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

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ABSTRACT

Background: Severe, steroid-insensitive asthma is a substantial clinical problem accounting for >50% of asthma-associated health-care costs. 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. The mechanisms that underpin infection-induced, severe, neutrophilic, steroid-insensitive (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 and Results: Novel mouse models of *Chlamydia*, *Haemophilus influenzae*, influenza and respiratory syncytial virus respiratory infection and ovalbumin-induced, severe, neutrophilic, steroid-insensitive allergic airway disease (SSIAAD) in BALB/c mice were developed. They were interrogated to discover a new infection-induced microRNA (miR)-21-dependent, phosphoinositide-3-kinase (PI3K)-mediated pathway that promotes steroid-insensitive airway inflammation and airway hyper-responsiveness (AHR) in AAD. This involves the suppression of nuclear HDAC2 levels. The potential for targeting of infection-induced miR-21 expression and PI3K activity in the lung were examined using therapeutic treatments with a specific miR-21 inhibitor (antagomir, Ant-21) and the pan-PI3K inhibitor LY294002. Treatment with Ant-21 or LY294002 reduced nuclear pAkt (an indicator of PI3K activity) and restored HDAC2 levels, which suppressed AHR and restored steroid sensitivity to inflammation, AHR and AAD.

Conclusion: We identify a previously unrecognized pathogenic 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.

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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 antagomir
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

76	Flu:	A/PR/8/34 H1N1 mouse-adapted influenza
77	GR:	Glucocorticoid receptor
78	Hdac/HDAC:	Histone deacetylase
79	Hinf:	Non-typeable *Haemophilus influenzae* (NTHi-289)
80	Ifng/IFNγ:	Interferon gamma
81	Il/IL:	Interleukin
82	ISH:	*In situ* hybridisation
83	i.n.:	Intranasally
84	i.p.:	Intraperitoneally
85	i.t.:	Intratracheally
86	LY29:	LY294002
87	MCh:	Methacholine
88	miRNA:	MicroRNA
89	Nr3c1/NR3C1:	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
90	Ova:	Ovalbumin
91	pAKT:	Phosphorylated AKT
92	PBS:	Phosphate-buffered saline
93	PI3K:	Phosphoinositide-3-kinase
94	Pten/PTEN:	Phosphatase and tensin homolog
95	qPCR:	Quantitative PCR
96	RSV:	Respiratory syncytial virus
97	Scram:	Scrambled control antagomir
98	SPG:	Sucrose phosphate glutamate buffer
99	SSIAAD:	Severe, steroid-insensitive allergic airway disease
100	Stat/STAT:	Signal transducer and activator of transcription

TBP:	TATA binding protein
T_H:	T-helper type lymphocyte
Tgfb/TGFβ:	Transforming growth factor, beta 1
Tnf/TNF:	Tumor necrosis factor
UV:	Ultra-violet

Corticosteroids are broad-acting anti-inflammatory agents and the mainstay treatments for asthma.[1] However, 5-10% of asthmatics do not respond to steroid treatment. These patients typically have more severe disease, account for ≥50% of asthma-associated health-care costs, and urgently require effective therapies.[2, 3]

Asthma is an inflammatory condition of the airways archetypally mediated by aberrant T-helper type (T_H)2 lymphocyte responses[4] that drive eosinophilic airway inflammation, mucus hypersecretion, and airway hyper-responsiveness (AHR).[5] Recent clinical evidence shows that asthma is a heterogeneous condition. Indeed, increased T_H1- and/or T_H17-responses,[6] and non-eosinophilic, predominantly neutrophilic airway inflammation prevail in moderate-to-severe asthma.[7] Severe asthma is often steroid-insensitive (SSI asthma) and is associated with non-eosinophilic endotypes of disease, particularly neutrophilic asthma.[8, 9]

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])[10] and the recruitment of histone deacetylase (HDAC)2 that deacetylates histones and suppresses gene transcription.[11] Reduced HDAC2 activity is associated with both SSI asthma and chronic obstructive pulmonary disease (COPD).[12-14] 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.[14, 15] 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.[16-19] *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.[20-22] These infections induce neutrophilic, T_H1 and/or T_H17 responses in experimental asthma models (allergic airway disease [AAD]) replicating the effects in patients.[23-26] Respiratory infections with influenza and respiratory syncytial virus (RSV) induce asthma exacerbations that are steroid insensitive.[27, 28] 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.[29-33]

Several microRNAs (miRNAs) are implicated in asthma pathogenesis, and miR-21 is important in murine AAD.[34-37] miR-21-deficient mice exhibit reduced eosinophilic inflammation and interleukin (IL)-4 levels with a concomitant increase in interferon (IFN)γ during ovalbumin (Ova)-induced AAD.[38] miR-21 can also down-regulate the expression of phosphatase and tensin homolog (PTEN), which antagonizes PI3K activity.[39, 40]

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[23, 29, 37, 41-49] are described in the Methods section, and Figs E1-E4 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 *C. muridarum* (Cmu; Fig E1). This is a natural mouse respiratory pathogen and the most appropriate *Chlamydia* strain for studying host:pathogen relationships in mice.[26, 44, 50-54] Infection and inflammation peak at d10 and 15, respectively.[44, 50] 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, *A*-*G*). Resolved *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, *F* and *G*). Resolved infection alone (Sal/Cmu) did not induce persistent airway inflammation or AHR compared to Sal/SPG (Fig 1, *A*-*G*), suggesting that *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 T_H1-(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 [*Tnf*]; Fig 1, *H*) and T_H17- (interleukin [*Il1*]*7*, *Il6*, transforming growth factor, beta 1 [*Tgfb*], *Il1b*; Fig 1, *H*), but reduced expression of T_H2- (*Il5*, *Il13*; data not shown) associated factors in the lungs compared to Ova/SPG controls. Thus, infection promoted a switch from T_H2-dominated, eosinophilic inflammation to T_H1/T_H17-dominated, neutrophilic responses in AAD. Thus, *Chlamydia* respiratory infection induces T_H1/T_H17-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[34-38] 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. This suggests that miR-21 is an infection-induced factor that may affect the lung environment prior to allergen challenge in *Chlamydia*-infected, allergic (Cmu/Ova) groups and play a role in the induction of SSIAAD.

We then showed that miR-21 expression was widespread occurring in airway epithelial, endothelial and infiltrating immune cells[9, 55] (Fig E2, *A* and *B*). 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.

206

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

209 To examine this potential, we next examined the *Chlamydia* infection-induced lung
210 environment on d32 immediately before DEX treatment and Ova re-challenge. *Chlamydia*-
211 infected, allergic (Ova/Cmu) mice exhibited increased miR-21 expression (Fig 2, *A*) with
212 concurrent decreases in the expression of *Pten*, *Nr3c1*, and *Hdac2* (Fig 2, *B-D*) compared to
213 Ova/SPG groups. These factors are involved in PI3K-dependent and steroid-mediated
214 responses. To determine whether these *Chlamydia*-induced effects were associated with
215 increased PI3K function we also assessed the levels of pAKT, a surrogate marker of PI3K-
216 dependent activity. Ova/Cmu mice had increased pAKT, and reduced HDAC2, protein levels
217 in their lung nuclear fractions (Fig 2, *E* and *F*). In contrast these mice had decreased pAKT
218 (Fig E3) levels in lung cytoplasmic fractions suggesting that infection resulted in increased
219 nuclear translocation of pAKT. Collectively, these data show that *Chlamydia*-induced miR-21
220 expression at the time of steroid treatment in SSIAAD is associated with attenuated *Nr3c1*
221 expression, increased PI3K responses and reduced HDAC2 levels.

222

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

224 We next assessed the role and potential for therapeutic targeting of increased miR-21
225 expression in *Chlamydia*-induced, SSIAAD. Administration of miR-21-specific inhibitor
226 (antagomir; Ant-21) on d32 (Fig E1) ablated lung miR-21 expression on d35 with or without
227 steroid treatment (Ova/Cmu/Ant-21±DEX) compared to scrambled antagomir (Scram)-treated
228 controls (Ova/Cmu/Scram±DEX; Fig 3, *A*). Again DEX had no statistically significant effects
229 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*) 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 E4). 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, and in the induction of steroid-insensitive airway inflammation and 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 and in the induction of steroid-insensitive airway inflammation and 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 T_H1/T_H17 responses and steroid-insensitive airway inflammation and AHR. By interrogating our models and using an antagomir 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.[26] 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[56] 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 *10*, *Tnf*) and T_H17-(*Il17*, *Il6*, *Tgfb*, *Il1b*) associated factors in the lung that are also increased in severe, neutrophilic asthma (Fig 1, *I*).[6] Importantly, inflammation

330 and AHR are steroid-insensitive in this model indicating that infection drives pathogenic
331 processes that are not suppressed by anti-inflammatory steroid treatment. These data extend
332 our understanding of clinical studies that associate respiratory infections with non-
333 eosinophilic forms of asthma that are steroid insensitive. Indeed, substantial clinical evidence
334 links *Chlamydia* respiratory infection in asthma with increased neutrophil numbers in the
335 lungs during exacerbations and steroid insensitivity.[16-19]

336 Several different mechanisms have been implicated in the pathogenesis of SSI asthma,
337 including altered immune responses, increased activity of transcription factors, and defective
338 GR function.[3, 57-59] However, notably many patients with SSI asthma have normal nuclear
339 translocation of GR and no deficit in GR:glucocorticoid response element binding affinity.[60]
340 This indicates that steroid insensitivity in asthma can be driven by mechanisms outside of the
341 canonical steroid-response pathway. Thus, targeting specific factors that control multi-
342 functional pathways may be the most effective therapeutic approach.

343 miRNAs can have potent effects on immunity and increasing evidence shows that they
344 have pathogenic roles in asthma.[35, 36, 61] miR-21 is highly induced in inflamed lungs and can
345 promote eosinophilic inflammation and T_H2 responses, whilst suppressing T_H1 immunity
346 through the disruption of IL-12p35.[34, 38, 62] Consequently, miR-21 studies in asthma have
347 focused on its role in immune polarization during allergic sensitization.[63] In this study, we
348 demonstrate a previously unrecognized role for miR-21 in steroid insensitivity. miR-21
349 expression was increased in both steroid-sensitive and SSIAAD. However, sham-infected,
350 allergic mice only exhibited increased miR-21 expression after the recapitulation of AAD.
351 This suggests that its up-regulation in steroid-sensitive AAD is a transient phenomenon
352 acutely induced by the allergic inflammatory response. In contrast, infected, allergic mice had
353 persistently increased miR-21 expression on d32 immediately before steroid treatment and
354 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, and PTEN antagonizes PI3K activity.[39, 40] 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.[64] Here we show that *Chlamydia* infection increased levels of nuclear pAKT, a well-established indicator of PI3K activity.[64] 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.[2, 14, 15] Non-selective inhibition of PI3K activity with LY294002 restored HDAC2 activity and steroid sensitivity in experimental COPD. Furthermore, smoke-exposed PI3Kδ dead knock-in transgenic mice have reduced tyrosine nitration of HDAC2 with no deficit in steroid sensitivity.[14, 15] 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 *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 *in vivo* with miR-21-specific antagomir treatment. This approach has been shown to specifically and potently reduce the levels of targeted miRNAs.[35, 37] Treatment inhibited *Chlamydia*-induced miR-21 expression, restored *Pten*, reduced nuclear pAKT and increased *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 suggesting that infection-induced miR-21 directly mediates AHR, but not inflammation, in SSIAAD through mechanisms that are independent of pathways that are affected by steroids. Our findings suggest that steroid-insensitive airway inflammation and AHR have different etiologies but require the overexpression of miR-21 to maintain steroid insensitivity. Many studies show that pulmonary inflammation and AHR may be linked but may also be independent (reviewed in[65]).

To substantiate the existence of a pathogenic miR-21/PI3K axis, we then blocked PI3K activity *in vivo* with the pan-PI3K inhibitor LY294002,[15, 64] 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 *Hdac2*/HDAC2 mRNA expression and nuclear protein levels, and restored steroid sensitivity also similar 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.

H. influenzae respiratory infection is commonly associated with neutrophilic asthma that is steroid insensitive.[22] 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,[25] and that inflammation and AHR in this model are steroid-insensitive.[24] 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,[27, 28] 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.[66, 67] Furthermore, these viral infections enhance eosinophilic airway inflammation and T_H2 immune responses in other murine models of allergic asthma.[68, 69] 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.

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 more specific and may broadly affect steroid-insensitive as well as steroid-independent networks.

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630

FIGURE LEGENDS

FIG 1. *Chlamydia* respiratory infection induces severe, steroid-insensitive (SSI), neutrophilic, allergic airway disease (AAD; SSIAAD) that has similar features to human SSI asthma and is associated with increased miR-21 expression. **(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 *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD with or without steroid (DEX) treatment compared to non-allergic controls (Sal) (≥two experiments; n=4-10 in total). 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 1, *F*]) was also determined in all allergic groups on d35. **(H)** Lung mRNA expression of T_H1- and T_H17-associated factors were measured. **(I)** Common features of SSI asthma and SSIAAD. Expression of miR-21 in whole lung tissue **(J)** was assessed on d35 in Cmu and sham-infected groups with Ova-induced AAD with or without DEX treatment compared to non-allergic controls (≥two experiments; n=4-6 in total).

FIG 2. *Chlamydia* respiratory infection induces a persistent increase in miR-21 expression and primes steroid-insensitive responses in severe, allergic airway disease (AAD). Lung mRNA expression of **(A)** miR-21, **(B)** *Pten*, **(C)** *Nr3c1*, and **(D)** *Hdac2* were assessed on d32 by qPCR prior to steroid treatment and recapitulation of AAD in *Chlamydia* (Cmu) and sham (SPG)-infected, allergic mice (Fig E1) (one experiment; n=8). Nuclear protein levels of **(E)** pAKT, AKT and pAKT:AKT ratio, and **(F)** HDAC2 were also determined by immunoblot (top panels) and densitometry (bottom panels) (≥two experiments; n=4). 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 cardinal features of severe, steroid-insensitive (SSI), allergic airway disease (AAD; SSIAAD). Lung expression of **(A)** miR-21 was determined by qPCR on d35 of the study protocol (Fig E1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD with or without steroid (DEX) and/or anti-miR-21 (Ant-21) or scrambled (Scram) antagomir treatment (≥two experiments; n=4-5). The effect of Ant-21 treatment on lung mRNA expression of **(B)** *Pten* and **(C)** *Hdac2* was assessed on d35 after the second set of Ova challenges in allergic groups not treated with DEX (≥two experiments; n=4-5). Nuclear protein levels of **(D)** pAKT, AKT and pAKT:AKT ratio, and **(E)** HDAC2 were determined on d35 by immunoblot (top panels) and densitometry (bottom panels) (≥two experiments; n=5) in all allergic groups with Ova-induced AAD with or without DEX and/or Ant-21 or Scram antagomir treatments. **(F)** Total leukocyte, **(G)** eosinophil, **(H)** neutrophil, **(I)** macrophage, and **(J)** lymphocyte numbers were enumerated in bronchoalveolar lavage fluid (BALF) in the same groups (≥two experiments; n=4-6). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) **(K)** in response to increasing doses of methacholine (MCh), and **(L)** 10 mg/mL of MCh (shows statistics at maximal dose from AHR curves [Fig 3, *K*]) was also determined (≥three experiments; n=5-10). 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 (SSI), allergic airway disease (AAD; SSIAAD). Lung mRNA expression of **(A)** *Hdac2* was determined by qPCR on d35 of the study protocol (Fig E1) in *Chlamydia* (Cmu) and sham (SPG)-infected groups with ovalbumin (Ova)-induced AAD that were treated with LY294002 (LY29) or vehicle (DMSO)

(≥two experiments; n=4-5). Nuclear protein levels of **(B)** pAKT, AKT and pAKT:AKT ratio, and **(C)** HDAC2 were determined by immunoblot (top panels) and densitometry (bottom panels) (≥two experiments; n=5) in all allergic groups with Ova-induced AAD with or without steroid (DEX) and/or LY29 or vehicle (DMSO) treatment. **(D)** Total leukocyte, **(E)** eosinophil, **(F)** neutrophil, **(G)** macrophage, and **(H)** lymphocyte numbers were enumerated in bronchoalveolar lavage fluid (BALF) in the same groups (≥two experiments; n=4-8). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn) **(I)** in response to increasing doses of methacholine (MCh), and **(J)** 10 mg/mL of MCh (shows statistics at maximal dose from AHR curves [Fig 4, *I*]) was also determined (≥two experiments; n=5-8). Data are mean±SEM. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

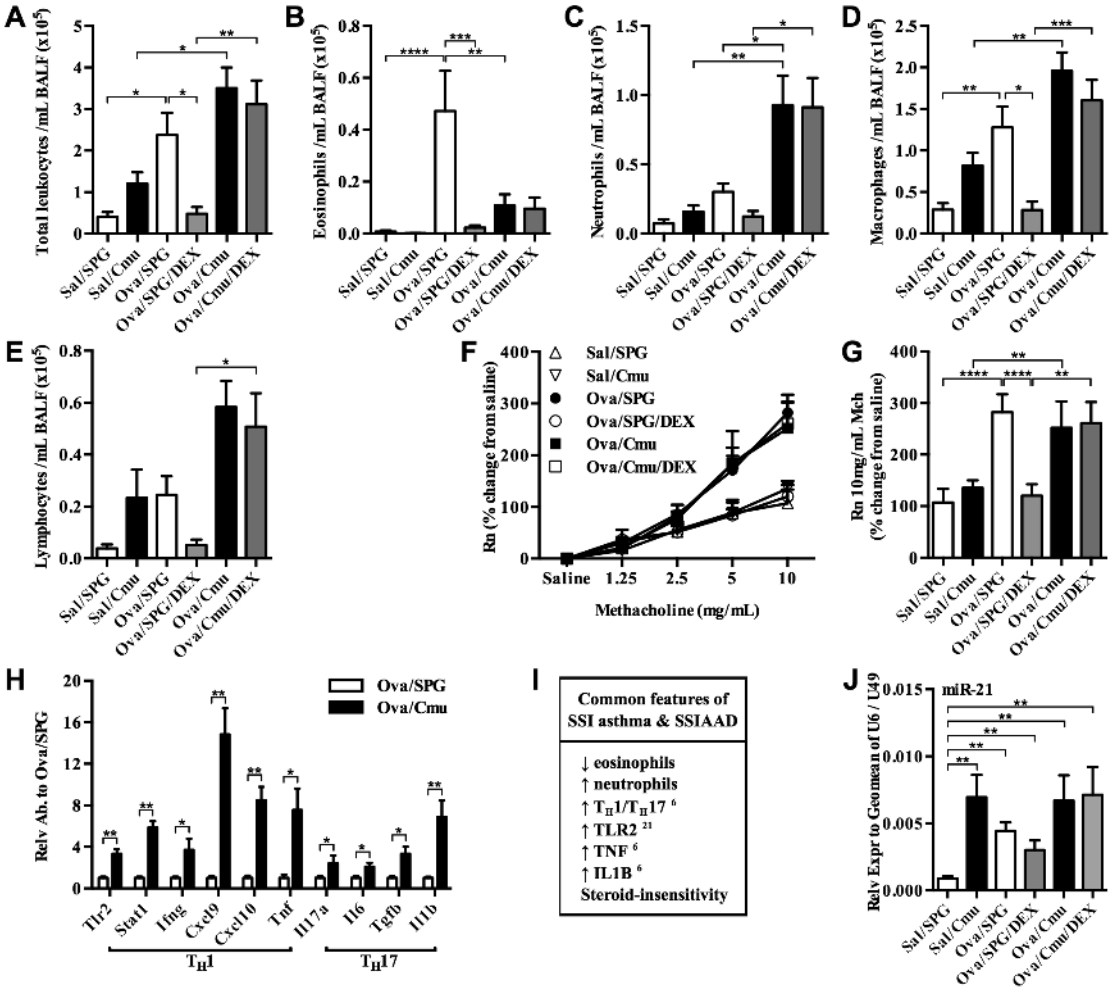
FIG 5. Inhibition of miR-21 suppresses cardinal features of *Haemophilus influenzae* respiratory infection-induced, severe, steroid-insensitive (SSI), neutrophilic, allergic airway disease (AAD; SSIAAD). **(A)** Total leukocyte, **(B)** eosinophil, **(C)** neutrophil, **(D)** macrophage, and **(E)** lymphocyte numbers were enumerated in bronchoalveolar lavage fluid (BALF) on d35 of the study protocol (Fig E1) in *H. influenzae* (Hinf) and sham (PBS)-infected groups with ovalbumin (Ova)-induced AAD with or without steroid (DEX) and/or anti-miR-21 (Ant-21) or scrambled (Scram) antagomir treatment (one experiment; n=5-6). 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 5, *F*]) was also determined (one experiment; n=5-6). 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 respiratory infection-induced, severe, steroid-insensitive (SSI), eosinophilic, allergic airway disease

706 (AAD; SSIAAD). **(A)** Total leukocyte, **(B)** eosinophil, **(C)** neutrophil, **(D)** macrophage, and
707 **(E)** lymphocyte numbers were enumerated in bronchoalveolar lavage fluid (BALF) on d35 of
708 the study protocol (Fig E1) in influenza (Flu) and sham (Media)-infected groups with
709 ovalbumin (Ova)-induced AAD with or without steroid (DEX) and/or anti-miR-21 (Ant-21)
710 or scrambled (Scram) antagomir treatment (one experiment; n=5-8). Airway hyper-
711 responsiveness (AHR) in terms of airway resistance (Rn) **(F)** in response to increasing doses
712 of methacholine (MCh), and **(G)** 10 mg/mL of MCh (shows statistics at maximal dose from
713 AHR curves [Fig 6, *F*]) was also determined (one experiment; n=7-8). Data are mean±SEM.
714 *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
715
716 **FIG 7.** Inhibition of miR-21 suppresses cardinal features of respiratory syncytial virus
717 infection-induced, severe, steroid-insensitive (SSI), eosinophilic, allergic airway disease
718 (AAD; SSIAAD). **(A)** Total leukocyte, **(B)** eosinophil, **(C)** neutrophil, **(D)** macrophage, and
719 **(E)** lymphocyte numbers were enumerated in bronchoalveolar lavage fluid (BALF) on d35 of
720 the study protocol (Fig E1) in respiratory syncytial virus (RSV) and sham (UV-inactivated
721 RSV; UV-RSV)-infected groups with ovalbumin (Ova)-induced AAD with or without steroid
722 (DEX) and/or anti-miR-21 (Ant-21) or scrambled (Scram) antagomir treatment (one
723 experiment; n=3-6). Airway hyper-responsiveness (AHR) in terms of airway resistance (Rn)
724 **(F)** in response to increasing doses of methacholine (MCh), and **(G)** 10 mg/mL of MCh
725 (shows statistics at maximal dose from AHR curves [Fig 7, *F*]) was also determined (one
726 experiment; n=5-6). Data are mean±SEM. *P<0.05; **P<0.01; ****P<0.0001.
727
728 **FIG 8.** Mechanisms and potential treatment of severe, steroid-insensitive, asthma. Infection in
729 allergic airway disease/asthma induces miR-21 that inhibits PTEN and promotes PI3K-

730 mediated suppression of HDAC2 and steroid insensitivity. This pathway may be targeted
731 therapeutically by inhibition of miR-21 and/or PI3K.

Figure 1


A
Total leukocytes /mL BALF (x10^5)
B
Eosinophils /mL BALF (x10^5)
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Neutrophils /mL BALF (x10^5)
D
Macrophages /mL BALF (x10^5)
E
Lymphocytes /mL BALF (x10^5)
F
Rn (% change from saline)
Saline
1.25
2.5
5
10
Methacholine (mg/mL)
G
Rn 10mg/mL Mch (% change from saline)
Sal/SPG
Sal/Cmu
Ova/SPG
Ova/SPG/DEX
Ova/Cmu
Ova/Cmu/DEX
H
Relv Ab. to Ova/SPG
Ova/SPG
Ova/Cmu
Tlr2
Stat1
Ifng
Cxcl9
Cxcl10
Tnf
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Il6
Tgfb
Il1b
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I
Common features of SSI asthma & SSIAAD
↓ eosinophils
↑ neutrophils
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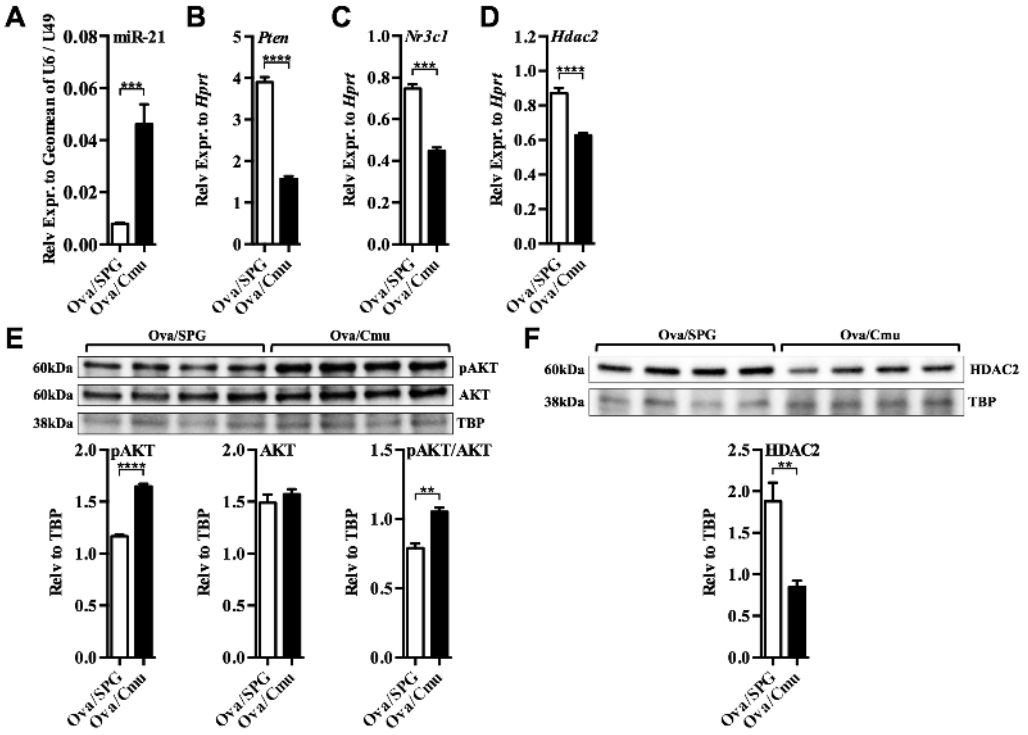
Figure 2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**
Ova/SPG
Ova/Cmu

Figure 3

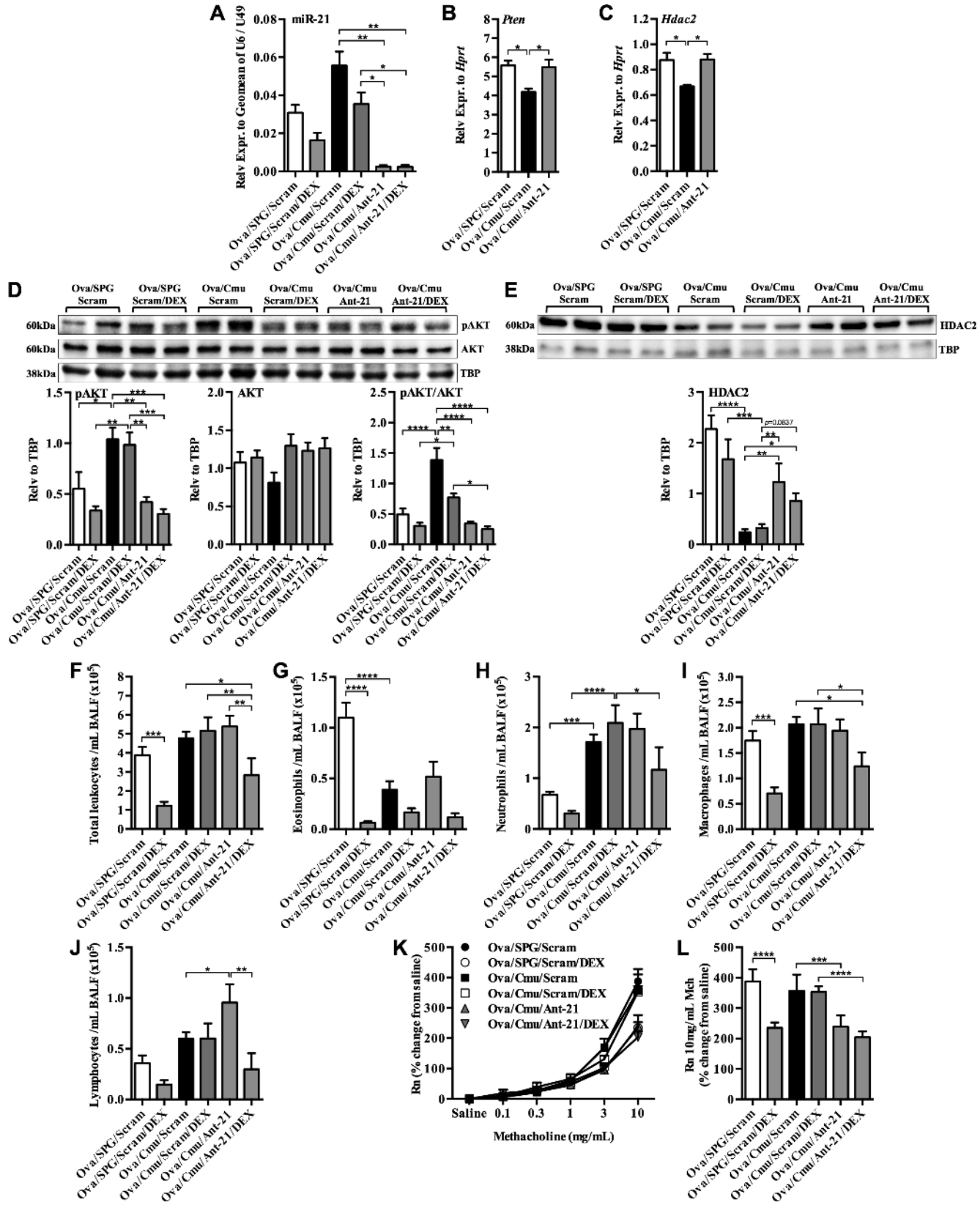
A
miR-21
Relv Expr. to Geomean of U6 / U49
B
Pten
Relv Expr. to Hprt
C
Hdac2
Relv Expr. to Hprt
Ova/SPG/Scram
Ova/SPG/Scram/DEX
Ova/Cmu/Scram
Ova/Cmu/Scram/DEX
Ova/Cmu/Ant-21
Ova/Cmu/Ant-21/DEX
D
Ova/SPG Scram
Ova/SPG Scram/DEX
Ova/Cmu Scram
Ova/Cmu Scram/DEX
Ova/Cmu Ant-21
Ova/Cmu Ant-21/DEX
60kDa
pAKT
60kDa
AKT
38kDa
TBP
pAKT
AKT
pAKT/AKT
Relv to TBP
E
60kDa
HDAC2
38kDa
TBP
HDAC2
p=0.0837
F
Total leukocytes /mL BALF (x10^5)
G
Eosinophils /mL BALF (x10^5)
H
Neutrophils /mL BALF (x10^5)
I
Macrophages /mL BALF (x10^5)
J
Lymphocytes /mL BALF (x10^5)
K
Rn (% change from saline)
Ova/SPG/Scram
Ova/SPG/Scram/DEX
Ova/Cmu/Scram
Ova/Cmu/Scram/DEX
Ova/Cmu/Ant-21
Ova/Cmu/Ant-21/DEX
Saline
0.1
0.3
1
3
10
Methacholine (mg/mL)
L
Rn 10mg/mL Mch
(% change from saline)

Figure 4

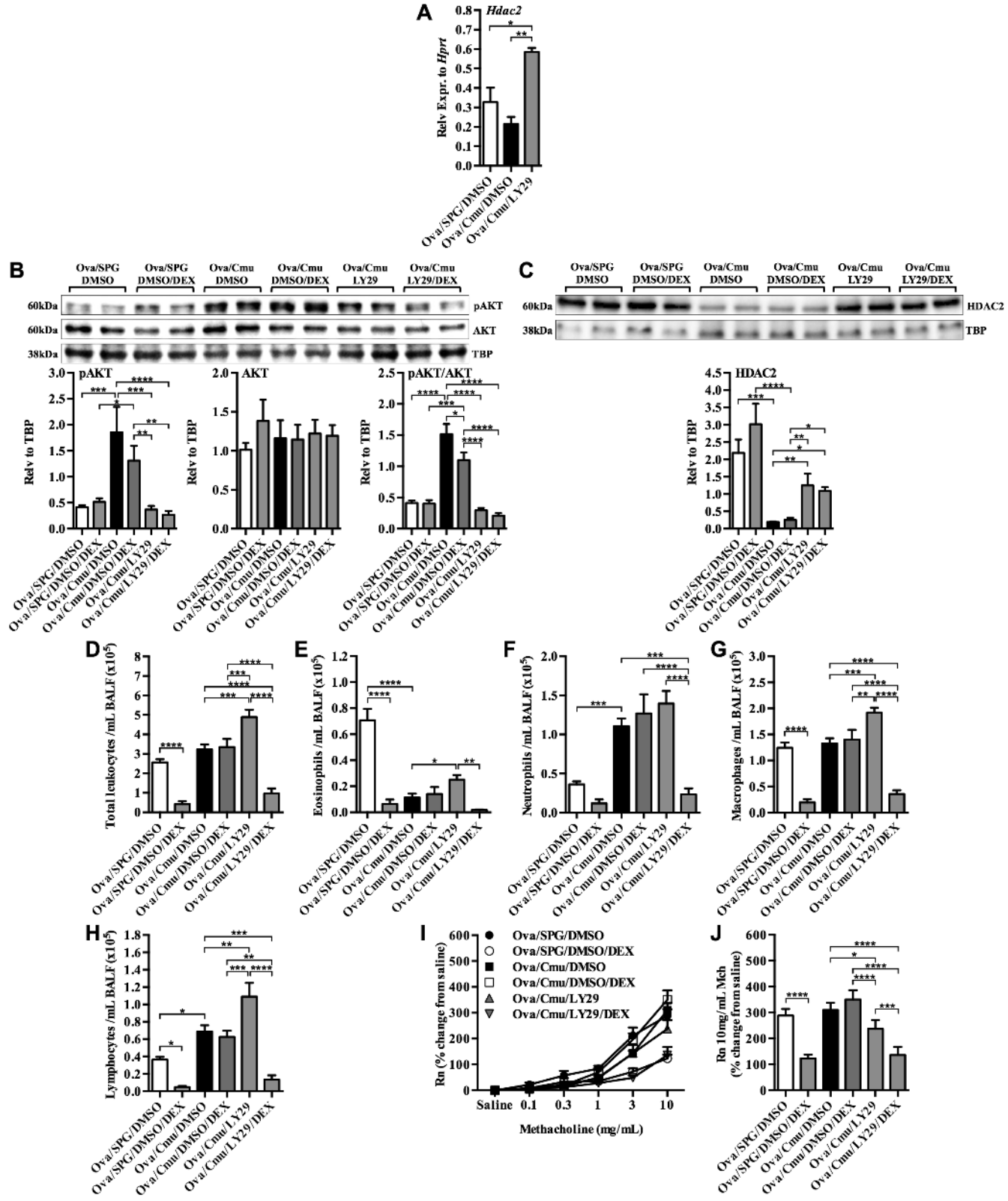
A
Hdac2
Relv Expr. to Hprt
Ova/SPG/DMSO
Ova/Cmu/DMSO
Ova/Cmu/LY29
B
Ova/SPG DMSO
Ova/SPG DMSO/DEX
Ova/Cmu DMSO
Ova/Cmu DMSO/DEX
Ova/Cmu LY29
Ova/Cmu LY29/DEX
60kDa
pAKT
AKT
38kDa
TBP
pAKT
AKT
pAKT/AKT
Relv to TBP
C
HDAC2
HDAC2
D
Total leukocytes /mL BALF (x10⁵)
E
Eosinophils /mL BALF (x10⁵)
F
Neutrophils /mL BALF (x10⁵)
G
Macrophages /mL BALF (x10⁵)
H
Lymphocytes /mL BALF (x10⁵)
I
Rn (% change from saline)
Ova/SPG/DMSO
Ova/SPG/DMSO/DEX
Ova/Cmu/DMSO
Ova/Cmu/DMSO/DEX
Ova/Cmu/LY29
Ova/Cmu/LY29/DEX
Saline
0.1
0.3
1
3
10
Methacholine (mg/mL)
J
Rn 10mg/mL Mch (% change from saline)

Figure 5

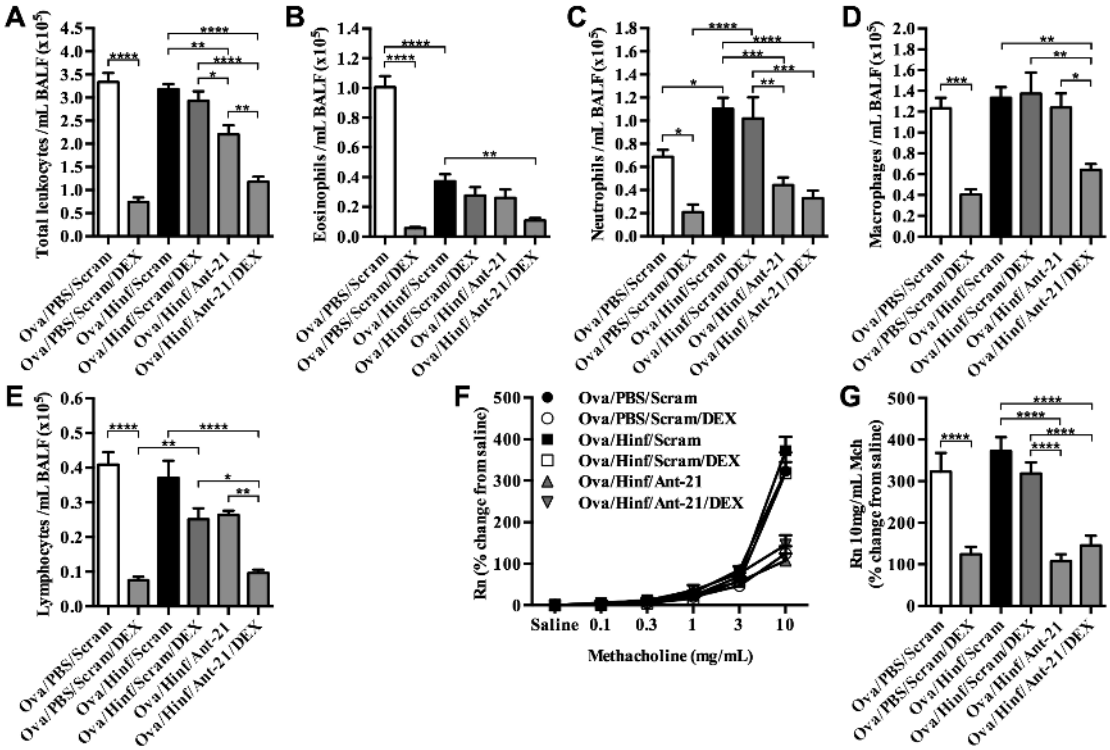
A
Total leukocytes /mL BALF (x10^5)
B
Eosinophils /mL BALF (x10^5)
C
Neutrophils /mL BALF (x10^5)
D
Macrophages /mL BALF (x10^5)
E
Lymphocytes /mL BALF (x10^5)
F
Rn (% change from saline)
Ova/PBS/Scram
Ova/PBS/Scram/DEX
Ova/Hinf/Scram
Ova/Hinf/Scram/DEX
Ova/Hinf/Ant-21
Ova/Hinf/Ant-21/DEX
Saline
0.1
0.3
1
3
10
Methacholine (mg/mL)
G
Rn 10mg/mL Mch
(% change from saline)

Figure 6

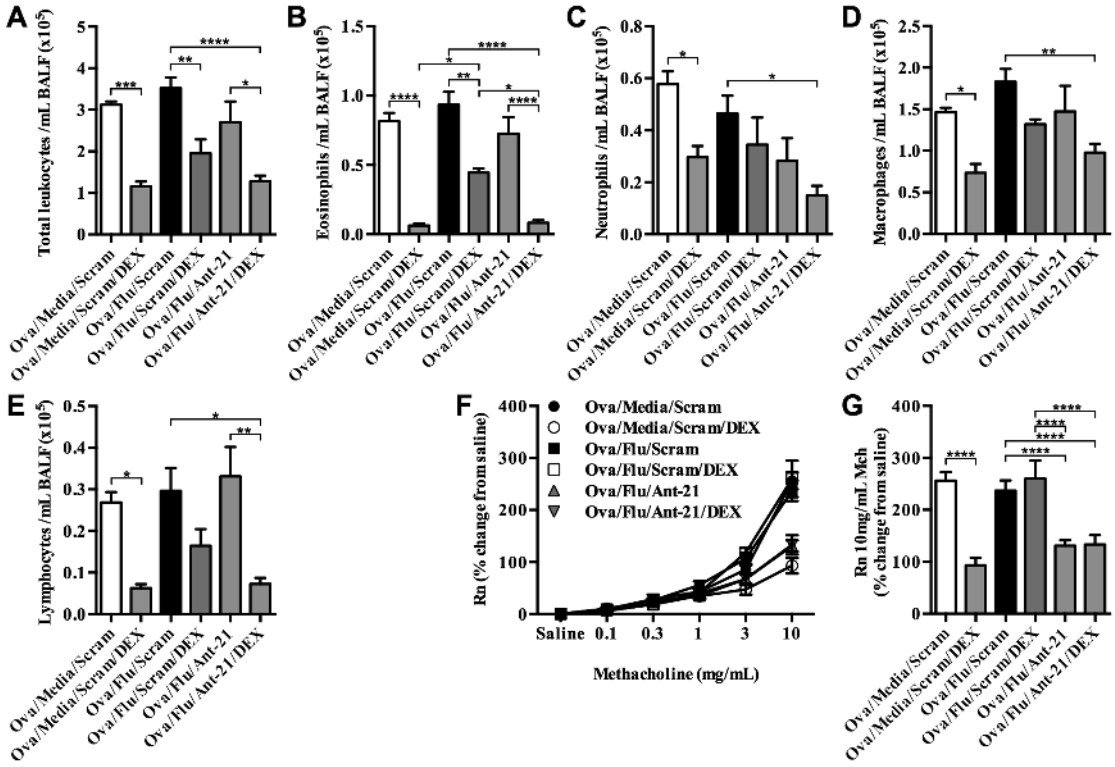
A
Total leukocytes /mL BALF (x10^5)
B
Eosinophils /mL BALF (x10^5)
C
Neutrophils /mL BALF (x10^5)
D
Macrophages /mL BALF (x10^5)
E
Lymphocytes /mL BALF (x10^5)
F
Rn (% change from saline)
Saline 0.1 0.3 1 3 10
Methacholine (mg/mL)
G
Rn 10mg/mL Mch (% change from saline)
Ova/Media/Scram
Ova/Media/Scram/DEX
Ova/Flu/Scram
Ova/Flu/Scram/DEX
Ova/Flu/Ant-21
Ova/Flu/Ant-21/DEX

Figure 7

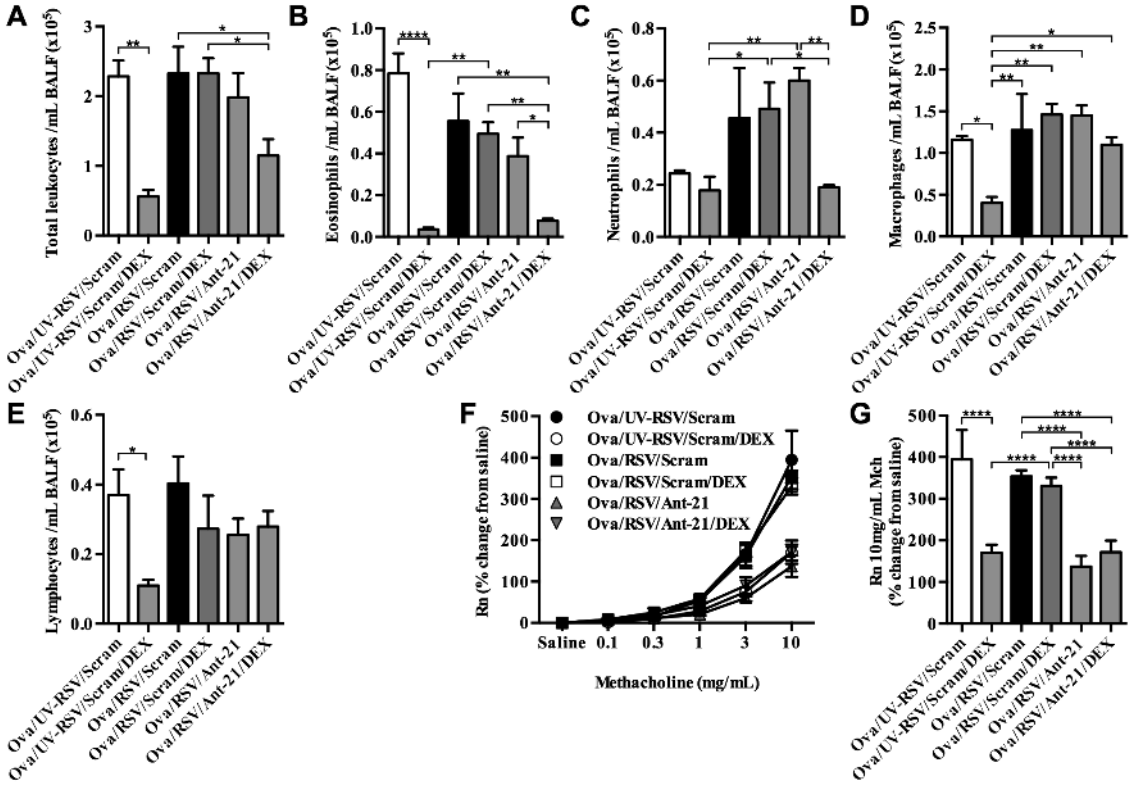
A
Total leukocytes /mL BALF (x10⁵)
B
Eosinophils /mL BALF (x10⁵)
C
Neutrophils /mL BALF (x10⁵)
D
Macrophages /mL BALF (x10⁵)
E
Lymphocytes /mL BALF (x10⁵)
F
Rn (% change from saline)
Ova/UV-RSV/Scram
Ova/UV-RSV/Scram/DEX
Ova/RSV/Scram
Ova/RSV/Scram/DEX
Ova/RSV/Ant-21
Ova/RSV/Ant-21/DEX
Saline 0.1 0.3 1 3 10
Methacholine (mg/mL)
G
Rn 10mg/mL Mch
(% change from saline)

Figure 8

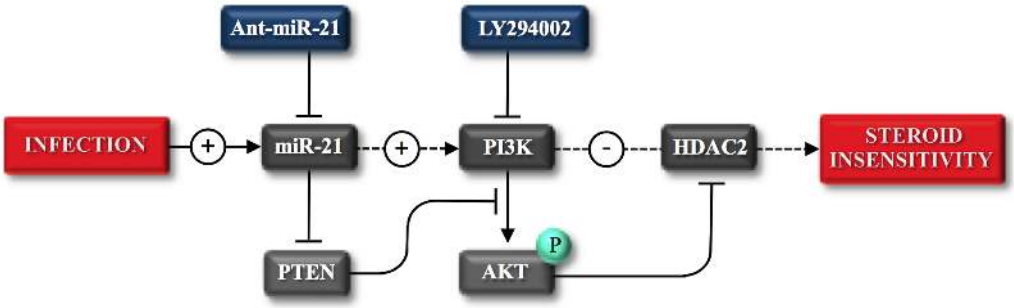
Ant-miR-21
LY294002
INFECTION
+
miR-21
+
PI3K
-
HDAC2
STEROID
INSENSITIVITY
PTEN
AKT
P

Figure E1

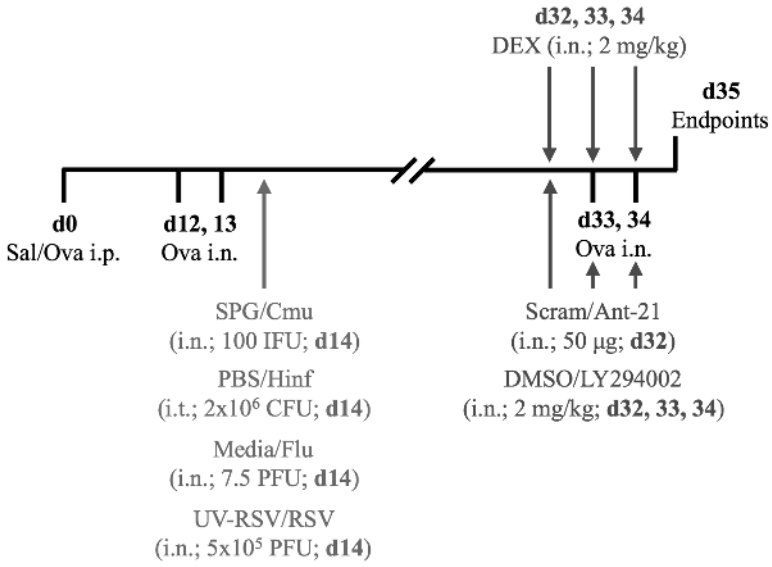
d32, 33, 34
DEX (i.n.; 2 mg/kg)
d35
Endpoints
d0
Sal/Ova i.p.
d12, 13
Ova i.n.
d33, 34
Ova i.n.
SPG/Cmu
(i.n.; 100 IFU; d14)
PBS/Hinf
(i.t.; 2x10^6 CFU; d14)
Media/Flu
(i.n.; 7.5 PFU; d14)
UV-RSV/RSV
(i.n.; 5x10^5 PFU; d14)
Scram/Ant-21
(i.n.; 50 μg; d32)
DMSO/LY294002
(i.n.; 2 mg/kg; d32, 33, 34)

Figure E2

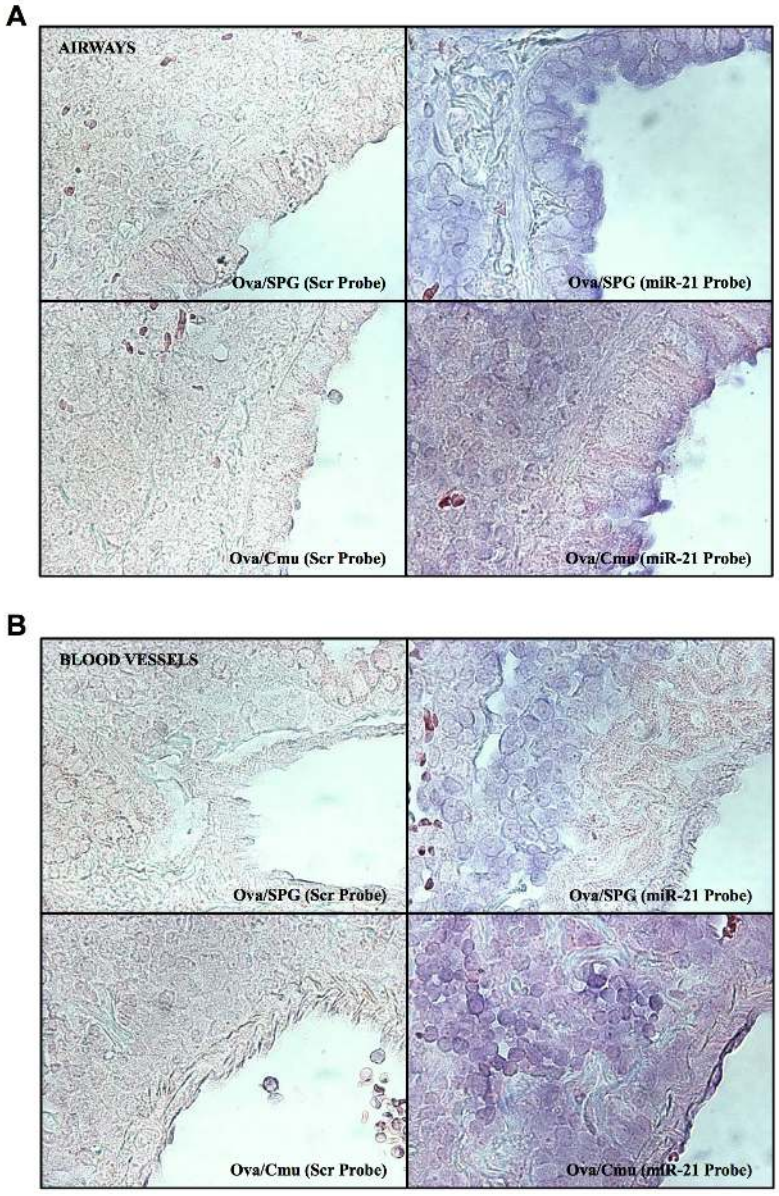
A
AIRWAYS
Ova/SPG (Scr Probe)
Ova/SPG (miR-21 Probe)
Ova/Cmu (Scr Probe)
Ova/Cmu (miR-21 Probe)
B
BLOOD VESSELS
Ova/SPG (Scr Probe)
Ova/SPG (miR-21 Probe)
Ova/Cmu (Scr Probe)
Ova/Cmu (miR-21 Probe)

Figure E3

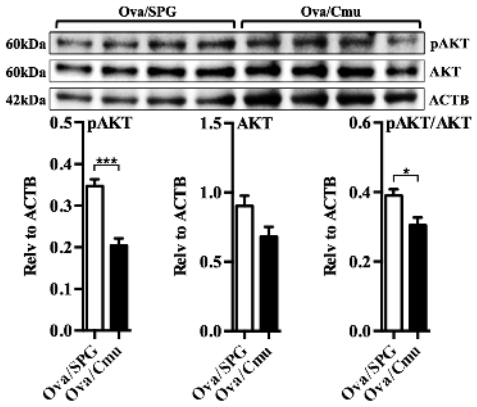
Ova/SPG
Ova/Cmu
60kDa
pAKT
60kDa
AKT
42kDa
ACTB
pAKT
AKT
pAKT/AKT
Relv to ACTB
***
*
Ova/SPG
Ova/Cmu

Figure E4

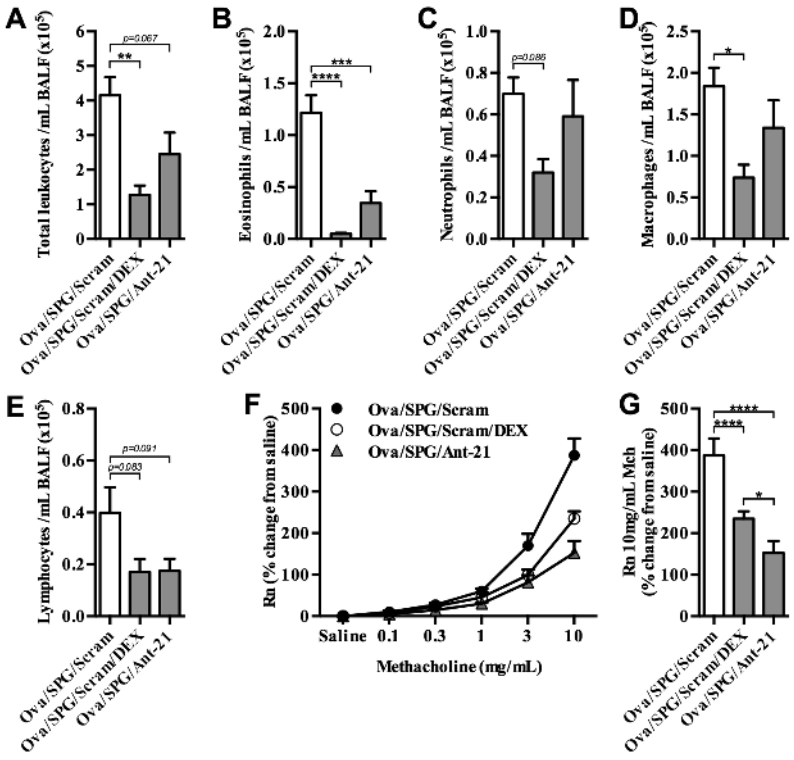
A
Total leukocytes /mL BALF (x10^5)
p=0.067
**
B
Eosinophils /mL BALF (x10^5)
***
****
C
Neutrophils /mL BALF (x10^5)
p=0.086
D
Macrophages /mL BALF (x10^5)
*
Ova/SPG/Scram
Ova/SPG/Scram/DEX
Ova/SPG/Ant-21
E
Lymphocytes /mL BALF (x10^5)
p=0.091
p=0.083
F
Rn (% change from saline)
Ova/SPG/Scram
Ova/SPG/Scram/DEX
Ova/SPG/Ant-21
Saline
0.1
0.3
1
3
10
Methacholine (mg/mL)
G
Rn 10mg/mL Mch (% change from saline)
****
****
*

Table E1

Primer	Nucleotide sequence	Target
Cxcl9 forward	5'-TTCCTGGAGCAGTGTGGAGTTCGA-3'	Chemokine (C-X-C motif) ligand 9
Cxcl9 reverse	5'-TGTAGTGGATCGTGCCTCGGCT-3'	Chemokine (C-X-C motif) ligand 9
Cxcl10 forward	5'-CCAAGTGCTGCCGTCATTTTC-3'	Chemokine (C-X-C motif) ligand 10
Cxcl10 reverse	5'-TCCCTATGGCCCTCATTCTCA-3'	Chemokine (C-X-C motif) ligand 10
Nr3c1 forward	5'-AAAGAGCTAGGAAAAGCCATTGTC-3'	Nuclear receptor subfamily 3, group C, member 1
Nr3c1 reverse	5'-TCAGCTAACATCTCTGGGAATTCA-3'	Nuclear receptor subfamily 3, group C, member 1
Hdac2 forward	5'-CGCGTGATGACCGTCTCATTCCA-3'	Histone deacetylase 2
Hdac2 reverse	5'-CACCGCGCTAGGCTGGTACA-3'	Histone deacetylase 2
Hprt forward	5'-AGGCCAGACTTTGTTGGATTTGAA-3'	Hypoxanthine guanine phosphoribosyl transferase
Hprt reverse	5'-CAACTTGCGCTCATCTTAGGCTTT-3'	Hypoxanthine guanine phosphoribosyl transferase
Ifng forward	5'-CTGGAGGAACTGGCAAAAGG-3'	Interferon gamma
Ifng reverse	5'-TTGCTGATGGCCTGATTGTC-3'	Interferon gamma
Il1b forward	5'-TGGGATCCTCTCCAGCCAAGC-3'	Interleukin 1 beta
Il1b reverse	5'-AGCCCTTCATCTTTTGGGGTCCG-3'	Interleukin 1 beta
Il17a forward	5'-GTGTCTCTGATGCTGTTGCT-3'	Interleukin 17A
Il17a reverse	5'-GTTGACCTTCACATTCTGGA-3'	Interleukin 17A
Il6 forward	5'-AGAAAACAATCTGAAACTTCCAGAGAT-3'	Interleukin 6
Il6 reverse	5'-GAAGACCAGAGGAAATTTTCAATAGG-3'	Interleukin 6
Pten forward	5'-TGGATTCGACTTAGACTTGACCT-3'	Phosphatase and tensin homolog
Pten reverse	5'-GCGGTGTCATAATGTCTCTCAG-3'	Phosphatase and tensin homolog
Stat1 forward	5'-CCCGAATTTGACAGTATGATGA-3'	Signal transducer and activator of transcription 1
Stat1 reverse	5'-GAAGGAACAGTAGCAGGAAGGA-3'	Signal transducer and activator of transcription 1
Tgfb forward	5'-CCCGAAGCGGACTACTATGCTA-3'	Transforming growth factor, beta 1
Tgfb reverse	5'-GGTAACGCCAGGAATTGTTGCTAT-3'	Transforming growth factor, beta 1
Tlr2 forward	5'-TGTAGGGGCTTCACTTCTCTGCTT-3'	Toll-like receptor 2
Tlr2 reverse	5'-AGACTCCTGAGCAGAACAGCGTTT-3'	Toll-like receptor 2
Tnf forward	5'-TCTGTCTACTGAACTTCGGGGTGA-3'	Tumor necrosis factor
Tnf reverse	5'-TTGTCTTTGAGATCCATGCCGTT-3'	Tumor necrosis factor
U6 forward	5'-CGGCAGCACATATACTAAAATTGG-3'	Small nuclear RNA (snRNA) U6
U6 reverse	5'-GCCATGCTAATCTTCTCTGTATC-3'	Small nuclear RNA (snRNA) U6
U49 forward	5'-ATCACTAATAGGAAGTGCCGTC-3'	Small nucleolar RNA (snoRNA) U49
U49 reverse	5'-ACAGGAGTAGTCTTCGTCAGT-3'	Small nucleolar RNA (snoRNA) U49
miR-21 forward[1]	5'-T+AGCTTATCAGACTG-3'	Mus musculus microRNA 21
miR-21 reverse	5'-GTAAAACGACGGCCAGTTCAACAT-3'	Mus musculus microRNA 21

[1] LNA™-modified bases are preceded by a [+] symbol

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

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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.[E1-8]

Treatment with DEX in AAD

Some groups were treated with DEX (2mg/kg, Sigma-Aldrich) i.n. on days 32-34.[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, 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.[E1-12]

miRNA inhibition with antagomirs

The miR-21 sequence was downloaded from miRBase University of Manchester, UK (http://www.mirbase.org/). Ant-21 and scrambled antagomir 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.[E13]

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.[E14]

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]).[E3, 4] All samples were coded and counts were performed in a blinded fashion.[E1-10, 15, 16]

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).[E3-10, 17] 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)[E9] in response to increasing doses of nebulized methacholine (Sigma-Aldrich). This combination of anesthesia and ventilation is common and recommended by the manufacturer.[E4, 9, 18] 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*).[E5] Expression of miR-21 was assessed by real-time quantitative PCR, as described previously.[E19] 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^2 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

142 equivalent.[E20] Analyses were performed using GraphPad Prism Software (San Diego,
143 California).
144
145 **REFERENCES**

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SUPPLEMENTAL FIGURE LEGENDS

FIG E1. Experimental protocol for the investigation and treatment of respiratory infection-induced, severe, steroid-insensitive (SSI), allergic airway disease (AAD; 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 $2x10^6$ 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 $5x10^5$ PFU of respiratory syncytial virus (d14). Controls were sham-infected with sucrose phosphate glutamate (SPG), phosphate buffered saline (PBS), Media, or $5x10^5$ PFU of UV-inactivated RSV (UV-RSV), respectively. Steroid responses were assessed by i.n. treatment with dexamethasone (DEX, d32-34). miR-21-depleting antagomir (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 antagomir (Scram) or DMSO vehicle.

FIG E2. Localization of miR-21 in lung tissues and cells in *Chlamydia* infection-induced, severe, steroid-insensitive (SSI) allergic airway disease (AAD; SSIAAD). Representative photomicrographs (100X magnification under immersion oil) 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 ovalbumin (Ova)-induced AAD (Ova/Cmu) compared to sham (SPG)-infected, allergic (Ova/SPG) controls. Localization of miR-21 in lung sections was achieved using *in situ* hybridization analyses with a miR-21-specific locked nucleic acid (LNA^{TM}) probe. miR-21-positive signal (blue color) is visible in luminal epithelial and immune cells associated with **(A)** airways, and immune cells associated with **(B)** blood vessels. miR-21-positive signal is not evident when scrambled (Scr) LNA^{TM} miRNA probe is employed. Nuclear Fast Red™ was used as a counterstain.

FIG E3. 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) prior to

241 steroid treatment and recapitulation of allergic airway disease (AAD) in *Chlamydia* (Cmu)
242 and sham (SPG)-infected, allergic mice. Data are mean±SEM. *P<0.05; ***P<0.001.
243
244 **FIG E4.** Inhibition of miR-21 suppresses cardinal features of steroid-sensitive, eosinophilic,
245 allergic airway disease (AAD). **(A)** Total leukocyte, **(B)** eosinophil, **(C)** neutrophil, **(D)**
246 macrophage, and **(E)** lymphocyte numbers were enumerated in bronchoalveolar lavage fluid
247 (BALF) on d35 of the study protocol (Fig E1) in sham (SPG)-infected groups with ovalbumin
248 (Ova)-induced AAD with or without steroid (DEX) and/or anti-miR-21 (Ant-21) or scrambled
249 (Scram) antagomir treatment (two experiments; n=6). Airway hyper-responsiveness (AHR) in
250 terms of airway resistance (Rn) **(F)** in response to increasing doses of methacholine (MCh),
251 and **(G)** 10 mg/mL of MCh (shows statistics at maximal dose from AHR curves [Fig E4, *F*]
252 was also determined (two experiments; n=5-10). Data are mean±SEM. *P<0.05; **P<0.01;
253 ***P<0.001; ****P<0.0001.