MicroRNA-21 drives severe, steroid-insensitive experimental asthma by amplifying PI3K-mediated suppression of HDAC2

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Respiratory infections drive severe, steroid-insensitive AAD through a miR-21/PI3K/HDAC2 axis.

Legend

Ova: Ovalbumin
AAD: Allergic airway disease
Cmu: Chlamydia muridarum
Hinf: Non-typeable Haemophilus influenzae
Flu: A/PR/8/34 H1N1 mouse-adapted influenza
RSV: Respiratory syncytial virus
MicroRNA-21 drives severe, steroid-insensitive experimental asthma by amplifying PI3K-mediated suppression of HDAC2

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ABSTRACT

Background: Severe, steroid-insensitive (SSI) asthma is a substantial clinical problem. Effective treatments are urgently required, however, their development is hampered by a lack of understanding of the mechanisms of disease pathogenesis. Steroid-insensitive asthma is associated with respiratory infections, and non-eosinophilic endotypes, including neutrophilic forms of disease. However, steroid-insensitive patients with eosinophil-enriched inflammation have also been described. The mechanisms that underpin infection-induced, SSI asthma may be elucidated using mouse models of disease.

Objective: To develop representative mouse models of SSI asthma, and to use them to identify pathogenic mechanisms and investigate new treatment approaches.

Methods: Novel mouse models of *Chlamydia, Haemophilus influenzae, influenza* and respiratory syncytial virus respiratory infections and ovalbumin-induced, severe, steroid-insensitive allergic airway disease (SSIAAD) in BALB/c mice were developed, and interrogated.

Results: Infection induced increases in the levels of microRNA (miR)-21 expression in the lung during SSIAAD, whereas the miR-21 target phosphatase and tensin homologue (PTEN) was reduced. This was associated with an increase in pAkt, an indicator of phosphoinositide-3-kinase (PI3K) activity, and decreased nuclear HDAC2 levels. Treatment with a specific miR-21 inhibitor (antagomir, Ant-21) increased PTEN. Treatment with Ant-21, or the pan-PI3K inhibitor LY294002, reduced PI3K activity and restored HDAC2 levels. This led to the suppression of airway hyper-responsiveness (AHR) and restored steroid sensitivity to AAD. These observations were replicated with SSIAAD associated with four different pathogens.

Conclusion: We identify a previously unrecognized role for a miR-21/PI3K/HDAC2 axis in SSIAAD. Our data highlights miR-21 as a novel therapeutic target for the treatment of this form of asthma.
Abstract word count: 250

Clinical Implications: Respiratory infections drive SSIAAD through a miR-21/PI3K/HDAC2 axis. Targeting miR-21 or PI3K suppresses disease and restores steroid-sensitivity, indicating the therapeutic potential of miR-21/PI3K-targeted therapies in combination with steroids in SSI asthma.

Capsule Summary: Respiratory infections drive severe, neutrophilic, steroid-insensitive AAD through a miR-21/PI3K/HDAC2 axis. Inhibition of increased miR-21 or PI3K responses suppresses disease and restores steroid sensitivity, identifying a role for this axis in infection-associated, SSI asthma.

Key words: severe asthma; corticosteroids; airway hyper-responsiveness; miR-21; PI3-kinase; HDAC2; Chlamydia; Haemophilus influenzae; influenza; respiratory syncytial virus

Abbreviations used:

AAD: Allergic airway disease
AHR: Airway hyper-responsiveness
Ant-21: miR-21-specific antagonim
BALF: Bronchoalveolar lavage fluid
Cmu: Chlamydia muridarum
COPD: Chronic obstructive pulmonary disease
Cxcl/CXCL: Chemokine (C-X-C motif) ligand
DEX: Dexamethasone
DMSO: Dimethyl sulfoxide
Flu: A/PR/8/34 H1N1 mouse-adapted influenza
GR: Glucocorticoid receptor
Hdac/HDAC: Histone deacetylase
Hinf: Non-typeable *Haemophilus influenzae* (NTHi-289)
Ifng/IFNγ: Interferon gamma
Il/IL: Interleukin
ISH: *In situ* hybridization
i.n.: Intranasally
i.p.: Intraperitoneally
i.t.: Intratracheally
LY29: LY294002
MCh: Methacholine
miRNA: MicroRNA
Nr3c1/NR3C1: Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
Ova: Ovalbumin
pAKT: Phosphorylated AKT
PBS: Phosphate-buffered saline
PI3K: Phosphoinositide-3-kinase
Pten/PTEN: Phosphatase and tensin homolog
qPCR: Quantitative PCR
RSV: Respiratory syncytial virus
Scram: Scrambled control antagomir
SPG: Sucrose phosphate glutamate buffer
SSIAAD: Severe, steroid-insensitive allergic airway disease
Stat/STAT: Signal transducer and activator of transcription
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<th>No.</th>
<th>Acronym</th>
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<tr>
<td>101</td>
<td>TBP</td>
<td>TATA binding protein</td>
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<tr>
<td>102</td>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T-helper type lymphocyte</td>
</tr>
<tr>
<td>103</td>
<td>Tgfβ/TGFβ</td>
<td>Transforming growth factor, beta 1</td>
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<tr>
<td>104</td>
<td>Tnf/TNF</td>
<td>Tumor necrosis factor</td>
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<td>105</td>
<td>UV</td>
<td>Ultra-violet</td>
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Corticosteroids are broad-acting anti-inflammatory agents and the mainstay treatments for asthma.\textsuperscript{1} However, 5-10\% of asthmatics do not respond to steroid treatment. These patients typically have more severe disease, account for $\geq 50\%$ of asthma-associated healthcare costs, and urgently require effective therapies.\textsuperscript{2,3}

Asthma is an inflammatory condition of the airways archetypally mediated by aberrant T-helper type (T\textsubscript{H})2 lymphocyte responses\textsuperscript{4} that drive eosinophilic airway inflammation, mucus hypersecretion, and airway hyper-responsiveness (AHR).\textsuperscript{5} Recent clinical evidence shows that asthma is a heterogeneous condition. Indeed, increased T\textsubscript{H}1- and/or T\textsubscript{H}17-responses,\textsuperscript{6} and non-eosinophilic, predominantly neutrophilic airway inflammation prevail in moderate-to-severe asthma.\textsuperscript{7} Severe asthma is often steroid-insensitive (SSI asthma) and is associated with non-eosinophilic endotypes of disease, particularly neutrophilic asthma.\textsuperscript{8,9} However, persistent eosinophilic airway inflammation in steroid-insensitive asthma patients has been described,\textsuperscript{10,11} suggesting that SSI asthma may also be associated with this type of inflammation.

The anti-inflammatory effects of corticosteroids are largely mediated through the activation of the nuclear receptor subfamily 3, group C, member 1 (NR3C1; commonly termed the cytosolic glucocorticoid receptor [GR])\textsuperscript{12} and the recruitment of histone deacetylase (HDAC)2 that deacetylates histones and suppresses gene transcription.\textsuperscript{13} Reduced HDAC2 activity is associated with both SSI asthma and chronic obstructive pulmonary disease (COPD).\textsuperscript{14-16} Steroid insensitivity and reduced HDAC2 activity are both linked to aberrant phosphoinositide-3-kinase (PI3K)-activity. Pharmacologic and genetic interruption of PI3K function reinstated steroid-sensitivity and HDAC2 activity in experimental COPD.\textsuperscript{16,17} Thus, exaggerated PI3K activity may promote steroid-insensitivity by reducing HDAC2 responses.
Substantial clinical and experimental evidence links respiratory bacterial and viral infections, with SSI asthma. *Chlamydia*-associated asthma has increased airway neutrophils that predict the presence of the bacterium, and is resistant to steroid treatment.\textsuperscript{18-21} *Haemophilus influenzae* is commonly isolated from the airways of SSI asthma patients, and its presence correlates with more severe airflow obstruction, neutrophilic inflammation and steroid insensitivity.\textsuperscript{22-24} These infections induce neutrophilic, $T_H1$ and/or $T_H17$ responses in experimental asthma models (allergic airway disease [AAD]) replicating the effects in patients.\textsuperscript{25-28} Respiratory infections with influenza and respiratory syncytial virus (RSV) induce asthma exacerbations that are steroid insensitive.\textsuperscript{29, 30} PI3K activity promotes $T_H17$ immune responses and facilitates the entry of these pathogens into host cells, and promotes their replication that further activates PI3K.\textsuperscript{31-35}

Several microRNAs (miRNAs) are implicated in asthma pathogenesis, and miR-21 is important in murine AAD.\textsuperscript{36-39} miR-21-deficient mice exhibit reduced eosinophilic inflammation and interleukin (IL)-4 levels with a concomitant increase in interferon (IFN)$\gamma$ during ovalbumin (Ova)-induced AAD.\textsuperscript{40} miR-21 can also down-regulate the expression of phosphatase and tensin homolog (PTEN), which antagonizes PI3K activity.\textsuperscript{41-43}

We assessed the roles of miR-21 and PI3K in the pathogenesis of SSIAAD. We first developed novel mouse models of *Chlamydia, Haemophilus influenzae,* influenza and RSV infection-induced SSIAAD that recapitulate the hallmark features of SSI asthma. We then interrogated them to show that infection-induced miR-21 promotes SSIAAD by reducing PTEN, amplifying PI3K-dependent activity and suppressing HDAC2. These effects were attenuated, and steroid-sensitivity restored, by inhibiting miR-21 and/or PI3K. Thus, we define a novel miR-21/PI3K/HDAC2 axis in a previously unrecognized pathogenic role, and identify miR-21 as a novel therapeutic target in SSI asthma.
METHODS

Murine model of established AAD, Dexamethasone treatment, Respiratory infections in established AAD, miRNA and PI3K inhibition, Airway inflammation, AHR, Quantification of mRNA and miRNA expression, miRNA in situ hybridization, Immunoblot analyses, and Statistics\textsuperscript{25, 31, 39, 44-52} are described in the Methods section, and Figs E1-E5 and Table E1 are in this article’s Online Repository at www.jacionline.org.

RESULTS

Chlamydia respiratory infection induces SSIAAD

Ova-induced AAD was established in BALB/c mice, which were then infected with \textit{C. muridarum} (Cmu; Fig E1). This is a natural mouse respiratory pathogen and the most appropriate \textit{Chlamydia} strain for studying host:pathogen relationships in mice.\textsuperscript{28, 47, 53-57} Infection and inflammation peak at d10 and 15, respectively.\textsuperscript{47, 53} Disease features in Ova-induced AAD wane over time (unpublished data), therefore to assess the impact of infection we recapitulated the asthma phenotype with two additional Ova challenges 19-20d post-infection (d33-34 of the model). Hallmark AAD features were assessed on d35 with or without corticosteroid (dexamethasone; DEX) treatment.

In the absence of infection, AAD (Ova/SPG) was characterized by predominantly eosinophilic airway inflammation and AHR compared to non-allergic (Sal/SPG) controls (Fig 1, \textit{A-G}). Resolved \textit{Chlamydia} infection suppressed eosinophilic, and increased neutrophilic, airway inflammation in AAD and had no effect on the magnitude of AHR (Ova/Cmu vs Ova/SPG; Fig 1, \textit{F and G}). Resolved infection alone (Sal/Cmu) did not induce persistent airway inflammation or AHR compared to Sal/SPG (Fig 1, \textit{A-G}), suggesting that \textit{Chlamydia}-induced, neutrophilic AAD results from a change in AAD phenotype rather than having additive effects on inflammation and AHR. DEX treatment (Fig E1) inhibited airway
inflammation and AHR in AAD (Ova/SPG/DEX vs Ova/SPG; Fig 1, A-G) to baseline levels observed in Sal/SPG mice. By contrast treatment did not suppress Chlamydia-induced, AAD (Ova/Cmu/DEX) where neutrophilic inflammation and AHR were completely steroid insensitive.

Chlamydia-induced, SSIAAD was associated with increased mRNA expression of TH1-(toll-like receptor [Tlr]2, signal transducer and activator of transcription [Stat]1, interferon gamma [Ifng], chemokine [C-X-C motif] ligand [Cxcl]9 and 10 and tumor necrosis factor [Tnfi]; Fig 1, H) and TH17- (interleukin [Il17], Il6, transforming growth factor, beta 1 [Tgfb], Il1b; Fig 1, H), but reduced expression of TH2- (Il5, Il13; data not shown) associated factors in the lungs compared to Ova/SPG controls. Thus, infection promoted a switch from TH2-dominated, eosinophilic inflammation to TH1/TH17-dominated, neutrophilic responses in AAD. Thus, Chlamydia respiratory infection induces TH1/TH17-dominated, neutrophilic SSIAAD that closely resembles neutrophilic SSI asthma in humans (Fig 1, I).

Chlamydia infection induces a persistent increase in miR-21 expression in SSIAAD

Since miR-21 is implicated in the pathogenesis of asthma we assessed its expression in Chlamydia-induced, SSIAAD (Ova/Cmu). Lung miR-21 expression was increased on d35 compared to Sal/SPG controls (Fig 1, J). However, all allergic and/or infected groups had increased expression with a trend toward higher levels in infected groups. Importantly, all infected groups, regardless of allergic status, exhibited increased miR-21 expression on d35 compared to sham-infected, non-allergic (Sal/SPG) controls. Thus, Chlamydia infection substantially and chronically increases miR-21 expression even in the absence of allergic responses. Interestingly, the increased expression of miR-21 in several TH1 and TH17-associated factors (i.e. Stal, Ifng, Tnfi, Il17 and Il6) identified in Fig 1,
H, compared to sham-infected (Sal/SPG) controls (Fig E2, A). This suggests that miR-21 is an infection-induced factor that may potently affect the lung environment prior to allergen challenge in Chlamydia-infected groups and play a role in the induction of SSIAAD. We also conducted miR-21 qPCR analyses on lung tissue from C57BL/6 mice that were subjected to OVA-induced AAD or infected with Cmu. We show that miR-21 expression is increased by AAD and infection in a similar manner to that observed in BALB/c mice (Fig E2, B & C). These data show that the induction of miR-21 by AAD and infection is not mouse strain-specific.

We then showed that miR-21 expression was widespread occurring in airway epithelial, endothelial and infiltrating immune cells\(^9, 58\) (Fig E3). DEX had no effect on miR-21 expression in allergic groups (Ova/SPG/DEX and Ova/Cmu/DEX), indicating that its expression is steroid insensitive irrespective of the presence of infection.

**Chlamydia infection primes steroid-insensitive responses in AAD that is associated with increased miR-21 expression**

To examine this potential, we next examined the Chlamydia infection-induced lung environment on d32 immediately before DEX treatment and Ova re-challenge. Chlamydia-infected, allergic (Ova/Cmu) mice exhibited increased miR-21 expression (Fig 2, A) with concurrent decreases in the expression of Pten, Nr3c1, and Hdac2 (Fig 2, B-D) compared to Ova/SPG groups. These factors are involved in PI3K-dependent and steroid-mediated responses. To determine whether these Chlamydia-induced effects were associated with increased PI3K function we also assessed the levels of pAKT, a surrogate marker of PI3K-dependent activity. Ova/Cmu mice had increased pAKT, and reduced HDAC2, protein levels in their lung nuclear fractions (Fig 2, E and F). In contrast these mice had decreased pAKT (Fig E4) levels in lung cytoplasmic fractions suggesting that infection resulted in increased
nuclear translocation of pAKT. Collectively, these data show that *Chlamydia*-induced miR-21 expression at the time of steroid treatment in SSIAAD is associated with attenuated *Nr3c1* expression, increased PI3K responses and reduced HDAC2 levels.

**miR-21 increases pAKT and reduces HDAC2 levels to induce SSIAAD**

We next assessed the role and potential for therapeutic targeting of increased miR-21 expression in *Chlamydia*-induced, SSIAAD. Administration of miR-21-specific inhibitor (antagomir; Ant-21) on d32 (Fig E1) ablated lung miR-21 expression on d35 with or without steroid treatment (Ova/Cmu/Ant-21±DEX) compared to scrambled antagomir (Scram)-treated controls (Ova/Cmu/Scram±DEX; Fig 3, A). Again DEX had no statistically significant effects on miR-21 expression in the allergic groups (Ova/SPG/Scram/DEX and Ova/Cmu/Scram/DEX). Inhibition of miR-21 restored *Pten* and *Hdac2* (Fig 3, B and C), but not *Nr3c1* (the gene encoding the glucocorticoid receptor, data not shown), expression in SSIAAD (Ova/Cmu/Ant-21).

SSIAAD groups that were sham treated (Ova/Cmu/Scram) had increased pAKT, and reduced HDAC2, levels in lung nuclear fractions compared to Ova/SPG/Scram controls (Fig 3, D and E). Ant-21 suppressed pAKT, and restored HDAC2, protein levels in SSIAAD with or without steroid treatment (Ova/Cmu/Ant-21±DEX vs Ova/Cmu/Scram±DEX). Steroid treatment, without Ant-21, had no effects and did not suppress pAKT or increase HDAC2 levels. Thus, the inhibition of miR-21 in SSIAAD suppresses PI3K responses and restores HDAC2 levels independently of steroid treatment.

DEX again reduced airway inflammation and AHR in AAD (Ova/SPG/Scram/DEX vs Ova/SPG/Scram) but not in SSIAAD (Ova/Cmu/Scram/DEX; Fig 3, F-L). Ant-21 treatment, in the presence but not the absence of steroids, suppressed inflammation in SSIAAD (Ova/Cmu/Ant-21/DEX vs Ova/Cmu/Scram±DEX). Ant-21 also completely inhibited AHR
to baseline levels observed in Ova/SPG/Scram/DEX controls irrespective of the presence of steroids. These data show that infection-induced miR-21 expression promotes steroid-insensitive airway inflammation and inflammation- and DEX-independent AHR in SSIAAD.

We then assessed the role and potential for therapeutic targeting of miR-21 in steroid-sensitive AAD (Ova/SPG/Ant-21; Fig E5). Ant-21 treatment induced a close to statistically significant decrease in inflammation (p=0.067 difference for total leukocytes, significant reduction in eosinophils) and inhibited AHR to baseline levels observed in steroid-treated (Ova/SPG/Scram/DEX) controls.

These data demonstrate that *Chlamydia*-induced miR-21 expression plays important roles in increasing pAKT and reducing HDAC2 levels, which regulate the steroid sensitivity of airway inflammation and the induction of steroid-insensitive AHR.

**PI3K activity increases pAKT and reduces HDAC2 levels to induce SSIAAD**

We next examined the role of PI3K activity in *Chlamydia*-induced SSIAAD. Administration of the pan-PI3K inhibitor, LY294002, increased *Hdac2* mRNA expression in SSIAAD (Ova/Cmu/LY29 vs Ova/Cmu/DMSO; Fig 4, A). LY294002 also suppressed pAKT, and restored HDAC2, protein levels in lung nuclear fractions in SSIAAD with or without steroids (Ova/Cmu/LY29±DEX) compared to sham-treated controls (Ova/Cmu/DMSO±DEX; Fig 4, B and C). LY294002 treatment, in the presence of steroids, suppressed inflammation in SSIAAD (Fig 4, D-H). However, this suppression did not occur in the absence of steroids where inflammation was increased (Ova/Cmu/LY29 vs Ova/Cmu/DMSO). LY294002 alone, like with Ant-21, suppressed AHR in SSIAAD with greater effects in combination with steroids where responsiveness was inhibited to baseline levels observed in steroid-treated, sham-infected AAD (Ova/SPG/DMSO/DEX).
Thus, *Chlamydia*-induced, PI3K activity also plays important roles in increasing pAKT and reducing HDAC2 levels in the regulation of steroid sensitivity of airway inflammation and the induction of steroid-insensitive AHR in SSIAAD. Taken together our data demonstrate that *Chlamydia* respiratory infection drives a miR-21-dependent, PI3K-mediated axis that induces SSIAAD.

**Inhibition of miR-21 suppresses hallmark features of *Haemophilus*-induced, SSIAAD**

To assess the broader applicability of our findings to SSI asthma induced by other bacterial infections, we developed a novel model of *Haemophilus influenzae*-induced, SSIAAD and examined the role and potential of therapeutic targeting of miR-21 (Fig E1). Like with *Chlamydia*, *Haemophilus* infection induced the key features of neutrophilic, SSIAAD, with increased neutrophilic airway inflammation and AHR that were steroid insensitive (Fig 5). Ant-21 treatment, in the presence of steroids, suppressed inflammation in *Haemophilus*-induced, SSIAAD (Ova/Hinf/Ant-21/DEX vs Ova/Hinf/Scram±DEX). Interestingly, unlike with *Chlamydia*, Ant-21 also suppressed inflammation in the absence of steroids (Ova/Hinf/Ant-21 vs Ova/Hinf/Scram±DEX). Again Ant-21 completely inhibited AHR in the presence and absence of steroids to baseline levels observed in steroid-treated, sham-infected AAD (Ova/PBS/Scram/DEX). These data demonstrate that the key features of both *Chlamydia-* and *Haemophilus*-induced, SSIAAD are induced by a miR-21-dependent mechanism.

**Inhibition of miR-21 suppresses hallmark features of influenza- and RSV-induced, SSIAAD**

We next investigated the wider applicability of our observations to SSI asthma induced by viral respiratory infections. We developed novel models of influenza- and RSV-
induced, SSIAAD and examined the role and potential of therapeutic targeting of miR-21 (Fig E1). Unlike with bacteria, both influenza and RSV infections had no effect on the numbers of total or individual leukocyte cell types in the airways in AAD (Ova/Flu/Scram vs Ova/Media/Scram, and Ova/RSV/Scram vs Ova/UV-RSV/Scram; Fig 6 and 7). Eosinophilic inflammation in influenza- and RSV-induced AAD was partially, or completely steroid-insensitive, respectively. AHR in virus infection-induced AAD was also steroid-insensitive. Like with bacteria, Ant-21 treatment, in the presence of steroids, suppressed inflammation in virus infection-induced SSIAAD (Ova/Flu/Ant-21/DEX vs Ova/Flu/Scram, and Ova/RSV/Ant-21/DEX vs Ova/RSV/Scram±DEX). Similarly, Ant-21 completely inhibited AHR in virus infection-induced SSIAAD in the presence or absence of steroids to baseline levels observed in steroid-treated, sham-infected AAD (Ova/Media/Scram/DEX and Ova/UV-RSV/Scram/DEX).

Collectively, our data demonstrate that miR-21 and a miR-21/PI3K/HDAC2 axis play important roles in the induction of steroid-insensitive airway inflammation and AHR in bacteria (*Chlamydia* and *Haemophilus*) and virus (influenza and RSV) infection–induced SSIAAD.

**DISCUSSION**

We developed novel experimental models of SSI asthma that are driven by bacterial (*Chlamydia* and *Haemophilus*) and viral (influenza and RSV) respiratory infections. These models recapitulate the hallmark features of this form of human asthma including exaggerated Th1/Th17 responses and steroid-insensitive airway inflammation and AHR. By interrogating our models and using an antagonir that specifically depletes miR-21 and the pan-PI3K inhibitor, LY294002, we demonstrate that infection-induced miR-21 expression promotes
PI3K-mediated phosphorylation and nuclear translocation of pAKT that suppresses HDAC2 levels and leads to steroid-insensitivity (Fig 8).

We previously showed that an ongoing Chlamydia respiratory infection during systemic sensitization to Ova leads to exaggerated T\(_h1\) (Ifng)/T\(_h17\) (Il17) responses and neutrophilic inflammation in AAD.\(^{28}\) In SSI asthma we propose that it is infection in patients with established asthma that drives the development of this form of disease. Here, we advance our previous studies\(^{59}\) by developing a model that more accurately reflects the human scenario. We established AAD in mice and then induced a Chlamydia respiratory infection. AAD wanes over 20d (unpublished observations), and so to test the impact of a resolved infection on disease we recapitulated the AAD phenotype with a second set of Ova challenges. This is representative of asthmatics that are exposed to respiratory infection and allergens and is reflective of what occurs in the community. We show that Chlamydia infection-induced AAD is characterized by exaggerated expression of a range of T\(_h1\)-(Tlr2, Stat1, Ifng, Cxcl9 and Il10, Tnf) and T\(_h17\)-(Il17, Il6, Tgf\(\beta\), Il1b) associated factors in the lung that are also increased in severe, neutrophilic asthma (Fig 1, I).\(^6\) Importantly, inflammation and AHR are steroid-insensitive in this model indicating that infection drives pathogenic processes that are not suppressed by anti-inflammatory steroid treatment. These data extend our understanding of clinical studies that associate respiratory infections with non-eosinophilic forms of asthma that are steroid insensitive. Indeed, substantial clinical evidence links Chlamydia respiratory infection in asthma with increased neutrophil numbers in the lungs during exacerbations and steroid insensitivity.\(^{18-21}\)

Several different mechanisms have been implicated in the pathogenesis of SSI asthma, including altered immune responses, increased activity of transcription factors, and defective GR function.\(^3, 60-62\) However, notably many patients with SSI asthma have normal nuclear translocation of GR and no deficit in GR:glucocorticoid response element binding affinity.\(^{63}\)
This indicates that steroid insensitivity in asthma can be driven by mechanisms outside of the canonical steroid-response pathway. Thus, targeting specific factors that control multifunctional pathways may be the most effective therapeutic approach.

miRNAs can have potent effects on immunity and increasing evidence shows that they have pathogenic roles in asthma. miR-21 is highly induced in inflamed lungs and can promote eosinophilic inflammation and T_H2 responses, whilst suppressing T_H1 immunity through the disruption of IL-12p35. Consequently, miR-21 studies in asthma have focused on its role in immune polarization during allergic sensitization. In this study, we demonstrate a previously unrecognized role for miR-21 in steroid insensitivity. miR-21 expression was increased in both steroid-sensitive and SSIAAD. However, sham-infected, allergic mice only exhibited increased miR-21 expression after the recapitulation of AAD. This suggests that its up-regulation in steroid-sensitive AAD is a transient phenomenon acutely induced by the allergic inflammatory response. In contrast, infected, allergic mice had persistently increased miR-21 expression on d32 immediately before steroid treatment and Ova re-challenge and when Chlamydia-induced inflammation has subsided to baseline levels. Significantly, this effect was accompanied by concomitant reductions in the mRNA expression of Pten, Nr3c1 and Hdac2. miR-21 has been shown to directly inhibit PTEN in both human and murine cells, which was shown using PTEN reporter luciferase activity assays and inhibitors (human and mouse) and mimics (mouse) of miR-21. Furthermore, under normal conditions, PTEN antagonizes PI3K activity by catalyzing the dephosphorylation of phosphatidylinositol 3,4,5-bisphosphate (PIP3) into phosphatidylinositol 4,5-bisphosphate (PIP2), which inhibits the recruitment and activation (by phosphorylation) of Akt. Thus, we hypothesized that infection-induced miR-21 expression drives steroid-insensitivity in AAD by suppressing PTEN, thereby potentiating PI3K activity (Fig 8).
Several studies link PI3K activity with inflammation and AHR in AAD, and one showed that rhinovirus infection induced PI3K-dependent neutrophilic airway inflammation.\textsuperscript{70} Here we show that \textit{Chlamydia} infection increased levels of nuclear pAKT, a well-established indicator of PI3K activity.\textsuperscript{70} This effect was associated with decreased levels of nuclear HDAC2. Our findings are consistent with studies showing that PI3K-mediated reduction in HDAC2 expression and activity promotes steroid insensitivity.\textsuperscript{2, 16, 17} Non-selective inhibition of PI3K activity with LY294002 restored HDAC2 activity and steroid sensitivity in experimental COPD. Furthermore, smoke-exposed PI3K\(\delta\) dead knock-in transgenic mice have reduced tyrosine nitration of HDAC2 with no deficit in steroid sensitivity.\textsuperscript{16, 17} Thus, infection-induced miR-21 expression may disrupt PTEN activity and amplify PI3K activity, which mediates the phosphorylation and nuclear translocation of AKT, resulting in reduced HDAC2 levels and steroid insensitivity. To our knowledge our study is the first to identify the axis encompassing the miR-21-dependent, PI3K-mediated suppression of HDAC2 in the pathogenesis of steroid insensitivity, and miR-21 as a therapeutic target whose activity can be attenuated \textit{in vivo} with specific inhibitors to reverse its effects.

We used two approaches to investigate the role and potential for therapeutic targeting of the miR-21-dependent, PI3K-mediated axis in SSIAAD. First, we inhibited miR-21 \textit{in vivo} with miR-21-specific antagomir treatment. This approach has been shown to specifically and potently reduce the levels of targeted miRNAs.\textsuperscript{37, 39} Treatment inhibited \textit{Chlamydia}-induced miR-21 expression, restored \textit{Pten}, reduced nuclear pAKT and increased \textit{Hdac2}/HDAC2 mRNA expression and protein levels in SSIAAD. Ant-21 suppressed steroid-insensitive airway inflammation when co-administered with steroids, demonstrating that targeted inhibition of miR-21 restored steroid sensitivity. Interestingly, AHR was attenuated by antagomir treatment alone in both steroid-insensitive and -sensitive AAD suggesting that miR-21 is an important mediator of the AHR in AAD. However, \textit{Chlamydia}-infected, non-
allergic (Sal/Cmu) mice have increased miR-21 expression in the lungs but do not have AHR. This suggests that increased miR-21 expression does not induce AHR in the absence of AAD. Elucidating the miR-21-dependent mechanism of action that leads to AHR in AAD warrants further investigation and will be the focus of future studies. In contrast, antagonir treatment alone had no effect on inflammation in SSIAAD. Together, our findings suggest that steroid-insensitive airway inflammation and AHR have different etiologies but require the overexpression of miR-21 to maintain steroid insensitivity. Our findings are consistent with several studies that show that treatment with inhaled steroids does not cause equivalent reductions in airway inflammation and AHR. It is now well established that there is a disconnect between inflammation and AHR in asthma and that they are regulated by different processes. Indeed, in a study by Crimi and colleagues, no correlation was shown between the number of inflammatory cells present in sputum, BAL or bronchial biopsies and the level of AHR in patients with chronic asthma. This study, as well as others, postulate that other non-canonical factors, such as airway remodeling and autonomic dysfunction that leads to persistently constricted airways may drive the inter-individual variation in AHR in patients with asthma. Our studies show that inhibiting miR-21 alone does not affect inflammation but suppresses AHR. However, the combination of miR-21 inhibition and DEX treatment suppresses both of these features. This suggests that these two interventions are targeting the disconnected processes that drive inflammation and AHR in SSIAAD.

Characterization of the pathways that underpin miR-21-induced AHR independently of inflammation is not within the scope of the current study but warrants future investigation.

To substantiate the existence of a pathogenic miR-21/PI3K axis, we then blocked PI3K activity in vivo with the pan-PI3K inhibitor LY294002, and assessed the impact on SSIAAD. Inhibition reduced nuclear pAKT back to sham-infected levels similar to Ant-21 treatment. Steroid treatment alone had no effect on nuclear pAKT levels, indicating that PI3K
activity is steroid insensitive. LY294002 also restored lung \textit{Hdac2}/HDAC2 mRNA expression and nuclear protein levels, and restored steroid sensitivity in a similar manner to Ant-21. The comparable effects of Ant-21 and LY294002 treatments suggest that infection initiates and maintains the activation of a pathogenic signaling axis comprised of both miR-21 and PI3K, which suppresses HDAC2 that leads to the induction of SSIAAD. Whilst miR-21 has many potential targets in the lung other than PTEN that could contribute to the induction of AHR in AAD in a non-PI3K-dependent manner, we show that both Ant-21 and LY294002 treatments suppress AHR in the absence of DEX, which suggests that miR-21 may drive AHR in AAD through a PI3K-mediated pathway.

Whilst the current study demonstrates a novel pathogenic miR-21-dependent, PI3K-mediated pathway in the pathogenesis of SSIAAD, it does not identify specific cell types that express miR-21 that are important for the regulation of the miR-21/PTEN/PI3K/HDAC2 pathway, and thus glucocorticoid insensitivity. Our \textit{in situ} hybridization analyses show that miR-21 is expressed in several cell types within the lung during SSIAAD (Fig E3). Thus, it is likely that multiple cell types are mediating these effects. Elucidating the relative contributions of individual cell types involved would further our understanding of how this pathway generates the cardinal features of SSIAAD. However, this is a major undertaking that will form the basis for future studies. In addition to the miR-21-dependent responses that are described in the current study, miR-21 and/or PTEN/PI3K responses have also been implicated in other disease processes in the lung, including pro-fibrotic responses in idiopathic pulmonary fibrosis, enhanced accumulation of myofibroblasts, and allergen-induced bronchial inflammation and AHR.\textsuperscript{76-78} Thus, especially since miR-21 is expressed in multiple structural cells of the lung, it is likely that the miR-21/PI3K/HDAC2 axis is involved in these processes and requires further study.
H. influenzae respiratory infection is commonly associated with neutrophilic asthma that is steroid insensitive. To assess the widespread applicability for targeting miR-21 in SSI asthma, we examined the effects of its inhibition in Haemophilus-induced SSIAAD. We previously showed that H. influenzae infection induces T\(_{H17}\)-dominant immunity that drives neutrophilic, rather than eosinophilic, inflammatory responses in AAD, and that inflammation and AHR in this model are steroid-insensitive. Thus, we hypothesized that Haemophilus, like Chlamydia, induces SSIAAD through a miR-21-dependent mechanism.

We first developed a novel and refined model of Haemophilus-induced SSIAAD, where infection is induced in established AAD. We showed that Ant-21 treatment also restored steroid sensitivity to inflammation and AHR in this model. Again treatment suppressed AHR in the absence of steroids.

Since influenza virus and RSV respiratory infections have also been linked to SSI asthma, we developed novel models of influenza- and RSV-induced SSIAAD and assessed the wider applicability for targeting miR-21. Unlike bacteria-induced SSIAAD, both influenza- and RSV-induced AAD were characterized by steroid-insensitive eosinophilic airway inflammation and AHR. Some studies have shown that steroid-insensitive asthma may also be associated with persistent eosinophilic inflammation despite moderate-to-high dose steroid treatment. Furthermore, these viral infections enhance eosinophilic airway inflammation and T\(_{H2}\) immune responses in other murine models of allergic asthma. Ant-21 treatment, in the presence of steroids, suppressed viral infection-induced, steroid-insensitive eosinophilic inflammation. These data suggest that respiratory bacterial and viral infection-induced miR-21 primes for steroid-insensitive responses but has minimal influence over the chemoattraction of specific immune cell types. Similar to bacteria-induced SSIAAD, Ant-21 treatment alone suppressed AHR in influenza- and RSV-induced SSIAAD. These data
indicate that infection-induced miR-21 may also regulate inflammation- and steroid-independent pathways to induce steroid-insensitive AHR in AAD.

We also showed that Ant-21 treatment suppressed the key features of $T_{H2}$-mediated steroid-sensitive AAD, i.e. eosinophilic inflammation and AHR.

In summary, our study demonstrates for the first time that miR-21 promotes steroid-insensitive inflammation and AHR in respiratory infection-induced SSIAAD. We define the functional relevance of infection-induced activation, and maintenance, of a novel miR-21/PI3K/HDAC2 axis in steroid insensitivity. Our study indicates that the inhibition of miR-21 may have broad therapeutic relevance to respiratory infection-induced SSI asthma, and also steroid-sensitive, eosinophilic, asthma. This is more attractive than targeting PI3K pathways as inhibition of miR-21 is upstream and more specific, and may broadly affect steroid-insensitive as well as steroid-independent networks such as those that drive AHR that is independent of inflammation in the asthmatic lung.
REFERENCES


30


FIGURE LEGENDS

FIG 1. *Chlamydia* infection induces severe, steroid-insensitive (SSI), allergic airway disease (AAD) and increases miR-21 expression. (A) Total leukocyte, (B) eosinophil, (C) neutrophil, (D) macrophage, and (E) lymphocyte numbers in bronchoalveolar lavage fluid (BALF) on d35 (Fig E1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD +/- dexamethasone (DEX) treatment compared to non-allergic controls (Sal) (≥two experiments; n=4-10 per group). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) (F) to increasing doses of methacholine (MCh) and (G) 10 mg/mL of MCh (statistics at maximal dose from AHR curves [Fig 1, F]). (H) Lung mRNA expression of T_{H}1- and T_{H}17-associated factors. (I) Common features of SSI asthma and SSIAAD. Expression of miR-21 in lung tissue (J) (≥two experiments; n=4-6 per group). Data are mean±SEM. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

FIG 2. *Chlamydia* infection persistently increase in miR-21 expression and primes steroid-insensitive responses in severe, steroid-insensitive, allergic airway disease (SSIAAD). Lung mRNA expression of (A) miR-21, (B) *Pten*, (C) *Nr3c1*, and (D) *Hdac2* were assessed on d32 by qPCR in *Chlamydia* (Cmu) and sham (SPG)-infected, allergic mice (Fig E1) (one experiment; n=8 per group). Nuclear protein levels of (E) pAKT, AKT and pAKT:AKT ratio, and (F) HDAC2 determined by immunoblot (top panels) and densitometry (bottom panels) (≥two experiments; n=4 per group). Data are mean±SEM. **P<0.01; ***P<0.001; ****P<0.0001.

FIG 3. *Chlamydia*-induced miR-21 increases PI3K activity and decreases HDAC2 levels, and drives features of severe, steroid-insensitive, allergic airway disease (SSIAAD). Lung expression of (A) miR-21 determined by qPCR on d35 (Fig E1) in *Chlamydia* (Cmu) and
sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD +/- dexamethasone (DEX) +/- anti-miR-21 (Ant-21) or scrambled (Scram) antagonist (≥two experiments; n=4-5 per group). Effect of Ant-21 treatment on lung mRNA expression of (B) *Pten* and (C) *Hdac2* on d35 in allergic groups not treated with DEX (≥two experiments; n=4-5 per group). Nuclear protein levels of (D) pAKT, AKT and pAKT:AKT ratio, and (E) HDAC2 on d35 determined by immunoblot (top panels) and densitometry (bottom panels) (≥two experiments; n=5 per group). (F) Total leukocyte, (G) eosinophil, (H) neutrophil, (I) macrophage, and (J) lymphocyte numbers in bronchoalveolar lavage fluid (BALF) (≥two experiments; n=4-6 per group). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) (K) to increasing doses of methacholine (MCh), and (L) 10 mg/mL of MCh (statistics at maximal dose from AHR curves [Fig 3, K]) (≥three experiments; n=5-10 per group). Data are mean±SEM. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

**FIG 4.** Chlamydia-induced PI3K activity suppresses *Hdac2*/HDAC2 levels and drives cardinal features of severe, steroid-insensitive, allergic airway disease (SSIAAD). Lung mRNA expression of (A) *Hdac2* determined by qPCR on d35 (Fig E1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD treated with LY294002 (LY29) or vehicle (DMSO) (≥two experiments; n=4-5 per group). Nuclear protein levels of (B) pAKT, AKT and pAKT:AKT ratio, and (C) HDAC2 determined by immunoblot (top panels) and densitometry (bottom panels) (≥two experiments; n=5 per group). (D) Total leukocyte, (E) eosinophil, (F) neutrophil, (G) macrophage, and (H) lymphocyte numbers in bronchoalveolar lavage fluid (BALF) (≥two experiments; n=4-8 per group). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) (I) to increasing doses of methacholine (MCh), and (J) 10 mg/mL of MCh (statistics at maximal dose from AHR
curves [Fig 4, I] (≥two experiments; n=5-8 per group). Data are mean±SEM. *P<0.05;  
**P<0.01; ***P<0.001; ****P<0.0001.

**FIG 5.** Inhibition of miR-21 suppresses cardinal features of *Haemophilus influenzae* infection-induced, severe, steroid-insensitive, allergic airway disease (SSIAAD). (A) Total leukocyte, (B) eosinophil, (C) neutrophil, (D) macrophage, and (E) lymphocyte numbers in bronchoalveolar lavage fluid (BALF) on d35 (Fig E1) in *H. influenzae* (Hinf) and sham (PBS)-infected groups with ovalbumin (Ova)-induced AAD +/- dexamethasone (DEX) +/- anti-miR-21 (Ant-21) or scrambled (Scram) antagomir (one experiment; n=5-6 per group). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) (F) to increasing doses of methacholine (MCh), and (G) 10 mg/mL of MCh (statistics at maximal dose from AHR curves [Fig 5, F]) (one experiment; n=5-6 per group). Data are mean±SEM. *P<0.05;  
**P<0.01; ***P<0.001; ****P<0.0001.

**FIG 6.** Inhibition of miR-21 suppresses cardinal features of influenza virus infection-induced, severe, steroid-insensitive, allergic airway disease (SSIAAD). (A) Total leukocyte, (B) eosinophil, (C) neutrophil, (D) macrophage, and (E) lymphocyte numbers in bronchoalveolar lavage fluid (BALF) on d35 (Fig E1) in influenza (Flu) and sham (Media)-infected groups with ovalbumin (Ova)-induced AAD +/- dexamethasone (DEX) +/- anti-miR-21 (Ant-21) or scrambled (Scram) antagomir (one experiment; n=5-8 per group). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) (F) to increasing doses of methacholine (MCh), and (G) 10 mg/mL of MCh (statistics at maximal dose from AHR curves [Fig 6, F]) (one experiment; n=7-8 per group). Data are mean±SEM. *P<0.05;  
**P<0.01; ***P<0.001; ****P<0.0001.
FIG 7. Inhibition of miR-21 suppresses cardinal features of respiratory syncytial virus infection-induced, severe, steroid-insensitive, allergic airway disease (SSIAAD). (A) Total leukocyte, (B) eosinophil, (C) neutrophil, (D) macrophage, and (E) lymphocyte numbers in bronchoalveolar lavage fluid (BALF) on d35 (Fig E1) in respiratory syncytial virus (RSV) and sham (UV-inactivated RSV; UV-RSV)-infected groups with ovalbumin (Ova)-induced AAD +/- dexamethasone (DEX) +/- anti-miR-21 (Ant-21) or scrambled (Scram) antagonir (one experiment; n=3-6 per group). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) (F) to increasing doses of methacholine (MCh), and (G) 10 mg/mL of MCh (statistics at maximal dose from AHR curves [Fig 7, F]) (one experiment; n=5-6 per group). Data are mean±SEM. *P<0.05; **P<0.01; ****P<0.0001.

FIG 8. Mechanisms and potential treatment of severe, steroid-insensitive, asthma. Infection in allergic airway disease/asthma induces miR-21 that inhibits PTEN and promotes PI3K-mediated suppression of HDAC2 and steroid insensitivity. This pathway may be targeted therapeutically by inhibition of miR-21 and/or PI3K.
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1 LNA™-modified bases are preceded by a [+] symbol
A network diagram showing experimental protocols with timepoints and treatments:

- **d0**: Sal/Ova i.p.
- **d12, 13**: Ova i.n.
- **d33, 34**: Ova i.n.
- **d32, 33, 34**: DEX (i.n.; 2 mg/kg)
- **d35**: Endpoints

### Treatments:

- **spg/cmu** (i.n.; 100 IFU; d14)
- **PBS/Hif** (i.t.; 2x10⁶ CFU; d14)
- **Media/Flu** (i.n.; 7.5 PFU; d14)
- **UV/RSV/RSV** (i.n.; 5x10⁵ PFU; d14)

### Additional Treatments:

- **Scrata/Ant-21** (i.n.; 50 μg; d32)
- **DMSO/LY294002** (i.n.; 2 mg/kg; d32, 33, 34)
A

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MicroRNA-21 drives severe, steroid-insensitive experimental asthma by amplifying PI3K-mediated suppression of HDAC2

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* These authors contributed equally to this work

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SUPPLEMENTARY METHODS

Ethics statement

This study was performed in accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committee of The University of Newcastle, Australia.

Murine model of established AAD

Female wild-type BALB/c mice, 6-8 weeks old, were sensitized to Ova (50µg, intraperitoneal [i.p.] injection, Sigma-Aldrich, Sydney, Australia) in Rehydragel® (1mg, Reheis, Berkeley Heights, New Jersey, USA) in sterile saline (200µl) under isoflurane anesthesia. They were subsequently challenged intranasally (i.n.) with Ova (10µg/50µL sterile saline) on d12-13 to induce AAD and again on d33-34 to recapitulate AAD. Sham-sensitized controls received saline sensitization with Rehydragel® and the subsequent Ova challenges. AAD was characterized on d32 or d35.\textsuperscript{E1-8} Some AAD experiments were conducted on female wild-type C57BL/6 mice, 6-8 weeks old in order to show that miR-21 expression is not strain-specific.

Treatment with DEX in AAD

Some groups were treated with DEX (2mg/kg, Sigma-Aldrich) i.n. on days 32-34.\textsuperscript{E1, 9, 10}

Chlamydia, Haemophilus, influenza and respiratory syncytial virus respiratory infection-induced, SSIAAD

Mice with established AAD were inoculated under isoflurane anesthesia on d14 i.n. with C. muridarum (ATCC VR-123 [Cmu], 100 inclusion-forming units [ifu], in 30µL sucrose phosphate glutamate [SPG] buffer), intratracheally (i.t.) with non-typeable H. influenzae
(NTHi-289 [Hinf], 2x10^6 colony forming units, in 30µL phosphate-buffered saline [PBS]), or i.n. with influenza (A/PR/8/34 H1N1 mouse-adapted [Flu], 7.5 plaque forming units [PFU], in 50µL UltraMDCK media), or respiratory syncytial virus (human RSV, long strain, type A [RSV], 5x10^5 PFU, in 50µL Dulbecco's Modified Eagle Medium [DMEM] supplemented with 10% fetal bovine serum). Sham-inoculated controls received SPG, PBS, Media or UV-inactivated (UV)-RSV in DMEM, respectively. Some mice were treated with DEX (2mg/kg, Sigma-Aldrich) i.n. on d32-34. Some Cmu respiratory infections were induced in female wild-type C57BL/6 mice, 6-8 weeks old (300 ifu) in order to show that miR-21 expression is not strain-specific.

**miRNA inhibition with antagonirs**

The miR-21 sequence was downloaded from miRBase University of Manchester, UK (http://www.mirbase.org/). Ant-21 and scrambled antagonist control (Scram, nonspecific RNA VIII, BLAST searched against the mouse genome) were designed and purchased from Sigma-Aldrich. The sequence of Ant-21 was:

5’mU.*.mC.*.mA.mA.mC.mA.mU.mC.mA.mG.mU.mC.mU.mG.mA.mU.mA.mA.mG.*.mC.*.mU.*.mA.*.3’-Chl, where (m) denotes 2’-O-methyl-modified nucleotides, (*) denotes phosphorothioate linkages, and (–Chl) denotes hydroxyprolinol-linked cholesterol. Groups of mice were treated with Ant-21 (50µg delivered in 50µL sterile saline i.n.) or an equivalent amount of Scram on day 32 with or without DEX, as described previously.¹¹³

**PI3K inhibition**

Groups were treated i.n. with the class I pan-PI3K inhibitor LY294002 (2mg/kg, Selleck, Houston, USA, in 3% dimethyl sulfoxide [DMSO] vehicle) on d32-34 with or without DEX. Controls were treated with vehicle.¹¹⁴
Airway inflammation

Airway inflammation was assessed in cytospin preparations of cells in bronchoalveolar lavage fluid (BALF, 2x1 mL washes with Hank’s Balanced Salt Solution, Life Technologies, Australia) that were stained with May-Grunwald-Giemsa. Differential leukocyte counts were determined using morphological criteria (=175 cells by light microscopy [x40]). All samples were coded and counts were performed in a blinded fashion.

Lung function

Mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) and their tracheas were cannulated (tracheostomy with ligation). FlexiVent apparatus (FX1 System, SCIREQ, Montreal, Canada) was used to assess airway-specific resistance (Rn, tidal volume of 8mL/kg at a respiratory rate of 450 breaths/min) in response to increasing doses of nebulized methacholine (Sigma-Aldrich). This combination of anesthesia and ventilation is common and recommended by the manufacturer. Assessments were performed at least three times per dose of saline/methacholine and the average calculated.

Quantification of mRNA and miRNA expression by real-time quantitative PCR

Total RNA was isolated from homogenized lungs with TRIzol® Reagent (Invitrogen, Life Technologies, Australia). Random-primed reverse transcriptions were performed for mRNA real-time quantitative PCRs. Gene expression was normalized to the transcript of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (Hprt). Expression of miR-21 was assessed by real-time quantitative PCR, as described previously. Briefly, multiplex reverse transcriptions were performed on DNase I-treated total RNA using a
combination of reverse primers specific for mature mmu-miR-21 and the endogenous controls
U6 small nuclear RNA (snRNA) and U49 small nucleolar RNA (snoRNA), to a final
concentration of 40 nM each. The relative abundance of miR-21 was calculated against the
geometric mean of U6 and U49. For primer sequences refer to Table E1. All reactions were
performed using BioScript™ reverse transcriptase in 1x first-strand buffer according to
manufacturer’s instructions (Bioline Pty. Ltd., NSW, Australia). Real-time quantitative PCR
assays were performed with SYBR Green Supermix (KAPA Biosystems, Inc., MA, USA) and
a Mastercycler® ep realplex™ system (Eppendorf South Pacific, NSW, Australia).

miRNA in situ hybridization (ISH)
miR-21 was localized in histological sections of formalin-fixed, paraffin-embedded lungs
using a miRCURY LNA™ miRNA ISH optimization kit (miR-21, Exiqon, Vedbæk, Denmark) in accordance with the manufacturer’s protocol. Briefly, lung sections were de-
paraffinized, rehydrated in an ethanol:RNase free water gradient, protease-treated (15µg/mL
of Proteinase K for 10 min in Proteinase K buffer), washed in PBS, dehydrated in ethanol, air-
dried, and pre-hybridized in 1x ISH buffer at 55°C for 1 hr in a humidifying chamber. miR-
21-specific, and scrambled (negative control), double-digoxigenin (DIG) LNA™ probes (40
nM) were then applied to the lung sections and hybridized at 55°C overnight in a humidifying
chamber. Hybridized sections were then washed (5x-0.2x saline-sodium citrate [SSC] buffer
gradient) and blocked (2% lamb serum in PBS-Tween [PBS-T, 0.1% Tween20, Ajax,
Finechem, NSW, Australia]) at room temperature (RT) for 15 min. Sheep-anti-DIG antibody
conjugated with alkaline phosphatase (AP, Roche, Life Science, Australia, 1:800 in 2% lamb
serum in PBS-T) was then applied to the sections and probe:target complexes were detected
with an AP substrate solution (containing BM Purple [1:3, Roche, Life Science] and
Levamisole [endogenous AP activity inhibitor, 0.2 mM, Sigma-Aldrich]) that produces a dark
blue precipitate in the presence of AP activity. Nuclear Fast Red™ (Vector laboratories, CA, USA) was used as a counterstain.

**Immunoblot assays**

Nuclear protein fractions were isolated from lung tissues using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL, USA) with added Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Sample protein and Precision Plus Protein™ WesternC™ Standards (Bio-Rad, CA, USA) were resolved on 4-15% gradient Mini-PROTEAN® TGX Stain-Free™ polyacrylamide gels (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Australia). The blots were then blocked with 5% bovine serum albumin in Tris-buffered saline and Tween 20 and incubated with primary antibodies overnight before adding the relevant secondary antibody with Precision Protein™ StrepTactin-horseradish peroxidase (HRP) Conjugate (Bio-Rad). Primary antibodies employed were; anti-pAKT (Ser473) and anti-AKT antibodies (Cell Signaling Technology, MA, USA), and anti-HDAC2 and anti-TATA binding protein (TBP, 1TBP18) antibodies (Abcam, MA, USA), and were used according to manufacturer’s instructions. Secondary antibodies used were anti-Rabbit IgG HRP (R&D Systems, MN, USA) and anti-Mouse IgG (whole molecule)-Peroxidase antibodies (Sigma-Aldrich). SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used to develop and visualize membranes by chemiluminescence (Bio-Rad, ChemiDoc MP System).

**Statistics**

Comparisons between two groups were made using unpaired t-Tests or a non-parametric equivalent where appropriate. Comparisons between multiple groups were made using a One-way ANOVA and a Post Test or non-parametric equivalent where appropriate. Lung function
data were assessed using a Two-way ANOVA and an appropriate Post Test or non-parametric equivalent. Analyses were performed using GraphPad Prism Software (San Diego, California).

REFERENCES


E11. Petersen BC, Dolgachev V, Rasky A, Lukacs NW.


SUPPLEMENTAL FIGURE LEGENDS

FIG E1. Experimental protocol for the investigation and treatment of respiratory infection-induced, severe, steroid-insensitive, allergic airway disease (SSIAAD). Mice were intraperitoneally (i.p.) sensitized to ovalbumin (Ova, d0) and AAD was induced by intranasal
(i.n.) Ova challenge (d12, 13) followed by re-challenge (d33, 34). Non-allergic controls were sham-sensitized with saline (Sal). Some groups were inoculated i.n. with 100 inclusion-forming units (IFU) of *Chlamydia muridarum* (Cmu, d14), intratracheally (i.t.) with $2 \times 10^6$ colony-forming units (CFU) of non-typeable *Haemophilus influenzae* (Hinf, d14), or i.n. with 7.5 plaque forming units (PFU) of influenza (Flu, d14) or $5 \times 10^5$ PFU of respiratory syncytial virus (d14). Controls were sham-infected with sucrose phosphate glutamate (SPG), phosphate buffered saline (PBS), Media, or $5 \times 10^5$ PFU of UV-inactivated RSV (UV-RSV), respectively.

Steroid responses were assessed by i.n. treatment with dexamethasone (DEX, d32-34). miR-21-depleting antagonim (Ant-21, d32) and pan-phosphoinositide-3-kinase inhibitor (LY294002, d32-34) were administered i.n. to suppress miR-21 and PI3K, respectively. Controls received scrambled antagonim (Scram) or DMSO vehicle.

**FIG E2.** Expression of T<sub>H1</sub> and T<sub>H17</sub>-associated factors in *Chlamydia* (Cmu)- and sham (SPG)-infected, non-allergic controls and miR-21 expression in C57BL/6 mice. (A) Lung expression of T<sub>H1</sub>- and T<sub>H17</sub>-associated factors on d35 (Fig E1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups in the absence of ovalbumin (Ova)-induced allergic airways disease (AAD) (two experiments; n=6 per group). Lung miR-21 expression during (B) Ova-induced AAD and (C) Cmu respiratory infection in C57BL/6 mice (one experiment; n=4-8 per group). Data are mean±SEM. *P<0.05; **P<0.01; ***P<0.001.

**FIG E3.** Localization of miR-21 in lung tissues and cells in *Chlamydia* infection-induced, severe, steroid-insensitive (SSI) allergic airway disease (AAD; SSIAAD). Representative photomicrographs (20X magnification) showing tissue and cellular localization of miR-21 in histological sections of mouse lung collected on d35 of the study protocol (Fig E1) in *Chlamydia* (Cmu)-infected groups with and without ovalbumin (Ova)-induced AAD.
(Ova/Cmu and Sal/Cmu, respectively) compared to sham (SPG)-infected, allergic and non-allergic (Ova/SPG and Sal/SPG, respectively) controls. Localization of miR-21 in lung sections was characterized using in situ hybridization analyses with a miR-21-specific locked nucleic acid (LNA™) probe. miR-21-positive signal (blue color) is visible in luminal epithelial and immune cells associated with airways and blood vessels. miR-21-positive signal is not evident when scrambled (Scr) LNA™ miRNA probe is employed. Nuclear Fast Red™ was used as a counterstain.

FIG E4. pAKT and AKT levels in lung cytoplasmic fractions. Cytoplasmic protein levels of pAKT, AKT and pAKT:AKT ratio were determined on d32 of the study protocol (Fig E1) by immunoblot (top panels) and densitometry (bottom panels) (>two experiments; n=4 per group) prior to steroid treatment and recapitulation of allergic airway disease (AAD) in Chlamydia (Cmu) and sham (SPG)-infected, allergic mice. Data are mean±SEM. *P<0.05; **P<0.01; ***P<0.001.

FIG E5. Inhibition of miR-21 suppresses cardinal features of steroid-sensitive, eosinophilic, allergic airway disease (AAD). (A) Total leukocyte, (B) eosinophil, (C) neutrophil, (D) macrophage, and (E) lymphocyte numbers were enumerated in bronchoalveolar lavage fluid (BALF) on d35 of the study protocol (Fig E1) in sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD with or without steroid (DEX) and/or anti-miR-21 (Ant-21) or scrambled (Scram) antagonim treatment (two experiments; n=6 per group). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) (F) in response to increasing doses of methacholine (MCh), and (G) 10 mg/mL of MCh (shows statistics at maximal dose from AHR curves [Fig E4, F] was also determined (two experiments; n=5-10 per group). Data are mean±SEM. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.