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Luke Hatchwell
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I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of my thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

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Luke Hatchwell
Thesis by Publication

I hereby certify that this thesis is submitted in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author; and endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the jointly authored publications.

Luke Hatchwell
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List of publications included as part of thesis

Publication 1:

THE E3 UBIQUITIN LIGASE MIDLINE 1 PROMOTES ALLERGEN AND RHINOVIRUS-INDUCED ASTHMA BY INHIBITING PROTEIN PHOSPHATASE 2A ACTIVITY

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Publication 2:

TOLL-LIKE RECEPTOR 7 GOVERNS INTERFERON AND INFLAMMATORY RESPONSES TO RHINOVIRUS AND IS SUPPRESSED BY IL-5-INDUCED LUNG EOSINOPHILIA

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Publication 3:

SALMETEROL ATTENUATES CHEMOTACTIC RESPONSES IN RHINOVIRUS-INDUCED EXACERBATION OF ALLERGIC AIRWAYS DISEASE BY MODULATING PROTEIN PHOSPHATASE 2A

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Adam Collison and Luke Hatchwell designed and performed mouse and cell culture experiments, analysed data, generated figures and edited the manuscript. Nicole Verrills and Helen Carpenter performed and analysed PP2Ac quantification and immunoprecipitation and designed *in-vitro* experiments. Nicole Verrills also edited the manuscript. Peter Wark and Melinda Tooze performed and supervised studies on clinical samples collected from healthy subjects and subjects with asthma and performed cell culture experiments. Ana Pereira de Siqueira coordinated and supervised mouse and human studies. Anthony Don and Jonathan Morris synthesized AAL$_{4}$ for use as an activator of PP2A and developed the dosing regiment. Nives Zimmermann and Marc Rothenberg coordinated and assisted in microarray array analysis. Nathan Bartlett and Sebastian Johnston assisted in design of experiments, provided stocks of RV1B for further propagation and cDNA standards and edited the manuscript. Paul Foster supervised mouse studies, interpreted data and edited the manuscript. Joerg Mattes conceptualized, coordinated, designed and supervised mouse and human studies, interpreted and analysed data, and drafted and edited the manuscript. All authors contributed to data discussion and revised the manuscript during the resubmission period.

Joerg Mattes
**Publication 2:**

Luke Hatchwell and Adam Collison designed and performed mouse and cell culture experiments, analysed data, generated figures and edited the manuscript. Jason Girkin and Junyao Li performed experiments and analysed data. Jie Zhang assisted in supervision. Peter Wark and Kristy Parsons performed and supervised studies on healthy subjects and subjects with asthma, collected and processed biopsies, and performed cell culture experiments. Simon Phipps assisted in the design and conceptualization of some mouse experiments. Darryl Knight supervised and interpreted cell culture experiments. Nathan Bartlett and Sebastian Johnston assisted in design of mouse experiments, provided RV1B for further propagation and cDNA standards. Paul Foster assisted in design, supervision and interpretation of mouse studies. Joerg Mattes conceptualized, coordinated, designed and supervised mouse and human studies, interpreted and analysed data, and drafted and edited the manuscript. All authors contributed to data discussion and revised the manuscript during the resubmission period.

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Publication 3:

Luke Hatchwell designed and performed mouse and cell culture experiments, analysed data, generated figures, drafted and edited the manuscript. Jason Girkin and Matthew Morten performed experiments and analysed data. Matthew Dun and Nicole Verrills designed experiments and performed and analysed PP2Ac measurements and immunoprecipitations. Nicole Verrills also edited the manuscript. Hamish Toop and Jonathan Morris synthesized AAL(S) for use as an activator of PP2A and developed the dosing regiment. Sebastian Johnston assisted in design of mouse experiments, provided RV1B for further propagation and cDNA standards. Paul Foster assisted in design, supervision and interpretation of mouse studies. Adam Collison designed and performed mouse and cell culture experiments, analysed and interpreted data, and edited the manuscript. Joerg Mattes conceptualized, coordinated, designed and supervised mouse and cell culture studies, analysed and interpreted data, and edited the manuscript. All authors contributed to data discussion and revised the manuscript during the resubmission period.

Joerg Mattes
Statement of Contribution of Others

The co-authors listed below attest that Research Higher Degree candidate Luke Michael Hatchwell was the primary contributor (first or co-first author) to the following papers/publications:

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    *These authors contributed equally to this work.

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    *These authors contributed equally to this work.

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Abstract of Thesis

Asthma is a chronic inflammatory disease of the airways, associated with debilitating reversible airflow obstruction. The majority of healthcare costs from asthma-related hospitalisations are attributed to exacerbations by respiratory viruses, with rhinoviruses (RV) being the most commonly detected. This thesis presents original research papers detailing investigations to elucidate the mechanisms underlying RV-induced exacerbations of allergic airways disease (AAD).

The first manuscript (see Chapter 2) details the elucidation of a novel TRAIL signalling pathway where the TRAIL-regulated gene product Midline-1 (MID1), which inhibits protein phosphatase 2A (PP2A), was found to promote AAD through increased homing of myeloid dendritic cells (mDCs) to the airway via CCL20 release. Notably, inhibition of MID1 or reactivation of PP2A abolished airway hyperresponsiveness (AHR) and attenuated airways inflammation and mucus hypersecretion in mouse models of AAD and RV-induced exacerbation.

The second manuscript (see Chapter 3) investigates the importance of Toll-like receptor (TLR) 7-elicited interferon (IFN) responses during RV infection in an asthmatic setting. We show that following exposure to house dust mite (HDM), mice deficient in TLR7 display exaggerated eosinophilic inflammation and attenuated anti-viral responses when challenged with RV. TLR7 expression in the lungs of mice was found to be suppressed by interleukin-(IL)-5-induced eosinophilia, while human asthmatics with eosinophilic but not neutrophilic airways inflammation also showed reduced TLR7 and IFN expression.

The third manuscript (see Chapter 4) revisits established therapeutic agents, long-acting β2 agonists (LABAs), in light of recently described interactions with PP2A. This study extends those findings by reporting that administration of salmeterol, or other β2 agonists, protected mice against HDM- and RV-induced lung inflammation as effectively as the corticosteroid dexamethasone. Salmeterol but not dexamethasone mediated this via increased PP2A activity, the inflammatory phenotype recapitulated when PP2A was targeted by siRNA.

Taken together, these studies have identified new targets for the therapeutic intervention of asthma and RV-induced exacerbation.
CHAPTER 1: Literature review and study design
1.1 Asthma

1.1.1 Clinical presentation

Asthma is a heterogeneous chronic disorder of the conducting airways, generally defined by the recurring appearance of variable and reversible airflow obstruction, a heightened sensitivity or irritability of the bronchi termed airway hyperreactivity (AHR), oversecretion of mucus into the airway lumen and the persistence of underlying inflammation that leads to structural remodelling (Figure 1.1)\(^1\)\(^-\)\(^3\). Due to the variable nature of these features, numerous clinical subtypes exist that share the common symptoms of chest-tightness, cough, breathlessness, intermittent wheezing and life-threatening airway occlusion\(^4\)\(^-\)\(^6\). Unfortunately these signs cause asthma to overlap with other respiratory conditions upon presentation.

Confirming a diagnosis of asthma necessitates multiple avenues of evidence to ensure delivery of appropriate therapies to patients\(^7\). These most commonly come from patient questionnaires, assessment of pulmonary function and identification of patient allergies\(^8\). Diagnostic assessment of pulmonary function is performed by spirometry and agent-based bronchial provocation to identify AHR\(^8\)\(^,\)\(^9\). Spirometry allows the non-invasive measurement of inhaled/exhaled volumes of air over time, the most relevant being forced vital capacity (FVC), the maximum volume that can be manually inspired and expired, and forced expiratory volume in 1 second (FEV\(_1\)), the maximum volume forcibly exhaled in 1 second after full inspiration\(^10\)\(^,\)\(^11\). Bronchial provocation with compounds such as methacholine are used to identify AHR, a cornerstone of the disease, as the airways of asthmatics are more responsive to these bronchoconstrictors compared to those of non-asthmatics\(^12\)\(^,\)\(^13\).

Positive indications of asthma are defined by congruent description of symptoms from patients, FEV\(_1\)/FVC ratio below predicted values, a reversible airflow limitation following inhalation of a rapidly-acting bronchodilator (≥12% increase in FEV\(_1\) from baseline), as well as an increased airway responsiveness to methacholine (agent concentration inducing a 20% decrease in FEV\(_1\) of <8 mg/ml)\(^14\)\(^,\)\(^15\). Additionally, due to the strong association between asthma and atopy (elevated levels of total and allergen-specific immunoglobulin-(Ig)-E in serum), skin-prick testing for common aeroallergens
is recommended to help identify environmental factors that can trigger asthma symptoms\textsuperscript{16,17}.

The diagnostic use of these measurements is in-part made possible by the persistence of episodic airway inflammation over a chronic period causing non-reversible structural changes to the airways termed ‘remodelling’, which will be expanded upon in Chapter 1.1.3\textsuperscript{18,19}.

\textbf{Figure 1.1. Changes to the airways in asthma and during an asthma attack.} A histologically normal airway (left) retains optimal functionality due to a maintained airway lumen diameter and relaxed airway smooth muscle. Persistence of underlying inflammation in asthmatics causes structural remodelling of the airways (middle), leading to a thickened airway wall and deceased lumen calibre. When an asthma attack is triggered by aeroallergens, the airways become functionally restricted and sometimes occluded by smooth muscle constriction and mucus hypersecretion, induced by the activation of infiltrating immune cells. (Adapted from NIH MedlinePlus the Magazine, Fall Issue, 2011)\textsuperscript{20}.
1.1.2 Disease burden

The degree of disability and poor quality of life suffered by asthmatics, together with the attributed financial drain of treatment, places this disease among the most costly chronic pathologies plaguing modern society\textsuperscript{21,22}. Coupled with increasing global prevalence during the 20\textsuperscript{th} century, asthma has become a principal healthcare and economic concern to the Western world, with greater humanitarian concern in developing countries\textsuperscript{23,24}.

Based on the findings of Masoli et al. in 2004 for the Global Initiative of Asthma (GINA), global prevalence of asthma is estimated to be approximately 300 million worldwide, with an additional 100 million cases predicted by 2020\textsuperscript{25}. Numerous studies have flagged the United Kingdom, Australia and North America as asthma hot-spots, in spite of slightly differing criteria for asthma diagnosis (Figure 1.2)\textsuperscript{25,26}.

Australian prevalence of asthma, defined as individuals with clinically-relevant symptoms such as intermittent wheeze and abnormal airway function, is estimated at approximately 9\text% in children and 6\text% in adults\textsuperscript{27,28}. 
Figure 1.2. Global pattern of asthma prevalence. In 2004, Masoli *et al.* developed a report on the global burden of asthma for the Global Initiative for Asthma (GINA)\(^2\). Based on their data, the distribution of asthma prevalence worldwide (defined as individuals that have received a clinical diagnosis of asthma) is presented as a % proportion of the county’s total population. Of those surveyed, developed countries with a profile of westernised lifestyles appeared to have the highest disease prevalence. (Adapted from Graham-Rowe, Nature: Outlook, 2011)\(^2\).
1.1.3  Pathogenesis

1.1.3.1  Asthma pathology and predisposition

Asthma is a complex pathology that manifests over time with airway inflammation and alterations to airway structure, neuronal innervation and vascularisation\textsuperscript{30}. Histological abnormalities observed within the airway wall of an asthmatic include an activated epithelial cell layer, infiltrating eosinophils and CD4\textsuperscript{+} T helper lymphocytes, as well as activated neutrophils, mast cells and basophils\textsuperscript{31,32}. When this airway inflammation occurs over a chronic period, persistent changes to airway structure, termed ‘remodelling’, result following tissue repair\textsuperscript{33}. These alterations can include mucous gland and goblet cell hyperplasia and hypersecretion, angiogenesis, thickened basement membrane, subepithelial collagen deposition, airway smooth muscle hypertrophy weakening of pulmonary cartilage and structural compromise of the epithelial layer\textsuperscript{34,35}. These changes collectively impair respiratory function and are worsened by repeated exposure to common airborne triggers such as allergens and viruses, the cycle of pathology progression summarised in Figure 1.3\textsuperscript{36}.

Yet, compared to other chronic diseases, asthma has a relatively early onset and is the foremost chronic condition affecting children and young adults\textsuperscript{37}. Given this timeframe, the ideology regarding asthma initiation focuses on the interaction between environmental cues and genetic susceptibility of the host prior to manifestation of symptoms\textsuperscript{38,39}. Of the identified genetic susceptibilities linked to asthma, atopy (elevated serum IgE specific to environmental allergens) is the most prevalent in the Western world and still increasing\textsuperscript{40}. Atopy manifests as sensitisation against innocuous environmental allergens at a young age, with remaining individuals eliciting a measured response to aeroallergens by generating allergen-specific IgG1 and IgG4 antibodies\textsuperscript{41}. T cells isolated from these individuals also proliferate and produce interleukin-(IL)-2 and interferon-(IFN)-\(\gamma\) when exposed to their cognate allergens, this type 1 helper T cell (T\textsubscript{H1}) response promoting an “immunotolerant state”\textsuperscript{42}. This is the case for most inhaled aeroallergens, with no development of inflammation upon chronic exposure\textsuperscript{43,44}. 
Figure 1.3. Inflammation and tissue repair cycle leading to asthma pathology. Following exposure to environmental triggers such as allergens or viruses (top left), asthma-prone individuals will experience airway inflammation and associated respiratory symptoms (right). Resolution of inflammation and tissue repair contributes to airway remodelling with repeated cycles of environmental stimulation, until a threshold of histological changes is reached and airway function abnormalities persist (bottom). (Adapted from Holt & Sly, Nature Medicine, 2012).³⁶

Atopic individuals, in contrast, will produce exaggerated levels of allergen-specific IgE and their T cells preferentially secrete IL-4, -5 and -13 (a type 2 helper T cell (T\(_H\)2) response), when stimulated with allergens like plant pollen, house dust mites (HDM), animal dander and molds⁴⁵-⁴⁷. This ubiquitous low-level aeroallergen exposure results in chronically active T\(_H\)2 cells in the lung and a proinflammatory cytokine environment that promotes cellular infiltration by inducing chemokine expression⁴⁸.
1.1.3.2 Early-phase response to allergen by resident innate immune cells and structural lung cells

Airway epithelial cells (AECs) have come to light as crucial initiators of programmed immune cascades to airborne allergens through their antigen-sensing repertoire and networks of cytokines and chemokines\(^{49-51}\). Upon contact with aeroallergens, granulocyte macrophage colony-stimulating factor (GM-CSF), calcitonin-gene-related-peptide (CGRP), IL-25, IL-33, thymic stromal lymphopoietin (TSLP), tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and chemokine (C-C motif) ligand-20 (CCL-20) are secreted to induce homing of immature dendritic cells (DCs) to the contact site\(^{52-54}\). DCs pick up and process these antigens at the mucosal site before migrating to draining peribronchial lymph nodes via direction from chemokine gradients, such as CCL19 and CCL21 (Figure 1.4)\(^{52,55}\). These DCs form immunological synapses with naïve T cells through T cell receptor (TCR):major histocompatibility complex (MHC) class II interactions and co-stimulatory signals to stimulate CD4\(^+\) T helper cell differentiation\(^{56}\). In recent years the identification of Type-2 innate lymphoid cells (ILC2) in mucosal tissues, and how they provide an early source of IL-13 in the presence of IL-25 and -33, has provided an additional link between innate antigen detection and priming of Type 2 adaptive responses\(^{57}\).

Myeloid DCs (mDCs) found in the airway wall skew naïve CD4\(^+\) T cells toward the Th2 phenotype and attract them to sites of allergen contact by releasing CCL17 and CCL22 \(^{58,59}\), although this DC response against allergens is dependent on innate immune signalling in the presence of pathogen-associated molecular patterns (PAMPs) like the gram negative bacterial component lipopolysaccharide (LPS), or damage-associated molecular patterns (DAMPs)\(^{60}\). This is further regulated by the influence of plasmacytoid DCs (pDCs), which are reported to promote immune tolerance to allergens, in addition to their IFN-producing potential\(^{61}\). Thus the relative abundance of mDCs versus pDCs in the airways, together with the presence of danger signals at the mucosal surface critically determine the generation of allergen-specific effector T cells.

Sustained inflammation against allergens in allergic asthma can be further exacerbated by viral infections, such as rhinovirus (RV) and respiratory syncytial virus (RSV)\(^{62}\), which will be expanded upon in Chapter 1.3.
Figure 1.4. Sensitisation to aeroallergens in the airways of atopic individuals. Aeroallergens are captured and processed by DCs, in co-ordination with epithelial-derived cytokines, leading to activation and migration of mature DCs to draining lymph nodes. Allergen peptides are presented thought MHC class II to naïve T cells, in the presence of costimulatory molecules and resident IL-4, polarising them into the $T_{H2}$ phenotype. IL-4 and -13, together with appropriate costimulatory molecules, initiates B cell immunoglobulin class-switching to produce IgE, which diffuses into local lymphatics and enters systemic blood. Allergen-specific or non-specific IgE binds to the high-affinity receptor for IgE (FceRI) on tissue-resident mast cells, sensitising them to respond when the host is later re-exposed to the allergen. (Adapted from Galli et al., Nature, 2008)
1.1.3.3 Adaptive immune elements during the late-phase response

The phenotypical cytokines released by T\textsubscript{H}2 cells, IL-4, -5 and -13, are heavily involved in the processes of cellular influx, chronic inflammation and airway remodelling, as displayed in Figure 1.5\textsuperscript{63,64}. Labelling asthma as a T\textsubscript{H}2-driven disease emerged primarily from clinical studies showing elevated T\textsubscript{H}2 cytokines in the airways and blood of asthmatics, and from mouse studies using models of allergic airway disease\textsuperscript{65-67}.

![Diagram of immune response](image)

**Figure 1.5. Roles of the T\textsubscript{H}2 cytokines in the pathogenesis of allergic asthma.** Asthma initiation and progression results from persistent airway inflammation mediated by allergen-specific T\textsubscript{H}2 cells responding to allergen peptides displayed through MHC class II. Allergen inhalation induces production of the cytokines: IL-4, that promotes T\textsubscript{H}2 differentiation, IgE production and mast cell priming; IL-5, which releases, matures and recruits eosinophils to lung tissue; and IL-13, which induces mucus hypersecretion, smooth muscle constriction and AHR. IL-10-producing Tregs possess the ability to suppress and resolve T\textsubscript{H}2 responses. (Adapted from Broide, Journal of Allergy and Clinical Immunology, 2008)\textsuperscript{68}.

Much of our knowledge regarding function of IL-4, -5 and -13 is derived from inhibition studies using neutralising antibodies, small RNA interference, or gene targeting, which all prevent some aspect of allergic airway disease\textsuperscript{69}. IL-4 can cause B lymphocyte immunoglobulin gene segments to undergo class-switch recombination, resulting in rearrangement of immunoglobulin heavy chain gene segments and the subsequent production of IgE\textsuperscript{70}. This IgE makes its way to the blood stream where it binds to tissue-resident mast cells via the high affinity IgE receptor (FceRI)\textsuperscript{71}. This
primes the mast cells and enables immediate response and pro-inflammatory mediator release upon allergen re-exposure.

IL-5 is known to be necessary for the maturation and release of eosinophils from the bone marrow, as well as tissue eosinophilia in concert with eotaxins. However its role in AHR was somewhat disparate as mouse studies found IL-5 necessary for AHR but anti-IL-5 treatment of stable asthmatics had no effect on AHR despite partial reduction in sputum and blood eosinophilia. This required characterisation of concert roles for eotaxins and IL-13 in regulating lung eosinophilia and AHR together with IL-5, explaining clinical findings of humanised IL-5 antibody not altering lung function or AHR in stable asthmatics. Interestingly, IL-5 inhibition prevents exacerbation in patients with severe unstable eosinophilic asthma, a monoclonal therapy for these patients receiving approval from the FDA in 2015.

IL-13 has been implicated in all hallmark features of allergic asthma in both human and mouse studies. In fact, introducing IL-13 exogenously to the airways of mice induces AHR, airways inflammation and mucus hypersecretion in the absence of allergen, in a signal transducer and activator of transcription 6 (STAT6)-dependent manner. Conversely, IL-13 inhibition after allergen sensitisation precludes development of experimental asthma despite subsequent and repeated allergen challenges. IL-13 inhibition during clinical trials resulted in reduced symptoms and a lower drop in lung function after allergen challenge of asthmatics.

Thus there is strong evidence that T\textsubscript{H}2 cytokines, particularly IL-13, are important effector molecules in the perpetuation of allergic inflammation and AHR.
1.1.4 Asthma phenotypes and treatments

While it is tempting to settle that asthma is a T\textsubscript{H}2-driven disease linked strongly with atopy and allergy, meta-analysis of many clinical studies suggest that while this is the case for most asthmatics, not all cases fit this rigid view\textsuperscript{84,85}. As asthma has evolved from describing a single disease into one encompassing multiple subgroups, the use of phenotype classification has required redefinition that unifies natural patient history, consistent clinical presentation, identifiable pathobiology with biomarkers and predictable therapy responsiveness, criteria that is summarised in Figure 1.6\textsuperscript{86,87}. Although there is no standardised system of subgrouping these phenotypes without criteria overlap, the use of multiple avenues of inflammometry-based evidence can still provide some degree of treatment tailoring for patients\textsuperscript{88,89}.

![Figure 1.6. Asthma phenotypes and their defining characteristics.](image)

The sub-classification of asthma phenotypes or endotypes can be a dynamic process, requiring multiple avenues of evidence with some overlap between them. Five generalised asthma phenotypes have been described: (1) Early-onset allergic is the most prevalent and is strongly linked with T\textsubscript{H}2-mediated responses and allergic syndromes, remaining responsive to corticosteroid treatment. (2) Late-onset eosinophilic manifests in adulthood and while also appearing T\textsubscript{H}2 in nature, is usually more severe and refractory to corticosteroids. (3) Exercise-induced asthma appears intermittently with exercise, has a small T\textsubscript{H}2 profile and is responsive to granulocyte-targeting therapies. (4) Obesity-related asthma displays a lack of T\textsubscript{H}2-associated biomarkers, with signalling from adipokines and oxidative stress being proposed as patients are reported to respond to antioxidants and weight loss. (5) Neutrophilic asthmatics display a T\textsubscript{H}17-associated pathobiology, sputum neutrophilia and moderate-to-severe respiratory dysfunction. This group represents a significant healthcare burden as they are refractory to corticosteroids but potentially responsive to macrolide therapy. (Adapted from Wenzel, Journal of Allergy and Clinical Immunology, 2012)\textsuperscript{90}.
Based on early evidence of the T cell response to inhaled allergens, it was hypothesised that successful therapies would promote Th1-dominant responses instead of Th2, although it is now known that release of Th1 cytokines also contributes to exacerbation of established asthma\(^{91,92}\). This is further challenged by proposed roles of Th17 and regulatory T cells (Tregs) in the immune response against aeroallergens. Th17 cells have been shown to augment experimental airway inflammation and the IL-17 cytokines are elevated in severe asthmatics, however the specific role of Th17 cells in allergic asthma is still being elucidated\(^{93,94}\). Allergic asthmatics show functional deficits in their Treg subsets\(^95\), and adoptive transfer of Tregs can suppress established AAD in animal models\(^{96,97}\). This phenomenon can be partly exploited by the current therapies of corticosteroids and long-acting β\(_2\) agonists (LABAs) to enhance IL-10 production from Tregs\(^95\).

Ultimately our limited understanding of the molecular basis that underpins asthma restricts treatment options largely to reversing acute airflow obstruction with short-acting β\(_2\) agonists (SABAs), controlling symptoms with LABAs, and trying to inhibit the multitude of pro-inflammatory cascades non-selectively with inhaled corticosteroids (ICS)\(^98\). Although this strategy is effective in controlling symptoms of most asthmatics, like those displaying eosinophilic airways inflammation, with mild to moderate disease\(^99,100\), such treatments does not selectively target the pro-inflammatory signals which are crucial in the regulation of other hallmark features of asthma, including the exacerbation of AHR by respiratory viruses or neutrophilic inflammation\(^101\).
1.2 Rhinovirus

1.2.1 Virology

Human Rhinoviruses (HRVs) are small non-enveloped viruses of the Picornaviridae family that have an icosahedral capsid formation, comprising 60 copies of each of the four viral proteins, VP1-VP4. Their positive-sense single-stranded RNA genome is approximately 7200bp in length, a single gene whose translated protein is cleaved into 11 proteins by viral proteases. Since their discovery in the 1950’s, over 100 distinct serotypes have been isolated, compromising the three species HRV-A, -B and -C, with HRV-A and -B historically grouped further into major (90%) or minor strains (10%) based on their cell entry receptor specificity, intracellular adhesion molecule 1 (ICAM-1) or low density lipoprotein receptor (LDLR) respectively. This distinction has largely fallen by the wayside since the introduction of molecular identification techniques.

Upon contact and uptake into AECs, virions undergo conformational changes in response to an endosomal pH drop that yields hydrophobic subviral particles (Figure 1.7). The RNA genome moves to the cytosol through viral protein-mediated pores or membrane rupture, where host ribosomes translate the positive-sense RNA into a polyprotein that undergoes processing into its individual viral proteins. Negative-strand parental RNA is also transcribed and new virions are assembled out of VP1-VP4 and packaged with new HRV genomes and viral proteins prior to cellular export via cell lysis.

HRV infections are very common in the human population, at a rate of 5-12 per year during childhood, with infections continuing throughout life, indicating a lack of effective cross-serotype immunity. Infections in healthy individuals cause common cold-like symptoms, while those with existing lung pathology like asthma or chronic obstructive pulmonary disease (COPD), experience much more severe disease, which will be discussed in Chapter 1.3.

No approved antiviral agents exist for the prevention or treatment of HRV infection due to limitations of use in clinical trials arising from drug toxicities, drug interactions and
low efficacy when applied to a natural setting\(^{110}\). Vaccine development is similarly hindered by the number of HRV serotypes together with high-level sequence variability in its antigenic sites, the elucidation of common antigenic epitopes are a necessary step to ensure induction of efficacious cross-reactive antibodies\(^{111}\). To this end, there has been recent work with VP1 peptides, the capsid protein involved in receptor binding and cell entry, as it is recognised by HRV-neutralising antibodies naturally however no studies have progressed past the \textit{in-vitro} stage\(^{112}\). Hence, treatment of infection remains primarily supportive and focuses on symptom relief\(^{113}\).

\textbf{Figure 1.7. HRV replication in airway epithelial cells.} Viral uptake occurs via clathrin-dependent or independent endocytosis or via micropinocytosis, depending on the receptor type used. Lowering of pH in the endosomal space triggers viral uncoating and the genomic RNA undergoes translation into viral proteins or replication into negative strand (parental) RNA. Progeny virions are then assembled and packaged prior to cellular export via cell lysis. (Adapted from Jacobs \textit{et al.}, Clinical Microbiology Reviews, 2013)\(^{113}\).
1.2.2 Host response to infection

1.2.2.1 Detection and innate immune responses

Unlike influenza virus and RSV, HRV infection is not associated with upper respiratory tract cytopathology, with cell linings and borders remaining intact\(^{114}\). Lower respiratory tract infection is also supported, with pro-inflammatory responses including alveolar damage and secretion of IL-6 and IL-8\(^{115}\). HRV also disrupts epithelial tight junctions, facilitating the transmigration and exposure of bacteria and bacterial components to the basolateral membrane\(^{116}\).

Innate pattern recognition receptors (PRRs) are often the first point of pathogen detection, with HRV capsid components being detected by Toll-like receptor (TLR) 2 on the cell surface and the ssRNA genome by TLR7/8 in the endosomal space\(^{117}\). During replication in the cytosol, dsRNA is detected by TLR3, melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid-inducible gene 1 (RIG-1), which function in concert through interferon regulatory factor (IRF)-3 and -7 and nuclear factor kappa B (NFkB) to maximise the induction of IFN-β and IFN-λ, CCL5/RANTES, chemokine (C-X-C motif) ligand (CXCL)-10/IP-10, IL-6 and CXCL8/IL-8\(^{118-120}\). This sets in motion the recruitment of monocytes, neutrophils and lymphocytes to the airway wall where rate of viral clearance is affected by presence of HRV-reactive or cross-reactive T cells and levels of Th1 cytokine production, particularly IFN-γ and IL-2\(^{121,122}\).
Figure 1.8. Cellular and cytokine response to HRV-infected airway epithelial cells. HRV-infected airway epithelial cells secrete a milieu of cytokines and chemokines, including IFN-β, IFN-λ, IL-6, GM-CSF, CXCL10/IP-10, CCL5/RANTES and CXCL8/IL-8. These cytokines attract various inflammatory cells such as dendritic cells (DC), lymphocytes (Lym), neutrophils (Neut) and macrophages (Mφ). (Adapted from Saraya et al., Frontiers in Microbiology, 2014)\textsuperscript{123}.
1.2.2.2 Humoral responses to HRV

Following infection of an antibody-naïve individual with HRV, serotype-specific neutralising serum (IgG) and airway secretory (IgA) antibodies are produced. These antibodies are detectable 1-2 weeks post-infection and remain elevated for up to a year, persisting high-titre serotype-specific antibodies being associated with improved protection from infection and reduced symptom severity\textsuperscript{124,125}. Yet the occurrence of cross-neutralisation between serotypes is very hit-and-miss, often giving little to no protection against related but distinct HRV strains\textsuperscript{126}. Further supporting the role of humoral immunity in preventing and controlling HRV infection, patients with primary hypogammaglobulinemia (reduced levels of all antibody classes) experience more frequent and severe HRV infections despite receiving replacement Ig therapy\textsuperscript{127}. 

1.3 Rhinovirus-induced asthma exacerbation

1.3.1 Prevalence and healthcare burden

Viral respiratory tract infections have been reported as triggers for exacerbation of asthma since the 1970’s\textsuperscript{128}, the advent of molecular detection equipment, like Reverse transcription - polymerase chain reaction (RT-PCR), facilitating the discovery that viruses are found in approximately 80% of wheezing children and half of adult acute wheezing episodes\textsuperscript{129}. Of the respiratory viruses detected in these circumstances, HRV’s are identified approximately 65% of the time\textsuperscript{130,131} and are the only respiratory virus significantly associated with asthma exacerbation in children (Figure 1.9)\textsuperscript{132}. Asthma exacerbations contribute the majority of hospitalisations and healthcare costs associated with HRV infection\textsuperscript{133,134}.

Figure 1.9. Prevalence of viruses and bacteria species associated with asthma exacerbation. Presented by proportion are the prevalence of viral and bacterial species most commonly detected during asthma exacerbation in young children (<2 years old), older children (6-17 years old) and adults, displayed as the median percentages from several studies reviewed in Papadoloulos et al., 2011\textsuperscript{135}. (Adapted from Edwards et al., Nature Reviews Microbiology, 2012)\textsuperscript{136}. 
1.3.2 Clinical observations

Understanding the provoking mechanisms of RV-induced exacerbation offers a significant opportunity for improved disease management and patient quality of life. To this end, both human and murine models of RV-induced asthma exacerbation have been developed with the goal of arriving at these novel therapies in the future\textsuperscript{137,138}.

Experimental infection of asthmatics and non-asthmatics with HRV was observed to increase lower respiratory tract symptoms akin to a mild exacerbation in asthmatic subjects with little change noted in non-asthmatics (Figure 1.10), including a more protracted duration of virus-induced symptoms\textsuperscript{139}. This was accompanied with increased AHR and decreased peak expiratory flow and FEV\textsubscript{1}, supporting that respiratory tract viral infections lead to greater morbidity in asthmatics\textsuperscript{137}.

![Figure 1.10. Lower respiratory symptom scores during HRV infection.](image)

Experimental infection with RV16 in asthmatic (top) and healthy individuals (bottom) results in more severe lower respiratory (chest) symptom scores in asthmatics, with scores significantly above baseline on days 1 and 3-7. (Adapted from Message et al., PNAS, 2008)\textsuperscript{137}. 
1.3.3 Experimental findings

Based on observations from clinical studies, it has been suggested that there are inherent differences in how asthmatics respond to RV compared to healthy individuals. Subsequent efforts to elucidate the underpinning molecular mechanisms having been largely centred on first-contact responses at the broncho-epithelial barrier, such as pathogen detection by TLRs and innate interferon signalling, and how this influences the responsiveness of circulating peripheral blood mononuclear cells (PBMCs).

TLR activation is a critical initiator of anti-microbial pathways that ultimately result in the clearance of invading pathogens, yet this can ameliorate or exacerbate chronic lung inflammation, as shown in a mouse model of AAD where activation of TLR3&4 (by dsRNA and LPS respectively) increased airways inflammation\(^{140}\). However there is mounting evidence that activation of TLR7 or TLR9 may be protective in asthma with emerging studies reporting that pre-treatment with agonists reduce the cardinal signs of lung pathology in acute and chronic asthma models \(^{141-143}\). Although it remains to be seen if these agonists have efficacy for the prevention or treatment of virally-induced asthma exacerbation.

Type I interferons, type III interferons and the interferon-stimulated genes (ISGs) they activate have diverse anti-viral and immunomodulatory activities, so the emergence of defects in this pathway in asthmatics was not completely surprising and has spurred on a number of investigations. Wark et al. were the first to report that bronchial epithelial cells isolated by bronchoscopy from asthmatics and infected with RV \textit{ex-vivo} produced lower levels of IFN-β and had higher levels of viral replication (though equal IL-6 and CCL5 responses) compared to non-asthmatics (Figure 1.11)\(^{144}\). This phenotype was independent of patient steroid treatment and was supressed by exogenous IFN-β, indicating a deficiency in IFN production and not responsiveness\(^{144}\). The findings of Contoli et al. supported this by reporting that bronchoalveolar lavage (BAL) cells from asthmatics produced less IFN-λ in response to RV compared to non-asthmatics, and that the magnitude of IFN response negatively correlated with airway symptom scores, viral load and markers of inflammation\(^{145}\). Bullens et al. also demonstrated that IFN-λ1 expression in asthmatic sputum cells negatively correlated with asthma symptoms, further highlighting a potentially protective role for IFN in asthma\(^{146}\).
These phenotypic differences at the site of infection may also skew the functional programming of circulating myeloid and lymphoid cells, as signals from mucosal surfaces during infection are known to influence more than just their release and migration from the bone marrow\textsuperscript{147}. To this end, PBMCs from asthmatic and non-asthmatic individuals have been compared following stimulation with RV, with multiple defects in their IFN signalling pathways found \textsuperscript{148}. In a study looking at regulation of T\textsubscript{H}2 responses by IFNs, Pritchard \textit{et al.} published that type I IFNs from pDCs are integral to controlling RV infection and limiting deleterious immune activation\textsuperscript{149}. This same group also found increased secretion of IL-5 and other T\textsubscript{H}2 cytokines from PBMCs when exposed to both RV and type III IFN, a phenotype reversed by addition of IFN-β\textsuperscript{150}. Attempting to bring these studies full circle, a randomised controlled trial found that administration of nebulised IFN-β prevented the progression of upper respiratory tract infection to the manifestation of wheezing symptoms in asthmatics\textsuperscript{151}. Taken together, the larger picture of signalling alterations in asthmatics is starting to come into focus, with potential targets for modulation slowly being identified.
Figure 1.1. Immune response to viral infection in healthy and asthmatic individuals. Respiratory viral infection in healthy individuals (left) results in robust IFN and T\(_H\)1 cell responses, leading to rapid control of viral replication and minimal lung inflammation. In an asthmatic (right), impaired IFN responses in a T\(_H\)2 dominated micro-environment results in poorly-controlled viral replication and an exaggerated inflammatory response. (Adapted from Singanayagam et al., BMC Medicine, 2012)\(^{152}\).
1.3.4 Therapeutic options

There are a number of therapeutic strategies that can potentially alter the occurrence of virus-induced asthma exacerbations. Infection prevention with vaccines, monoclonal antibodies, viral attachment inhibitors or viral protease inhibitors are sound in theory, however they require serotype and temporal knowledge to be administered successfully. β₂ agonists remain the go to for acute exacerbation by relaxing airway smooth muscle and promoting bronchodilation at symptom onset, although usually paired with an ICS for their anti-inflammatory effects, a mainstay since the 1970’s. Despite optimal corticosteroid use, refractory patient populations have driven development of alternate therapies, such as the classes of leukotriene antagonists. These target the cognate receptors for the inflammatory lipid mediators produced by granulocytes, which increase airway smooth muscle contraction and vascular permeability during exacerbation. Additionally, type-2 cytokines have been the subject of monoclonal antibody development, with multiple generations of IL-4/IL-13 and IL-5 neutralising antibodies reaching phase II and phase III clinical trials. There are also studies documenting the experimental administration of macrolide/ketolide antibiotics, exogenous IFN, which with the others mentioned, remain the most viable therapies against virus-induced asthma exacerbation (Figure 1.12).
Figure 1.12. Available strategies to prevent virus-induced asthma exacerbation. As AECs are the initial site of viral infection and host response, using anti-viral approaches to either block infection or boost viral immunity are attractive but hard to design. Following infection and release of AEC-produced cytokines (IL-25, IL-33, TSLP and TRAIL), type-2 innate lymphoid cells (ILC2) and T_{H}2 cells are primed to activate either directly or by DCs, becoming the primary source of the type-2 cytokines that underpin asthma pathology. Monoclonal antibodies (mAbs) against IL-5 (mepolizumab) and IgE (omalizumab) have recently been licensed and approved for use in asthma treatment. (Adapted from Tay et al., Expert Rev Respir Med, 2015).
1.4 Study rationale

1.4.1 Publication 1

As detailed in Chapter 1.1.3.2, AECs are the initial site of allergen and viral responses in the lung, producing a milieu of cytokines and chemokines to initiate cellular and antiviral responses. One of these cytokines is TRAIL, a TNF superfamily member that shares significant homology with TNF-α and Fas ligand (FasL)\textsuperscript{160,161}. It’s ability to initiate apoptosis in transformed tumour cells, while having little cytotoxic effect on most primary cells has driven research into this cytokine for decades\textsuperscript{162,163}. TRAIL expression is activation-dependent and is produced by AECs, DCs, T cells, NK cells, neutrophils, eosinophils and monocytes, all cells relevant to allergic and anti-viral responses\textsuperscript{164-166}. In clinical studies, TRAIL was found to be elevated in the BAL of allergic asthmatics following allergen challenge, which correlated with eosinophilic inflammation\textsuperscript{167}. Eosinohils and alveolar macrophages isolated from asthmatics also expressed more TRAIL on their surface and had prolonged lifespans \textit{in-vitro}\textsuperscript{164,167}.

The role of TRAIL in asthma pathogenesis was further investigated by Weckmann \textit{et al.}, where it was reported that mice deficient in TRAIL were protected from developing all the hallmark features of AAD\textsuperscript{53}. TRAIL was found to be necessary and adequate for the development of AAD, with TRAIL directly upregulating CCL20 release from AECs, promoting the homing of mDCs and \(T_{H2}\) cells to the airways. However the signalling pathway downstream of TRAIL in this context was yet to be identified.

To this end, a whole transcriptome microarray study was performed (ArrayExpress accession no.E-MEXP-2960) on RNA isolated from the airway tissue of wildtype and TRAIL-deficient mice. It was found that Midline-1 (MID1) was the most dysregulated transcript, with expression significantly higher in the presence of TRAIL and further increased during AAD, suggesting that this gene could be downstream of TRAIL in a pro-inflammatory setting. MID1 encodes a microtubule-associated E3 ubiquitin ligase, which is a member of the RBCC protein family\textsuperscript{168}. Midline-1 is primarily known to interact with the regulatory \(\alpha4\) subunit of protein phosphatase 2A (PP2A), where in this complex Midline-1 initiates the ubiquination of the catalytic subunit of PP2A (PP2Ac), targeting it for proteosomal degradation, as shown in Figure 1.1\textsuperscript{169,170}. 

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PP2A is natively a homotrimer (A, B and C subunits) of three groups of ubiquitously expressed phosphoserine-threonine specific enzymes. A and C are highly conserved scaffolding and catalytic subunits, with B a varied class conferring substrate specificity to many signalling molecules, such as mitogen-activated protein (MAP) kinases.

Figure 1.13. Regulation of PP2Ac and PP2A activity by MID1/α4. MID1, α4 and PP2Ac form a ternary complex (1) when instructed, α4 initially thought to facilitate MID1-dependent polyubiquitination of PP2Ac and subsequent proteosomal degradation (2A). Recent studies however promote the paradigm where MID1 acts as the ligase for α4 (2B), leading to a conformational change and cleavage of α4 (3 and 4), allowing the polyubiquitination of PP2Ac by a currently unknown E3 ligase (5). (Adapted from Watkins et al., J Biol Chem, 2012).

This observation of PP2A dephosphorylating molecules like MAP kinase (M KK) 3/6 and inhibitors of NFκB (IκB) kinase (IKK), which regulate the activation of NFκB and p38, is highly relevant for the role of TRAIL signalling in AAD as activation of MAPKs and NFκB result in the downstream production of proinflammatory cytokines (such as the eotaxins, IFN-γ, GM-CSF, CCL20 etc.) under chronic conditions.

Hence, the purpose of this first study was to identify the stimuli required to see upregulation of MID1 in an in-vivo model of AAD, link this to suppression of PP2A activity and then artificially modulate this phenomenon and measure changes to the
inflammatory phenotype. This was extended into responses against RV in a non-allergic and allergic setting, finishing with the translational work of documenting these responses in primary AECs isolated from healthy and asthmatics individuals.
1.4.2 Publication 2

RV can be sensed at mucosal barriers by a conserved number of PRRs, such as those of the TLR and retinoid acid-induced gene 1-like receptor (RLR) families. Those specifically capable of detecting RV; TLR3, TLR7, RIG-I and MDA-5, whom are endosomally localised and recognise viral nucleic acids, are critical in initiating anti-viral IFN production. However, as described in Chapter 1.3.3 there are documented deficits in IFN responses to RV in some asthmatics, with little understanding as to why this is. Given the upstream importance of these virus-sensing receptors in anti-viral pathways, various groups are investigating the relative contribution of these receptors during RV infection, a story only beginning to be elucidated.

These recent studies have shown that expression of TLR3, RIG-I and MDA-5 in AECs and BAL cells is comparable between asthmatics and non-asthmatics (Figure 1.14), yet there remains a reduced anti-viral responsiveness to RV in asthmatics, with lower production of IFNs$^{175,176}$. The contributions of TLR3 and MDA-5 during RV infection in-vivo has been investigated by Wang et al. using mouse models, where they reported that deficiency of MDA-5 but not TLR3 delayed IFN responses and viral clearance, while absence of either receptor attenuated lung inflammation and AHR$^{177}$. In contrast, the in-vivo role of functional TLR7 signalling in mounting anti-viral responses to RV has not yet been determined. TLR7 is widely expressed in innate immune cells such as pDCs and macrophages, as well as in structural lung cells, including well-differentiated AECs$^{178}$. Interestingly, the treatment of mice with the TLR7 agonist R848 lead to a long-lasting protection from the development of AAD$^{179}$ and TLR7 activation also reduced airway smooth muscle contractility in mice$^{180}$. PBMCs isolated from asthmatics also exhibit sup-optimal production of IFN-α and IFN-λ following TLR7 stimulation$^{181,182}$, indicating functional deficits in this pathway as well. Yet, the in-vivo role of functional TLR7 signalling in RV infection and RV-induced exacerbation of AAD has yet to be defined.
Figure 1.4. Baseline expression of virus-sensing receptors in the airways of healthy and asthmatic individuals. Baseline mRNA expression of MDA5, TLR3 and RIG-I in (left) primary bronchial epithelial cells (pBEC) and (right) endobronchial biopsies isolated from 8 healthy controls (HC) and 12 asthmatic individuals. Expression was determined by qRT-PCR and presented relative to housekeeper 18S rRNA using the ΔΔCT method. (Adapted from Parsons et al., Clin Exp Allergy, 2014) \(^{175}\).

To address these gaps in knowledge, this study was designed to identify the in-vivo contribution of intact TLR7 signalling during RV infection in an allergic lung setting by assessing the magnitude of anti-viral responses, viral replication and tissue inflammation. This was further dissected through the application of exogenous IFN therapy and transfer of TLR7-competent pDCs to mice deficient in TLR7 signalling, in order to elucidate whether these responses could be modulated and the cellular contribution of IFN producing cells. Finally, the signals required to modulate the expression of TLR7 in mouse lung tissue was investigated and then compared to TLR7/IFN expression in human lung tissue biopsies collected from non-asthmatic and asthmatic individuals of varying clinical phenotypes.
1.4.3 Publication 3

Following the elucidation of a novel TRAIL signalling pathway and its relevance to asthmatic lung inflammation in Chapter 2, as well as identifying PP2A as a target in this context, it was serendipitous that two studies were published around this time linking deficits in PP2A activity to severe asthma and then describing a novel pharmacological modulation of PP2A activity by an established therapeutic agent normally prescribed to asthmatics.

In the first of these two studies, Kobayashi et al. found that PP2A promoted the nuclear translocation of the glucocorticoid receptor (GR) and corticosteroid sensitivity of U937 cells when stimulated with TNF-α or LPS in-vitro, these cells becoming steroid resistant when PP2A was targeted for inhibition. PBMCs isolated from severe asthmatics also exhibited reduced PP2A activity compared to healthy controls ex-vivo, supporting the idea that PP2A is suppressed in asthmatics with active inflammation. 183

In their following study, Kobayashi et al showed that the LABA formoterol could also restore corticosteroid sensitivity in-vitro, an effect that was lost when PP2A was inhibited. This was investigated further by showing that formoterol enhanced PP2A activity in cell lines and human PBMCs, as well as in a cell free system, and that this effect was also exhibited by the LABA salmeterol (Figure 1.15). 184

The focus of this study henceforth was to extend the findings of Publication #1 and Kobayashi et al. into an in-vivo model of AAD (with a clinically relevant allergen) and RV-induced exacerbation, as well as provide insight into the mechanisms by which these anti-inflammatory effects are being mediated, outside of the known bronchodilatory effects of LABAs.
Figure 1.15. Agonistic effects of LABAs on recombinant or immunopurified PP2A. Recombinant (left) or immunopurified PP2A from U937 cells (right) was treated with formoterol (FM), salmeterol (SM) or salbutamol (SB) for 20 minutes before activity was assessed by phosphatase assay. Okadiac acid (OA) was added to some groups for 60 minutes prior to assessment to inhibit PP2A activity. Activity expressed as fold change relative to non-treatment (NT). (Adapted from Kobayashi et al., Pulm Pharmacol Ther, 2012)\textsuperscript{184}. 
CHAPTER 2: The E3 ubiquitin ligase midline 1 promotes allergen and rhinovirus-induced asthma by inhibiting protein phosphatase 2A activity

2.1 Manuscript

The E3 ubiquitin ligase midline 1 promotes allergen and rhinovirus-induced asthma by inhibiting protein phosphatase 2A activity

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Allergic airway inflammation is associated with activation of innate immune pathways by allergens. Acute exacerbations of asthma are commonly associated with rhinovirus infection. Here we show that, after exposure to house dust mite (HDM) or rhinovirus infection, the E3 ubiquitin ligase midline 1 (MID1) is upregulated in mouse bronchial epithelium. HDM regulates MID1 expression in a Toll-like receptor 4 (TLR4)- and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–dependent manner. MID1 decreases protein phosphatase 2A (PP2A) activity through association with its catalytic subunit PP2AC. siRNA-mediated knockdown of MID1 or pharmacological activation of PP2A using a nonphosphorylatable FTY720 analog in mice exposed to HDM reduces airway hyperreactivity and inflammation, including the expression of interleukin-25 (IL-25), IL-33 and CCL20, IL-5 and IL-13 release, nuclear factor (NF)κB activity, p38 mitogen-activated protein kinase (MAPK) phosphorylation, accumulation of eosinophils, T lymphocytes and myeloid dendritic cells, and the number of mucus-producing cells. MID1 inhibition also limited rhinovirus-induced exacerbation of allergic airway disease. We found that MID1 was upregulated in primary human bronchial epithelial cells upon HDM or rhinovirus exposure, and this correlated with TRAIL and CCL20 expression. Together, these findings identify a new role of MID1 in allergic airway inflammation and links innate immune pathway activation to the development and exacerbation of asthma.

Allergic airway inflammation and asthma are associated with the activation of innate and adaptive immune cells1. The cytokines thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-25, IL-33 and tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) are released by bronchial epithelial cells upon allergen exposure, activating dendritic cells and promoting Thelper type 2 (Th2) cell differentiation1. Th2 cells then release IL-13, which induces airway hyperreactivity (AHR) and mucus production in a signal transducer and activator of transcription 6 (STAT6)-dependent manner6–8. Respiratory infections, which are predominantly caused by rhinovirus in people with asthma, exacerbate airway inflammation and further contribute to disease burden and healthcare costs9–11. Some individuals with asthma have deficiencies in their antiviral epithelial response, predisposing them to exaggerated inflammatory responses12,13. This places the bronchial epithelium at the center of asthma pathogenesis and makes it a target for advanced therapeutics14–16.

To identify new signaling pathways activated by allergens, we determined gene transcripts that were differentially expressed in blunted diseased airway wall tissue of wild-type (WT) mice and mice deficient for TRAIL (Tnfsf10−/−) (ArrayExpress accession no. E-MEXP-2960), which are protected from ovalbumin-4 and HDM-induced (Supplementary Fig. 1) allergic airway disease. Among other mRNA sequences, we found that the microRNA-associated E3 ubiquitin ligase MID1 (also known as tripartite motif containing protein [TRIM] 18) was upregulated in WT mice sensitized and challenged with HDM (allergic mice) as compared to WT mice sensitized and challenged with normal saline only (nonallergic control mice) and allergic Tnfsf10−/− mice (Fig. 1a). We observed increased MID1-specific staining in allergic WT mice primarily in bronchial epithelial cells (Fig. 1a). TLR4 signaling is required for the development of allergic airway inflammation in response to HDM extract14–16. Upregulation of MID1 was attenuated in mice deficient in TLR4 (Tlr4−/−) or the...
adaptor molecule MyD88 (MyD88-/-) in response to allergen exposure as compared to allergic WT mice (Fig. 1a). Although mice deficient in Stat6 (Stat6-/-), like Tlr4-/-, MyD88-/- and TnfR1-/- mice, show reduced airway inflammation in response to allergen exposure6,13,18, we found high MID1 expression in Stat6-/- mice sensitized and challenged with HDM (Fig. 1a), suggesting HDM promotes MID1 expression in a TLR4-dependent manner upstream of Stat6.

To investigate whether the induction of MID1 required pre-existing allergic inflammation, we administered one dose of either HDM, recombinant rTRAIL or a low or high dose of the TLR4 ligand...
Lipopolysaccharide (LPS) intranasally to naive WT mice (Fig. 1b). MID1 expression was significantly upregulated in the airway wall 24 h after HDM, rTRAIL, and high-dose but not low-dose LPS exposure (Fig. 1b).

The MID1 gene is located at locus Xp12.3 in humans, and mutations in MID1 have been associated with X-linked Opitz/GBBB syndrome, an inherited malformation characterized by midline defects such as cleft lip and/or palate[7,8]. Mutations found in individuals with Opitz syndrome disrupt transport of MID1 and migration of neural crest cells[9,10]. Beyond embryonic development, MID1 interacts with the oct regulatory subunit of the protein phosphatase PP2A and is required for the ubiquitin-specific modification and proteasome-mediated degradation of its catalytic subunit PP2Ac[11-13]. In HDM-challenged WT and Stat6−/− mice, induction of MID1 was associated with decreased PP2A activity and PP2Ac protein expression (Fig. 1a,c). MID1 expression, PP2A activity, and PP2Ac expression remained unchanged in Tg(Epo) mice sensitized and challenged with HDM (Fig. 1a,c), suggesting that MID1 regulates PP2A activity upstream of Stat6 in vivo.

The PP2A holoenzyme is composed of three subunits; the PP2A-B subunit has multiple isoforms and contains substrate specificity, whereas PP2A-A and PP2A-C are the highly conserved scaffolding and catalytic subunits, respectively[14]. PP2A is the most abundantly expressed protein phosphatase and has been shown to dephosphorylate mitogen-activated protein kinases (MAPKs) and inhibitor of κB (IκB) protein, thereby limiting p38 MAPK, c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) activity[15-17]. p38 MAPK signaling activity is high in the airway wall of individuals with severe asthma[18,19] and promotes airway inflammation in mice[20,21], whereas NF-κB has a key role in TLR-mediated allergic airway disease[22]. Dephosphorylation of JNK by PP2A has been shown to regulate glucocorticoid receptor nuclear translocation, which may be relevant for steroid-resistant asthma[23].

To assess the role of MID1 in allergic airway disease, we reduced MID1 expression by siRNA in sensitized mice 24 h before the first challenge with HDM and then every second day during challenge to levels observed in nonallergic mice (Fig. 1d). MID1 silencing attenuated AHR (Fig. 1e), reduced the accumulation of eosinophils in the lungs and the number of Aclain blue–periodic acid–Schiff (PAS)–positive mucus-producing epithelial cells (Fig. 1f), reduced IL-5 and IL-13 release from ex vivo HDM-stimulated lung cells isolated from the draining lymph nodes of the lungs (Fig. 1f), and lowered mRNA expression of CCL20, IL-25, and IL-33 expression (Fig. 1g) but not of TSLP, GM-CSF, CCL17 and CCL22 in the airway wall (Supplementary Fig. 2) as compared to allergic mice treated with a nonsense siRNA. Conversely, treatment of naive mice with rTRAIL increased IL-25 and IL-33 expression (Fig. 1h), which suggests that these factors are regulated by MID1 downstream of TRAIL. MID1 silencing also increased PP2A activity (Fig. 1i) and reduced the levels of phosphorylated IκBα, the NF-κB
expression in the lungs, and reduced the amounts of phosphorylated IkBα, activated NF-κB subunits and phosphorylated JNK compared to nonsense siRNA treatment (Fig. 3a-f). MID1 inhibition also impaired virus replication and, consequently, interferon-α (IFN-α) and IFN-β mRNA expression in the lung of RV1B-infected mice (Supplementary Fig. 6). Next, we silenced MID1 expression in allergic mice with one dose of siRNA given after the last HDM challenge and then infected them with RV1B 24 h later. MID1 silencing reduced rhinovirus-induced exacerbation of AHR, eosinophilic inflammation and the number of mucus-producing epithelial cells (Fig. 3g,h). MID1 inhibition also raised P2A2 activity, impaired chemokine release and lowered levels of phosphorylated IkBα, activated NF-κB subunits and phosphorylated JNK in the lung of RV1B-infected allergic mice (Fig. 3i). After ex vivo recall stimulation of peripheral lymph node cells isolated from RV1B-infected allergic mice with HDM, production of IL-5 (nonsense siRNA, 8.6 ± 0.6 ng ml⁻¹ versus MID1 siRNA, 3.2 ± 0.6 ng ml⁻¹; mean ± s.e.m., P < 0.01) but not IL-13 (data not shown) was reduced. Virus replication and IFN expression in the airway wall was not altered in allergic mice in response to MID1 inhibition (Supplementary Fig. 6).

Next, we incubated transformed human bronchial epithelial cells (BEAS-2B) with tTRAIL or HDM and found they increased MID1 and CCL20 mRNA expression (Fig. 4a) and suppressed P2A2 activity (Fig. 4b). Western blotting and immunoprecipitation with a P2A2-specific antibody suggested that MID1 was associated with P2A2 and the trimer subunit (Fig. 4c).

Differences between transformed and primary human epithelial cell responses to rhinovirus have been described. We therefore also collected primary human bronchial epithelial cells from subjects with asthma (n = 14) and healthy subjects (n = 14). As expected, lung function was lower in individuals with asthma (P < 0.05), and the majority of those subjects were treated with inhaled corticosteroids (Supplementary Table 1). In vitro infection with RV1B as well as exposure to tTRAIL or HDM increased MID1 expression in epithelial cells from both healthy subjects and those with asthma (Fig. 4d). tTRAIL and CCL20 expression were also upregulated upon RV1B infection (Fig. 4d) and positively correlated with MID1 expression (Fig. 4e). We found impaired IFN-κ production by bronchial epithelial cells from asthmatics as compared to cells from healthy subjects as previously reported (22,23) (Fig. 4f). Intracellular RV1B RNA levels isolated from epithelial cells 24 h after infection were not different between asthmatics and healthy subjects (Supplementary Fig. 5), which is in line with previous reports demonstrating in vitro differences in RV1B RNA levels at 8 h only when virus replication peaks (21,22). Thus, MID1 activates proinflammatory signaling in bronchial epithelial cells from human subjects, which may act in concert with other aberrant responses to allergen and virus exposure in asthma to promote exaggerated airway inflammation and rhinovirus-induced exacerbation.

More than 500 E3 ubiquitin ligases have been identified to date that regulate diverse cellular processes through targeting specific substrates for degradation by the proteasome. The ubiquitin system has been linked to cancer, neurodegenerative and muscle wasting disorders, diabetes, infection and inflammation. Here we have identified MID1 as an E3 ubiquitin ligase that regulates airway inflammation by limiting P2A2 activity (Supplementary Fig. 7) suggesting both MID1
and PP2A activity may be targeted for the treatment of asthma and rhinovirus–induced exacerbations.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.C. and L.H. performed and designed mouse and cell culture experiments, analyzed data, generated figures and edited the manuscript. P.A, B.N.E. and M.T. performed and supervised studies on healthy subjects and subjects with asthma and performed cell culture experiments. N.V. and I.C. performed and analyzed PP2A measurements and immunoprecipitation and designed experiments. N.V. edited the manuscript. A.S.D. and C.M. synthesized and analyzed NPPs for use as an activator of PP2A and developed the dosing regimen. N.Z. and M.R.R. coordinated and assisted in microarray gene analysis. N.W.B. and S.L.J. assisted in design of experiments, provided RVBl for further propagation and CDNAs and edited the manuscript. A.P.S. coordinated and supervised mouse and human studies. P.S.E supervised mouse studies, interpreted data and edited the manuscript. I.M. conceptualized, coordinated, designed and supervised mouse and human studies, interpreted and analyzed data, and drafted and edited the manuscript. All authors contributed to data discussion and revised the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests are available in the online version of the paper.

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ONLINE METHODS
Mice: WT, Tg(Thy1-hChRNA1), Thy1−/−, Mdb88−/− and Stat6−/−; all on a BALB/c background (male, 6–14 weeks of age) were obtained from the special pathogen-free facility of the University of Newcastle. Mice were housed with ad libitum access to food and water with a 12-h light and dark cycle. The Animal Care and Ethics Committee of the University of Newcastle, Australia approved all experiments which were conducted and reported in accordance with the ARRIVE guidelines.

Induction of allergic airway disease and rhinovirus infection. We sensitized and challenged mice by exposing them intranasally to crude HDM extract (50 µg daily at days 0, 1, and 2 followed by four exposures of 5 µg HDM daily from day 14 to 1 day delivered in 50 µl of sterile saline) from Geer Laboratories (allergic mice). The single dose of HDM extract given to naive mice in some experiments was 50 µg in 50 µl of sterile saline. Control nonallergic mice received sterile saline only during sensitization and challenge instead of HDM extract. In some experiments, we infected allergic (day 18, 3, 5 and 7 after last HDM extract challenge) or nonallergic mice with 103 plaque-forming units (PFU) of Rhinovirus 2 (NY84) intranasally or with ultraviolet light (UV)-inactivated Rhinovirus 2 (UV-Rh) (2.5 × 103 PFU of median tissue culture infective dose) intranasally. Mice were killed 24 h after the last allergen or rhinovirus challenge by pentobarbital sodium (Vitaxan) overdose.

Airway hyperreactivity measurement (AHR). We assessed AHR innovatively in separate groups of ketamine xylazine (iLum)−anesthetized mice by measure- ment of total lung resistance and dynamic compliance (Beneck). Percentage increase over baseline (PBR) in response to nebulized methacholine (Sigma) was calculated.

Isolation of mRNA. We isolated total RNA from the mouse lung using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

Quantitative RT-PCR. We performed quantitative RT-PCR with SYBR Green premixed POX (Invitrogen). We quantified mRNA copies using cDNA standards for all genes of interest. We normalized expression to the housekeeper genes Hprt for mouse and GAPDH for human mRNAs. Primers are listed in the Supplementary Methods.

Airway inflammation. We calculated the trachea of mice and lavaged their lungs with 1 ml PBS (Gibco) to collect bronchoalveolar cells, which were enumerated and differentiated by cytospin and May–Grunwald staining under blinded conditions.

Airway morphology studies. We stained paraffin-fixed lung tissues with Alcan blue–periodic acid–Schiff for the enumeration of mucus-producing airway epithelial cells. Charcoal chro-matotope hematoxylin for the identification of eosinophilic or toluidine blue for mast cells. We identified cells by morphological criteria, and we counted ten 100 µm fields in each slice under blinded conditions.

Cytokines and chemokines analysis. We excised parotid lymph node cells filtered through a 100 µm cell sieve (BD) and cultured 5 × 106 cells per ml in RPMI 1640 medium (HyClone) with 10% (v/v) FCS (SAFC Biosciences), 2 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin–streptomycin (Gibco), 0.1 mM sodium pyruvate (HyClone) and 50 µM 2-mercaptoethanol in the presence or absence of 50 µm m31-HDM (optimal concentration) for 6 days. We determined IL-4, IL-5, IL-13 and IFN-γ levels in supernatants by ELISA (BD Biosciences Pharmingen). Lungs were homogenized using a TissuTear stick homogenizer (BioSpec Products). Homogenate levels of CCL5/MIP-1α, CCL4/MIP-1β, CCL11/ eotaxin, CXC10/IP10 and CXC11/IL8 were measured by employing a Multiplex Immunoassay (Millipore), whereas CCL2/MCP-1 and CXC12/MIP-3β intranasally at day 13 (after HDM sensitization and 24 h before the first HDM challenge) and then every second day until mice were killed. In all rhinovirus studies, mice were treated 24 h before and killed 24 h after HRV18 challenge.

siRNA. The antisense strand sequence of siRNA-MID1 from Ambion was: 5′-UGAGGCUAUUGCAAACAUCAUA-3′. A second siRNA, MID1-2, was ordered from Dharmacon to evaluate off target effects (target sequence 5′-UGAGGCCUAUACAAUGUAUG-3′). We ordered the two non-sense siRNA (chosen to have an equivalent GC content) with no similarities to other sequences from Ambion (Opti-2) and Dharmacon. We administered 3.75 nmol siRNA in 25 µl of sterile saline intranasally at day 13 (after HDM sensitization and 24 h before first HDM challenge) and then every second day until mice were killed. In all rhinovirus studies, mice were treated 24 h before and killed 24 h after HRV18 challenge.

AA203 treatment. We treated mice with 10 µg of AA203 or 2% 2-hydroxypropyl-β-cyclodextrin (vehicle) intranasally at day 13 (after HDM sensitization and 24 h before the first HDM challenge) and then daily through the HDM challenge period until mice were killed.

Immunofluorescent detection. Formalin-fixed lung sections were blocked with 25% (v/v) normal sheep serum (SAFC Biosciences) for 1 h before being incubated with either an MID1-specific antibody (Santa Cruz Biotechnology, cat. no. sc-55247, 1:2000) followed by a secondary PE-conjugated antibody (Santa Cruz Biotechnology, cat. no. sc-2743, 2 µg/ml) or an Alexa Fluor 488-conjugated antibody against phosphorylated p70 (Cell Signalling Technology, cat. no. 49185, 25 µg/ml). Nuclei were counterstained with DAPI (Sigma). We analyzed stained slides with an Olympus BX51 UV microscope using DP Controller 3.1.1.267 software (Olympus). Fluorescent intensity was quantified using Image ProPlus 6.0 software, measuring red channel (phycocerythrin-stained MID1) or green channel (Alexa Fluor 488-stained Phos-p38 intensity) in the airway epithelial cells of ten high powered fields per slide under blinded conditions.

Immunoprecipitation. We lysed BEAS 2B cells at 80% confluence in the presence of protease inhibitors (papain, leupeptin, aprotinin and PMSF, Sigma). Protein lysate (500 µg) was incubated with 4 µg PP2A-C monoclonal antibody (clone 1D5, 1:5000, Millipore) and protein A agarose beads (Millipore) at 4°C overnight followed by three washes in lysis buffer. We separated immunoprecipi- tated proteins on 12% (v/v) polyacrylamide gels and transferred them to nitrocellulose. We probed immunoblots with primary polyclonal antibodies to PP2A C ( affinity-purified rabbit antibodies raised against a PP2A C peptide (HBVEETPTDPY41, 1:10000), cat (Novus Biologicals, cat. no. N9109, 487, 1:500) or MID1 (Santa Cruz Biotechnology, cat. no. sc-55248, 1:200) and appropriate secondary antibodies as described above.

Flow cytometry. We dissociated mouse lungs mechanically and stained whole lung cell suspensions with FITC-conjugated anti-TCR B chain (BD, cat. no. 553171), clone J557-907, phycocerythrin-conjugated anti-CD4 (BD, cat. no. 553562, clone H129.19, 1:500), PE-conjugated anti-CD11b (BD, cat. no. 561092, clone 53-67), PerCP-Cy5.5-conjugated anti-CD11b (BD, cat. no. 561092, clone M170), FITC-conjugated anti-CD11c (BD, cat. no. 553801, clone HL3), phycocerythrin-conjugated anti-MHCII (eisocytosis, cat. no. 12-5521, clone M5114E.2.5) and allophycocyanin-conjugated anti-mPDCA-1.
Bronchial epithelium cell cultures. We cultured transformed human bronchial epithelial cells (BEAS-2B) in complete DMEM (Thermo Scientific) with 5% (vol/vol) FCS and primary human bronchial epithelial cells in bronchial epithelial cell growth medium (BEGM, Clonetics) as previously described. After one passage, we seeded 2 × 10^6 BEAS-2B cells onto 12-well trays, cultured them until 80% confluence, serum-starved them for 24 h and incubated them with HDM (50 μg ml⁻¹) or sTRAIL (1 μg ml⁻¹) for 24 h in serum-free DMEM. Primary bronchial epithelial cells were obtained from patients with stable persistent asthma and healthy controls by bronchoscopy using a single sheathed nylon cytology brush. Primary bronchial epithelial cells were seeded, cultured, and incubated under the same conditions as BEAS-2B cells with the exception of using different growth medium (BEGM). Primary bronchial epithelial cells were also infected with RV1B (multiplicity of infection of 2) and cultured for 24 h in serum-free BEGM media. mRNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. TRAIL, MDPI and CCL20 mRNA expression were quantified by quantitative RT-PCR. We measured the concentration of IFN-α in the cell supernatant by ELISA (IFN-α/β DuoSet ELISA, R&D Systems). The Hunter-New England Health and University of Newcastle Human Research Ethics Committees approved all human studies, and written informed consent was obtained from all subjects before participation.

Statistical analysis. The significance of differences between groups was analyzed using Student's t-test, Mann-Whitney test or two-way analysis of variance as appropriate using Graphpad Prism 5. A value of P < 0.05 is reported as significant. We cultured at least 13 pairs of primary bronchial epithelial cell samples per group (healthy subjects and asthmatics) in the presence or absence of RV1B to detect a significant difference in MFI expression of CD13 with a power of 90%.

TRAIL regulates house dust mite induced allergic airways disease, MID1, and PP2A activity. (a) Total lung resistance (RI) as percentage change of baseline measurement (PBS) in response to inhaled methacholine in allergic (HDM) versus non-allergic (SAL) wild-type (WT) and Tnfαf10−/− mice. (n=4-12 mice per group). (b) Number of cells in bronchoalveolar lavage fluid (BALF) (n=3-4 mice per group). (c) Number of peribronchial perivascular eosinophils (x1000) and mucus-producing cells per 100μm². (n=3 mice per group). (d) Cytokine release from in-vitro house dust mite stimulated peribronchial lymphnode cells and (e) CCL20 levels in lung homogenates. (n=4 mice per group). Results are mean±s.e.m. *, P < 0.05, **, P < 0.01, and ***, P < 0.001.
MID1 inhibition does not affect TSLP, GM-CSF, CCL17, and CCL22 RNA expression. (a) TSLP, (b) GM-CSF, (c) CCL17, (d) CCL22 mRNA expression in the airway wall of non-allergic (SAL) versus allergic (HDM) mice treated with a scrambled siRNA (nonsense siRNA) or a MID1 targeting siRNA (MID1 siRNA-2) every second day during the allergen challenge period intranasally. (n=4-6 mice per group). Results are mean±s.e.m.
Supplementary Fig 3

Phos-p38 MAPK

Phosphorylated p38 MAPK protein expression in the airway wall. Scale Bar, 50 µm. p38 MAPK (green); DNA (DAPI, blue).
MID1 inhibition employing a different siRNA abolishes airways hyperreactivity, reduces airway inflammation and increases PP2A activity.

(a) MID1 mRNA in the airway wall of non-allergic (SAL) versus allergic (HDM) mice treated with a scrambled siRNA (nonsense siRNA) or a MID1 targeting siRNA (MID1 siRNA-2) every second day during the allergen challenge period intranasally. (n=3-5 mice per group). (b) Total lung resistance as percentage change of baseline measurement (PBS) in response to inhaled methacholine. (n=5-7 mice per group). (c) Number of cells in bronchoalveolar lavage fluid (BALF). (n=3 mice per group). (d) Number of peribronchial perivascular eosinophils, mucus-producing cells, and mast cells per 100μm². (n=3-4 mice per group). (e) Cytokine release from in-vitro house dust mite stimulated peribronchial lymphnode cells. (f) PP2A activity and PP2Ac protein concentrations in lung homogenates. (n=4 mice per group). Results are mean±s.e.m. *, P < 0.05 and ***, P < 0.01.
PP2A activation does not affect TSLP, GM-CSF, CCL17, and CCL22 RNA expression. (a) TSLP, (b) GM-CSF, (c) CCL17, (d) CCL22 mRNA expression in the airway wall of non-allergic (SAL) versus allergic (HDM) mice treated with 2% 2-hydroxypropyl-cyclodextrin (vehicle) or AAL(S) each day during the allergen challenge period intranasally. (n=3-5 mice per group). Results are mean±s.e.m.
**Supplementary Fig 6**

**A**

- RV1B + nonsense siRNA
- RV1B + MID1 siRNA

**B**

- RV1B + nonsense siRNA
- RV1B + MID1 siRNA

**C**

- RV1B + nonsense siRNA
- RV1B + MID1 siRNA

**D**

- RV1B + nonsense siRNA
- RV1B + MID1 siRNA

**E**

- HDM + RV1B + nonsense siRNA
- HDM + RV1B + MID1 siRNA

**F**

- HDM + RV1B + nonsense siRNA
- HDM + RV1B + MID1 siRNA

**G**

- HDM + RV1B + nonsense siRNA
- HDM + RV1B + MID1 siRNA

**H**

- HDM + RV1B + nonsense siRNA
- HDM + RV1B + MID1 siRNA

**I**

- HIV / RV1 RNA in primary airway epithelial cells from healthy subjects and asthmatics 24hrs after inoculation.

---

**MID1 inhibition does not affect RV1B replication and IFN release in allergic mice.**

(a) Positive strand RV1B RNA, (b) RV1B titre, (c) IFNα and (d) IFNβ mRNA in the airway wall of naïve mice treated with a scrambled siRNA (nonsense siRNA) or a MID1 targeting siRNA (MID1 siRNA) 24hrs before RV1B challenge intranasally. (e) Positive strand RV1B RNA, (f) RV1B titre, (g) IFNα and (h) IFNβ mRNA in the airway wall of house dust mite allergic mice treated with a scrambled siRNA (nonsense siRNA) or a MID1 targeting siRNA (MID1 siRNA) 24hrs before RV1B inoculation intranasally. (n=3-6 mice per group). (i) Positive strand RV1B RNA in primary airway epithelial cells from healthy subjects and asthmatics 24hrs after infection. Results are mean ± s.e.m. *P < 0.05, and **P < 0.01.
Supplementary Fig 7

Proposed role of MID1 in allergic airways disease.
**Supplementary Table 1 online: Characteristics of healthy and asthmatic subjects.**

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<th>Asthmatic</th>
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*, P < 0.05
Supplementary methods online: Primer list.

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CHAPTER 3: Toll-like receptor 7 governs interferon and inflammatory responses to rhinovirus and is suppressed by IL-5-induced lung eosinophilia

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3.1 Manuscript

**Respiratory infection**

**ORIGINAL ARTICLE**

Toll-like receptor 7 governs interferon and inflammatory responses to rhinovirus and is suppressed by IL-5-induced lung eosinophilia

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**ABSTRACT**

**Background** Asthma exacerbations represent a significant disease burden and are commonly caused by rhinovirus (RV), which is sensed by Toll-like receptors (TLR) such as TLR7. Some asthmatics have impaired interferon (IFN) responses to RV, but the underlying mechanisms of this clinically relevant observation are poorly understood.

**Objectives** To investigate the importance of intact TLR7 signalling in vivo during RV exacerbation using mouse models of house dust mite (HDM)-induced allergic airways disease exacerbated by a superimposed RV infection.

**Methods** Wild-type and TLR7-deficient (Th7−/−) BALB/c mice were intranasally sensitised and challenged with HDM prior to infection with RV/18. In some experiments, mice were administered recombinant IFN or adoptively transferred with plasmacytoid dendritic cells (pDC).

**Results** Allergic Th7−/− mice displayed impaired IFN release upon RV/18 infection, increased virus replication and exaggerated eosinophilic inflammation and airways hyper-reactivity. Treatment with exogenous IFN or adoptive transfer of TLR7-competent pDCs blocked these exaggerated inflammatory responses and boosted IFNγ release in the absence of host TLR7 signalling. TLR7 expression in the lungs was suppressed by allergic inflammation and by interleukin (IL)-5-induced eosinophilia in the absence of allergy. Subjects with moderate-to-severe asthma and eosinophilic but not neutrophilic airways inflammation, despite inhaled steroids, showed reduced TLR7 and IFNγR2 expression in bronchial biopsies. Furthermore, TLR7 expression inversely correlated with percentage of sputum eosinophils.

**Conclusions** This implicates IL-5-induced airways eosinophilia as a negative regulator of TLR7 expression and antiviral responses, which provides a molecular mechanism underpinning the effect of eosinophil-targeting treatments for the prevention of asthma exacerbations.

**INTRODUCTION**

Asthma is a complex and heterogeneous inflammatory disease of the airways with increasing global prevalence. The most common trigger for asthma symptoms is immune cell activation against innocuous antigens (allergens) and respiratory viral infections.

**Key messages**

- What is the key question?
  - What underlies impaired interferon responses to rhinovirus in asthmatics?

- What is the bottom line?
  - Suppression of Toll-like receptor (TLR7) by interleukin-5-induced lung eosinophilia impairs interferon responses to rhinovirus, leading to exaggerated inflammatory responses and exacerbation.

- Why read on?
  - This provides a mechanism whereby therapies modulating TLR7 or targeting eosinophils may ameliorate virus-induced asthma exacerbations.

Upon antigen exposure, cytokines such as thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor, interleukin (IL)-25, IL-33 and T cell cytokines factor-related apoptosis-inducing ligand are released by the airway epithelium, resulting in the activation of innate immune cells that promote T helper 2 (Th2) cell differentiation, leading to the release of Th2 cytokines such as IL-4, IL-5 and IL-13. IL-13 is a potent inducer of airway hyper-reactivity (AHR) and mucus production in a signal transducer and activator of transcription-6 (STAT6)-dependent manner. IL-5 regulates maturation of eosinophils in the bone marrow and in concert with chemokines such as eotaxin the recruitment of these cells into the airways. Thus, Th2 cell activation underpins many clinical phenotypes including allergic and eosinophilic asthma. The proportion of asthmatics with high eosinophil numbers in their airways represent the majority of patients, and some of those unresponsive to corticosteroids have severe therapy-refractory asthma with a disproportionally large burden of disease. Importantly, they are also prone to asthma exacerbations, which can be partially alleviated by therapies that block IL-4, IL-5 or IL-13 and reduce eosinophilic inflammation in the lungs. However, the molecular mechanisms that link Th2-induced eosinophilia with susceptibility to exacerbation are yet to be defined.
Viral respiratory infections are detected in up to 85% of asthma exacerbations and two-thirds of those are caused by respiratory viruses (RV). Notably, a group of asthmatics has impaired release of innate interferons (IFNa, IFNb and IFNγ) upon experimental RV infection and IFNα inversely correlated with induced sputum eosinophils on day 3 of acute infection. IFNα 2/3 inversely correlated with induced sputum eosinophils on day 3 of the acute infection (p=0.05), when subjects were experimentally infected with RV16 in vivo. RV is sensed by a limited number of pattern recognition receptors, including Toll-like receptors (TLRs) and retinoid acid-induced gene 1 like receptors. TLR3 and TLR7 are endosomally localised and recognise viral nucleic acids, critically regulating antiviral IFN production. Recent studies have shown that impaired TLR3 function does not affect RV replication in vivo and TLR3 expression is not reduced in asthma. The in vivo role of functional TLR7 signalling in mounting antiviral responses to RV has yet to be determined. TLR7 is widely expressed in innate immune cells such as dendritic cells (DCs) and macrophages, as well as in structural lung cells, including airway epithelia. Treatment of mice with TLR7 agonists leads to a long-lasting protection from the development of allergic airways disease (AAD) and TLR7 activation also reduced airway smooth muscle contractility in mice. And others have found reduced IFNα and IFNγ release upon TLR7 stimulation of peripheral blood mononuclear cells from asthmatics and a trend towards lower TLR7 expression was observed in bronchoalveolar lavage fluid (BALF) cells from asthmatics. However, the role of TLR7 in RV-induced exacerbation of AAD has yet to be fully elucidated.

**METHODS**

**Patient biopsies**

Endobronchial biopsies were obtained by bronchoscopy with samples taken from third-generation bronchi. Biopsies were stored in RNALater (Ambion) at −20°C until needed. RNA was extracted following the miRNeasy Mini Handbook (Qiagen)—purification of total RNA from tissue via homogenisation (Qwashredder). RNA was quantified by spectrophotometry (NanoDrop) and 200 ng of extracted RNA reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; ABI). The inflammatory phenotype was determined by induced sputum count, and patients with a RAST ImmunoCAP Specific IgG-positive blood test were designated as atopic.

**Animals**

Wild-type (WT), Thr7−/−, Thr4−/−, MyD88−/−, Stat6−/− and IL-5 transgenic mice, all on a BALB/c background (6-14 weeks of age), were obtained from Australian BioResources (Moore Vale, Australia) and housed in approved containment facilities within the HMRI Building, University of Newcastle (Newcastle, Australia). Mice had ad libitum access to food and water under a 12 h light and dark cycle. All experiments were approved by the Animal Care and Ethics Committee of the University of Newcastle.

**Induction of AAD and RV-induced exacerbation**

AAD and RV-induced exacerbation were performed as previously described. Briefly, lyophilised crude horse dust mite (HDM) (Dermatophagoides pteronyssinus) extract (Greer Laboratories) was reconstituted in sterile saline (SAL) and intranasally delivered to mice under light isoflurane anaesthesia. Sensitisation on days 0, 1 and 2 (HDM 30 μg/50 μL) was followed by daily challenge (HDM 5 μg/50 μL) on days 14, 15, 16 and 17 to induce AAD. On day 18, mice were euthanised by pentobarbitural sodium overdose (Vibrac) and samples collected. In other experiments, mice were intranasally infected with live minor group RV (RV1B), 50 μL containing 5×10⁶ virions median tissue culture infective dose (TCID50) or UV-inactivated RV1B 24 h after last HDM exposure to exacerbate pre-existing AAD. Samples were collected 24 h after RV1B infection.

**Administration of recombinant cytokines and LPS**

Naive mice were intranasally administered recombinant mouse IL-1β (15 μg/μL; BioLegend), IL-5 (15 μg/μL; BioLegend) or a low or high dose (0.12 μg/μL and 4 μg/μL, respectively) of lipopolysaccharide (LPS) (Escherichia coli, 0111:B4; Sigma-Aldrich), with samples collected 24 h later. HDM-sensitised and challenged Thr7−/− mice, which also received live RV1B, were intranasally administered either recombinant mouse IFNα2 (10,000 IU/μL; R&D Systems) or a vehicle control (phosphate buffered saline) on day 18 (2 h following RV1B infection). Mice were sacrificed 24 h postinfection and samples collected.

**AHR measurement**

AHR was measured as previously described. Briefly, AHR was invasively assessed in separate groups of anaesthetised mice by measurement of total lung resistance and dynamic compliance (Buxco). Mice were mechanically ventilated, and AHR to nebulised methacholine (increased lung resistance) was expressed as a percentage change from control (baseline).

**Analysis of lung inflammation**

BALF was collected and analysed as previously described. Enumeration of peribronchial/perivascular eosinophils and PAS-positive cells was performed as previously described.

**Flow cytometry**

Single lung cell suspensions were prepared and stained as previously described. Antibodies used were FITC-anti-CD11b (BD, cat. no. 553171, clone H57-597), PE-anti-CD4 (BD, cat. no. 553632, clone HL29.19), PerCPCantI-CD8a (BD, cat. no. 561092, clone 53-67.7), PerCPCy5.5-anti-CD11b (BD, cat. no. 561092, clone M1/70), FITC-anti-CD11c (BD, cat. no. 553801, clone HI30), PE-anti-MHCII (BD, cat. no. 12-5321, clone M5/114.15.2), all at 1:15 dilution. Positive cells were identified using a FACSCanto (BD) by the following criteria: mDCs—CD11b+ CD11c− MHCII−; T cells—TCRαβ chain+ with CD4+ or CD8α+. Data analysed with BD FACSDiva.

**Quantitative RT-PCR**

Trachea and lungs were extracted from euthanised mice and forceps used to separate the airways from the parenchyma by blunt dissection. Total mRNA was extracted using Trizol (Ambion; Carlsbad, USA). cDNA was generated via reverse transcription using BioScript (Bioline; Alexandria, Australia). Quantitative PCRs (qPCR) were performed on cDNA generated from mouse airway tissue and human endobronchial biopsies with SYBR Green (Invitrogen; Mulgrave, Australia) using primers detailed in online supplementary table S1. Ct’s of the genes of interest were referenced HPRT or GAPDH for mouse and human tissue, respectively. All steps were performed according to manufacturer’s instructions.

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Generation of BM-derived pDCs and adoptive transfer

WT and TL7-deficient bone marrow-derived plasmacytoid DCs (pDCs) were generated as previously described.20 pDCs were resuspended at 1x10^7 cells/mL and 0.5x10^7 pDCs were administrated intranasally to allergic Thr7^(-/-) mice, 2 h prior to RVIB infection.

Quantification of lung cytokines and IFNs

A single lung lobe from each mouse was excised and snap frozen before being homogenised in buffers recommended in the manufacturer’s instructions. Levels of IL-5, IL-13, IFNγ (BD Biosciences; PharMingen), IFNα, IFNβ (R&D Systems) were determined in clarified lung lysates by ELISA. CCL2/MCP1, CCL3/ MIP1α, CCL4/MIP1β, CXCL9/MIG and CXCL10/IP10 were measured by employing a Multiplex Immunoassay (Millipore). All concentrations were normalised to lung weight.

pDC culture and TCID50

Primary mouse pDCs were isolated from pooled WT or TL7-deficient spleens via mechanical dissection and isolation with PDCa-1 magnetic beads on an AutoMACS platform (Miltenyi Biotec) according to manufacturer’s instructions. Following two isolation runs, positively selected cells were seeded into 96-well plates at 1x10^5 cells/well and cultured in the absence or presence of RVIB (multiplicity of infection = 5).20 Supernatant was harvested 48 h postinfection and IFN production assessed with ELISA. Number of live RVIB virions was determined by standard TCID50 serial dilution on HELA H1 cells, calculated via the Spearman–Karber method.

Statistical analysis

The significance of differences between groups was analysed using Student’s t test, Mann–Whitney test, analysis of variance or Kruskal–Wallis with Dunn’s test for multiple comparisons as appropriate using Graphpad Prism 6. A value of p<0.05 is reported as significant. Data are expressed as mean±SEM.

RESULTS

To investigate TL7 as a regulator of AAD and antiviral responses in vivo, we repeatedly challenged WT and TL7-deficient (Thr7^(-/-)) mice with HDM, then superinoculated a RV infection to exacerbate their established AAD as described previously.32 Exposure of allergic Thr7^(-/-) mice to RV compared with WT controls, resulted in impaired production of type I (α, β), II (γ) and III (λ) IFNs with higher RV replication in the lower airways (Figure 1A,B). This was despite a lack of an increase in type II (γ) and III (λ) IFNs to RV infection in allergic WT mice. Deficient IFN responses coincided with exaggerated AHR (figure 1C), eosinophilic airway inflammation (figure 1D,E), accumulation of CD4+, CD8+ and myeloid (m) DCs but not pDCs in the lungs (figure 1F), and production IL-5 and CCL11 (eotaxin-1) (figure 1G). We also observed increased levels of the TL2-prime cytokines IL-25 and TSLP in allergic Thr7^(-/-) mice infected with RV (see online supplementary figure S1). CCL2, CCL3, CCL4 and CCL7 but not CCL9 and CXCL10 were also increased in the absence of TL7 signaling (see online supplementary figure S2). There was no change in IL-13 levels or numbers of mucus-producing cells (see online supplementary figure S3a), suggesting a novel and critical role of TL7 signaling in RV-induced asthma exacerbation.

Allergic Thr7^(-/-) mice received recombiant type I and III IFNs 2 h after infection with RV. Notably, one dose of IFNα2, β or γ suppressed RV-induced eosinophilic inflammation (figure 2A), as well as IL-5 and CCL11 production (figure 2B) but not IL-13 (see online supplementary figure S3b). All IFN treatments induced IFNγ in the lungs (figure 2C) and limited RV replication (figure 2D). There was no effect of IFN treatment on TSLP IL-25 or IL-33 expression (data not shown). Thus IFN treatment promotes IFNγ release and impairs IL-5 and CCL11 production and RV replication in the absence of TL7.

pDCs release large quantities of IFN during viral infection, and here we show that splenic pDCs exposed to RV in vitro display impaired release of IFNα and IFNβ but not IL-25 in the absence of TL7 (figure 3A). Adoptive transfer of TL7-expressing pDCs into allergic Thr7^(-/-) mice re-established IFNα, IFNβ and IFNγ but not IFNα, release in the lungs upon RV infection and limited RV replication as compared to allergic Thr7^(-/-) mice that received TL7-deficient pDCs (figure 3B, C). Transfer of WT pDCs also limited RV-induced AHR (figure 3D), eosinophilic airway inflammation (figure 3E) and production of IL-5 and CCL11 (figure 3F) but not IL-13 release (see online supplementary figure S3c). Thus adoptive transfer of TL7-expressing pDCs—like exogenous IFN treatment—promotes IFNγ release and impairs eosinophilic airways inflammation and RV replication in the absence of TL7.

TL7 expression was assessed in a number of knockout mouse strains as it was suppressed in allergic WT mice with Thr7^(-/-) mediated AAD (figure 4A). Notably this allergen-induced reduction in TL7 expression was not observed in Thr4^(-/-), MyD88^(-/-) or STAT6^(-/-)-deficient mice, suggesting that intact Thr7-promoting signalling pathways and the presence of eosinophilic airways inflammation are required for suppression of TL7. To investigate the specific role of Thr7 cytokines in this in vivo observation, we delivered one dose of recombinant IL-13 or IL-5 intranasally to WT mice and compared those responses to ones challenged with LPS (a TL7 agonist). Interestingly, in mice that constitutively overexpress IL-5 (IL-5^K52^ mice) TL7 expression was significantly reduced (figure 4B) and was associated with accumulation of eosinophils in the airways in the absence of allergy (figure 4D). Intranasal administration of one dose of IL-5 or IL-13, however, had no effect on TL7 expression or eosinophil recruitment although IL-13 did induce AHR and increased expression of Muc5AC (data not shown). In addition, one low or high dose of LPS increased TL7 expression and resulted in the accumulation of neutrophils but not eosinophils in the lungs (figure 4C,D). Thus eosinophilic airways inflammation due to chronic IL-5 release is associated with a reduction in TL7 expression while a single administration of IL-5 or IL-13 had no effects on TL7.

We next analysed TL7 expression in bronchial biopsies collected from healthy subjects and patients with moderate-to-severe persistent asthma (clinical and demographic data in online supplementary table S2). Importantly, asthmatics with an eosinophilic airways inflammation, as determined by bronchial lavage,21 displayed significantly reduced TL7 (figure 4E) and IFN0,2,3 expression (figure 4E), independent of atopic status (figure 4G). Furthermore, levels of TL7 expression positively correlated with IFNα2 and IL-3 expression (figure 4I–K) and inversely correlated with percentage of sputum eosinophils (figure 4H) but not macrophages or neutrophils (vs=0.04; p=0.81; n=53 and r=0.31; p=0.08; n=33, respectively). These results suggest that suppression of TL7 and IFN
expression in moderate-to-severe asthmatics is specifically tied to eosinophilic airways inflammation in this cohort.

DISCUSSION

Impaired innate IFN responses to respiratory viruses have been proposed as one mechanism underlying the clinical observation of asthmatics being susceptible to RV-induced exacerbation.7 8 Innate IFN responses are instigated by a limited number of pattern recognition receptors, such as TLR3 whose cognate ligands include the RV viral genome.10 11 Experiments in human immortalised epithelial cell lines (BEAS-2B) have supported a role of TLR3 in mediating an antiviral and anti-inflammatory response.7 However, TLR3 expression in the airways22 and TLR3-induced responses did not vary in PBMCs derived from asthmatic or atopic patients by comparison to healthy subjects.22 We show here, for the first time in vivo, that a lack of TLR7 signalling under conditions that model a viral asthma exacerbation leads to impaired IFN production and exaggerated Th2-driven inflammation. Our findings, such as increased levels of the Th2-priming cytokines IL-25, IL-33 and TSLP in Threonine−/− mice, mirror those published by Kaiko et al.,14 infecting non-allergic mice with mouse pneumovirus, a mouse pathogen similar to human respiratory syncytial virus. These studies suggest that intact TLR7 signalling is required for sufficient IFN induction and restraint of proinflammatory responses in the lung.

As TLR7 activation is upstream of IFN production, we hypothesised that administration of exogenous IFN would protect allergic Threonine−/− mice from RV-induced exacerbation. We observed that treatment with type I or III IFNs resulted in the Threonine−/− mice developing a suppressed phenotype similar to that of the WT controls, whose TLR7 signalling is active. All IFN treatments induced the release of IFNγ in the lung, with type I and III IFNs promoting themselves exclusively, indicative of their known distinct signalling pathways.14

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Figure 2  Exogenous interferon can protect Toll-like receptor (TLR7)-deficient mice from rhinovirus (RV)-induced exacerbation. Exacerbated TLR7-deficient mice received recombinant interferon IFNα2 (α), IFNβ (β), and a vehicle control (−) 2 h post-RV18 infection on day 18. Samples were collected 24 h post infection. (A) Eosinophils present in bronchoalveolar lavage (BAL) fluid and per 100 μm² of lung tissue. Levels of interleukin (IL)-5 (B) and interferon (C) in clarified lung homogenates as assessed by ELISA. CCL11 expression (D) and viral RV18 RNA (D) in lower airway tissue was quantified by quantitative RT-PCR. Results are means±SEM (n=3–7 mice per group) and are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001 as compared to vehicle control group determined by Student’s t test.

RV-induced pDC-derived IFN production was dependent upon intact TLR7 signalling in vitro. Additionally, adoptive transfer of TLR7-expressing pDCs into allergic Th7−/− hosts re-established type I and II IFN responses, limiting RV replication and eosinophilic airways inflammation. Notably TLR7 deficiency did not impair pDC recruitment into the lungs. This implicates activation of TLR7 signalling on pDCs and increasing host IFN release as important therapeutic

Figure 3  Adoptive transfer of Toll-like receptor (TLR7)-competent plasmacytoid dendritic cells (pDCs) to TLR7-deficient mice limits exacerbation of allergic airways disease (AAD). Purified splenic pDCs from TLR7-deficient (−/−) and TLR7-competent (+/+ ) bone marrow were adaptively transferred to allergic Th7−/− recipients. Mice were inoculated with RV18 2 h later and endpoints measured 24 h post infection. (A) Spleen-isolated pDCs were infected in vitro with RV18 and interferon (IFN) release in cell supernatants assessed by ELISA. Levels of IFNs (B), as well as interleukin (IL)-5 (F) in clarified lung homogenates as assessed by ELISA. Positive-strand RV18 RNA (C) and CCL11 expression (E) from lower airway tissue quantified by quantitative RT-PCR. (D) Total lung resistance presented as percentage change in response to methacholine (n=7–8 mice per group). (E) Eosinophils present in bronchoalveolar lavage (BAL) fluid. Results are means±SEM (n=3–5 mice per group). *p<0.05, **p<0.01, ***p<0.001 determined by Student’s t test except for (D) where analysis of variance compared the RI curves. All p values as compared to exacerbated mice that received TLR7-deficient pDCs.


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Figure 4  Toll-like receptor (TLR7) expression is reduced during eosinophilic lung inflammation. (A) Wildtype (WT), TLR4−/−, MyD88−/− and Stat6−/− mice were sensitized and challenged with house dust mite (HDM) over 10 days and gene expression of TLR7 in lower airway tissue was quantified by quantitative RT-PCR. TLR7 airway expression in allergic airways as a percentage of sterile saline (SAL) expression for each strain, (B) as well as lung tissue eosinophils and (C) bronchoalveolar lavage (BAL) fluid neutrophils and eosinophils (D) from rIL-5, rIL-13, IL-5 Tg and lipopolysaccharide (LPS)-treated mice 24 h post treatment. Results are means±SEM (n=6 mice per group), gene expression in mice expressed as a % compared to non-allergic SAL treated wildtype mice. TLR7 (E) and interferon (IFN) gene expression in bronchial biopsies collected from non-asthmatic (n=13) and asthmatic (n=20) subjects stratified into eosinophilic (<3% eosinophils) or non-eosinophilic (<3% eosinophils) phenotypes based on BAL cell counts. (F) TLR7 expression in bronchial biopsies collected from non-asthmatic (n=13) and asthmatic (n=20) subjects stratified according to atopic status. Lines indicate the median, boxes extend from 25th to the 75th percentile, and error bars extend to 10th and 90th percentiles. (G) Correlation between TLR7 expression from bronchial biopsies and percentage of sputum eosinophils. (H) Correlation between TLR7 and IFN expression from patient biopsies. *p<0.05, **p<0.01, ***p<0.001 as determined by Student's t test (A-D) or Kruskal–Wallis with Dunn's multiple comparisons test.

strategies, either through the use of TLR7 agonists or targeting pathways upstream of IFN production. Other IFN-producing cells may also be relevant. We have recently shown that anti-CCL7 treatment is sufficient to inhibit IRF-7-dependent IFNB expression in RV infection, which was associated with reduced macrophage inflammation but not pDC inflammasome. This highlights the complexity of the inflammatory and antiviral immune response on a cellular level, particularly in an allergic environment.

WT allergic mice with TLR2−/−-driven AAD had significantly lower levels of TLR7 expression compared with non-allergic SAL-treated mice in the absence of RV infection, which is in line with the pattern seen clinically in non-infected eosinophilic asthmatics. TLR4 signalling is required for the development of a robust TLR2-mediated allergic airways inflammation in response to LIDM extract, which also contains low amounts of endotoxins. Allergic airways inflammation suppresses TLR7 expression, which is prevented by disruption of TLR2-promoting signalling pathways governed by TLR4, MyD88 and STAT6. In contrast to allergic mice, TLR4 activation by LPS led to an upregulation of TLR7 expression in non-allergic mice. Thus the effects of TLR4 signalling on the regulation of TLR7 expression are determined by the presence or absence of TLR2-dominant allergic airways inflammation. Interestingly, LPS also upregulated TLR3
expression in human monocytes, which was critical for antiviral responses. TLR7−/− mice infected with RV1B, however, displayed normal type I IFN responses, unchanged viral titers and reduced inflammatory responses. This is in marked contrast to our data generated in allergic TLR7−/− mice and previous data in non-allergic TLR7−/− mice. Further studies are now required to elucidate the clinical effect of endotoxins on TLR7-mediated responses in asthma.

In the absence of TLR2, dominant allergic airways inflammation, no suppression of TLR7 expression was observed by a single exposure to IL-13 or IL-5. However, chronic exposure to IL-5 alone, which is associated with lung cosinophilia and the development of AHR, markedly impaired TLR7 lung expression in the absence of allergy. This finding was of clinical relevance because we observed reduced TLR7 expression in endobronchial biopsies from asthmatic patients with eosinophilic inflammatory profiles. Eosinophilic asthmatics also expressed lower levels of innate IFNs in addition to TLR7, a difference that was not observed for expression of TLR3, retinoic acid-inducible gene I or melanoma differentiation-associated gene 5. These results are congruent with a recent study that mapped TLR7 expression in the airways of severe asthmatics.

Our results suggest a reciprocal regulation between IL-5-induced eosinophilia and TLR7 expression that affects antiviral IFN responses to RV (summary illustrated in figure 5). In the absence of virus, it has been shown that TLR7 stimulation with synthetic agonists in vitro resulted in inhibition of IL-5 through IFNγ and Notch signalling pathways in antigen-presenting cells and upregulation of IFNγ production by memory CD4+ T cells and natural killer cells. Consistent with these findings, we show impaired IFNγ release in the absence of TLR7 promotes TGF-β immune response, which can be rescued by type I and III IFN therapy or adoptive transfer of TLR7-sufficient pDCs. This is directly relevant to subjects with moderate-to-severe asthma with a predominantly eosinophilic airways inflammation as these individuals have suppressed—but not fully deficient—TLR7 expression in their lungs, which correlated with reduced IFN expression. Notably, a human monoclonal antibody to IL-5 (mpolizumab) reduced exacerbation frequency in moderate-to-severe asthmatics with a predominantly eosinophilic airways inflammation, a strategy that may be of specific benefit to that clinical population as not all asthmatics appear to exhibit impaired IFN responses to RV. Our data suggest that TLR7 and its downstream signalling pathway limits TLR2 responses in RV-induced exacerbations and may be a promising therapeutic target for the prevention and treatment of viral exacerbation in eosinophilic asthmatics.

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Contributors

LH and AC contributed equally: performed and designed mouse and cell culture experiments, analysed data, generated figures and edited the manuscript. KS and II performed experiments and analysed data. LJ assisted in supervision. PABW and KP performed and supervised studies on healthy subjects and subjects with asthma, collected and processed biopsies, and performed cell culture experiments. SP assisted in the design and conceptualisation of some mouse experiments. OK supervised and interpreted cell culture experiments. NVB and SLJ assisted in design of mouse experiments, provided RV1B for further propagation and cDNA standards. PSF assisted in design, supervision and interpretation of mouse studies. JM conceptualised, coordinated, designed and supervised mouse and human studies, interpreted and analysed data, and drafted and edited the manuscript. All authors contributed to data discussion and revised the manuscript.

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Competing interests

SLJ has received consulting fees from GlaxoSmithKline, Chiesi, Boehringer Ingelheim and Novartis. PABW and SLJ are authors on patents.
relating to use of PIs in treatment of exacerbations of asthma disease and holds some options in Sputnik, a company developing PIS for treatment of exacerbations of asthma disease.

Patient consent obtained.


Provenance and peer review. Not commissioned; externally peer reviewed.

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REFERENCES

3.2 Supplementary material

Supplementary Figure 1. Expression of T₃₂-priming epithelial cytokines IL-25 and TSLP but not IL-33 are higher in exacerbated TLR7-deficient mice. Total RNA was extracted from lower airway tissue of mice following RV-induced exacerbation and expression of IL-25 (A), IL-33 (B), and thymic stromal lymphopoietin (TSLP) (C) assessed by qRT-PCR. Copy numbers are displayed as % compared to WT HDM+UV group. Results are Mean±SEM (n=3-6 mice per group). *, P < 0.05, **, P < 0.01, ***, P < 0.001 as compared to strain-matched HDM+UV group or otherwise indicated as determined by students t-test.
Supplementary Figure 2. TLR7-deficiency leads to heightened local production of chemokines upon RV-induced exacerbation. Lung protein levels of CCL2/MCP1, CCL3/MIP1α and CCL4/MIP1β (A), as well as CXCL9/MIG and CXCL10/IP10 (B) was quantified by bead-based Multiplex Immunoassay in clarified tissue homogenates. (A) CCL7/MCP3 was quantified from the same tissue by sandwich ELISA. Results are Mean±SEM (n=3-4 mice per group) with all protein concentrations normalised to lung weight. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ as compared to strain-matched HDM+UV group or otherwise indicated as determined by students t-test.
Supplementary Figure 3. TLR7-deficiency does not alter IL-13 production or number of PAS-positive cells following exacerbation. Levels of IL-13 protein and number of PAS⁺ epithelial cells per 100μm² of lung tissue, assessed by ELISA and histological analysis respectively. Data from experiments displayed in Figure 1 (A), Figure 2 (B), and Figure 3 (C). Results are Mean±SEM (n=3-6 mice per group). *, P < 0.05, **, P < 0.01, ***, P < 0.001 as compared to strain-matched HDM+UV group or otherwise indicated as determined by students t-test.
<table>
<thead>
<tr>
<th><strong>Gene</strong></th>
<th><strong>Forward primer (5’-3’)</strong></th>
<th><strong>Reverse primer (3’-5’)</strong></th>
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<td>Murine <em>HPRT</em></td>
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<td>Murine <em>IL25</em></td>
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<td>CTAAGCCATGACCAGGCGGCG</td>
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<tr>
<td>Murine <em>IL33</em></td>
<td>TCCTTGCTTGGGCAGTATCCA</td>
<td>TGCTCAATGTTGCAACAGACG</td>
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<tr>
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<td>AGGCCATCCTGAGAACTTGAAG</td>
<td>CGGAATTCATGAAGGAATACCC</td>
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<tr>
<td>Murine <em>TLR7</em></td>
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<td>TGGAAGCTTTGTGCAATACAGAAA</td>
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<tr>
<td>Human <em>GAPDH</em></td>
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<td>(+)-strand hrRV1B</td>
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*HPRT* = Hypoxanthine-guanine phosphoribosyltransferase  
*GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase
**Supplementary Table 2 online: Characteristics of healthy and asthmatic subjects.**

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Asthmatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50.8 (26, 69)</td>
<td>57.8 (30, 77)</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>6 (46.2)</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Non-smoker, n (%)</td>
<td>13 (100)</td>
<td>17 (85)</td>
</tr>
<tr>
<td>FEV₁% predicted</td>
<td>98.4 ± 4.1</td>
<td>67.4 ± 5.4***</td>
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<tr>
<td>ICS, n (%)</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Mean ICS dose –</td>
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<td>505.26</td>
</tr>
<tr>
<td>(µg, Beclomethasone equivalent)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LABA, n (%)</td>
<td>0 (0)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>OCS, n (%)</td>
<td>0 (0)</td>
<td>1 (7.1)</td>
</tr>
</tbody>
</table>

***, *P* < 0.001
CHAPTER 4: Salmeterol attenuates chemotactic responses in rhinovirus-induced exacerbation of allergic airways disease by modulating protein phosphatase 2A

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4.1 Manuscript

Salmeterol attenuates chemotactic responses in rhinovirus-induced exacerbation of allergic airways disease by modulating protein phosphatase 2A

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Newcastle and Sydney, Australia, and London, United Kingdom

Background: β-Agonists are used for relief and control of asthma symptoms by reversing bronchoconstriction. They might also have anti-inflammatory properties, but the underlying pathophysiologies remain poorly understood. Recently, a direct interaction between formoterol and protein phosphatase 2A (PP2A) has been described in vitro.

Objective: We sought to elucidate the molecular mechanisms by which β-agonists exert anti-inflammatory effects in allergen-driven and rhinovirus 1B–exacerbated allergic airways disease (AAD).

Methods: Mice were sensitized and then challenged with house dust mite to induce AAD while receiving treatment with salmeterol, formoterol, or salbutamol. Mice were also infected with rhinovirus 1B to exacerbate lung inflammation and therapeutically administered salmeterol, dexamethasone, or the

PP2A-activating drug (S)-2-amino-4-(4-[heptoxyl]phenyl)-2-methylbutan-1-ol (AAL1/S). Results: Systemic or intranasal administration of salmeterol protected against the development of allergen- and rhinovirus-induced airway hyperreactivity and decreased eosinophil recruitment to the lungs as effectively as dexamethasone. Formoterol and salbutamol also showed anti-inflammatory properties. Salmeterol, but not dexamethasone, increased PP2A activity, which reduced CCL11, CCL26, and CXCL12 expression and reduced levels of phosphorylated extracellular signal–regulated kinase 1 and active nuclear factor κB subunits in the lungs. The anti-inflammatory effect of salmeterol was blocked by targeting the catalytic subunit of PP2A with small RNA interference. Conversely, increasing PP2A activity with AAL1/S abolished rhinovirus-induced airway hyperreactivity, eosinophil influx, and CCL11, CCL26, and CXCL12 expression. Salmeterol also directly activated immunoprecipitated PP2A in vitro isolated from human airway epithelial cells.

Conclusions: Salmeterol exerts anti-inflammatory effects by increasing PP2A activity in AAD and rhinovirus-induced lung inflammation, which might potentially account for some of its clinical benefits. (J Allergy Clin Immunol 2014;133:1720-7)

Key words: Long-acting β2-agonist, salmeterol, formoterol, salbutamol, asthma, allergy, rhinovirus, exacerbation, chemokine, dexamethasone, (S)-2-amino-4-(4-[heptoxyl]phenyl)-2-methylbutan-1-ol, protein phosphatase 2A, nuclear factor κB

Asthma is characterized by airway hyperreactivity (AHR), mucous hypersecretion, and, commonly, airways inflammation.1,2 The degree of eosinophilic, neutrophilic, and lymphocytic accumulation in the airways correlates with disease severity, and clinical treatments aim to control and ameliorate episodic airways obstruction, the clinical hallmark of asthma.3,4 Airway inflammation in asthmatic patients is tightly regulated and involves the release of IL-4, IL-5, and IL-13 by Th2 cells, as well as chemokines, such as CCL11 (eotaxin-1), CCL20 (macrophage inflammatory protein 3α), and CXCL2 (macrophage inflammatory protein 2α), by resident lung and immune cells.5,6 A majority of all health care costs generated by the estimated 300 million asthmatic patients worldwide is attributed to exacerbations caused by respiratory viruses,1,7 and rhinoviruses are the most commonly detected.8,9

Current treatments for controlling asthma symptoms consist of—if indicated—combination therapy of inhaled corticosteroids and long-acting β2-agonists (LABAs).3,10 Inhaled corticosteroids have broad immunomodulatory effects through glucocorticoid receptor (GR) activation, whereas LABAs relax airway smooth muscle through activation of β2-adrenoceptors to reopen obstructed
Abbreviations used
AAD: Allergic airways disease
AA(S): 2-(S)-2-methoxyethoxy-4,6-dimethyl-5-(2-naphthyl)-3(2H)-pyridinone-1-ol
AHR: Airway hyperreactivity
CR: Glucocorticoid receptor
DHM: Horse dust mite
LABA: Long-acting β₂-agonist
NF-κB: Nuclear factor κB
OVA: Ovalbumin
PAS: Periodic acid–Schiff
p-ERK1: Phosphorylated extracellular signal-regulated kinase 1
PP2A: Protein phosphatase 2A
RVI1: Rhesus virus 1B
siRNA: Small interfering RNA

Airways,17,18 The LABA salmeterol also possesses an anti-
flammatory mode of action in models of ovalbumin (OVA)-
driven allergic airways disease (AAD).19,20 but the molecular
mechanisms of these observations remain elusive. Interestingly,
LABAs increase the activity of protein phosphatase 2A (PP2A)
through direct interaction independent of β₂-adrenocep-
tor signaling in vitro.21 PP2A is one of the most abundantly
distributed cellular proteins and regulates kinase-driven
signaling through dephosphorylation of numerous signaling
molecules.22,23

In this study we show that salmeterol attenuated AHR,
ecothoracic inflammation, and local chemokine expression by
increasing PP2A activity and suppression of downstream
mitogen-activated protein kinase and nuclear factor κB
(NF-κB) signaling pathways.

MICE AND INDUCTION OF AAD AND RHINOVIRUS
EXACERBATION

Specific-pathogen-free male BALB/c mice were intranasally sensitized and
challenged with ovalbumin (OVA) (50 µg/mL, respectively; Greer Laboratories,
Lenoir, NC) for 17 days, with β₂-agonists administered before and during the challenge phase (see Fig E1, A–C, in this
article’s Online Repository at www.jacionline.org). Non-sensitized mice received sterile endotoxin-free saline (0.9%) only. In some experiments sensi-
tized mice were intranasally infected with 1 × 10³ viruses of the minor group rhinovirus 1B (RVI1) or UV-inactivated RVI1 on day 18 (see Fig E1, D and
E, in this article’s Online Repository at www.jacionline.org).24 All mice were
killed 24 hours after the final challenge. The Animal Care and Ethics Commit-
tee of the University of Newcastle, Australia, approved all experiments, which
were conducted in accordance with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines.

AHR MEASUREMENT

AHR to nebulized methacholine (increasing lung resistance) was
measured, as previously described.1 Results were expressed as % change
in lung resistance and percentage change from control (baseline).

ENUMERATION OF LUNG INFLAMMATION

Bronchoalveolar lavage fluid and blood smears were collected and stained
with May-Grünwald–Giemsa. Lung slices were stained with Congo-Red
(CR), periodic acid–Schiff (PAS), or toluidine blue to identify peribronchial
eosinophils, goblet cells, and mast cells, respectively, as previously
described.25

QUANTITATIVE RT-PCR

Trachea and lungs were extracted from killed mice, and forensic were used
to separate the airway from the parenchyma by means of blunt dissection.26
Total mRNA was then extracted with TRIzol (Ambion, Carlsbad, Calif), and
cDNA was generated with BioScript (BioLabs, Alexandria, Australia). Quantita-
tive PCR was performed with SYBR Green (Life Technologies, Mulgrave,
Australia) by using primers detailed in Table E1 in this article’s Online
Repository at www.jacionline.org.

LYMPH NODE CELL CULTURE AND ELISA

Single-cell suspensions of peribronchial lymph node cells were cultured for 6
days in the presence or absence of HDM (50 µg/mL), as previously described.27
ELISA (BD Biosciences Pharmingen, San Jose, Calif) was used to determine
concentrations of IL-9 and IL-13 in the supernatants of cell cultures.

QUANTIFICATION OF LUNG CHEMOKINES, PHOSPHORYLATED
EXTRACELLULAR SIGNAL-REGULATED KINASE 1, AND ACTIVE
NF-κB SUBUNITS

Snap-frozen lung lobes were homogenized in buffers recommended by the
manufacturer’s instructions. Levels of CCL11, CCL20, CXCL2, and p-
phosphorylated extracellular signal-regulated kinase 1 (p-ERK1) were
measured by using the Immulite 2000 (Siemens, Orange, Calif) assay (Active
Med, Carlsbad, Calif). All concentrations were normalized to lung weight.

BEAS-2B CELL CULTURE AND PP2A ACTIVITY

BEAS-2B cells, an SV40-transformed airway bronchial epithelial cell line,
were grown in Basal Epithelial Cell Growth Medium (REGM Clio-
nectsTM) until 80% confluence. After serum starvation, they either received
dexamethasone, salmeterol, or both preceding HDM stimulation (50
µg/mL) or were left untreated, and PP2A activity was measured by using the Active PP2A DoSai; IC activity assay (R&D Systems), according to the manufacturer’s instructions.

STATISTICAL ANALYSIS

Statistical significance was analyzed with the Student t test or Mann-
Whitney U test. All statistical analyses shown are comparisons with vehicle
control groups, unless otherwise stated. Data are expressed as means ± SEMs.

RESULTS

Systemic salmeterol suppresses HDM-induced AHR AND INFLAMMATION BUT NOT NUMBERS OF PAS-POSITIVE CELLS

We administered salmeterol or vehicle control intraperitoneally for 24 hours before and during the challenge phase in a mouse model of
HDM-induced AAD (see Fig E1, A). Data collection was performed 24 hours after the last salmeterol administration to account for possible bronchodilator effects inflating airways resistance. This was based on the documented duration of action of salmeterol in human subjects,29 as well as our own data from time course studies in naive mice showing protective effects on methacholine-induced AHR at 2 hours (see Fig E2, A, in this arti-
cle’s Online Repository at www.jacionline.org), but not 24 hours (see Fig E3, B), after treatment. However, in allergic mice salme-
toler significantly suppressed AHR compared with the vehicle
control 24 hours after treatment (Fig 1, A) concurrently with a reduction in the numbers of neutrophils and eosinophils in bronchoalveolar lavage fluid (Fig 1, B). This decrease in lung eosinophil numbers was also observed within the peribronchial tissue surrounding the airways (Fig 1, C) but associated with higher numbers of blood eosinophils (Fig 1, D). This suggests that salmeterol may exert its anti-inflammatory function by limiting eosinophil chemoattractants from the blood compartment to the lung tissue. Interestingly, salmeterol did not attenuate the number of PAS-positive cells (see Fig E3, A-D, in this article’s Online Repository at www.jacionline.org) in the airways after HDM challenge.

**Salmeterol suppresses chemokines but not \( \text{Th}_{12} \)-associated cytokines**

Salmeterol treatment had no effect on IL-4, IL-5, and IL-13 expression in the airways (Fig 2, A) nor did it attenuate \( \text{Th}_{12} \) responses of peribronchial lymph node cells cultured \textit{ex vivo} (see Fig E4 in this article’s Online Repository at www.jacionline.org). In contrast, CCL11, CCL20, and CXCL2 levels were significantly reduced by salmeterol treatment (Fig 2, B) concurrently with a partial restoration of PP2A activity (Fig 2, C), as well as decreased levels of p-ERK1 and active NF-κB subunits (Fig 2, D and E).

**Local administration of other long- and short-acting β-agonists have comparable effects to salmeterol treatment**

To investigate whether anti-inflammatory effects could be elicited by other β-agonists, we administered salmeterol, formoterol, and salbutamol via the airway route to mice during allergen challenge (see Fig E1, B). An identical suppression of AHR and inflammation was observed for all 3 compounds (see Fig E5, A and B, in this article’s Online Repository at www.jacionline.org), with numbers of PAS-positive cells remaining unaffected by treatment (see Fig E5, C). Consistent with our prior findings, local expression of chemokines was attenuated (see Fig E5, D), and there was a moderate level of PP2A activity restoration (see Fig E5, E).

**Salmeterol-induced anti-inflammatory action is dependent on PP2A reactivation**

To demonstrate a causal link between salmeterol and PP2A reactivation, we treated mice with salmeterol in combination with PP2Ac-targeting small interfering RNA (siRNA) or a nonsense control siRNA via the airway route during allergen challenge (see Fig E1, C). The efficacy of siRNA-mediated targeting of PP2Ac was confirmed by means of measurement of PP2A activity and PP2Ac protein levels (Fig 3, A). Inhibition of PP2Ac abolished salmeterol-induced suppression of chemokine expression (Fig 3, B) and eosinophil recruitment to the airways (Fig 3, C), whereas \( \text{Th}_{12} \)-associated cytokines were unaffected (Fig 3, D). This confirms that salmeterol modulates PP2A activity to suppress airways inflammation.

**Salmeterol suppresses AHR and inflammation in rhinovirus-exacerbated AAD**

In a mouse model of RV1B-induced exacerbation of AAD (see Fig E1, D), we found salmeterol to be as effective as dexamethasone in suppressing rhinovirus-induced AHR (Fig 4, A) and eosinophil recruitment to the airways (Fig 4, B). However, salmeterol treatment did not attenuate \( \text{Th}_{12} \)-associated cytokines, whereas...
Fig 2. Salmeterol attenuates chemokine but not T3,2 cytokine expression. A and B, Cytokine/chemokine expression in lower airway tissue (Fig 2, A and B) and protein levels of CXCL2 (Fig 2, B). C-E, PP2A activity (Fig 2, C), p-ERK1 (Fig 2, D), and activated NF-κB subunits (Fig 2, E) quantified from lung lysates by using ELISA. Results are presented as means ± SEMs (n = 3-6 mice per group). *P < .05, **P < .01, and ***P < .001 compared with the vehicle control group. SAL, Sterile saline.

Fig 3. Inhibition of PP2A reverses the therapeutic effect of salmeterol. A and B, PP2A activity and PP2Ac protein (Fig 3, A) and chemokines (Fig 3, B) in lung lysates. C, Cells in bronchoalveolar lavage (BAL) fluid. Infl, Eosinophils; Lyn, lymphocytes; Mac, macrophages; Neu, neutrophils. D, Cytokine expression in lower airway tissue. Results are presented as means ± SEMs (n = 3-6 mice per group). *P < .05, **P < .01, and ***P < .001 compared with the vehicle control group or otherwise indicated. SAL, Sterile saline.

dexamethasone significantly suppressed IL-13 (Fig 4, C). Both treatments reduced CCL11 expression, but only salmeterol attenuated CCL20 and CXCL2 production (Fig 4, D). Salmeterol had no effect on IFN-α and IFN-β expression (see Fig E6, A), in this article’s Online Repository at www.jacionline.org, which correlated with rhinovirus replication (see Fig E6, B). In contrast,
dexamethasone treatment led to increased rhinovirus replication, which resulted in an increase in IFN-α expression.

**Salmeterol modulates phosphatase activity through direct interaction with the PP2A complex**

As observed in models of HDM-induced AAD, salmeterol treatment correlated with a partial restoration of PP2A activity in the lungs of rhinovirus-exacerbated mice, whereas dexamethasone had no effect (Fig 5, A). Next, we stimulated human epithelial cells (BEAS-2B) with HDM after pretreatment with salmeterol and dexamethasone (Fig 5, B). As observed in vivo, salmeterol, but not dexamethasone, partially restored PP2A activity. To confirm a direct molecular interaction between salmeterol and PP2A independent of, for example, β2-adrenoceptors, we immunopurified PP2Ac from BEAS-2B cells, preserving the
bound protein complexes only, and treated them with salmeterol in a cell-free system (Fig 5, C). Importantly, we observed that salmeterol increased PP2A activity directly and in a dose-dependent manner comparable with the effect of the direct PP2A agonist (S)-2-amino-4-(di-[heptyloxy]phenyl)-2-methylbutan-1-ol (AAL[S]).

Reactivation of PP2A protects against AHR and suppresses eosinophilia in rhinovirus-exacerbated AAD

To provide evidence for the therapeutic potential of PP2A modulation by other drugs in rhinovirus-induced exacerbation of AAD, we treated mice with the nonphosphorylatable FTY720 analogue AAL(S) (see Fig E1, E). AAL(S) cannot be phosphorylated by sphingosine kinase 2 and therefore cannot bind to S1P receptors like phosphorylated FTY720.20 We have previously shown that AAL(S) increases PP2A activity in vivo and is therapeutically effective in models of HDM-induced AAD.21 Here we confirm these findings in rhinovirus-infected allergic mice (Fig 6, A). Notably, AAL(S) limited rhinovirus-induced AHR (Fig 6, B) and eosinophilic airways inflammation (Fig 6, C) in association with reduced CCL11, CCL20, and CXCL2 expression (Fig 6, D).

There was no effect on type 1 interferon expression and viral replication of AAL(S) treatment (see Fig E7 in this article’s Online Repository at www.jacionline.org).

Reactivation of PP2A prevented rhinovirus-induced neutrophilic inflammation

Naive mice received AAL(S) 24 hours before rhinovirus infection, which restored PP2A activity up to 24 hours after infection (see Fig E8, A; in this article’s Online Repository at www.jacionline.org) and conferred protection against rhinovirus-induced AHR 24 hours after infection (see Fig E8, B), neutrophilic inflammation (see Fig E8, C), and CXCL2 production (see Fig E8, D).

DISCUSSION

Using a well-characterized model of HDM-induced AAD,26,27 we observed suppression of AHR, as well as reduced eosinophilic airways inflammation, caused by systemic and local salmeterol treatment, whereas blood eosinophil levels mildly increased. This suggests that the anti-inflammatory effect of salmeterol might be associated with impaired eosinophilic chemotaxis rather than proliferation or maturation. This was supported by reduced levels of CCL11, which is crucial for eosinophil tissue homing,28,29 whereas levels of TGF-β-associated cytokines, such as IL-5 and IL-13, were not suppressed by salmeterol treatments. The singular and cooperative role of IL-5 and CCL11 in the regulation of eosinophils has previously been dissected in an OVA-induced model of AAD,30 and our data support a role of salmeterol in CCL11-mediated eosinophilic chemotaxis. Salmeterol also reduced CCL20 levels in this study, which is a critical signal for the expression of AAD through regulation of dendritic cell and T-cell recruitment.31 Interestingly, neutrophilic inflammation was also limited on salmeterol treatment, and this was associated with reduced levels of CXCL2, which is a potent chemoattractant for neutrophils.32,33

IL-13 has been identified as a critical cytokine signal for the development of mucus hypersecretion and goblet cell hyperplasia.
in patients with allergic asthma. In this study, as assessed by PAS staining concurrently with IL-13 expression, it was not altered by salmeterol treatment. These data support previous findings showing that salmeterol treatment of OVA-sensitized and OVA-challenged mice did not attenuate mucus production in the airways.

PP2A activity was reduced in PBMCs from patients with severe asthma, and synthetic knockdown in vitro conferred resistance to corticosteroids. This was associated with hyperphosphorylation of the GR, in addition to modulating GR nuclear translocation. In this study salmeterol partially restored PP2A activity and suppressed in vitro levels of p-EAK1, which is known to play a key role in inflammatory gene expression in asthmatic patients and is dephosphorylated by PP2A. Similarly, salmeterol treatment led to lower quantities of active NF-κB, which is a key inflammatory mediator sequestered by IκBα, another molecule dephosphorylated by PP2A.

Notably, Hu et al. observed suppressed mitogen-activated protein kinase and NF-κB activation in salmeterol-treated dendritic cells.

Rhinovirus-induced exacerbations of asthma are common and only partially responsive to corticosteroid treatment. In this study we used an established model of HDM-induced AAD with superimposed rhinovirus infection, leading to an exacerbation of AHR and inflammation greater than the level seen in allergic mice exposed to UV-inactivated rhinovirus. Protection against the development of AHR after salmeterol treatment was equal to dexamethasone treatment in rhinovirus-exacerbated allergic mice. Although both treatments suppressed lung eosinophilia, the mechanisms were distinct. Namely, dexamethasone suppressed both IL-13 and CCL11, as well as mucus hypersecretion (data not shown) but did not inhibit other inflammatory chemokines or increase PP2A activity in vivo or in vitro. Additionally, only dexamethasone treatment was found to promote rhinovirus replication, leading to increased IFN-α production (see Fig E7). The effects of corticosteroids on interleukins and rhinovirus replication remain largely unexplored, but our results suggest that this treatment might adversely affect rhinovirus clearance, an observation reported previously.

To confirm a causal link between PP2A activity and the anti-inflammatory effects exerted by salmeterol, we used siRNA directed against PP2A, which completely blocked the therapeutic effect of salmeterol. In support, the PP2A agonist AAL(S) yielded an identical response in rhinovirus-exacerbated mice as salmeterol treatment. Additionally, increasing PP2A activity suppressed neutrophilic inflammation and CXCL2 release induced by rhinovirus infection in naive mice. Finally, we demonstrated a direct modulation of PP2A activity by salmeterol in a cell-free system, suggesting that the anti-inflammatory effects of salmeterol-induced PP2A activation in vivo can occur, at least in part, independent of β2-adrenergic receptor activation. Further studies are now required to determine the precise interaction between PP2A subunits and salmeterol on a molecular level.

In summary, we provide a novel molecular mechanism for salmeterol-mediated suppression of AHR and airways inflammation that can be further exploited by optimized PP2A-activating drugs and β-agonists. We hypothesize that salmeterol and other β-agonists used as an add-on to steroid treatment have the potential to target distinct proinflammatory pathways unresponsive to corticosteroids in patients with asthma and virus-induced exacerbations.

We thank Ana Faresin deSilvestro, Heather MacDonald, Jane Grehan, and the staff from the Animal Care Facility of the University of Newcastle for their technical assistance.

Key messages
- Salmeterol suppresses allergen- and rhinovirus-induced airways hyperreactivity, inflammation, and chemokine release by increasing PP2A activity.
- Increasing PP2A activity with AAL(S) also suppresses hallmark features of allergic and rhinovirus-induced airways disease.
- Salmeterol-induced modulation of PP2A activity might be of therapeutic benefit for the treatment of asthma and rhinovirus-induced exacerbation.

REFERENCES


4.2 Supplementary material

ONLINE METHODS

Animals
Male BALB/c mice, 6-8 weeks old, were obtained from the Special Pathogen Free Facility of the University of Newcastle. Mice were housed in approved containment facilities within the David Maddison Clinical Sciences Building and the HMRI Building, University of Newcastle (Newcastle, Australia), with ad libitum access to food and water under a 12 hr light and dark cycle. The Animal Care and Ethics Committee of the University of Newcastle, Australia approved all experiments which were conducted and reported in accordance with the ARRIVE guidelines.

Induction of AAD and RV-induced exacerbation
Crude house dust mite (*Dermatophagoides pteronyssinus*) extract was obtained from Greer Laboratories (Lenoir, NC) as a lyophilized preparation of milled mites. HDM was resuspended in sterile saline (SAL) and mice were treated intranasally under light isoflurane anaesthesia with 50µg protein/50µl (Der p 1 constituted ~10% of protein weight) or sterile endotoxin-free saline on days 0, 1, and 2 for sensitization. Sensitization was followed by daily HDM exposure (5µg/50µl) on days 14, 15, 16, and 17 to induce AAD (Fig E1A). Nonsensitized mice (saline-treated) received sterile endotoxin-free saline (0.9%) only. Mice were euthanized via pentobarbital sodium (Virbac) overdose on day 18, 24 hours after the last HDM exposure (and salmeterol treatment where applicable). In other experiments mice were intranasally infected with minor group RV (RV1B), 50µl containing 1x10⁷ virions, or an equivalent amount of UV-inactivated RV1B on day 18, which was 24hrs after the last HDM challenge to exacerbate preexisting AAD (Fig E1D)[E1]. Mice were euthanized 24hrs after RV1B infection.

Administration of beta agonists, siRNA, dexamethasone and the PP2A activator (S)-2-amino-4-(4-(heptyloxy)phenyl)-2-methylbutan-1-ol (AAL(S))
Salmeterol (3mg/kg; Sigma-Aldrich, New South Wales, Australia) or vehicle control (sterile saline) was administered by intraperitoneal injection (200µl) either to naïve mice, to HDM sensitized mice on day 13 (24 hours before the first HDM challenge) and then every day during challenge, or once only on day 17 following last HDM challenge but at least 18 hours before inoculation with RV1B. In other experiments, mice received salmeterol (0.4mg/kg), formoterol (0.4mg/kg; Sigma-Aldrich, New South Wales, Australia), salbutamol (2mg/kg; Sigma-Aldrich, New South Wales, Australia) or vehicle control (sterile saline) via a 50µl intranasal application daily through days 13-17. Some mice in combination with HDM and salmeterol also received a PP2Ac-α-targetted siRNA intranasally (3.75nmol/25µl) on days 13, 15 and 17 at the same time salmeterol was administered, or a nonsense control siRNA (chosen to have an equivalent CG content) with no similarities to other sequences. Both siRNA were purchased from Dharmacon. Other mice received dexamethasone (6mg/kg; Sigma-Aldrich, New South Wales, Australia) or AAL(S) dissolved in a 2% 2-hydroxypropyl-cyclodextrin vehicle (10μg/50μl: Sigma-Aldrich, New South Wales, Australia) on day 17 prior to RV1B inoculation via intraperitoneal injection or intranasal application respectively.

**AHR measurement**

Airway hyperreactivity was measured as previously described [E2]. Briefly, AHR was invasively assessed in separate groups of ketamine-xylene (Illum)–anesthetized mice by measurement of total lung resistance and dynamic compliance (Buxco). Mice were mechanically ventilated and AHR to nebulised methacholine (increased lung resistance) was expressed as a percentage change from control (baseline).

**Analysis of bronchoalveolar lavage and circulatory leukocytes**

Mice were cannulated and lungs flushed twice with 1ml of Hank's Buffered Salt Solution (HBSS). Bronchoalveolar lavage fluid (BALF) was centrifuged at 800 x g for 10 min at 4°C and cell-free supernatant was collected and frozen at -80°C. Cells were treated with Red Blood Cell Lysis buffer (12mM NaHCO₃, 0.1mM EDTA, 155mM NH₄Cl, pH 7.35) for 5 min before being centrifuged as above and resuspended in 100µl of HBSS and total number of viable cells determined by trypan blue exclusion in a Neubauer cell chamber. Cytospins were prepared and slides were stained with May-
Grunwald-Giemsa. Slides were blinded and differential cell counts determined from a total of 200 cells per slide. Blood was retrieved from euthanized mice via insertion of a 25-gauge needle into the right ventricle (returns were between 0.4-0.6ml per mouse) and blood smears performed. These were also stained and counted in the same manner as above to determine differential blood leukocyte proportions.

**Histology**
A single lung lobe was excised and fixed in 10% Neutral Buffered Formalin for 24 hours, then stored in 70% Ethanol. 5µm slices were taken and stained with Carbol’s chromotrope-hematoxylin (CR), Periodic acid-Schiff (PAS), or Toluidine blue for the enumeration of peribronchial eosinophils, PAS-positive cells, and mast cells respectively. For each mouse, 10 high powered fields were counted at x1000 original magnification [E3].

**Quantitative RT-PCR**
Trachea and lungs were extracted from euthanized mice and forceps were used to separate the airways from the parenchyma by blunt dissection [E4]. This effectively resulted in the isolation of several generations of airways containing the resident airway epithelial cells, fibroblasts, smooth muscle cells, the basement membrane and infiltrating inflammatory cells. Total mRNA was then extracted using TRIzol® (Ambion, Carlsbad, USA) and concentrations were determined by spectrophotometry (Nanodrop). cDNA was generated via reverse transcription using BioScript™ (Bioline, Alexandria, Australia). Quantitative PCRs (qPCR) were performed with SYBR® Green (Invitrogen, Mulgrave, Australia) using primers detailed in Table E1. CTs of the genes of interest were referenced to a standard curve of known concentration to obtain copy numbers and normalised to the endogenously expressed control gene, HPRT. All steps were performed according to manufacturers instructions.

**Lymph node cell culture and ELISA**
Peribronchial lymph nodes were extracted, filtered into a single cell suspension and cultured for 6 days in the absence or presence of HDM (50µg/ml) as previously described [3]. Briefly, cells were cultured at 1.25 x 10⁶ cells per well per 250µl of complete RPMI (RPMI-1640, 0.1mM sodium pyruvate, 10% heat-inactivated FCS,
2mM L-Glutamine, 20mM HEPES, 100U/ml Pen/Strep, and 50µM 2-mercaptoethanol). ELISA (BD Biosciences, PharMingen, USA) was then used to determine the concentration of IL-5 and IL-13 in the supernatants of cell cultures.

**Quantification of lung chemokines, phosphorylated-ERK1 and active NFκB subunits**

A single lung lobe from each mouse was excised and snap frozen in liquid nitrogen before being homogenised in buffers recommended in the manufacturers instructions. Levels of CCL11, CCL20, CXCL2, and phosphorylated-ERK1 were determined in clarified lung lysates by ELISA (R&D Systems) whereas quantitation of activate NFκB subunits was performed with the TransAM NFκB Transcription Factor Assay (Active Motif) and data expressed as protein concentration (p50 and p65) or optical density at 450nm (p52 and RelB) according to the manufacturers instructions. All concentrations were normalised to lung weight.

**BEAS-2B cell culture and PP2A activity**

BEAS-2B human bronchial epithelial cells, a SV40-transformed airway bronchial epithelial cell line, were grown in complete Bronchial Epithelial cell Growth Medium (BEGM, Clonetics) as previously described [E5] until 80% confluent, at which time they were serum-starved overnight and either pre-treated with dexamethasone (1µM), salmeterol (1µM), a combination of the two, or vehicle control only (0.1% DMSO) for 1 hour preceding HDM stimulation (50µg/ml). After 24 hours cell lysates were harvested and PP2A activity assessed by Active PP2A DuoSet IC activity assay (R&D Systems), according to the manufacturer’s instructions and data expressed as percentage change from media control. PP2A activity was also assessed in mouse lung lysate, expressed as percentage change from non-allergic sterile saline-treated mice and normalised to lung weight. In another experiment, untreated BEAS-2B cells, following serum-starvation, were lysed under non-reducing conditions (20mM Imidazole, 2mM EDTA, 2mM EGTA, 0.01% Triton-X 100) and PP2Ac complexes were immunoprecipitated (IP) and subjected to in-vitro activation using either; vehicle control (0.1% DMSO), 0.01µM, 0.1µM and 1µM salmeterol, or 2.5µM AAL(S) for 30 minutes at 30°C. PP2A complexes were then incubated with a PP2A specific phosphopeptide and subsequent measurement of free phosphate (PO₄) in solution was performed according to the manufacturer’s instructions. PP2A activity was normalised to western blot band intensity of PP2Ac
protein (37 kDa) from the same experiment for all groups including total protein lysate prior to IP and also in the absence of IP capture antibody (Neg PP2Ac ab).

**Statistical analysis**

GraphPad Prism version 6.0 software (GraphPad Software, La Jolla, California) was used to determine statistical significance of differences between groups through the use of the Student’s t-test or Mann-Whitney test as appropriate. All statistical analysis shown are comparisons to vehicle control groups unless otherwise stated. Data are expressed as Mean±SEM, with a P value of less than 0.05 considered statistically significant.

**References**


Online Repository Figure 1. Models of AAD and RV-induced exacerbation. Mice were sensitized to and challenged with HDM over 17 days while receiving treatments prior to and during the challenge phase as shown (A-C). In other experiments, mice were additionally infected with live or UV-inactivated RV1B on day 18 and treatments administered following final HDM challenge (D-E). All mice were euthanized 24 hours after final challenge for analysis.
**Online Repository Figure 2.** Duration of salmeterol-induced bronchodilation in mice. Naïve mice were intraperitoneally injected with salmeterol (3mg/kg) or a vehicle control and total lung resistance invasively assessed at 2 hours and 24 hours-post treatment. Total lung resistance presented as raw baseline values (A) and percentage change in response to methacholine (B). Results are Mean±SEM (n=6-7 mice per group). *, P < 0.05. ns, non-significant.
Online Repository Figure 3. Numbers of PAS-positive epithelial cells and mast cells are not effected by salmeterol treatment. Fixed lung sections stained with Periodic acid-Schiff (A-C) or Toluidine blue (E-G) from allergic vehicle (A, E), salmeterol treated (B, F), or non-allergic SAL treated mice (C, G) (micrographs x100 magnification, scale bar = 50μm). PAS-positive epithelial cells (D) and mast cells (H) were counted from ten high-powered fields (100 μm²). Results are Mean±SEM (n=3-4 mice per group).
Online Repository Figure 4. Production of T\textsubscript{n}2 cytokines from lymph node cells is not affected by salmeterol treatment. Single-cell suspensions of peribronchial-draining lymph node cells were cultured for 6 days in the absence or presence of HDM (50µg/ml). Supernatants were collected and IL-5 and IL-13 quantified by ELISA. Data are presented as Mean±SEM (cells pooled from n=8 mice per group).
Online Repository Figure 5. Intranasal administration of beta agonists suppresses AAD.

(A) Total lung resistance presented as raw baseline values and percentage change in response to methacholine (n=7-10 mice per group). (B) Cells in bronchoalveolar lavage (BAL). (C) PAS-positive epithelial cells per 100µm². Levels of chemokines (D) and PP2A activity (E) in lung lysates. Results are Mean±SEM (n=3-6 mice per group). *, P < 0.05, **, P < 0.01, ***, P < 0.001 as compared to vehicle control group.
Online Repository Figure 6. Salmeterol does not attenuate type I IFN responses or RV replication in exacerbated mice. (A) Gene expression of IFN-α and IFN-β in lower airway tissue, mRNA copy numbers normalized to HPRT. (B) Copy numbers of positive-strand RV1B RNA from lower airway tissue of infected mice. Results are Mean±SEM (n=3-6 mice per group). *, P < 0.05 as compared to vehicle control group.
Online Repository Figure 7. AAL(S) does not attenuate type 1 IFN responses or RV replication in exacerbated mice. (A) Gene expression of IFN-α and IFN-β in lower airway tissue, mRNA copy numbers normalized to HPRT. (B) Copy numbers of positive-strand RV1B RNA from lower airway tissue of infected mice. Results are Mean±SEM (n=3-4 mice per group). ns = non-significant.
Online Repository Figure 8. PP2A agonist AAL(S) suppressed RV-induced inflammation.

PP2A activity (A) and CXCL2 protein levels (D) in lung lysates. (B) Total lung resistance at 1 dpi presented as raw baseline values and percentage change in response to methacholine (n=4-6 mice per group). (C) Cells in bronchoalveolar lavage (BAL). Results are Mean±SEM (n=4-6 mice per group), dpi = days post-infection. *, P < 0.05, **, P < 0.01 as compared to vehicle control group.
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HPRT = Hypoxanthine-guanine phosphoribosyltransferase
CHAPTER 5: Discussion
5.1 Elucidating the TRAIL-MID1-PP2A pathway

For the first time it has been demonstrated that there is an upregulated expression of the E3 ubiquitin ligase midline-1, with a concordant down regulation of PP2A activity and total PP2Ac protein, in the murine airway wall following repeated HDM challenges. This phenotype was dependent upon intact TRAIL/TLR4/MyD88 but not STAT6 signalling. Subsequent experiments demonstrated that MID1 and PP2A could be synthetically modulated in-vivo by direct application of targeted siRNA or by a non-phosphorylatable FTY720 analogue. This had the effect of abolishing AHR and reducing the cardinal signs of allergic lung inflammation, attenuating mucus hypersecretion, airway eosinophilia, Th2 cytokine production, as well as suppressing NFκB activation and phosphorylation of MAP kinases. AAL(s) also reduced the number of CD4+ T cells and mDCs recruited into the airway upon HDM challenge.

Modulating MID1 also attenuated RV-induced lung inflammation in both a non-allergic and allergic setting by preventing the development of AHR, neutrophilic and eosinophilic recruitment, chemokine secretion and NFκB activation. Finally, this pathway was investigated in-vitro through the use of immortalised bronchial epithelial cells (BECs). Following stimulation with recombinant TRAIL or HDM, these BECs increased MID1 expression, while reducing PP2A activity. Immunoprecipitation performed on these cells also found MID1/α4/PP2Ac to be associated, confirming that this complex exists in this cell type. This was extended clinically by analysing responses in BECs isolated from a cohort of healthy and asthmatic individuals after stimulation ex-vivo with rTRAIL, HDM or infection with RV. MID1 was upregulated in both groups and significantly correlated with TRAIL and CCL20 expression, one of the effector chemokines in this pathway.

The impact that this study has made in the fields of asthma, allergic diseases and lung dysfunction has been quite notable, for instance by way of citations in peer-reviewed articles and literature reviews. A number of studies published since this paper have also been strongly influenced by its work, such as the investigation of the requirement of TRAIL signalling in a chronic model of allergen-driven AAD. In this study it was
reported that TRAIL-deficient mice, who exhibited no upregulation of MID1 expression in their airways, were protected from the cardinal signs of AAD inflammation, as well as features of structural remodelling such as airway smooth muscle hypertrophy and sub-epithelial collagen deposition\textsuperscript{185}. TRAIL signalling was also found to be necessary for the development of persistent pathological lung changes in mice following neonatal infection with \textit{Chlamydia muridarum}, another pathogen strongly linked to the development of chronic lung disease\textsuperscript{186}.

The TRAIL-MID1-PP2A pathway has also recently been implicated to underlie other allergic diseases, such as eosinophilic esophagitis (EoE), where TRAIL and MID1 were found to be upregulated in esophageal biopsies from EoE patients compared to healthy individuals. This was further investigated in a mouse model of \textit{Aspergillus fumigatus}-induced EoE, where TRAIL-deficiency or MID1 knockdown attenuated eosinophilic inflammation and esophageal remodelling\textsuperscript{187}. TRAIL was also very recently reported to be critical for pro-inflammatory responses against RV infection in a non-allergic lung environment but not anti-viral IFN production. In an allergic lung setting, TRAIL-deficiency prevented RV-induced exacerbation\textsuperscript{188}.

The identification and subsequent modulation of the TRAIL-MID1-PP2A axis in the context of AAD had not been explored \textit{in-vivo} prior to these studies, targeting PP2A in particular showing promise given its promiscuous de-phosphorylation of many signalling molecules, including those already described to be important for allergic inflammation. Modulation of TRAIL levels and/or the signalling pathways downstream of TRAIL may represent novel therapeutic strategies to impair aberrant inflammatory responses associated with asthma, allergies and viral exacerbation.
5.2 Suppression of TLR7-mediated IFN responses in asthma

This study marks the first *in-vivo* investigation of the impact of TLR7 signalling during RV infection in AAD. Allergic TLR7-deficient mice exhibited sub-optimal IFN responses following RV infection, leading to increased viral replication and exacerbated AHR. This was accompanied by increased IL-5 secretion, eosinophilic tissue inflammation, mDC and T cell recruitment, as well as higher chemokine production (eotaxin-1 notably) compared to wildtype controls.

Exogenous IFN therapy prevented this exaggerated phenotype in TLR7-deficient mice, IFN-α, -β and -λ2 suppressing eosinophil-associated cytokines and recruitment to the airways. IFN-α and -β treatment restored type I IFN production, IFN-λ2 signalled in an autocrine fashion, while each of the three when administered alone acted to increase expression of IFN-γ and reduce viral replication. Adoptive transfer of TLR7-competant pDCs (the prototypical IFN producing cell that expresses very high amounts of TLR7) into the airways of allergic TRL7-deficient mice restored type I and type III IFN production when challenged with RV, supressed viral replication, AHR and eosinophilic inflammation compared to mice transferred TLR7-deficient pDCs.

In the process of dissecting this phenotype, it was discovered that mice that had been repeatedly challenged with HDM had lower expression of TLR7 in their airways and that intact TLR4/MyD88/STAT6 signalling was required for this suppression. Furthermore, singular stimulation with recombinant IL-5, IL-13 or LPS could not replicate this but IL-5 transgenic mice (whom express IL-5 consistently and show persistent tissue eosinophilia) did, suggesting that TLR7 was suppressed in the presence of eosinophils and IL-5. This specificity was mirrored by the minimal alterations to IL-13 production or mucus hypersecretion seen in the exacerbated TLR7-deficient mice.

Finally, using cells from a cohort of non-asthmatics and asthmatics of multiple inflammatory phenotypes, endobronchial biopsy samples showed lower TLR7 expression in the asthmatic group, especially from those with eosinophilic lung inflammation regardless of atopic status. There was also a significant inverse correlation
between TLR7 expression and percentage of sputum eosinophils, as well as TLR7 correlating with expression of IFN-α and -λ2/3.

The suggestion of a reciprocal relationship between IL-5-driven eosinophilia and TLR7 expression has been hinted at previously in the literature, with TLR7 agonists being shown to inhibit IL-5 expression via the production of IFN-γ by Notch signalling pathways\textsuperscript{189} and by CD4\textsuperscript{+} and NKT cells\textsuperscript{190}. Clinically, a trialled human monoclonal antibody to IL-5 (mepolizumab) was reported to reduce exacerbation frequency in moderate-to-severe asthmatics with a predominantly eosinophilic airways inflammation\textsuperscript{191}, although it was unknown if this subgroup exhibited suppressed TLR7 or IFN responses. Very recently a study by Duerr \textit{et al.} in Nature Immunology has added to this picture by demonstrating that type I and type II IFNs via IL-27 regulate ILC2 cells and restrict type 2 immunopathology, helping bolster a number of findings in Chapter 3, despite the investigation of ILC2 cells being outside the scope of this study\textsuperscript{192}. Additionally, in the last six months these findings were largely mirrored in alveolar macrophages isolated from severe asthmatics, where suppressed TLR7 expression drove impaired antiviral responses, a phenotype that in-part could be explained by alterations in microRNA expression profiles\textsuperscript{193}.

Therapeutic strategies that target the early cellular and molecular signals leading to exaggerated \textit{T}H\textsubscript{2} responses may be of particular benefit for asthma patients, with our data suggesting that TLR7 signalling limits these responses in RV-induced asthma exacerbations and may be a promising therapeutic target for the prevention and treatment of viral exacerbation in eosinophilic asthmatics. However further work is needed to determine if the mechanisms of IFN deficiencies in these asthmatics are conserved between studies looking at BECs, PBMCs and DCs.
5.3 PP2A-agonising effects of long-acting B\textsubscript{2} agonists

With the exploration of PP2A modulation during AAD in Chapter 2, along with the timely publication of a link between supressed PP2A in severe asthmatics and agonistic effects of LABAs, this presented a novel opportunity to extend these reports into an \textit{in-vivo} study using a clinically complex and relevant allergen.

We found that allergic mice repeatedly challenged with HDM were protected from developing AHR and lung eosinophilia if they received treatment with the LABA salmeterol. These effects appeared to be due to retention of eosinophils in the blood compartment and were independent on the well-described bronchodilatory effects of LABAs. Chemokines, phosphorylated-ERK1 and activated NFκB but not type 2 cytokines or mucus hypersecretion were supressed by salmeterol treatment, as well as a partial restoration of PP2A activity. This phenotype was conserved across other LABAs (formoterol) and SABAs (salbutamol), and was lost if PP2Ac was targeted for knockdown by siRNA.

Salmeterol also supressed AHR and cellular inflammation during RV-induced exacerbation of AAD, to the same magnitude as the corticosteroid dexamethasone, however only salmeterol supressed chemokine release and increased PP2A activity. Human BECs also exhibited this salmeterol-specific PP2A reactivation \textit{in-vitro}, the combination of salmeterol and dexamethasone not improving this efficacy. Remarkably, salmeterol was able to agonise immunopurified PP2A complexes directly in a cell-free system, to the same degree as the drug AAL(s). This also translated \textit{in-vivo} with AAL(s) preventing exacerbation of AHR, eosinophil recruitment and chemokine production by partially restoring PP2A activity.

The findings presented in Chapter 4 have influenced the course of literature since their publication with Li \textit{et al.} reporting that the mechanism whereby microRNA miR-9 induced IFN-γ/LPS-mediated corticosteroid insensitivity in pulmonary macrophages was due to miR-9 targeting PPP2RD5 (a PP2A subunit). Antagonism of miR-9 or treatment with AAL(s) increased PP2A activity and GR nuclear translocation in macrophages and restored corticosteroid sensitivity in multiple models of steroid-
resistant AHR\textsuperscript{194}. PP2A modulation was further extended a year later into a mouse model of egg allergen-driven EoE, where the application of salmeterol, dexamethasone, or a combination of the two, attenuated eosinophilic inflammation and cytokine release in the esophagus, as well as tissue fibrosis, yet again only those groups treated with salmeterol exhibited increased PP2A activity\textsuperscript{195}. Restoring PP2A activity to ameliorate models of allergic and inflammation-driven pathology has at this point become a reproducible phenomenon, although further studies are required to determine the precise interaction between PP2A subunits and LABAs on a molecular level.

We have described here a novel mechanism where salmeterol-mediated attenuation of AHR and inflammation can potentially be optimised by exploiting off-target PP2A-agonising effects, under the hypothesis that $\beta_2$ agonists can be utilised to not only sensitise asthmatics to corticosteroid therapy in combination, but target distinct pro-inflammatory pathways to prevent or reduce virally-induced asthma exacerbation.
5.4 Thesis summary

The research compiled within this thesis was conducted over a four year period and encompasses three published journal articles. These papers focus on the identification and modulation of novel inflammatory and anti-viral pathways involved in AAD, RV infection and RV-induced asthma exacerbation, primarily using mouse models with some translational aspects using human clinical samples.

The parallel focus on elucidating the molecules downstream of TRAIL signalling that play instrumental roles in what is a complex dysregulated immune response, paired with investigating deficits in IFN responses against a highly ubiquitous human pathogen was done to address two emerging fields of interest in asthma research.

The identification and subsequent modulation of the TRAIL-MID1-PP2A axis in the context of AAD had not been explored in-vivo prior to these studies, targeting PP2A in particular showing promise given its promiscuous de-phosphorylation of many signalling molecules, including those already described to be important for allergic inflammation.

Concurrently, finding a link between IL-5-driven inflammation and suppressed TLR7 expression in both mouse and human tissue, as well as functional loss of TLR7 resulting in reduced IFN production, prolonged viral clearance and heightened lung inflammation in an allergic setting, strengthens the current dogma surrounding viral exacerbation.

Taken together, these studies investigate the pathogenesis of AAD, RV infection and RV-induced exacerbation of asthma, and demonstrate that synthetically modulating novel targets by siRNA or pharmacologically with the small molecule AAL(s) or the commercially available LABA salmeterol, was able to significantly abrogate hallmark features of disease within the models. These techniques were applied to highlight new potential targets for therapeutic intervention and enrich the field of study they were aimed towards.
APPENDICES
Appendix A – Copyright policies

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Appendix B – List of additional publications

Journal Articles

2017 1) TRAIL signalling is pro-inflammatory and pro-viral in a murine model of rhinovirus 1B infection.
Girkin, J.*, Hatchwell, L.*, Collison, AM., Hansbro, PM., Starkey, MR., Yagita, H., Foster, PS., Mattes, J.
*These authors contributed equally to this work.

2015 2) TNF-related apoptosis-inducing ligand (TRAIL) regulates midline-1, thymic stromal lymphopoietin, inflammation, and remodelling in experimental eosinophilic esophagitis.
Collison, AM., Sokulsky, LA., Sherrill, JD., Nightingale, S., Hatchwell, L., Talley, NJ., Walker, MM., Rothenberg, ME., Mattes, J.
doi: 10.1016/j.jaci.2015.03.031.

3) MicroRNA-9 regulates steroid-resistant airway hyperresponsiveness by reducing protein phosphatase 2A activity.
Li, JJ., Tay HL., Maltby, S., Xiang, Y., Eyers, F., Hatchwell, L., Zhou H., Toop, HD., Morris, JC., Nair, P., Mattes, J., Foster, PS., Yang, M.
4) **CCL7 and IRF-7 mediate hallmark inflammatory and IFN responses following Rhinovirus 1B infection.**

Girkin, J., **Hatchwell, L.**, Foster, PS., Johnston, SL., Bartlett, N., Collison, A., Mattes, J.

doi: 10.4049/jimmunol.1401362.

2014 5) **Tumor necrosis factor-related apoptosis-inducing ligand translates neonatal respiratory infection into chronic lung disease.**

Starkey, MR., Nguyen, DH., Essilfie, AT., Kim, RY., **Hatchwell, LM.**, Collison, AM., Yagita, H., Foster, PS., Horvat, JC., Mattes, J., Hansbro, PM.

doi: 10.1038/mi.2013.65.

2013 6) **Inhibiting AKT phosphorylation employing non-cytotoxic anthraquinones ameliorates TH2 mediated allergic airways disease and rhinovirus exacerbation.**

de Souza Alves, CC., Collison, A., **Hatchwell, L.**, Plank, M., Morten, M., Foster, PS., Johnston, SL., da Costa, CF., de Almeida, MV., Teixeira, HC., Ferreira, AP., Mattes, J.

doi: 10.1371/journal.pone.0079565.
Conference Abstracts

2014  1)  *Reduced TLR7 expression may underpin impaired response to viral infection in asthma.*
Collison, A., **Hatchwell, L.**, Girkin, J., Li, J., Parsons, K., Bartlett, NW., Johnston, SL., Pereira de Siqueira, A., Foster, PS., Phipps, S., Wark, PA., Mattes, J.
Thoracic Society of Australia and New Zealand Annual Scientific Meeting: Adelaide, SA, Australia.

Girkin, J., Sokulsky, L., **Hatchwell, L.**, Starkey, M., Collison, A., Hansbro, P., Mattes, J.
Thoracic Society of Australia and New Zealand Annual Scientific Meeting: Adelaide, SA, Australia.

2013  3)  *Interferon regulatory factor 7 mediates host responses to rhinovirus infection and exacerbation of allergic airways disease.*
Girkin, J., **Hatchwell, L.**, Foster, PS., Johnston, SL., Collison, A., Mattes, J.
The 43rd Annual Scientific Meeting of the Australasian Society for Immunology: Wellington, New Zealand.

4)  *CCL7 (MCP-3) facilitates rhinovirus-induced lung inflammation and exacerbation of allergic airway disease.*
**Hatchwell, L.**, Collison, A., Phipps, S., Bartlett, NW., Foster, PS., Johnston, SL., Mattes, J.
International Conference of the American Thoracic Society; Philadelphia, PA, USA.
5) *CCL7 (MCP-3) mediates rhinovirus-induced lung inflammation and exacerbation of allergic airway disease.*

**Hatchwell, L.,** Collison, A., Phipps, S., Foster, PS., Johnston, SL., Mattes, J.

**Thoracic Society of Australia and New Zealand Annual Scientific Meeting:** Darwin, NT, Australia.

6) *Salmeterol attenuates chemotaxis in rhinovirus-induced exacerbation of asthma via modulation of PP2A.*

Girkin, J., **Hatchwell, L.**, Foster, PS., Johnston, SL., Collison, A., Mattes, J.

**Thoracic Society of Australia and New Zealand Annual Scientific Meeting:** Darwin, NT, Australia.

2012 7) *Deficient Toll-like receptor 7-mediated anti-viral responses underpin rhinovirus-induced asthma exacerbation.*

**Hatchwell, L.,** Collison, A., Phipps, S., Johnston, SL., Foster, PS., Mattes, J.

**The 42nd Annual Scientific Meeting of the Australasian Society for Immunology:** Melbourne, VIC, Australia.

8) *Salmeterol attenuates chemotactic responses in rhinovirus-induced asthma exacerbation via modulation of PP2A.*

Girkin, J., **Hatchwell, L.**, Foster, PS., Johnston, SL., Collison, A., Mattes, J.

**The 42nd Annual Scientific Meeting of the Australasian Society for Immunology:** Melbourne, VIC, Australia.
9) Antagonism of microRNA is comparable to azithromycin treatment in a mouse model of rhinovirus-induced exacerbation of allergic airways disease.
Collison, A., Hatchwell, L., Pereira de Siqueira, A., Bartlett, NW., Johnston, SL., Foster, PS., Mattes, J.
International Conference of the American Thoracic Society;
San Francisco, CA, USA.

10) TRAIL regulates inflammatory and anti-viral responses to rhinovirus and rhinovirus-induced exacerbation of asthma.
Hatchwell, L., Collison, A., Bartlett, NW., Johnston, SL., Foster, PS., Pereira de Siqueira, A., Mattes, J.
International Conference of the American Thoracic Society;
San Francisco, CA, USA.

11) Antagonism of microRNA-122 is comparable to azithromycin treatment in a mouse model of rhinovirus-induced exacerbation of allergic airways disease.
Collison, A., Hatchwell, L., Pereira de Siqueira, A., Bartlett, NW., Johnston, SL., Foster, PS., Mattes, J.
Thoracic Society of Australia and New Zealand Annual Scientific Meeting; Canberra, ACT, Australia.

12) Toll-like receptor 7 mediates anti-viral responses to rhinovirus while suppressing exacerbation of asthma.
Hatchwell, L., Collison, A., Pereira de Siqueira, A., Bartlett, NW., Johnston, SL., Foster, PS., Mattes, J.
Thoracic Society of Australia and New Zealand Annual Scientific Meeting; Canberra, ACT, Australia.
13) *TRAIL regulates inflammatory responses to rhinovirus and rhinovirus-induced exacerbation of asthma.*

Hatchwell, L., Collison, A., Pereira de Siqueira, A., Bartlett, NW., Johnston, SL., Foster, PS., Mattes, J.
Thoracic Society of Australia and New Zealand Annual Scientific Meeting; Canberra, ACT, Australia.

2011 14) *TRAIL regulates anti-viral and inflammatory responses in rhinovirus infection and rhinovirus-induced asthma exacerbation.*

Hatchwell, L., Collison, A., Pereira de Siqueira, A., Wark, PA., Bartlett, NW., Johnston, SL., Foster, PS., Mattes, J.
The 41st Annual Scientific Meeting of the Australasian Society for Immunology; Adelaide, SA, Australia.

15) *A novel E3 ubiquitin ligase-phosphatase interaction links rhinovirus infection and exacerbation of asthma.*

Hatchwell, L., Collison, A., Pereira de Siqueira, A., Don, A., Foster, PS., Verrills, N., Mattes, J.
International Conference of the American Thoracic Society; Denver, CO, USA.

16) *The development of House Dust Mite-induced allergic airways disease is regulated by a novel E3 ubiquitin ligase-dependent deactivation of a protein phosphatase.*

Collison, A., Hatchwell, L., Pereira de Siqueira, A., Don, A., Zimmerman, N., Rothenberg, ME., Verrills, N., Foster, PS., Mattes, J.
International Conference of the American Thoracic Society; Denver, CO, USA.
17) **Role of TRAIL in rhinovirus infection and exacerbation of asthma.**

**Hatchwell, L.**, Collison, A., Pereira de Siqueira, A., Wark, PA., Bartlett, NW., Johnston, SL., Foster, PS., Mattes, J.

The 7th Symposium of the International Eosinophil Society:
Québec City, Québec, Canada.

18) **A novel E3 ubiquitin ligase links rhinovirus infection to exacerbation of asthma.**

**Hatchwell, L.**, Collison, A., Pereira de Siqueira, A., Foster, PS., Verrills, N., Don, A., Wark, PA., Mattes, J.

Thoracic Society of Australia and New Zealand Annual Scientific Meeting: Perth, WA, Australia.

2010 19) **Regulation and modulation of protein phosphatases in House Dust Mite-induced allergic airways disease.**

**Hatchwell, L.**, Collison, A., Verrills, N., Don, A., Foster, PS., Mattes, J.

The 40th Annual Scientific Meeting of the Australasian Society for Immunology: Perth, WA, Australia.

20) **The development of House Dust Mite-induced allergic airways disease is dependent on a novel ubiquitin ligase.**

Collison, A., **Hatchwell, L.**, Pereira de Siqueira, A., Verrills, N., Foster, PS., Mattes, J.

The 40th Annual Scientific Meeting of the Australasian Society for Immunology: Perth, WA, Australia.
Appendix C – List of abbreviations & figures

Acronyms and abbreviations

AAD: Allergic airways disease
AEC: Airway epithelial cell
AHR: Airways hyperreactivity
BAL: Bronchoalveolar
BEC: Bronchial epithelial cell
CCL: Chemokine (C-C motif) ligand
CGRP: Calcitonin gene-related peptide
COPD: Chronic obstructive pulmonary disease
CXCL: Chemokine (C-X-C motif) ligand
DAMP: Damage-associated molecular pattern
DC: Dendritic cell
EoE: Eosinophilic esophagitis
FEV₁: Forced expiratory volume in 1 second
FcεRI: Type 1 high affinity IgE receptor
FVC: Forced vital capacity
GINA: Global Initiative of Asthma
GM-CSF: Granulocyte macrophage colony-stimulating factor
GR: Glucocorticoid receptor
HDM: House dust mite
ICAM-1: Intracellular adhesion molecule 1
ICS: Inhaled corticosteroids
IFN: Interferon
Ig: Immunoglobulin
IKK: Inhibitor of NFκB (IκB) kinase
IL: Interleukin
ILC2: Type-2 innate lymphoid cell
IRF: Interferon regulatory factor
ISG: Interferon stimulated gene
LABA: Long-acting β2 agonist
LDLR: Low density lipoprotein receptor
LPS: Lipopolysaccharide
mAb: Monoclonal antibody
MAPK: Mitogen-activated protein kinase
MDA-5: Melanoma differentiation-associated gene 5
mDC: Myloid dendritic cell
MID1: Midline-1
MHC: Major histocompatibility complex
RIG-I: Retinoic acid-inducible gene I
RT-PCR: Reverse transcription - polymerase chain reaction
PAMP: Pathogen-associated molecular pattern
PBMC: Peripheral blood mononuclear cell
pDC: Plasmacytoid dendritic cell
PP2A: Protein phosphatase 2A
PPR: Pattern recognition receptor
NFκB: Nuclear factor kappa B
RSV: Respiratory syncytial virus
RV: Rhinovirus
SABA: Short-acting β2 agonist
siRNA: Short-interfering RNA
STAT6: Signal transducer and activator of transcription 6
TCR: T cell receptor
T\(_H\): T helper cell
TLR: Toll-like receptor
TSLP: Thymic stromal lymphopoietin
TRAIL: TNF-related apoptosis-inducing ligand
Treg: T regulatory cell
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184. Kobayashi, Y., Mercado, N., Miller-Larsson, A., Barnes, P. & Ito, K. Increased corticosteroid sensitivity by a long acting beta(2) agonist formoterol via beta(2)


