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Neutrophilic inflammation in asthma and defective epithelial translational control

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Take home message:

Defective translational control of neutrophil-driving cytokines in bronchial epithelium leads to exaggerated, corticosteroid-insensitive production *in vitro* and correlates with neutrophilic inflammation in airway lumen and FEV_1 % reversibility.

Plain language summary:

Asthma is a heterogeneous disease, where treatment of neutrophilic, corticosteroid-insensitive asthma is still a major challenge, not in the least as the underlying mechanism is largely unknown. Bronchial epithelial cells contribute to neutrophilic inflammation by release of pro-inflammatory mediators such as CXCL-8, interleukin-6 and G-CSF. We report that the translational control of these pro-inflammatory cytokines in bronchial epithelial cells is defective in asthma patients, which results in their exaggerated and also corticosteroid-insensitive production. The degree of *in vitro* CXCL-8 production correlates with CXCL-8 levels and neutrophil numbers in the airway lumen from the patients from which the epithelial cells were obtained. Even more so, this *in vitro* CXCL-8 production correlated with their bronchodilator-induced improvement of FEV_1.

Abstract

Neutrophilic inflammation in asthma is associated with interleukin-17A (IL-17A), corticosteroid-insensitivity and bronchodilator-induced FEV_1 reversibility. IL-17A synergizes with tumour necrosis factor-α (TNF-α) in the production of the neutrophil chemokine CXCL-8 by primary bronchial epithelial cells (PBECs).

We hypothesized that local neutrophilic inflammation in asthma correlates with IL-17A and TNF-α-induced CXCL-8 production by PBECs from asthma patients.

PBECs from most asthma patients displayed an exaggerated CXCL-8 production to TNF-α and IL-17A, but not to TNF-α alone, and that was also insensitive to corticosteroids. This hyperresponsiveness of PBECs strongly correlated with CXCL-8 levels and neutrophil numbers in bronchoalveolar lavage from the corresponding patients, but not with that of eosinophils. This hyperresponsiveness also correlated with bronchodilator-induced FEV_1% reversibility. At the molecular level epithelial hyperresponsiveness was associated with failure of the translational repressor T-cell internal antigen-1 related protein (TiAR) to translocate to the cytoplasm to halt CXCL-8 production, as confirmed by TiAR knockdown. This is in line with the finding that hyperresponsive PBECs also produced enhanced levels of other inflammatory mediators.

Hyperresponsive PBECs in asthma patients may underlie neutrophilic and corticosteroid-insensitive inflammation and a reduced FEV_1, irrespective of eosinophilic inflammation. Normalizing cytoplasmic translocation of TiAR is a potential therapeutic target in neutrophilic, corticosteroid-insensitive asthma.

Introduction

Asthma is a heterogeneous disease characterized by chronic airway inflammation and airway hyperresponsiveness culminating in episodes of reversible airway obstruction. This heterogeneity is reflected by differences in pathology, clinical course and response to standard treatment (1, 2). An understanding of molecular mechanisms underlying this heterogeneity may provide a rationale for targeted treatment of patients. This is exemplified by successful therapeutic interventions with anti-interleukin(IL)-5 (3, 4) and anti-IgE (5) in severe asthma patients with a marked eosinophilic inflammation (6) and T_H2-high signature respectively. Treatment strategies for asthma with a predominant neutrophilic inflammation are not available, which relates to the poor understanding of mechanisms that drive asthma pathogenesis in these patients.

IL-17A has been associated with neutrophilic inflammation (7, 8) and corticosteroid-insensitivity in allergic asthma (9), in severe asthma and in exacerbations of asthma (10-12). IL-17A and neutrophilic inflammation have also been linked to the post-bronchodilator FEV_1 (forced expiratory volume in 1 second) response (13, 14). IL-17A targets among others stromal cells (7) like primary bronchial epithelial cells (PBECs), which are one if not the most abundant cell type in the airways that produce many inflammatory mediators relevant to asthma and therefore may, at least in part, drive airway inflammation (15). In addition, IL-17A has been shown to decrease the responsiveness of CXCL-8 production by bronchial epithelium to corticosteroids (16). IL-17A synergizes with pro-inflammatory stimuli like TNF-α in the production of CXCL-8 and IL-6 by attenuating degradation of *CXCL8* and *IL6* mRNA (17-19). Previously, we have shown that *CXCL8* and *IL6* mRNA degradation is promoted by microRNA16 (miR16) expression, which is attenuated by IL-17A (20). Hence, we

hypothesized that PBECs from asthma patients release more CXCL-8 and IL-6 in response to IL-17A and TNF-α than PBECs from healthy individuals because of reduced *CXCL8* and *IL6* mRNA degradation.

PBECs collected by bronchial brush were expanded and the production of mediators relevant to neutrophilic inflammation and other mediators were determined after *in vitro* stimulation of PBECs with IL-17A and TNF-α. Despite enhanced CXCL-8 and IL-6 responses and high miR16 levels by PBECs from most asthma patients, the half-lives of *CXCL8* and *IL6* mRNA were not different in PBECs from healthy controls. Instead we found a defective cytoplasmic translocation of the translational repressor TiAR in PBECs from asthma patients, which related to corticosteroid insensitivity, *in vivo* neutrophilic inflammation and the post-bronchodilator FEV_1 response.

Methods

Study design

Bronchial epithelial brushes were obtained from three registered trials, the RESOLVE (NCT1677) study with healthy and mild asthma patients, the MATERIAL (NTR01520051) study with mild asthma patients and TASMA (NCT02225392) study with severe asthma patients. After inclusion, BALF and bronchial brushes were collected by standard bronchoscopy procedures (21) on the same day. Lung function parameters, FEV_1 % reversibility and methacholine challenge test (PC_{20}) were performed 4 days prior to bronchoscopy.

Subjects

The study protocols were reviewed and approved by the ethical review committee and were in accordance with the declaration of Helsinki. All patients and controls provided written

informed consent. The studies were conducted in one center only, at the Department of Respiratory Medicine of the Academic Medical Center, Amsterdam, The Netherlands. Patients with mild allergic asthma (RESOLVE and MATERIAL studies) met the following criteria: a history of episodic chest symptoms, baseline FEV_1 >80% predicted, airway responsiveness to methacholine (provocative concentration causing 20% fall in FEV_1, PC_{20} < 9.8 mg/mL), skin prick test positive for at least one of 12 common aeroallergens. Patients were not allowed to use inhaled or systemic corticosteroids or treatment other than inhaled short-acting β2-agonists within 2 weeks prior to the start of the study. Healthy individuals from the RESOLVE study had a FEV_1 >80% predicted, PC_{20} >16 mg/mL, skin prick test negative for 12 common aeroallergens. All mild asthma patients and healthy controls were non-smoking or had stopped smoking 12 months ago with ≤5 pack years.

Patients with severe asthma (TASMA study), fulfilling the World Health Organization or modified innovative medicines initiative criteria of severe refractory asthma, were included (22, 23). Patients using inhaled corticosteroid (ICS) at a dosage ≥500µg fluticasone equivalent per day and long acting ß2-agonist (LABA) at a dosage of ≥100µg per day salmeterol dose aerosol or equivalent for the past 6 months and systemic corticosteroid use (≤20mg/day prednisone equivalent) were allowed. Furthermore, their ACQ >1,5 for 2 weeks and they were non-smokers for 1 year or more (former smoker ≤15 pack years).

Culturing PBECs and stimulation

PBECs were obtained by brush biopsy during bronchoscopy (P0), from healthy individuals and asthmatics, plated on 6-well plates pre-coated with purecol (Advanced Biomatrix) and grown until confluence in BEBM medium (Lonza) supplemented with growth factors (Lonza) and Ciproxin (Sigma) at 2 µg/ml. The cells were then passaged into 24-well plates (P1) in

equal amounts and when confluent stimulated with recombinant human (rh) TNF-α (5 ng/ml) (R&D systems), rhIL-17A (100 ng/ml) (R&D systems), rhTNF-α plus rhIL-17A (5 ng/ml and 100 ng/ml, respectively) or no stimulus for 2 hrs or 16 hrs for the assessment of expressed RNA and released protein, respectively. We used the corticosteroid dexamethasone, which is a recognized reference standard for corticosteroids and also is highly soluble in water. When indicated 3.8 μM dexamethasone (Sigma) was used. The concentrations of TNF-α, IL-17A and dexamethasone were optimized in earlier studies (18). As there was limited availability of PBECs the dexamethasone and other experiments were not performed with PBECs from all patients.

Detailed description of luminex, miRNA16 analysis and LNA knockdown, mRNA and half-life analysis, immunohistochemistry, immunopurification and western blot, TiAR knockdown and alveolar macrophages isolation and stimulation are provided in the online data supplement.

Results

PBECs from most mild and severe asthma patients are hyperresponsive to IL-17A and TNF-α and insensitive to corticosteroids

PBECs were obtained from 43 mild, 16 severe asthma patients and 16 healthy controls, the characteristics and clinical parameters of whom are summarized in table 1. Previously we have shown that human lung epithelial-like NCI-H292 cells (CRL 1848; American Type Culture Collection, Manassas, VA) and PBECs in response to IL-17A and TNF-α displayed a synergistic production of CXCL-8 and IL-6 (18). This was due to stabilization of the encoding transcripts, which for NCI-H292 cells was shown to be dependent on an attenuated production of miR16 (20). We confirmed the synergistic effect of TNF-α plus IL-17A on CXCL-8 production by PBECs from all individuals, but the synergistic effect was far more pronounced in PBECs from

most asthma patients (Figure E1A). In line herewith, PBECs from mild and severe asthma patients, compared to those from healthy controls, produced significantly higher levels of CXCL-8, IL-6, CXCL-10, G-CSF and MIF (Figure 1A to E) upon stimulation with TNF-α and IL-17A. No synergistic effect was seen on the production of VEGF-a, CXCL-1, IL-1α, IL-1RA, SDS-1a (data not shown). PBECs that showed a synergistic CXCL-8 production (< 10,000 pg/ml of CXCL-8) similar to those from most healthy controls are referred to as normoresponsive hereafter (marked in black), whereas those with a higher response (≥10,000 pg/ml of CXCL-8) are referred to as hyperresponsive (marked in red for mild and severe asthmatics, and in blue for healthy individuals; Figure 1A). Variation for example in cell numbers and in metabolic activity for PBECs from both asthma groups and healthy individuals may underlie differences in CXCL-8 production. Hence, we plotted CXCL-8 production against the synergistic effect calculated as CXCL-8 production in response to TNF-α and IL-17A divided by CXCL-8 production to TNF-α alone plus that of CXCL-8 production to IL-17A alone, which is less dependent on these variables (Figure E1B). There was a strong correlation between CXCL-8 levels and synergism for PBECs from all groups, indicative of little variability between PBECs from the various groups. It is clear from figure E1B that the normoresponsive PBECs can be separated well from the hyperresponsive PBECs by using the 10,000 pg/ml of CXCL-8, rather than by the synergistic factor.

Hyperresponsive PBECs from healthy controls were markedly less hyperresponsive than PBECs from asthma patients (Figure 1A). Most PBECs hyperresponsive for CXCL-8 were also hyperresponsive for IL-6, G-CSF, CXCL-10, and MIF (the red and blue symbols illustrating hyperresponsive CXCL-8 production were maintained in the graphs for the other cytokines). In a sub-study with PBECs from 5 healthy and 4 asthmatics, PBECs hyperresponsive to IL-17A and TNF-α were also hyperresponsive to IL-17A and LPS (5 μg/ml; data not shown).

To determine sensitivity to corticosteroids, PBECs from mild and severe asthmatics were also exposed to TNF-α plus IL-17A in the presence of dexamethasone (3.8μM, titrated as the optimal concentration). With dexamethasone, normoresponsive PBECs (*n*=7) showed significantly reduced levels of CXCL-8, IL-6, G-CSF (Figure 1F to H) as opposed to no significant changes by hyperresponsive PBECs (*n*=17). CXCL-8 production by both normo- and hyperresponsive PBECs exposed to TNF-α alone was significantly attenuated by dexamethasone, indicating that it is IL-17A that induces corticosteroid unresponsiveness in hyperresponsive PBECs (Figure E1C). MIF and IP-10, however, did not decrease in response to dexamethasone, not even in normoresponsive PBECs (Figure E1D). Therefore, in addition to an exaggerated CXCL-8 production, hyperresponsive and normoresponsive PBECs are also differentiated by dexamethasone-insensitive *versus* dexamethasone-sensitive CXCL-8 production, respectively.

Hyperresponsive PBECs correlate with neutrophilic inflammation and response of FEV_1 to salbutamol

To determine whether this intrinsic feature of PBEC hyperresponsiveness may underlie key features in asthma, hyperresponsiveness was correlated to mediators in bronchoalveolar lavage fluid (BALF) and clinical parameters. As clinical and inflammatory parameters differ between mild and severe asthma, for example through the use of corticosteroids, we analysed mild and severe asthma separately. CXCL-8 production by PBECs to IL-17A and TNF-α, significantly correlated with CXCL-8 measured in BALF of the respective mild asthma patients (Figure 2A). BALF recovery (73% ± 6.6%; mean ± SD) was similar for patients allowing for direct comparison. CXCL-8 BALF levels from healthy controls were below the lower limit of detection. There was also a significant positive correlation with the neutrophil

percentage (Figure 2B) and absolute neutrophil numbers (data not shown) in BALF. No correlation was observed with eosinophil percentage (Figure 2C) or with absolute eosinophil numbers.

Both baseline FEV_1% predicted and bronchodilator reversibility are clinical parameters that allow sub-phenotyping of asthma (24, 25). Mild and severe asthma patients with hyperresponsive PBECs had significantly higher FEV_1% reversibility compared to those with normoresponsive PBECs (Figure 2D). In line herewith, hyperresponsiveness significantly correlated with the FEV_1% reversibility in mild and severe asthma patients (Figure 2E). There was, however, no correlation with PC_{20} (provocative concentration of methacholine causing a 20% fall in FEV_1) methacholine challenge test (Figure E2).

Enhanced miR16 levels in PBECs from asthma patients

We previously demonstrated that miR16 promoted *CXCL8* and *IL6* mRNA degradation in NCI-H292 cells (20). miR16 levels were significantly higher in PBECs from asthma patients compared to that from healthy controls, both at baseline for unstimulated cells and after TNF-α and IL-17A exposure for 16 hrs (Figure 3A and B, respectively). The mRNA levels of *CXCL8* and *IL6* in PBECs from mild and severe asthma patients were significantly higher after 2 hrs with TNF-α and IL-17A compared to TNF-α alone (Figure 3C), indicative of a reduced mRNA degradation. In line with our previous findings (20), *CXCL8* and *IL6* mRNA half-lives were significantly enhanced upon exposure to TNF-α and IL-17A. However, the degradation of *CXCL8* and *IL6* mRNA was not different between normo- and hyperresponsive PBECs (Figure 3D). Knock-down of miR16 in PBECs with a 75% transfection efficiency had no effect on *CXCL8* and *IL6* mRNA (Figure 3E) and protein levels (Figure 3F) measured after 2 hrs and

16 hrs, respectively. This indicated that *CXCL8* and *IL6* mRNA and protein were not modulated by miR16, contrary to what was observed earlier in NCI-H292 epithelial cells (20).

Defective cytoplasmic translocation of TiAR in hyperresponsive PBECs leads to exaggerated production of inflammatory mediators

The afore-mentioned results indicated that the synergistic and corticosteroid-insensitive CXCL-8 and IL-6 production by hyperresponsive PBECs to TNF-α and IL-17A was not due to attenuated mRNA degradation. An alternative explanation for the enhanced production is an altered translational control. The AU-rich elements at the 3'-untranslated region of mRNAs, to which AU-binding proteins and likely miR16 bind (20), are also involved in controlling translation, such as by the AU-binding protein TiAR. TiAR immunoprecipitation from the cytoplasmic fraction of normoresponsive PBECs after 2 hrs of TNF-α and IL-17A stimulation contained *CXCL8* and *IL6* mRNA, measured by qPCR (Figure 4A), indicating that TiAR controls translation of *CXCL8* and *IL6* mRNA. Normo- and hyperresponsive PBECs were immuno-stained for TiAR and eif3η (required for translational arrest) before and after stimulation for 2 hrs with TNF-α and IL-17A. TiAR co-localised with eiF3η in normoresponsive PBECs in the cytoplasm, whereas TiAR was confined to the nucleus in hyperresponsive PBECs (Figure 4B). This was also true for 2 hrs of exposure to TNF-α and IL-17A in the presence of dexamethasone (Figure E3A). Identical staining's were seen after 16 hrs of exposure with TNF-α and IL-17A, with or without dexamethasone (data not shown). To clarify whether the failure of TiAR to translocate to cytoplasm in hyperresponsive PBECs was due to TiAR or a regulatory process facilitating TiAR translocation, hyperresponsive PBECs were exposed to arsenite that should enforce the formation of cytoplasmic stress granules containing TiAR. Treatment with relatively low arsenite concentrations resulted in TiAR translocation and at higher concentrations led to stress granules (Figure E3B). These stress granules were further

confirmed by co-localisation of eiF3η with TiAR, and also were obtained in normoresponsive PBECs (Figure E3C). PBECs were also stained for RBP1, an endoplasmic reticulum (ER) marker, which appeared as triangles around the nucleus whereas TiAR appeared as streaks, indicating that TiAR was not associated with ER (Figure E3D).

To show that TiAR halts translation we knocked down TiAR in hyperresponsive and normoresponsive PBECs. When TiAR expression was knocked down, the levels of CXCL-8 increased only in normoresponsive PBECs, whereas in hyperresponsive PBECs the knock-down had no effect on CXCL-8 production after TNF-α and IL-17A exposure (Figure 4C). The knockdown of TiAR was confirmed by western blot in both hyperresponsive and normoresponsive PBECs (Figure 4D).

IL-17RA mRNA expression does not differ between normo- and hyperresponsive PBECs

In a sub-study we determined whether normo- and hyperresponsive PBECs could be distinguished on basis of differential expression of the IL-17A receptor, IL-17RA. Similar IL-17RA mRNA expression in both normo- and hyperresponsive PBECs from mild asthma patients and healthy controls was found (Figure E4A). Staining of normo- and hyperresponsive PBECs on cytospins with anti-IL-17RA showed no differences in IL-17RA protein expression by immunohistochemistry, but these analyses were hampered by relatively large background staining (data not shown).

Alveolar macrophages do not display exaggerated responses to IL-17A and TNF-α

Alveolar macrophages like bronchial epithelial cells are key players in driving immune and inflammatory responses. To determine whether alveolar macrophages showed synergistic responses to IL-17A combined with a pro-inflammatory stimulus, we purified alveolar

macrophages from sputum of asthmatics and healthy controls. The macrophages and monocytes did not show any synergistic increase in CXCL-8 levels with TNF-α and IL-17A stimulations compared to TNF-α alone (Figure E4B).

Discussion

Asthma is a heterogeneous disease, indicative of various mechanisms underlying the disease. Here we report a defective cytoplasmic translocation of TiAR in PBECs from asthma patients, which is paralleled by an exaggerated CXCL-8 production that is corticosteroid-insensitive when PBECs are exposed to TNF-α and IL-17A. Notably, the defect correlated with CXCL-8 levels and neutrophil numbers in BALF and with bronchodilator-induced reversibility of FEV_1 of asthma patients. The extent of the defect varies between patients, in line with the heterogeneity in asthma. Of equal importance, the defect was found in PBECs from both mild and severe asthma patients. Together this indicates that the defective TiAR translocation is a clinically relevant abnormality in asthma and underlies neutrophilic inflammation.

IL-17A in asthma has been implicated in neutrophilic and corticosteroid-insensitive responses, which at least in part may relate to the synergistic production of CXCL-8 by PBECs exposed to IL-17A and TNF-α (18). We found that PBECs from most asthma patients showed an exaggerated synergistic effect to IL-17A and TNF-α, referred here to as hyperresponsiveness, contrasting with the normoresponsive PBECs from most healthy controls and some patients. This hyperresponsiveness of PBECs, which is paralleled by corticosteroid insensitivity, is intriguing for a number of reasons. First, the IL-17A and TNF-α-induced CXCL-8 release by hyperresponsive PBECs *in vitro* correlates with CXCL-8 levels and percentage neutrophils in BALF of mild asthmatics, which strengthens the clinical relevance

of hyperresponsive PBECs. Secondly, cluster analysis in the Severe Asthma Research Program (SARP) study showed that sputum neutrophilia is associated with corticosteroid-insensitivity (25), substantiating earlier findings (9). In line herewith, our results show that IL-17A triggers corticosteroid insensitivity in hyperresponsive PBECs only, indicating that there is a difference in the signalling cascade downstream of the IL-17A receptor, but distinct from the cascade involved in mRNA stabilization. Whether the corticosteroid-insensitive inflammation by hyperresponsive PBECs fully underlies the corticosteroid-insensitive inflammation *in vivo* is unknown, although the specific cytokines produced by the abundant PBECs are likely to contribute significantly to airway inflammation. Thirdly, the clinical relevance of hyperresponsive PBECs is strengthened further by its correlation with bronchodilator FEV_1% reversibility. IL-17A has been linked to bronchodilator reversibility and, in line herewith, blocking the IL-17 receptor improved ACQ (Asthma Control Questionnaire) scores only in the group with high FEV_1% reversibility (13). A similar association between post-bronchodilator FEV_1 and sputum neutrophils has been shown, but not with eosinophils (14). So these findings suggest that hyperresponsive PBECs may underlie neutrophilic inflammation in asthma. Whereas we tend to distinguish eosinophilic from neutrophilic inflammation in asthma it is important to realise that in patients with eosinophilic inflammation (usually with ≥ 3% sputum eosinophils), neutrophils are also present (26). Therefore, the TiAR translocation defect is likely to be present even in asthma patients with eosinophilic inflammation, although its contribution to asthma pathophysiology may be less than that by eosinophils.

In a recent deep-phenotyping study, asthma patients with a high IL-17A profile and neutrophilic inflammation were found to experience more exacerbations (27). IL-1ß was one of the genes abundantly overexpressed in these patients. Previously we described that TNF-

α combined with IL-17A can be substituted for by IL-1ß, which gave similar exaggerated CXCL-8 responses by PBECs (17). In another recent murine study IL-17A was found to synergize with IL-33 in corticosteroid-insensitive neutrophilic inflammation and enhanced airway hyperresponsiveness (29). Together this indicates that similar analyses should be performed with IL-1ß and IL-33, which may reveal a role for hyperresponsive PBECs in exacerbations. Rodhe and colleagues (28) showed enhanced neutrophilic inflammation upon a rhinovirus-induced exacerbation. More recently Toussaint et al. (30) showed that neutrophil extracellular DNA traps were formed during virus-induced exacerbations, similar to that found for RSV (31). These traps are potentially anti-viral as they do bind viruses (31), but at the same time also can cause airway obstruction.

Previously, we have shown that IL-17A attenuates *CXCL8* mRNA degradation by reducing miR16 levels, which leads to an exaggerated CXCL-8 production. In the present study we confirmed the attenuated *CXCL8* mRNA degradation upon exposure to IL-17A. Contrary to our expectations, however, IL-17A-induced changes in the half-lives of *CXCL8* and *IL6* mRNA were similar to that in normoresponsive PBECs, whereas miR16 levels were markedly higher in hyperresponsive PBECs. These findings led us to hypothesize that the exaggerated production of CXCL-8 was due to an aberrant translational control, which made us focus on TiAR. TiAR can bind to *CXCL8* mRNA and thus its failure to translocate to the cytoplasm in hyperresponsive PBECs suggests that TiAR does not halt translation of *CXCL8* mRNA. Similar TiAR-binding sites are present in mRNAs encoding other cytokines, which may explain similar exaggerated production of the other cytokines. We consider it unlikely that the hyperresponsiveness of PBECs is related to differential IL-17RA expression. IL-17RA mRNA expression was similar for hyperresponsive and normoresponsive PBECs. Although the IL-

17RA stainings were less conclusive, hyperresponsive and normoresponsive PBECs did not show different responses to IL-17A alone. On top of that, IL-17A equally stabilised *CXCL8* and *IL6* mRNA in both normoresponsive and hyperresponsive PBECs, indicating that at least part of the IL-17A downstream signalling is not different between hyper- and normoresponsive PBECs. Macrophages do not show this synergistic response to IL-17A and TNF-α, making the existence of hyperresponsive macrophages less likely. We, however, cannot exclude this yet for fibroblasts, which do show a synergistic response to IL-17A and TNF-α (18).

In this study, patients and controls were well-characterized, the procedures were well standardized in the subsequent studies and we have gone through great lengths to characterize the associated molecular defect, but there are a couple of limitations to this study. Although all brushes and PBECs were treated identically and there were no obvious differences in cell growth, we cannot exclude the possibility of differential growth between hyper- and normoresponsive PBECs. However, hyperresponsive PBECs, based upon CXCL-8 release by TNF-α and IL-17A were also hyperresponsive when expressed relative to TNF-α and IL-17A alone, i.e. the synergistic effect. Therefore, we conclude that the differences obtained in responsiveness to IL-17A are genuine. In severe asthma patients there were some bronchial brushes that could not be successfully cultured, so we cannot exclude a minor selection bias. The use of oral and inhaled corticosteroids by severe asthmatics on a regular basis might have influenced the mediators released by PBECs. However, PBECs were cultured a couple of weeks *in vitro* before stimulation with IL-17A and TNF-α, which suggests that hyperresponsiveness is an intrinsic defect. The use of corticosteroids in severe asthmatics may have affected lung function. Nevertheless, we found a correlation between hyperresponsive PBECs and FEV_1 reversibility for severe asthma patients as well. There was no correlation observed between pre-bronchodilator FEV_1 % and hyperresponsiveness in

PBECs from both mild and severe asthma patients nor with % BALF neutrophils (data not shown). Therefore, we cannot confirm the findings by Shaw et al. (14) for which there may be various explanations. Most likely, the number of severe asthma patients used in this correlation analysis is very low compared to their study.

For the current studies PBECs were grown submerged as opposed to the more differentiated air-liquid interphase (ALI) cultures. This was done to limit the time span between sampling and testing as we appeared to lose the defect with more passages of the PBECs (data not shown). Experiments with submerged cultures required around 10 days whereas ALI cultures would have taken around 28 days. Furthermore, at least in our hands, the success rate of submerged cultures was higher than for ALI cultures. And finally, adding stimuli and additional compounds to ALI cultures, with apical and basolateral domains may have complicated these quite complex studies further. As PBECs from both healthy and asthmatic individuals were treated in exact the same manner, the current differential findings with the submerged cultures are genuine. The relevance of which is clear from the correlation of hyperresponsiveness with *in vivo* parameters (BALF CXCL-8 and neutrophils, and FEV_1) from the individuals from whom PBECs were obtained.

In summary, PBECs in the majority of mild and severe asthma patients are hyperresponsive to IL-17A and pro-inflammatory stimuli like TNF-α and LPS, reflected by an enhanced production of CXCL-8 and other inflammatory mediators, and corticosteroid insensitivity. A defective cytoplasmic translocation of TiAR underlies this hyperresponsiveness and relates to neutrophilic inflammation and bronchodilator-induced FEV_1 reversibility. Unravelling the

underlying mechanisms would facilitate the development of therapeutic interventions that could reduce the burden of corticosteroid-insensitive neutrophilic inflammation in asthma.

Author contributions: A.R. conducted experiments, analyzed data and prepared the manuscript. S.C. conducted experiments and analyzed data. A.D. conducted experiments. P.I.B. performed bronchoscopies, established the TASMA study and reviewed the manuscript. P.J.S. contributed to study design and reviewed the manuscript. R.L. devised the study, contributed to the experimental set-up definition and prepared the manuscript. All authors approved the final version of the manuscript.

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Figure legends

Figure 1. Enhanced and dexamethasone-insensitive production of inflammatory mediators in response to TNF-α and IL-17A by primary bronchial epithelial cells (PBECs) from mild and severe asthma patients. PBECs from mild (*n*=43) and severe asthmatics (*n*=16) as compared to those from healthy individuals (*n*=16), produced significantly higher levels of CXCL-8 (*A*), IL-6 (*B*), CXCL-10 (*C*), G-CSF (*D*) and MIF (*E*) after stimulation with TNF-α plus IL-17A for 16 hrs. PBECs with relative high CXCL-8 production were denoted hyperresponsive (blue for healthy individuals and red for asthma patients) as opposed to normoresponsive PBECs with relative lowest CXCL-8 production (black symbols). Data are shown as mean ± SD. For statistical analyses two-tailed Mann-Whitney U t-tests were used: ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. The symbols for normo- and hyperresponsive CXCL-8 production were used in all graphs, showing that normo- and hyperresponsiveness extends to various mediators. CXCL-8 (*F*), IL-6 (*G*) and G-CSF (*H*) production by normoresponsive PBECs (*n*=7) exposed to TNF-α and IL-17A for 16 hrs was significantly reduced by dexamethasone (3.8 μM), whereas hyperresponsive PBECs (*n*=15) did not respond to dexamethasone. For statistical analyses two-tailed paired t-test was used: *P<0.05.

Figure 2. PBEC hyperresponsiveness relates to neutrophilic airway inflammation and bronchodilator-induced FEV_1 reversibility in asthma. CXCL-8 released *in vitro* by PBECs from mild asthma patients (*n*=24) after 16 hrs stimulation with TNF-α and IL-17A showed a strong positive correlation with CXCL-8 (*A*), percentage of neutrophils (*B*), but not with percentage of eosinophils (*C*) in their corresponding bronchoalveolar lavage fluid (BALF). (*D*) Mild and severe asthma patients (*n*=38) with hyperresponsive PBECs had significantly higher bronchodilator-induced FEV_1 % reversibility compared to patients with normoresponsive

PBECs. (*E*) Bronchodilator-induced FEV_1 % reversibility correlated positively with CXCL-8 released after 16 hrs stimulation with TNF-α and IL-17A, in mild (*n*=25) and severe (*n*=13) asthma patients (red - hyperresponsive ; black – normoresponsive). Pearson's correlation test was performed with two-tailed analysis: ***P<0.001, **P<0.01, *P<0.05.

Figure 3. *CXCL8* and *IL6* mRNA half-lives are identical in normo-responsive and hyper-responsive PBECs despite enhanced levels of microRNA16 (miR16). Relative miR16 expression was increased in PBECs at baseline (*A*) and after 16 hrs of stimulation with TNF-α and IL-17A (*B*) in asthmatics (*n*=20) compared to that in healthy controls (*n*=14). Explanation of the colored symbols is provided in Figure 1. Data are shown as mean ± SD. For statistical analyses two-tailed Mann-Whitney U t-tests were used: ***P<0.001, **P<0.01.. (*C*) *CXCL8* mRNA and *IL6* mRNA, normalized for *GAPDH* mRNA, were increased after 2hrs of stimulation with TNF-α and IL-17A compared to TNF-α alone, both in normo- and hyperresponsive PBECs from asthma patients (*n*=12). (*D*) Half-lives of *CXCL8* mRNA (*n*=13) and *IL6* mRNA (*n*=9) were significantly higher after 2hrs with TNF-α and IL-17A stimulation compared to TNF-α alone, both in normo- and hyperresponsive PBECs from asthma patients. For statistical analyses two-tailed paired t-test was performed: **** P<0.0001, **P<0.01, *P<0.05. (*E*) miR16 knockdown in PBECs with complementary locked nucleic acid (LNA)-miR16 and LNA-scr (scrambled control) or non-transfected (nt) as a control showed no effect on *CXCL8* mRNA and *IL6* mRNA 2 hrs after stimulation with TNF-α or TNF-α plus IL-17A, compared to not stimulated (ns) PBECs (*n*=6). (*F*) As E, but for released CXCL-8 after 16 hrs of incubation, showing no differences with LNA-miR16 and LNA-scr or non-transfected (nt) after stimulation with TNF-α or TNF-α plus IL-17A, or no stimulation (ns) (*n*=3). For statistical analyses 2-way ANOVA, Bonferroni's multiple comparison post-test was used.

Figure 4. TiAR interacts with *CXCL8* mRNA and *IL6* mRNA, but fails to translocate in hyperresponsive PBECs leading to exaggerated production of CXCL-8. (*A*) Western blot showing TiAR bands in cytoplasmic fraction (CyF), pre-cleared lysate (PCL) and in the eluate but not in the flow through (FT). In the eluate the presence of *CXCL8* and *IL6* mRNA was detected by qPCR indicating these mRNA's co-immunoprecipitated with TiAR from the cytoplasmic fraction of normoresponsive PBECs collected after 2hrs of TNF-α and IL-17A stimulation. (*B*) Confocal images showing the co-localization of TiAR (red) and eiF3η (green), representative of hyper- (*n*=8) and normoresponsive (*n*=4) PBECs stimulated with TNF-α and IL-17A for 2 hrs. Nuclei are stained with DAPI. (*C*) Knockdown of TiAR using three lenti-viral constructs, where the TiAR-2 construct failed to knockdown TIAR (not shown). Enhanced CXCL-8 production (TNF-α plus IL-17A for 16 hrs) after transfection with TiAR constructs TiAR-1 and TiAR-3 in normoresponsive PBECs (*n*=4), whereas this did not occur in hyperresponsive PBECs (*n*=5). Controls are transfections with scrambled (scr) construct and non-transfected (nt) cultures. For statistical analyses 2-way ANOVA, Bonferroni's multiple comparison post-test was used. (*D*) Representative western blots showing effective knockdown of TiAR using the lenti-viral TiAR construct TiAR-3 as compared to a scrambled construct and non-transfected PBECs for both normo- and hyper-responsive PBECs. Quantified band intensities are represented in bar graph.

Table 1. Patient characteristics of mild and severe asthma patients and healthy controls.

Characteristics	**Healthy**	**Mild asthma**	**Severe asthma**
Subjects (n)	17	43	16
Age (years) *	22.26 (19-31)	21.36 (18-29)	44.53 (25-66)
Sex ratio (M:F)	0.13	0.47	0.44
FEV_1 pre-bronchodilator (L) †	4.17 (0.70)	4.10 (0.69)	2.72 (0.72)
FEV_1 post-bronchodilator (L) †	4.29 (0.69)	4.20 (0.77)	2.99 (0.73)
FEV_1 pre-bronchodilator (% pred) †	108.2 (10.51)	101.12 (11.59)	86.61 (21.29)
FEV_1 post-bronchodilator (% pred) †	111.6 (10.58)	107.96 (11.67)	94.76 (21.43)
FEV_1 reversibility †	3.4 (2.60)	6.84 (4.47)	8.23 (4.91)
PC_{20} (mg/ml) †	>16.0	2.57 (2.3)	1.42 (1.64)
FeNO (ppb) †	20.8 (11.09)	52.34 (30.15)	61.79 (71.02)

*Mean (min-max)

† Mean (SD)

Figure 1

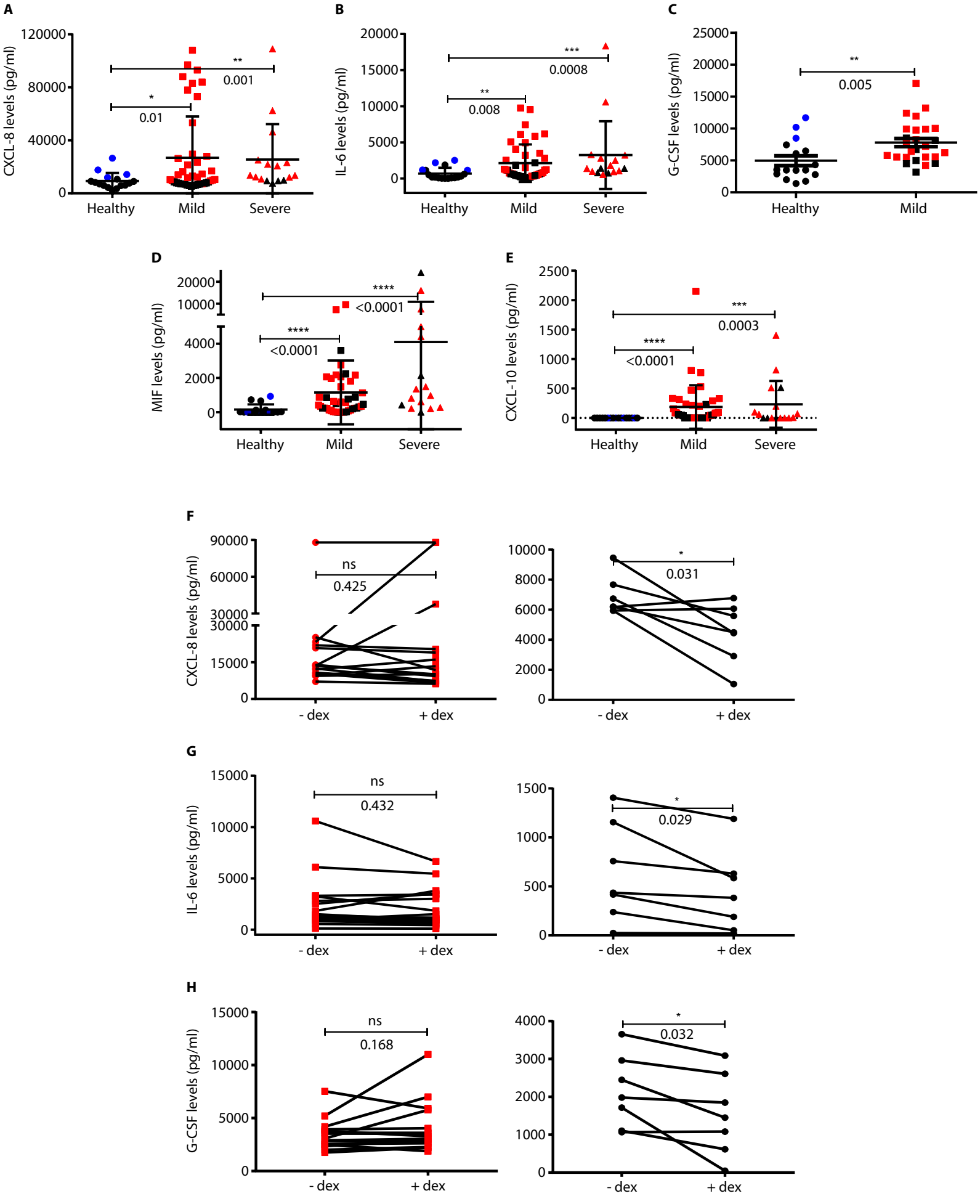

A
CXCL-8 levels (pg/ml)
120000
80000
40000
0
**
0.001
*
0.01
Healthy
Mild
Severe
B
IL-6 levels (pg/ml)
20000
15000
10000
5000
0
***
0.0008
**
0.008
Healthy
Mild
Severe
C
G-CSF levels (pg/ml)
25000
20000
15000
10000
5000
0
**
0.005
Healthy
Mild
D
MIF levels (pg/ml)
20000
10000
4000
2000
0
****
<0.0001
****
<0.0001
Healthy
Mild
Severe
E
CXCL-10 levels (pg/ml)
2500
2000
1500
1000
500
0
***
0.0003
****
<0.0001
Healthy
Mild
Severe
F
CXCL-8 levels (pg/ml)
90000
60000
30000
30000
15000
0
ns
0.425
- dex
+ dex
10000
8000
6000
4000
2000
0
*
0.031
- dex
+ dex
G
IL-6 levels (pg/ml)
15000
10000
5000
0
ns
0.432
- dex
+ dex
1500
1000
500
0
*
0.029
- dex
+ dex
H
G-CSF levels (pg/ml)
15000
10000
5000
0
ns
0.168
- dex
+ dex
4000
3000
2000
1000
0
*
0.032
- dex
+ dex

Figure 2

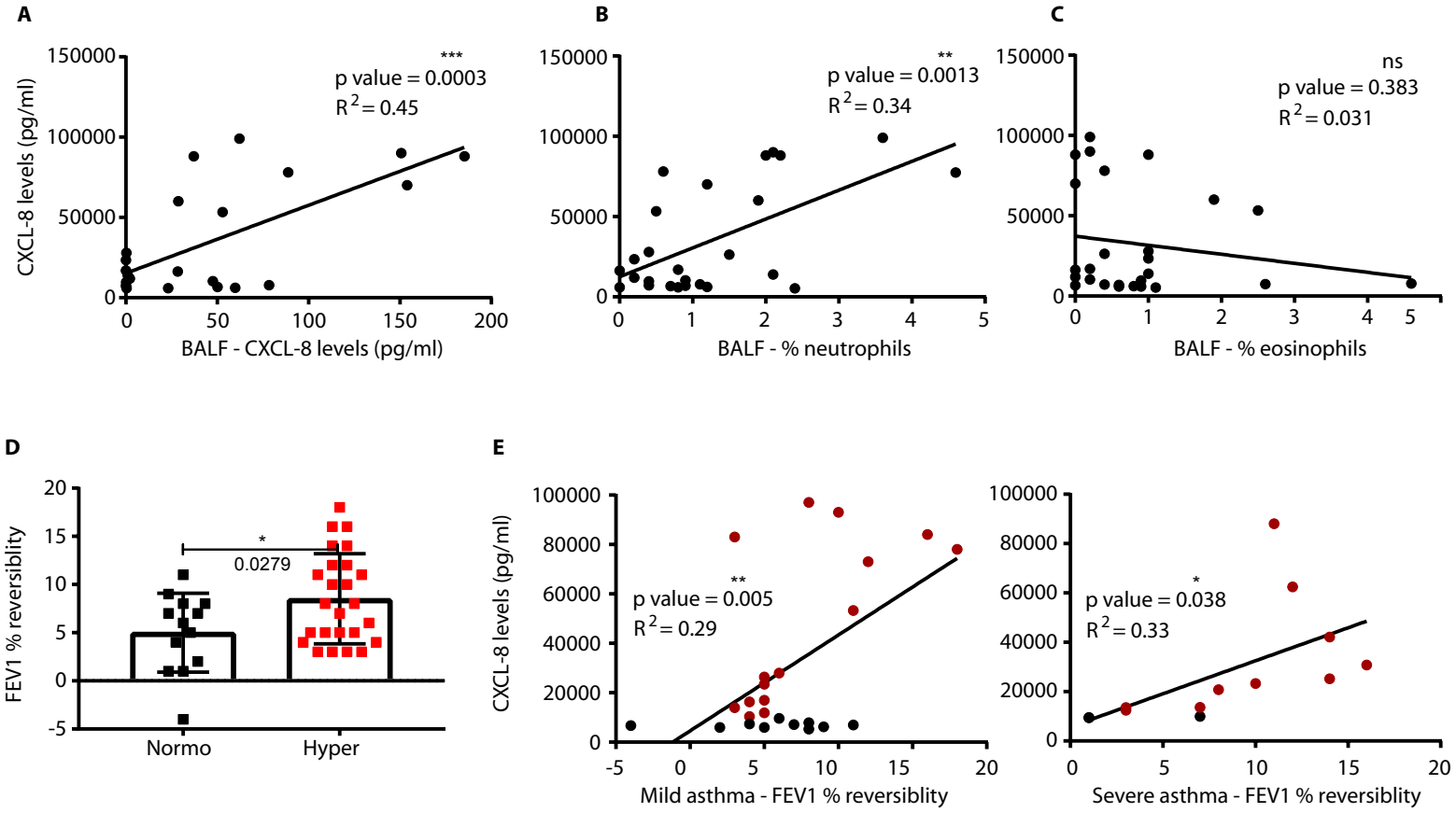

A
CXCL-8 levels (pg/ml)
150000
100000
50000
0
0
50
100
150
200
BALF - CXCL-8 levels (pg/ml)
***
p value = 0.0003
R 2 = 0.45
B
150000
100000
50000
0
0
1
2
3
4
5
BALF - % neutrophils
**
p value = 0.0013
R 2 = 0.34
C
150000
100000
50000
0
0
1
2
3
4
5
BALF - % eosinophils
ns
p value = 0.383
R 2 = 0.031
D
FEV1 % reversiblity
20
15
10
5
0
-5
*
0.0279
Normo
Hyper
E
CXCL-8 levels (pg/ml)
100000
80000
60000
40000
20000
0
-5
0
5
10
15
20
Mild asthma - FEV1 % reversiblity
**
p value = 0.005
R 2 = 0.29
100000
80000
60000
40000
20000
0
0
5
10
15
20
Severe asthma - FEV1 % reversiblity
*
p value = 0.038
R 2 = 0.33

Figure 3

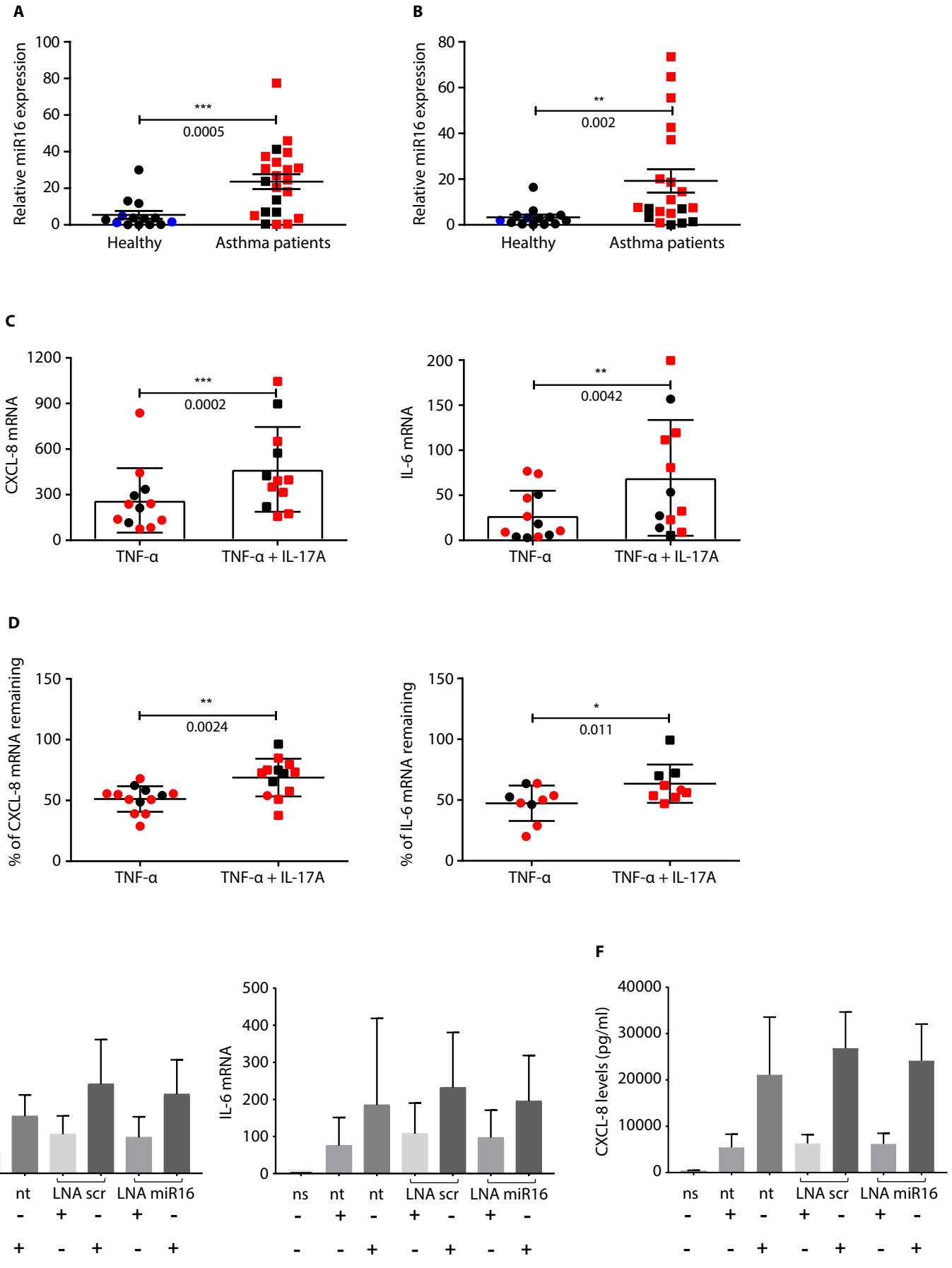
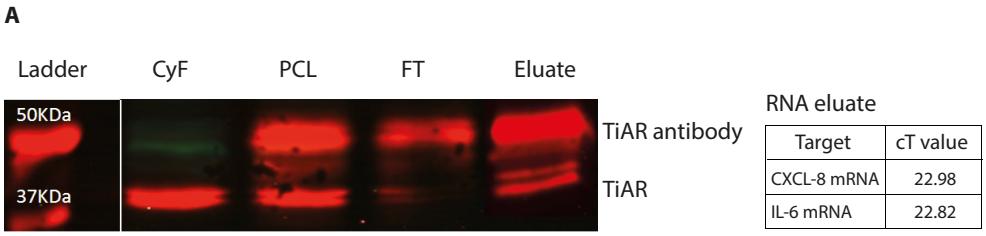
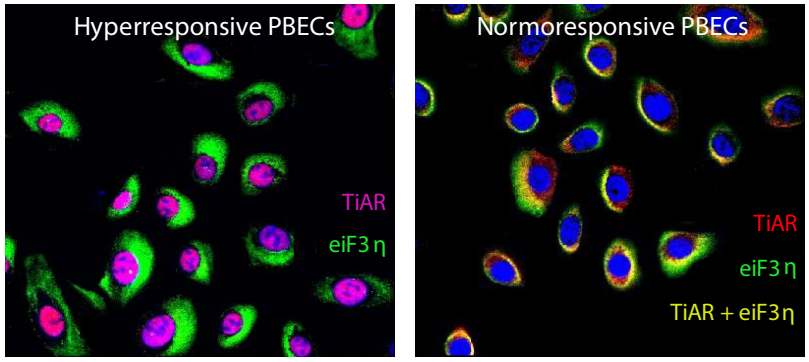

A
Relative miR16 expression
100
80
60
40
20
0
***
0.0005
Healthy
Asthma patients
B
Relative miR16 expression
80
60
40
20
0
**
0.002
Healthy
Asthma patients
C
CXCL-8 mRNA
1200
900
600
300
0
***
0.0002
TNF-α
TNF-α + IL-17A
IL-6 mRNA
200
150
100
50
0
**
0.0042
TNF-α
TNF-α + IL-17A
D
% of CXCL-8 mRNA remaining
150
100
50
0
**
0.0024
TNF-α
TNF-α + IL-17A
% of IL-6 mRNA remaining
150
100
50
0
*
0.011
TNF-α
TNF-α + IL-17A
E
CXCL-8 mRNA
2000
1500
1000
500
0
ns
nt
nt
LNA scr
LNA miR16
TNF-α
- + - + - + -
TNF-α + IL-17A
- - + - + - +
IL-6 mRNA
500
400
300
200
100
0
ns
nt
nt
LNA scr
LNA miR16
- + - + - + -
- - + - + - +
F
CXCL-8 levels (pg/ml)
40000
30000
20000
10000
0
ns
nt
nt
LNA scr
LNA miR16
- + - + - + -
- - + - + - +

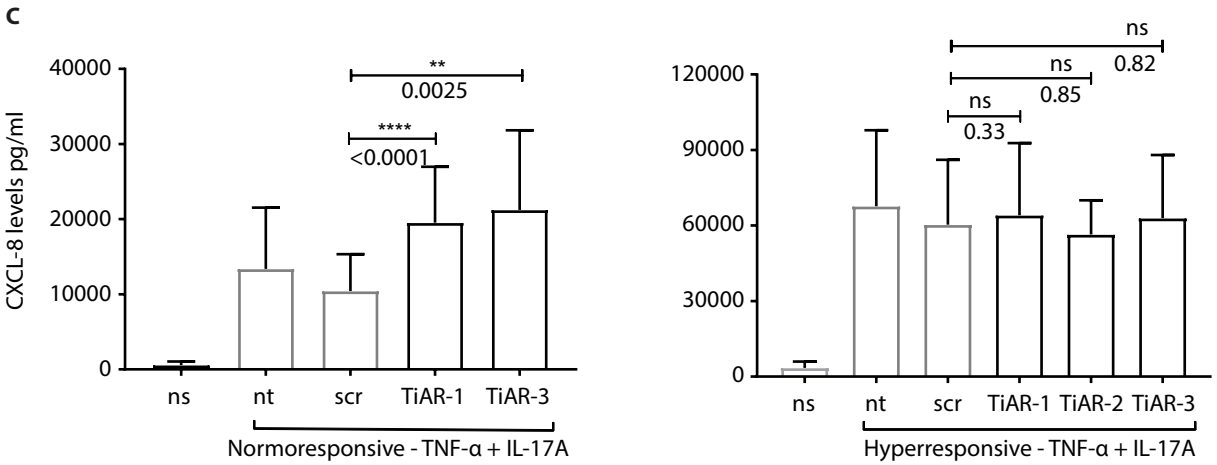
Figure 4

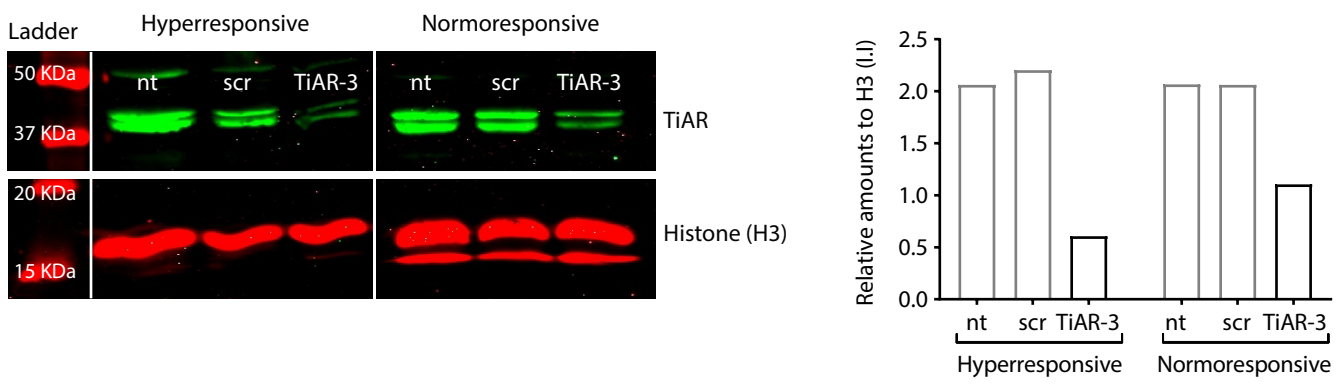


RNA eluate

Target	cT value
CXCL-8 mRNA	22.98
IL-6 mRNA	22.82







Supplementary methods

Luminex

Experiments were done in triplicate and equal volumes from triplicates were combined into one sample for cytokine measurement by multiplex assays (BioRad) and read with a Bioplex (BioRad).

miR16 analysis and LNA knockdown

miR16-1 stem-loop primer was used to prepare cDNA and miR16 primers used to determine its levels by qPCR. Expression of miR16 was normalized to an ubiquitously expressed small non-coding RNA, RNU6B as performed earlier and sequences provided in table S1 (19). PBECs were plated on 24-well plates (500 µl) and were grown till 30% confluence. Cells were transfected with LNA-miR16 (hsa-miR-16 miRCURY LNA inhibitor probe – Exiqon) or scrambled LNA (LNA-con), with sequences provided in table S1, in the presence of Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions, at a final concentration of 50 nM in serum-free medium (Opti-MEM, Invitrogen). FAM-labelled LNA-miR16 and scrambled (LNA-con) were used at a final concentration of 50 nM as controls for si-RNA and LNA-mediated knockdown, respectively, and to determine transfection efficiency. After transfection, cells were left to recover for 48 hrs followed by stimulation with recombinant human (rh) TNF-α (5 ng/ml) (R&D systems,

rhTNF-α plus rhIL-17A (5 ng/ml and 100 ng/ml, respectively) or no stimulus for 2 hrs for the assessment of RNA and 16 hrs for the assessment of protein production.

mRNA and half-life analysis

Total RNA was extracted from cells with Trizol and cDNA was synthesized with Revert AidTM H Minus Reverse Transcriptase (Fermentas). Quantitative PCR reactions were done with Power Sybr green PCR master mix (Applied Biosystems) and run on Step One Plus Real Time PCR (Applied Biosystems). Expression of the *CXCL8* and *IL6* mRNA was normalized to *GAPDH* mRNA as performed earlier (19), with sequences provided in table Sf1. mRNA half-life was measured by stimulating PBECs for 2 hrs with TNF-α or TNFα and IL-17A followed by addition of actinomycin D (ActD; 5 μg/ml, Sigma) for 1 hr and RNA isolation using Trizol reagent (Invitrogen).

Immunohistochemistry

PBECs were seeded overnight at 5,000 cells in 200 μl per well in chambered slides (Lab-Tek, Thermo Fisher Scientific Inc.). The cells were stimulated with or without TNF-α plus IL-17A for 2 hrs. The cells were then fixed with 4% formaldehyde (Merck) in PBS for 10 minutes, permeabilized with 0.1% Tween-20 (Merck) in 4% formaldehyde for 10 minutes and blocked with 10% donkey serum (#017-000-121 Jackson Immunoresearch) for 1 hr. Primary antibodies were then added: TiAR (#D32D3 – CST; 1:250), eif3η (sc-16377 – Santa Cruz;

1:250), RBP1 (#GTX101844 – GeneTex; 1:250) and incubated at 4°C overnight followed by 1 hr incubation with fluorescent tagged secondary antibody, Alexa Fluor donkey anti-goat 647, red, (A21245 Life Technologies) for TiAR (1:250) and RBP1 (1:400) and Alexa Fluor donkey anti-goat 488, green, (A11055 Life Technologies). Nuclei were counterstained with DAPI (Invitrogen). The samples were then analysed with Leica Confocal Microscope SP-8 X SMD using LAS AF Lite software.

Immuno-purification and western blot

PBECs were plated in T75 (15 ml) flasks and grown until confluent. The cells were then left unstimulated or stimulated with TNF-α and IL-17A for 2 hrs after which cells were harvested and cytoplasmic lysates were prepared on ice using 10% Nonidet P-40 (NP-40) as mentioned elsewhere (24). The cytoplasmic fraction was pre-incubated with an isotype control antibody (Rb-anti-human-α2 macroglobulin, Dako A0033) and protein A beads to remove non-specific bound proteins and loaded on to MAC's separation columns. The pre-cleared lysate then obtained was incubated with rabbit monoclonal TiAR antibody (#D32D3 -CST) and protein A beads. After incubation it was then loaded onto the columns and washed with high and low salt buffers resulting in the flow through. The protein fraction (eluate) with bound RNA was eluted using urea lysis buffer. The detailed description of the protocol and buffers used has been described here (19). Total protein in each fraction was measured by BCA protein assay

kit (Thermo Scientific). Equal amounts of protein were separated by electrophoreses on 13% SDS-PAGE on Bio-Rad Mini Protean II and Hoefer SE600 systems and transferred to PVDF membrane (Millipore). Blots were blocked with 0.4% (w/v) skimmed milk powder in phosphate-buffered saline and incubated with primary antibodies in the ratio rabbit anti-TiAR (1:1000) and with rabbit anti-histone H3 (1:3000) and incubated at 4°C overnight followed by 1 hr incubation at room temperature with IR dye-anti-Rabbit secondary antibodies (1:10,000) (LI-COR Biosciences). The membranes were scanned and quantified using the Odyssey Infrared Imaging System (Li-COR Biosciences).

TiAR knockdown

PBECs were plated on 24-well plates (500 µl) and were grown till 30% confluence. Cells were transduced with the lenti-viral system with pLV-H1-EF1α-green vector ligated with TiAR or scrambled constructs, provided in table S1. The protocols of ligation, transformation, screening inserts in the vector and production of lenti-viral particles were done using BiOSETTIA manual for gene silencing. Polybrene was used to increase the transfection efficiency. Phenotypic screening with green fluorescence showed that 70% of the cells were transfected. The cells were transfected for 72 hrs and stimulated with TNFα and IL-17A to measure the release of cytokine after 16 hrs in the supernatants. To confirm the knockdown by western blots, whole cell lysis was done with RIPA buffer and analysed for TiAR as mentioned above.

Alveolar macrophages isolation and stimulation

The alveolar macrophages were obtained from adult-onset asthma patients (23). After sputum induction in asthma patients and healthy controls, sputum was processed 1:1 with 10 mM Dithiothreitol (Sigma), for 30 minutes at 4°C on a rotator. DNAse (150 units/ml; Sigma; #D5025-150KU) was added 1:1000 only when there were still some cellular aggregates, 10 minutes on ice after which cells were pelleted (10 min at 400g) and resuspended in 1 ml PBS. Then 3 ml PBS/2% FCS/1 mM EDTA, 30 µl of an erythrocyte pellet and 50 µl RosetteSep Human Monocyte Enrichment Cocktail (Stem cell Technologies) was added for 20 minutes and incubated at room temperature. Subsequently, cells were layered on top of 3 ml LymphoPrep and centrifuged for 22 minutes at 1355g. The mononuclear layer was collected and washed in 25 ml ice-cold IMDM (1% FCS, 100 units penicillin/ml and 10mg/ml streptomycin) and centrifuged for 10 min at 500g) and the pellet was dissolved in 1 ml IMDM (1% FCS, 100 units penicillin/ml and 10mg/ml streptomycin). Macrophages were allowed to adhere to the surface of the plates for 24 hrs and then stimulated with TNF-α and IL-17A, TNF-α alone or not stimulated in fresh medium.

Statistical methods

GraphPad Prism 7 was used for statistical analyses (t-tests, one-way and two-way ANOVA). For t-tests of data with normal distribution parametric t-tests were used whereas Mann-

Whitney U non-parametric t-test was performed for data not normally distributed. P values ≤0.05 were considered statistically significant. For associations, Pearson's correlation test was performed with two-tailed analysis. Scatter plot analysis was done in IBM SPSS.

Supplementary tables

Table E1. Oligonucleotide sequences provided for the respective analysis.

Analysis	Oligonucleotide sequences
mRNA analysis	Hu IL-8 PPH00568A, SA Biosciences Hu IL-6 PPH00560B, SA Biosciences Hu GAPDH (forward) : TCA TCT CTG CCC CCT CTG C Hu GAPDH (reverse) : GAG TCC TTC CAG GAT ACC AA
miR 16 analysis	miR16-1 (Stem loop) GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GCC AAT AT miR 16-1 (Forward) TGC GGT AGC AGC ACG TAA AT miR16-1 (Reverse) TGC AGG GTC CGA GGT AT
miRCURY™ LNA inhibitor	miR16 - CGC CAA TAT TTA CGT GCT GCT A scrambled control – TAA CAC GTC TAT ACG CCC A
BiOSETTIA TiAR knockdown	TiAR 1 - AAAA TGACAGAAGTCCTTATACT TTGGATCCAA AGTATAAGGACTTCTGTCA TiAR 2 - AAAA GAAAGGAGGTCAAAGTAAA TTGGATCCAA TTTACTTTGACCTCCTTTC TiAR 3 - AAAA AAGGGCTATTCATTTGTCAGA TTGGATCCAA TCTGACAAATGAATAGCCCTT

Supplementary figure legends

Figure E1. (*A*) **IL-17A with TNF-α distinguishes hyperresponsive PBECs**. PBECs from 43 mild and 16 severe asthmatics and 16 healthy controls were stimulated with TNF-α plus IL-17A for 16 hrs inducing significantly higher levels of CXCL-8 production compared TNF-α or IL-17A alone, in all groups, but more pronounced in asthmatics. Data are shown as mean ± SD. For statistical analyses two-tailed Wilcoxon matched pairs signed rank t-test was used: ****P<0.0001. (*B*). **Correlation between TNF-α and IL-17A-induced CXCL-8 production and the synergistic effect distinguishes hyperresponsive PBECs.** CXCL-8 release upon TNF-α and IL-17A stimulation showed a strong positive correlation with the synergistic effect, calculated as CXCL-8 levels with TNF-α+IL-17A stimulations divided by CXCL-8 levels with TNF-α stimulation alone plus that by IL-17A stimulation alone. Explanations for colored symbols are provided in Figure 1. Pearson's correlation test was performed with two-tailed analysis. (*C*). **TNF-α stimulation alone with dexamethasone reduces CXCL-8 production in bronchial epithelium**. Normo- (in black; *n*=7) and hyperresponsive (in red; *n*=15) PBECs stimulated for 16hrs with TNF-α in the presence or absence of dexamethasone (3.8 μM). For statistical analysis paired t-tests (parametric) were used: *P<0.05. (*D*) **CXCL-10 and MIF levels are not affected by dexamethasone in PBECs.** As (C).

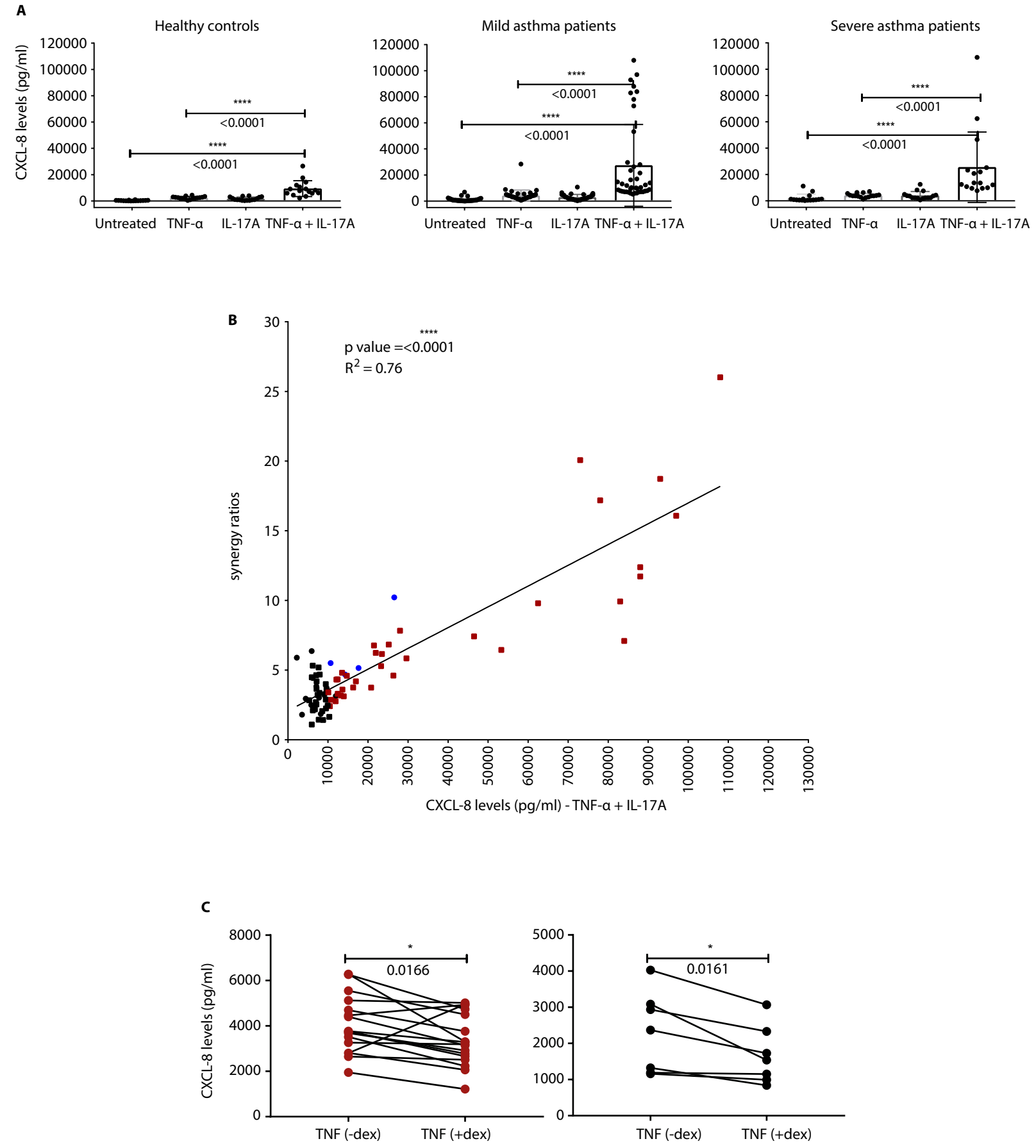
Figure E2. Hyperresponsiveness of PBECs is not associated with PC_{20}. CXCL-8 levels produced by PBECs from mild and severe asthmatics (*n*=35) after TNF-α and IL-17A stimulation did not correlate with PC_{20} (mg/ml) measured 3 days prior to collection of bronchial brushes. Pearson's correlation test was performed with two-tailed analysis.

Figure E3. TiAR in hyperresponsive PBECs does not translocate to the cytoplasm with dexamethasone, but translocates upon exposure to arsenite. (*A*) Immunohistochemistry of

hyper- (*n*=5 ; upper panel) and normoresponsive (*n*=3 ; lower panel) PBECs shown as TiAR (red) and eiF3η (green) after stimulation with TNF-α and IL-17A for 2 hrs in the presence (right) and absence (left) of dexamethasone. Nuclei are stained with DAPI. (*B*) TiAR (red) is shown by confocal microscopy to translocate to the cytoplasm in hyperresponsive PBECs treated for 30 minutes with 50 μM arsenite and to form stress granules (arrows) with 500 μM after 30 minutes of arsenite. (*n*=3). (*C*) Co-localization of TiAR (red) and eiF3η (green) in cytoplasm of normoresponsive PBECs stimulated for 16 hrs with TNF-α and IL-17A and treatment with 50 μM arsenite for 30 minutes. At 500 μM of arsenite for 30 minutes stress granules are formed. Nuclei are stained with DAPI. (*D*) TiAR is not associated with endoplasmic reticulum as shown by immunohistochemistry of localization of TiAR (green) in the nucleus and RBP1 (red) in cytoplasm stimulated TNF-α and IL-17A for 2 hrs in hyperresponsive PBECs.

Figure E4. (*A*) **IL-17RA mRNA expression remains unchanged between hyper- and normoresponsive PBECs**. IL-17RA mRNA expression is similar in normo- (*n*=3) and hyperresponsive PBECs (*n*=3), from mild asthmatics (*n*=6) and healthy controls (*n*=6), left unstimulated or stimulated with TNF-α, IL-17A or TNF-α with IL-17A for 16 hrs and normalized to GAPDH mRNA expression. (*B*) **Alveolar macrophages from asthmatics and healthy controls do not display hyperresponsiveness.** CXCL-8 measured in healthy controls (*n*=5) and asthma patients (*n*=6) did not show any synergistic increase with TNF-α and IL-17A compared to TNF-α alone.

Figure E1


A
Healthy controls
Mild asthma patients
Severe asthma patients
CXCL-8 levels (pg/ml)
120000
100000
80000
60000
40000
20000
0
****
<0.0001
Untreated
TNF-α
IL-17A
TNF-α + IL-17A
B
p value =<0.0001
R2 = 0.76
synergy ratios
CXCL-8 levels (pg/ml) - TNF-α + IL-17A
C
CXCL-8 levels (pg/ml)
*
0.0166
0.0161
TNF (-dex)
TNF (+dex)

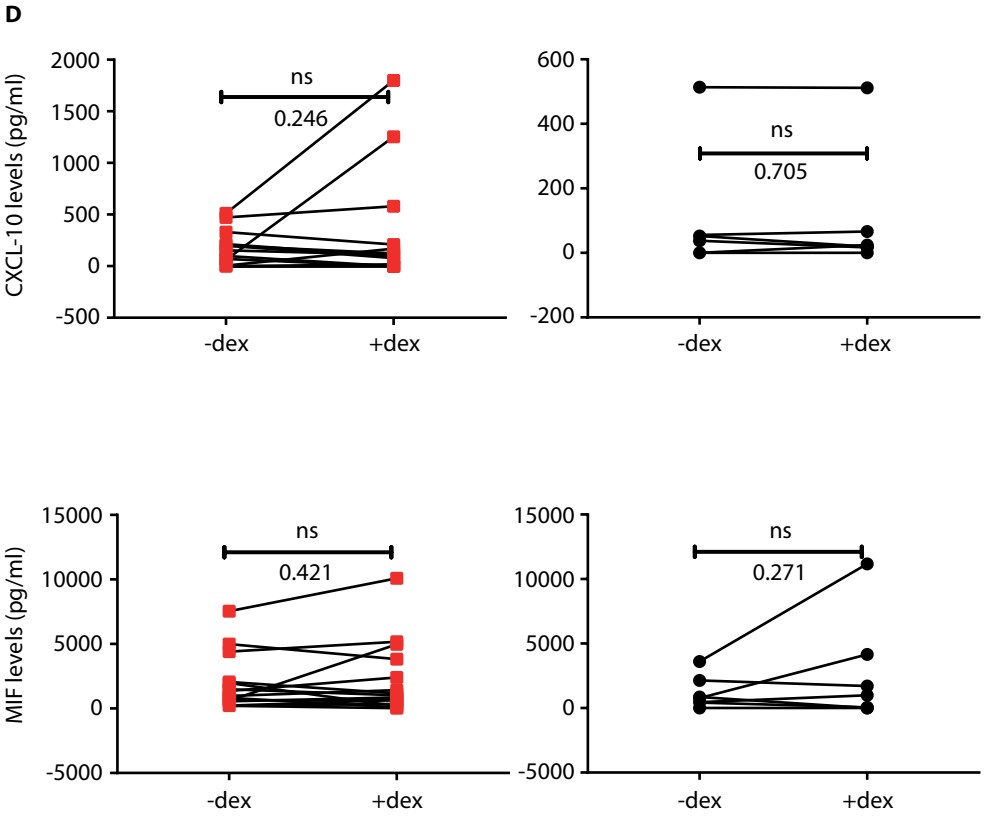

D
CXCL-10 levels (pg/ml)
2000
1500
1000
500
0
-500
ns
0.246
-dex
+dex
600
400
200
0
-200
ns
0.705
-dex
+dex
MIF levels (pg/ml)
15000
10000
5000
0
-5000
ns
0.421
-dex
+dex
15000
10000
5000
0
-5000
ns
0.271
-dex
+dex

Figure E2

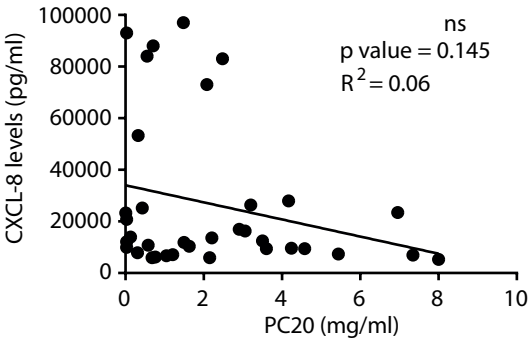

CXCL-8 levels (pg/ml)
100000
80000
60000
40000
20000
0
ns
p value = 0.145
R 2 = 0.06
0
2
4
6
8
10
PC20 (mg/ml)

Figure E3

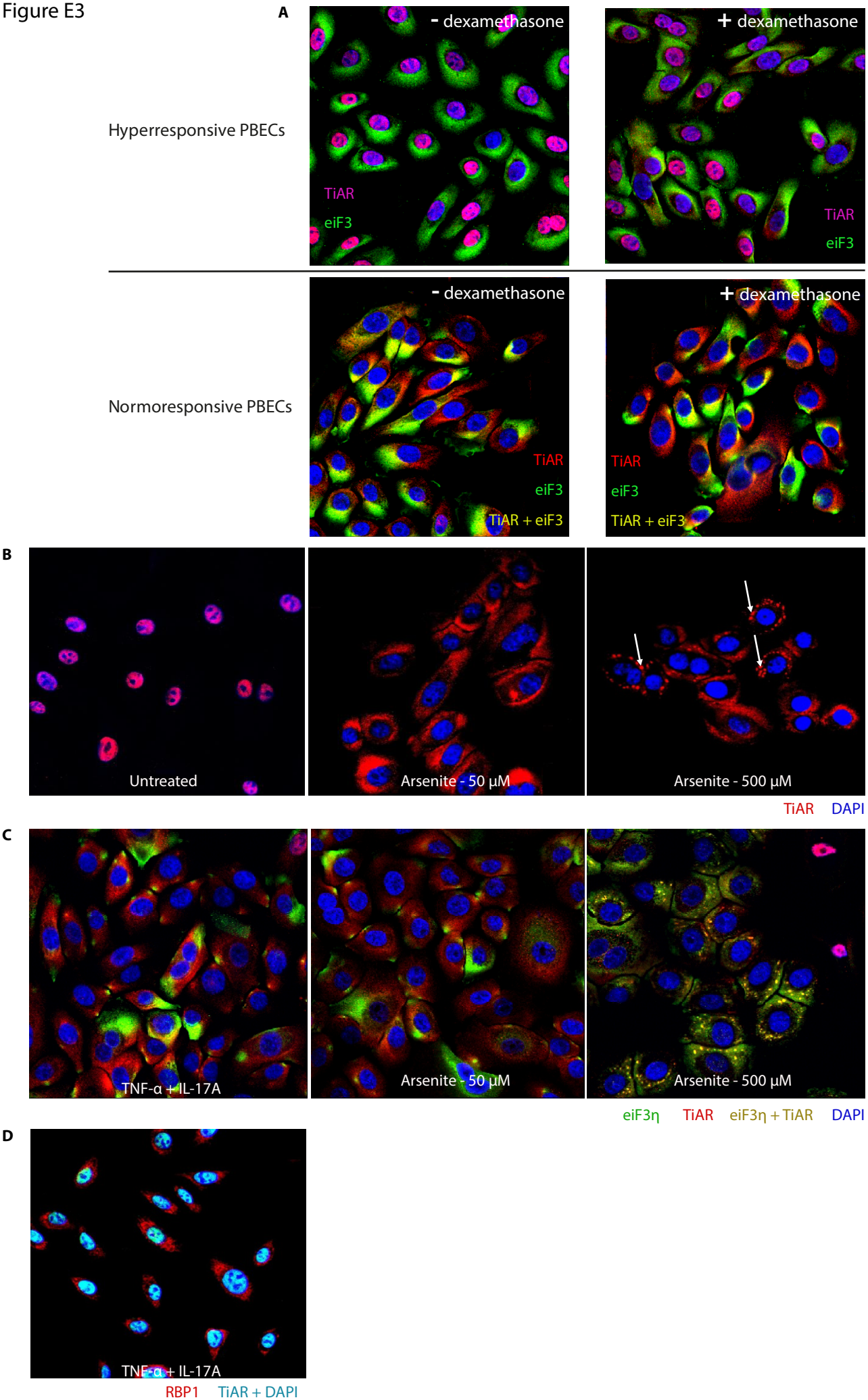
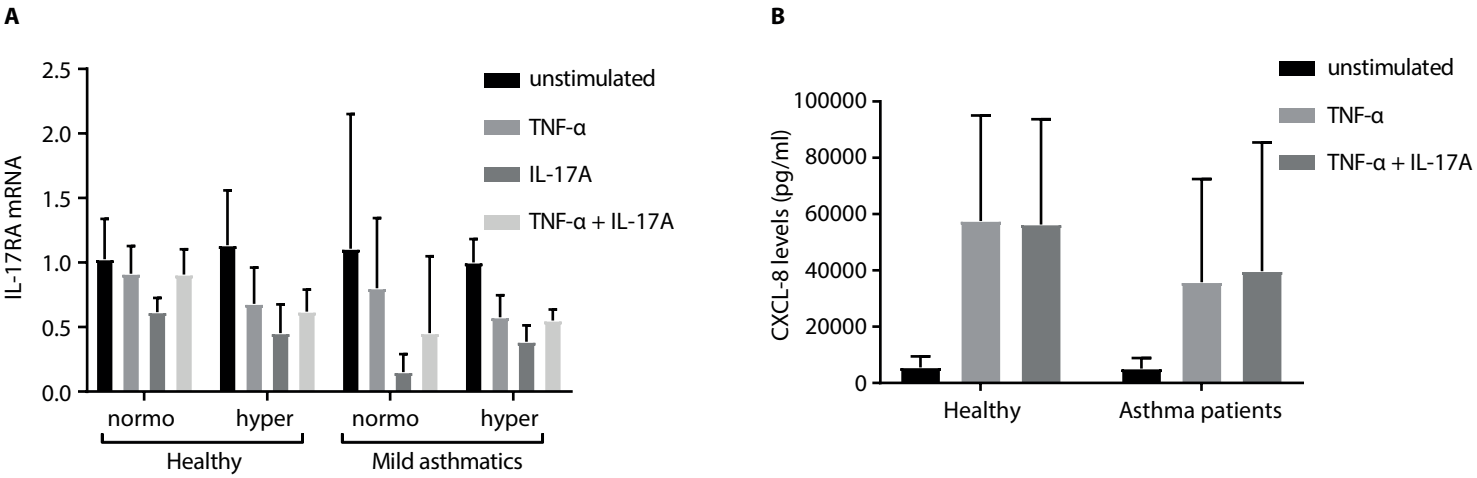

A
Hyperresponsive PBECs
- dexamethasone
TiAR
eiF3
+ dexamethasone
TiAR
eiF3
Normoresponsive PBECs
- dexamethasone
TiAR
eiF3
TiAR + eiF3
+ dexamethasone
TiAR
eiF3
TiAR + eiF3
B
Untreated
Arsenite - 50 µM
Arsenite - 500 µM
TiAR DAPI
C
TNF-α + IL-17A
Arsenite - 50 µM
Arsenite - 500 µM
eiF3η TiAR eiF3η + TiAR DAPI
D
TNF-α + IL-17A
RBP1 TiAR + DAPI

Figure E4


A
IL-17RA mRNA
2.5
2.0
1.5
1.0
0.5
0.0
unstimulated
TNF-α
IL-17A
TNF-α + IL-17A
normo
hyper
normo
hyper
Healthy
Mild asthmatics
B
CXCL-8 levels (pg/ml)
100000
80000
60000
40000
20000
0
unstimulated
TNF-α
TNF-α + IL-17A
Healthy
Asthma patients