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# The Complex Relationship Between Inflammation And Lung Function In Severe Asthma

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## Abstract

Asthma is a common respiratory disease affecting approximately 300 million people worldwide. Airway inflammation is thought to contribute to asthma pathogenesis, but the direct relationship between inflammation and airway hyperresponsiveness remains unclear. This study investigates the role of inflammation in a steroid-insensitive, severe allergic airway disease model and in severe asthmatics stratified by inflammatory profile. First, we utilized the $T_H17$ cell adoptive transfer mouse model of asthma to induce pulmonary inflammation, which was lessened by TNFα neutralization or neutrophil depletion. While decreased airspace inflammation following TNFα neutralization and neutrophil depletion rescued lung compliance, neither intervention improved airway hyperresponsiveness to methacholine, and tissue inflammation remained elevated when compared to control. Further, sputum samples were collected and analyzed from 41 severe asthmatics. In severe asthmatics with elevated levels of sputum neutrophils, but low levels of eosinophils, increased inflammatory markers did not correlate with worsened lung function. This subset of asthmatics also had significantly higher levels of $T_H17$-related cytokines in their sputum compared to other severe asthmatics with other inflammatory phenotypes. Overall, this work suggests that lung compliance may be linked with cellular inflammation in the airspace, while T cell-driven airway hyperresponsiveness may be associated with tissue inflammation and other pulmonary factors.



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Supplementary Material

Supplementary material is linked to the online version of the paper at http://www.nature.com/mi.

**Disclosure**

The anti-TNFα, anti-IL-17A, and control IgG1 antibodies used in these studies were provided by Janssen Research & Development.

**Keywords**

neutrophils; lung; T cells; allergy

## Introduction

Asthma is a complex heterogeneous disorder, with a broad spectrum of phenotypes ranging from mild to severe disease with varying degrees of responsiveness to standard steroid therapy. Traditionally, asthma is viewed as an eosinophilic airway disorder and research has focused on the role of T helper ($T_H$) 2 cells and cytokines (IL-4, IL-5, and IL-13) in promoting these pathological features. Most patients with asthma have disease that can be well controlled with inhaled corticosteroids and long-acting β2-agonists, but approximately 5–10% of asthmatics have severe refractory disease.[1–3] Severe asthma differs from mild or moderate persistent asthma as it can often be characterized by neutrophilic inflammation in the presence or absence of classical $T_H2$-induced eosinophilic inflammation. In addition, $T_H17$ cells, which mediate neutrophil recruitment, are thought to play an influential role in asthma pathogenesis, especially in asthmatics that fail to respond to glucocorticoid therapy[4]. However, the presence of this phenotype in patients is yet to be clearly defined.

Atopic asthma is thought to be driven by the recruitment of $CD4^+$ T lymphocytes to the lungs and is characterized by pulmonary inflammation, reversible airflow limitation, mucus hypersecretion, and airway hyperresponsiveness (AHR). However, the relationship between inflammation and physiologic changes is complex. Several studies have shown that the extent of neutrophilia correlates to asthma severity.[5–7] Indeed, it has been reported that subsets of asthmatics characterized by neutrophilic inflammation exhibit decreased improvement in $FEV_1$ (forced expiratory volume in 1 second) and airway responsiveness following glucocorticoid treatment.[8] Furthermore, neutrophils are known to be largely steroid-insensitive and glucocorticoids inhibit neutrophil apoptosis.[9, 10] Although steroid-resistant asthma is often neutrophil dependent[11], lavage or biopsy eosinophils were also related to severe therapy-resistant asthma and lung function changes in children and adults.[12] Despite such correlative studies, the mechanistic role of airway inflammation in relation to AHR is currently unclear. We therefore investigated the inflammatory phenotype of severe asthma and involvement of inflammation in the physiologic changes in the lung.

## Results

### Pulmonary inflammation is suppressed by anti-TNFα treatment in $T_H17$ cell transfer, OVA challenged mice

The pro-inflammatory cytokine, TNFα, is thought to play an important role in the pathogenesis of asthma. To date, studies have mainly focused on the role of TNFα in $T_H2$-based, eosinophil-dominant, steroid sensitive asthma and little is known about its role in $T_H17$-induced, neutrophil-dominant, steroid-resistant disease. Evidence supports a role for TNFα in severe asthma as systemic and lung levels of TNFα are increased in patients with severe refractory asthma.[13, 14] TNFα is also known to synergize with IL-17A in a number of inflammatory models. Specifically, IL-17A is thought to contribute to asthma pathogenesis

by influencing neutrophil influx into the lungs and airway remodeling, which are more prominent in chronic steroid-resistant disease.[15] To determine whether TNFα or IL-17A mediates airway inflammation and AHR in $T_H17$-dependent allergic airway disease, an adoptive transfer model of airway antigen-induced inflammation was used[4] (Figure 1A). Briefly, *in vitro* polarized $T_H17$ cells were adoptively transferred into BALB/c SCID mice that were challenged with OVA intratracheally one day prior to adoptive transfer and then again challenged with OVA following cell transfer for three consecutive days. Mice were also treated with anti-TNFα, anti-IL-17A, or IgG1 control on Days 1 and 3. Control mice did not receive T cell transfer, but were challenged with OVA (No cell control). Other control groups included mice that received PBS intratracheally instead of OVA and IgG1 (PBS+ IgG) or anti-TNFα (PBS + Anti-TNFα) as well as naïve BALB/c SCID mice. Twenty-four hours after the last OVA challenge, $T_H17$-induced allergic airway responses were assessed (Figure 1A). This model was chosen to mimic the high neutrophil, low eosinophil allergic airway disease identified from stratification of severe asthmatics.

As expected, adoptive transfer of $T_H17$ cells into OVA-challenge BALB/c SCID mice resulted in increased inflammatory cell recruitment into the lungs (Figure 1B). Differential counting of the bronchoalveolar lavage (BAL) fluid cells revealed predominantly neutrophils and macrophages were elevated in $T_H17$ cell transfer, OVA challenged mice when compared to control mice (Figure 1C). This $T_H17$-induced cell influx was markedly attenuated by anti-TNFα treatment, but not significantly reduced by anti-IL-17A treatment (Figure 1B). Specifically, anti-TNFα treatment following $T_H17$ cell transfer and OVA challenge reduced the number of neutrophils in the airspaces, but had no effect on the number of macrophages (Figure 1C). Anti-IL-17A treatment slightly decreased the number of neutrophils and increased macrophages present in the airspaces when compared to $T_H17$ cell transfer, OVA challenged mice (Figure 1C). Histological analyses of the lung also confirmed that cellular inflammation was significantly increased in the lung tissue of $T_H17$ cell transfer, OVA challenged mice when compared to OVA-challenged mice that did not receive $T_H17$ cell transfer (No cell control). Further, both anti-TNFα and anti-IL-17A treatments significantly lessened tissue inflammation when compared to $T_H17$ cell transfer, OVA challenged mice. However, the amount of tissue inflammation present in the lungs of $T_H17$ cell transfer, OVA challenged mice treated with anti-TNFα and anti-IL-17A was still significantly increased above control levels (Figure 1D and E). Tissue inflammation was further characterized based on the location in the pulmonary tissue as perivascular, peribronchial, or parenchymal-associated inflammation (Supplemental Figure 1). Perivascular, peribronchial, and parenchymal associated inflammation was higher in $T_H17$ cell transferred, OVA challenged mice regardless of antibody treatment when compared to all control mice (Naïve, PBS + IgG, PBS + Ant-TNFα, and no cell control mice). Further, OVA-challenge alone induced tissue inflammation above control levels in BALB/c SCID mice (Supplemental Figure 1). Anti-TNFα treatment significantly reduced tissue inflammation in all three compartments, while anti-IL-17A treatment only reduced perivascular inflammation versus TH17 transferred control mice. Overall, although airspace inflammation was significantly lessened by anti-TNFα treatment in $T_H17$ cell transfer, OVA challenged mice, a significant amount of tissue inflammation remained.

### Neutralization of TNFα markedly attenuates $T_H17$-induced cytokine production in the lung

To further characterize the inflammation observed in these mice, cytokine and chemokine levels in the lungs were measured. As expected, $T_H17$ cell transfer, OVA challenged mice expressed elevated levels of two primary $T_H17$ cytokines, IL-17A (0.3 ± 0.3 vs. 68.4 ± 19.9) and IL-22 (not detected vs. 56.5 ± 20.8), when compared to no cell controls. OVA-challenged mice reconstituted with $T_H17$ cells showed increased levels of $T_H1$- (Figure 2A), $T_H2$- (Figure 2B), and $T_H17$-related cytokines and chemokines (Figure 2C) in the lungs, while anti-TNFα and anti-IL-17A treatment in $T_H17$ cell transfer, OVA challenged mice significantly inhibited this response. Anti-TNFα treatment reduced cytokine levels to control levels for the majority of the cytokines measured, although some cytokines and chemokines increased or did not significantly change when compared to $T_H17$ cell transfer, OVA challenged mice (Supplemental Table 1). All control mice (Naïve, PBS + IgG, PBS + Ant-TNFα, and no cell control mice) had low levels of all cytokines and chemokines measured (Figure 2, Supplemental Table 1) except IL-13, IL-17A, IL-1α, and GM-CSF, which were induced by antibody treatment alone. Since TNFα and IL-17A may regulate cytokine levels by impacting translation and secretion, we also examined the effect of anti-TNFα and anti-IL-17A on cytokine mRNA levels (Figure 2D). TNFα neutralization inhibited transcription of IL-4, IL-6, and IFNγ, while IL-17A antibody only impacted IL-6 (Figure 2D). Overall, these results show that anti-TNFα attenuates protein and gene expression of many cytokines in the lungs of $T_H17$ cell transfer, OVA challenged mice. Anit-IL-17A had lesser effects on the inflammatory cytokine milieu compared to anti-TNFα.

### Lung compliance is restored, while AHR does not consistently change following anti-TNFα treatment in $T_H17$ transfer, OVA challenged mice

Having observed that the neutralization of TNFα or IL-17A could alter pulmonary inflammation, we next wanted to determine if anti-TNFα or anti-IL-17A treatment were sufficient to lessen AHR and mucus production in $T_H17$-dependent allergic airway disease. $T_H17$ cell transfer was found to significantly induce AHR above the levels found in control mice as expected based on previous findings[4] (Figure 3A–E). Despite abrogating airspace inflammation and reducing tissue inflammation, AHR was not consistently altered in $T_H17$ cell transfer, OVA challenged mice following TNFα neutralization. $T_H17$ cell transfer, OVA-challenged mice treated with anti-TNFα had significantly less airway resistance (Rn) (Figure 3A), while greater tissue damping (G) (Figure 3B), when compared to $T_H17$ cell transfer, OVA-challenged mice treated with IgG control. In addition, no change in lung elasticity (H) between these groups was noted (Figure 3C). Although anti-TNFα did not have a consistent effect on AHR, anti-TNFα did significantly improve static lung compliance and hysteresis (Figure 3D and E respectively). Anti-IL-17A treatment in $T_H17$ cell transfer, OVA challenged mice did not significantly change lung function when compared to $T_H17$ cell transfer, OVA challenged mice treated with IgG control (Figure 3A–E). Together, these data show that lung compliance (which is rescued by anti-TNFα) may be linked with airspace cellular inflammation, while tissue inflammation may be one mechanism by which AHR is altered.

### Neutralization of TNFα and IL-17A increase mucus hypersecretion in $T_H17$ cell transfer, OVA challenged mice

Pulmonary expression of *Clca3* (Figure 4A), *Muc5ac* (Figure 4B), and *Muc5b* (Figure 4C) was measured using real-time PCR as these genes are associated with goblet cell hyperplasia and mucus production in allergic airway disease.[16, 17] Neutralization of TNFα significantly increased expression of *Muc5ac* and *Clca3*, but not *Muc5b* in the lungs of $T_H17$ cell transfer, OVA challenged mice when compared to control mice. Neutralization of IL- 17A in $T_H17$ cell transfer, OVA challenged mice also elevated *clca3* and mucus gene expression in the lungs when compared to $T_H17$ cell transfer, OVA challenged mice (Figure 4A–C). *Clca3* and *Muc5ac* expression were elevated to a lesser extent than anti-TNFα, while *Muc5b* expression was increased following IL-17A neutralization. Periodic acid-Schiff (PAS) staining of lung sections followed by histologic scoring showed increased mucus production was present in the lungs following neutralization of TNFα in $T_H17$-induced allergic airway disease (Figure 4D and E). Positive PAS staining was present in airway epithelial cells of all mice that received $T_H17$ cell transfer, but not in the OVA-challenged control mice that did not receive a T cell transfer, mice that received only IgG or anti-TNFα without T cell transfer or OVA challenge, and naïve BALB/c SCID mice. Overall, neutralization of TNFα significantly decreased neutrophilic inflammation, rescued lung compliance, and worsened mucus secretion, while having a small effect on AHR. Neutralization of IL-17A had lesser effects on inflammation, minimally impacted AHR, and worsened mucus secretion.

### Neutrophil depletion reduces $T_H17$-induced pulmonary inflammation

To determine whether the airway responses of $T_H17$ reconstituted mice were mediated by neutrophils, BALB/c SCID mice were treated as outlined above. To selectively deplete neutrophils, mice were treated on Day 1 and 3 during $T_H17$ cell transfer and OVA challenge with an intraperitoneal injection of Ly6G-specific monoclonal antibody, 1A8 (Figure 5A). At the time of sacrifice on Day 5, total cells in the BAL fluid and cell differentials were used to evaluate $T_H17$-induced cellular recruitment into the lungs (Figure 5A). Consistent with the present experiments and previous finding[4], $T_H17$ cell transfer induced inflammatory cell influx into the lungs (Figure 5B), specifically neutrophils and macrophages (Figure 5C). As expected, the neutrophilic inflammation was significantly diminished with specific depletion using anti-Ly6G, 1A8 (Figure 5C). Histological analyses of the lungs also confirmed that treatment with 1A8 decreased inflammation in $T_H17$ cell transfer, OVA-challenged mice (Figure 5D–E). Tissue inflammation was further characterized based on the location in the pulmonary tissue as perivascular, peribronchial, or parenchymal-associated inflammation (Supplemental Figure 2). Perivascular, peribronchial, and parenchymal-associated inflammation was higher in $T_H17$ cell transfer, OVA challenged mice regardless of neutrophil depletion when compared to OVA-challenged mice that did not receive T cell transfer (No cell control, Supplemental Figure 2). Neutrophil depletion significantly reduced peribronchial and parenchymal inflammation, but not perivascular inflammation.

Lastly, neutrophil depletion lead to increased production of $T_H17$ cytokines and chemokines (CXCL1, G-CSF, and IL-6, Figure 5F); as well as CXCL10 (Supplemental Table 2) in the lungs. TNFα, CXCL2, CCL2, CCL3, CXCL5, GMCSF, IFN-γ IL-17A, IL-13, IL-10, IL-12p40, IL-1α and IL-4 were unchanged by neutrophil depletion in $T_H17$ cell transfer,

OVA challenged mice (Supplemental Table 2). OVA-challenged mice that did not receive T cell transfer (No Cell Control) had negligible or low levels of all cytokines and chemokines measured (Supplemental Table 2). These data show that neutrophil depletion alters the $T_H17$-induced inflammatory milieu present in the lung.

### Neutrophil depletion rescues lung compliance, but not AHR during $T_H17$-induced allergic airway disease

To determine whether neutrophils are necessary to induce $T_H17$-dependent AHR, AHR to methacholine was measured. $R_n$ and G were found to be significantly decreased in $T_H17$ cell transfer, OVA challenged, 1A8 treated mice when compared to mice the received $T_H17$ cell transfer and OVA challenge (Figure 6A–B), although $T_H17$ cell transfer and OVA challenge still induced AHR (vs. control mice, Figure 6A and B) in these mice. No significant changes were noted in H (Figure 6C). In addition, neutrophils are partially responsible for lung stiffening in $T_H17$-dependent allergic airway disease as neutrophil depletion trended to increase static lung compliance (Figure 6D) and decrease hysteresis (Figure 6E). Together, these data indicate that neutrophils play a partial role in the development of AHR in $T_H17$-dependent allergic airway disease.

### Depletion of neutrophils attenuates $T_H17$-induced mucus production

Pulmonary expression of *Clca3* (Figure 7A), *Muc5ac* (Figure 7B), *Muc5b* (Figure 7C) was measured using real-time PCR. $T_H17$ cell adoptive transfer again increased mucus gene expression and PAS staining. Neutrophil depletion significantly reduced mucus production in the lungs of $T_H17$ cell transfer, OVA challenged mice (Figure 7A–C). PAS staining of lung sections further confirmed this marked decrease in mucus production following depletion of neutrophils in $T_H17$-induced allergic airway disease (Figure 7D and E). Positive PAS staining was present in airway epithelial cells of all mice that received $T_H17$ cell transfer and OVA challenge, but not in the OVA-challenged control mice that did not receive a T cell transfer. These data suggest that mucus production in this model may be dependent upon neutrophil products.

### Asthma subject characteristics

Since we have shown that AHR is present in a severe asthma model independent of neutrophilic inflammation in mice, we wanted to examine if there is evidence for a subset of severe asthmatics that resembles this phenotype. Sputum samples were collected from 41 subjects with severe asthma, as defined by American Thoracic Society guidelines[18] (Table 1). Severe asthmatics were stratified based on the percentage of neutrophils and eosinophils in the sputum using cutoffs of greater than 30% neutrophils or 1.8% eosinophils (based on University of Pittsburgh SARP site median splits) to designate “high” or “low” resulting in the following groups: patients with high levels of both neutrophils and eosinophils (H/H, N=11), with high levels of neutrophils and low levels of eosinophils (H/L, N=10), with low levels of neutrophils and high levels of eosinophils (L/H, N=11), and with low levels of both cell types (L/L, N=9). The groups did not differ by age, gender, race, age at onset, duration of symptoms, or body mass index. In regards to lung function, there were no significant differences in $FEV_1$ % predicted, forced vital capacity (FVC) % predicted, or $FEV_1$/FVC

between the groups of severe asthmatics. Use of corticosteroids did not differ between groups.

### Severe asthmatics with neutrophilia have a distinct inflammatory profile

$T_H17$ cells produce IL-17A, IL-17F, and IL-22, which have proinflammatory functions resulting in production of high levels of granulocyte and monocyte chemokines and growth factors, such as CXCL1, CCL2, IL-6, IL-8, and G-CSF. Cytokine levels in the sputum from the patient populations described above were examined to determine whether certain cytokines identified with these pre-specified cellular phenotypes in severe asthmatics. H/L severe asthmatics had increased levels of CXCL1 (Figure 8A), CXCL10 (Figure 8B), CCL2 (Figure 8C), IL-6 (Figure 8D), and IL-8 (Figure 8E). Levels of G-CSF and IL-22 (Figure 8F and G) were not different amongst severe asthma groups, although H/L severe asthmatics displayed a trend towards elevated IL-22 and G-CSF. There were no significant differences in sputum levels of CCL7, CCL3, CCL4, TNFα, IL-1α, and IL-1β between groups (data not shown). Other cytokines and chemokines investigated included GM-CSF, IFN-α2, IFN-γ, IL-4, IL-5, IL-9, IL-10, IL-12p40, IL-13, IL-17A, sCD40L, but amounts in the sputum were below the levels of detection for the majority of the samples collected (data not shown).

Sputum cytokine levels were then tested for correlation with pulmonary function (Table 2). Interestingly, sputum CXCL1 levels ($r_s$=0.640, p=0.046) and IL-6 ($r_s$=0.766, p=0.01) were found to positively correlate to $FEV_1$ % predicted in the H/L asthmatics. In addition, IL-6 was positively correlated with $FEV_1$/FVC ($r_s$=0.733, p=0.02) in these patients. These data indicate that high cytokine inflammation correlate with improved lung function in H/L asthmatics. Unlike H/L asthmatics, poor lung function correlated with greater cytokine levels in all other severe asthmatic groups. Among H/H asthmatics, IL-8 inversely correlated with $FEV_1$ % predicted ($r_s$=−0.627, p=0.04) and FVC % predicted ($r_s$=−0.664, p=0.03). CCL2 ($r_s$=−0.850, p=0.004) inversely correlated with $FEV_1$ % predicted and inversely correlated ($r_s$=−0.733, p=0.03) with FVC % predicted in L/L asthmatics. Lastly, there were no significant correlations between lung function and sputum cytokine level in the L/H asthmatics. These findings in severe asthmatics were similar to the murine studies in that cytokine levels for H/L severe asthmatics did not predict declining lung function (in fact it was the opposite). Overall, in severe asthma that is characterized by high levels of neutrophils and low levels of eosinophil in the lungs, levels of inflammatory cytokines may not be indicative of poor lung function.

## Discussion

Asthma is a complex heterogeneous disorder, with a broad spectrum of phenotypes ranging from mild to severe disease with varying degrees of responsiveness to standard steroid therapy. Severe asthmatics often do not display canonical $T_H2$ or eosinophilic inflammation and have difficult to control disease by conventional corticosteroids. Many studies have attempted to define phenotypes of severe asthma as reviewed by S.E. Wenzel.[19] Unfortunately, these clinically characterized phenotypes provide little insight into the underlying pathogenic mechanisms of disease.

Much of the research in the field of allergic asthma has focused on $T_H2$-dominant, eosinophilic disease. The murine model herein is a model of non-eosinophilic, steroid-insensitive asthma.[4] In this model, adoptive transfer of OVA-specific $T_H17$ cells to donor mice is sufficient to promote the hallmark characteristics of severe asthma *in vivo*, such as neutrophilic inflammation, AHR, and mucus metaplasia, which are not attenuated by glucocorticoid treatment.[4] Current asthma models also involve antigen sensitization and subsequent antigen challenge for asthma development. As clinical presentation of disease is long after antigen sensitization and T cell polarization in humans, one cannot intervene in these stages of asthma development. Therefore, focusing on T cell effector mechanisms may be the key to effective therapies and the need for animal models to investigate mechanisms of established asthma is evident. This innovative adoptive transfer model allows for antigen challenge responses to be investigated independent of antigen sensitization. Unlike existing methodologies, this model is instrumental in facilitating investigations into critical T cell effector mechanisms and in allowing for experimental intervention at this stage.

Although the development of allergen-induced airway inflammation has been extensively studied, few studies have directly addressed the causal relationship between allergic inflammation and $T_H17$-induced allergic airway disease. The goal of this work was to determine if allergic inflammation is directly related to airway hyperresponsiveness in a model of $T_H17$-induced allergic airway disease. In the present study, two approaches were utilized to study the role of inflammation in the $T_H17$-induced allergic airway disease in mice. First, TNFα or IL-17A was neutralized in $T_H17$ cell transfer, OVA challenged mice.

TNFα is a potent pro-inflammatory cytokine with immunoregulatory activities and is principally produced by macrophages, while IL-17A is produced by T cells and known to drive AHR in mice.[20] Dysregulated TNFα responses have been implicated in several inflammatory conditions, including rheumatoid arthritis[21, 22], Crohn's disease[23], Beçhet's disease[24], and $T_H2$-dominant asthma. In this study, such treatment attenuated $T_H17$ inflammatory disease in terms of airspace inflammation and cytokine production. However, neutralizing TNFα only rescued lung compliance, not AHR, and actually worsened mucus production in our *in vivo* studies. Further, anti-IL-17A treatment had less effect on inflammation, lung compliance, and AHR. In our previous work, deletion of IL-17RA inhibited $T_H17$ induced disease to a greater extent than anti-IL-17A antibody did herein.[4] It is possible that blocking Il-17A and/or multiple $T_H17$-related cytokines (IL-17F and/or IL-25) may be required to have greater effects on allergic airway disease.

The apparent disconnect between airspace inflammation-dependent lung compliance and airspace inflammation-independent AHR is intriguing. These data suggest that the therapeutic potential of anti-TNFα may be limited to targeting airspace inflammation. Overall, lung compliance (which is rescued by anti-TNFα) may be linked with airspace cellular inflammation, while AHR may be associated with tissue inflammation in this model of $T_H17$-mediated allergic airway disease. Our data suggest that TNFα may be a critical cofactor for $T_H17$-driven inflammation, perhaps through its interaction with IL-17A and IL-17F (and/or additional $T_H17$ products; IL-9, IL-21, or IL-22). This work illustrates that airspace inflammation and tissue inflammation are not equivalent in this model of allergic

airway disease and reveals the complex relationship between lung function and cellular inflammation in severe asthma.

Secondly, neutrophils were depleted in $T_H17$-induced allergic airway disease. There is increasing evidence that neutrophils are associated with severe asthma, although the functional relevance of these cells in disease progression remains unclear. In this study, neutrophil depletion had minimal effects on $T_H17$-driven AHR and compliance, while it reduced mucus production. We did observe elevated lung production of neutrophil factors G-CSF, CXCL1, IL-6 and CXCL10. We believe this is due to the lack of feedback from neutrophils. As the lung is attempting to recruit neutrophils to the airspaces and neutrophils are depleted, more cytokines are produced to overcome this defect. Once again, lung compliance may be closely linked to airspace inflammation, while AHR may not. The importance of each cell type in allergic inflammation and the development of AHR is not clear. One study showed that resolution of AHR and mucus hypersecretion correlated well with clearance of $T_H2$ cells from the lung, but not eosinophils in the lung.[25] $T_H2$ cells were required for the development of allergic inflammation and the maintenance of an allergic response.[25] Similarly, others found that reduction of neutrophils did not affect the development of later inflammatory changes in the airways or the development of AHR, suggesting the early and transient neutrophil response does not play a direct role in the development of allergen-induced AHR.[26] However, another study revealed that AHR following allergic sensitization and challenge was neutrophil-dependent because it was abrogated in CXCR2-deficient mice and in wild-type mice receiving neutrophil depleting antibody, Gr-1.[27] Unfortunately, Gr-1 antibody also depletes macrophages and dendritic cells, which may explain this discrepancy. Depletion of antigen presenting cells may attenuate AHR by blocking T cell-dependent mechanisms independent of neutrophils.

This work reports the novel finding that severe asthma phenotypes segregated by inflammatory cell profile can be associated with an immunologically distinct cytokine profile detectable in sputum. Based on multiplex analyses for cytokines in the sputum; severe asthmatics with high levels of neutrophils and low levels of eosinophils display characteristics of neutrophil-mediated, $T_H17$-like disease. Furthermore, elevated cytokine levels in the sputum of high neutrophil, low eosinophil severe asthmatics did not predict worsened lung function. In fact, a number of cytokines significantly and directly correlated to $FEV_1$ % predicted, FVC % predicted, and $FEV_1$/FVC in this asthma endotype, suggesting this severe asthma phenotype is driven by a different mechanism or perhaps a different cycle of the asthma inflammatory process. Conversely, sputum cytokine levels inversely correlated with lung function in the other severe asthma groups, further suggesting a different mechanism of disease within those phenotypes. Overall, these findings further define the immunopathology that underlies severe asthma and may help identify a target population of severe asthmatics who have difficult to control disease for a specific approach to therapy.

AHR is a defining clinical feature of asthma; however, the mechanisms underlying the development of AHR are still under investigation. Research has focused on understanding the effects of TNFα, IL-13, IL-17A, and other cytokines on mediating immune responses in asthma.[13, 14, 28–34] However, most therapeutic strategies in asthma that target the

inflammatory processes have had variable success. Indeed, clinical studies using various TNFα neutralizing antibodies showed no benefit or improvements in lung mechanics in asthmatics.[35–38] A recent clinical study also shows brodalumab, a human anti- IL-17RA monoclonal antibody, which blocks the activity of IL-17A, IL-17F, and IL-25, did not consistently lessen disease in patients with asthma.[39] Based on our findings, these clinical studies may not have shown benefits from these anti-inflammatory therapies for two reasons. One reason is that inhibiting inflammation by limiting TNFα and IL-17A is not sufficient as it only limits airspace inflammation, while not completely impacting tissue inflammation or AHR and increasing mucus production. More importantly, we believe that these treatments were not found to be efficacious in clinical studies, as our results suggest that not all asthmatics may respond based on the inflammatory composition of their sputum. The results of this work suggest that inflammation might not be linked directly to physiology and that patient disease phenotype needs to be considered. In support of this, targeting of TNFα in severe, steroid resistant asthmatics showed more promising efficacy.[37]

Current literature and these results also imply an inflammation–independent process, possibly related to direct effects on epithelial cells and the lung matrix. Epithelial and extracellular matrix changes may account for the elevation in mucus production and inconsistent change to AHR. Indeed, a study has shown the IL-17A induced changes in airway smooth muscle contraction alters airway hyperresponsiveness.[20] Further, we recognize that IL-33 is also known to be sufficient and required for severe allergic airway disease[40] and may also contribute to the sustained tissue inflammation, variable AHR, and increased mucus production in these studies.

Based on the apparent disconnect between airway inflammation, AHR, and lung compliance in neutrophil-dominant severe asthma, it is clear that both innate and adaptive immune response in the lung are likely contributing to different aspects of disease. The clinical significance of this disparate regulation of AHR and compliance is unclear. It is not surprising that lung stiffening may be mechanistically distinct from methacholine responsiveness, as Changes in lung compliance in humans in either direction can be associated with decreased $FEV_1$. A better understanding of these underlying immune mechanisms is required. It is likely the T cell responses and/or the structural status of the airway, not necessarily inflammation, is important to AHR. Further, $T_H17$-induced allergic airway disease is comprised of distinct inflammatory, mucus, and AHR mechanisms that are not necessarily driven by common pathways. These observations are likely critical to designing appropriate therapy for patients with severe asthma.

## Methods

### Adoptive transfer model

Six- to eight-week-old female BALB/c SCID and C.DO11.10 ovalbumin (OVA) specific TCR-transgenic mice (Jackson Laboratory) were housed at the Children's Hospital of Pittsburgh in a pathogen-free environment. Experiments were approved by The University of Pittsburgh Institutional Animal Care and Use Committee. $CD4^+CD62^+$ naïve T cells from the spleens of DO11.10 mice were cultured as previously reported [4]. BALB/c SCID mice were challenged with 50 μg OVA (Sigma-Aldrich) via oropharyngeal aspiration and $1\times10^6$

$T_H17$ cells were adoptively transferred by retro-orbital injection. Control mice received OVA challenges, but phosphate buffered saline retro-orbitally at time of cell transfer. Mice were treated with either 300 μg of control IgG1, 100 μg anti-TNFα antibody (CNTO 5048, Janssen R&D), 300 μg of anti-IL17A antibody (CNTO 8096, Janssen R&D), or 250 μg of control rat IgG2a or murine Ly-6G antibody, clone 1A8 (BioXCell, West Lebanon, NH) or 200 μL of phosphate buffered saline, via intraperitoneal injection.

### Murine AHR and Allergic Airway Disease Measurements

AHR to methacholine challenge was done as previously described [41–43]. Following these analyses, BAL fluid was collected and total cells were counted using a hemocytometer. Cytospin preparations of BAL cells were used for differential counting. Lung lobes were separated and processed as follows: flash frozen in liquid nitrogen for cytokine analysis by Lincoplex or gene expression analyses by real-time PCR (Applied Biosystems Taqman) or inflation fixed with 10% buffered formalin and paraffin embedded for histology. Inflammation and mucus production were assessed by H&E and PAS staining, respectively, and scored by a pathologist who was blinded to the sample groups

### Human Subjects

Sputum collection was completed on 41 subjects, ranging from age 9 through 64 years, participating in the Severe Asthma Research Program (SARP) at The University of Pittsburgh. Subjects included severe asthmatics (as defined by the American Thoracic Society Workshop on Refractory Asthma[18]) who had sputum induction, or, if safety criteria were not met, a spontaneous sputum collected (n=3). Samples were collected with approval from The University of Pittsburgh Institutional Review Board.

### Sputum induction and processing

Sputum induction method was adopted from the Asthma Clinical Research Network method described in detail[44]. Briefly, after establishing a post-bronchodilator baseline (safety criterion of >/=60%), subjects inhaled nebulized 3% buffered saline to produce induced sputum. Collections and pulmonary functions were done every 4 minutes for a maximum of 12 minutes. If subject $FEV_1$ dropped below 80% of their post-bronchodilator $FEV_1$, then induction was terminated for patient safety. Induced sputum was processed using equal amounts of 10% Sputolysin (Calbiochem, La Jolla, CA) immediately after collection. Sputum supernatant was obtained by centrifugation and cell cytospins were stained for differential count of leukocytes, bronchial epithelial, and squamous cells.

### Cytokine Analyses

Human sputum supernatant and murine lung homogenate samples were analyzed using custom Lincoplex multiplex kits according to manufacturer’s instructions. IL-22 levels in human sputum supernatants were measured by ELISA (R&D Systems).

### Statistical analyses

Data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). Experiments involving 2 variables (AHR measurement, Lincoplex results, BAL fluid

differentials) were analyzed by two-way analysis of variance with a Bonferonni post hoc test. Data with one variable were analyzed using one-way analysis of variance with Tukey's post-hoc test. Spearman's correlation analyses were conducted using SPSS 20 (IBM Corporation, Armonk, NY) where appropriate. Data shown are mean ± SEM. A value of $p<0.05$ was considered to be statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **AHR** | airway hyperresponsiveness |
| **BAL** | bronchoalveolar lavage |
| **$FEV_1$** | forced expiratory volume in one second |
| **FVC** | forced vital capacity |
| **G** | tissue damping |
| **H** | tissue elasticity |
| **OVA** | ovalbumin |
| **PAS** | Periodic acid-Schiff |
| **$R_n$** | airway resistance |
| **SARP** | Severe Asthma Research Program |
| **$T_H$** | T helper |

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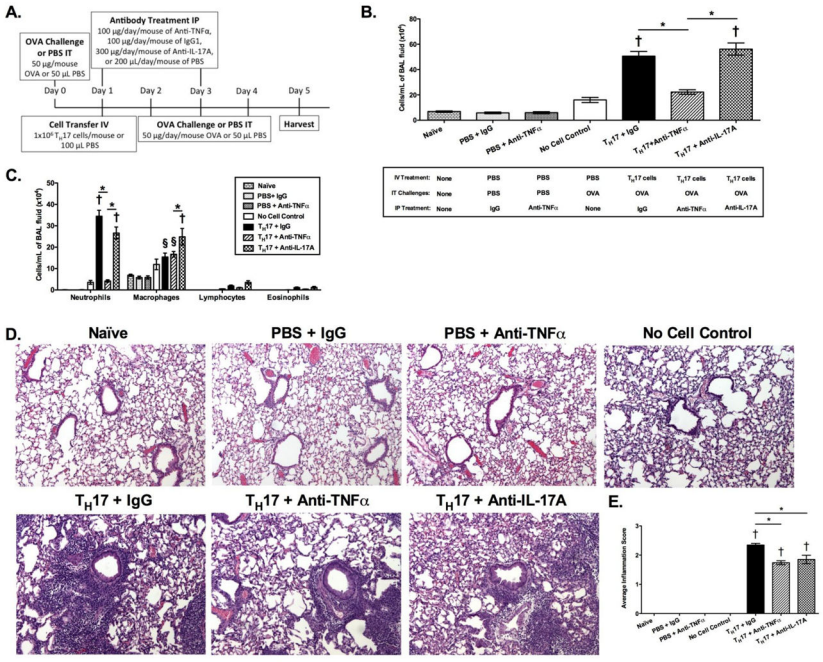


**Figure 1.**

$T_H17$-mediated airway inflammation is attenuated by TNFα neutralization in $T_H17$ cell transferred, OVA-challenged mice. BALB/c SCID mice were treated as previously described to induce $T_H17$-mediated allergic airway disease and treated with anti-TNFα or anti-IL-17A antibodies (A). Total cells/mL in the BAL fluid (B), cell differentials (C), and H&E stained lung sections (D) were used to assess cellular inflammation in the lung. Cellular inflammation was also quantified by a pathologist (M.L.M.) blinded to the groups (E). Mean±SEM, naïve (n=6), PBS + IgG (n=4), PBS + Anti-TNFα (n=4), no cell control (n=3), $T_H17$ + IgG (n=9–10), $T_H17$ + Anti-TNFα (n=4–6), $T_H17$ + Anti-IL-17A (n=6), one-way ANOVA with Tukey's post-test (B and D) and two-way ANOVA with Bonferroni's post-test (C), *$p<0.01$ for comparison shown, †$p<0.001$ when compared to all controls (naïve, PBS + IgG, PBS + Anti-TNFα, and no cell control), §$p<0.001$ when compared to naïve, PBS + IgG, PBS + Anti-TNFα.

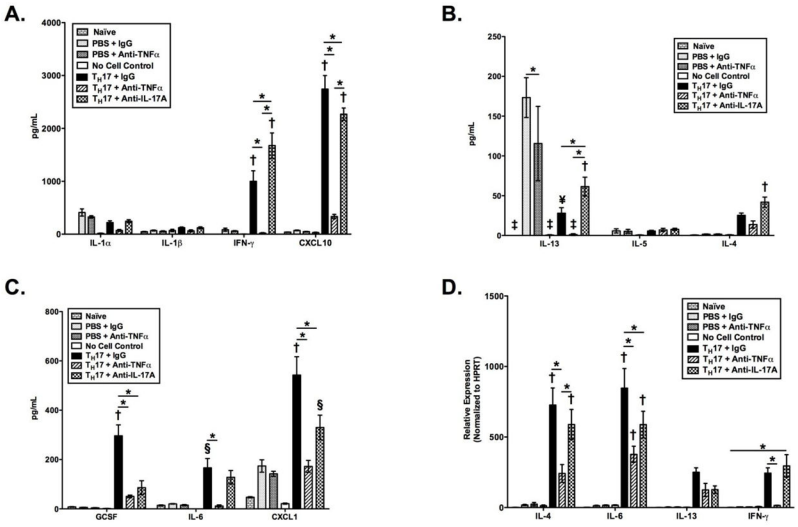


**Figure 2.**

Cytokine and chemokine levels in the lung are attenuated by TNFα neutralization in $T_H17$ cell transferred, OVA-challenged mice. $T_H1$- (A), $T_H2$- (B), and $T_H17$-related (C) inflammatory cytokines/chemokines levels and mRNA expression (D) were also measured in the lung. Mean±SEM, naïve (n=5–6), PBS + IgG (n=4), PBS + Anti-TNFα (n=4), no cell control (n=3), $T_H17$ + IgG (n=10), $T_H17$ + Anti-TNFα (n=4–7), $T_H17$ + Anti-IL-17A (n=6), *$p<0.05$ for comparison shown, †$p<0.05$ when compared to all controls (naïve, PBS + IgG, PBS + Anti-TNFα, and no cell control), §$p<0.05$ when compared to naïve, PBS + Anti-TNFα, and no cell control, ‡$p<0.05$ when compared to PBS + IgG and PBS + Anti-TNFα, ¥$p<0.05$ when compared to naïve, PBS + IgG, and PBS + Anti-TNFα, two-way ANOVA with Bonferroni's post-test

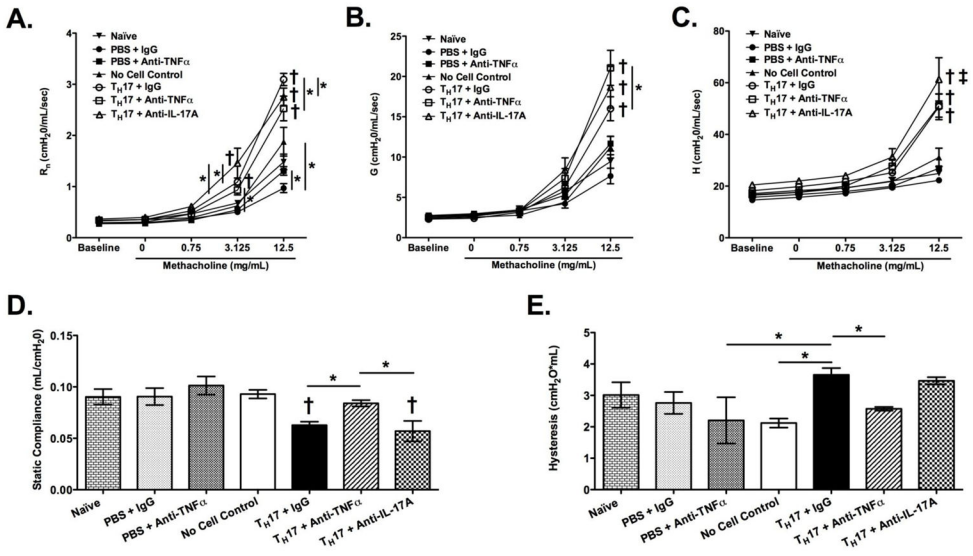


**Figure 3.**

TNFα neutralization rescues $T_H17$-induced lung stiffening, but not AHR in $T_H17$ cell transferred, OVA-challenged mice. Airway hyperresponsiveness to methacholine challenge (A–C) was evaluated in naïve mice (n=6), mice treated with PBS and IgG antibody (n=4), mice treated with PBS and anti-TNFα antibody (n=4), control mice with no cell transfer (n=3), mice that received $T_H17$ cells and IgG antibody (n=12–14), mice that received $T_H17$ cells and anti-TNFα antibody (n=8–9), or mice that received $T_H17$ cells and anti-IL-17A antibody (n=5–6). Static lung compliance (D) and hysteresis (E) were also assessed in these mice. Mean±SEM, two-way ANOVA with Bonferroni's post-test (A–C), and one-way ANOVA with Tukey's post-test (D, E), *$p<0.05$ for comparisons shown, †$p<0.05$ when compared to all controls (naïve, PBS + IgG, PBS + Anti-TNFα, and no cell control), ‡$p<0.05$ when compared to $T_H17$ + IgG and $T_H17$ + Anti-TNFα.

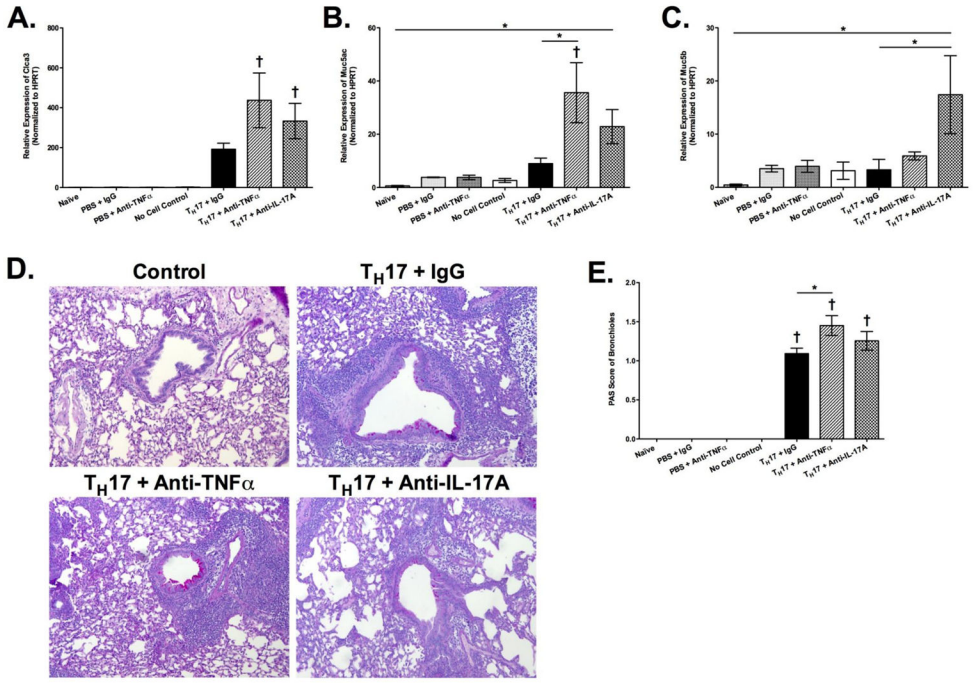


**Figure 4.**

Mucus production induced by $T_H17$ cell transfer and OVA challenge is worsened by TNFα neutralization in mice. Mucus production was assessed by real-time PCR for *Clca3* (A), *Muc5ac* (B), and *Muc5b* (C) and PAS staining of lung sections (D) following $T_H17$ cell transfer with anti-TNFα or IgG treatment and following control treatments. Control shown is representative of all controls (naïve, PBS + IgG, PBS + Anti-TNFα, and no cell control) (D). Mucus production was quantified by a pathologist (M.L.M.) blinded to the groups (E). Mean±SEM, naïve (n=5–6), PBS + IgG (n=4), PBS + Anti-TNFα (n=4), no cell control (n=3), $T_H17$ + IgG (n=10), $T_H17$ + Anti-TNFα (n=4–6), $T_H17$ + Anti-IL-17A (n=6), one-way ANOVA with Tukey's post-test, *$p<0.05$ for comparison shown and †$p<0.05$ when compared to all controls (naïve, PBS + IgG, PBS + Anti-TNFα, and no cell control)

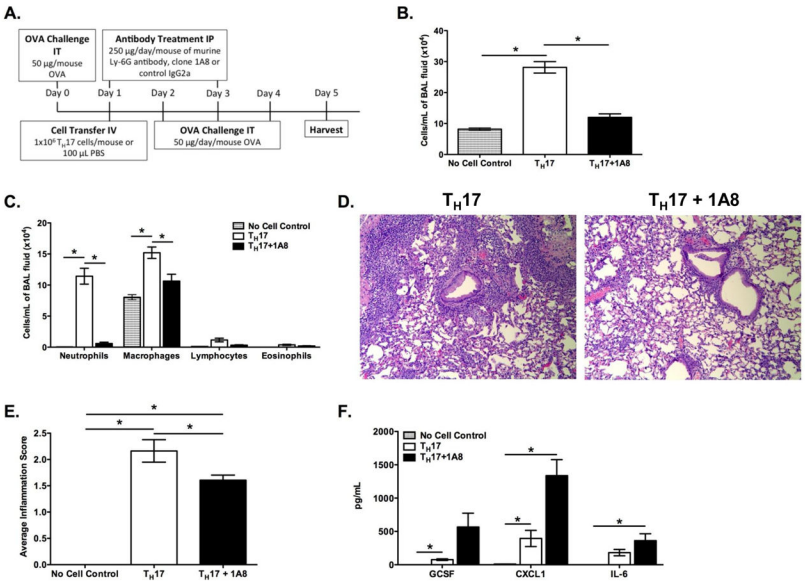


**Figure 5.**

$T_H17$-induced inflammatory responses are altered by neutrophil depletion by 1A8 antibody. $T_H17$-mediated allergic airway disease was induced in BALB/c SCID and neutrophils were depleted by 1A8 antibody treatment as described (A). Total cells/mL in the BAL fluid (B), cell differentials (C), and H&E stained lung sections (D) were used to assess cellular inflammation in the lung. Cellular inflammation was also quantified by a pathologist (M.L.M.) blinded to the groups (E). $T_H17$ cytokine and chemokine levels were measured using multiplex analyses (F). Control mice did not receive T cell transfer, but were challenged with OVA (No Cell Control). Mean ± SEM, n=7–8, two-way ANOVA with Bonferroni's post-test (C, F) and one-way ANOVA with Tukey's post-test (B, E), *$p<0.05$

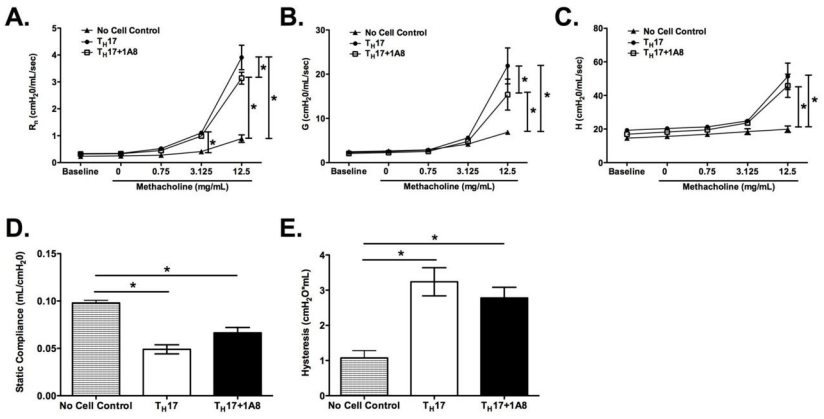


**Figure 6.**

Neutrophil depletion rescues $T_H17$-induced lung stiffening, but only partially affects AHR in $T_H17$ cell transferred, OVA-challenged mice. Dose response of methacholine in mice that received $T_H17$ cells or mice that received $T_H17$ cells treated with anti-Ly6G (1A8) antibody was determined using flexiVent analyses (A–C). Static lung compliance (D) and hysteresis (E) were also assessed in these mice. Mean±SEM, n=7–8, two-way ANOVA with Bonferroni's post-test (A–C) and one-way ANOVA with Tukey's post-test (D, E), *p<0.05

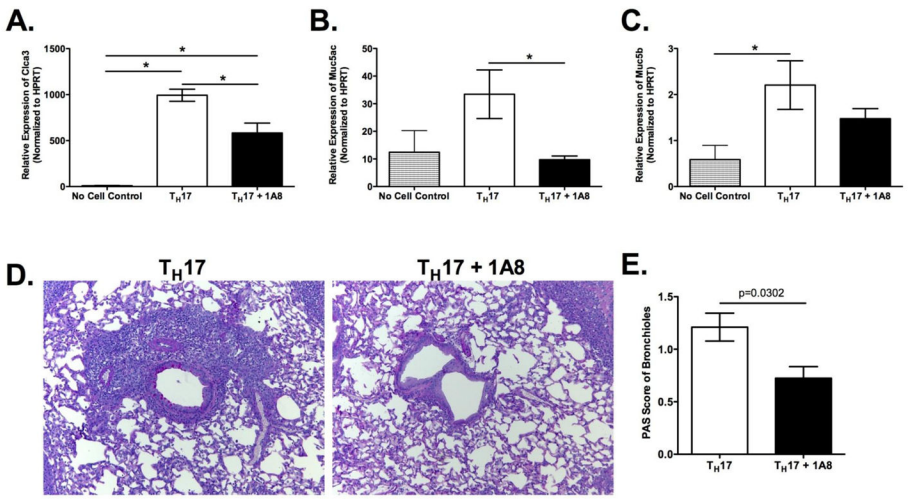


**Figure 7.**

Neutrophil depletion by 1A8 antibody decreases mucus production in $T_H17$ cell transferred, OVA-challenged mice. Mucus production was assessed using real-time PCR for *Clca3 (A)*, *Muc5ac (B)*, and *Muc5b* (C) and PAS staining of lung sections (D). Mucus production was also quantified by a pathologist (M.L.M.) blinded to the groups (E). Mean±SEM, n=6–8/group except PAS quantification (n=4/group), one-way ANOVA with Tukey's post-test (A–C), unpaired t-test (E), *$p<0.05$ unless otherwise noted.

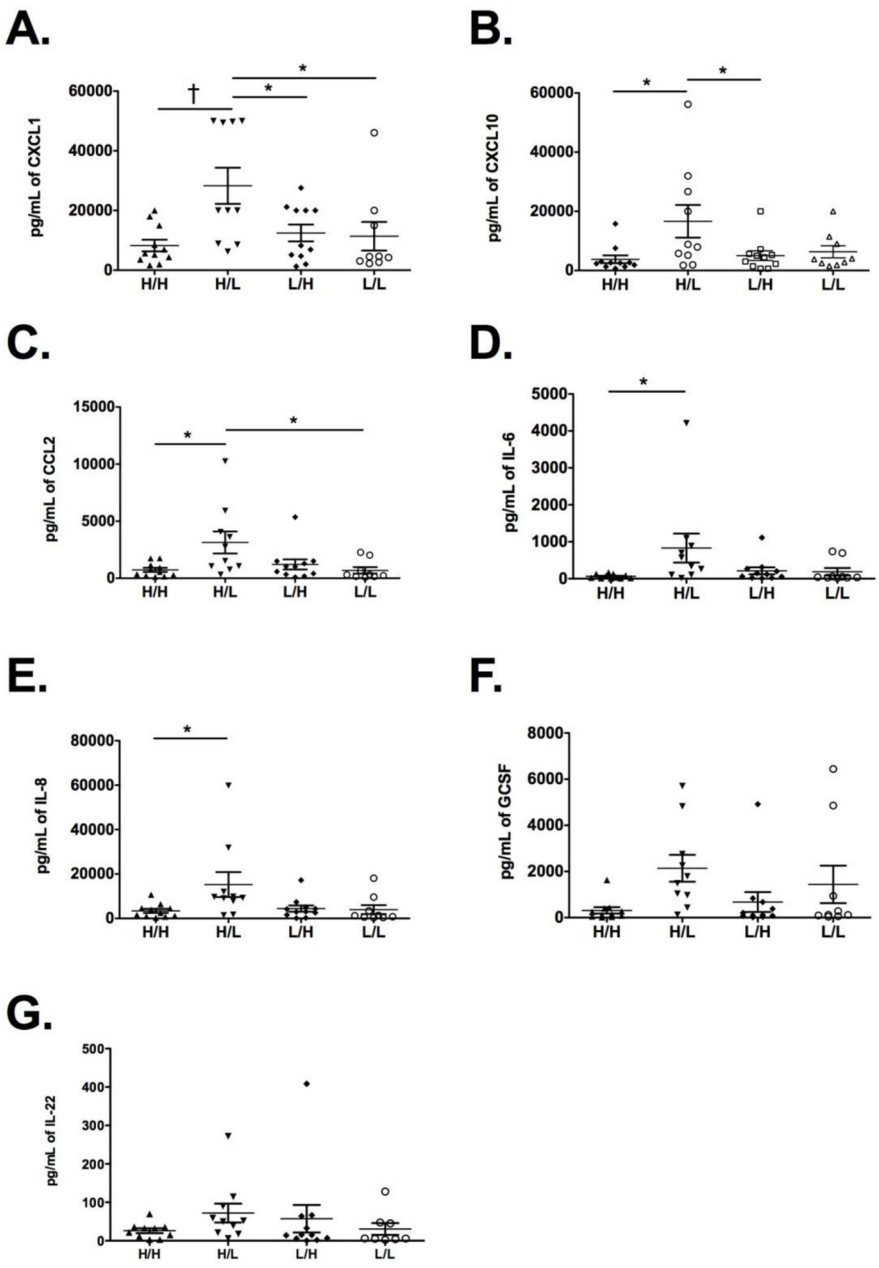


**Figure 8.**

Severe asthmatics with high sputum neutrophilia and low eosinophilia have a distinct inflammatory profile. Asthmatics with high levels of neutrophils and low levels of eosinophils (H/L, N=10) had increased sputum CXCL1 (A), CXCL10 (B), CCL2 (C), IL-6 (D), and IL-8 (E) when compared to those with high neutrophils and eosinophils (H/H, N=11), with low neutrophils and high eosinophils (L/H, N=11), and low levels of both cells (L/L, N=9). G-CSF (F) and IL-22 (G) levels were not significantly different among the groups. Each point represents one patient. Mean±SEM, *p<0.05, †p<0.01

**TABLE 1**

SUBJECT CHARACTERISTICS

| | Severe Asthmatics | | | | |
|---|---|---|---|---|---|
| | **H/H** | **H/L** | **L/H** | **L/L** | **P value** |
| **N= 41** | 11 | 10 | 11 | 9 | |
| **Gender** | | | | | |
| **Male/Female** | 5/6 | 3/7 | 5/6 | 4/5 | 0.88 |
| **Race** | | | | | |
| **Caucasian/ African American/ Other** | 9/2/0 | 7/2/1 | 8/3/0 | 7/1/1 | 0.93 |
| **Age (yr)** | 34.6±4.9 | 36.9±3.7 | 43.0±5.8 | 31.7±6.0 | 0.47 |
| **Age Onset** | 12.7±3.9 | 11.8±5.7 | 23.7±6.7 | 10.9±6.5 | 0.35 |
| **Duration** | 21.8±5.5 | 27.0±3.3 N=9 | 19.3±3.9 | 20.7±4.9 | 0.68 |
| **BMI** | 28.1±1.4 | 31.3±2.1 | 28.4±2.1 | 29.1±2.0 | 0.64 |
| **Lung Function:** | | | | | |
| **$FEV_1$ % predicted** | 56.3±7.5 | 54.6±7.7 | 68.9±7.0 | 66.7±9.3 | 0.47 |
| **FVC % predicted** | 76.3±7.7 | 72.5±8.7 | 80.3±6.9 | 89.4±10.5 | 0.56 |
| **$FEV_1$/FVC** | 0.6±0.04 | 0.6±0.04 | 0.7±0.03 | 0.6±0.03 | 0.19 |
| **Corticosteroid Use (Yes/No):** | | | | | |
| **Inhaled** | 11/0 | 9/1 | 10/1 | 9/0 | 0.60 |
| **Systemic** | 8/3 | 9/1 | 5/6 | 6/3 | 0.19 |

*Definition of Abbreviations:* BMI= body mass index, $FEV_1$= forced expiratory volume in 1 second, FVC= forced vital capacity

Mean ± SEM are shown and the number of subjects is indicated if information was not available for all subjects in the group.

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## TABLE 2

CORRELATIONS BETWEEN PULMONARY FUNCTION AND SPUTUM CYTOKINE LEVELS IN SEVERE ASTHMATICS

| | Comparison | Test Statistics |
|---|---|---|
| **H/H (N=11)** | IL-8 vs. $FEV_1$ % predicted | $r_s$=−0.627 (p=0.04) |
| | IL-8 vs. FVC % predicted | $r_s$=−0.664 (p=0.03) |
| **H/L (N=10)** | CXCL1 vs. $FEV_1$ % predicted | $r_s$=0.640 (p=0.046) |
| | IL-6 vs. $FEV_1$ % predicted | $r_s$=0.766 (p=0.01) |
| | IL-6 vs. $FEV_1$/FVC | rs=0.733 (p=0.02) |
| **L/H (N=11)** | No significant correlations (p>0.05) | |
| **L/L (N=9)** | CCL2 vs. $FEV_1$ % predicted | $r_s$=−0.850 (p=0.004) |
| | CCL2 vs. FVC % predicted | $r_s$=−0.733 (p=0.03) |

*Definition of Abbreviations:* $FEV_1$= forced expiratory volume in 1 second, FVC= forced vital capacity

Data shown are Spearman's correlation coefficient values ($r_S$) and were determined by a Spearman's correlation using SPSS statistical software. p values are shown in parentheses.