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Macrophage migration inhibitory factor promotes glucocorticoid resistance of neutrophilic inflammation in a murine model of severe asthma

Venkata Sita Rama Raju Allam,[1,2] Stelios Pavlidis,[3,4] Gang Liu,[5] Nazanin Zounemat Kermani,[3] Jennifer Simpson,[6] Joyce To,[7] Sheila Donnelly,[7] Yi-Ke Guo,[3] Philip M Hansbro,[5] Simon Phipps,[6] Eric F Morand,[8] Ratko Djukanovic,[9] Peter Sterk,[10] Kian Fan Chung,[11,12] Ian Adcock,[11,12] James Harris,[8] Maria B Sukkar[1]

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For numbered affiliations see end of article.

Correspondence to
Associate Professor Maria B Sukkar, Graduate School of Health, Faculty of Health, University of Technology Sydney, Sydney, New South Wales, Australia; maria.sukkar@outlook.com

JH and MBS contributed equally.

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ABSTRACT

Background Severe neutrophilic asthma is resistant to treatment with glucocorticoids. The immunomodulatory protein macrophage migration inhibitory factor (MIF) promotes neutrophil recruitment to the lung and antagonises responses to glucocorticoids. We hypothesised that MIF promotes glucocorticoid resistance of neutrophilic inflammation in severe asthma.

Methods We examined whether sputum MIF protein correlated with clinical and molecular characteristics of severe neutrophilic asthma in the Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED) cohort. We also investigated whether MIF regulates neutrophilic inflammation and glucocorticoid responsiveness in a murine model of severe asthma in vivo.

Results MIF protein levels positively correlated with the number of exacerbations in the previous year, sputum neutrophils and oral corticosteroid use across all U-BIOPRED subjects. Further analysis of MIF protein expression according to U-BIOPRED-defined transcriptomic-associated clusters (TACs) revealed increased MIF protein and a corresponding decrease in annexin-A1 protein in TAC2, which is most closely associated with airway neutrophilia and NLRP3 inflammasome activation. In a murine model of severe asthma, treatment with the MIF antagonist ISO-1 significantly inhibited neutrophilic inflammation and increased glucocorticoid responsiveness. Coimmunoprecipitation studies using lung tissue lysates demonstrated that MIF directly interacts with and cleaves annexin-A1, potentially reducing its biological activity.

Conclusion Our data suggest that MIF promotes glucocorticoid-resistance of neutrophilic inflammation by reducing the biological activity of annexin-A1, a potent glucocorticoid-regulated protein that inhibits neutrophil accumulation at sites of inflammation. This represents a previously unrecognised role for MIF in the regulation of inflammation and points to MIF as a potential therapeutic target for the management of severe neutrophilic asthma.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Macrophage migration inhibitory factor (MIF) is an immunomodulatory molecule that promotes neutrophil recruitment to the lung. It also acts as an endogenous inhibitor of glucocorticoid activity.

WHAT THIS STUDY ADDS

⇒ Data from the U-BIOPRED cohort and experimental severe asthma suggest that MIF reduces the biological activity of annexin-A1, a glucocorticoid-regulated protein that potently inhibits neutrophil accumulation at sites of inflammation.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ MIF represents a promising therapeutic target for the management of glucocorticoid-resistant neutrophilic inflammation in severe asthma. Further investigation of MIF activity in this context warrants further study.

INTRODUCTION

Asthma is a heterogeneous disorder associated with discrete endotypes that arise from distinct pathobiological mechanisms. Although the cellular and molecular pathways that underpin asthma endotypes are still emerging, several observable clinical phenotypes are evident. Patients with eosinophil-dominant airway inflammation respond well to treatment with glucocorticoids or monoclonal antibodies directed against type-2 cytokines.[1] However, approximately half of patients with asthma have non-eosinophilic disease that is not adequately managed with current therapies. Hence, there is an unmet treatment need for this subgroup of patients, particularly those with severe, non-eosinophilic asthma.[1–3]

Non-eosinophilic asthma is often associated with persistent neutrophilic inflammation, increased disease severity and resistance to treatment with glucocorticoids.[1–3] In clinical trials, the antibiotic azithromycin reduces asthma exacerbations and improves quality of life in those with severe non-eosinophilic disease.[4] It also reduces severity in a mouse model of severe asthma.[5] However, this therapy is associated with increased antibiotic resistance in respiratory bacteria, emphasising the need for alternative approaches.[1–3] While several

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neutrophil-directed therapies have been developed and trialled in asthmatic subjects,[6] none have progressed to clinical use due to limited efficacy.[1 2] Incomplete understanding of the mechanisms that regulate neutrophil recruitment and clearance from the lung is arguably the most significant barrier to the development of effective therapies for non-eosinophilic asthma.

Macrophage migration inhibitory factor (MIF) is an immunomodulatory molecule that promotes neutrophil recruitment to the lung.[7–10] Importantly, MIF also acts as an endogenous inhibitor of glucocorticoid activity and is thought to diminish the clinical response to glucocorticoid treatment in a number of rheumatic diseases.[11 12] Moreover, we and others have shown that MIF can mediate activation of the NLRP3 inflammasome,[13 14] a molecular complex that regulates the processing and secretion of interleukin (IL)-1 family cytokines implicated in severe glucocorticoid-resistant neutrophilic asthma.[15–18] Accordingly, we hypothesised that MIF promotes the development of neutrophilic inflammation and glucocorticoid resistance by augmenting NLRP3/IL-1β signalling and simultaneously antagonising the anti-inflammatory and/or proresolving effects of glucocorticoids in asthmatic subjects.

In this study, we examined the relationship between MIF protein abundance, neutrophilic inflammation, NLRP3 inflammasome activation and the expression of several glucocorticoid-inducible genes in the U-BIOPRED severe asthma cohort.[18] We also examined whether MIF inhibition protects against airway neutrophilia and IL-1β release and concomitantly increases glucocorticoid responsiveness in a murine model of severe asthma.[19]

METHODS

Analysis of U-BIOPRED data

The U-BIOPRED project was established to identify multidimensional phenotypes of asthma and new treatment targets using a combination of omics technologies and systems biology approaches.[20] We analysed data across all subjects in the U-BIOPRED adult cohort who provided sputum samples (n=120 subjects). Based on hierarchical clustering of differentially expressed genes between eosinophilic and non-eosinophilic subjects, three transcriptomic-associated clusters (TACs) were described.[18] These were divided in to 30 TAC1 subjects, 22 TAC2 subjects and 52 TAC3 subjects divided across 84 severe asthmatics and 20 mild–moderate asthmatics. All asthmatics were on >800 µg (fluticasone propionate equivalents) inhaled corticosteroid with 57% of TAC1 subjects; 36% of TAC2 subjects; and 25% of TAC3 subjects on oral or injectable corticosteroids. Protein expression in sputum samples was measured using the SOMAscan proteomic assay (SomaLogic, Boulder, Colorado, USA). Analysis of genes in sputum samples was performed using Array Studio software (Accession number: GSE76262, Omicsoft Corporation, Research Triangle Park, North Carolina, USA). Detailed methodology for protein and gene expression analysis has been described previously.[18]

Murine model of severe asthma

Female C57BL/6 mice (8 weeks of age) were purchased from the Australian Resource Centre (Perth, Australia) and housed under specific pathogen-free conditions. Mice were acclimatised for 1 week prior to the start of the experiment. On day 0, mice were sensitised to house dust mite (HDM) allergen (100 µg) (*Dermatophagoides pteronyssinus*; Citeq Biologics, Groningen, Netherlands) emulsified with an equal volume of complete Freund's adjuvant (CFA) (Sigma-Aldrich, St Louis, Missouri, USA) via subcutaneous injection. On day 14, mice were challenged with HDM (100 µg) via the intranasal route. Control mice were sensitised and challenged with phosphate buffered saline (PBS) only. Previous studies have shown that a single administration of the MIF inhibitor ISO-1 (4,5-dihydro-3-(4-hydroxyphenyl)-5-isoxaz oleacetic acid methyl ester) at a dose of 35 mg/kg inhibits airway neutrophilia induced by intratracheal administration of recombinant MIF in naïve mice.[8] We tested two dosing regimens. In the first, ISO-1 (35 mg/kg, Tocris Bioscience) or its vehicle (VEH, 5% dimethyl sulfoxide in PBS) were administered via intraperitoneal injection 30 min before HDM challenge (denoted HDM+ISO-1 and HDM+VEH, respectively), while in the second, it was administered 30 min before and 6 hours after HDM challenge (denoted HDM+ISO-1 bid). Dexamethasone (DEX, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione, 1 mg/kg, Sigma Aldrich) was administered 30 min prior to HDM challenge via oral gavage either alone or in combination with ISO-1 (denoted HDM+DEX and HDM+ISO-1+DEX, respectively). Randomisation was not used to allocate mice to control or treatment groups nor were potential confounders controlled for. A total of 96 mice were used with the following numbers of mice allocated to each treatment group: n=17 (PBS), n=17 (HDM), n=15 (HDM+VEH), n=18 (HDM+ISO-1), n=9 (HDM+ISO-1 bid), n=10 (HDM+DEX) and n=10 (HDM+ISO-1+DEX). Measurement of airway hyperreactivity (AHR) and analysis of tissue samples for all experimental endpoints is described in the online supplemental data.

A single researcher (VSRRA) was responsible for conducting all experimental procedures, outcome measurements (except histological analysis and immunoprecipitation studies) and data analysis: this person was aware of group allocation during all stages of the experiment. The researcher who performed the histological analysis (JS) was blind to group allocation. An a priori decision was made to exclude mice from experiments if they experienced >10% decrease in body weight during the course of the experiment. Prior studies using a similar murine model of experimental severe asthma indicated that a minimum sample size of four mice per group would be sufficient to achieve statistical significance for the primary outcome of neutrophil numbers in the airway lumen.[16]

This work was conducted in accordance with the ARRIVE guidelines.

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of the distribution of gene of interest expression in U-BIOPRED data. To determine the association of gene of interest expression with categorical variables, Kruskal-Wallis test with Dunn's posthoc multiple comparison analysis was used for non-normally distributed data, and pairwise Student's t-test was used for normally distributed data. P values were adjusted for multiple testing using the false discovery rate (FDR). Spearman's rank-order correlation was used to measure and test the association between gene of interest expression and numerical variables.

For studies in mice, all data were expressed as mean±95% CI, except for lung function measurments which were expressed as mean±SEM, and analysed using GraphPad Prism V.7. Normality of distribution of outcomes measured was examined by the Shapiro-Wilk test. For normally distributed data, between group differences were compared using one-way analysis of variance (ANOVA) with Bonferroni post hoc multiple comparison analysis. For non-normally distributed data, between-group differences were compared using the Kruskal-Wallis test with Dunn's

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post hoc multiple comparison analysis. Two-way ANOVA was conducted to compare in vivo methacholine (MCh) dose–response relationships with Bonferroni post hoc analysis of individual doses. Outliers in the data were identified using the outlier test in GraphPad Prism and excluded from the analysis.

RESULTS

MIF protein abundance correlates with airway neutrophilia and oral corticosteroid use in the U-BIOPRED cohort

We examined whether MIF protein abundance in sputum correlated with clinical characteristics of the U-BIOPRED cohort, a well-characterised cohort consisting of healthy volunteers, mild–moderate asthmatics, non-smokers with severe asthma and smokers with severe asthma.[16 18] MIF protein levels were positively correlated with the number of exacerbations in the previous year and sputum neutrophils and were negatively correlated with lung function impairment (forced expiratory volume in 1 s (FEV_1 % predicted) and sputum macrophages (figure 1A–D and online supplemental table S1). When each of these correlations were examined within individual subject groups, MIF protein levels were negatively correlated with sputum macrophages in non-smokers with severe asthma only (online supplemental figure S1A–D). Notably, oral corticosteroid use across all U-BIOPRED subjects was associated with significantly higher levels of MIF protein (figure 1E and online supplemental table S1), but this relationship was not observed when examined within the severe asthma groups individually (online supplemental figure S2A).

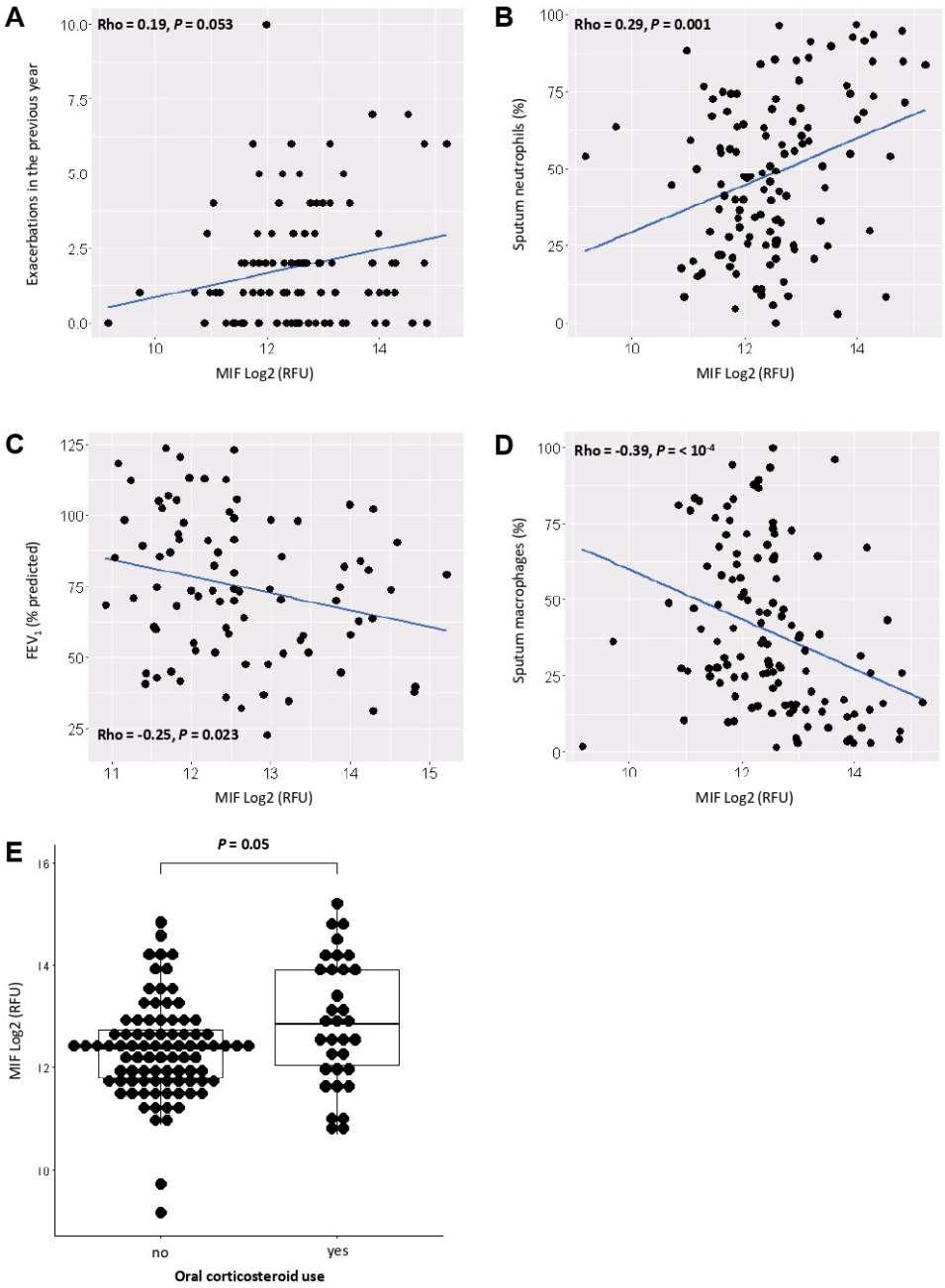

Figure 1 Correlations between MIF protein and selected clinical characteristics across all U-BIOPRED subjects. Associations were measured and tested using Spearman's rank-order correlation. P values were corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) procedure. MIF, macrophage migration inhibitory factor; RFU, relative fluorescent unit; Rho, Spearman's correlation coefficient.

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MIF is constitutively expressed and stored in intracellular pools and therefore does not require de novo synthesis for secretion, necessitating studies at the protein level to understand its function. Nevertheless, for completeness, we report that *MIF* mRNA negatively correlated with the degree of lung function impairment (FEV1 % predicted) and sputum neutrophils across all U-BIOPRED subjects (online supplemental table S1). Within individual subject groups, *MIF* mRNA was positively correlated with FEV1 % predicted in healthy volunteers and mild-moderate asthmatics. There was no significant correlation between *MIF* mRNA, the number of exacerbations in the previous year, sputum neutrophils or sputum macrophages within each of the U-BIOPRED subject groups (online supplemental figure S1E–H). Further, *MIF* mRNA was not associated with oral corticosteroid use across all U-BIOPRED subjects (online supplemental table S1) nor within the severe asthma groups (online supplemental figure S2B).

MIF protein abundance is increased in U-BIOPRED molecular phenotypes characterised by neutrophilic inflammation and NLRP3 inflammasome activation

Previous analyses from U-BIOPRED identified three TACs based on unsupervised hierarchical clustering of sputum mRNA expression data. Compared with TAC1 and TAC3, TAC2 is associated with neutrophilia and inflammasome activation.[18] In neutrophils, MIF colocalises with the S100A8/A9 heterodimeric complex which makes up ~40% of the cytosolic content.[21] Thus, to determine whether there is an association between neutrophilic inflammation and MIF expression, we examined sputum protein abundance of MIF and S100A9 measured by the SOMAscan Assay platform across the three TACs. S100A8 is not available on this platform. Compared with TAC1 and TAC3, subjects in TAC2 had significantly elevated levels of MIF and S100A9 protein (figure 2A,B). Analysis of gene expression data also revealed significantly higher levels of *S100A8* and *S100A9* in TAC2 compared with TAC1 and TAC3 (figure 2C,D). However, subjects in TAC2 had similar levels of *MIF* mRNA compared with subjects in TAC1 and significantly lower levels compared with TAC3 (online supplemental figure S3). MIF positively regulates the expression of the pattern-recognition receptor toll-like receptor 4 (TLR4),[22] which lies upstream of NLRP3 inflammasome activation.[23] Consistent with our previous analysis demonstrating a highly significant positive correlation between NLRP3 and sputum neutrophil counts in U-BIOPRED and other subjects,[16] *TLR4* and *NLRP3 (CIAS1)* mRNA were also significantly higher in TAC2 compared with TAC1 and TAC3 (figure 2E,F).

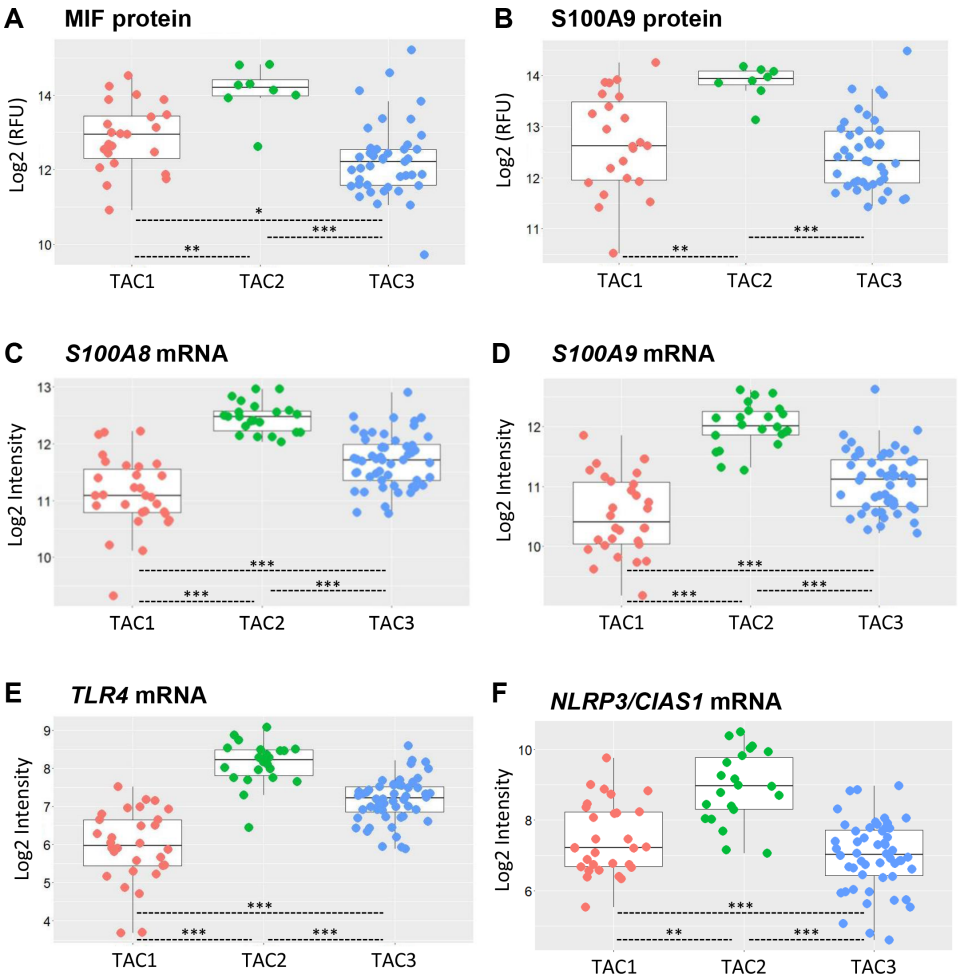

Figure 2 Expression of innate immune mediators in sputum according to TAC status. Protein levels of MIF (A) and S100A9 (B) measured by SOMAscan in log2 RFUs. Gene expression levels of S100A8 (C), S100A9 (D), TLR4 (E) and NLR family pyrin domain containing 3 (NLRP3) (F) measured by microarray and presented as $\log_2$ signal intensity values. Note: there was no complete overlap between sputum samples used for protein and mRNA measurements. *P<0.05, **P<0.01, ***P<0.001. MIF, macrophage migration inhibitory factor; RFU, relative fluorescent unit; TAC, transcriptomic-associated cluster; TLR4, toll-like receptor 4.

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To corroborate these findings, we examined correlations between MIF protein abundance and molecular markers of neutrophilic inflammation and NLRP3 inflammasome activation across all U-BIOPRED subjects (online supplemental table S2). MIF protein levels significantly positively correlated with S100A9 protein, *NLRP3* mRNA expression and IL-1β gene and protein expression (online supplemental table S2). For completeness, we also report that *MIF* mRNA expression significantly negatively correlated with *TLR4* and *IL1B* gene expression (online supplemental table S2). Collectively, these data suggest an underlying role for MIF in the development of neutrophilic responses in a subgroup of asthmatic individuals represented by the TAC2 molecular phenotype. However, there was no significant correlation between MIF protein abundance and sputum neutrophils in TAC2, possibly due to the small sample size (online supplemental figure S4A).

Increased MIF protein abundance is associated with reduced expression of the glucocorticoid-inducible proresolving mediator annexin-A1

To examine the relationship between MIF, airway neutrophilia and the glucocorticoid response at a molecular level, we compared the expression of three important glucocorticoid-regulated anti-inflammatory genes across the three TACs, namely, dual-specificity phosphatase 1 (DUSP1), the glucocorticoid-inducible leucine zipper (GILZ, encoded by *TSC22D3*) and annexin-A1. Compared with TAC1 and TAC3, subjects in TAC2 expressed significantly higher levels of *DUSP1* mRNA (figure 3A). *TSC22D3* was similarly expressed in TAC1 and TAC2, which had significantly higher levels compared with TAC3 (figure 3B). However, compared with TAC1 and TAC3, subjects in TAC2 expressed significantly lower levels of annexin-A1 mRNA and protein (figure 3C,D). Annexin-A1 is a potent proresolving mediator that limits neutrophil accumulation at sites of inflammation[24]; thus, lower levels of annexin-A1 in TAC2 might contribute to persistence of the neutrophilic response in this molecular subgroup. We therefore examined correlations between annexin-A1 gene and protein expression and sputum neutrophils within each of the three TACs. Annexin-A1 protein expression was negatively correlated with sputum neutrophils in both TAC1 and TAC2, while *ANXA1* mRNA was negatively correlated with sputum neutrophils in TAC1 only (online supplemental figure S4B, C).

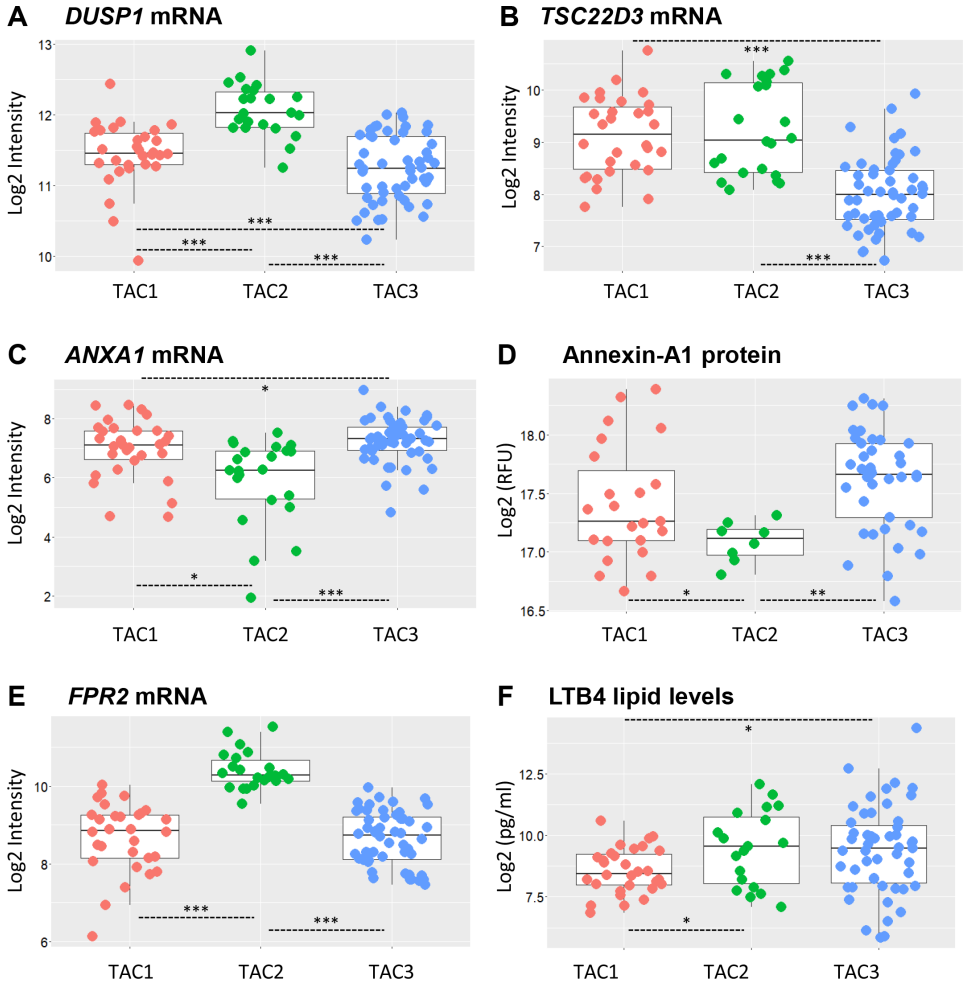

Figure 3 Expression of glucocorticoid-regulated genes and other mediators in sputum according to TAC status. Gene expression levels of DUSP1 (A), TSC22D3 (B), ANXA1 (C) and FPR2 (E) measured by microarray and presented as $\log_2$ signal intensity values. Protein levels of annexin-A1 (D) measured by SOMAscan in log2 RFUs. The level of LTB4 (F) was determined by ELISA and is presented as picogram per millilitre in each patient sample. Note: there was no complete overlap between sputum samples used for protein, mRNA and lipid measurements. *P<0.05, **P<0.01, ***P<0.001. ANXA1, denotes annexin A1 gene; DUSP1, dual-specificity phosphatase 1; FPR2, formyl peptide receptor 2; RFU, relative fluorescent unit; TAC, transcriptomic-associated cluster; TSC22D3, TSC22 domain family protein 3.

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Annexin-A1 signals via formyl peptide receptor 2 (FPR2), which is also the proresolving receptor for lipoxin A_4. Notably, FPR2 is reportedly expressed at lower levels in people with severe asthma[25]; thus, we also examined FPR2 expression across the three TACs. Compared with TAC1 and TAC3, TAC2 had significantly higher levels of *FPR2* mRNA, indicating that reduced annexin-A1 expression, rather than reduced signalling via this receptor is more likely to explain enhanced neutrophil infiltration in this molecular subgroup (figure 3E). Moreover, annexin-A1 mediates its anti-inflammatory effects, in part, by inhibiting the activation of cytosolic phospholipase A2, a rate limiting enzyme in eicosanoid synthesis. However, sputum levels of the potent neutrophil chemoattractant LTB4 were similar across TAC2 and TAC3, arguing against a specific role for this eicosanoid in mediating neutrophilic responses in TAC2 (figure 3F).

MIF acts as an endogenous inhibitor of glucocorticoid activity. Thus, lower levels of annexin-A1 coupled with evidence of increased MIF protein abundance in TAC2 could suggest that MIF sustains the neutrophilic response through inhibitory effects on glucocorticoid-regulated production of annexin-A1. Indeed, although numbers were small, *ANXA1* mRNA expression tended to be lower, while MIF protein levels tended to be higher in TAC2 subjects using oral corticosteroids compared with those who were not (online supplemental figure S5). Thus, we examined correlations between glucocorticoid-regulated genes and molecular markers of neutrophilic inflammation, as identified in TAC2, across all U-BIOPRED subjects. Consistent with the TAC-based analysis, *ANXA1* mRNA levels negatively correlated with MIF and S100A9 protein, *TLR4* and *NLRP3* mRNA and IL-1βmRNA and protein expression. Annexin-A1 protein expression negatively correlated with *NLRP3* mRNA and IL-1β protein only (online supplemental table S2). In contrast and as expected based on the TAC analysis, *DUSP1* mRNA levels positively correlated with MIF and S100A9 protein, *TLR4* mRNA and molecular markers of inflammasome activation. Similarly, *TSC22D3* positively correlated with MIF and S100A9 protein, *NLRP3* mRNA and IL-1β protein. Moreover, *DUSP1* and *TSC22D3* mRNA positively correlated with each other, while expression of each of these genes negatively correlated with either annexin-A1 mRNA or protein expression or both (online supplemental table S2).

Lower levels of annexin-A1 are associated with airway neutrophilia and oral corticosteroid use in U-BIOPRED subjects with severe asthma

Collectively, our aforementioned findings indicate that lower levels of annexin-A1 might potentially underlie airway neutrophilia and glucocorticoid resistance in severe asthma. Thus, we examined correlations between glucocorticoid-regulated genes and clinical characteristics of all U-BIOPRED subjects. Consistent with the TAC-based analysis, annexin-A1 gene and protein expression negatively correlated with sputum neutrophils. They were also negatively correlated with blood eosinophils and other markers of eosinophilic inflammation, including FeNO and serum periostin. Annexin-A1 gene and protein expression positively correlated with sputum macrophages (online supplemental table S1). In contrast to annexin-A1, *DUSP1* and *TSC22D3* mRNA both positively correlated with sputum and blood neutrophils, again reflecting outcomes of the TAC-based analysis. There were also positive correlations between both *DUSP1* and *TSC22D3* mRNA with sputum and blood eosinophils and total IgE, although both were negatively correlated with sputum macrophages. Of note, both *DUSP1* and *TSC22D3* mRNA were associated with severe asthma and features of severe disease, including oral corticosteroid use, the number of exacerbations in the previous year and the degree of lung function impairment (online supplemental table S1).

Finally, to extend the aforementioned findings, we sought to investigate correlations between annexin-A1 gene and protein expression and selected clinical characteristics within individual U-BIOPRED subject groups (online supplemental figures S6 and S7). Notably, annexin-A1 protein was negatively correlated with sputum neutrophils in the severe asthma groups only, while *ANXA1* mRNA was negatively correlated with sputum neutrophils in mild–moderate asthmatics and non-smokers with severe asthma (online supplemental figure S6B,F). On the other hand, annexin-A1 protein was positively correlated with sputum macrophages in both smokers and non-smokers with severe asthma, while *ANXA1* mRNA was positively correlated with sputum macrophages in non-smokers with severe asthma only (online supplemental figure S6D,H). Significantly, in the group of smokers with severe asthma, compared with those who were not using oral corticosteroids, those who were using oral corticosteroids had lower levels of annexin-A1 protein (online supplemental figure S7B).

MIF inhibition abrogates airway neutrophilia and increases glucocorticoid responsiveness in experimental severe asthma

Considering our aforementioned findings, we investigated whether MIF inhibition attenuates neutrophilic responses and increases glucocorticoid responsiveness in a murine model of experimental severe asthma. C57BL/6 mice were sensitised with HDM in the presence of CFA, then 14 days later challenged with HDM.[19] This protocol elicited a mixed granulocytic response without significantly modulating macrophage numbers in the bronchoalveolar lavage fluid (BALF) (figures 4A and 5A and online supplemental figure S8). A single dose of the MIF inhibitor ISO-1 given 30 min prior to HDM challenge had no significant effect on the numbers of total cells infiltrating the airway lumen, including eosinophils and neutrophils (figure 4A). It also had no significant effect on allergen-induced increases in airway contraction to methacholine (referred to as AHR, figure 4B). However, when given 30 min prior to and 6 hours post HDM challenge, ISO-1 significantly decreased airway neutrophil numbers and AHR (figure 4A,B). Meanwhile, a single dose of the glucocorticoid dexamethasone (1 mg/kg) given 30 min prior to HDM challenge reduced eosinophil infiltration and AHR but had no significant effect on neutrophil infiltration, indicating glucocorticoid resistance of the neutrophilic response (figure 5A,B). However, combined administration of dexamethasone and ISO-1 30 min prior to HDM challenge was associated with a striking reduction in airway neutrophil numbers and further inhibition of AHR, indicating that MIF inhibition increases sensitivity to the anti-inflammatory effects of glucocorticoids (figure 5A,B).

To further evaluate the airway inflammatory response, we examined cellular tissue infiltration by H&E staining and airway mucus production by periodic acid–Schiff staining. Consistent with the aforementioned findings, a single dose of ISO-1 given 30 min prior to allergen challenge had no effect on lung inflammation scores, while two doses significantly reduced lung inflammation scores. Similarly, treatment with dexamethasone alone had no effect on lung inflammation scores, whereas combined treatment with dexamethasone and ISO-1 significantly inhibited lung inflammation scores (figure 6A). While we observed

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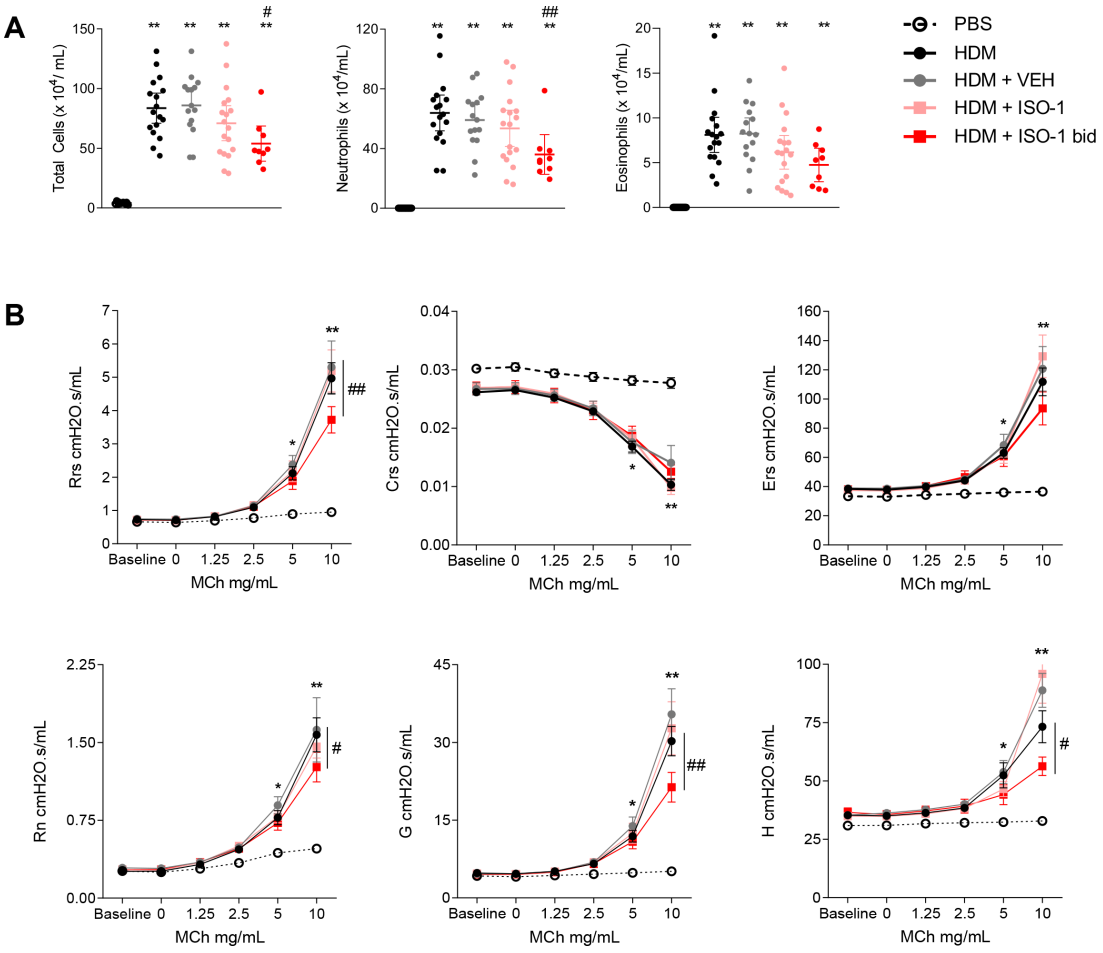

Figure 4 ISO-1 inhibits neutrophilic inflammation and AHR in experimental severe asthma. Mice were treated with VEH or with ISO-1 30 min prior to HDM challenge (HDM+VEH and HDM+ISO-1, respectively). Alternatively, mice were treated with two doses of ISO-1 given 30 min prior to and 6 hours post HDM challenge (HDM+ISO-1 bid). Control mice were treated with PBS only. (A) Total cells, neutrophils and eosinophils were measured in BALF. (B) Total respiratory system resistance (Rrs), compliance (Crs) and elastance (Ers), proximal airway resistance (Rn) and distal tissue dampening (G) and elastance (H) were measured using forced oscillation technique. Data represent mean±95% CI (A) or mean±SEM (B). *P<0.05, **P<0.01 vs PBS group. #P<0.05, ##P<0.01 vs HDM group. n=7–18 mice per group. AHR, airway hyper-reactivity; BALF, bronchoalveolar lavage fluid;HDM, house dust mite; MCh, methacholine; PBS, phosphate buffered saline; VEH, vehicle.

a significant increase in airway mucus production following allergen challenge in this model, airway mucus scores were not significantly altered under any of the treatment conditions tested, likely reflecting the variability in these data (figure 6B).

MIF inhibition synergises with glucocorticoid-mediated suppression of inflammatory protein expression in experimental severe asthma

Recent studies have shown that MIF promotes NLRP3 inflammasome assembly and IL-1β secretion in macrophages,[13 14] which is potentially corroborated by our findings demonstrating increased MIF protein abundance in TAC2 subjects, characterised by NLRP3 inflammasome activation (figure 2F). Thus, we investigated whether the protective effects of MIF inhibition in experimental severe asthma were associated with concomitant inhibition of IL-1β secretion. Treatment of mice with ISO-1 at the dose which inhibited airway neutrophilia and AHR had no effect on lung NLRP3 protein levels or IL-1β release in BALF (figure 7A,B). To identify other potential pathways that might be impacted by MIF inhibition, we measured 21 analytes in the BALF, including type 1, type 2 and type 17 mediators. Treatment of mice with ISO-1 alone 30 min prior to HDM challenge had no modulatory effect on mediator release (figure 8A–I and online supplemental table S3). In contrast, treatment with ISO-1 30 min prior to and 6 hours post allergen challenge led to a significant reduction in the concentrations of S100A8 and CCL11 (eotaxin-1), consistent with the observed reduction in neutrophil and eosinophil numbers at this dose, respectively (figure 8A,B and online supplemental table S3). However, this treatment regimen had no significant effect on the secretion of other mediators measured (figure 8C–I and online supplemental table S3).

Treatment of mice with dexamethasone alone had no significant effect on NLRP3 protein levels or IL-1β release (figure 7A,B), suggesting glucocorticoid resistance of NLRP3 inflammasome signalling in experimental severe asthma, as previously demonstrated.[16 17] Moreover, with the exception of S100A8 and IL-1α (figure 8A,D) treatment of mice with dexamethasone alone had no effect on the secretion of all other mediators measured (figure 8B,C,E–I, online supplemental table S3). However, combined treatment with dexamethasone and ISO-1 30 min prior to HDM challenge led to significant inhibition of NLRP3 protein levels and IL-1β secretion (figure 7A,B) and several additional mediators, including CCL11, CCL3, TNF-α, IFN-γ, IL-33 and granulocyte macrophage-colony stimulating

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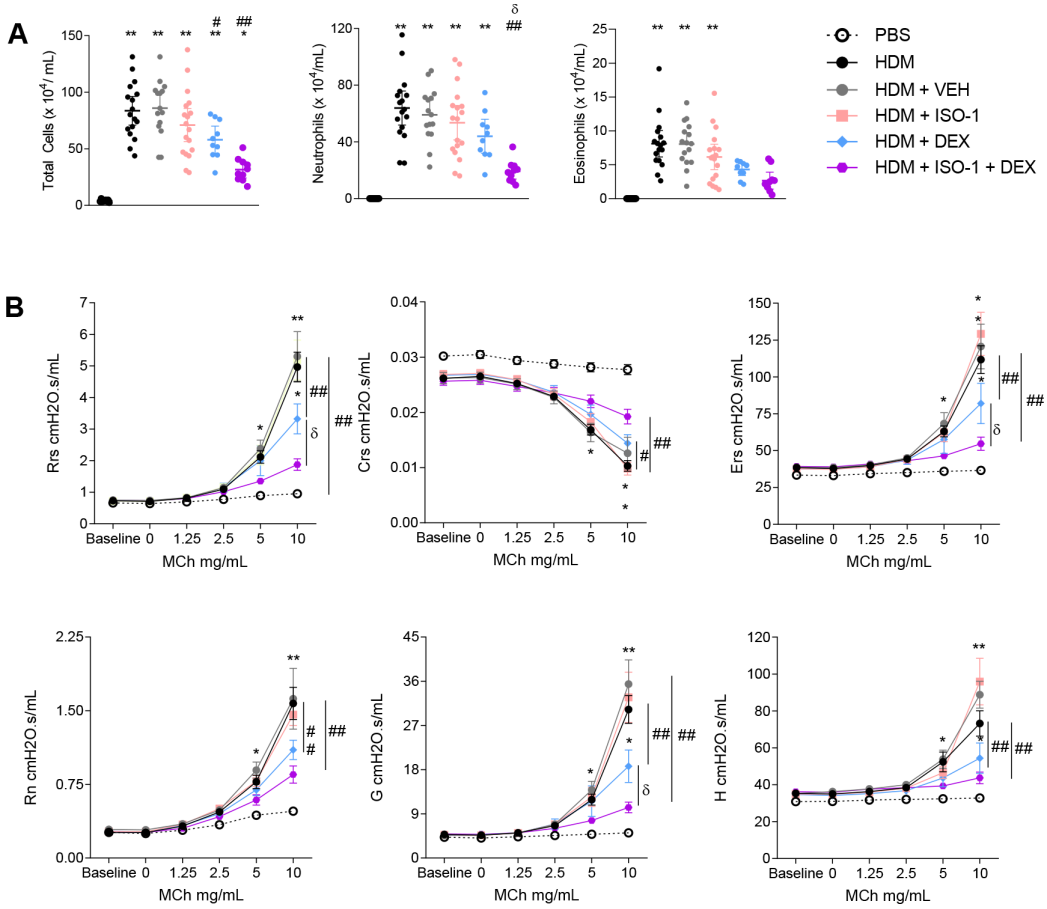

Figure 5 ISO-1 renders glucocorticoid-insensitive neutrophilic inflammation sensitive to the anti-inflammatory effects of glucocorticoids in experimental severe asthma. Mice were treated with VEH, ISO-1, DEX, or ISO-1 and DEX 30 min prior to HDM challenge. Control mice were treated with PBS only. (A) Total cells, neutrophils and eosinophils were measured in BALF. (B) Total respiratory system resistance (Rrs), compliance (Crs) and elastance (Ers), proximal airway resistance (Rn) and distal tissue dampening (G) and elastance (H) were measured using forced oscillation technique. Data represent mean±95% CI (A) or mean±SEM (B). *P<0.05, **P<0.01 vs PBS group. #P<0.05, ##P<0 .01 vs HDM group. δP<0.05 vs HDM+DEX group. n=7–18 mice per group. BALF, bronchoalveolar lavage fluid; DEX, dexamethasone; HDM, house dust mite; MCh, methacholine; PBS, phosphate buffered saline; VEH, vehicle.

factor (GM-CSF) (figure 8B,C,E–H and online supplemental table S3), further indicating that MIF antagonism increases sensitivity to the anti-inflammatory effects of glucocorticoids in experimental severe asthma. Importantly, while allergen challenge in this model was associated with significant release of MIF in BALF, treatment of mice with dexamethasone either alone or together with ISO-1 had no effect on BALF MIF concentrations (figure 8I and online supplemental table S3), indicating that enhanced glucocorticoid efficacy in the context of MIF inhibition was not due to an inhibitory effect of dexamethasone on MIF secretion.

MIF inhibition protects against annexin-A1 cleavage in experimental severe asthma

Subjects in TAC2 presented with higher levels of MIF and lower levels of annexin-1 mRNA and protein. Thus, we examined whether MIF augments the neutrophilic response by inhibiting the expression and/or activity of annexin-A1. Following cellular activation, annexin-A1 is externalised on the cell surface. However, within this microenvironment, annexin-A1 is cleaved at its N-terminal region by neutrophil-derived proteases, resulting in a loss of potency.[26 27] Consequently, we used immunoblotting to examine expression levels of both the full-length and cleaved protein. We detected robust levels of full-length 37 kDa annexin-A1 protein in lung tissue lysates under basal conditions. There was no change in its abundance following allergen challenge or treatment with ISO-1 and/or dexamethasone (figure 9A). In the BALF, full-length annexin-A1 was not detected; however, we detected a protein band at ~33 and ~28 kDa in allergen-challenged but not PBS-challenged mice, indicating that annexin-A1 externalisation and cleavage predominantly occurs in the airway lumen (figure 9A). Thus, we quantified the overall extent of annexin-A1 cleavage in BALF under all experimental conditions (figure 9B). To do this, we performed densitometric analysis on each of the ~33 kDa and ~28 kDa protein bands separately and added the values together. This analysis revealed a significant increase in the total amount of cleaved annexin-A1 in BALF following HDM challenge. Strikingly, administration of ISO-1 30 min prior to and 6 hours post allergen was associated with a significant reduction in the total amount of cleaved annexin-A1. Moreover, while administration of either ISO-1 or dexamethasone alone 30 min prior to allergen challenge did not significantly alter the total amount of cleaved annexin-A1, the combined administration of ISO-1 and dexamethasone ablated the presence of cleaved annexin-A1 products (figure 9B).

These findings suggest that MIF contributes to the externalisation and/or cleavage of annexin-A1 in experimental severe asthma. To determine if this occurred through a direct interaction,

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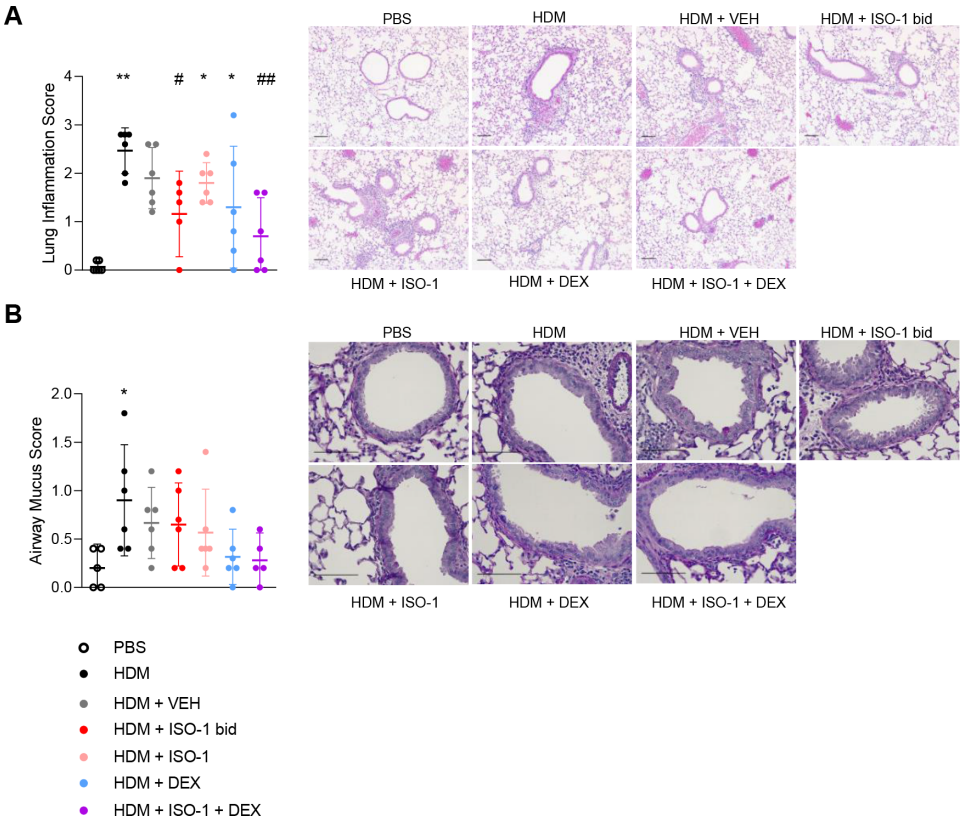

Figure 6 ISO-1 renders glucocorticoid-insensitive tissue inflammation sensitive to the anti-inflammatory effects of glucocorticoids in experimental severe asthma. Mice were treated with VEH, ISO-1, DEX or ISO-1 and DEX 30 min prior to HDM challenge. Alternatively, mice were treated with two doses of ISO-1 given 30 min prior to and 6 hours post HDM challenge (HDM+ISO-1 bid). Control mice were treated with PBS only. Lung inflammation (A) and mucus production (B) were assessed by H&E and PAS staining, respectively. Data represent mean±95% CI. *P<0.05, **P<0.01 vs PBS group. #P<0.05, ##P<0.01 vs HDM group. n=5–6 mice per group. Representative images for H&E (×10 original magnification) and PAS (×40 magnification) are shown. Scale bars, 60 µm. DEX, dexamethasone; HDM, house dust mite; H&E, hematoxylin and eosin; PAS, periodic acid–Schiff;PBS, phosphate buffered saline; VEH, vehicle.

we immunoprecipitated MIF from lung tissue lysates of PBS and HDM treated mice and performed an immunoblot to determine whether MIF and annexin-A1 were components of the same protein complex. We detected a single protein band at ~12 kDa in PBS and HDM challenged mice, but not IgG control samples, confirming successful immunoprecipitation of MIF protein (figure 9C and online supplemental figure S9). Moreover, we detected the presence of a~37 kDa annexin-A1 protein band in these samples, indicating that MIF and annexin-A1 directly interact. Notably, there was a marked increase in the intensity of the 28 kDa band, relative to the 37 kDa band, in HDM but not PBS treated mice, indicating that MIF promotes the cleavage of annexin-A1 in experimental severe asthma. Finally, to examine whether MIF inhibition protects against annexin-A1 cleavage by interfering with the MIF–annexin-A1 interaction, we immunoprecipitated MIF from lung tissue lysates of mice treated

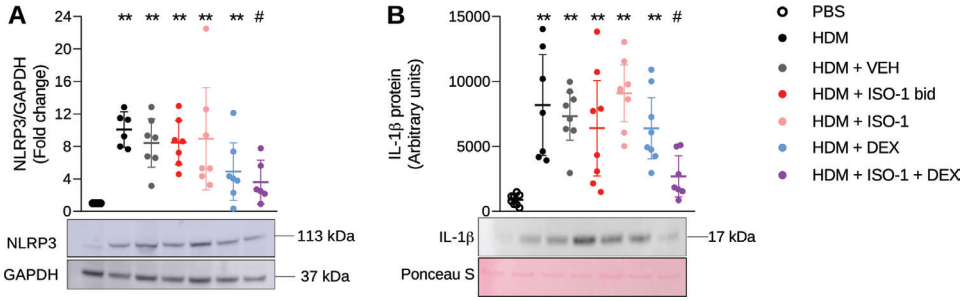

Figure 7 ISO-1 renders glucocorticoid-insensitive NLRP3 inflammasome activation sensitive to the anti-inflammatory effects of glucocorticoids in experimental severe asthma. Mice were treated with VEH, ISO-1, DEX or ISO-1 and DEX 30 min prior to HDM challenge. Alternatively, mice were treated with two doses of ISO-1 given 30 min prior to and 6 hours post HDM challenge (HDM+ISO-1 bid). Control mice were treated with PBS only. (A) NLRP3 protein measured in lung tissue lysates by immunoblotting. Data were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold change relative to the PBS group. (B) IL-1β protein measured in BALF by immunoblotting. Data represent mean±95% CI. **P<0.01 vs PBS group. #P<0.05 vs HDM group. n=6–8 mice per group. BALF, bronchoalveolar lavage fluid; DEX, dexamethasone; HDM, house dust mite; IL, interleukin; PBS, phosphate buffered saline; VEH, vehicle.

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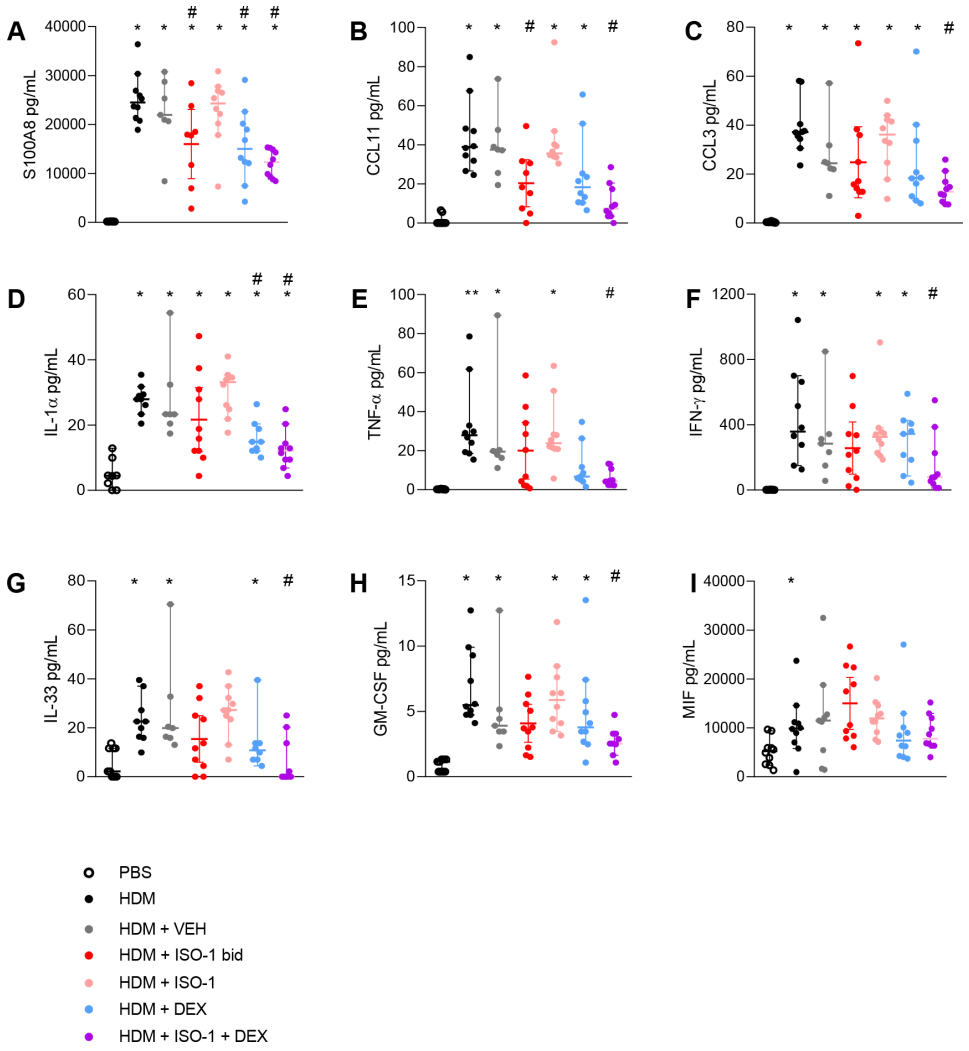

Figure 8 ISO-1 renders glucocorticoid-insensitive inflammatory mediator release sensitive to the anti-inflammatory effects of glucocorticoids in experimental severe asthma. Mice were treated with VEH, ISO-1, DEX or ISO-1 and DEX 30 min prior to HDM challenge. Alternatively, mice were treated with two doses of ISO-1 given 30 min prior to and 6 hours post HDM challenge (HDM+ISO-1 bid). Control mice were treated with PBS only. The concentration of cytokines and chemokines in BALF were determined using a customised Magnetic Luminex assay using the MAGPIX System. Data represent mean±95% CI. *P<0.05, **P<0.01 vs PBS and #P<0.05 vs HDM. n=7–10 mice per group. BALF, bronchoalveolar lavage fluid; DEX, dexamethasone; HDM, house dust mite; PBS, phosphate buffered saline; VEH, vehicle.

with a single dose of ISO-1 or dexamethasone alone or their combination 30 min prior to HDM challenge. Treatment with either dexamethasone, ISO-1 or the combination of ISO-1 and dexamethasone ablated annexin-A1 cleavage at the tissue level (figure 9D and online supplemental figure S10).

DISCUSSION

We provide evidence of increased MIF protein abundance and reduced annexin-A1 gene and protein expression in the neutrophil-associated TAC2 molecular phenotype of asthmatic subjects in the U-BIOPRED cohort. We also demonstrated that MIF promotes lung neutrophil recruitment and proteolytic cleavage of annexin-A1, potentially attenuating its biological activity in a mouse model of severe asthma in vivo. Furthermore, MIF inhibition rendered the neutrophilic response sensitive to glucocorticoid inhibition in this model. Together, our findings suggest that MIF promotes glucocorticoid resistance of the neutrophilic response by limiting the anti-inflammatory and proresolving functions of the glucocorticoid-regulated protein annexin-A1. While this mechanism may potentially be most relevant to the TAC2 molecular phenotype, further studies are needed to establish whether this mechanism underpins, or is associated with, one or more molecular phenotypes (or endotypes) of severe asthma.

The MIF inhibitor ISO-1 (35 mg/kg) inhibits lung neutrophil recruitment induced by intratracheal administration of recombinant MIF in naïve mice.[8] This was associated with reduced lung CXCL1 and CXCL2 levels, indicating that MIF induces the release of proneutrophilic chemokines.[8] In contrast, in experimental severe asthma, administration of ISO-1 (35 mg/kg) 30 min prior to and 6 hours post allergen challenge protected against airway neutrophilia and AHR without significantly affecting the levels of CXCL1 and other mediators including IL-1β, IL-1α, IL-6, TNF-α and IL-17A involved in lung neutrophil recruitment. However, ISO-1 inhibits NLRP3 activation, IL-1β, IL-6 and TNF-α secretion at 10-fold lower doses in other experimental models.[28] We previously reported that sputum levels of IL-1β in patients with severe asthma correlate with NLRP3, NLRP1 and NLRC4.[16] We have demonstrated a highly specific role for MIF in activating the NLRP3 inflammasome[13] and the

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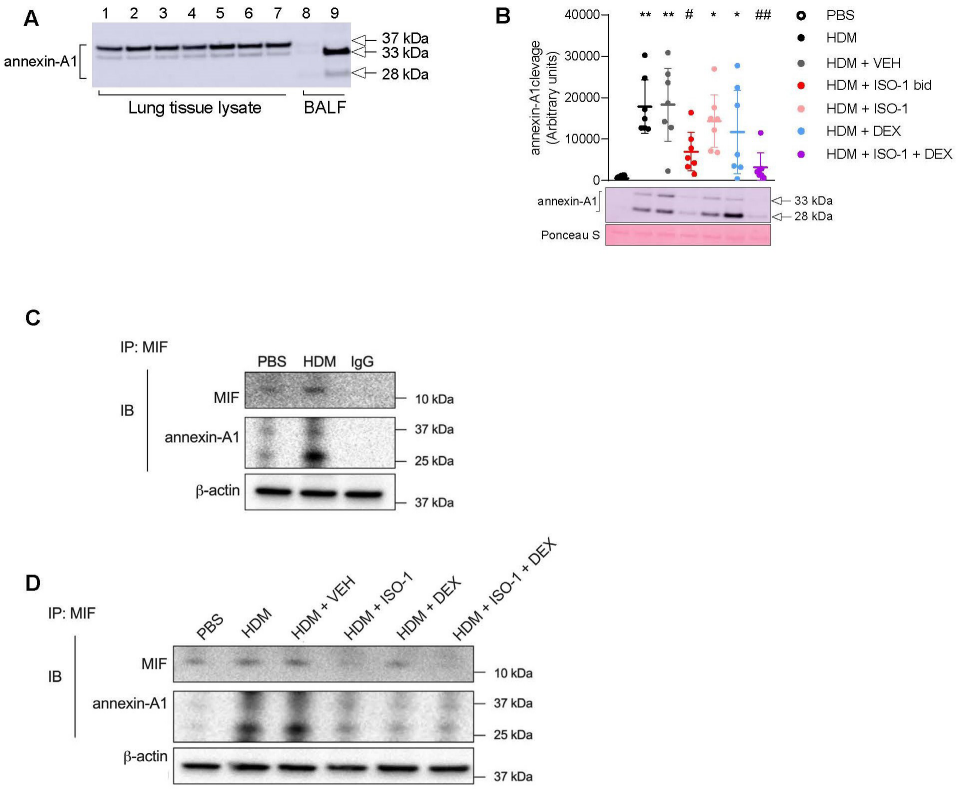

Figure 9 ISO-1 protects annexin-A1 against proteolytic cleavage in experimental severe asthma. Mice were treated with VEH, ISO-1, DEX or ISO-1 and DEX 30 min prior to HDM challenge. Alternatively, mice were treated with two doses of ISO-1 given 30 min prior and 6 hours post HDM challenge (HDM+ISO-1 bid). Control mice were treated with PBS only. (A) Annexin A1 protein expression was measured by immunoblotting in lung tissue lysate (lanes 1–7) and BALF (lanes 8 and 9). Lanes 1 and 8: PBS, lanes 2 and 9: HDM, lane 3: HDM+VEH, lane 4: HDM+ISO-1 bid, lane 5: HDM+ISO-1, lane 6: HDM+DEX, lane 7: HDM+ISO-1+DEX. Image representative of data from four mice. (B) Sum total of annexin-A1 cleavage products (33 and 28 kDa) measured in BALF by immunoblotting. Ponceau S staining was used to confirm equal protein loading for measurements made in cell-free BALF. Data represent mean±95% CI. (C,D) Immunoblots demonstrating MIF, full-length ~37 kDa annexin-A1 and cleaved ~28 kDa annexin-A1 protein bands in immunoprecipitated MIF–protein complexes from lung tissue lysates. MIF and annexin-A1 were not detected in protein complexes immunoprecipitated with an isotype control antibody confirming antibody specificity, as seen in (C).β-actin was used to confirm equal protein loading. *P<0.05, **P<0.01 vs PBS group. #P<0.05, ##P<0.01 vs HDM group. n=7 mice per group. DEX, dexamethasone; HDM, house dust mite; MIF, macrophage migration inhibitory factor; PBS, phosphate buffered saline; VEH, vehicle.

lack of effect of ISO-1 on IL-1β release in our model may suggest that other inflammasomes mediate IL-1β secretion. Further studies with different dosing regimens for ISO-1, different MIF inhibitors and/or *Mif*$^{-/-}$ mice are warranted to confirm this.

In line with our findings, endothelial cell-specific deletion of MIF significantly protects against lipopolysaccaride-induced airway neutrophilia in mice without impacting airway levels of CXCL1, IL-1α and IL-1β. Inhibition of airway neutrophilia was due to reduced relaxation of perivascular pericytes and reduced neutrophil transmigration across the vessel wall.[10] Inhibition of airway neutrophilia by ISO-1 in our study was associated with relatively selective inhibition of S100A8. Since the S100A8/A9 heterocomplex regulates neutrophil rolling and adhesion to the vessel wall via autocrine activation of TLR4 signalling[29], increased MIF, S100A8/A9 and TLR4 expression in patients with TAC2 asthma point to the existence of a previously unrecognised MIF-S100A8A/9-TLR4 inflammatory axis that may play a crucial role in the development of the airway neutrophilic response in severe asthma.

Airway neutrophilia in severe asthma may be due to impairments in the active resolution of inflammation.[30–32] Annexin-A1 is a mediator of the resolution of inflammation and inhibits neutrophil transmigration across the endothelium, and promotes neutrophil clearance from tissues by inducing apoptosis and macrophage efferocytosis.[24] Under basal conditions, annexin-A1 is predominantly intracellular; however, on cell activation, it is externalised onto the cell surface where it is susceptible to proteolytic cleavage by neutrophil-derived proteinase 3 (PR3).[33] Two cleavage products are seen in the BALF 24 hours post allergen challenge in experimental severe asthma, the classical ~33 kDa product and an additional ~28 kDa product.[34 35] The latter may reflect additional proteolysis under conditions of severe inflammation, as it is not detected in a model of mild–moderate ovalbumin-induced asthma.[36] PR3-resistant mutants induce longer lasting anti-inflammatory effects and more rapid disease resolution in experimental models of inflammation, indicating that proteolytic cleavage terminates the proresolving activities of annexin-1.[26 27] Moreover, annexin-A1 proteolysis by specific proteases generates proteolytic fragments that promote neutrophil transendothelial migration.[37] In our study, inhibition of airway neutrophilia in response to ISO-1 was associated with a concomitant reduction in the overall extent of annexin-A1 cleavage, indicating an active role for annexin-A1 cleavage in the neutrophilic response.

We identify a previously unknown role for MIF in annexin-A1 cleavage involving direct protein–protein interactions. MIF is a molecular chaperone[14] that may potentially induce conformational changes promoting annexin-A1 externalisation and

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susceptibility to proteolytic cleavage. Patients with severe asthma have raised levels of IFN-γ and lower levels of secretory leucocyte protease inhibitor (SLPI) in their airway epithelium. IFN-γ augments AHR through inhibition of SLPI,[38] and SLPI protects annexin-A1 from proteolysis.[39] These data support increased annexin-A1 cleavage in severe asthma and our proposed mechanism.

Although there were greater numbers of airway neutrophils in severe asthma versus non-severe asthma, there was no difference in BALF annexin-A1 protein levels between these two groups in the SARP-3 cohort.[31] The differences in findings may be due to the different methods used to measure annexin-A1. However, reduced annexin-A1 expression at both the gene and protein level in TAC2 and overall negative correlation between annexin-A1 protein and sputum neutrophils in patients with severe asthma suggest it is unlikely to be a spurious finding. Moreover, while our experimental findings indicate that MIF acts at the level of annexin-A1 cleavage, evidence of reduced annexin-A1 gene and protein expression in TAC2 suggests that MIF may also inhibit annexin-A1 gene and protein expression. In support, exogenous MIF downregulates annexin-A1 protein in RAW 264.7 macrophages.[40] Further studies should examine effects of MIF on annexin-A1 mRNA and protein expression and post-translational modifications in the context of the airway inflammatory response.

MIF also inhibits glucocorticoid-induced expression of annexin-A1[40] and other glucocorticoid-regulated genes, namely, GILZ[41] and DUSP-1,[41 42] in certain cell types in vitro. We previously reported that annexin-A1 is required for glucocorticoid-mediated upregulation of GILZ and DUSP-1 in macrophages and fibroblasts, respectively, indicating that annexin-A1 activation lies upstream of these proteins.[43–45] Consistent with this, mice deficient in GILZ respond to glucocorticoids and resolve lung neutrophilic inflammation due to endogenous annexin-A1 activity.[46] Our findings from U-BIOPRED, however, provide clear evidence of divergent regulation of annexin-A1 and GILZ/DUSP1 in asthma. In addition, contrary to evidence that MIF inhibits GILZ and DUSP1 expression in specific cell types in vitro, we observed significant positive correlations between MIF protein abundance and these genes in asthmatic subjects. Expression levels of *TSC22D3* and *DUSP1* were also positively correlated with oral corticosteroid use, suggesting these pathways are most likely intact. These findings highlight the need for further investigation of the molecular interactions between MIF and major effectors of the glucocorticoid response in severe asthma.

In conclusion, we demonstrate that reduced glucocorticoid responsiveness is related to endogenous mechanisms that potentially impair the proresolving activities of glucocorticoids. Thus, MIF impairs glucocorticoid-mediated resolution of the neutrophilic response by inhibiting the expression and activity of annexin-A1 (figure 10). Complete characterisation of annexin-A1 cleavage and its functional significance is an important area for further investigation as this will establish whether excessive and/or dysregulated annexin-A1 cleavage is significant in the persistence of airway neutrophilia in severe asthma.

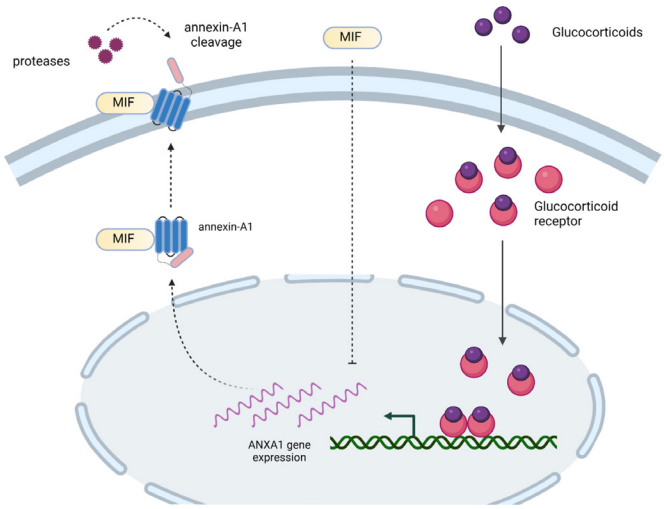

Figure 10 MIF impairs glucocorticoid responses by targeting annexin-A1. Our collective findings suggest that MIF impairs glucocorticoid-mediated resolution of the neutrophilic response in severe asthma by inhibiting the expression and activity of annexin-A1. Potential mechanisms include inhibition of glucocorticoid-mediated induction of annexin-A1 gene expression. Additionally, it is possible that MIF directly binds to annexin-A1 to promote externalisation and cleavage of the N-terminal domain by proteases. Image created with BioRender. ANXA1 denotes annexin-A1 gene; MIF, macrophage migration inhibitory factor.

Author affiliations
[1]Graduate School of Health, Faculty of Health, University of Technology Sydney, Sydney, New South Wales, Australia
[2]Department of Medical Biochemistry and Microbiology, Biomedical Centre, Uppsala University, Uppsala, Sweden
[3]Data Science Institute, Imperial College London, London, UK
[4]Computational Sciences, Janssen Research and Development, High Wycombe, UK
[5]Centre for Inflammation, Centenary Institute and University of Technology Sydney, Sydney, New South Wales, Australia
[6]Respiratory Immunology, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia
[7]School of Life Sciences, Faculty of Science, University of Technology Sydney, Sydney, New South Wales, Australia
[8]Centre for Inflammatory Diseases, Monash University, Melbourne, Victoria, Australia
[9]NIHR Southampton Biomedical Research Centre, Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK
[10]Department of Respiratory Medicine, Amsterdam University Medical Centres, University of Amsterdam, Amsterdam, The Netherlands
[11]Airways Disease, National Heart & Lung Institute, Imperial College London, London, UK
[12]Biomedical Research Unit, Royal Brompton & Harefield NHS Trust, London, UK

Contributors Guarantor: MBS. Concept and design: VSRRA, KFC, IM, JH and MBS. Acquisition of data, analysis and interpretation: VSRRA, SPa, GL, NZK, JS, JT, SD, Y-KG, PMH, SPh, EFM, RD, PS, KFC, IM, JH and MBS. Drafting of the manuscript: VSRRA, KFC, IM, EFM, JH and MBS. All authors revised the manuscript critically.

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Union Innovative Medicines Initiative during the conduct of the study. RD reports receiving fees for lectures at symposia organised by Novartis, AstraZeneca and TEVA; consultation for TEVA and Novartis as member of advisory boards; and participation in a scientific discussion about asthma organised by GlaxoSmithKline. RD is a cofounder and current consultant and has shares in Synairgen, a University of Southampton spin-out company.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the ethics committees of each of the 16 clinical recruiting centres (NCT01982162). The participants gave informed consent to participate in the study before taking part. All procedures were performed at the University of Technology Sydney (UTS) under protocols compliant with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the UTS Animal Care and Ethics Committee.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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