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REVIEW

Elucidating novel disease mechanisms in severe asthma

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Corticosteroids are broadly active and potent anti-inflammatory agents that, despite the introduction of biologics, remain as the mainstay therapy for many chronic inflammatory diseases, including inflammatory bowel diseases, nephrotic syndrome, rheumatoid arthritis, chronic obstructive pulmonary disease and asthma. Significantly, there are cohorts of these patients with poor sensitivity to steroid treatment even with high doses, which can lead to many iatrogenic side effects. The dose-limiting toxicity of corticosteroids, and the lack of effective therapeutic alternatives, leads to substantial excess morbidity and healthcare expenditure. We have developed novel murine models of respiratory infection-induced, severe, steroid-resistant asthma that recapitulate the hallmark features of the human disease. These models can be used to elucidate novel disease mechanisms and identify new therapeutic targets in severe asthma. Hypothesis-driven studies can elucidate the roles of specific factors and pathways. Alternatively, 'Omics approaches can be used to rapidly generate new targets. Similar approaches can be used in other diseases.

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ASTHMA

The prevalence of asthma has been increasing globally over the past three decades. There is an estimated 300 million people suffering the disease worldwide, with 1–18% of the population affected depending on the countries involved.[1] In Australia, >10% of the population suffers from asthma.[2] This disease imparts a large healthcare burden costing $58 billion per year in the USA, Europe and Australia.[3] Asthma is characterised by airway inflammation, remodelling and hyperresponsiveness (AHR). Chronic mild to moderate asthma involves frequent recurrent exacerbations of eosinophil-mediated immunopathological processes inducing hallmark symptoms such as coughing, wheezing, dyspnoea and tightening of the chest in response to bronchoconstriction.[4,5] Asthma severity is clinically characterised according to symptoms and with impaired lung function as a lower than predicted forced expiratory volume in 1 s (FEV_1). Mild to moderate asthmatics have intermittent symptoms with exacerbations 2–7 times per week and a baseline FEV_1 of 60–80% predicted. Severe asthmatics have multiple exacerbations per day with one hospitalisation in the previous year, a baseline FEV_1 of <60% predicted, poor symptom control and worsening symptoms with tapering of corticosteroids.[4–6]

Corticosteroids and bronchodilators are used in combination to control symptoms and improve lung function. These therapies are highly effective in patients with mild to moderate eosinophilic asthma.[4,5] However, a large population-based survey within Australia highlighted that almost 45% of patients had either uncontrolled symptoms despite good self-reported adherence to medication or uncontrolled symptoms with no preventer or poor adherence.[7] In larger studies, figures show that between 10 and 15% of asthmatics are refractory to corticosteroids even with high-dose therapy and their symptoms are not well managed despite adherence to currently available medication. These patients are said to have severe, steroid-resistant (SSR) or insensitive (SSI) asthma, and typically have a non-eosinophilic-associated disease profile in contrast to the classic eosinophil-dominated response. This form of asthma is currently the major unmet clinical need in regard to asthma pathology.[8]

This review aims to provide (1) a background of the immunopathological processes behind an example disease in asthma, (2) a description of current therapies and how severe asthma differs, (3) discuss potential mechanisms driving severe asthma, and (4) demonstrate how murine models can be utilised in the discovery of novel mechanistic or functional targets for the development of therapeutic interventions for this difficult-to-treat form of asthma, and other diseases.

PATHOLOGY OF ASTHMA

In asthma, insults to the respiratory epithelium induce the release of pro-inflammatory mediators that cause inflammatory cell influx.[9] The infiltration of inflammatory cells initiates the additional release of cell mediators that contribute to narrowing of the bronchial lumen and mucus-secreting cell hyperplasia leading to pulmonary oedema and subsequent vascular leakage.[10,11] These events lead to AHR and airway

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obstruction.[4,5] Repeated insults lead to cycles of injury that results in the remodelling of airways characterised by collagen deposition, epithelial membrane thickening, increased airway smooth muscle (ASM) mass, and scar formation within the basement membrane, which is characteristic of chronic asthma.[12,13]

Eosinophilic mild to moderate asthma

T helper lymphocyte type (Th)2 cell-mediated, eosinophilic allergic asthma affects approximately half of the asthmatic population.[14] Although it is more pronounced in mild to moderate asthmatics, it is also present in persistent, moderate to severe asthmatics.[15] It is associated with the excessive production of Th2 cytokines, interleukin (IL)-4, IL-5, IL-13, and eosinophilic inflammation in the airways upon subsequent exposure to aeroallergens. Eosinophilic asthma is defined by high sputum eosinophil levels with macrophage and neutrophil levels equivalent to healthy individuals.[16]

The process of disease development is not well understood, but is thought to occur through abnormal signalling events leading to the differentiation of naïve T helper (Th0) cells into antigen-specific effector and memory Th2 cells.[4,5,17] Normally innocuous environmental antigens are captured by dendritic cells, fragmented and presented to naive $CD4^+$ T helper lymphocytes in lung draining lymph nodes.[18] Under normal non-pathological conditions (Figure 1), this leads to the maturation of tolerogenic regulatory T helper cells (Tregs). However, in allergic asthma, antigen presentation drives the development of antigen-specific Th2 cells. These Th2 cells release pro-inflammatory Th2 cytokines, IL-4, IL-5 and IL-13, which are involved in the activation and differentiation of immunoglobulin (Ig) class switching and production of IgE antibodies from B lymphocytes.[19,20] Increased levels of extracellular IgE are correlated with the severity of allergic sensitisation involving bronchial hyperresponsiveness,[21] in which it readily reacts with Fcε receptors on the surface of inflammatory mast cells to activate them and cause subsequent degranulation to release histamines and prostaglandins.[22] This leads to increased vascular permeability, vascular leakage, oedema and AHR. IL-13 is involved in the chemoattraction and activation of eosinophils, IgE class switching on B cells, and induction of AHR and mucus hyper-secretion.[23,24] Clinically, IL-13 has been shown to induce both AHR and mucus secretion, which interestingly, is suppressed in both humans and mice by IL-13 antibody neutralisation.[25,26]

Non-eosinophilic endotypes of asthma

The mild to moderate asthmatics with eosinophil-rich airway inflammation (described above) are termed eosinophilic asthmatics. Research over the past decade has shown that distinct asthma phenotypes exist that are characterised by neutrophil and/or macrophage-dominated inflammation and increased Th1 and/or Th17 associated responses. Up to 25% of asthmatics have high sputum neutrophil levels with low to normal levels of eosinophils, and are described as neutrophilic asthmatics.[16] As many as one-third of all asthmatics have higher than average macrophage levels with no change in neutrophils or eosinophils, and are referred to as paucigranulocytic asthmatics.[27] Mixed granulocytic asthmatics are those with high-sputum neutrophil and eosinophil numbers.[23] Patients with non-eosinophilic asthma typically are older and have more severe disease that is resistant to steroid therapy. Importantly, non-eosinophilic endotypes of asthma are associated with increased innate immune responses including increased CXCL8/IL-8 expression, and toll-like receptor (TLR)-2 and TLR-4 activity (Figure 2). CXCL8 is involved in the chemoattraction and activation of neutrophils, and TLRs respond to pathogen-associated molecular patterns (PAMPs) on the surface of pathogens. Interestingly, TLR-2 and TLR-4 orchestrate the infiltration of neutrophils and macrophages during inflammation, and initiate Th1/Th17-associated, pathogen-targeted adaptive immune responses.[28,29]

The role of neutrophils and macrophages in the pathogenesis of non-eosinophilic asthma is not fully understood, but may involve the release of pro-inflammatory cytokines, including IL-1β, interferon (IFN)-γ and tumour necrosis factor (TNF)α. New evidence suggests that the NLRP3 inflammasome may be an important driver of these SSR forms of asthma.[30,]

Late onset and severe forms of asthma have been associated with incompletely reversible airway obstruction and a mixed Th1/Th17 cytokine profile.[31,32] Increased Th1/Th17 cytokine expression correlates with increased neutrophils in asthmatic patients[32] and

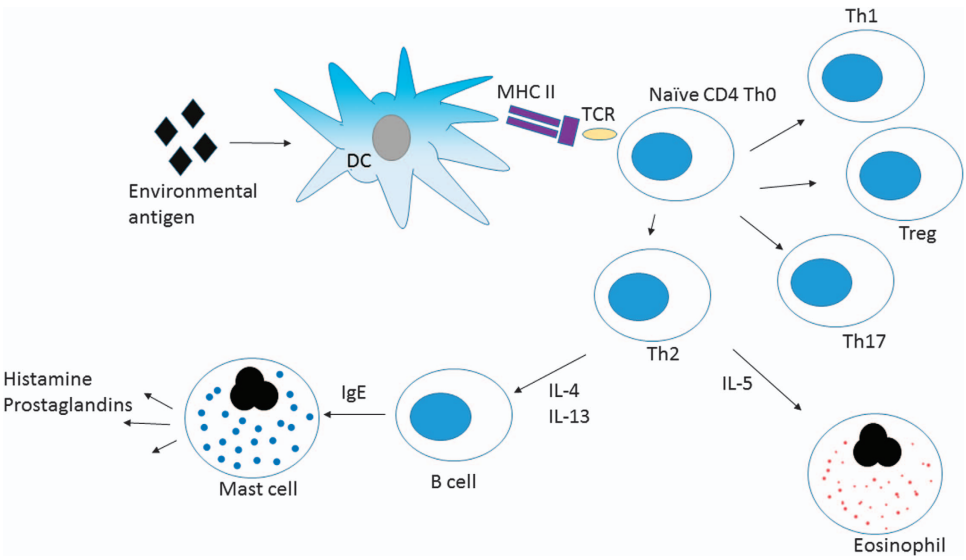

Figure 1 Inflammatory pathways in typical allergic asthma. Environmental allergens can stimulate dendritic cells (DCs), which in turn activate naïve CD4 T helper 0 (Th0) cells. These can differentiate into Th1, Th2, Treg, Th17 cells depending on the cytokine environment. A plethora of cytokines are then secreted including; IL-5 that activates eosinophils; IL-4 and IL-13 that activate B cells. B cells can then release IgE that acts on mast cells to release histamine and prostaglandins and other mediators.

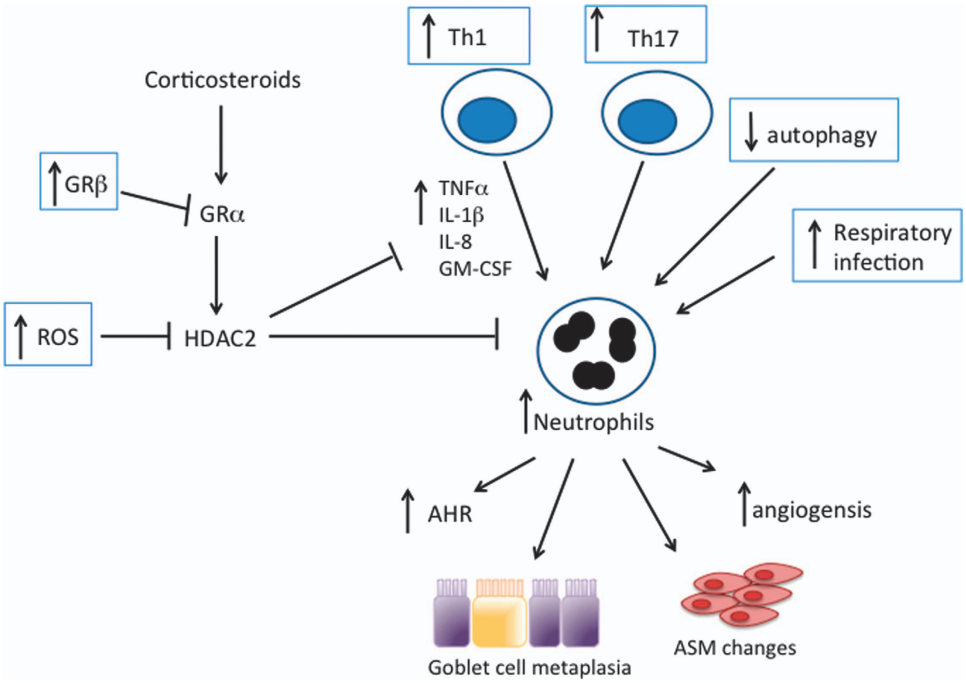

Figure 2 Inflammatory pathways in severe, steroid-resistant (SSR) asthma. In SSR asthma insults such as respiratory infection can lead to increased neutrophilic inflammation. Neutrophils contribute to increased airway hyperresponsiveness (AHR), goblet cell hyperplasia, changes in airway smooth muscle (ASM) phenotype and increased angiogenesis. In addition, increases in Th1 and Th17 cell numbers, and decreases in autophagy, also occur. Increased levels of glucocorticoid receptor (GRβ) can inhibit GRα, and reactive oxygen species (ROS) can decrease histone deacetylase (HDAC)-2 levels leading to impaired corticosteroid activity.

severe, neutrophilic asthma is characterised by extensive tissue remodelling.[32,33] Surprisingly, clinical trials assessing the efficacy of the anti-IL-17 receptor A antibody, Brodalumab, showed no significant clinical benefit in moderate to severe asthmatics.[34] Clinical and experimental evidence has identified that the Th2 cytokines, IL-4 and IL-13, increase Th17-driven inflammation.[35] Thus, blockade of both Th2 and Th17 cytokines may provide synergistic therapeutic benefit in some patients.

SSR asthma

The situation with SSR asthma is complex and there is variation in how individual studies describe populations of non-eosinophilic, neutrophilic, and SSR or SSI asthma. The majority of SSR asthmatics have predominantly neutrophilic inflammation and many neutrophilic asthmatics have steroid-resistant disease. We discuss the relevant studies according to how individual study populations were described.

Structural changes in asthma have been shown to be associated with Th2-mediated eosinophilic inflammation, rather than Th1/Th17-associated neutrophilic inflammatory responses.[36] However, the degree of airway remodelling is directly correlated with asthma severity, and severe asthma is predominantly non-eosinophilic, with increased Th1/Th17-associated responses and sputum neutrophils.[31,37] Together, these data suggest that the mechanisms underpinning severe asthma are multifactorial, including resistance to steroid treatment.

Steroid-resistant asthma is defined as the inability to achieve greater than 15% improvement in FEV_1 following a seven day course of 20 mg per day prednisone or equivalent corticosteroid.[38] These patients have a poor quality of life owing to a reduced ability to perform simple daily tasks, such as walking up flights of stairs, and frequency of hospitalisation.[6] Steroid-resistant asthmatics disproportionately contribute to over 50% of total healthcare costs associated with the disease. Importantly, SSR asthma is strongly associated with infection (see below) and up to 50% of patients are obese.[39,40]

CURRENT ASTHMA THERAPIES AND LIMITATIONS

Glucocorticoids and β-adrenoceptor agonists

The primary objective of current asthma therapies is to reduce symptoms and prevent exacerbations of the disease without necessitating emergency department visits and/or hospitalisations. In most patients, asthma is controlled, and exacerbations prevented, through combined therapy with inhaled corticosteroids and long-acting β-agonists (LABAs).[41,42] Although the symptomatic relief of the onset of asthma symptoms is through administration of short-acting β-agonists (SABAs), corticosteroids are used to reduce inflammation through the suppression of pro-inflammatory gene transcription.[43]

Inhaled corticosteroids comprised of glucocorticoids act through the binding of glucocorticoid receptors (GR).[42] Activation of the GR results in increased expression of anti-inflammatory genes, decreased expression of pro-inflammatory genes and non-genomic mechanisms.[44] Binding of glucocorticoids to intracellular GRα, complexed with heat shock proteins and immunophilins, leads to a conformational receptor change allowing nuclear trans-localisation. Two GR proteins interact to form a homodimer and bind to DNA at glucocorticoid response elements located within the regulatory regions of glucocorticoid responsive genes and act as enhancer elements, leading to the transcription of anti-inflammatory genes.[43] Direct anti-inflammatory effects are most likely due to glucocorticoid-induced repression of pro-inflammatory and immune genes that are normally activated by the transcription factors nuclear factor-κB and

activating protein-1.[8] The additional anti-inflammatory actions of glucocorticoids are broad as they have indirect effects that include the downregulation of a number of cytokines, adhesion molecules, enzymes, and receptors such as GRβ.[45] Therefore, glucocorticoids are both directly and indirectly responsible for inhibiting the development, infiltration, and activation of a variety of immune cells involved in airways inflammation, including macrophages, T cells, eosinophils and airway epithelial cells. These broad effects allow glucocorticoids to not only suppress airway inflammation, but also reduce subsequent effects on AHR and lumen narrowing.[43]

There are numerous issues with steroid treatments. Their efficacy wanes over time, there are side effects of long term use (for example, osteoporosis) and issues with inhaler use and delivery, and people naturally do not like taking steroids (for example, pregnant women). Furthermore, steroid therapy temporarily alleviates the symptoms of disease and is not a cure. Therefore, not surprisingly, there are a plethora of new therapies being tested for allergic asthma that attempt to provide long terms benefits, such as targeted therapeutics in the form of monoclonal antibodies (for example, against Th2 cytokines, IL-4, IL-5 and IL-13, Figure 1) (reviewed in refs 4 and 5). This includes clinical trials of the IL-4 receptor antagonist, Dupilumab. One study showed improved lung function, reduced levels of Th2-associated inflammatory markers and fewer asthma exacerbations in a specific patient group of persistent, moderate to severe asthmatics with elevated eosinophils.[15] There are a wide range of other avenues that are being investigated such as the induction of Treg responses with bacterial components.[46–50]

There are currently no other effective treatments for SSR asthma. Macrolides are antibiotics that also have anti-inflammatory properties, which have been shown to be effective in experimental models and may, therefore, have beneficial effects in chronic respiratory disease. These are currently in clinical trials.[51,52] Elucidating the mechanisms involved in the pathogenesis of SSR asthma may identify new therapeutic targets.

MECHANISMS OF PATHOGENESIS OF SSR ASTHMA

The development of new therapies is facilitated by improving our understanding of the underlying mechanisms that promote disease pathogenesis. SSR and SSI asthma are multifactorial and elucidating the pathogenesis of these forms may identify novel therapeutic targets. Potential factors include oxidative stress, altered immune responses, impaired autophagy, angiogenesis, changes in ASM phenotype and respiratory infections (Figure 2).

Increased oxidative stress and mitochondrial dysfunction

Oxidative stress may be induced by free radicals that induce reactive oxygen species (ROS), and is strongly associated with inflammatory responses in the airways in asthma.[53,54] Mitochondrial dysfunction may also contribute to production of ROS and has been observed in both human and mouse asthmatic bronchial epithelial cells.[55,56] Increases in mitochondrial respiratory complex III mediates higher production of ROS[57] and treatment with mitochondrial-targeted antioxidant (mitoTEMPO) neutralised mitochondrial ROS in both mouse and cultured human epithelial cells.[58] In ovalbumin-induced allergic airways disease (AAD), mitochondrial dysfunction was also evident by reduced expression of cytochrome c oxidase and complex I,[55] with function restored with anti-IL-4, but not anti-IFNγ, monoclonal antibodies.

In regard to steroid resistance, ROS has a suppressive effect on steroid efficacy. This most likely occurs by reducing functional HDAC-2 activity and dampening of its anti-inflammatory effects that occur during GR signalling.[59] By combating oxidative stress with the use of antioxidants it may be possible to reverse these effects and increase glucocorticoid efficacy, making disease more manageable.

Altered immune factors

Th1- and Th17-dominated responses seen in SSR asthma are associated with induction of pro-inflammatory cytokines such as IL-1β and TNFα. It is likely that increased production of these factors leads to the induction of inflammatory responses that are unresponsive to steroid treatment. Increased expression of IL-1β has been associated with a refractory response to steroid therapy alongside TNFα in a number of inflammatory conditions.[39,60] A single study has assessed the effects of the blocking IL-1β with the monoclonal antibody, Canakinumab (ACZ885), in mild to moderate asthmatics.[61] This study with a small patient cohort ($n=21$), demonstrated that ACZ885 significantly attenuated the late asthmatic response to allergen by 28%.[61] An additional phase I clinical trial assessed the IL1R antagonist, Anakinra, which blocks the receptor for both IL-1α and IL-1β, in healthy subjects treated with inhaled lipopolysaccharide to induce a neutrophilic response. Subcutaneous administration of Anakinra significantly decreased neutrophilia, IL-1β, IL-6 and IL-8 levels without increasing the rate of infections.[62] Work from Stampfli[63] and others[64] has demonstrated that sensitisation to house dust mite (HDM) is dependent on IL-1α and not IL-1β in mouse models and is supported by clinical evidence of ACZ885 and Anakinra in patients. However to date, no studies have assessed IL-1β monoclonal antibodies or Anakinra in SSR asthma or *via* different routes of administration.

In addition, GRβ is known to inhibit the activation of anti-inflammatory genes by GRα when present in high concentrations.[65] TNFα enhances GRβ expression, and both factors have been identified in steroid-resistant asthma.[45,66] Interestingly, a recent study has shown anti-TNFα therapy reduced steroid resistance in an animal model of asthma, but clinical trials have been stopped as a result of increased susceptibility to infection.[4] There may be a synergistic interaction between IL-27 and the archetypal Th1 cytokine IFNγ that induces steroid-resistant AHR by inhibiting glucocorticoid signalling in macrophages, as shown in experimental models.[67]

Impaired autophagy

Autophagy is a normal homeostatic process that clears proteins in a cell. Recent evidence has shown that diminished autophagy leads to increased steroid-resistant AHR and neutrophilic inflammation in mice in an experimental model of HDM-induced AAD.[68] This increased neutrophilic inflammation was associated with impaired autophagy in pulmonary $CD11c^+$ cells.[68]

Increased angiogenesis

Angiogenesis is a prominent feature of airway remodelling that occurs in asthmatic airways in which expansion in blood vessel formation, accompanied by fibrosis of inflamed tissue, leads to further aggravation of chronic inflammation.[69] It is currently unknown whether inflammation leads to remodelling or vice versa, however, it is clear these two processes influence the outcome of chronic asthma. Reducing angiogenesis through endogenous inhibitors may result in the reduction of accompanying airway inflammation and fibrosis that contribute to AHR. For example, collagen IV is found to be decreased in the airway basement membrane in asthma.[70] The non-collagenous domain-1 α3 chain of collagen IV is an endogenous anti-angiogenic agent known as tumstatin, and is depleted in asthmatic airways.[69] Use of recombinant tumstatin, and synthetic

tumstatin-derived peptides, inhibits angiogenesis, inflammation and suppresses AHR in murine models.[69,70]

Changes in ASM phenotype

Narrowing of the airways, although multifactorial, is strongly associated with ASM abnormalities. ASM cells are thought to contribute to the pathogenesis of asthma by providing a link between airway inflammation and remodelling, and they are the major controllers of bronchoconstriction.[71,72] ASM contraction (Figure 3) begins with a contractile agonist, such as histamine, binding to its G protein-coupled receptor. This interaction activates phospholipase C (PLC), and the Ca^{2+}-oscillation pathway forming inositol triphosphate (IP_3) through phosphatidylinositol biphosphate (PIP_2) hydrolysation. IP_3 binds to its receptors on the sarcoplasmic reticulum membrane, releasing intracellular calcium ions (Ca^{2+}).[73] Ca^{2+} forms a complex with calmodulin, activating myosin light chain kinase (MLCK), which phosphorylates regulatory myosin light chains forming phosphorylated-MLC (p-MLC). This activates actin and myosin cross-bridges, resulting in shortening and contraction of ASM cells.[74] Ca^{2+} returns to homoeostatic levels through plasma membrane Ca^{2+} ATPase pumps, uptake by mitochondria, and reuptake into the sarcoplasmic reticulum by action of sarco/endoplasmic reticulum Ca^{2+} ATPase. In addition, Ca^{2+}-sensitisation can occur whereby signalling through the agonist receptor activates protein kinase C or Rho-activated kinase pathways, leading to phosphorylation of myosin light chain phosphatase (MLCP). Once MLCP is phosphorylated, it is unable to dephosphorylate MLC, making the contractile apparatus more sensitive to Ca^{2+} from the Ca^{2+} oscillation pathway. Although the understanding of Ca^{2+} signalling is established in healthy ASM, changes to ASM under disease conditions are less well understood.[75] A greater understanding of these mechanisms that underpin hyper-contractility and airway remodelling due to ASM abnormalities, and its subsequent effect on steroid resistance in severe asthma, could provide more specific targets to reverse steroid resistance and/or increase steroid sensitivity.

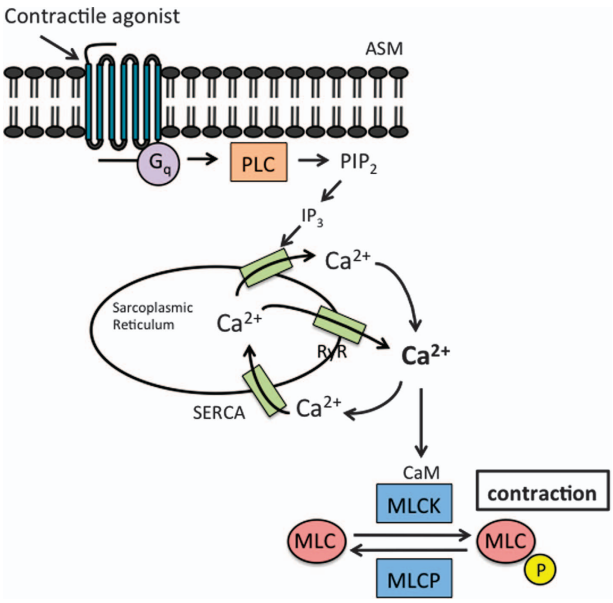

Figure 3 Airway smooth muscle contraction. Bronchoconstriction occurs when intracellular Ca^{2+} levels and Ca^{2+} sensitivity are increased. Ca^{2+} release from the sarcoplasmic reticulum, either through IP_3 or Ca^{2+} activation of the IP3R or RyR, respectively, increases intracellular Ca^{2+} to activate CaM and MLCK, resulting in the phosphorylation of MLC and subsequent contraction. Ca^{2+} oscillations contribute to this contraction, whereby intracellular Ca^{2+} is released via IP3 or RyR or taken up into the sarcoplasmic reticulum through SERCA. CaM, calmodulin; $[Ca^{2+}]i$, intracellular calcium; IP_3, inositol trisphosphate; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

Respiratory infections

Although a number of mechanisms have been identified that may have a role in driving SSR asthma, many studies demonstrate a strong link between respiratory infections and the pathogenesis of the disease.

Chlamydia pneumoniae is linked clinically and experimentally with the induction and development of asthma, particularly severe asthma, in children and adulthood.[76–82] In asthmatics with evidence of previous *Chlamydia* infection (elevated *Chlamydia*-specific serum IgE, IgG and IgA), disease tends to be more severe, with more frequent acute exacerbations, and increased neutrophilia.[77,83] In relation to steroid resistance, asthmatics requiring high-dose steroid therapy are more likely to have evidence of *Chlamydia* infection, compared with those on low dose steroid therapy.[84] Furthermore, increased airway neutrophilia, along with high-dose steroid treatment, has been shown to predict the presence of respiratory *Chlamydia* infection in a cohort of adolescent asthmatics.[83] *Chlamydia* is able to infect immune cells altering their function and promoting the development of AAD.[85,86]

Haemophilus influenzae is commonly isolated from the lungs of severe, neutrophilic asthmatic patients that are refractory to steroid therapy.[87] Increased neutrophil levels during *H. influenzae* infection correlates with decreased lung function, and increased airway obstruction and IL-8 responses.[87] Importantly, *H. influenzae* respiratory infection in murine models of asthma has been shown to induce an inflammatory phenotype resembling neutrophilic asthma that is IL-17 dependent.[88,89]

Asthmatics with viral infections (i.e. rhinovirus, respiratory syncytial virus and influenza) have increased exacerbations, significantly reduced FEV_1, and increased neutrophilic inflammatory responses compared with non-infected asthmatics.[90,91] These features and virus-exacerbated wheeze do not respond to steroid therapy.[92] Elucidating the effect of respiratory infections on the asthmatic lung may uncover the mechanisms that underpin the pathogenesis of steroid resistance, and highlight novel therapeutic targets for this form of the disease.

ASSESSMENT OF THE PATHOGENESIS OF SSR ASTHMA

Animal models of steroid-resistant asthma

To uncover the mechanisms linking respiratory infection with SSR asthma, we have sought to model this association. This has led to the development of murine models that recapitulate the key features (airway inflammation and AHR) of human asthma, termed AAD (Table 1).[51,88,93] We then established *Chlamydia*, *H. influenza* and influenza respiratory infections. These models have the hallmark features of infection. We then combined them to show that infection induced SSRAAD.[80,88,89] Clinical studies indicate that it is infections in established asthma that drive more severe disease and steroid resistance. Thus, we refined our models so that AAD is first established and then respiratory infection is induced, which better models the human scenario.[51] Mice are sensitised with Ova and alum (d0, 50 μg Ova, intraperitoneally (i.p.)), followed by Ova challenge (d12–13, 10 μg Ova, intransally (i.n.)) to induce acute AAD. Mice are left for 20 days and then re-challenged (d33–34, 10 μg Ova, i.n.). This represents an exacerbation of established disease. AAD is assessed 1 day after the final challenge (d35). To induce SSR disease mice are

Table 1 Mouse models of steroid-resistant asthma

Reference	*Mouse strain*	*Experimental protocol*	*Outcomes*
93	Balb/C	Sensitisation OVA i.p. −21 days, −7 days; aerosolized OVA 3 days per week, 4 weeks	*cf.* Saline ↑ Eosinophils, ↑ Neutrophils
93	Balb/C	Sensitisation OVA i.p. −21 days, −7 days; aerosolized OVA 3 days per week, 4 weeks; dexamethasone 24 h and 2 h before last challenge	*cf.* OVA ↓ Eosinophils, ↑ **Neutrophils**
68	C57Bl/6	Sensitisation HDM intranasal d1; HDM intranasal d8, d15	*cf.* Saline ↑ AHR, ↑ Eosinophils, ↑ Neutrophils
68	C57Bl/6	Dexamethasone d1; sensitisation HDM intranasal d1; HDM intranasal d8, d15	*cf.* OVA ←→ **AHR**, ↓ Eosinophils, ←→ **Neutrophils**
51	Balb/C	Sensitisation OVA i.p. d0; OVA IN d12–13; OVA IN d33–34	*cf.* Saline ↑ AHR, ↑ Eosinophils, ↑ Neutrophils
51,88	Balb/C	Sensitisation OVA i.p. d0; OVA IN d12–13; OVA IN d33–34; dexamethasone IN d32–34	*cf.* OVA ↓ AHR, ↓ Eosinophils, ↓ Neutrophils
51	Balb/C	Sensitisation OVA i.p. d0; OVA IN d12–13; Chlamydia muridarum IN d14; OVA IN d33–34	*cf.* OVA ↑ AHR, ↑ Eosinophils, ↑ Neutrophils
51	Balb/C	Sensitisation OVA i.p. d0; OVA IN d12–13; Chlamydia muridarum IN d14; OVA IN d33–34; dexamethasone IN d32–34	*cf.* OVA/cmu ←→ **AHR,** ←→ **Eosinophils,** ←→ **Neutrophils**
51	Balb/C	Sensitisation OVA i.p. d0; OVA IN d12–15	*cf.* Saline ↑ AHR, ↑ Eosinophils, ↑ Neutrophils
51,88	Balb/C	*H. influenzae* IT d-10; sensitisation OVA i.p. d0; OVA IN d12–15	*cf.* OVA ←→/↓ AHR, ↓ Eosinophils, ↑ Neutrophils
88	Balb/C	*H. influenzae* IT d-10; sensitisation OVA i.p. d0; OVA in d12–15; dexamethasone d13–15	*cf.* HI OVA ←→ **AHR**, ←→ **Eosinophils,** ←→ **Neutrophils**
51	Balb/C	*H. influenzae* IT d-10; Clarithromycin d8–d-6; sensitisation OVA i.p. d0; OVA in d12–15; dexamethasone d13–15	*cf.* HI Clar OVA ←→ **AHR,** ↓ Eosinophils, ←→ **Neutrophils**

Abbreviations: AHR, airway hyperresponsiveness; Clar, clarithromycin; *cf.*, compared with; HI, *haemophilus influenzae*; HDM, house dust mite; OVA, ovalbumin.
Bold entities signify steroid-resistant features in various models.

infected after AAD is established (d14) but before re-challenge. Some groups are treated with dexamethasone (DEX; d32–34, 2 mg kg^{-1} in PBS, i.n.). Infection suppresses eosinophilic and Th2-associated responses whilst increasing neutrophilic airway inflammation and Th1/Th17-associated responses. It also results in steroid resistance of inflammation and AHR in AAD. Thus, infection induces severe neutrophilic AAD that is steroid-resistant (SSRAAD; Table 1). The effects of each of these three infections on an AAD model results in different immune and inflammatory profiles that represent different subsets of SSR asthma observed clinically, including neutrophilic and eosinophilic subsets. *Chlamydia* infection leads to an increase in neutrophilic, and a decrease in eosinophilic, airway inflammation. It also increases the expression of Th1- and Th17-associated cytokines. *Haemophilus* infection also leads to an increase in neutrophilic inflammation, and a decrease in eosinophilic inflammation, while increasing the expression of Th17-associated cytokines. Influenza infection does not alter neutrophilic or eosinophilic inflammation, but does increase the expression of Th1-associated cytokines. We have also infected neonatal mice with these pathogens and then induced AAD in later life. These early life infections also increase the severity of AAD and induce steroid resistance of key disease features. The overarching feature of steroid-resistant AHR and inflammation in each of the three infection models suggests that they are a valuable platform for the investigation of universal mechanisms of steroid resistance.

Factor-targetted studies

These and other models of SSR asthma can be used in traditional ways to explore the contributions of specific factors of interest. Their levels can be measured and factor-deficient or -overexpressing mice with SSRAAD can be assessed compared to wild-type and control mice. Furthermore, targeted interventions with siRNA, antibodies or potential new drugs can be used to intervene in candidate processes to elucidate their roles and potential for therapeutic targeting. Using such approaches roles have been identified for neutrophils and macrophages, IL-13, IL-17, IL-27/IFNγ and microRNAs (miRNAs) in SSRAAD in adulthood,[67,80,86,89,94–97] and bone marrow changes, IL-13, TRAIL and PD-1 in early life infection-induced disease.[98–101]

Microarray, RNA-Seq and discovery-driven studies

A large body of evidence indicates that functional abnormalities may be broadly important in the development of steroid resistance in chronic inflammatory diseases, including asthma. However, as each disease is characterised by a unique set of pathogenic mechanisms it is likely that the processes that lead to steroid resistance are context-dependent and many different processes may be involved. Thus, novel discovery and global 'Omics studies are valuable in identifying new factors and the elucidation of multi-component pathways. Genome-wide association studies utilise rapidly scanning markers across multiple sets of DNA, or genomes, to find genetic variations associated with a disease. Genetic predisposition may have a role in steroid resistance in asthma, and so genome-wide association studies can be used to identify any variations that underpin this.[102,103] Previously, susceptibility loci/genes in severe asthma have been identified (ORMDL3/GSDMB, IL1RL1/IL18R1)[103] but few, if any, represent targets for therapeutic strategies with real-world applications.

Functional genomics uses data gained from genomic projects to describe gene (and subsequently protein) function and interaction. This field of molecular biology has been used to undertake discovery-driven research regarding asthma pathology. This is a powerful tool, finding novel factors, mechanisms and pathways involved in different disease states. DNA microarrays have been used to advance our understanding of the mechanisms that underpin abnormalities in asthma pathology, showing their capacity to accelerate the discovery process and provide a more complete understanding of the molecular basis of disease.[104] Quantitative detection of reverse-transcribed RNA transcripts in many studies has shown that microarray-based profiling can identify novel factors that are altered within the disease. Also, gene expression (GEx) microarray is a well-established, high-throughput

technology that is designed to analyse the transcriptional profile (largely mRNA but also miRNA) of an entire genome. Despite the mainstream availability of technologically superior approaches, microarray-based profiling remains as a readily accessible and powerful tool in the characterisation of transcriptional responses. Microarrays consist of embedded probes, each representing a gene in a genome of interest, using fluorescence to quantify the degree of sample hybridisation to complementary array probes, in which the data undergoes transformation, normalisation, and multiple test corrections to minimise the chance of false positives and type II errors. However, microarray-based gene expression profiling is limited as a pure discovery platform since probe libraries are generated against databased genes with a strong representation from mRNAs or transcripts thereof.

Recent advances in sequencing technology have given researchers unprecedented access to next-generation sequencing as a pure discovery tool in the field of transcriptomics. Transcriptomics is the study of the transcriptome, which is the full complement of all RNA species in a given cell or population of cells. RNA sequencing (RNA-seq) is an approach that is gaining popularity in disease research and employs next-generation sequencing technology to map the genome of a disease state. RNA-seq provides the sequence of RNA molecules within a sample and relative abundance of each. In contrast to microarray-based approaches, RNA-seq is not limited to detecting transcripts represented in a prepared probe library. RNA-seq has the sensitivity to detect transcripts over six orders of magnitude with low background noise, which minimises the likelihood of obtaining false results. RNA-seq has been shown to be highly accurate in determining gene expression levels with a high level of reproducibility across technical and biological replicates. This approach has been used to identify three clusters of asthma endotypes in sputum and blood samples; first cluster with low pre-bronchodilator FEV_1 and high post-bronchodilator response; second cluster with asthma hospitalisation; third cluster with normal lung function and lower inhaled steroid requirements.[105] In addition, transcriptomic analysis of severe asthmatics has identified activated $CD8^+$ T cells compared with non-severe asthma and healthy controls.[106]

Interactions of the identified genes from these processes may then be explored through bioinformatics profiling that allows differentially expressed genes found within disease states to be visualised, showing not only interactions between altered genes, but also through the grouping of genes into molecular processes and pathways using commercially available platforms such as Ingenuity Pathway Analysis. The application of GEx microarray and RNA-seq technologies with subsequent bioinformatics analysis to models of infection-induced, SSR asthma can facilitate the identification of novel factors and/or signalling pathways that are potentially underpinning the pathogenesis and/or development of steroid resistance.[107]

Translational human studies

The analysis of animal models is a powerful tool for discovery of the involvement of new pathogenic factors and their potential for therapeutic targeting. However, in order to ascertain the human relevance of these findings, the pathways, mechanisms and potential therapeutic targets identified must be pursued further in clinical studies. The same factors can be assessed in cells and tissues from humans where their potential role/s in the target disease can be assessed, and relationships with infections can be tested.[69,87,107] Once the critical cells and tissues have been identified *in vitro* studies with representative cell lines or primary cells can be performed with and without allergen, infectious or other challenge and the relevant interventions.[108] However, great care needs to be taken during translation and, ideally, well-characterised patients with the relevant disease and disease endotype/s are recruited for such studies. Roles for IL-5 and IL-13 in asthma pathogenesis were discovered using mouse models and assessment of human tissues.[4,5,109] Monoclonal antibodies for human use were developed against these cytokines and tested in a cohort of asthma patients in a safety and efficacy trial.[4,5] However, the trials were not designed as treatment trials and the patients were not tested prior to entering the trials for elevated IL-5 and IL-13, and the treatments were deemed to have failed as they did not improve patient symptoms. Upon stratification of patients with high levels of eosinophilic inflammation despite inhaled glucocorticoid use, anti-IL-5 treatment (Mepolizumab and Reslizumab) significantly reduced asthma exacerbations and eosinophil levels.[110,111] Mepolizumab is now FDA approved and Reslizumab is recommended for approval. This highlights how direct mouse to human clinical translation can lead to registered new drugs. Furthermore, development of new techniques, including precision cut lung slicing enables the assessment of lung function and the effects of drugs in live tissues. Both mouse and human lung tissues can be interrogated using this technique.[112–114] Comparisons of responses to factors and treatments in diseased and normal tissues from mouse and human origin using the same method will facilitate translation of experimental findings.

Infectious exacerbations and other diseases

The studies described above are related to understanding the pathogenesis of asthma. Asthma and other chronic respiratory diseases are prone to sporadic acute increases in symptoms termed attacks or exacerbations. Respiratory infections are the primary cause of these exacerbations. Mouse models of infectious exacerbations have been developed whereby infections are induced after AAD is established.[115] They enable the investigation of the pathogenesis of infectious exacerbations in asthma and other chronic respiratory diseases. In similar ways many other mouse models of other respiratory (for example, chronic obstructive pulmonary disease, pollution exposure[116–119]) and other conditions (for example, colitis, arthritis, clotting defects[118,120,121]) have been developed for which there are no treatments in humans. These models can be interrogated in similar ways in order to develop new therapies for these diseases.

CONCLUSION AND FUTURE DIRECTIONS

The development of animal models that accurately recapitulate the hallmark features of human diseases, such as SSR asthma, are rapidly being developed. They are valuable in identifying and elucidating the roles of pathogenic factors, identifying new treatment targets and testing new therapies. Individual factors can be selected from other studies or from hypothesis-driven research. In relation to the ever growing array of bioinformatics technology and sequencing events, the application of these technologies has the capacity to unlock a greater understanding of SSR asthma and other diseases in conjunction with representative animal models and identify novel targets and therapeutic strategies. Data need to be carefully applied to well designed human studies to validate and translate findings into humans and clinical trials.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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