Title

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

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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 hypothesized 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 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. Co-immunoprecipitation 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.

Key Messages

What is the key question?

Does the immunomodulatory protein macrophage migration inhibitory factor (MIF) promote glucocorticoid resistance of neutrophilic inflammation in severe asthma?

What is the bottom line?

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.

Why read on?

Pharmacological inhibition of MIF protects against neutrophilic inflammation and enhances glucocorticoid responsiveness in an experimental model of severe asthma, thus MIF represents a promising therapeutic target for the management of severe neutrophilic asthma.

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 sub-group 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, emphasizing the need for alternative approaches.[1-3] While several 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 IL-1 family cytokines, which are implicated in severe glucocorticoid-resistant neutrophilic asthma.[15 16 17 18] Accordingly, we hypothesized that MIF promotes the development of neutrophilic inflammation and glucocorticoid resistance by augmenting NLRP3/IL-1β signaling and simultaneously antagonizing the anti-inflammatory and/or pro-resolving 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 multi-dimensional phenotypes of asthma and new treatment targets using a combination of omics technologies and systems biology approaches.[20] This study was approved by the Ethics Committees for each of the 16 clinical recruiting centres (NCT01982162). All participants gave written and signed informed consent. We analyzed 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 >800μg (FP equivalents) inhaled corticosteroid with 57% of TAC1 subjects on oral or injectable corticosteroids, 36% of TAC2 subjects on oral or injectable corticosteroids and 25% of TAC3 patients were on oral or injectable corticosteroids. Protein expression in sputum samples was measured used the SOMAscan proteomic assay (SomaLogic Inc., Boulder, CO). Analysis of genes in sputum samples was performed using Array Studio software (Accession number: GSE76262, Omicsoft Corporation, Research Triangle Park, NC, 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. 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 approved by the UTS Animal Care and Ethics Committee. Mice were acclimatized for 1 week prior to the start of the experiment. On day 0, mice were sensitized to HDM allergen (100μg) (Dermatophagoides pteronyssinus, Greer Laboratories, Lenoir, NC, USA) emulsified with an equal volume of complete Freund's adjuvant (CFA) (Sigma-Aldrich, St Louis, MO, USA) via subcutaneous injection. On day 14, mice were challenged with HDM (100μg) via the intranasal route. Control mice were sensitized and challenged with PBS only. Previous studies have shown that a single administration of the MIF inhibitor ISO-1 (4,5-Dihydro-3-(4-hydroxyphenyl)-5-isoxazoleacetic acid methyl ester) at a dose of 35 mg/kg inhibits airway neutrophilia induced by intra-tracheal administration of recombinant MIF in naïve mice.[8] We tested two dosing regimens. In the first, ISO-1 (35mg/Kg, Tocris Bioscience) or its vehicle (5% DMSO in PBS) were administered via intraperitoneal injection 30 min before HDM challenge, whilst in the

second it was administered 30 min before and 6 h post HDM challenge ('ISO-1 bid'). Dexamethasone (9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione, 1mg/Kg, Sigma Aldrich) was administered 30 min prior to HDM challenge via oral gavage either alone or in combination with ISO-1. Randomization 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); 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 data supplement.

A single researcher (VSSR Allam) 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 (J Simpson) 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 4 mice per group would be sufficient to achieve statistical significance for the primary outcome of neutrophil numbers in the airway lumen.[16]

Statistical Analysis

For U-BIOPRED data, normality of distribution of genes of interest expression was examined by the Shapiro-Wilk test. Association of genes expressions with categorical variables was determined by the Kruskal-Wallis test with Dunn's post-hoc multiple comparison analysis for non-normally distributed gene expressions and pairwise Student's t-test for normally

distributed expressions. P values were adjusted for multiple testing using false discovery rate (FDR). Association of genes of interest expression with numerical variables was measured and tested using Spearman's rank-order correlation.

For studies in mice, data were expressed as mean ± 95 % CI and analysed using GraphPad Prism 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 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 post-hoc multiple comparison analysis. Two-way ANOVA was conducted to compare *in vivo* 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 (FEV_1 % predicted) and sputum macrophages (figure 1A-D, online supplementary Table S1). When each of these correlations were examined within individual patient groups, MIF protein levels were negatively correlated with sputum macrophages in non-smokers with severe

asthma only (online supplementary figure S1A-D). Notably, oral corticosteroid use across all U-BIOPRED subjects was associated with significantly higher levels of MIF protein (figure 1E, online supplementary Table S1), but this relationship was not observed when examined within the severe asthma groups individually (online supplementary figure S2A).

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 supplementary Table S1). *MIF* mRNA was also negatively 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 patient groups (online supplementary figure S1E-H). Further, *MIF* mRNA was not associated with oral corticosteroid use across all U-BIOPRED subjects (online supplementary Table S1) nor within the severe asthma groups (online supplementary 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 transcriptomic-associated clusters (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 co-localizes 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 to 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 to TAC1 and TAC3 (figure 2C, D). However, subjects in TAC2 had similar levels of *MIF* mRNA compared to subjects in TAC1 and significantly lower levels compared to TAC3 (online supplementary figure S3). MIF positively regulates the expression of the pattern-recognition receptor toll-like receptor 4 (TLR4)[22] which lies up-stream 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 to TAC1 and TAC3 (figure 2E, F).

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 supplementary Table S2). MIF protein levels significantly positively correlated with S100A9 protein, *NLRP3* mRNA expression and IL-1β gene and protein expression (online supplementary Table S2). For completeness, we also report that *MIF* mRNA expression significantly negatively correlated with *TLR4* and *IL1B* gene expression (online supplementary Table S2). Collectively, these data suggest an underlying role for MIF in the development of neutrophilic responses in a sub-group 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 supplementary figure S4A).

Increased MIF protein abundance is associated with reduced expression of the glucocorticoid-inducible pro-resolving 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 to 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 to TAC3 (figure 3B). However, compared to TAC1 and TAC3, subjects in TAC2 expressed significantly lower levels of annexin-A1 mRNA and protein (figure 3C, D). Annexin-A1 is a potent pro-resolving 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 sub-group. 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 supplementary figure S4B, C).

Annexin-A1 signals *via* the formyl peptide receptor 2 (FPR2) which is also the pro-resolving 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 to TAC1 and TAC3, TAC2 had significantly higher levels of *FPR2* mRNA, indicating that reduced annexin-A1 expression, rather than reduced signaling *via* this receptor is more likely to explain enhanced neutrophil infiltration in this subgroup of patients (figure 3E). Moreover, annexin-A1 mediates its anti-inflammatory effects, in part, by inhibiting the activation of cytosolic phospholipase A2 ($cPLA_2$), 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 to those who were not (online supplementary 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-1β mRNA and protein expression. Annexin-A1 protein expression negatively correlated with *NLRP3* mRNA and IL-1β protein only (online supplementary 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 supplementary 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 findings above 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 supplementary 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 positively correlated with features of severe disease, including oral corticosteroid use, the number of exacerbations in the previous year and the degree of lung function impairment (online supplementary Table S1).

Finally, to extend findings above, we sought to investigate correlations between annexin-A1 gene and protein expression and selected clinical characteristics within individual U-BIOPRED patient groups (online supplementary figure S6, 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 supplementary 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 supplementary figure S6D, H). Significantly, in the group of smokers with severe asthma, compared to those who were not using oral

corticosteroids, those who were using oral corticosteroids had lower levels of annexin-A1 protein (online supplementary figure S7B).

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

Considering our findings above, we investigated whether MIF inhibition attenuates neutrophilic responses and increases glucocorticoid responsiveness in a murine model of experimental severe asthma. C57BL/6 mice were sensitized with house dust mite (HDM) in the presence of complete Freund's adjuvant (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) (figure 4A, 5A and online supplementary 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 and 6 h post HDM challenge ('ISO-1 bid'), MIF inhibition significantly decreased airway neutrophil numbers and AHR (figure 4A, B). Meanwhile, a single dose of the glucocorticoid dexamethasone (Dex; 1 mg/kg)) given 30 min prior to HDM challenge significantly 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 Dex 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 hematoxylin and eosin (H&E) staining and airway mucus production by Periodic acid-Schiff staining. Consistent with findings above, 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 Dex alone had no effect on lung inflammation scores, whereas combined treatment with Dex and ISO-1 significantly inhibited lung inflammation scores (figure 6A). While we observed a significant increase in airway mucous production following allergen challenge in this model, airway mucous scores were not significantly altered under any of the treatment conditions tested, likely reflecting the variability in this data (figure 6B).

MIF inhibition synergizes 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[10 11], 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, online supplement Table S3). In contrast, treatment with ISO-1 30 min prior and 6 h 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). However, this treatment regimen had no significant effect on the secretion of other mediators measured (online supplementary Table S3).

Treatment of mice with Dex alone had no significant effect on NLRP3 protein levels or IL-1β release (figure 7A, B), suggesting glucocorticoid-resistance of NLRP3 inflammasome signaling in experimental severe asthma, as previously demonstrated.[16 17] Moreover, with the exception of S100A8, IL-1α and TNFα (figure 8A, D, E) treatment of mice with Dex alone had no effect on the secretion of all other mediators measured (online supplement Table S3). However, combined treatment with Dex and ISO-1 30 min prior to HDM challenge led to significant inhibition of NLRP3 protein levels and IL-1β secretion (figure 6A, B) and several additional mediators, including CCL11, CCL3, IFN-γ, IL-33 and GM-CSF (figure 8B, C, F, G, H), 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 Dex either alone or together with ISO-1 had no effect on BALF MIF concentrations (figure 8I), indicating that enhanced glucocorticoid efficacy in the context of MIF inhibition was not due to an inhibitory effect of Dex 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 externalized 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 Dex (figure 9A). In the BALF, full-length annexin-A1 was not detected, however we detected a protein band at ~33 kDa and ~28 kDa in allergen- but not PBS-challenged mice indicating that annexin-A1 externalization 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 and 6 h post allergen was associated with a significant reduction in the total amount of cleaved annexin-A1. Moreover, while administration of either ISO-1 or Dex 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 Dex 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, 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, online supplementary 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 with a single dose of ISO-1 or Dex alone or their combination 30 min prior to HDM challenge. Treatment with either Dex, ISO-1 or the combination of ISO-1 and Dex ablated annexin-A1 cleavage at the tissue level (figure 9D, online supplementary 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 pro-resolving 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 (35mg/kg) inhibits lung neutrophil recruitment induced by intra-tracheal 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 pro-neutrophilic chemokines.[8] In contrast, in experimental severe asthma, administration of ISO-1 (35 mg/kg)

30 min prior 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 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 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 LPS-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] Thus, 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, upon 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 h 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 pro-resolving 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 induces conformational changes promoting annexin-A1 externalisation and susceptibility to proteolytic cleavage. Patients with severe asthma have raised levels of IFN-γ and lower levels of secretory leukocyte protease inhibitor (SLPI) in their airway epithelium. IFN-γ augments AHR through inhibition of SLPI[38] and SLPI protects annexin-A1 from proteolysis.[39] This data supports 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 asthma

and healthy controls 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 suggests 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 down-regulates 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 up-regulation 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 pro-resolving 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.

COMPETING INTERESTS

V.S.R.R.A, S.P, G.L, N.Z.K, J.S, J.T, S.D, Y.G, P.M.H, S.P, J.H and M.B.S have nothing to disclose. E.F.M reports grants from Janssen, Bristol Myers Squibb, UCB, Merck Serono, and Eli Lilly outside the submitted work. In addition, E.F.M has patents 7,709,514 and 7,863,313 issued. K.F.C has received honoraria for participating in Advisory Board meetings of GlaxoSmithKline, AstraZeneca, Novartis, Merck, Boehringer Ingelheim and TEVA regarding treatments for asthma and chronic obstructive pulmonary disease and has also been renumerated for speaking engagements. I.M.A and P.J.S report grants from the public private European Union Innovative Medicines Initiative during the conduct of the study. R.D 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. R.D. is a co-founder and current consultant, and has shares in Synairgen, a University of Southampton spin out company.

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AUTHOR CONTRIBUTIONS

Concept and design: V.S.R.R.A, K.F.C, I.M.A, J.H, M.B.S. Acquisition of data, analysis, and interpretation: V.S.R.R.A, S.P, G.L, N.Z.K, J.S, J.T, S.D, Y.G, P.M. H, S.P, E.F.M, R.D, P.J.S, K.F.C, I.M.A, J.H, M.B.S. Drafting the manuscript, V.S.R.R.A, K.F.C, I.M.A, E.F.M, J.H, M.B.S. All authors revised the manuscript critically.

EXCLUSIVE LICENCE

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FIGURE LEGENDS

Figure 1. Correlations between MIF protein and selected clinical characteristics across all U-BIOPRED subjects

Spearman's rank correlation was used for correlation analysis. *P* values were corrected for multiple comparisons using Benjamini-Hochberg correction. Rho: Spearman's correlation coefficient.

Figure 2. Expression of innate immune mediators in sputum according to transcriptomic-associated cluster (TAC) status

Protein levels of macrophage migration inhibitory factor (MIF) (A) and S100A9 (B) measured by SOMAscan in log2 relative fluorescent units (RFU). Gene expression levels of S100A8 (C) S100A9 (D) NLR Family Pyrin Domain Containing 3 (NLRP3) (E) and Toll-like receptor 4 (TLR4) (F) measured by microarray and presented as $\log_2$ signal intensity values. Note, there was not a complete overlap between sputum samples used for protein and mRNA measurements. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001

Figure 3. Expression of steroid responsive genes and mediators in sputum according to transcriptomic-associated cluster (TAC) status

Gene expression levels of dual-specificity phosphatase 1 (DUSP1) (A) TSC22 Domain Family Protein 3 (TSC22D3) (B) annexin A1 (ANXA1) (C) and formyl peptide receptor 2 (FPR2) (E) measured by microarray and presented as $\log_2$ signal intensity values. Protein levels of annexin A1 (D) measured by SOMAscan in log2 relative fluorescent units (RFU). The level of LTB4 (E) was determined by ELISA and presented as pg/ml in each patient sample. Note,

there was not a complete overlap between sputum samples used for protein, mRNA and lipid measurements. *P < 0.05, **P < 0.01, and ***P < 0.001

Figure 4. ISO-1 inhibits neutrophilic inflammation and AHR in experimental severe asthma

Mice were treated with vehicle or with ISO-1 30 min prior to HDM challenge. Alternatively, mice were treated with 2 doses of ISO-1 given 30 min prior and 6 h post HDM challenge (ISO-1 bid). Control mice were treated with PBS only. (A) Total cells, eosinophils and neutrophils were measured in BALF. (B) Total lung resistance (Rrs), compliance (Crs) and elastance (Ers), and proximal airway resistance (Rn) distal airway dampening (G) and elastance (H) were measured using forced oscillation technique. Data represent mean ± 95% CI. *P < .05, **P < .01, and ***P < .001 vs PBS group. #P < .05 and ##P < .01 vs HDM group. N = 7 – 18 mice per group. HDM = house dust mite.

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 vehicle or with ISO-1 and/or Dex 30 min prior to HDM challenge. Control mice were treated with PBS only. (A) Total cells, eosinophils and neutrophils were measured in BALF. (B) Total lung resistance (Rrs), compliance (Crs) and elastance (Ers) and proximal airway resistance (Rn), distal airway dampening (G) and elastance (H) were measured using forced oscillation technique. Data represent mean ± 95% CI. *P < 0.05, **P < 0.01, and ***P < 0.001 vs PBS group. #P < 0.05 and ##P <0 .01 vs HDM group. δP < 0.05 vs HDM + DEX group. N = 7 – 18 mice per group. HDM = house dust mite.

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 vehicle or with ISO-1 and/or Dex 30 min prior to HDM challenge. Alternatively, mice were treated with 2 doses of ISO-1 given 30 min prior and 6 h post HDM challenge (ISO-1 bid). Control mice were treated with PBS only. Airway inflammation (A) and mucus production (B) were assessed by hematoxylin and eosin (H&E) and Periodic-Acid Schiff (PAS) staining, respectively. Data represent mean ± 95% CI. **P* < 0.05 vs PBS group. #*P* < 0.05 and ##*P* < 0.01 vs HDM group. N = 5 – 6 mice per group. Representative images for H&E (x10 original magnification) and PAS (x40 magnification) are shown. Scale bars, 60μm. HDM = house dust mite.

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 vehicle or with ISO-1 and/or Dex 30 min prior to HDM challenge. Alternatively, mice were treated with 2 doses of ISO-1 given 30 min prior and 6 h post HDM challenge (ISO-1 bid). Control mice were treated with PBS only. (A) NLRP3 protein measured in lung tissue lysates by immunoblotting. Data were normalized to 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* < 0.05 vs PBS group. #*P* < 0.05 and ##*P* < 0.01 vs HDM = house dust mite group. N = 6 – 8 mice per group.

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 vehicle or with ISO-1 and/or Dex 30 min prior to HDM challenge. Alternatively, mice were treated with 2 doses of ISO-1 given 30 min prior and 6 h post HDM challenge (ISO-1 bid). The concentration cytokines and chemokines in BALF were determined using a customized Magnetic Luminex assay using the MAGPIX® System. Data represent mean ± 95% CI. *P < 0.05 vs PBS and # P < 0.05 vs HDM. N = 7 – 10 mice per group.

Figure 9. ISO-1 protects annexin-A1 against proteolytic cleavage in experimental severe asthma

Mice were treated with vehicle or with ISO-1 and/or Dex 30 min prior to HDM challenge. Alternatively, mice were treated with 2 doses of ISO-1 given 30 min prior and 6 h post HDM challenge (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 to 7) and BALF (lanes 8 and 9). Lanes 1 and 8 represent mice treated with PBS; lanes 2 and 9 represent mice treated with HDM. Lanes 3 and 4 represent mice treated with vehicle or ISO-1 30 min prior and 6 h post HDM challenge, respectively; lanes 5, 6, and 7 represent mice treated with ISO-1, Dex, or ISO-1 + Dex 30 min prior to HDM challenge, respectively. Image representative of data from 4 mice. (B) Sum total of annexin-A1 cleavage products (33 kDa 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 ± SEM. *P < .05 vs PBS group. #P < .05 and ##P < .01 vs HDM = house dust mite group. N = 7 mice per group. (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.

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.

Supplementary Material

Title

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

Authors

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METHODS

Measurement of airway hyperreactivity

Airway hyperreactivity (AHR) was measured 24 h after allergen challenge on day 15 by forced oscillation technique using FlexiVent apparatus (SCIREQ, Montreal, Canada). Mice were anesthetized using a cocktail of xylazine (0.2mg/10gm) and ketamine (0.4mg/10gm body weight). An 18-gauge blunt needle was inserted into the trachea and mice were kept under mechanical ventilation at 200 breaths/min with a delivered tidal volume of 0.25 mL against a positive end-expiratory pressure (PEEP) of 3 cm H_2O. Total respiratory system resistance (Rrs), compliance (Crs) and elastance (Ers), proximal airway resistance (Rn) and distal tissue dampening (G) and elastance (H) were determined by administering increasing doses of nebulized methacholine (0 to 10 mg/mL) (Sigma-Aldrich, St Louis, MO, USA).

Analysis of bronchoalveolar lavage fluid

Once lung function measurements were completed, mice were exsanguinated after a lethal dose of pentobarbital (100 mg/kg). The lungs were lavaged twice with 0.5 mL sterile Hanks Balanced Salt Solution (HBSS). The collected fluid was spun at 3000 rpm for 10 min at 4^{o}C. Cell supernatants were retained for analysis of cytokine/chemokine expression, while cell pellets were resuspended in sterile HBSS for enumeration of total and differential cell counts. To perform differential cell counts, cells were spun on glass slides using a Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific) and stained with Diff-Quik®. A total of 200 cells were counted. The concentration of MIF and LTB4 in BALF was determined using commercial ELISA assays (R&D Systems). The concentration of other cytokines and chemokines in BALF were determined using a customized Magnetic Luminex assay (R&D systems) using the MAGPIX® System. Five parameter logistic regression was performed to predict the concentration of unknown samples.

Quantification of airway inflammation and mucus production

After collection of BALF, the left lung was inflated with neutral buffered formalin (NBF), excised and fixed in NBF. Lung sections were stained with hematoxylin and eosin (H&E) and airway inflammation (inflammatory cell infiltrate) semi-quantified by blinded scoring of the inflammatory cell infiltrate surrounding each airway. Scores ranged from 0-4 (0: no inflammatory cell infiltrates around airway, 1: low level cell infiltrates around part of airway, 2: moderate cell infiltrates around part of or entire airway, 3: significant inflammatory cell infiltrates around part of or entire airway, 4: airway completely surrounded by inflammatory cell infiltrates). Mucus producing cells were identified by Periodic acid-Schiff (PAS) staining and scored blindly from 0 to 5 based on percentage of PAS positive airway epithelial cells (AEC) (0: 0% of total AEC, 1: 1-10% of total AEC, 2: 10-30% of total AEC, 3: 30-50% of total AEC, 4: 50-80% of total AEC, 5: >80% of total AEC).[1] Five airways were scored per mouse. All sections were imaged on Aperio Scanscope XT and Leica DM750 Brightfield microscope.

Analysis of NLRP3, IL-1β and annexin-A1 protein expression by immunoblotting

NLRP3, IL-1β and annexin-A1 were measured in lung homogenates and/or BALF by immunoblotting. To prepare lung homogenates, the right lung was lysed using RIPA buffer containing protease and phosphatase inhibitors (cOmplete™ ULTRA and PhosSTOP™, Roche Diagnostics, Australia). Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions. Proteins were loaded onto 4-12% bis-tris gels and separated by electrophoresis at 200V for 50 min. 40 μg and 1 μg of total protein was loaded onto gels to measure NLRP3 and annexin-A1 in lung homogenates, respectively, while 10 μg and 1 μg of total protein was loaded onto gels to measure IL-1β and annexin-A1 in BALF, respectively. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot 2 Dry Blotting System (Life Technologies). PVDF membranes were then blocked with 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween (TBST) for 1 h at room temperature, prior to overnight incubation with primary antibodies for NLRP3 (Adipogen Life Science #AG-20B-0014-C100

1:500 dilution), IL-1β (BioVision INC #5129-100 1:500 dilution) or annexin-A1 (R&D Systems #MAB37701 1:20000 dilution) at 4^0C. To ensure even protein loading, PVDF membranes were incubated with primary antibody for GAPDH (Santa Cruz Biotechnology #sc-32233 1:1000 dilution) (lung homogenates) or stained with 0.1% Ponceau S (Fisher Biotech) in 5% acetic acid (BALF). PVDF membranes were then washed with TBST three times (5 min each) and incubated with HRP-linked anti-mouse IgG secondary antibodies (GE Healthcare #NA931-1ML 1:2000 dilution) for 1 hour at room temperature. PVDF membranes were then washed with TBST three times (5 min each). Protein bands were visualized using enhanced chemiluminescence (ECL) (Amersham, GE Healthcare) and densitometric analysis was performed using Image J software (v1.47).

Analysis of MIF-annexin-A1 protein complexes by immunoprecipitation and immunoblotting

MIF protein was immunoprecipitated from lung homogenates using the Dynabeads™ Protein G Immunoprecipitation Kit (Thermo Fisher Scientific #10007D) according to the manufacturer's instructions. Briefly, Dynabeads™ were incubated with anti-MIF antibody (Abcam #ab175189 2 mg) or control IgG_1 antibody (R&D Systems #MAB002) with rotation at room temperature for 10 min. The beads-antibody complexes were then incubated with lung homogenates (1 mg) with rotation at room temperature for a further 10 min. The bead-antibody-protein complexes were separated using a magnet. Antibody-protein complexes were then eluted by adding 20 μL elution buffer and 10 μL Laemmli sample containing β-mercaptoethanol (Bio-Rad #161-0737 & #161-0710) and heating at 70°C for 10 min. The beads were then removed using a magnet and eluents were collected for analysis by immunoblotting. To do this, eluents were loaded onto 4-15% Mini-PROTEN precast gels (Bio-Rad) and separated by electrophoresis at 110 V for 90 min. Following electrophoresis, proteins were transferred to a PVDF membrane using a PowerPac supply (Bio-Rad) on ice at 90V for 2 h. PVDF membranes were blocked with 5% BSA for 2 h at room temperature and incubated overnight with anti-MIF (Abcam ab175189 1:2000 dilution), anti-annexin-A1 (R&D Systems #MAB37701 1:20000 dilution) or

anti-β-actin (Abcam #ab8226, 1:10,000 dilution) antibodies at 4°C. PVDF membranes were then washed with TBST three times (10 min each) and incubated with HRP-linked anti-mouse or anti-rabbit IgG secondary antibodies for 2 h at room temperature. PVDF membranes were then washed with TBST three times (10 min each) and protein bands were visualized using SuperSignal™ West Femto Maximum sensitivity substrate (Thermo Fisher Scientific).

Table S1: Correlations between levels of selected mRNAs and proteins with clinical characteristics across all U-BIOPRED subjects

Clinical Characteristics	*Mif*	MIF	*ANXA1*	Annexin-A1	*DUSP1*	*TSC22D3*
Age years	rho = 0.04	rho = 0.01	rho = -0.13	rho = -0.06	rho = 0.08	rho = 0.15
Age of onset years	rho = 0.08	rho = -0.02	rho = -0.01	rho = 0.03	rho = -0.11	rho = -0.06
Gender(female/male)	$p^* = 0.6$	$p^* = 0.4$	$p^* = 0.5$	$p^* = 0.5$	p* = 0.6	p* = 0.7
BMI	rho = 0.03	rho = 0.03	rho = 0.09	rho = 0.1	rho = 0.06	rho = 0.13
Smoker (none smoker/smoker)	$p^* = 0.5$	$p^* = 0.4$	$p^* = 0.8$	$p^* = 0.9$	p* = 0.4	p* = 0.8
Nasal polyps (no/yes)	$p^* = 0.9$	$p^* = 0.2$	$p^* = 0.8$	$p^* = 0.8$	p* = 0.3	p* = 0.5
Allergic rhinitis (no/yes)	$p^* = 0.4$	$p^* = 0.2$	$p^* = 0.09$	$p^* = 0.1$	p* = 0.09	p* = 0.1
Eczema (no/yes)	$p^* = 0.5$	$p^* = 0.08$	$p^* = 0.09$	$p^* = 1$	p* = 0.7	p* = 0.3
Severe asthma (Healthy/MMA/SAns/SAs)	$p^* = 0.5$	$p^* = 0.2$	$p^* = 0.8$	$p^* = 0.5$	**p* = <0.001**	**p* = <0.001**
Oral corticosteroid use (0/1)	$p^* = 0.9$	**$p^* = 0.05$**	$p^* = 0.8$	$p^* = 0.1$	**p* = 0.001**	**p* = <0.001**
Atopy (+/-)	$p^* = 0.4$	$p^* = 0.9$	$p^* = 0.7$	$p^* = 0.4$	p* = 0.7	p* = 0.5
Exacerbations previous year	rho = 0.09	**$rho^{\dagger} = 0.19$**	rho = -0.11	rho = -0.07	rho = 0.1	**$rho^{\dagger} = 0.23$**
FEV1 % pred	*$rho^{\dagger} = -0.18$*	*$rho^{\dagger} = -0.25$*	rho = 0.11	rho = 0.05	*$rho^{\dagger} = -0.35$*	*$rho^{\dagger} = -0.46$*
Total IgE IU·mL1	rho = -0.17	rho = 0.04	rho = 0.11	rho = -0.09	**$rho^{\dagger} = 0.18$**	**$rho^{\dagger} = 0.21$**
Blood leukocytes (10^3/µl)	rho = 0.02	rho = 0.05	rho = 0.02	rho = -0.12	**$rho^{\dagger} = 0.36$**	**$rho^{\dagger} = 0.42$**
Blood eosinophils (10^3/µl)	rho = 0.05	rho = 0.03	rho = 0.08	*$rho^{\dagger} = -0.26$*	**$rho^{\dagger} = 0.18$**	**$rho^{\dagger} = 0.22$**
Blood neutrophils (10^3/µl)	rho = -0.01	rho = 0.04	rho = -0.02	rho = -0.09	**$rho^{\dagger} = 0.39$**	**$rho^{\dagger} = 0.44$**
Sputum eosinophils %	rho = 0.14	rho = 0.1	rho = -0.05	rho = -0.16	rho = 0.16	**$rho^{\dagger} = 0.34$**
Sputum neutrophils %	*$rho^{\dagger} = -0.21$*	**$rho^{\dagger} = 0.29$**	*$rho^{\dagger} = -0.42$*	*$rho^{\dagger} = -0.28$*	**$rho^{\dagger} = 0.65$**	**$rho^{\dagger} = 0.44$**
Sputum Macrophages %	rho = 0.08	*$rho^{\dagger} = -0.39$*	**$rho^{\dagger} = 0.46$**	**$rho^{\dagger} = 0.32$**	*$rho^{\dagger} = -0.74$*	*$rho^{\dagger} = -0.70$*

FeNO ppb	rho = -0.06	rho = 0.17	rho = 0.01	*rho† = -0.21*	rho = -0.06	rho = -0.04
Serum periostin (ng/mL)	rho = -0.04	rho = 0.13	*rho† = -0.25*	rho = -0.17	rho = 0.16	rho = 0.20
CRP (mg/L)	rho = -0.07	rho = 0.02	rho = 0.03	rho = -0.13	**rho† = 0.33**	rho = 0.40
Combined atopy regional aeroallergens (-/+)	p* = 0.8	p* = 0.5	p* = 0.6	p* = 0.1	p* = 0.4	p* = 0.8
History pneumonia (no/yes)	p* = 0.8	p* = 0.4	**p* = 0.04**	p* = 0.07	p* = 0.04	p* = 0.09

BMI: body mass index; FEV1: forced expiratory volume in 1 second; FeNO: exhaled nitric oxide fraction; CRP: C-reactive protein; MMA: mild and moderate asthma; SAns: severe asthma non-smokers; SAs: severe asthma smokers. Rho: Spearman's correlation coefficient. †: correlation is significant at the 0.05 level at least. Association of genes and proteins expression with asthma-associated variables were measured and tested using Spearman's rank-order correlation.

Table S2: Correlations between levels of selected mRNAs and proteins across all U-BIOPRED subjects

	Mif **mRNA**		**MIF**		***ANXA1*** **mRNA**		**Annexin-A1**		***DUSP1*** **mRNA**		***TSC22D3*** **mRNA**	
	Rho	*P*	*Rho*	*P*	*Rho*	*P*	*Rho*	*P*	*Rho*	*P*	*Rho*	*P*
Mif mRNA	n/a		- 0.07	*p* = 0.600	**0.24**	***p* = 0.009**	0.20	*p* = 0.060	*-0.17*	*p = 0.07*	0.00	*p* = 1.000
MIF	- 0.07	*p* = 0.600	n/a		*- 0.34*	*p = 0.001*	- 0.17	*p* = 0.070	**0.43**	***p* < 0.001**	**0.40**	***p* < 0.001**
S100A9	- 0.11	*p* = 0.300	**0.62**	***p* < 0.001**	*- 0.39*	*p < 0.001*	- 0.05	*p* = 0.600	**0.46**	***p* < 0.001**	**0.37**	***p* = 0.001**
TLR4 mRNA	*- 0.20*	*p = 0.030*	0.16	*p* = 0.100	*- 0.23*	*p = 0.010*	0.00	*p* = 1.000	**0.31**	***p* = 0.001**	0.09	*p* = 0.3
NLRP3 mRNA	- 0.15	*p* = 0.100	**0.34**	***p* = 0.001**	*- 0.33*	*p < 0.001*	*- 0.36*	*p = 0.001*	**0.75**	***p* < 0.001**	**0.68**	***p* < 0.001**
IL-1B mRNA	*- 0.18*	*p = 0.040*	**0.29**	***p* = 0.006**	*- 0.33*	*p < 0.001*	- 0.13	*p* = 0.200	**0.56**	***p* < 0.001**	0.16	*p* = 0.080
IL-1β	0.05	*p* = 0.700	**0.23**	***p* = 0.010**	*- 0.22*	*p = 0.040*	*- 0.23*	*p = 0.010*	**0.29**	***p* = 0.006**	**0.27**	***p* = 0.010**
ANXA1 mRNA					n/a		0.13	p = 0.200	*- 0.33*	*p < 0.001*	*- 0.37*	*p < 0.001*
Annexin-A1					0.13	*p* = 0.200	n/a		- 0.14	*p* = 0.100	*- 0.33*	*p = 0.002*
DUSP1 mRNA									n/a		**0.77**	***p* < 0.001**
TSC22D3 mRNA											n/a	

Spearman's correlation coefficient (Rho, left) and *p* value (right). Blue = positive correlation, Red = negative correlation.

Table S3: Inflammatory mediator levels in BALF

Mediator pg/mL	PBS	HDM	HDM + Veh	HDM + ISO-1 bid	HDM + ISO-1	HDM + Dex	HDM + ISO-1 + Dex
MIF	5145 ± 886	10287 ± 1866*	11934 ± 3184	13745 + 2196	12195 ± 1261	9218 + 2190	8894 + 1115
S100A8	187.79 ± 10.76	25313 ± 1612*	22413 ± 2756*	15992 ± 2994*#	22814 ± 2103*	15714 ± 2331*#	12092 ± 879*#
LTB4	101.73 ± 20.11	81.64 ± 10.49	84.27 ± 16.12	92.61 ± 24.53	112.10 ± 22.98	83.97 ± 15.88	92.16 ± 13.6
IFN-γ	1.20 ± 0.41	452.03 ± 89.49*	318.21 ± 96.10*	257.00 ± 70.55	354.87 ± 64.57*	295.39 ± 58.63*	153.45 ± 57.21#
TNF-α	0.26 ± 0.05	33.63 ± 6.44*	27.91 ± 10.32*	19.97 ± 6.42	28.73 ± 5.21	11.66 ± 3.75#	6.20 ± 1.39#
GM-CSF	0.87 ± 0.15	6.88 ± 0.95*	5.06 ± 1.32*	4.08 ± 0.64	6.12 ± 0.84*	4.85 ± 1.11*	2.72 ± 0.35#
CXCL1	11.77 ± 4.11	43.08 ± 5.08	31.21 ± 5.71	38.97 ± 6.24	43.26 ± 2.63*	30.83 ± 5.87	27.91 ± 6.04
CCL2	0.00 ± 0.00	167.67 ± 14.45*	215.10 ± 50.17*	161.38 ± 46.09*	200.68 ± 18.54*	120.45 ± 36.70*	47.32 ± 14.57*
CCL3	0.43 ± 0.08	39.22 ± 3.45*	27.66 ± 5.44*	24.84 ± 6.40*	33.51 ± 3.99*	24.58 ± 6.02*	14.01 ± 1.90#
CCL5	25.44 ± 4.29	194.22 ± 26.35*	247.52 ± 92.85*	187.56 ± 51.00*	201.37 ± 28.37*	113.89 ± 19.33*	101.41 ± 23.00
CCL11	13.5 ± 0.9	44.32 ± 5.98*	40.03 ± 6.62*	20.36 ± 5.22#	42.27 ± 5.76*	24.31 ± 6.09*	10.24 ± 2.86#
IL-1α	4.81 ± 1.61	27.92 ± 1.46*	27.80 ± 4.75*	21.67 ± 4.33*	30.24 ± 2.29*	15.82 ± 1.72*#	12.63 ± 1.93*#
IL-4	1.95 ± 0.80	62.52 ± 7.80	60.02 ± 19.15	66.36 ± 21.51	105.21 ± 17.36	131.90 ± 49.25	115.64 ± 32.40
IL-5	0.47 ± 0.08	13.10 ± 2.86*	10.86 ± 2.14*	10.72 ± 2.17*	7.66 ± 0.95	16.27 ± 2.80*	6.63 ± 0.65
IL-6	2.42 ± 0.52	131.08 ± 27.13*	102.46 ± 24.32*	122.45 ± 21.80*	164.01 ± 22.42*	124.85 ± 28.92*	130.48 ± 27.41*
IL-10	0.52 ± 0.23	2.75 ± 0.12*	3.24 ± 0.61*	1.95 ± 0.46	2.78 ± 0.20*	1.96 ± 0.36*	1.06 ± 0.31
IL-13	6.81 ± 0.77	20.22 ± 2.33*	25.70 ± 6.40*	16.88 ± 2.30*	22.02 ± 1.59*	18.64 ± 3.58*	14.60 ± 3.67*
IL-17A	0.84 ± 0.35	8.84 ± 1.03*	10.91 ± 2.90*	7.77 ± 1.67	10.965 ± 2.24*	8.55 ± 1.93*	4.15 ± 1.40
IL-17E	1.81 ± 1.20	38.72 ± 3.91*	43.71 ± 13.07*	24.09 ± 7.92	43.94 ± 7.46*	33.78 ± 9.56*	12.19 ± 5.30*
IL-23 p19	30.37 ± 6.62	105.15 ± 10.28*	125.87 ± 41.53*	82.95 ± 15.57	114.97 ± 9.01*	65.33 ± 8.16	56.15 ± 9.96
IL-33	5.89 ± 2.02	23.45 ± 3.25*	26.97 ± 7.63*	15.44 ± 4.20	26.47 ± 3.31*	13.38 ± 3.92	6.13 ± 3.08#

Data represent mean ± 95%. *P < .05 vs PBS and #p<0.05 vs HDM (shaded). N = 7-10 mice per group

REFERENCES

1. Tanaka A, Allam V, Tiberti N, et al. The Parasitic 68-mer Peptide FhHDM-1 reduces mixed granulocytic inflammation in allergic asthma. *J Allergy Clin Immunol* 2018;Accepted Jan 2018

FIGURE LEGENDS

Figure S1. Correlations between MIF protein or *MIF* mRNA and selected clinical characteristics within individual U-BIOPRED subject groups

Correlations were measured and tested using Spearman's rank-order correlation. *P* values were corrected for multiple comparisons using Benjamini-Hochberg correction. Rho: Spearman's correlation coefficient. HV: Healthy volunteers; MMA: mild and moderate asthma; SAns: severe asthma non-smokers; SAs: severe asthma smokers

Figure S2. Correlation between MIF protein or *MIF* mRNA and oral corticosteroid use in U-BIOPRED subjects with severe asthma

Correlations were measured and tested using Spearman's rank-order correlation within severe asthma groups. *P* values were corrected for multiple comparisons using Benjamini-Hochberg correction. Rho: Spearman's correlation coefficient.

Figure S3. *Mif* gene expression according to transcriptomic-associated cluster (TAC) status. *Mif* gene expression measured by microarray and presented as $\log_2$ signal intensity values. **P* < .05, ***P* < .01, and ****P* < .001

Figure S4. Correlations between glucocorticoid-regulated genes and/or proteins and sputum neutrophils in U-BIOPRED healthy volunteers (HV) and transcriptomic-associated clusters (TACs)

Correlations were measured and tested using Spearman's rank-order correlation within TAC groups. *P* values were corrected for multiple comparisons using Benjamini-Hochberg correction. Rho: Spearman's correlation coefficient.

Figure S5. Correlations between glucocorticoid-regulated genes and/or proteins and oral corticosteroid use in U-BIOPRED transcriptomic-associated clusters (TACs)

Associations were measured and tested using Spearman's rank-order correlation. P values were corrected for multiple comparisons using Benjamini-Hochberg correction. Rho: Spearman's correlation coefficient.

Figure S6. Correlations between Annexin-A1 protein or *ANXA1* mRNA and selected clinical characteristics within individual U-BIOPRED subject groups

Associations were measured and tested using Spearman's rank-order correlation. *P* values were corrected for multiple comparisons using Benjamini-Hochberg correction. Rho: Spearman's correlation coefficient.

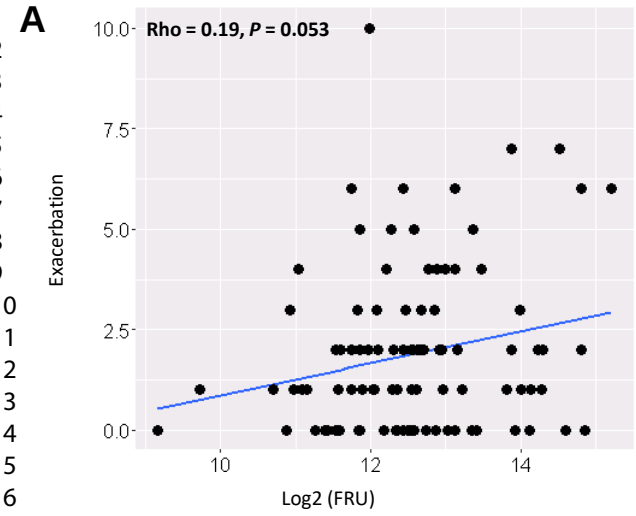
Figure S7. Correlation between Annexin-A1 protein or *ANXA1* mRNA and oral corticosteroid use in U-BIOPRED subjects with severe asthma

Associations were measured and tested using Spearman's rank-order correlation. P values were corrected for multiple comparisons using Benjamini-Hochberg correction. Rho: Spearman's correlation coefficient.

Figure S8. Macrophage cell numbers in BALF are not altered in experimental severe asthma. Macrophage numbers in BALF of mice treated with vehicle or with ISO-1 and/or Dex 30 min prior to HDM challenge or alternatively, mice treated with 2 doses of ISO-1 given 30 min prior and 6 h post HDM challenge (ISO-1 bid). Control mice were treated with PBS only. N = 7 – 18 mice per group. HDM = house dust mite.

Figure S9. MIF binds to and cleaves annexin-A1. Full immunoblot image demonstrating MIF, full-length ~37 kDa annexin-A1 and cleaved ~28 kDa annexin-A1 protein bands in immunoprecipitated MIF-protein complexes from PBS or HDM challenged mice. MIF and annexin-A1 were not detected in protein complexes immunoprecipitated with an isotype control antibody confirming antibody specificity. β-actin was used to confirm equal protein loading.

Figure S10. MIF binds to and cleaves annexin-A1. Full immunoblots demonstrating MIF, full-length ~37 kDa annexin-A1 and cleaved ~28 kDa annexin-A1 protein bands in immunoprecipitated MIF-protein complexes from mice treated with PBS or vehicle, ISO-1, Dex or ISO-1 and Dex 30 min prior to HDM challenge. β-actin was used to confirm equal protein loading.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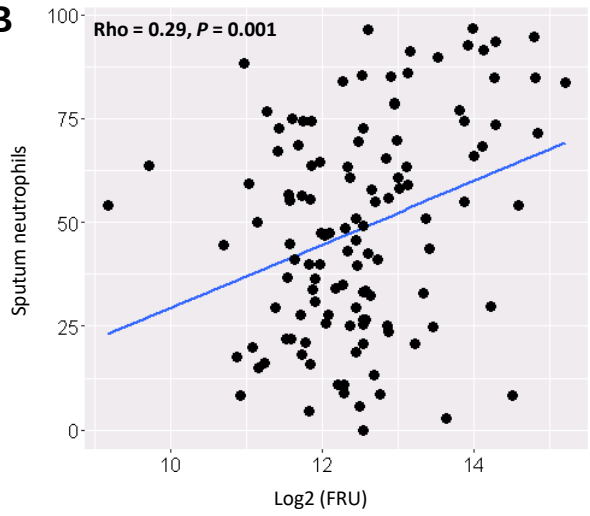
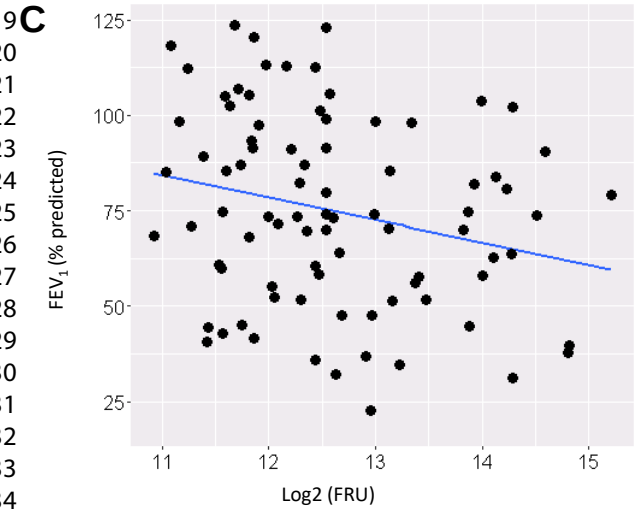
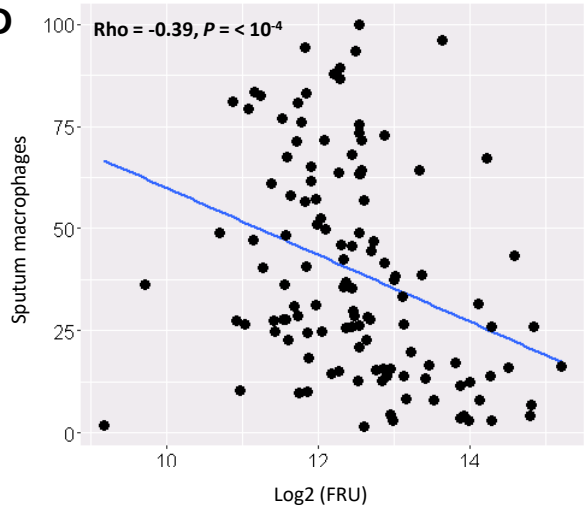
Exacerbation
Log2 (FRU)


B
Rho = 0.29, P = 0.001
Sputum neutrophils
Log2 (FRU)


C
FEV1 (% predicted)
Log2 (FRU)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Rho = -0.39, P = < 10-4
Sputum macrophages
Log2 (FRU)

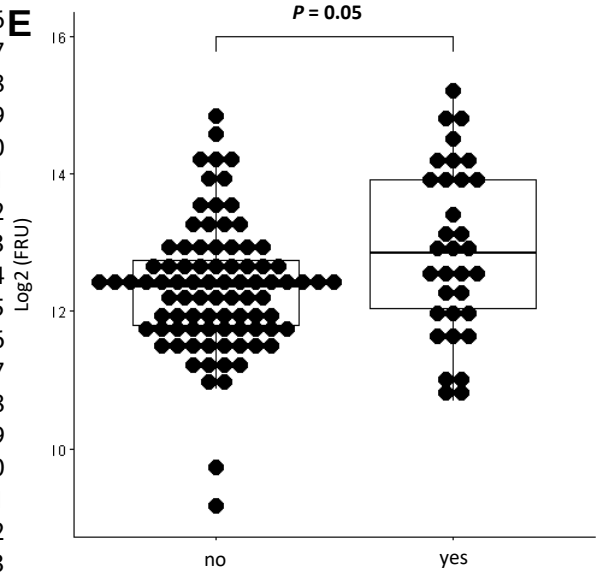

E
P = 0.05
Log2 (FRU)
no
yes
Oral corticosteroid use

Figure 2

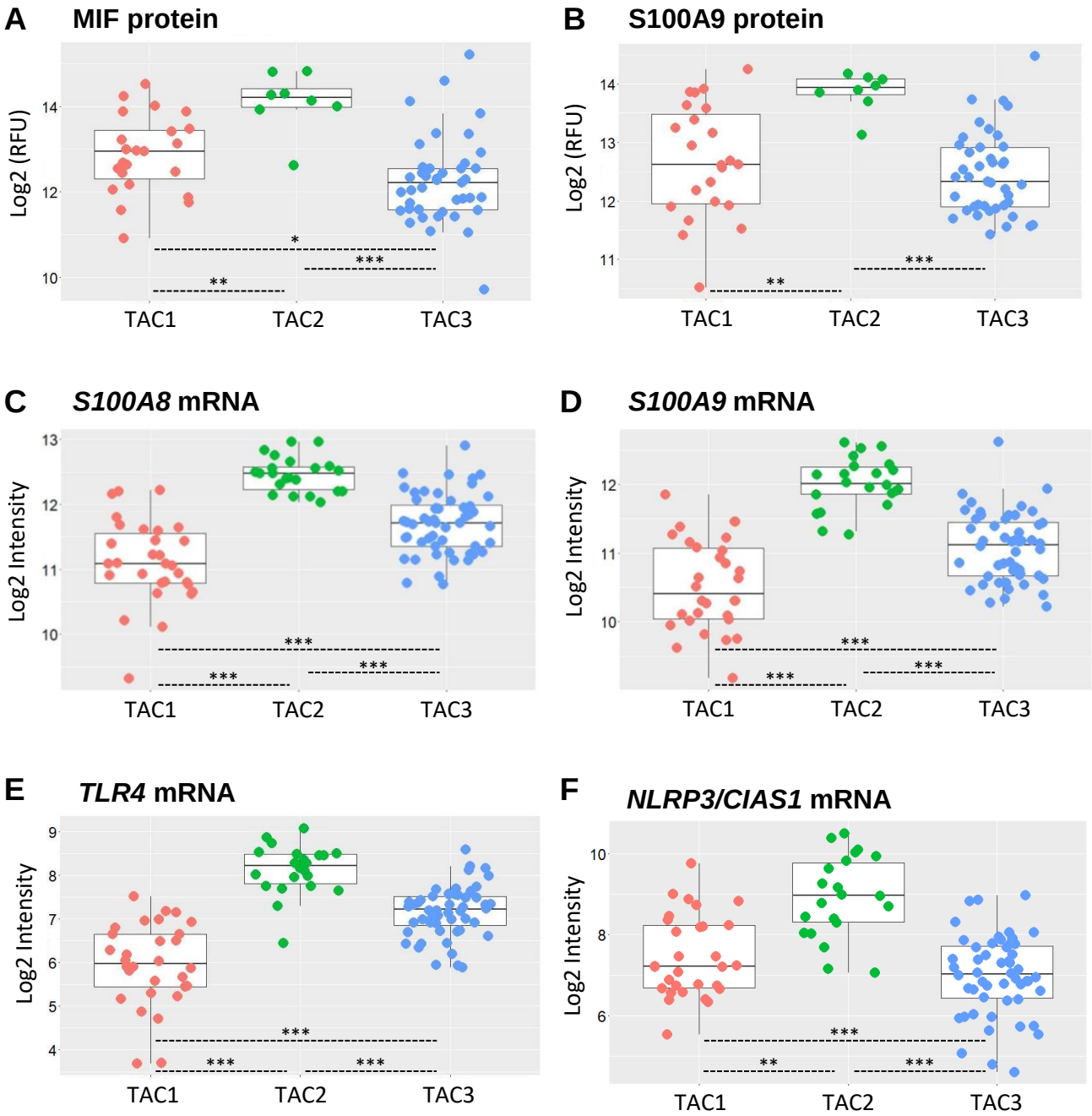

A
MIF protein
Log2 (RFU)
TAC1
TAC2
TAC3
B
S100A9 protein
Log2 (RFU)
C
S100A8 mRNA
Log2 Intensity
D
S100A9 mRNA
Log2 Intensity
E
TLR4 mRNA
Log2 Intensity
F
NLRP3/CIAS1 mRNA
Log2 Intensity

Figure 3

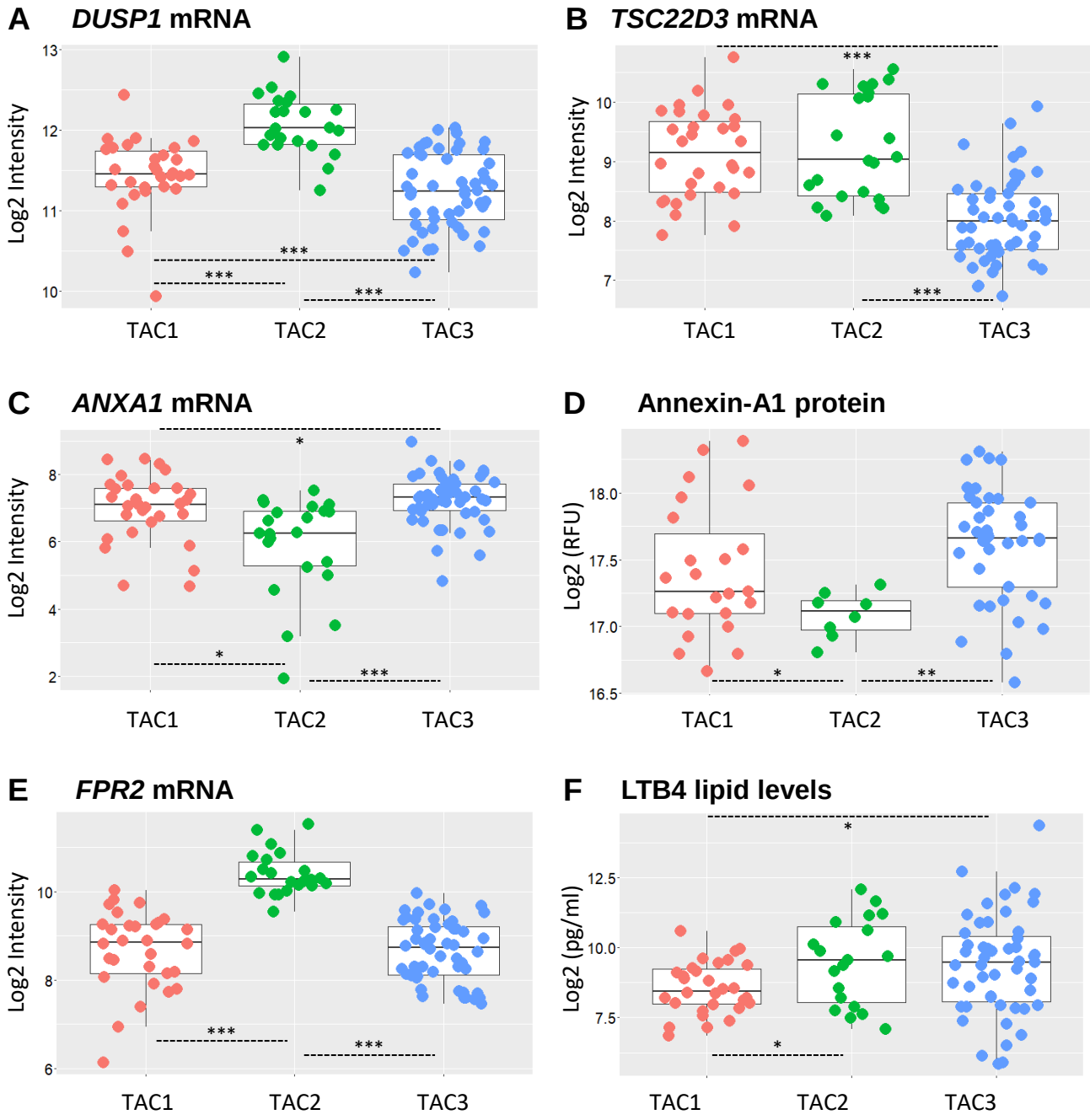
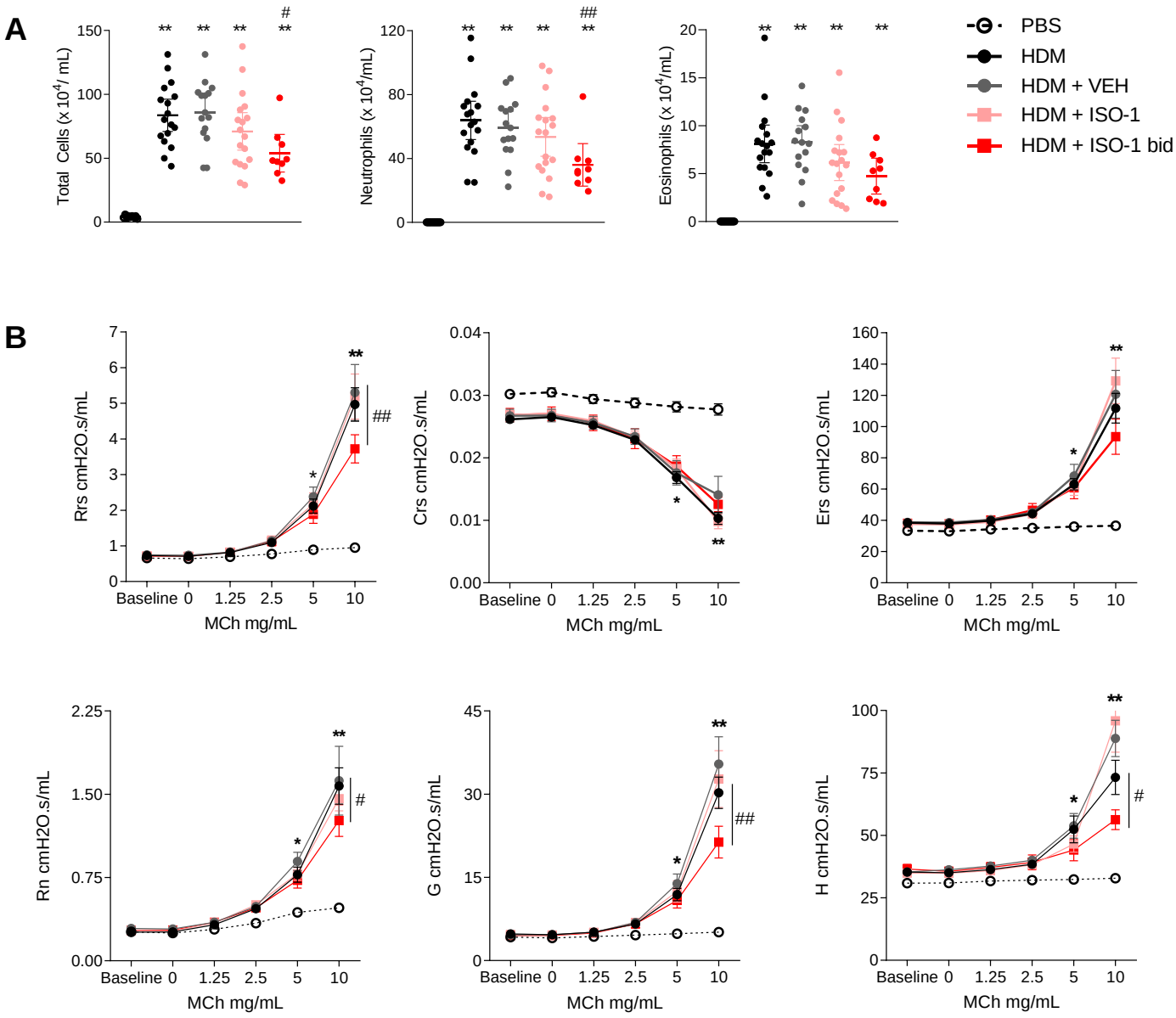

A
DUSP1 mRNA
Log2 Intensity
13
12
11
10
***
***
***
TAC1
TAC2
TAC3
B
TSC22D3 mRNA
Log2 Intensity
10
9
8
7
***
***
TAC1
TAC2
TAC3
C
ANXA1 mRNA
Log2 Intensity
8
6
4
2
*
*
***
TAC1
TAC2
TAC3
D
Annexin-A1 protein
Log2 (RFU)
18.0
17.5
17.0
16.5
*
**
TAC1
TAC2
TAC3
E
FPR2 mRNA
Log2 Intensity
10
8
6
***
***
TAC1
TAC2
TAC3
F
LTB4 lipid levels
Log2 (pg/ml)
12.5
10.0
7.5
*
*
TAC1
TAC2
TAC3

Figure 4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Total Cells (x 10^4/ mL)
Neutrophils (x 10^4/mL)
Eosinophils (x 10^4/mL)
PBS
HDM
HDM + VEH
HDM + ISO-1
HDM + ISO-1 bid
B
Rrs cmH2O.s/mL
Crs cmH2O.s/mL
Ers cmH2O.s/mL
Rn cmH2O.s/mL
G cmH2O.s/mL
H cmH2O.s/mL
Baseline 0 1.25 2.5 5 10
MCh mg/mL

Figure 5

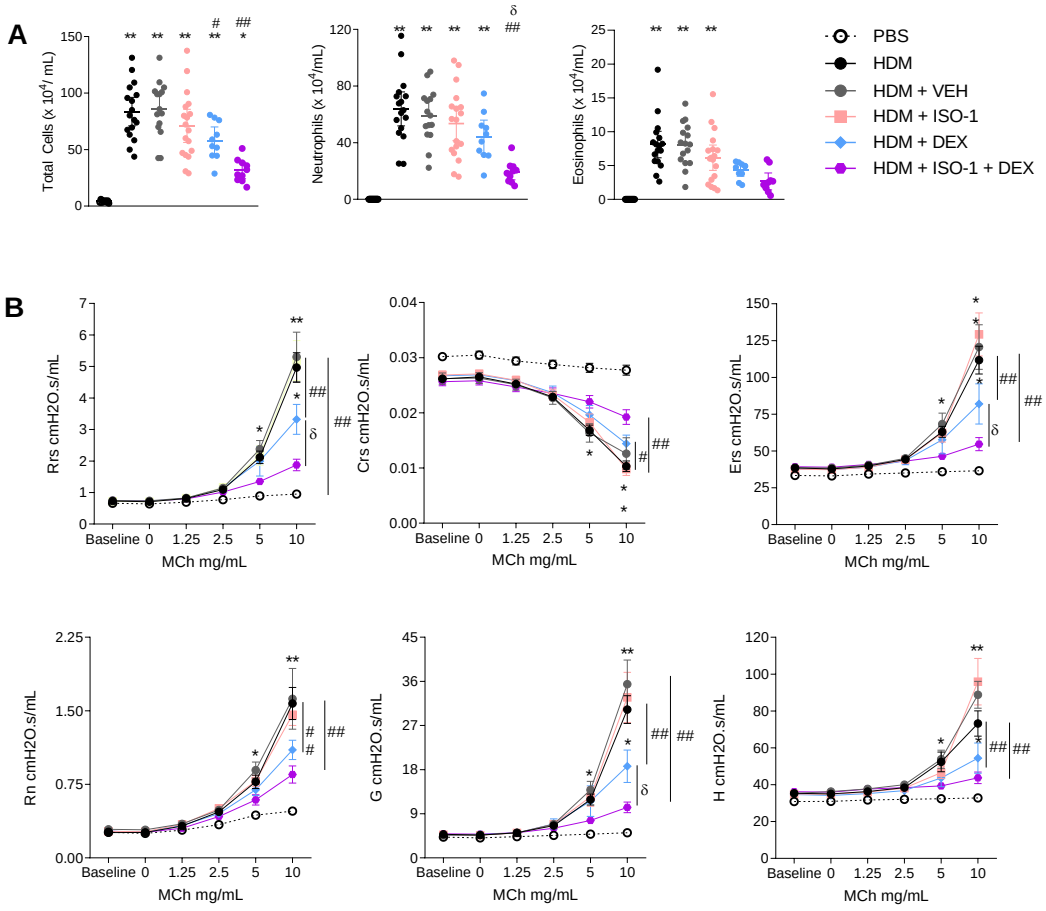

A
Total Cells (x 10⁴/mL)
Neutrophils (x 10⁴/mL)
Eosinophils (x 10⁴/mL)
PBS
HDM
HDM + VEH
HDM + ISO-1
HDM + DEX
HDM + ISO-1 + DEX
B
Rrs cmH2O.s/mL
Crs cmH2O.s/mL
Ers cmH2O.s/mL
Rn cmH2O.s/mL
G cmH2O.s/mL
H cmH2O.s/mL
Baseline 0 1.25 2.5 5 10
MCh mg/mL

Figure 6

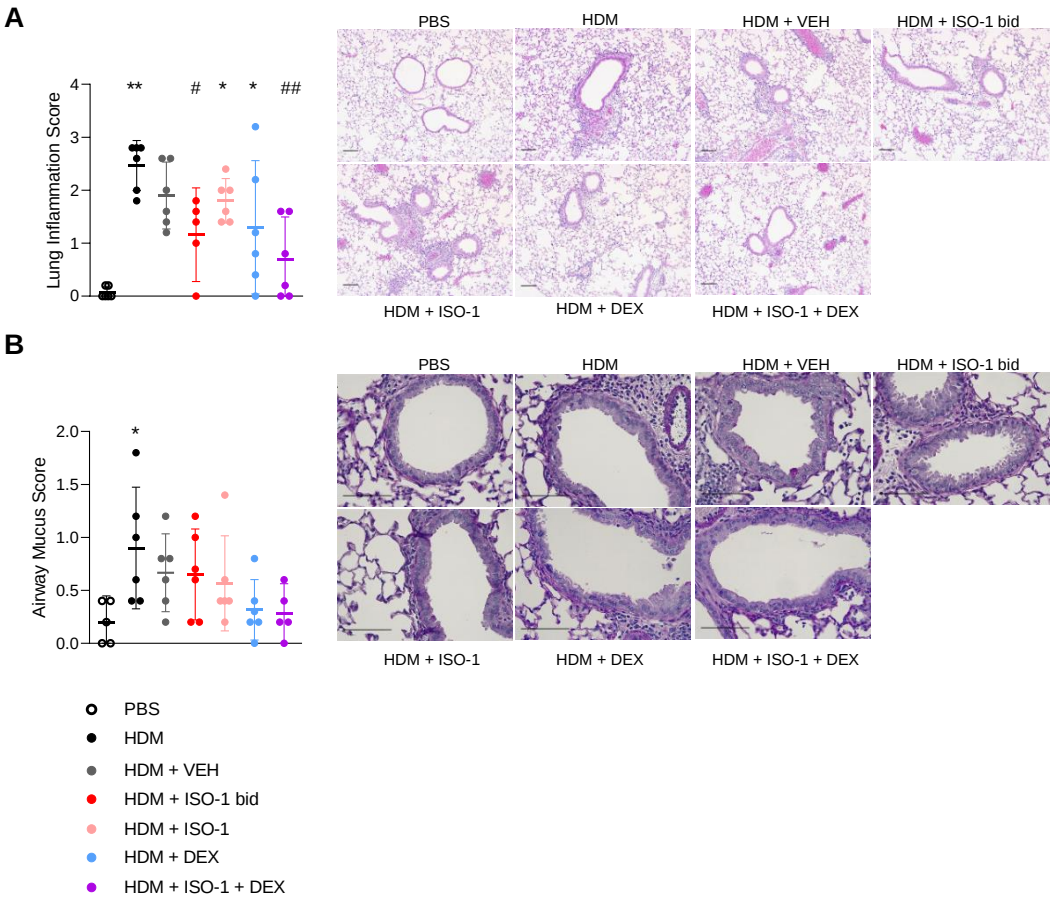
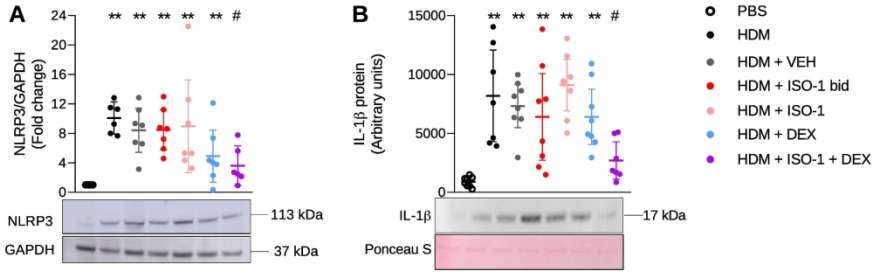

A
Lung Inflammation Score
0
1
2
3
4
**
#
*
*
##
PBS
HDM
HDM + VEH
HDM + ISO-1 bid
HDM + ISO-1
HDM + DEX
HDM + ISO-1 + DEX
B
Airway Mucus Score
0.0
0.5
1.0
1.5
2.0
*
PBS
HDM
HDM + VEH
HDM + ISO-1 bid
HDM + ISO-1
HDM + DEX
HDM + ISO-1 + DEX
PBS
HDM
HDM + VEH
HDM + ISO-1 bid
HDM + ISO-1
HDM + DEX
HDM + ISO-1 + DEX

Figure 7



190x254mm (300 x 300 DPI)

Figure 8

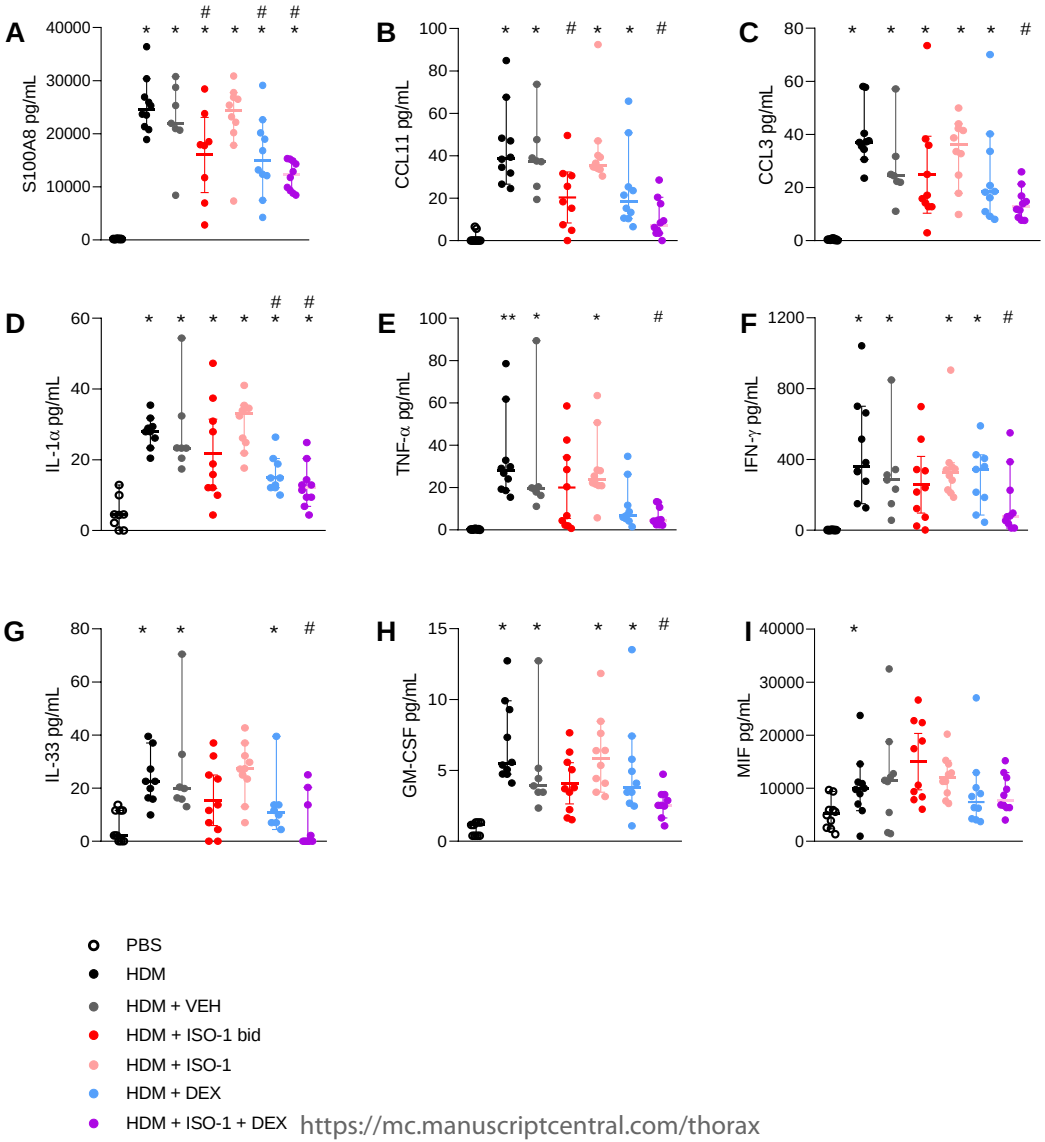
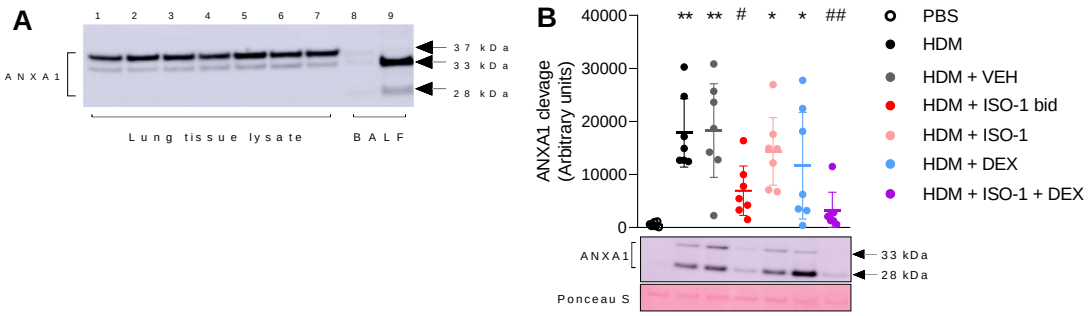
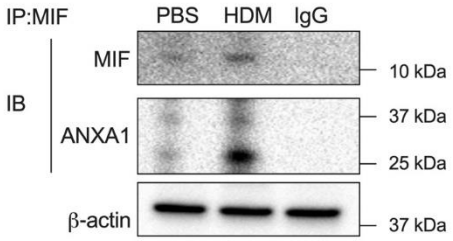

A
S100A8 pg/mL
B
CCL11 pg/mL
C
CCL3 pg/mL
D
IL-1α pg/mL
E
TNF-α pg/mL
F
IFN-γ pg/mL
G
IL-33 pg/mL
H
GM-CSF pg/mL
I
MIF pg/mL
PBS
HDM
HDM + VEH
HDM + ISO-1 bid
HDM + ISO-1
HDM + DEX
HDM + ISO-1 + DEX

Figure 9


A
1 2 3 4 5 6 7 8 9
ANXA1
37 kDa
33 kDa
28 kDa
Lung tissue lysate
BALF
B
ANXA1 clevage (Arbitrary units)
40000
30000
20000
10000
0
** ** # * * ##
PBS
HDM
HDM + VEH
HDM + ISO-1 bid
HDM + ISO-1
HDM + DEX
HDM + ISO-1 + DEX
ANXA1
33 kDa
28 kDa
Ponceau S


C
IP:MIF
PBS
HDM
IgG
IB
MIF
10 kDa
ANXA1
37 kDa
25 kDa
β-actin
37 kDa

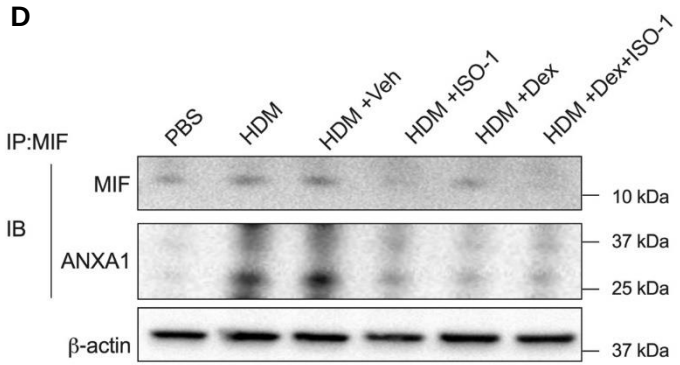
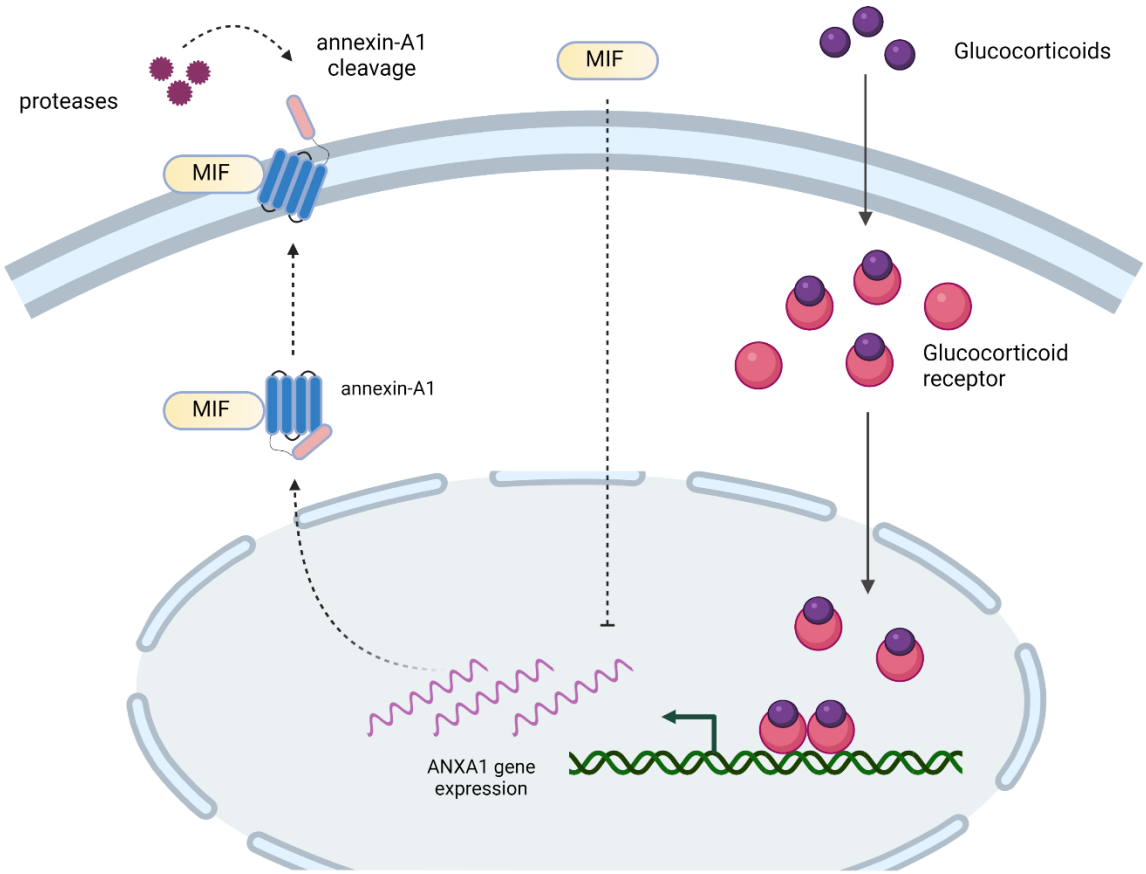

D
IP:MIF
PBS
HDM
HDM +Veh
HDM +ISO-1
HDM +Dex
HDM +Dex+ISO-1
IB
MIF
10 kDa
ANXA1
37 kDa
25 kDa
β-actin
37 kDa

Figure 10


proteases
annexin-A1 cleavage
MIF
MIF
Glucocorticoids
Glucocorticoid receptor
MIF
annexin-A1
ANXA1 gene expression

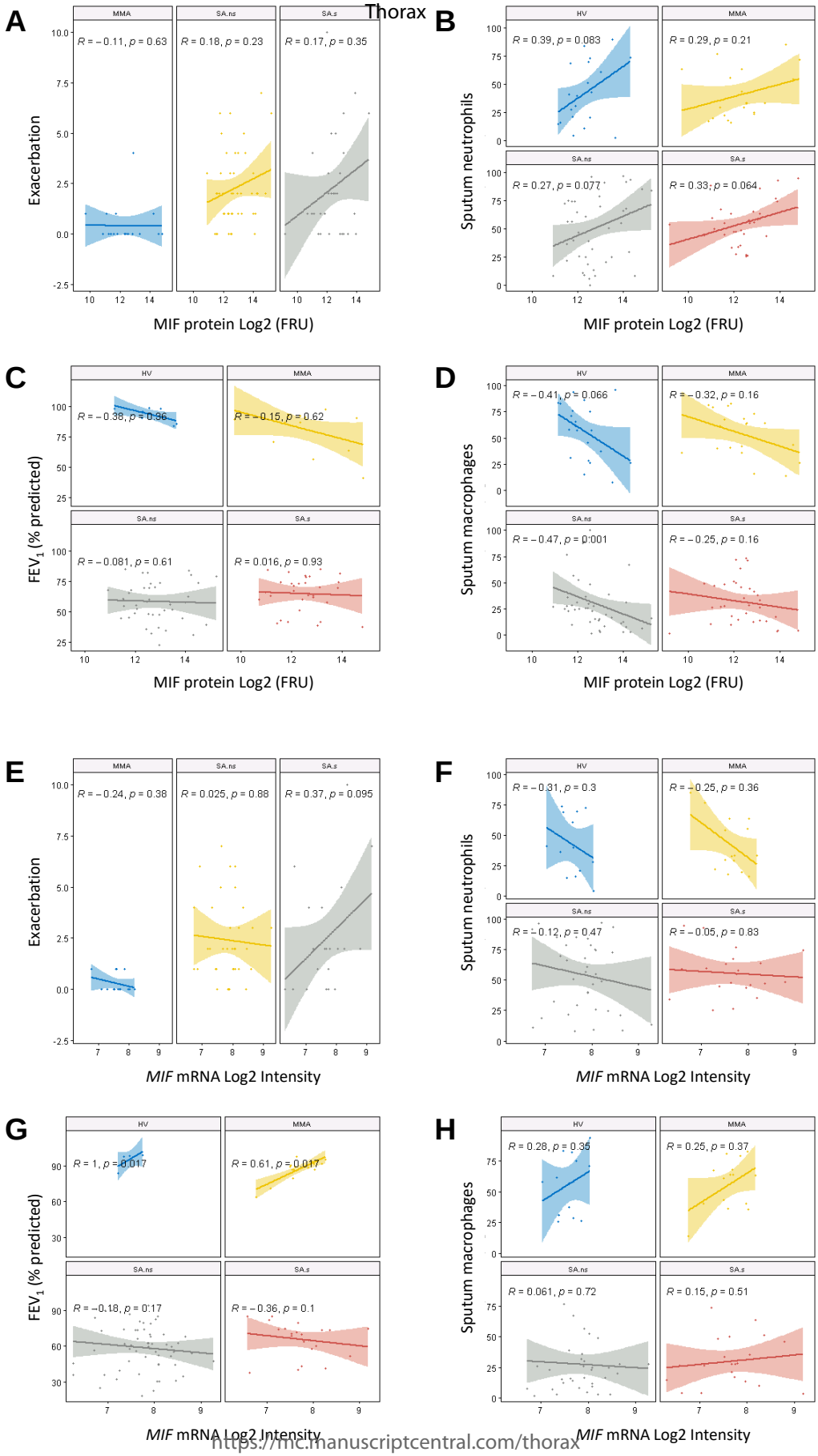

A
MMA
SA.ns
SA.s
R = −0.11, p = 0.63
R = 0.18, p = 0.23
R = 0.17, p = 0.35
Exacerbation
MIF protein Log2 (FRU)
B
HV
MMA
SA.ns
SA.s
R = 0.39, p = 0.083
R = 0.29, p = 0.21
R = 0.27, p = 0.077
R = 0.33, p = 0.064
Sputum neutrophils
MIF protein Log2 (FRU)
C
R = −0.38, p = 0.06
R = −0.15, p = 0.62
R = −0.081, p = 0.61
R = 0.016, p = 0.93
FEV1 (% predicted)
MIF protein Log2 (FRU)
D
R = −0.41, p = 0.066
R = −0.32, p = 0.16
R = −0.47, p = 0.001
R = −0.25, p = 0.16
Sputum macrophages
MIF protein Log2 (FRU)
E
R = −0.24, p = 0.38
R = 0.025, p = 0.88
R = 0.37, p = 0.095
Exacerbation
MIF mRNA Log2 Intensity
F
R = −0.31, p = 0.3
R = −0.25, p = 0.36
R = −0.12, p = 0.47
R = −0.05, p = 0.83
Sputum neutrophils
MIF mRNA Log2 Intensity
G
R = 1, p = 0.017
R = 0.61, p = 0.017
R = −0.18, p = 0.17
R = −0.36, p = 0.1
FEV1 (% predicted)
MIF mRNA Log2 Intensity
H
R = 0.28, p = 0.35
R = 0.25, p = 0.37
R = 0.061, p = 0.72
R = 0.15, p = 0.51
Sputum macrophages
MIF mRNA Log2 Intensity

Figure S2

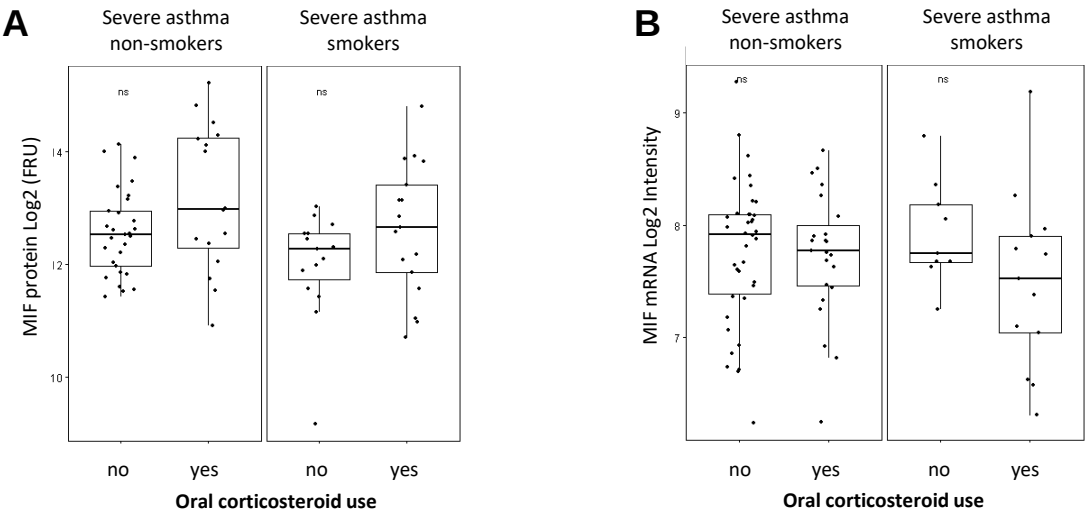

A
Severe asthma non-smokers
Severe asthma smokers
MIF protein Log2 (FRU)
ns
ns
no
yes
no
yes
Oral corticosteroid use
B
Severe asthma non-smokers
Severe asthma smokers
MIF mRNA Log2 Intensity
ns
ns
no
yes
no
yes
Oral corticosteroid use

Figure S3

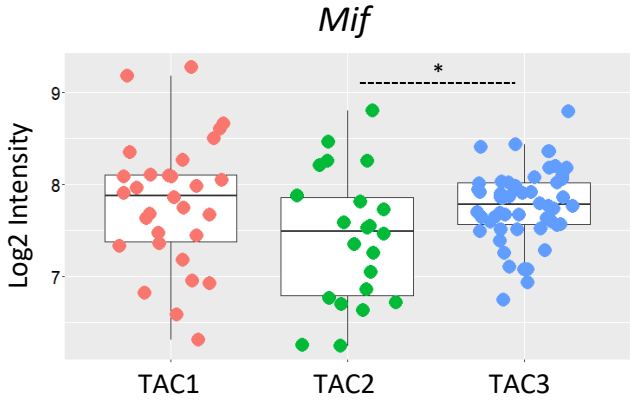


Figure S4

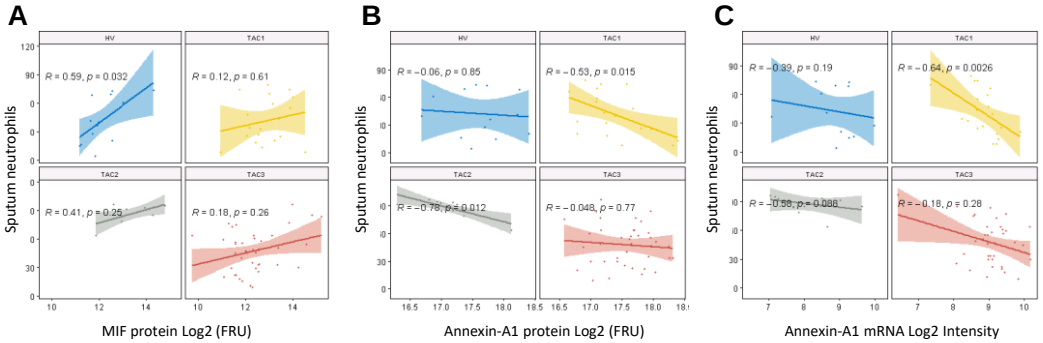
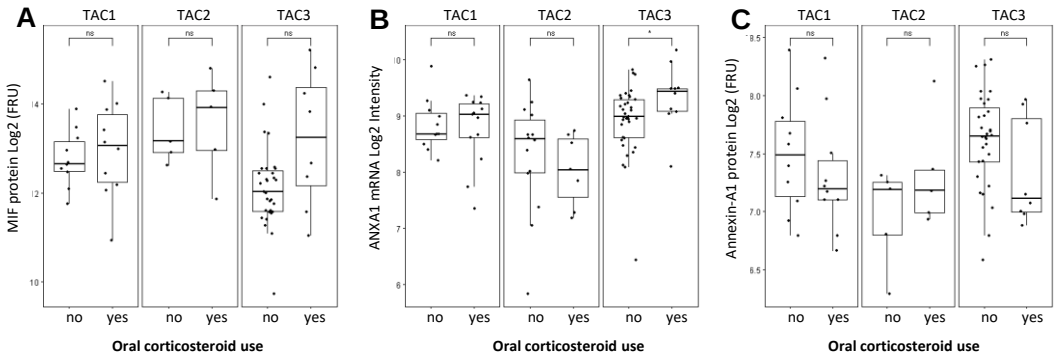
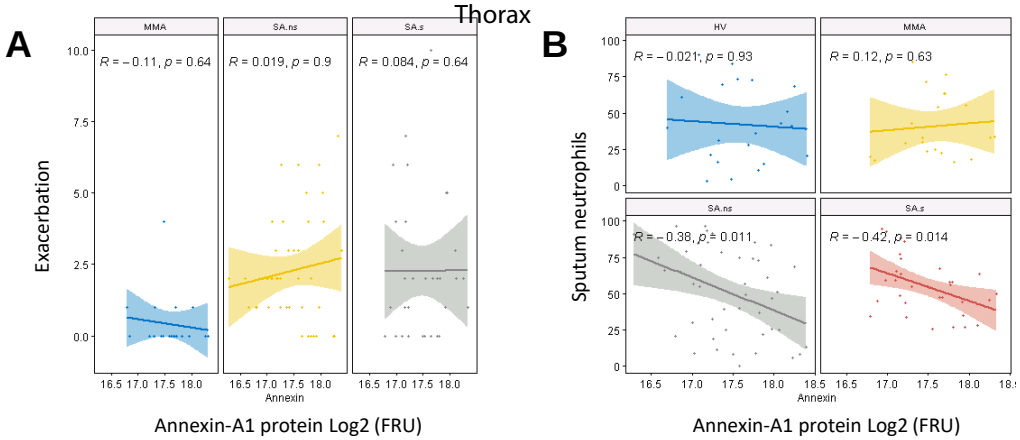
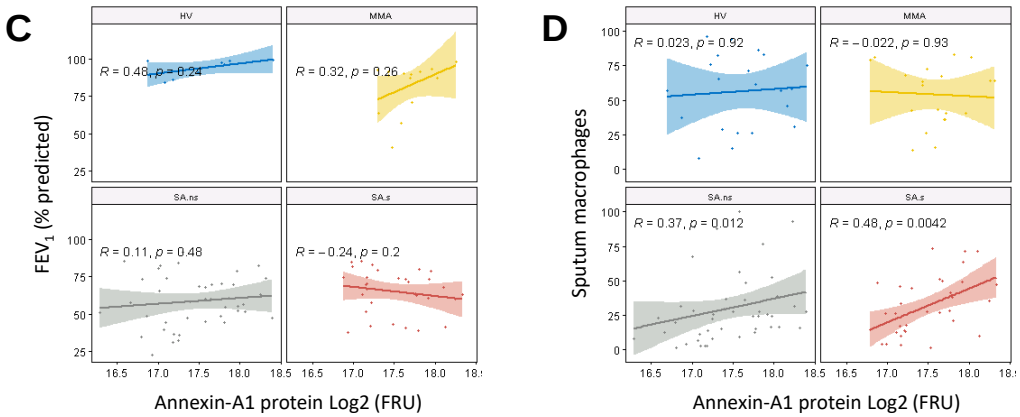
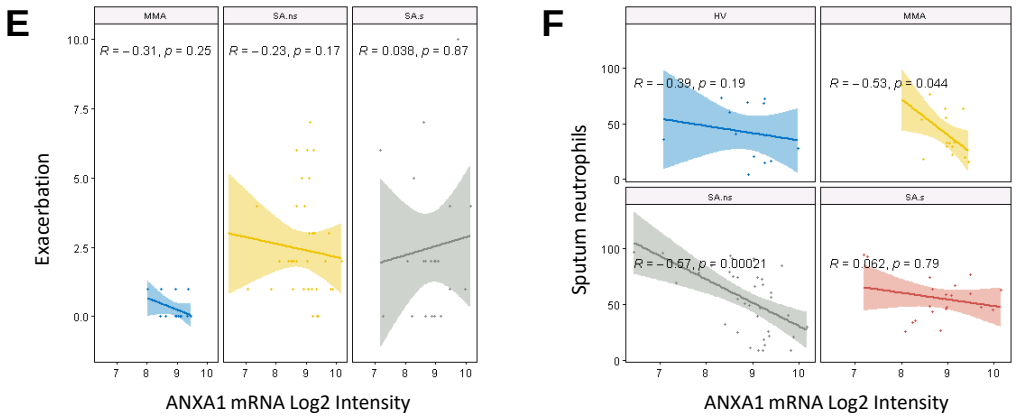

A
B
C
HV
TAC1
TAC2
TAC3
R = 0.59, p = 0.032
R = 0.12, p = 0.61
R = 0.41, p = 0.25
R = 0.18, p = 0.26
R = −0.06, p = 0.85
R = −0.53, p = 0.015
R = −0.78, p = 0.012
R = −0.048, p = 0.77
R = −0.33, p = 0.19
R = −0.64, p = 0.0026
R = −0.58, p = 0.088
R = −0.18, p = 0.28
Sputum neutrophils
MIF protein Log2 (FRU)
Annexin-A1 protein Log2 (FRU)
Annexin-A1 mRNA Log2 Intensity

Figure S5


A
TAC1
TAC2
TAC3
MIF protein Log2 (FRU)
no
yes
Oral corticosteroid use
B
ANXA1 mRNA Log2 Intensity
C
Annexin-A1 protein Log2 (FRU)


A
MMA
SA.ns
SA.s
R = −0.11, p = 0.64
R = 0.019, p = 0.9
R = 0.084, p = 0.64
Exacerbation
Annexin
Annexin-A1 protein Log2 (FRU)
B
HV
MMA
SA.ns
SA.s
R = −0.021, p = 0.93
R = 0.12, p = 0.63
R = −0.38, p = 0.011
R = −0.42, p = 0.014
Sputum neutrophils
Annexin
Annexin-A1 protein Log2 (FRU)


C
HV
MMA
SA.ns
SA.s
R = 0.48, p = 0.24
R = 0.32, p = 0.26
R = 0.11, p = 0.48
R = −0.24, p = 0.2
FEV1 (% predicted)
Annexin-A1 protein Log2 (FRU)
D
HV
MMA
SA.ns
SA.s
R = 0.023, p = 0.92
R = −0.022, p = 0.93
R = 0.37, p = 0.012
R = 0.48, p = 0.0042
Sputum macrophages
Annexin-A1 protein Log2 (FRU)


E
MMA
SA.ns
SA.s
R = −0.31, p = 0.25
R = −0.23, p = 0.17
R = 0.038, p = 0.87
Exacerbation
ANXA1 mRNA Log2 Intensity
F
HV
MMA
SA.ns
SA.s
R = −0.39, p = 0.19
R = −0.53, p = 0.044
R = −0.67, p = 0.00021
R = 0.062, p = 0.79
Sputum neutrophils
ANXA1 mRNA Log2 Intensity

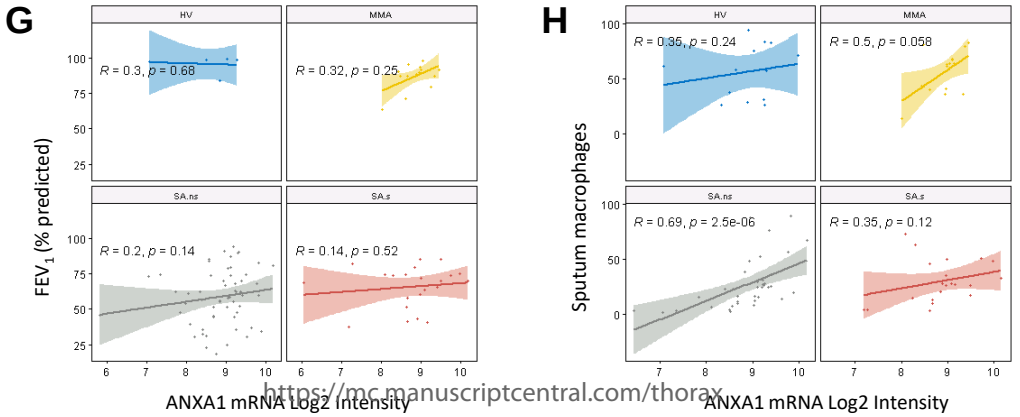

G
HV
MMA
SA.ns
SA.s
R = 0.3, p = 0.68
R = 0.32, p = 0.25
R = 0.2, p = 0.14
R = 0.14, p = 0.52
FEV1 (% predicted)
ANXA1 mRNA Log2 Intensity
H
HV
MMA
SA.ns
SA.s
R = 0.35, p = 0.24
R = 0.5, p = 0.058
R = 0.69, p = 2.5e-06
R = 0.35, p = 0.12
Sputum macrophages
ANXA1 mRNA Log2 Intensity

Figure S7

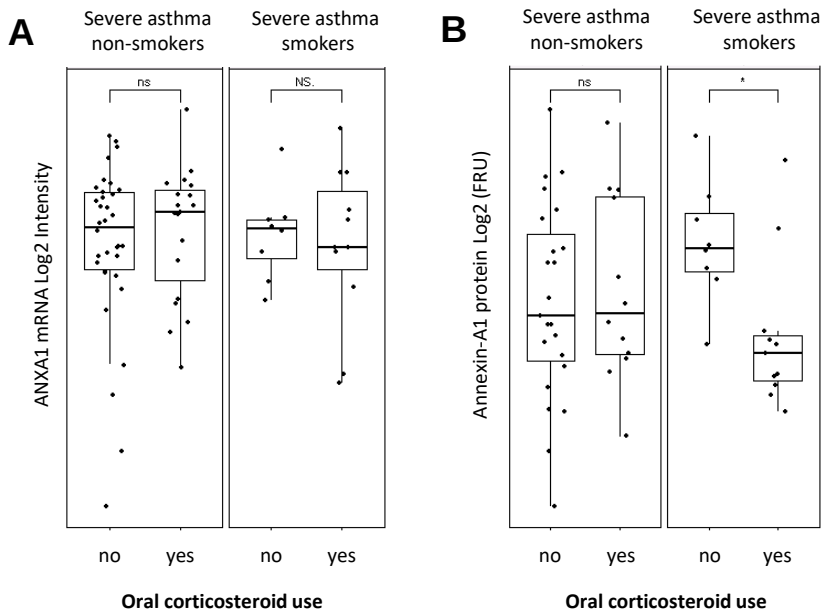

A
Severe asthma non-smokers
Severe asthma smokers
ANXA1 mRNA Log2 Intensity
ns
NS.
no
yes
no
yes
Oral corticosteroid use
B
Severe asthma non-smokers
Severe asthma smokers
Annexin-A1 protein Log2 (FRU)
ns
*
no
yes
no
yes
Oral corticosteroid use

Figure S8

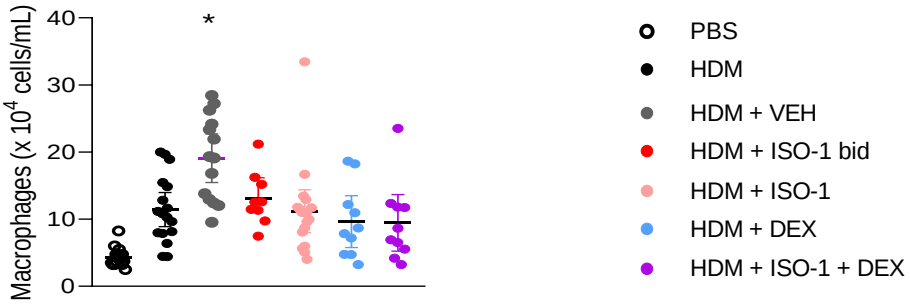

Macrophages (x 10^4 cells/mL)
40
30
20
10
0
*
PBS
HDM
HDM + VEH
HDM + ISO-1 bid
HDM + ISO-1
HDM + DEX
HDM + ISO-1 + DEX

Figure S9

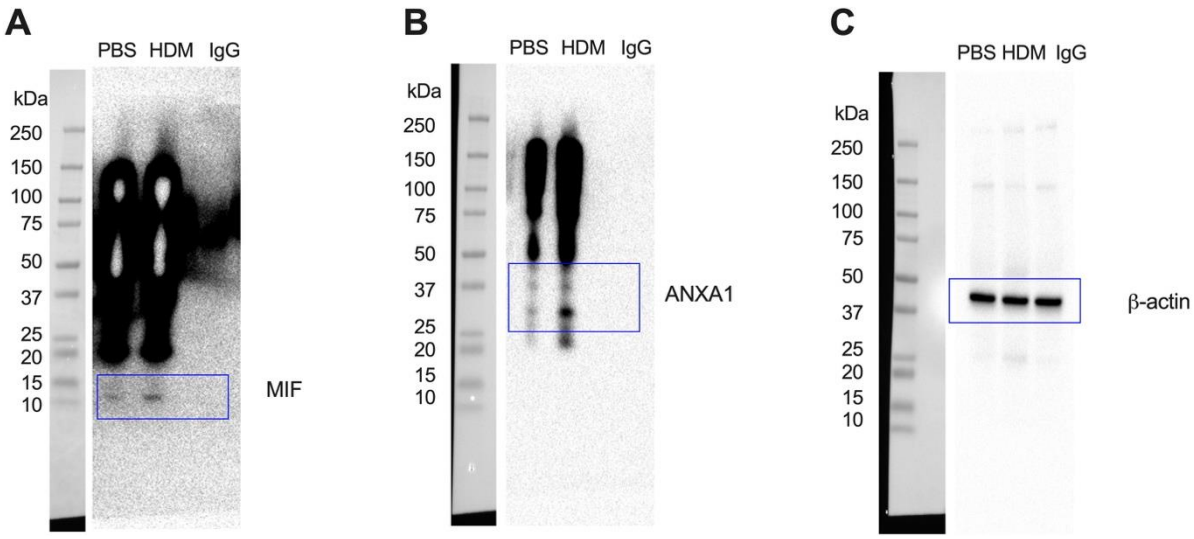
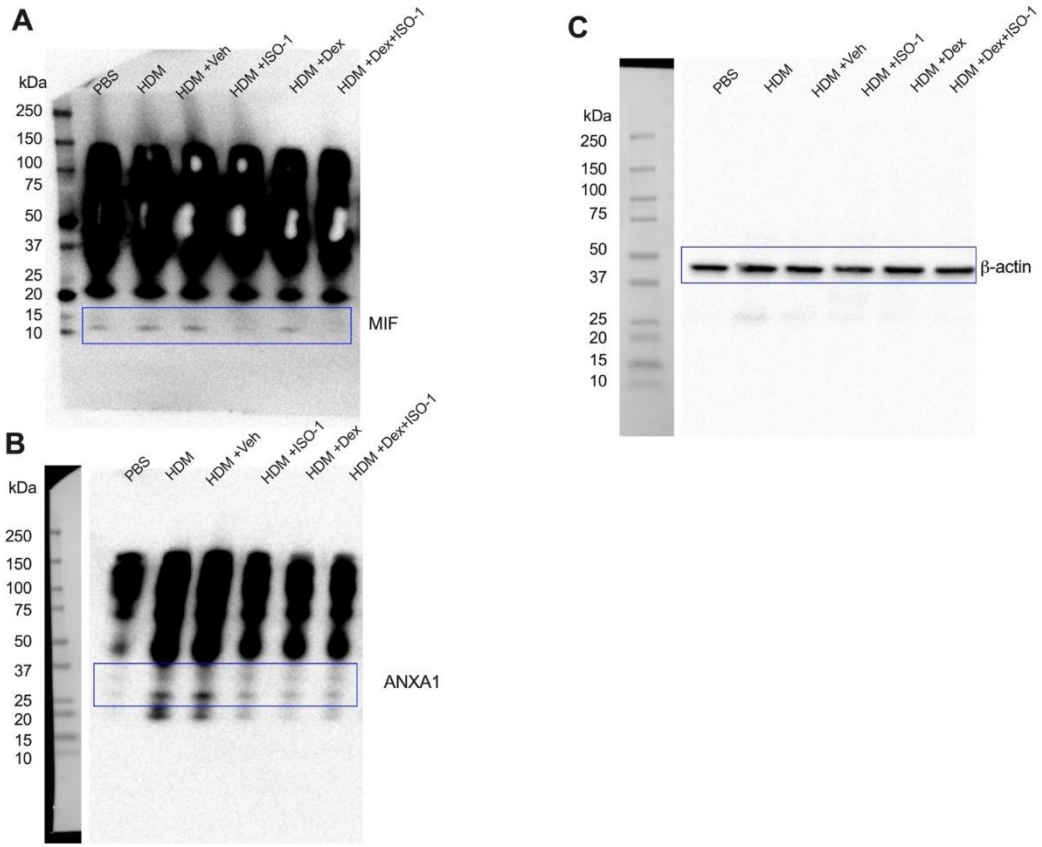

A
PBS HDM IgG
kDa
250
150
100
75
50
37
25
20
15
10
MIF
B
PBS HDM IgG
kDa
250
150
100
75
50
37
25
20
15
10
ANXA1
C
PBS HDM IgG
kDa
250
150
100
75
50
37
25
20
15
10
β-actin

Figure S10


A
kDa
PBS
HDM
HDM +Veh
HDM +ISO-1
HDM +Dex
HDM +Dex+ISO-1
250
150
100
75
50
37
25
20
15
10
MIF
B
ANXA1
C
β-actin