induce NLRP3 responses. This highlights the possibility that patients with asthma may encounter stimuli, such as respiratory infections, that induce innate immune responses that prime for NLRP3 inflammasome activity. Significantly, Darville et al.¹⁷⁶ have demonstrated that ATP-mediated P2X7R signaling is important for immunity against *Chlamydia* infection, which suggests that infection induces DAMPs signaling. Furthermore, both *Chlamydia* and *Haemophilus*, as well as influenza and RSV, respiratory infections can induce the release of active IL-1 β in an NLRP3 inflammasome-dependent, Caspase-1-mediated manner.^{177–180} These data highlight the possibility that respiratory infections can induce immune responses in the asthmatic lung that can prime for, and activate, NLRP3 inflammasome activity.

In order to investigate the role of the NLRP3 inflammasome, Caspase-1 and IL-1 β in severe, steroid-resistant asthma we performed a series of analyses on sputum samples from patients with asthma and our *Chlamydia*- and *Haemophilus*-induced models of severe, steroid-resistant AAD.¹³⁴ Our clinical team showed that the sputum expression of NLRP3 and IL-1 β positively correlated with sputum neutrophil numbers and negatively correlated with airflow obstruction and asthma control in a cohort of stable asthmatics, the majority of which were on ICSs. Importantly, NLRP3 and IL-1 β expression is higher in patients with severe asthma than those with mild asthma. These key clinical findings, for the first time, show a link between NLRP3 and IL-1 β responses and severe, steroid-resistant, neutrophilic asthma. We then extended upon these findings to show that NLRP3, Caspase-1, and IL-1 β responses, are all increased in infection-induced, severe, steroid-resistant AAD compared to steroid-sensitive AAD. To examine the functional roles of NLRP3, Caspase-1 and IL-1 β in the pathogenesis of disease, mice were treated intranasally with MCC950 (a highly selective inhibitor of the NLRP3 inflammasome¹⁸¹), ac-YVAD-cho (selective caspase-1 inhibitor), or anti-IL-1 β monoclonal antibody during infection-induced, severe, steroid-resistant AAD. We showed that all three treatments were effective for suppressing aberrant IL-1 β responses in our models and that this corresponded to the suppression of steroid-resistant neutrophilic inflammation and AHR in AAD. We then extended these findings by showing that the administration of recombinant IL-1 β to the airways of naïve mice or mice with steroid-sensitive AAD resulted in the induction of steroid-resistant neutrophilic inflammation and AHR. Finally, we showed that the depletion of neutrophils could suppress IL-1 β -induced, steroid-resistant AHR, which demonstrates an important role for neutrophilic inflammation

in the pathogenesis of IL-1 β -induced steroid-resistant disease. These findings demonstrate, for the first time, a novel role for NLRP3-dependent, Caspase-1-mediated, IL-1 β responses in the pathogenesis of severe, steroid-resistant asthma, and highlight that these responses may be targeted therapeutically for the treatment of this disease.

3.4 | miRNAs

miRNAs are highly conserved, single-stranded non-coding RNA molecules (approximately 22 nucleotides in length) that regulate gene expression at the post-transcriptional level, either through the inhibition of mRNA translation or the degradation of mRNA transcripts. Recent evidence suggests that each miRNA can target as many as 200 gene transcripts.^{182,183} A large body of evidence implicates miRNAs in a multitude of biological processes, including immune responses. However, defining their precise functional role is often challenging, and can be complicated by many factors, particularly the identification of their targets. Given that miRNAs can potentially regulate up to 30% of all protein-coding genes, and that they can be targeted with antisense inhibitors, it is essential to decipher their contributions to both innate and acquired immune function in disease states.¹⁸⁴

Increasing evidence implicates miRNAs in asthma pathogenesis, as well as other chronic respiratory diseases, and as potential therapeutic targets for treatments.^{1,185–190} Given the potential for miRNAs to regulate a large number of cellular processes that are crucial to the pathogenesis of disease, targeting alterations in miRNA expression in the asthmatic lung may have greater efficacy for the treatment of disease than steroids. Studies have shown that the expression patterns of miRNAs are markedly different in the bronchial epithelium of asthma patients compared to healthy controls and that steroids have only modest effects on these altered miRNA responses.¹⁹¹ A substantial amount of effort has been dedicated to characterizing the roles that miRNAs play in the pathogenesis of asthma in order to determine those that are the most likely therapeutic targets for the treatment of asthma.

miRNA (miR)-21 has been shown to be important in a number of murine models of AAD.^{192,193} miR-21 is highly upregulated during pulmonary inflammation¹⁹² and promotes T_H2 immune polarization through the suppression of IL-12p35.^{192,194} Moreover, miR-21-deficient mice have reduced eosinophilic inflammation and IL-4 levels with a concomitant increase in IFN- γ during Ova-induced AAD.¹⁹⁴ However, the inhibition of miR-21 during the challenge phase of house dust mite (HDM)-induced AAD in WT mice had no effect on the production of T_H2 cytokines or eosinophilic inflammation.¹⁹⁵ These studies suggest that miR-21 exerts potent immune polarizing effects during allergic sensitization rather than during the exacerbation of disease. We have defined potential roles of infection-induced miR-21 responses in the development of severe, steroid-resistant asthma.¹³³ We demonstrated that miR-21 expression is increased in the airways of *Chlamydia*-infected, allergic mice prior to Ova-induced exacerbation of severe, steroid-resistant AAD.¹³³ Importantly, we show that suppression of increased miR-21 expression by treatment with a miR-21-specific inhibitor (antagomir, Ant-21) restored steroid

sensitivity to airway inflammation and suppressed AHR in *Chlamydia*-, *Haemophilus*-, influenza-, and RSV-induced, severe, steroid-resistant AAD.¹³³ Importantly, since miR-21 downregulates the expression of PTEN,^{196,197} which antagonizes PI3K-dependent signaling,^{196–199} we hypothesized that overexpression of miR-21 and its attenuation of PTEN promotes steroid insensitivity by amplifying PI3K-mediated, suppression of HDAC2. We confirmed this by showing that PTEN responses are decreased, PI3K signaling is increased, and HDAC2 responses are impaired, in *Chlamydia*-induced, severe, steroid-resistant AAD.¹³³ Notably, we show that Ant-21 treatment restores PTEN expression, which corresponds with the suppression of PI3K signaling, and restoration of HDAC2 levels.¹³³ These findings demonstrate a novel miR-21/PTEN/PI3K/HDAC2 axis in pathogenesis and suggest that targeting aberrant miR-21 responses may be effective for the treatment of severe, steroid-resistant asthma. Importantly, our study shows that suppressing miR-21 may be effective in both neutrophil- and eosinophil-enriched subtypes of disease associated with bacterial or viral infections, indicating that miR-21-targeted therapeutic strategies may be widely applicable for the treatment of severe, steroid-resistant asthma.

miR-328 may also be a potential therapeutic target for *Haemophilus*-associated severe, steroid-resistant asthma. Our team has shown that airways tissue and macrophage expression of miR-328 is downregulated during *Haemophilus* respiratory infection.¹⁹⁰ We then showed that pre-incubation of macrophages and neutrophils with miR-328-specific antagomir (Ant-328) increased bacterial phagocytosis and intracellular killing processes by these cells upon exposure to *Haemophilus*, and that mice treated with Ant-328 were protected against infection in vivo.¹⁹⁰ We also show that Ant-328 treatment was capable of overcoming steroid- and COPD-induced susceptibility to worsened *Haemophilus* infection in mice.¹⁹⁰ Together, these findings suggest that miR-328 expression is decreased in the lung during *Haemophilus* infection in order to potentiate bacterial phagocytosis and destruction. They also highlight Ant-328 as a potent therapeutic agent for the treatment/prevention of *Haemophilus*-associated respiratory diseases, including severe, steroid-resistant asthma, where steroid therapy is likely to exacerbate bacterial infection while having no effect on disease.^{132,135}

Several studies highlight a potential role for miR-155 in the pathogenesis of asthma and also in severe, poorly controlled disease. Some studies have shown the importance of miR-155 in the pathogenesis of AAD.^{200,201} One used miR-155-deficient mice to show that miR-155 plays an important role in IL-33-dependent expansion of type 2 innate lymphoid cells (ILC2s) and the development of AAD.²⁰² Other evidence highlights a role for miR-155 in hypo-responsiveness to β 2 agonists, which is a clinical feature of severe asthma.²⁰³ Comer et al.²⁰⁴ showed that human airway smooth muscle cells (hASMCs) from patients with asthma that were exposed to a cocktail of pro-inflammatory mediators associated with severe asthma (IL-1 β , TNF- α , and IFN- γ), exhibited elevated miR-155 expression compared to hASMCs from non-asthmatics. They also found that the levels of miR-155 positively correlated with the expression and secretion of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2), respectively. COX-2 expression

and PGE2 secretion are linked with hypo-responsiveness to β 2 agonists.^{205–207} These data suggest that the increased levels of IL-1 β and type 1 (IFN- γ , TNF- α) cytokines observed in severe, steroid-resistant asthma may increase miR-155 responses that drive poor asthma control. We have previously shown that miR-155 is increased in steroid-sensitive AAD but that effectively targeting miR-155 expression for the treatment of disease is difficult due to differential expression and challenges associated with achieving miR-155 knockdown in different immune cell types.²⁰⁸ These findings highlight the need to examine the potential role and therapeutic targeting of miR-155 particularly in severe, steroid-resistant asthma in more detail.

Together, these studies highlight the potential role of miRNAs in mediating pathways associated with asthma and in the pathogenesis of severe, steroid-resistant asthma. Furthermore, they demonstrate the therapeutic potential of targeting altered miRNA responses for the treatment of disease.¹⁸⁵

3.5 | Cytokines and immune factors

We have also performed several studies to highlight the roles of individual cytokines and immune factors in the pathogenesis of early life infection-induced severe asthma in later life, and infection-induced, severe, steroid-resistant asthma.

We have conducted a series of studies that focused on the role of IL-13 signaling and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) responses, induced during early life *Chlamydia* infection, and the pathogenesis of severe AAD in later life.^{106,107} We showed that IL-13-deficient mice had reduced infection, inflammation and MSC hyperplasia during early life *Chlamydia* infection compared to infected WT controls. Importantly, IL-13-deficient mice were also protected against the permanent *Chlamydia*-induced AHR in later life, although they still had enlarged alveoli.¹⁰⁷ STAT6 deficiency had similar effects to IL-13 deficiency, suggesting that the IL-13-mediated effects on infection and infection-induced disease are dependent on IL-13 receptor signaling through the STAT6 pathway. We then showed that infection, and associated pathology, could be restored in IL-13-deficient mice through the administration of recombinant IL-13.¹⁰⁷ Finally, we showed that the neutralization of IL-13 during infection in WT mice was able to suppress early life *Chlamydia*-induced severe AAD in later life.¹⁰⁷ This identifies IL-13-mediated, STAT6 signaling as being critical for the induction of early life *Chlamydia* infection-induced severe AAD in later life. This indicates that targeting IL-13 signaling in children with evidence of *Chlamydia* infection may be effective in preventing infection-induced impairment of lung function and severe asthma in later life. Additionally, since we have also shown that IL-13 responses play an important role during infection in adult mice,^{2,209} IL-13-targeted therapies may also be effective for the treatment of severe, steroid-resistant asthma associated with respiratory infection in adult patients.²

TRAIL is an immune factor that regulates inflammation and apoptosis and has largely been studied in the context of tumor immunology. However, we, and others, have shown that TRAIL plays an important role in the pathogenesis of respiratory,^{210–212} and more recently gastrointestinal,²¹³ tract disease. Significantly, we show that TRAIL

plays an important role in early life *Chlamydia* infection-induced severe AAD in later life.¹⁰⁶ The expression of TRAIL and death receptor 5 (DR5; the apoptosis-inducing TRAIL receptor in mice) are significantly increased during neonatal *Chlamydia* lung infection that is associated with increased epithelial and endothelial cell apoptosis in the lung.¹⁰⁶ TRAIL-deficient mice are protected against increases in lung inflammation and MSC hyperplasia that are observed during neonatal *Chlamydia* infection in WT mice, despite showing similar DR5 expression.¹⁰⁶ Significantly, we show that TRAIL-deficient mice and WT mice treated with TRAIL-neutralizing antibody are protected against neonatal *Chlamydia* infection-induced alveolar enlargement, and increased AHR in later life.¹⁰⁶ These findings demonstrate an important pathological role for TRAIL in the development of early life infection-induced severe asthma in later life, and identify TRAIL signaling as a potential therapeutic target for the prevention of these events.

Numerous studies have linked increased type 17 responses with the pathogenesis of severe, steroid-resistant asthma.^{27,28,31,214,215} Indeed, we have shown that type 17 responses are increased in both *Chlamydia* and *Haemophilus*-induced, severe, steroid-resistant AAD.^{130–133,135} We extended these findings to show that these type 17 responses are critically important for induction of steroid-resistant neutrophilic inflammation and AHR in *Haemophilus*-induced, severe, steroid-resistant AAD.^{131,132} Importantly, we show that targeting increased IL-17 responses in *Haemophilus*-induced, severe, steroid-resistant AAD effectively suppresses disease.¹³¹

TNF- α responses are also associated with neutrophilic asthma³⁶ and TNF- α expression is refractory to steroid treatment in severe asthma.²⁷ Significantly, TNF- α responses are increased in *Chlamydia*-induced, severe, steroid-resistant AAD, and are responsible for the induction of steroid resistance in immune cells from *Chlamydia*-infected asthmatics.^{123,132,133} Importantly, we have recently shown that the depletion of TNF- α abolished steroid-resistant inflammation and AHR in our murine model of *Chlamydia*-induced, severe, steroid-resistant asthma.¹³² These findings suggest that anti-TNF- α treatments may be also beneficial in subpopulations of severe asthmatics with evidence of *Chlamydia* infection and/or excessive TNF- α responses.

In addition to IL-17 and TNF- α , targeting IFN- γ and/or IL-27 responses in patients with evidence of increased type 1 immunity, particularly in the presence of increased LPS levels and/or TLR4/MyD88 activity, may be effective for the treatment of steroid-resistant disease.^{30,153}

The findings identify several key factors that play important roles in the pathogenesis of severe forms of asthma. They also demonstrate the potential for targeting these factors for the treatment of disease. To date, studies targeting specific cytokines, including IL-13 and TNF- α , in severe, steroid-resistant asthma, have produced mixed results, however, these may not have been conducted in the most appropriate patient cohorts.²⁷ Our studies suggest that targeting specific cytokines and immune factors may be most effective in well-defined patient cohorts, where evidence for increased cytokine production is evident. New factors and mechanisms could be identified through 'Omics analysis (RNA-seq, proteomics) of human tissues and cells and representative animal models.²¹⁶

4 | BENEFICIAL INFECTIONS

Not all infections are deleterious in asthma. Probiotics may have beneficial effects,²¹⁷ and we have performed a series of studies that show widespread beneficial effects of *S. pneumoniae* and its vaccines and components in AAD and, potentially, asthma. Regulatory T-helper cells (T_{REG}) have anti-inflammatory properties and patients with allergic asthma have reduced levels of these cells that accompanies their allergic airway inflammation.²¹⁸ *S. pneumoniae* is unusual among bacteria in that its infections induce the outgrowth of T_{REGS}. We showed that low pathogenic *S. pneumoniae* infection suppressed the development of AAD when administered before, during, or after sensitization. Eosinophilic airway inflammation, T_H2 responses, mucus hypersecretion, and AHR were all suppressed. These effects were associated with increases in T_{REGS}, that suppress T_H2 responses, and the effects were removed with anti-CD25 treatment, that suppresses T_{REG} responses.²¹⁹ Infection is not necessary, since we observed similar effects on AAD with killed bacteria.²²⁰ This affect of killed bacteria has a partial requirement for TLR4 responses.¹⁵⁰ We have identified the components involved in *S. pneumoniae*-mediated suppression of disease, as protection can be achieved with combinations of capsular polysaccharide and the detoxified exotoxin pneumolysoid, which suppress established disease induced by systemic (Ova in alum) or airway (HDM) sensitization.²²¹ The combination of capsular polysaccharide and protein toxoid modulate the function of dendritic cells to induce T_{REGS} and suppress T_H2 and NK T-cell responses.²²² Fortunately, a current vaccine against *S. pneumoniae* (Prevenar) is also comprised of the capsular polysaccharides conjugated to a toxoid (diphtheria toxoid) that induces T-cell responses. Therapeutic use of this vaccine, including during established disease, again, suppressed the hallmark features of AAD.²²³ The roles of T_{REGS} in severe, steroid-resistant asthma have not been widely studied. It is possible that the beneficial anti-inflammatory effects of T_{REGS} induced by these and other bacterial components,²¹⁸ may also be effective in suppressing the pathogenic mechanisms that drive the pathogenesis of severe asthma. These possibilities would be valuable to study in the future.

5 | POTENTIAL ROLES FOR OBESITY AND HIGH-FAT DIET AND AIR POLLUTION IN SEVERE, STEROID-RESISTANT ASTHMA

5.1 | Obesity

Obesity is an important risk factor for asthma, with asthma risk being increased by 50% in overweight and obese individuals.²²⁴ Obesity appears to have a causal role in asthma, as prospective studies have reported that weight gain and obesity are associated with asthma onset.^{225–228} Obesity complicates asthma management as it leads to changes in respiratory physiology, including reduced dynamic (FEV₁, forced vital capacity)²²⁷ and static (expiratory reserve volume, functional residual capacity)²²⁹ lung volumes and reduced respiratory muscle strength.²³⁰ Obese patients also have reduced exercise capacity,²³¹ and increased dyspnoea and wheezing.²³² Thus, it is likely that

obesity not only plays a role in the development of asthma but also in the induction of more severe forms of disease.

Airway inflammation in obese asthmatics is not associated with type 2 responses²³³ as neither exhaled nitric oxide^{234–236} nor sputum eosinophils are increased in obese asthma.^{237,238} However, there is emerging evidence of increased neutrophilic airway inflammation in obese asthmatics,²³⁹ particularly in females.^{240,241} Systemic inflammation is also increased with elevated circulating levels of C-reactive protein (CRP) and IL-6 observed in obese patients, which positively correlate with neutrophilic airway inflammation.²³⁹ This pattern of inflammation is consistent with the observation that obese asthmatics have reduced responses to conventional asthma pharmacotherapy and are less likely to achieve asthma control using corticosteroids.^{242,243} As a result, obese asthmatics are prescribed higher doses of corticosteroids in attempts to manage their asthma symptoms, which results in increased healthcare expenditure and iatrogenic side effects. It is possible that the nature of the inflammation that develops in the presence of excess adipose tissue in obese asthmatics may result in reduced responsiveness to mainstay asthma medications. Obesity induces a chronic low-grade systemic inflammatory state through a variety of mechanisms that likely contribute to increased disease severity (Figure 2). Adipose tissue stores energy and plays an important role in regulating metabolic homeostasis by buffering lipid metabolism.²⁴⁴ After the intake of dietary lipids, adipose tissue clears blood lipids from the circulation. However, in the obese state, adipose tissue fails to store the excess triglycerides and free fatty acids. High-fat meals, which are commonly consumed by obese individuals, exacerbate this increase in circulating blood lipids, as the host is unable to adequately clear a bolus dose of fatty acids in the postprandial phase. As a result, obese individuals have chronically elevated levels of circulating saturated free fatty acids, which act as ligands for pattern recognition receptors, such as TLR4 and the NLRs. Upon activation, these pattern recognition receptors trigger pro-inflammatory processes such as the assembly of the NLRP3 inflammasome, and the release of Caspase-1, IL-1 β , and IL-18. In adipose tissue, IL-1 β responses promote the influx of pro-inflammatory (M1) macrophages²⁴⁵ that become hypoxic and undergo apoptosis, which results in the release of chemokines that perpetuate macrophage recruitment and their polarization into the M1 phenotype.²⁴⁶ Excess fatty acids can also bind to fatty acid-binding proteins and contribute to fat-induced inflammation²⁴⁷ potentially through the suppression of the anti-inflammatory action of nuclear receptors such as peroxisomal proliferator-activated receptors (PPAR) and liver X receptors (LXR).²⁴⁷ Endoplasmic reticulum (ER) stress also occurs in response to fatty acid overload, triggering the unfolded protein response, which activates inflammatory pathways, such as JNK/AP-1 and IKK/NF- κ B.²⁴⁸ Cyclic-AMP-responsive-element-binding protein H is also activated by ER stress, which stimulates the production of acute phase proteins, such as CRP, by the liver.²⁴⁹ Furthermore, ER stress leads to the production of reactive oxygen species, which can further augment the activity of NF- κ B and AP-1.²⁵⁰ Thus, multiple inflammatory mechanisms are induced by the presence of excess fatty acids.

Several clinical studies have shown the ability of excess fatty acids to directly increase oxidative stress^{251–253} and inflammation in

the systemic circulation through the upregulation of TLR2 and TLR4 expression,²⁵⁴ increased NF- κ B activity,^{251,252} and elevated TNF- α ,²⁵⁵ IL-6^{255–257}, and CRP²⁵¹ levels. Increased blood neutrophil numbers have also been associated with impaired endothelial function.²⁵⁸ We have previously extended these observations to the airways and reported that consumption of a high-fat meal also induces airway inflammation in asthma, with an increase in induced sputum percentage of neutrophils and TLR4 mRNA expression in sputum cells, that are associated with reduced improvement in lung function following bronchodilator use.²⁵⁹

The obvious approach to treating obese asthma is weight loss. This can be achieved through behavioral interventions involving dietary restriction and increased physical activity,^{229,239,260,261} or bariatric surgery, which is indicated for the morbidly obese. However, despite the convincing evidence demonstrating the efficacy of weight loss in improving outcomes for obese asthma patients, some individuals are not able to achieve this goal. Hence, alternative pharmacotherapies targeting this population are needed. The non-eosinophilic pattern of inflammation that derives from excess adipose tissue and fatty acid-induced postprandial neutrophilia highlights the need to consider alternative treatment options as this type of inflammation responds poorly to glucocorticoid treatment. Since many of the factors that we have identified as playing important roles in infection-associated, severe, steroid-resistant asthma, are also increased with obesity (e.g. increased TLR signaling, TNF- α , NLRP3, IL-1 β responses), therapies that target these responses may be effective for the treatment of obesity-associated disease.

5.2 | Air pollution

Air pollution is comprised of gaseous and particle phases, the composition of which is influenced by production source, seasonal variations, and weather patterns.^{262,263} The gaseous component includes volatile organic hydrocarbons, ozone, nitrogen oxides, carbon monoxide and dioxide, and other gases. The particulate component is categorized based on mean diameter. Coarse particulate matter (PM₁₀) is 10 μ m to 2.5 μ m in size, fine particulate matter (PM_{2.5}) is 2.5 μ m to 0.1 μ m, and ultrafine particulate matter is less than 0.1 μ m. The different sizes of particulate matter induce different immune responses *in vitro*²⁶⁴ and *in vivo*.²⁶⁵ Although air pollution is frequently associated with the external environment, indoor air pollution may also be important in lung disease.²⁶⁶ With both outdoor and indoor air pollution, the particulate matter component may act as a vehicle for biological components, including endotoxin, pollen, and fungal spores, leading to complex multi-exposures.^{262,267–270} Both gaseous and particle components of air pollution have been implicated in allergic sensitization and related mucosal immune responses.

The respiratory mucosa, consisting of airway epithelial cells and immune cells, is the first site of contact for inhaled air pollution.^{271–273} Air pollution activates innate immune receptors, including the TLRs and NLRs,²⁷⁴ expressed by these cells leading to the production of a variety of immune mediators that are important in allergic sensitization, exacerbations, and severe asthma (Figure 2).²⁷⁵ TLR2 and TLR4 can be

activated,^{276,277} which signal through NF- κ B,^{278,279} to induce IL-6 and IL-8 production.²⁶² The NLRP3 inflammasome can also be activated, leading to IL-1 β release,²⁸⁰ and is linked to the downstream production of GM-CSF and CCL-20^{281–283} via IL-1R1 signaling.²⁸⁴ Airway epithelial production of IL-8 may also occur through a TNF- α -converting enzyme (TACE)/epidermal growth factor/epidermal growth factor receptor pathway.^{285–288} Air pollution may also induce the production of thymic stromal lymphopoietin (TSLP), which can activate dendritic cells and induce skewing toward a type 2 cytokine profile.^{289–291}

In vivo mouse models have been used to assess the impact of air pollution on (i) sensitization phase, (ii) early inflammatory phase, or (iii) chronic disease phase.²⁹² The first report of air pollution-facilitated allergic sensitization of mice to Ova was performed by Muranaka et al.²⁹³, who demonstrated that diesel exhaust particles co-injected into the intraperitoneal cavity with Ova could lead to systemic elevations in Ova-specific IgE levels. Subsequently, they performed an intranasal instillation model with Ova and diesel exhaust particles, and reproduced the results of elevated Ova-specific IgE levels.²⁹⁴ The observation of air pollution-facilitated allergic sensitization in mice has been shown to be conserved for a variety of gaseous and particulate components of air pollution.^{265,284,295–298} In mice, air pollution exposure is frequently accompanied by activation of dendritic cells, irrespective of the composition.^{280,296,299–301} TLR4 and NLRP3 can play a role in the activation of dendritic cells,^{280,295} although the contribution of these receptors to allergen-specific IgE responses and allergic sensitization are not consistent between different compositions of air pollution.^{295,298} Kuroda et al.³⁰², demonstrated that the intratracheal instillation of particulate matter resulted in the death of alveolar macrophages and release of IL-1 α , which mediated the development of type 2 responses and allergic sensitization upon subsequent exposure to allergens. Furthermore, the induction of TSLP³⁰³ and IL-33³⁰⁴ by components of air pollution may also augment the function of ILCs^{298,305} that are important in allergic airway sensitization.^{306–308}

Clinical studies that investigate the impact of air pollution on allergic sensitization and exacerbations are limited by ethical considerations on de novo allergic sensitization, the requirement for specialized controlled human exposure systems,³⁰⁹ and the reduced level of mechanistic interrogation possible in human systems. Despite these limitations, controlled exposures to diesel exhaust,^{310–314} ozone,^{315,316} and particulate matter^{270,317,318} have been performed in healthy subjects and those with asthma. As real-world exposures are likely to be complex, clinical studies have also examined co-exposures to multiple environmental insults including combinations of diesel exhaust and ozone,³¹⁹ diesel exhaust and allergen,^{320,321} and particulate matter and allergen.³²²

The prevailing impact of air pollution exposure, irrespective of composition, or combination, is that markers of airway inflammation are increased in asthmatics and this may be important in asthma exacerbations. The airway epithelium is specifically impacted by air pollution exposures in clinical models, with evidence of activated EGFR activity³¹² and altered GM-CSF production,³¹⁵ observations that are conserved in vitro.

Similar to animal models of de novo sensitization to allergen, diesel exhaust particles have been used in humans to induce

allergic sensitization to neo-antigens. The seminal work by Diaz-Sanchez et al.³²³, demonstrated that exposure to diesel exhaust particles in the upper airways of healthy subjects was able to induce elevations in total IgE, elevations in allergen-specific IgE in allergic subjects,³²⁴ and allergic sensitization to neo-antigen.³²⁵ In cohort studies, genetic variants in TLR2, TLR4,³²⁶ and glutathione S transferases^{327–329} have been associated with increased asthma risk with air pollution exposure.

In vitro, in vivo, and clinical models of air pollution exposure all point toward an induction of immune responses in the respiratory mucosa that is consistent with the development of allergic sensitization and augmentation of inflammation associated with asthma pathology. Importantly, many of these innate immune processes overlap with those identified in clinical and experimental studies as being induced by infection in infection-associated, severe, steroid-resistant forms of asthma. Therefore, the role of pollution in the development of severe, steroid-resistant asthma requires further examination, particularly the effects of early life exposure to pollution on disease in later life.

6 | CONCLUSIONS

Severe, steroid-resistant asthma is a significant clinical problem, and the most important issue in asthma management. Patients do not respond to steroids through a variety of mechanisms, and there are no effective therapies. Severe, steroid-resistant asthma is linked to respiratory infection with bacteria and viruses, as well as obesity and, likely, air pollution, although the mechanisms of pathogenesis are not well understood. Understanding these mechanisms would lead to the identification of new therapeutic targets and pathways that may facilitate the development of new therapies. We have developed novel mouse models of severe, steroid-resistant AAD that are induced by the same bacteria (*Chlamydia* and *Haemophilus*) and viruses (influenza and RSV) that are associated with human severe asthma. We have used these to identify potentially important key drivers of pathogenesis involving PI3K, TLR signaling, the NLRP3 inflammasome, miRNAs, and cytokines and immune factors. Some of these factors may act together, and we discovered an infection-induced miR-21/PTEN/PI3K/HDAC2 axis that leads to steroid resistance. It is critically important that experimental studies are validated in well-characterized human clinical samples. We have also performed a series of studies that show not all respiratory infections are deleterious in asthma, and low pathogenic *S. pneumoniae* and its components suppress asthma features through the induction of T_{REGS} that may be used therapeutically, and could be applied to more severe disease. Emerging studies also show that obesity may drive the induction of severe asthma through altered macrophage function, TLRs, the NLRP3 inflammasome, and ER stress. The emergence of air pollution will inevitably become a major respiratory issue. In vitro and in vivo animal and human studies have linked air pollution with allergic sensitization involving TLR4 and the NLRP3 inflammasome that could also contribute to the development of more severe disease. Further examination of animal models and well-characterized human samples will continue to further the

understanding of the mechanisms of pathogenesis of severe asthma and enable the development of more effective therapies.

ACKNOWLEDGEMENTS

P. M. H. and J. C. H. have received grants from the National Health and Medical Research Council (NHMRC), the Hunter Medical Research Institute, and the Australian Research Council (ARC). Grant numbers: NHMRC (1043174, 1003565, 1023131, 1059238, 1045762, 1059238, 1079184, 1099095, 1118973, 1120252), and ARC (110101107). P. M. H. is supported by a NHMRC of Australia Principal Research Fellowship and a Brawn Fellowship from the Faculty of Health and Medicine, The University of Newcastle.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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How to cite this article: Hansbro PM, Kim RY, Starkey MR, et al. Mechanisms and treatments for severe, steroid-resistant allergic airway disease and asthma. *Immunol Rev.* 2017;278:41–62. <https://doi.org/10.1111/imr.12543>