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Mechanism of Th2/Th17-predominant and Neutrophilic, Th2/Th17-low Subtypes of Asthma

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Full Title: Mechanism of Th2/Th17-predominant and Neutrophilic, Th2/Th17-low

Subtypes of Asthma

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Running Title: Th2/Th17 and Neutrophilic Subtypes of Asthma

Abstract

Background: The mechanism of Th2/Th17-predominant and Th2/Th17-low asthma is unknown.

Objective: To study the immune mechanism of Th2/Th17-predominant and Th2/Th17-low asthma.

Methods: In a previously reported cohort of 60 asthmatic patients, 16 patients were immunophenotyped with Th2/Th17-predominant asthma and 22 patients with Th2/Th17-low asthma. We examined BAL leukocytes, cytokines, mediators, and epithelial cell function for these asthma subgroups.

Results: Th2/Th17-predominant asthma had elevated IL1 β , IL6, IL23, C3a and serum amyloid A (SAA) in BAL, and correlated with IL1 β and C3a. Th2/Th17 cells expressed higher levels of the IL1 receptor and p-p38 MAPK. Anakinra, an IL1 receptor antagonist protein, inhibited BAL Th2/Th17 cells. Th2/Th17-low asthma had two distinct subgroups—neutrophilic asthma (45%) and pauci-inflammatory asthma (55%). This contrasted with Th2/Th17-predominant and Th2-predominant asthma, which had neutrophilic asthma in 6% and 0% of patients, respectively. BAL neutrophils strongly correlated with BAL myeloperoxidase, IL8, IL1 α , IL6, G-CSF, and GM-CSF. Sixty percent of the patients with neutrophilic asthma had a pathogenic microorganism in BAL culture, which suggested a subclinical infection.

Conclusion: We uncovered a critical role for the IL1 β pathway in Th2/Th17-predominant asthma. A subgroup of Th2/Th17-low patients had neutrophilic asthma and elevated BAL IL1 α , IL6, IL8, G-CSF, and GM-CSF. IL1 α was directly involved in IL8 production and likely

contributed to neutrophilic asthma. Sixty percent of neutrophilic patients had a subclinical infection.

Clinical Implications

This study uncovers a critical role for the IL1 β pathway in development of Th2/Th17-predominant asthma. This subtype of asthma is likely to benefit from an IL1 inhibitor-like drug (e.g. anakinra). Neutrophilic asthma represents a subtype of Th2/Th17-low asthma. A subgroup of neutrophilic asthma is associated with subclinical infection and an infection-inducible cytokine profile. Anti-microbial agents and biologics targeting specific cytokines are likely to benefit this subgroup of asthma.

Capsule summary

This paper defines the molecular mechanisms of two subtypes of asthma—Th2/Th17-predominant asthma and Th2/Th17-low, neutrophilic asthma. The paper also delineates phenotype-specific therapeutic agents that are likely benefit these two subtypes of asthma.

Key words: Asthma endotype, Th2/Th17-predominant asthma, neutrophilic asthma, IL1, C3a, infection

Abbreviations: DAMP: danger-associated molecular pattern; SAA: serum amyloid A

Introduction

The variability in clinical manifestation and treatment response in asthma is considered to result from its heterogeneous mechanisms (1-3). There are many clinical phenotypes of asthma—atopic (includes both eosinophilic and non-eosinophilic), non-atopic, eosinophilic non-atopic, and neutrophilic (4). It is now generally accepted that atopic and eosinophilic non-atopic asthma are mediated by a type 2 immune response (an immune response that results in production of IL4, IL5 and IL13, among others) (5). Although Th2 cells are considered to play a central role in atopic asthma, it is likely that other type 2 cytokine producing cells such as type 2 innate lymphoid cells and CD8 T cells, and NKT cells also contribute to this phenotype (6-8).

Genome-wide transcriptomic analysis of airway epithelial cells led to identification a Th2-high and Th2-low endotypes of asthma (5). The Th2-high endotype manifested with increased expression of IL13 signature genes (periostin, CLCA1 and Serpin 2B) in airway epithelial cells. Their T cells produced increased amounts of IL5. Th2-low asthma did not show increased expression of type 2 cytokines. Another study reported increased expression of IFN γ + CD4 (Th1) cells in a subgroup severe asthma patients (8). Differentiated T helper cells manifest plasticity in their phenotype (9). Th2 cells can transdifferentiate into Th2/Th17 cells. The presence of dual positive Th2/Th17 has been described in the blood from asthmatic patients (10). Using flow cytometric detection of BAL cytokine-expressing T cells we recently reported 3 endotypes of asthma—Th2-predominant, Th2/Th17-predominant and Th2/Th17-low (11). The Th2/Th17-predominant asthma was characterized by the dominant presence of dual positive Th2/Th17 cells in bronchoalveolar lavage. These dual positive Th2/Th17 cells simultaneously produced IL4 and IL17. The cytokine milieu that favors the development of Th2/Th17 cells in the airways is unknown. In animal studies Th17 cells are associated with neutrophilic asthma

(12, 13). We anticipated a mixed eosinophilic and neutrophilic phenotype in Th2/Th17-predominant asthma. Instead, we observed increased eosinophils but not neutrophils in BAL from Th2/Th17-predominant asthma. This study suggested a different mechanism for neutrophilic asthma in humans. The objective of this study was to define the cytokine/mediator milieu that contributes to Th2/Th17-predominant, Th2/Th17-low, and neutrophilic asthma.

Materials and methods

Human subjects: The study subjects were recruited from the outpatient clinics of National Jewish Health. The study protocol for bronchoscopy and BAL was approved by the Institutional Review Board (IRB). An informed consent was obtained from the study subjects. Patients were allowed to continue their routine medication. The biological samples for this study (BAL fluid and cells) were from the same cohort of 60 patients that was described in the previously published paper (11). This cohort consisted of 22 Th2-predominant asthma, 16 Th2/Th17-predominant asthma and 22 Th2/Th17-low asthma patients. In this paper we further analyzed these existing samples to elucidate the mechanism of the identified endotypes. For experiments involving anakinra, p38 MAPK signaling and airway epithelial cells we recruited 20 new asthmatic patients as outlined in the text. Of these 20 patients 8 had Th2/Th17-predominant asthma, 6 patients had Th2-predominant asthma and 6 had Th2/Th17-low asthma.

Processing of BAL cells and flow cytometry: Bronchoscopy and BAL were performed as described previously (11, 14). BAL was processed immediately. Cells were isolated by centrifugation. Supernatant fluid was aliquoted into small samples and frozen. Cells were fixed immediately in 4% paraformaldehyde and processed for flow cytometry as described previously (11). In a small group of patients cells were cultured with anakinra (recombinant IL1 receptor antagonist protein from Prospe, Inc. East Brunswick, NJ) or medium for 3 days and then analyzed by flow cytometry.

Airway epithelial cell culture and function: The human immortalized airway epithelial cell line BEAS-2B and freshly isolated human bronchial epithelial cells from asthmatic patients (obtained by bronchial brushing) were grown in the BEBM medium (Lonza) with the addition of

the growth factor-enriched supplement BEGM (Cambrex, Inc., East Rutherford, NJ) and the medium was changed every 3 days as described previously (15). Before stimulation, cells were trypsinized and then plated at 4×10^4 /ml in a 6-well tissue culture plate. BEAS-2B cells were stimulated with a subthreshold dose of LPS (0.25 μ g/ml) with and without additional cytokines/growth factors (IL1 α , IL4, IL6, GM-CSF, IL17A, and C3a, 10 ng/ml each) for the indicated period of time. Freshly isolated human bronchial epithelial cells were cultured with IL1 α , IL6, GM-CSF, C3a (10 ng/ml each) and their combinations but without LPS for 48 hrs. In additional experiments cells were cultured with and without the p38 MAPK inhibitor SB212190 (10 μ M). Supernatant was collected and assayed for secreted cytokines by ELISA.

ELISA: We performed ELISA for the following cytokines and mediators: IL1 α , IL1 β , IL6, IL8, IL17A, IL21, IL23, G-CSF, GM-CSF, TNF α , C3a, myeloperoxidase, serum amyloid A, and uric acid. The foregoing cytokines/mediators were assayed in undiluted BAL fluid by ELISA kits or paired (capture and detection) antibodies as per the supplier's instruction. GM-CSF (sensitivity: 7.8 pg/ml), TNF- α (sensitivity: 7.8 pg/ml), IL 1 α (sensitivity: 7.8 pg/ml), IL 6 (sensitivity: 7.8 pg/ml), IL 8 (sensitivity: 15.6 pg/ml) and IL 17 A (sensitivity: 3.9 pg/ml), and IL21 (sensitivity: 31.3 pg/ml) were from Biolegend (San Diego, CA); IL 1 β (sensitivity: 3.9 pg/ml), G-CSF (sensitivity: 31.3 pg/ml), and myeloperoxidase (sensitivity 62.5 pg/ml) were from R&D Systems (Minneapolis, MN); C3a (sensitivity: 0.32ng/ml) was from eBioscience (San Diego, CA); Uric Acid Assay kit (sensitivity: 0.5 μ M) was from Cayman Chemical Company (Ann Arbor, MI); serum amyloid A kit (sensitivity: 0.41 ng/ml) was from Abcam (Cambridge, MA) and IL23 (sensitivity: 3 pg/ml) kit from MyBiosource (San Diego, CA).

132 **Statistical Analyses:** Comparison between the study groups was done by Mann-Whitney U test.
133 Comparison among multiple study groups was performed by Kruskal Wallis test. Pearson
134 correlation coefficient was used to calculate correlation coefficient.

135

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Results

Th17-inducing cytokine profile in BAL from asthmatic patients

Based upon flow cytometric analyses of the cytokine expression profile of BAL cells from 60 refractory asthmatic patients we previously reported three distinct immune endotypes of asthma—Th2-predominant asthma (the frequency of IL4⁺ CD4 T cells outnumbered that of IL4/IL17⁺ CD4 T cells and the IL4/IL17⁻ CD4 T cells) in 22 patients, Th2/Th17-predominant asthma (the frequency of IL4/IL17⁺ CD4 T cells outnumbered that of IL4⁺ CD4 T cells and the IL4/IL17⁻ CD4 T cells) in 16 patients, and Th2/Th17-low asthma (the frequency of Th2 and Th2/Th17 cells was below 5% of all CD4 T cells) in 22 patients (11). Flow cytograms representing each immune endotype are shown in the online repository figure E1. We were interested in stability of the Th2/Th17-predominant endotype over time. Repeat BAL analysis of Th2/Th17 cells on 4 asthmatic patients showed that the endotype persisted over a 3-5 year time period of observation (online repository table 1). We wanted to define the cytokine milieu that favored the development of dual positive Th2/Th17 cells in the airways. A previous study showed that IL1, IL6, IL21 and IL23 were capable of inducing Th2/Th17 cell differentiation (10). For this reason we assayed BAL fluid from this cohort of 60 asthmatic patients for these cytokines. IL21 was undetectable. IL1 α (Figure 1A) and IL23 (Figure 1D) were selectively increased in Th2/Th17-low and Th2/Th17-predominant asthma, respectively. IL1 β (Figure 1B) and IL6 (Figure 1C) were elevated in both Th2/Th17-predominant asthma and Th2/Th17-low asthma as compared to Th2-predominant asthma. The IL6 level was higher in Th2/Th17-predominant asthma as compared to Th2/Th17-low asthma.

Danger-associated Molecular Patterns (DAMPs) in the airways from the asthma subtypes

Next, we examined the mechanism of increased production of IL1 β and IL6 in Th2/Th17-predominant asthma. Many environmental toxicants induce airway stress and trigger the production of DAMPs (16, 17). DAMPs influence the production of immunomodulatory cytokines (18-22). We examined 3 DAMPs in BAL from the 3 asthma groups. The selection of these DAMPs was based upon their documented involvement in Th2- and Th17-type inflammation (19-22). C3a and serum amyloid A (SAA) but not uric acid was elevated in both Th2/Th17-predominant asthma and Th2/Th17-low asthma (Figure 2A-C). Their levels were higher in Th2/Th17-predominant asthma as compared to Th2/Th17-low asthma.

Clinical correlation of cytokines and DAMPs with Th2/Th17

Of the foregoing cytokines and DAMPs, only IL1 β and C3a significantly correlated with Th2/Th17 cells (Figure 3A & B). C3a also correlated ($r=0.69$, $P<0.001$) with IL1 β (Figure 3C). Since IL1 β strongly correlated with Th2/Th17, we sought to determine the expression level of the IL1 receptor (IL1R) on Th2/Th17 cells ex vivo and compared them with other cells. Th2/Th17 cells as well as Th17 cells demonstrated the highest level of expression of IL1R in BAL as compared to Th2 cells (Figure 3C). IL1 β activates multiple signaling pathways, one of which is the p38 MAPK pathway (23). We observed a high level expression of the phosphorylated form (activating phosphorylation) of p38 MAPK in Th2/Th17 and Th17 cells (Figure 3D). We recognize that p38 MAPK is not specific for IL1 β and is activated by many molecules. To define the role of IL1 β further, we cultured BAL cells from moderate to severe (as per NAEPP EPR3 guidelines) asthmatic patients with anakinra (recombinant IL1 receptor antagonist protein, brand name Kineret) and measured IL4/IL17+ cells. Anakinra inhibited the

expression of dual IL4/IL17⁺ CD4 T cells in BAL in a dose-dependent manner (Figure 3E). In order to determine if IL1 β was responsible for p38 MAPK signaling we cultured BAL cells from moderate to severe (as per NAEPP EPR3 guidelines) asthmatic patients with anakinra for 24 hr and analyzed the expression of p-p38 in CD4 T cells by flow cytometry. Anakinra significantly inhibited p-p38 in a dose-dependent manner (Figure 3F). These results in aggregate suggest that IL1 β plays an important role in differentiation/and or maintenance of Th2/Th17 cells in a subgroup of asthmatic patients.

Th2/Th17-low but not Th2/Th17-predominant asthma is associated with increased BAL neutrophils

In mouse studies a Th17 immune response was associated with neutrophilic asthma (12, 13). We anticipated increased airway neutrophils in Th2/Th17-predominant asthma. An analysis of BAL neutrophil counts revealed that Th2/Th17-low asthma but not Th2/Th17-predominant asthma was associated with neutrophilic asthma. Th2/Th17-low patients had significantly higher number of BAL neutrophils, $16.1 \pm 4.4\%$ as compared to $4 \pm 1.8\%$ in Th2/Th17-predominant and $2.5 \pm 0.5\%$ in Th2-predominant endotypes (Fig. 4A). An analysis of individual data points indicates clustering and separation of neutrophil-high patients from neutrophil-low patients (Fig. 4A). Based upon the clustering pattern of BAL neutrophils in these three groups we set a threshold number of 7 for neutrophilic asthma. When applied this threshold number, only 1 patient from the Th2/Th17-predominant asthma and none from the Th2-predominant asthma but 10 patients from the Th2/Th17-low asthma had neutrophilic asthma. In order to further confirm neutrophilic inflammation we examined BAL myeloperoxidase level in the same patients. The myeloperoxidase level was similarly elevated in Th2/Th17-low patients as compared to the other

two groups (Figure 4B). The myeloperoxidase level highly correlated ($r=0.89$, $P<0.001$) with the BAL neutrophil count.

IL8 (CXCL8) is a chemokine that critically drives neutrophilic inflammation (24-26). We measured IL8 level in the BAL fluid from the study groups. IL8 level was highest in the Th2/Th17-low group and lowest in the Th2-predominant group (Fig. 4C). The distribution of individual data points also suggested clustering and separation of IL8-high patients from IL8-low patients. When we used an arbitrary cut-off point of 200 pg/ml, only 1 patient from the Th2/Th17-predominant asthma, no patient from the Th2-predominant asthma and 10 patients from the Th2/Th17-low asthma groups were high for IL8, which was similar to that observed for neutrophils. As expected, BAL IL8 strongly correlated ($r=0.88$, $P=0.00001$) with BAL neutrophils (Figure 4D) suggesting that IL8 contributed to neutrophilic inflammation. The absence of neutrophilic inflammation in the Th2/Th17-predominant group was surprising. Since IL17 is produced by dual positive Th2/Th17 cells, which also produce high levels of IL4, we asked if IL4 & IL13 affected LPS induced epithelial production of IL8, the major inducer of neutrophilic inflammation. Both IL4 & IL13 significantly stimulated IL8 production at low doses but inhibited this production at high doses in BEAS-2B epithelial cells (Fig. 4E). Our results agree with a recent report (27), which suggested an inhibitory role of IL4/IL13. We asked if this relationship existed in vivo. To this goal we measured IL4 and IL8 in 42 BAL samples obtained from all three study groups. We found a negative correlation between IL4 and IL8 (Figure 4F). Note that the concentration of IL4 in BAL was relatively low, which is likely due to the dilution effect of the large volume (120 ml) of saline that was used for BAL. The results provide a mechanistic explanation for the lack of neutrophilic inflammation in IL4-high Th2/Th17-predominant asthma.

Role of cytokines and DAMPs in neutrophilic asthma

In addition to IL8 many other cytokines either directly or indirectly regulate neutrophilic inflammation. They include IL1 α & β , IL6, IL17A, G-CSF, GM-CSF and TNF α (28-32). IL1 α , IL1 β and IL6 were elevated in Th2/Th17-low asthma (Figure 1A-C). Next, we measured IL17A, G-CSF, GM-CSF and TNF α in BAL from the three study groups. TNF α was undetectable in BAL from the asthmatic patients. G-CSF and GM-CSF but not IL17A were elevated in Th2/Th17 low asthmatic patients as compared to Th2- and Th2/Th17-predominant asthmatic patients (Figure 5 A-C). IL1 α , GM-CSF, IL6, G-CSF and C3a (in order of the strength of correlation) correlated with IL8 (Table 1). G-CSF, IL1 α , IL6, GM-CSF and C3a correlated with BAL neutrophils. IL1 β , serum amyloid A and uric acid did not correlate with neutrophils and IL8.

Effect of cytokines and DAMPs in IL8 production by airway epithelial cells

Next we studied the effect of the BAL neutrophil-associated cytokines/mediators—IL1 α , IL6, GM-CSF and C3a (see Table 1) on epithelial IL8 production. We used two different cell types—the immortalized human bronchial epithelial cell line BEAS-2B and freshly isolated human bronchial epithelial cells from asthmatic patients obtained by bronchial brushing. The freshly isolated human bronchial epithelial cells spontaneously produced significant quantities of IL8 when cultured in the medium alone (Figure 5D). In contrast, the BEAS-2B cells required priming with a subthreshold dose of LPS to induce IL8 production. For this reason all BEAS-2B cultures were performed with the subthreshold dose of LPS. In both cell types IL1 α was the strongest inducer of IL8. IL6 and C3a induced modest levels of IL8 production by LPS-primed BEAS-2B cells whereas only C3a induced low levels of IL8 in freshly isolated airway epithelial

cells. GM-CSF did not induce IL8 in either cell types. A combination of IL1 α with the other biomolecules increased IL8 production but the increase was less than additive suggesting that epithelial IL8 production perhaps reached a peak. The results provide a mechanistic understanding of induction of neutrophilic inflammation by IL6, C3a, and especially, by IL1 α . As mentioned previously, IL1 (both α and β) activates p-p38 MAPK as one of the downstream signaling pathways. To determine if this pathway was involved in IL8 production, we incubated freshly isolated human airway epithelial cells with the p38 MAPK inhibitor SB212190 and then stimulated with IL1 α . SB212190 significantly inhibited IL8 production (Figure 5E), although the inhibition was modest suggesting a role for other signaling pathways. Nonetheless, the result demonstrated a role for the p38 MAPK pathway in IL1 α activation of epithelial cells.

Role of infection in neutrophilic asthma

BAL fluid from all study groups including Th2-predominant, Th2/Th17-predominant, and Th2/Th17-low groups were cultured for microbial growth (bacteria, fungi, and mycobacteria). In addition, a sample of the BAL cells and bronchial brushing was analyzed for atypical infection (*Mycoplasma pneumonia* and *Chlamydia pneumonia*) and respiratory viruses by quantitative PCR. The foregoing tests were performed as a part of the clinical work-up for refractory asthma at National Jewish Health. We analyzed these data for our study population. None of the patients from the Th2-predominant and Th2/Th17-predominant groups was positive for microbial organisms. The patients from the Th2/Th17-low group, whose BAL neutrophil count was above 7%, showed a higher frequency of microbial presence in the BAL (Table 2). Five of 10 patients from this neutrophilic subgroup of Th2/Th17-low asthmatics had bacterial growth and one had a viral infection. None of them was positive for fungal, mycobacterial or atypical microorganisms.

It should be noted that none of patients, who were positive for bacterial growth, had current or recent symptoms of an acute infection (fever, leukocytosis, sore throat, coryza, or myalgia).

Clinical characteristics of neutrophilic and non-neutrophilic Th2/Th17-low asthma

In the previous publication we compared the clinical data of the three study groups (11). In this paper we compared the clinical data of neutrophilic and non-neutrophilic asthmatic patients belonging to the Th2/Th17-low group. We compared age, sex, body mass index, blood neutrophils, BAL and blood eosinophils, total IgE, allergy skin test for environmental allergens, FEV1(%), FEV1/FVC ratio, PC20 for methacholine, and asthma control test (ACT) score (Table 3). There was no significant difference in all these parameters between neutrophilic and non-neutrophilic Th2/Th17-low asthma patients. Both study groups were characterized by low levels of total IgE, relatively (compared to Th2-predominant and Th2/Th17-predominant, ref. 11) lower levels of skin test positivity and blood eosinophils. Both groups had baseline airway obstruction and low abnormal ACT score.

Discussion

In this study we investigated putative mechanisms of Th2/Th17-predominant and Th2/Th17-low asthma using BAL samples from a previously immunophenotyped cohort of asthmatic patients. The Th2/Th17-predominant asthma represented 26% of the entire cohort of refractory asthma patients (online repository figure E2). Patients with Th2/Th17-predominant asthma had elevated eosinophils and IgE, and advanced median age (11). This group is largely similar to what has previously been described as late onset eosinophilic asthma (online repository figure E3). Here we show that the Th2/Th17-predominant asthma was associated with an increased level of IL1 β , IL6, IL23, C3a and SAA in BAL. All these biomolecules are known to induce Th17 (9). IL1 β is considered one of the most critical Th17-inducing cytokines in humans (33). IL1 β , IL6 and IL23 have been shown to induce human Th2/Th17 cells in vitro (10), which has direct relevance for this paper. Of these three cytokines only IL1 β correlated with Th2/Th17 cells in the airways. Th2/Th17 cells expressed higher levels of the IL1R and phospho-p38 MAPK indirectly supporting a role for IL1 β . Finally, the blockade of the IL1R by anakinra (Kineret) inhibited the expression of dual IL4/IL17+ cells in BAL from asthmatic patients. The latter data suggests that IL1 β is likely to play a causative role in Th2/Th17 development and/or maintenance. The results also suggest that IL1 β inhibition using anakinra or similar drugs such as rilonacept and kanakinumab could benefit this subtype of asthma.

Previous studies showed that C3a induced IL1 β (34) and conversely, IL1 β induced C3a (35) suggesting the existence of a positive feedback circuit under certain circumstances. Variants of the C3 gene were associated with asthma in African Caribbean people (36). Its level was reported to be increased in asthma (37) but it was unclear if C3a favored one particular subtype of asthma. We found that the level of C3a was highest in Th2/Th17predominant asthma. There

was a strong correlation between C3a and IL1 β and a significant correlation between C3a and Th2/Th17. The results suggest that C3a might contribute to Th2/Th17-predominant asthma through the induction of IL1 β . IL6 was elevated in Th2/Th17-predominant asthma. However, it did not correlate with Th2/Th17 cells. IL6 signals through STAT3. We reported increased pSTAT3, which co-localized with pSTAT6 in Th2/Th17-predominant asthma patients. We recognize that pSTAT3 signaling is not specific for IL6. Nonetheless, its increased level raises the possibility that IL6 was involved in Th2/Th17 development.

We identified a distinct subgroup of Th2/Th17-low asthma, which was characterized by increased BAL neutrophils (online repository figure E2) and increased frequency of infection. This group likely represents the previously identified neutrophilic asthma (online repository figure E3) (4). Animal studies suggested that neutrophilic asthma was associated with a Th17 immune response (12, 13). Although we and many other groups reported increased IL17A in BAL from asthmatic patients (11, 38, 39), we were unable to detect increased Th17 cells in BAL (11). Instead, IL17A was produced by dual positive Th2/Th17 cells as well as other cells (e.g. $\gamma\delta$ T cells) in asthma. We anticipated that Th2/Th17-predominant asthma would be associated with neutrophilic asthma. Instead, it was Th2/Th17-low asthma that was associated with neutrophilic asthma. We provided a mechanistic understanding for this unexpected finding. We showed IL4 and IL13 inhibited the production of IL8, a critical mediator of neutrophilic asthma. Our result is in agreement with the recent reports on the inhibition of proneutrophilic chemokines (27) and growth factors (40) by type 2 cytokines.

We found a strong positive correlation of BAL IL8 with BAL neutrophils in Th2/Th17 low, neutrophilic asthmatic patients. Previous studies demonstrated an association of sputum IL8 with neutrophilic asthma (41-43). IL8 frequently functions downstream of other cytokines, e.g.

IL1, IL6 and GM-CSF. IL1 α , IL6 and, to a lower extent, GM-CSF positively correlated with BAL IL8 and neutrophils. Of these cytokines only IL1 α strongly induced IL8 production by epithelial cells. The results of this study and a number of previous studies (42, 44-46) suggest that IL1 α , a mucosal alarmin, plays a central role in orchestrating neutrophilic inflammation in asthma. This is similar to IL33, another alarmin, triggering an ILC2 and /or Th2 type inflammation (47). Both IL1 α and IL33 are produced under basal conditions by human epithelial cells, localized to the nucleus and released as a result of epithelial stress. Like IL33, IL1 α is also produced by hematopoietic cells under inflammatory conditions, which further amplifies inflammation. An unexpected finding is the lack of correlation of IL1 β with neutrophilic asthma. Our data suggest a differential regulation of neutrophilic asthma and Th2/Th17-predominant asthma by IL1 α and IL1 β , respectively.

In an effort to investigate the mechanism of neutrophilic asthma we examined microbial organisms in the airway fluid and cells. Our studies detected a frequent association of bacterial infection with neutrophilic asthma. We believe that these patients had localized subclinical infection. This is further supported by the fact that despite having elevated neutrophil counts in the BAL these patients did not have blood neutrophilia. These findings have implications not only for pathogenesis but also for management of asthma. It should be noted that the frequency of Th2/Th17-low, neutrophilic asthma is relatively low and represents 16.6% of the entire cohort. Subclinical infection was present in 55% of these patients, which represents 10% of the entire cohort.

Although prolonged antibiotic therapy has been tried in refractory asthma with variable results (48-50), these studies did not target neutrophil-high or IL8-high asthma patients. Our co-

investigators published a study on refractory asthma, where they identified five clinical phenotypes (14). One phenotype was termed subacute bacterial infection which represented 25 of the 58 subjects. A pathogen identification was needed for inclusion into this phenotype. The group markedly improved both lung function and asthma control with prolonged directed antimicrobial therapy. It is likely that prolonged antibiotic therapy will be specifically beneficial to neutrophil-high Th2/Th17-low asthmatic patients. This study also raises questions about subclinical infection in patients with elevated airway neutrophils and IL8, who do not show a positive culture in conventional microbiological approaches due to their low sensitivity. It would be interesting to determine if PCR or next-generation sequencing approaches would detect subclinical infection in many of these culture-negative patients. It is likely that mild neutrophilic inflammation of the airways is independent of infection and reflects chronic local and/or systemic inflammation. Significant correlation of multiple cytokines with BAL neutrophilia supports this notion. The subgroup of patients with BAL neutrophilia and without infection is likely to benefit from therapies targeting IL8, IL6 and IL1 α and their receptors.

We recognize limitations of this study. The low sample size creates a major problem when probing a highly heterogeneous disease such as asthma. The patient population at National Jewish Health is highly skewed towards refractory asthma. Thus, the frequency of neutrophilic asthma in our study population may not reflect that of a general population. We studied patients at a single time point of their chronic illness. Thus, the observed abnormalities may not be linked to the pathogenesis but to the concurrent environmental factors and/or infection. The patient population was of advanced age, which could secondarily affect the outcome due to chronic inflammation. We recognize that phenotypes change over age, which may reflect the changing nature of the underlying mechanism. There are significant differences in FEV1, PC20 for

methacholine, blood eosinophils, and medication taken among study groups as reported previously (11), which could have affected the study outcomes. Although we used the best available assays and reagents, their sensitivity and specificity are far from being ideal. We recognize these limitations. Nonetheless, we believe that the results of this study shed new light on the mechanism of two immune endotypes of asthma—Th2/Th17-predominant asthma and neutrophilic, Th2/Th17-low asthma.

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Figures legends

Figure 1. Expression of Th2/Th17-inducing cytokines in BAL from three immunophenotypes of asthma. BAL fluid from Th2-predominant (N=22), Th2/Th17-predominant (N=16) and Th2/Th17-low (N=22) asthmatic patients were assayed for IL1 α (A), IL1 β (B), IL6 (C) and IL23 (D) by ELISA. Each symbol represents a single patient. P values are shown above the graphs.

Figure 2. DAMPs in three immunophenotypes of asthma. The levels of C3a (A), serum amyloid A (B) and uric acid (C) in BAL were measured in Th2-predominant (N=22), Th2/Th17-predominant (N=16) and Th2/Th17-low (N=22) asthma. Each symbol represents a single patient. P values are shown above the graphs.

Figure 3. A: Correlation of cytokines and DAMPs with Th2/Th17 cells. **B:** Correlation of IL1 β with Th2/Th17. **C:** Correlation of IL1 β with C3a. **D:** Expression of IL1 receptor (IL1R) and phosphor-p38 MAPK in IL4⁺ (Th2), IL17⁺ (Th17), IL4/IL17⁺ (Th2/Th17) and total cells. BAL cells from asthmatic patients were analyzed by flow cytometry for expression of CD4, and intracellular IL4, IL17, IL1R and p-p38 MAPK. *P<0.04, N=6. **E:** Inhibition of Th2/Th17 cells by anakinra. BAL cells were cultured with increasing concentrations of anakinra for 3 days and then analyzed for dual IL4/IL17⁺ CD4 T cells by flow cytometry. Each symbol represents an asthmatic patient. P value (on the top of the graph) was calculated by Kruskal Wallis test. **F:** Inhibition of p-p38 MAPK in BAL T cells by anakinra. BAL cells were cultured with anakinra for 24 hours and then examined for expression of p-p38 MAPK in CD4 T cells by flow cytometry. Each symbol represents an asthmatic patient. P value was calculated by Kruskal Wallis test.

Figure 4. BAL neutrophils in various immunophenotypes of asthma. **A:** BAL neutrophils in Th2-predominant (N=22), Th2/Th17-predominant (N=16) and Th2/Th17-low (N=22) asthma. Flow cytometric detection of IL4⁺ and IL4/IL17 dual positive BAL CD4 T cells was performed and the immunophenotype was assigned as reported in ref. 11. **B & C:** BAL myeloperoxidase (B) and IL-8 (C) in Th2-predominant, Th2/Th17-predominant and Th2/Th17 -low asthma. **D:** Correlation of BAL neutrophils and IL8 in Th2/Th17-low asthmatic patients. **E:** Effect of type 2 cytokines on IL8 production. Airway epithelial cells BEAS-2B were cultured with increasing concentration of IL4 and IL13 for 3 days and the culture supernatant was assayed for IL8 by ELISA (**P<0.05, N=4). **F:** Correlation between IL4 and IL8 in BAL from all 3 study groups (N=42).

Figure 5. **A-C:** Comparison of the levels of IL17A (A), GM-CSF (B) and G-CSF (C) in BAL from Th2-predominant, Th2/Th17-predominant and Th2/Th17-low asthmatic patients. **D:** Effect of cytokines/mediators on epithelial IL8 production. Freshly isolated human bronchial epithelial cells (HBE) and BEAS-2B cells were cultured with various cytokines/mediators (10 ng/ml) singly or in combination for 3 days. The BEAS-2B cell cultures were primed with a subthreshold dose of LPS (0.25 µg/ml). Culture supernatant was assayed for IL8 by ELISA. *P<0.05 (N=4). **E:** Freshly isolated human bronchial epithelial cells were cultured with DMSO or SB212190 (10 µM) in the presence of IL1α (10 ng/ml) for 3 days. The culture supernatant was assayed for IL8 by ELISA. *P<0.05 (N=3).

568 **Online repository figure E1:** Representative flow cytograms from Th2-predmonant, Th2/Th17-
569 predominant and Th2/Th17-low asthma. BAL cells were gated for CD4+ cells and analyzed for
570 expression of IL4 and IL17.

571 **Online repository figure 2:** Distribution of immune endotypes and neutrophilic asthma in a
572 cohort of 60 refractory asthma patients.

573 **Online repository figure 3:** The approximate relationship between the immune endotypes and
574 the conventional phenotypes of asthma.

Table 1. Correlation of BAL neutrophils and IL8 with inflammatory mediators

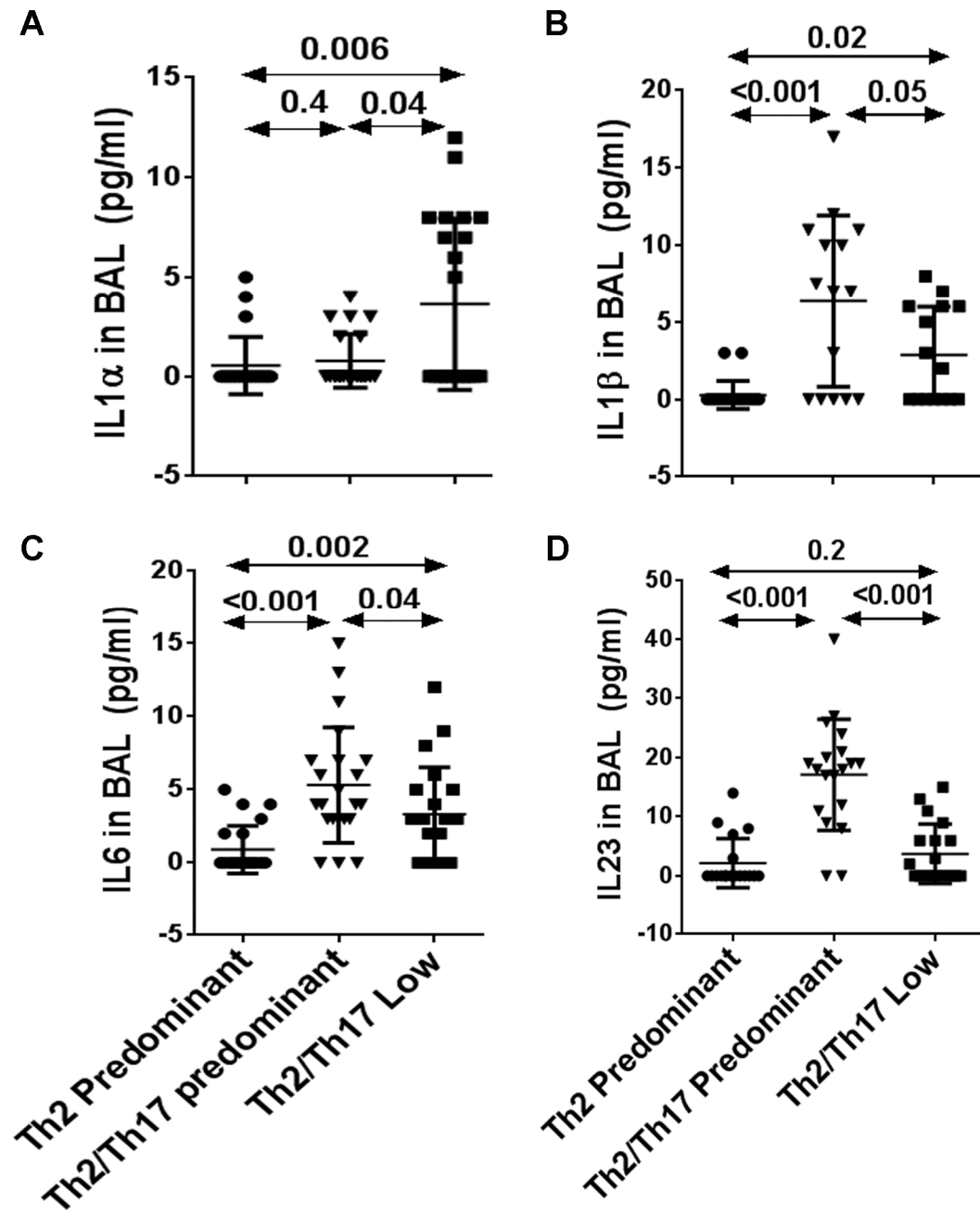
Variables in BAL	Correlation (r) with IL8	P	Correlation (r) with BAL neutrophils	P
IL1a	0.84	<0.0001	0.70	<0.0001
IL1b	0.07	NS	0.12	NS
IL6	0.68	<0.001	0.70	<0.0001
IL17A	-0.07	NS	-0.09	NS
G-CSF	0.43	0.02	0.75	0.001
GM-CSF	0.70	<0.001	0.56	0.001
C3a	0.36	0.03	0.48	0.007
Serum amyloid A	-0.14	NS	-0.15	NS
Uric acid	0.03	NS	-0.12	NS

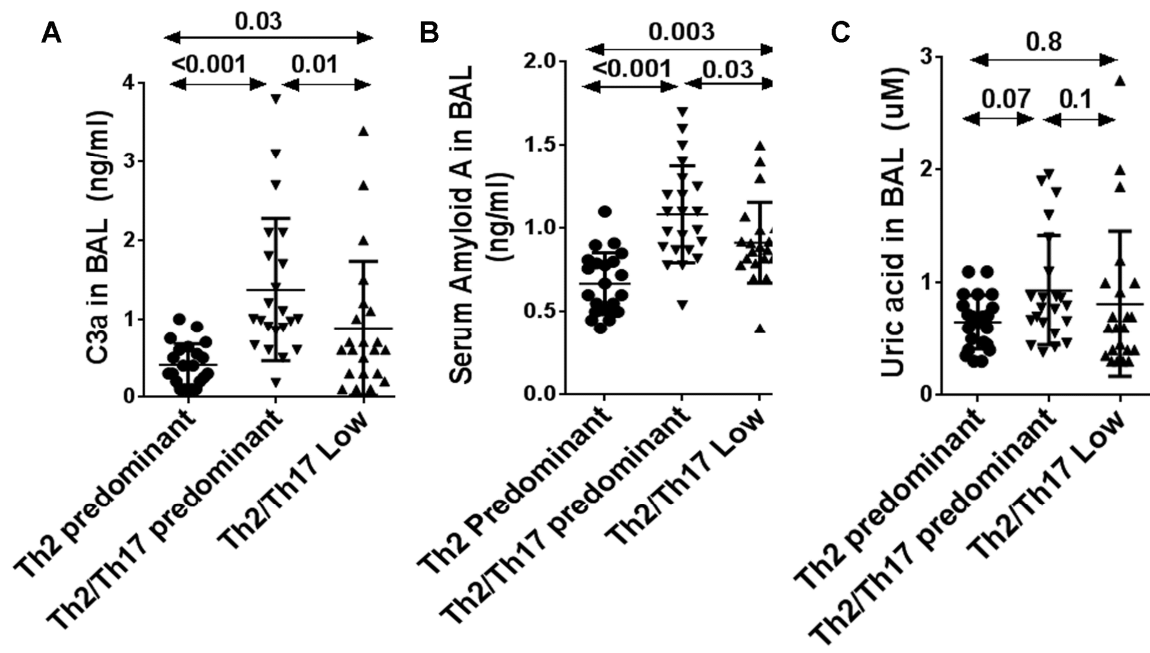
Table 2. Infection in Neutrophilic Asthma

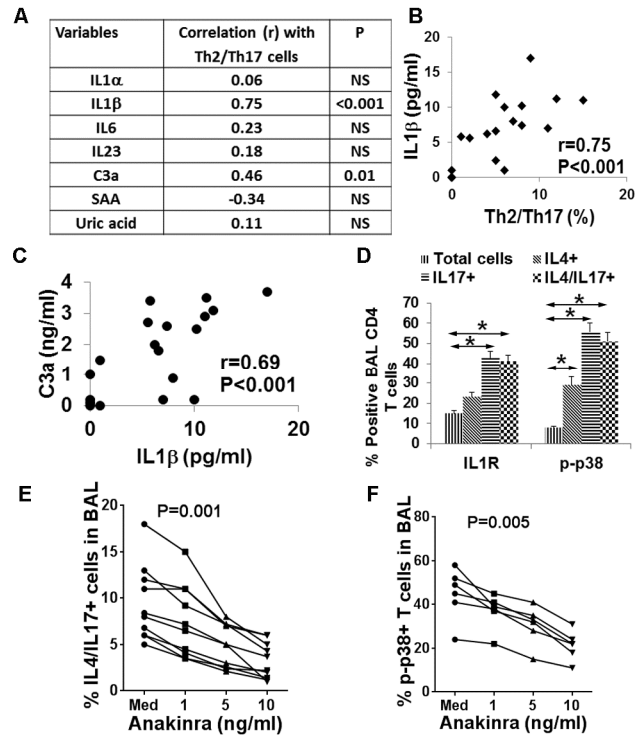
Patient #	BAL PMN (%)	BAL IL8 (pg/ml)	Mycoplasma, Chlamydophila PCR	12 virus panel	Bacterial culture	Fungal culture	Myco-bacteria culture
1	10	200	-	-	-	-	-
2	56	141	-	Rhinovirus	-	-	-
3	11	295	-	-	-	-	-
4	7	160	-	-	Pseudomonas Aeruginosa	-	-
5	84	2166	-	-	Hemophilus influenza	-	-
6	36	801	-	-	-	-	-
7	54	1790	-	-	Hemophilus influenza	-	-
8	11	430	-	-	Staph. Aureus	-	-
9	40	781	-	-	Staph. Aureus	-	-
10	31	566	-	-	-	-	-

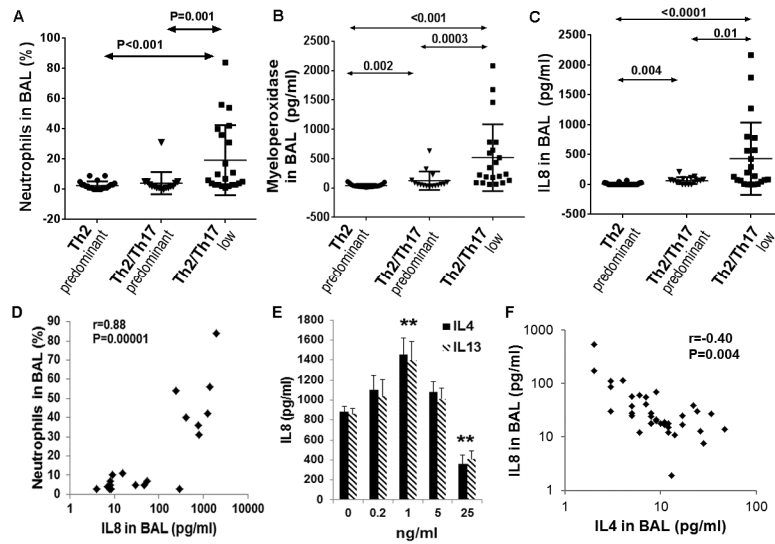
Table 3. Clinical Characteristics of Neutrophilic and Non-neutrophilic Th2/Th17-low Asthma

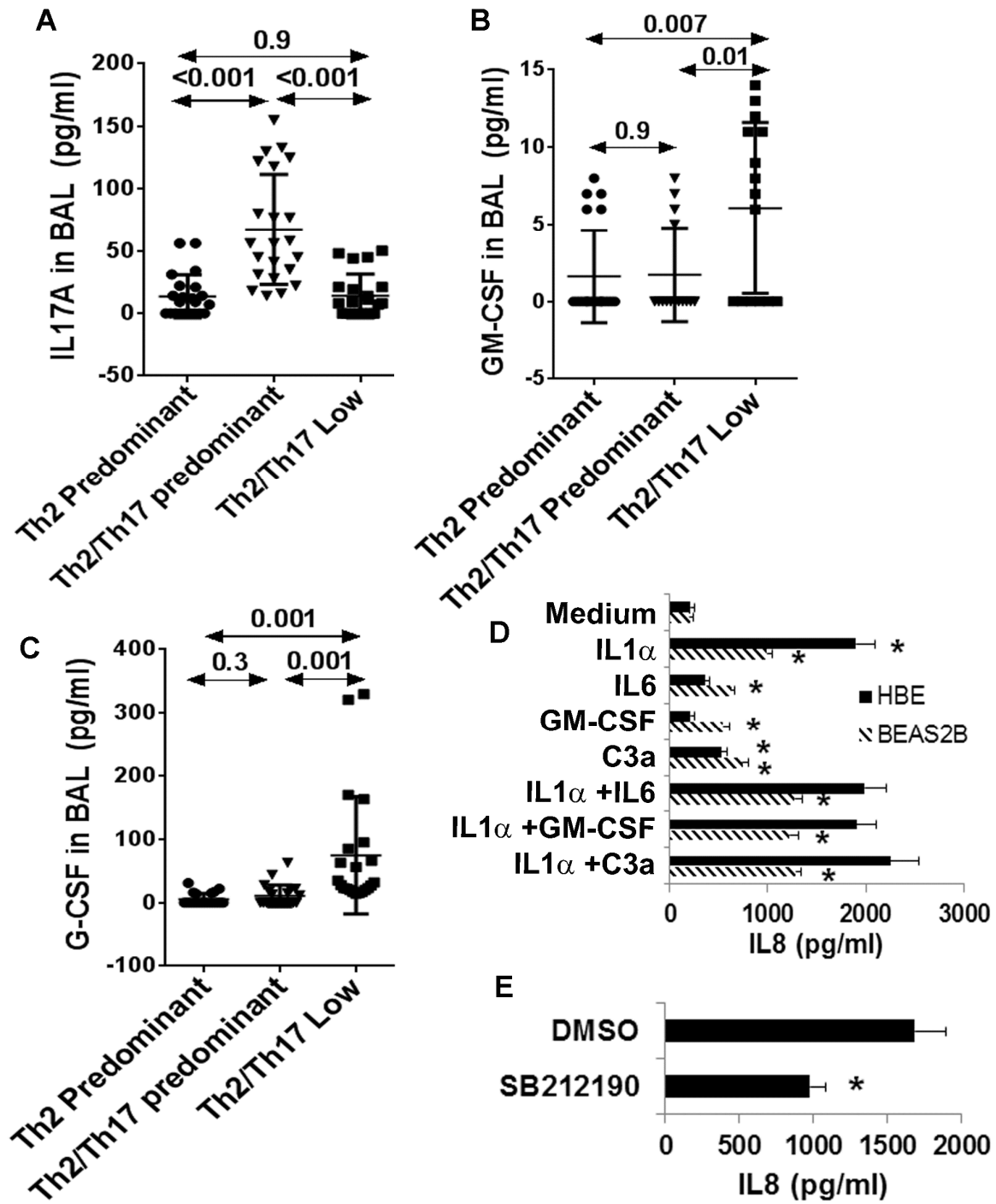
	Neutrophilic Asthma (N=10)	Non- neutrophilic Asthma (N=12)	
Variables	Median	Median	P value
BAL neutrophils (%)	33.5	3.5	<0.0001
F/M	6/4	8/4	NS
Age	52	54	0.9
BMI	27	25.5	0.9
Blood Neutrophils (per $\square$ L)	5.7	4.3	0.8
BAL eosinophils (%)	0	0	0.3
Blood eosinophils (per $\square$ L)	100	100	0.5
Total IgE	23.5	32.5	0.9
Skin test/RAST positive (%)	44%	44%	NS
FEV1 (%)	71.5	74	0.3
FEV1/FVC	67	74	0.3
PC20 for methacholine (mg/ml)	2.1	0.8	0.4
ACT score	13.5	16	0.5

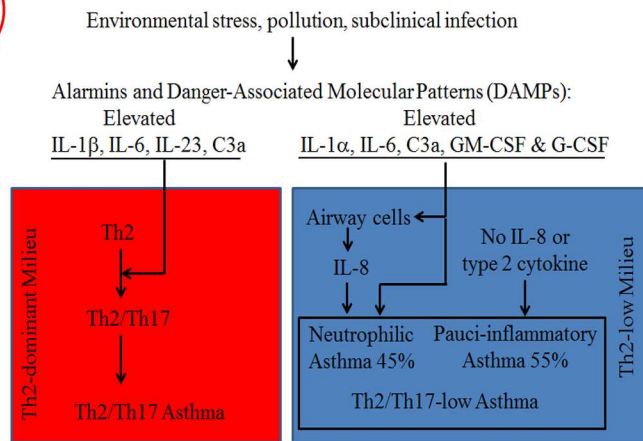












Online Repository Table 1**Persistence of Th2/Th17-predominant endotype**

Patient Number	Th2/Th17 cell (%)	
	First bronchoscopy & BAL	Repeat bronchoscopy & BAL (3-5 years later)
1	27%	17%
2	12%	18%
3	9%	6%
4	14%	9%

