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Th-17 regulatory cytokines IL-21, IL-23, and IL-6 enhance neutrophil production of IL-17 cytokines during asthma

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ABSTRACT

Background: In a subset of severe asthma patients, chronic airway inflammation is associated with infiltration of neutrophils, Th-17 cells and elevated expression of Th-17-derived cytokines (e.g., interleukin [IL]-17, IL-21, IL-22). Peripheral neutrophils from allergic asthmatics are known to express higher IL-17 cytokine levels than those from healthy subjects, but the regulatory mechanisms involved are not well understood. We hypothesize that Th-17 regulatory cytokines could modulate IL-17 expression in neutrophils. **Methods:** Peripheral blood neutrophils isolated from asthmatics were stimulated with IL-21, IL-23, and IL-6 cytokines and their ability to produce IL-17A and IL-17F was determined relative to healthy controls. Signal transducer and activator of transcription 3 (STAT3) phosphorylation levels were measured in stimulated neutrophil using flow cytometry. The requirement for STAT3 phosphorylation was determined by blocking its activation using a specific chemical inhibitor. **Results:** Stimulating asthmatic neutrophils with IL-21, 23, and 6 enhanced the production of IL-17A and IL-17F at significantly higher levels comparatively to healthy controls. Stimulating neutrophils with IL-21, IL-23, and IL-6 cytokines enhanced STAT3 phosphorylation, in all cases. Interestingly, inhibiting STAT3 phosphorylation using a specific chemical inhibitor dramatically blocked the ability of neutrophils to produce IL-17, demonstrating that STAT3 activation is the major factor mediating IL-17 gene expression. **Conclusions:** These findings suggest that neutrophil infiltration in lungs of severe asthmatics may represent an important source of pro-inflammatory IL-17A and -F cytokines, a production enhanced by Th-17 regulatory cytokines, and thus providing a feedback mechanism that sustains inflammation. Our results suggest that STAT3 pathway could be a potential target for regulating neutrophilic inflammation during severe asthma.

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Introduction

Studies from the past decade have confirmed the importance of neutrophils and Th-17 cells in the pathogenesis of severe asthma with persistent inflammation [1–4]. Asthma phenotypes can be classified into eosinophilic, neutrophilic, mixed (eosinophils and neutrophils), and paucigranulocytic (normal-like) based on sputum analysis [5]. Whereas the airway tissues of mild asthmatics are usually characterized by eosinophilic infiltration and Th-2 cytokine profile, the lung tissues of asthmatics with severe and persistent symptoms show a preferential infiltration of neutrophils with Th-17 cells and elevated levels of interleukin (IL)-4, IL-6, IL-8, IL-17, IL-21, IL-23, and transforming growth factor beta (TGF- β) cytokines [6–10]. In these patients, allergic sensitization can provoke a strong Th-17 inflammatory response with

airway neutrophilic infiltration and acute airway hyper-responsiveness [7,11–13]. Neutrophils are also associated with the corticosteroid-resistant asthma phenotype; in contrast to eosinophils, neutrophils are seemingly resistant to steroid-induced apoptosis, which correlates with the decreased response to therapy among severe asthmatics [14]; however, other studies have contradicted this view [15]. In fact, multiple molecular mechanisms have been proposed to explain steroid insensitivity, yet it remains a difficult clinical challenge up to date [16,17].

In general, neutrophils play a primary phagocytic defensive role, by rapidly moving into sites of infection where they engulf and destroy invading micro-organisms [18]. However, in the lung tissues of asthmatics, these cells along with Th-17 lymphocytes contribute to the persistent inflammation status by releasing mediator substances,

which directly or indirectly stimulate both proliferation and migration of smooth muscle cells of the airways, causing them to become narrow and thus breathing becomes difficult [19–21]. Therefore, there is continuing interest in elucidating the role of neutrophils in persistent airway inflammation of severe asthmatics and its complex interactions with neighboring cells. Importantly, evidence indicates that neutrophils do exchange directly and indirectly, biochemical signals with Th-17 cells, which characteristically release IL-17A, IL-17F, and IL-21 cytokines among others [22,23]. On one hand, Th-17 cells can indirectly induce neutrophil recruitment into the inflamed lung tissues via IL-17A and IL-17F, which stimulate airway epithelial cells to produce CXCL8 (IL-8), one of many neutrophil chemoattractants [7,24]. Th-17 cells also can induce a chemotactic response of neutrophils via STAT3-driven expression and release of cytokines other than IL-17 [25]. For instance, Th-17-derived IL-21 binds to IL-21Ralpha/common gamma chain heterodimeric receptors in macrophages and prompts them to produce and secrete CXCL8 chemokine [26]. However, it has also been reported that human peripheral neutrophils do express IL-21Ralpha receptors, and therefore, IL-21 can directly activate these cells [27]. IL-21, along with IL-6 and IL-23 cytokines, is important for the promotion of naïve CD4+ cells to differentiate toward the Th-17 cell lineage, and is itself a potent STAT3 activator [28].

On the other hand, as a feedback mechanism, activated airway neutrophils may induce a chemotactic infiltration of peripheral Th-17 cells via the production of CCL2 and CCL20 chemokines [29]. In the context of fungal infection, expression of IL-17 in bone-marrow neutrophils is dependent on IL-6 and IL-23 stimulation, which then functions in an autocrine fashion to maintain neutrophils in an activated state [30]. Hence, IL-21, and IL-23 are now considered as central signaling mediators in various inflammation contexts, including asthma [8,10], allergic dermatitis [31], liver injury induced by ischemia reperfusion [32], rheumatoid arthritis [33], and systemic lupus erythematosus [34], to mention just a few [23,25].

In vitro experiments have shown that neutrophils from asthmatic patients respond differently to stimulants in relation to those from non-asthmatic subjects, suggesting that physiological and/or biochemical changes have occurred in the diseased cells, perhaps as a result of repetitive stimulation by allergens or other inflammatory mediators during exacerbations, as observed for structural cells and peripheral leukocytes [35–38]. Therefore, since Th-17 regulatory cytokines, IL-21, IL-23, and IL-6, are known to be upregulated in asthma, we hypothesized that these cytokines may activate peripheral neutrophils in asthmatic patients to produce IL-17A and IL-17F cytokines. We also postulated that the response to stimulation could

be different to that of neutrophils from non-asthmatic subjects.

Materials and methods

Study subjects

A total of 28 asthmatic patients (14 males and 14 females, mean age: 32.5 ± 4.3 years) and 28 normal healthy subjects (14 males and 14 females, mean age: 34.1 ± 5.4 years) were included in the study. All patients were diagnosed with severe asthma as defined by the American Thoracic Society (ATS) [39] with high peripheral blood neutrophil count ($>65\%$). Patients were receiving high-dose inhaled corticosteroids or daily anti-leukotriene for $>50\%$ of the last year, and at least 1 other add-on therapy on a daily basis for the previous year. In addition, severe asthma patients were required to meet two of the following criteria: daily short-acting β -agonist, or persistent Forced expiratory volume (FEV1) $<60\%$ and FEV1/FVC (Forced vital capacity) $<75\%$ predicted, or 1 urgent visit, or at least 3 steroid bursts in the previous year, or prompt deterioration with $<25\%$ steroid dose reduction, or previous near-fatal asthma within the last 3 years. A summary of subjects' and patients' clinical data is presented in Table 1. Patients taking Omalizumab, those with smoking history or any other chronic pulmonary or other diseases, and/or having co-existing medical conditions, including gastroesophageal reflux disease (GERD), or obese patients (BMI above 30), were excluded from the study. The control subjects were required to be non-smokers, with normal lung function test, with no history of allergy or chronic respiratory/other illnesses and not taking any medications for the past 4 weeks. All recruited patients and subjects signed a written informed consent. This study was reviewed and approved by the Institutional Ethics Committee of King Khalid University Hospital, Riyadh, KSA.

Table 1. Principal demographic and clinical characteristics of patients and subjects.

	Asthmatics	Healthy controls
Age (years)	32.5 ± 4.3	34.1 ± 5.4
Males/females	14/14	14/14
Atopy (% of total)	100%	0
Duration of disease (years)	18.5 ± 7.6	n/a
FEV1 (mean ± SD)	56.53 ± 3.16	101.5 ± 4.36
FVC (mean ± SD)	74.54 ± 2.98	105.7 ± 5.77
FEV/FVC (mean ± SD)	67.28 ± 4.96	84 ± 3.81
Medications	-Inhaled corticosteroids: budesonide/formoterol (160/4.5 µg) (1–2 inhalations twice daily) -Anti-leukotriene: montelukast sodium (10 mg/d) -Albuterol (as needed)	No

Isolation of neutrophils

Peripheral blood samples (volume = 40 ml/subject) were collected in heparinized vials and peripheral blood neutrophils were isolated using density gradient separation method as previously described [40]. Briefly, equal volume of peripheral blood was layered on sodium metrizoate/Dextran 500 gradient mixture and centrifuged at 500 relative centrifugal force (RCF) for 35 minutes at 20–25°C. The polymorphonuclear layer, rich with neutrophils and eosinophils, was then collected. Cells were then stained with PE-conjugated antibodies for: CD3, CD4, CD8, CD14, CD20, and Seglic-8 (R&D Systems) to form a dump channel used to exclude eosinophils (Seglic-8⁺) and residual monocytes and lymphocytes. Neutrophils, the cell population negative for Phycoerythrin (PE) signal, were then sorted out using a cell sorter (Astraeus II, Beckman Coulter, USA) and suspended in Hanks' Balanced Salt Solution (HBSS) without Ca²⁺/Mg²⁺ for further use. Neutrophil purity was more than 95% as assessed by flow cytometry (LSRII, Becton Dickinson, USA) using neutrophil-specific markers CD66b and CD16 (R&D Systems).

Purified cells contained <0.5% monocytes/lymphocytes and no eosinophil contaminations as determined by Cytospin analysis. The expression of CD3, CD4, CD8, and CD161 mRNA was also determined using quantitative reverse transcription polymerase chain reaction (RT-PCR). No expression of CD161 mRNA was observed indicating the absence of Th-17 cells from the isolates. Neutrophil viability was >97% as determined by trypan blue exclusion test. Neutrophils were then cultured in complete Roswell Park Memorial Institute medium (RPMI)-1640 media (10% Fetal Calf Serum [FCS]), and used immediately.

RT-PCR assay

Neutrophils (300,000 cells per treatment) were stimulated, or not, with IL-21, IL-23, IL-21 + IL23, or IL-6 cytokines (20 ng/ml) for 4 hours. RNA was extracted using the RNA isolation kit (RNeasy Mini kit, Qiagen, CA, USA). The RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Maddison, WI, USA). cDNA was then amplified by real-time quantitative PCR (AB H7900 Fast R-T-PCR, Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. A SYBR Green Master PCR mix (Applied Biosystems) was employed to amplify IL-17A, IL-17F, IL-21R, IL23R, and GAPDH using specific cDNA primers (Integrated DNA Technologies, Leuven, Belgium) as previously described [41]. Triplicate measurement was conducted for each

patient. The quantification of gene expression relative to GAPDH and the fold changes in expression was determined using the RQ Manager Software (Applied Biosystems).

Flow cytometry analyses

Neutrophils obtained as described above were stimulated (300,000 cells per treatment) with cytokines IL-21, IL-23, IL-21 + IL23, and or IL-6 (20 ng/ml) for 12 hours. Cells were treated with Brefeldin A for the last 4 hours to enhance the detection of intracellular cytokines. Cells viability was >75% at 12 hours. Cells were stained with CD66b-PE (R&D Systems) for 15 minutes. They were then fixed with 4% PFA for 15 minutes, permeabilized with 0.25% Saponin for 1 hour, and stained for 30 minutes in dark with fluorophore-conjugated anti-IL-17A-APC (R&D Systems), anti-IL-17F-FITC, and anti-IgG isotype antibodies (R&D Systems), following standard techniques. Triplicate measurement was conducted for each patient. Cells were analyzed using LSR II flow cytometer and DIVA software (BD Biosciences, CA, USA).

Phosflow assay

A phosflow assay was performed to determine the percentage of tyrosine-phosphorylated STAT3 (pSTAT3) neutrophils. Briefly, neutrophils (3×10^6 cells) were starved for 2 hours in RPMI-1640 medium supplemented with 0.5% fetal bovine serum and then stimulated or not with IL-21 and IL-23 cytokines (20 ng/ml) in combination, in the absence or presence of Sc-202818 inhibitor (2.5 μ M), for 15 minutes at 37°C. Sc-202818 is a specific STAT3 inhibitor confirmed previously to efficiently inhibit the tyrosine-phosphorylation of STAT3 [42]. Cells were stained with CD66b-APC (R&D Systems) for 15 minutes. The cells were then fixed (4% PFA) for 15 minutes, permeabilized (methanol at –20°C) for 1 hour, and stained with PE-conjugated anti-STAT3 (directed to phospho-tyrosine Y705 residue) antibody; cells positive for pSTAT3 were quantified by flow cytometry. Triplicate measurement was conducted for each patient.

In another set of experiments, we tested whether pSTAT3 is required for IL-17 production. Neutrophils were stimulated as described above with IL-21 or IL-23 cytokines, in the presence or absence of Sc-202818 (2.5 μ M). After incubation for 12 hours, Brefeldin A was added to the cells and incubation was continued for one additional hour. Cells were stained with anti-IL-17A and -IL-17F antibodies as described above and quantified with BD LSR II flow cytometer and DIVA software.

Western blot analyses

Neutrophils (3×10^6 cells), starved for 2 hours in 0.5% FBS medium, were stimulated with IL-21, IL-23, IL-21+IL23, or IL-6 (20 ng/mL) for 18 hours. Cells viability was >70% at 18 hours. Total proteins were extracted using lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) containing protease and phosphatase inhibitors (Roche, Mannheim, Germany). Protein lysates (20 μ g) were resolved on 10% acrylamide Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels and transferred to Polyvinylidene difluoride (PVDF) membrane and blots were probed sequentially with anti-Actin and anti-IL-17A primary antibody (0.5 μ g/ml, R&D Systems) and IRDye-conjugated IgG secondary antibody. Signals were analyzed with an Odyssey CLx scanner using Odyssey imaging software 3.0 (Li-COR Biosciences, Inc).

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Expression of IL-17 cytokines was evaluated using Analysis of Variance (ANOVA) followed by Bonferroni–Dunn *post hoc* test. Two-way ANOVA was done to determine if significant differences existed in the means between the normal and severe asthmatic. Non-parametric Mann–Whitney *U* test (Systat, version 7.0, SPSS, Chicago, IL) was used to evaluate significance in differential phosphorylation of

STAT3. Values of $p < 0.05$ were considered statistically significant.

Results

Th-17 regulatory cytokines, IL-21 and IL-23, induce IL-17A and -F expression in neutrophils

IL-17A and IL-17F levels have been shown to increase in the lung tissue of some severe asthmatic patients [43]; we hypothesized that infiltrated neutrophils could be a major source of these cytokines, in response to stimulation by Th-17-regulatory IL-21 and IL23 cytokines. Therefore, we tested in vitro whether IL-17A and -F mRNA expression is induced in peripheral neutrophils in response to IL-21 and IL-23 stimulation, and whether this stimulatory response would differ between neutrophils isolated from severe asthmatics and from healthy subjects.

Peripheral blood neutrophils were isolated using metrizoate/Dextran density gradient separation method [40], which separates the polymorphonuclear cells into a separate layer. This cellular layer, rich with neutrophils and eosinophils, was then collected. Cells were stained with PE-conjugated antibodies for: CD3, CD4, CD8, CD14, CD20, and Seglic-8 to form a dump channel used to exclude eosinophils (Seglic-8⁺) and residual monocytes and lymphocytes (Figure 1A). Neutrophils, the cell population negative for PE signal, were then sorted out using a cell sorter and suspended in HBSS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ for further use. Neutrophil purity was more

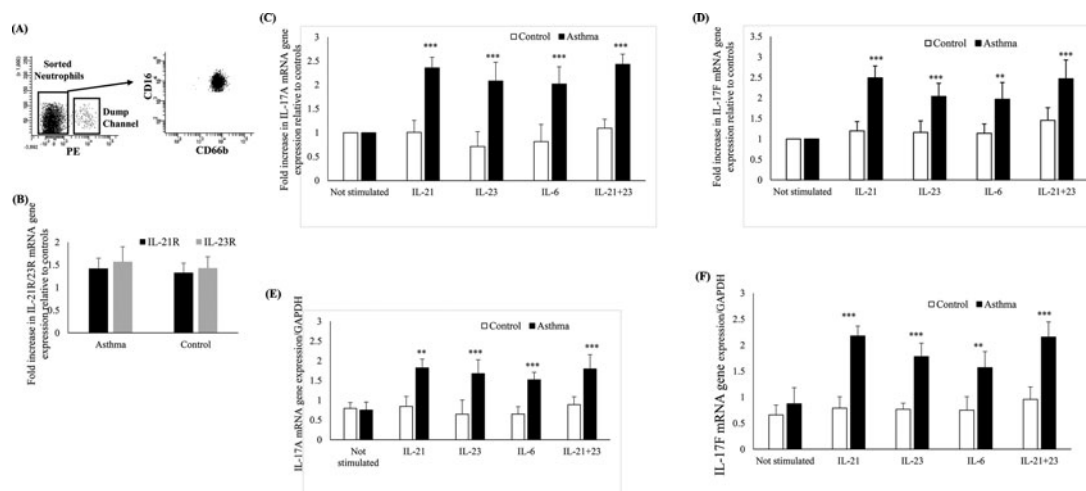


Figure 1. Gene expression of IL-17A and IL-17F in neutrophils obtained from asthmatic and normal healthy subjects. (A) Neutrophil isolation strategy from polymorphonuclear layer separated with a gradient, (B) Gene expression of IL-21R and IL23R in neutrophils isolated from healthy and asthmatic subjects. Following stimulation with Th-17 cytokines, (C) fold increase of IL-17A gene expression relative to non-stimulated baseline. (D) Fold increase of IL-17F gene expression relative to baseline. (E) IL-17A mRNA expression relative to GAPDH. (F) IL-17F mRNA expression relative to GAPDH. All assessments were performed following stimulation with IL-21, IL-23, IL-21 + IL-23, and IL-6 after 4 hours of stimulation. Data are expressed as means $\pm$ SEM ($n = 28$ for each asthmatic and controls). ** $p \leq 0.01$ stimulated vs non-stimulated cells; *** $p \leq 0.001$ stimulated vs non-stimulated cells.

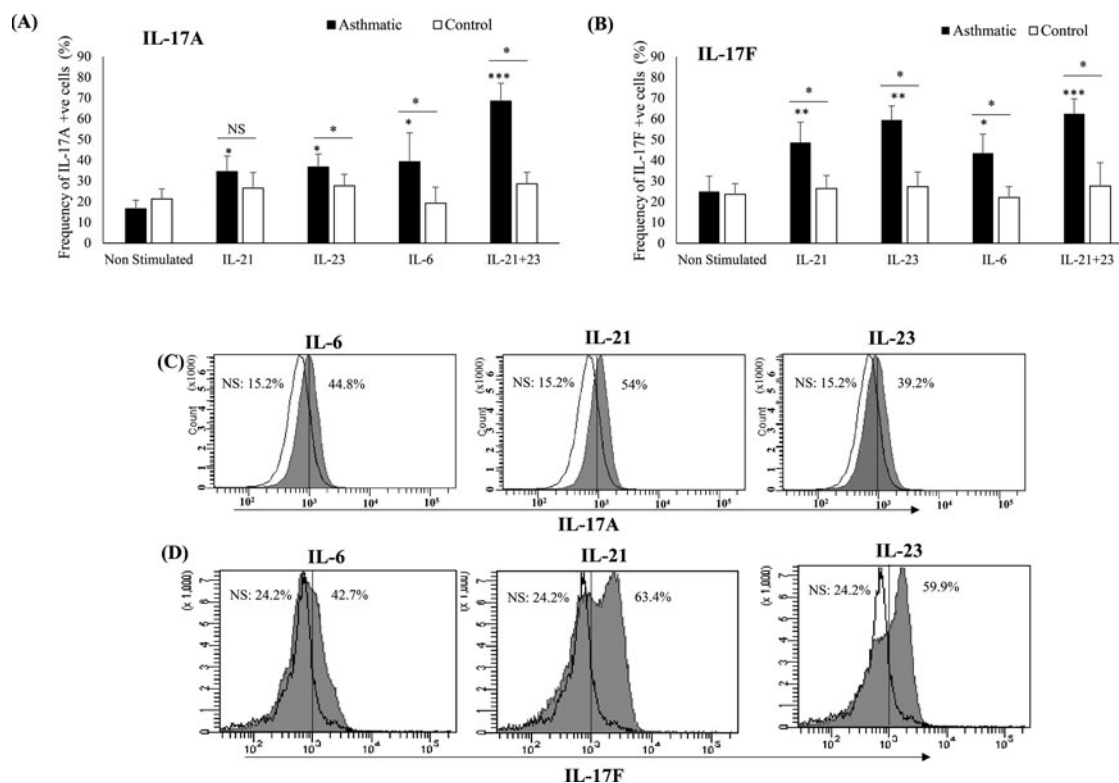


Figure 2. Frequency of asthmatic and healthy neutrophils expressing IL-17A and IL-17F following stimulation with IL-21, IL-23, IL-21 + IL-23, and IL-6 cytokines. Neutrophils isolated from both asthmatic and healthy subjects were stimulated for 12 hours with IL-21, IL-23, IL-6, and IL-21 + IL-23 cytokines and stained with anti-IL-17A-APC and anti-IL-17F-FITC conjugated antibodies. (A) The frequency of IL-17A positive asthmatic and healthy neutrophils following cytokine stimulation. (B) The frequency of IL-17F positive asthmatic and healthy neutrophils following cytokine stimulation. (C) Representative data for stimulated neutrophils expressing IL-17A. (D) Representative data for stimulated neutrophils expressing IL-17F. Data are expressed as means $\pm$ SEM ($n = 28$ for each asthmatics and controls). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

than 95% as assessed by flow cytometry using neutrophil-specific markers CD66b and CD16 (Figure 1A).

First, we measured the level of expression of IL-21R and 23R on peripheral blood neutrophils isolated from healthy and asthmatic subjects. As shown in Figure 1B, neutrophils isolated from both healthy and asthmatic patients express comparable levels of IL-21 and IL-23 receptors. Following stimulation of isolated neutrophils with IL-21 and 23 regulatory cytokines, a significant increase in IL-17A and IL-17F mRNA expression was evident in neutrophils isolated from asthmatic subjects whereas in cells from healthy subjects, these stimulations had no significant effect (Figure 1C and D). Stimulation with higher cytokine concentrations (up to 100 ng/ml) had similar effect on asthmatic and control neutrophils (data not shown). Approximately 2–2.2 fold increase in IL-17A expression was observed in asthmatic subjects, relative to the corresponding non-stimulated control, with IL-21 (2.35 fold, $p \leq 0.001$) and IL-23 (2.08 fold, $p \leq 0.001$) as well as in combination (IL-21 + IL-23: 2.43 fold, $p \leq 0.001$) (Figure 1C). IL-21 and IL-23 cytokines also induced a 2.0–2.5 fold increase in IL-17F expression relative to non-stimulated control in asthmatic subjects

(IL-21: 2.49 fold, $p \leq 0.001$; IL-23: 2.04 fold, $p \leq 0.001$; IL-21 + IL-23: 2.46 fold, $p \leq 0.001$) (Figure 1D). The positive control, IL-6, also significantly induced IL-17A and IL-17F mRNA expression from asthmatic neutrophils (Figure 1C and D). Figure 1E and F compare IL-17A and -F mRNA induced expression between neutrophils from severe asthmatics and those from healthy subjects, respectively. IL-17A-induced expression in neutrophils from asthmatics was significantly greater than that of healthy controls when stimulated with IL-21, IL-23, or in combination. Similarly, all stimulations enhanced significantly IL-17F expression in neutrophils from asthmatics in relation to healthy controls.

We also quantified the percent of neutrophils expressing both IL-17A and IL-17F upon cytokine stimulations by flow cytometry (Figure 2A–D). Contrary to neutrophils isolated from healthy subjects, asthmatic neutrophils responded to stimulation with Th-17 regulatory cytokines with a significant increase in IL-17 cytokine production. The frequency of both IL-17A and IL-17F positive cells was significantly elevated upon stimulating asthmatic neutrophils with IL-21 (IL-17A: 34.5% vs 16.5% [$p = 0.05$]; IL-17F: 48.6% vs 24.9% [$p = 0.01$]), IL-23

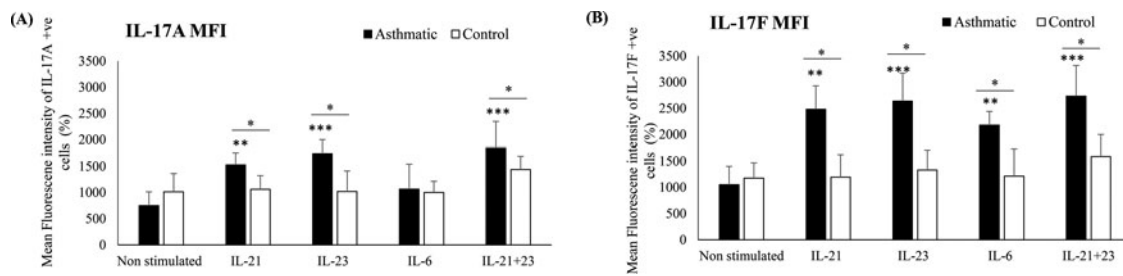


Figure 3. MFI of IL-17A and IL-17F within neutrophils stimulated with IL-21, IL-23, IL-21 + IL-23, and IL-6 cytokines. (A) MFI of IL-17A within asthmatic and healthy neutrophils following cytokine stimulation. (B) MFI of IL-17F within asthmatic and healthy neutrophils following cytokine stimulation. Data are expressed as means $\pm$ SEM ($n = 28$ for each asthmatics and controls). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

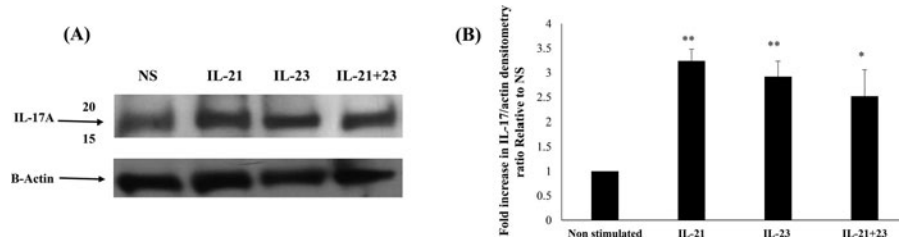


Figure 4. Western analysis of asthmatic neutrophil derived IL-17 proteins expression following stimulation with IL-21, IL-23, and IL-21 + IL-23 cytokines (A) Western blotting (B) Fold increase in IL-17/Actin densitometry ratio relative to non-stimulated. Data are expressed as means $\pm$ SEM ($n = 10$). * $p < 0.05$ vs non-stimulated cells, ** $p \leq 0.01$ vs non-stimulated cells.

(IL-17A: 36.7% vs 16.5% [$p = 0.05$]); IL-17F: 59.5% vs 24.9% [$p = 0.008$]; and IL-6 (IL-17A: 39.2% vs 16.5% [$p = 0.04$]; IL-17F: 43.5% vs 24.9% [$p = 0.04$]) relative to non-stimulated cells (Figure 2A and B). Stimulating neutrophils with a combination of cytokines resulted in the highest expression frequency for both IL-17A (68.5% vs 16.5%, $p = 0.003$) and IL-17F (62.4% vs 24.9%, $p = 0.003$) (Figure 2A and B). Neutrophils isolated from healthy controls had no significant elevations of IL-17 positive cells following stimulation with any of the Th-17 regulatory cytokines (Figure 2A and B). In fact, the response of asthmatic neutrophils to cytokine stimulation was significantly higher than that of healthy neutrophils in most of the conditions as indicated in Figure 2A and B. Representative data for IL-17A and IL-17F expression in asthmatic neutrophils is shown in Figure 2C and D, respectively.

Furthermore, the mean fluorescence intensity (MFI) of IL-17A and IL-17F was also analyzed as an estimate for the intensity of expression within IL-17 positive cells. MFI was significantly increased for both IL-17A (IL-21: 2.04 fold, $p = 0.003$; IL-23: 2.32 fold, $p \leq 0.001$; IL-6: 1.42 fold, $p = \text{NS}$; IL-21 + IL-23: 2.46 fold; $p \leq 0.001$) and IL-17F (IL-21: 2.35 fold, $p = 0.004$; IL-23: 2.50 fold, $p \leq 0.001$; IL-6: 2.07 fold, $p = 0.03$; IL-21 + IL-23: 2.59 fold, $p \leq 0.001$) in asthmatic neutrophils upon stimulation compared to baseline (Figure 3A and B). These IL-17 MFI levels of asthmatic neutrophils were also significantly higher than that of stimulated healthy neutrophils (Figure 3A and B). Furthermore, we analyzed neutrophil enhanced production of IL-17 cytokines upon

stimulation with Th-17 regulatory cytokines in whole cell lysates by Western immunoblotting. A 15–20 kDa IL-17A immunoreactive band was present at low intensity in non-stimulated cells while the expression substantially increased with IL-21, IL-23, IL-21 + IL-23, and IL-6 (positive control) cytokines stimulation (Figure 4A). The densitometry data further confirmed a significant 2–3 folds increase in IL-17A production following stimulation with IL-21 (3.24 folds increase and $p = 0.002$), IL-23 (2.92 fold increase and $p = 0.009$) and IL-21 + IL-23 cytokines (2.52 fold increase and $p = 0.015$) compared to non-stimulated cells (Figure 4B).

STAT3 activation is required for IL-21/IL-23 induced IL-17 production in neutrophils

To investigate the mechanism behind IL-21 and IL-23-induced IL-17 production, we examined whether STAT3 signaling pathway activation is required for neutrophil-derived IL-17 production. Asthmatic and healthy neutrophils were stimulated with IL-21 + 23 cytokines for 15 minutes and the percentage of positive cells for activated STAT3 by phosphorylation (pSTAT3) were quantified by flow cytometry. The frequency of asthmatic cells with activated pSTAT3 increased significantly upon stimulation with IL-21 + IL-23 (44.3%, $p = 0.005$) compared to non-stimulated cells (18.0%) (Figure 5A). This increase in pSTAT3 was significantly reduced upon stimulating the cells in the presence of Stattic (Sc-202818), a selective STAT3 inhibitor (20.1%, $p = 0.002$). Stimulating

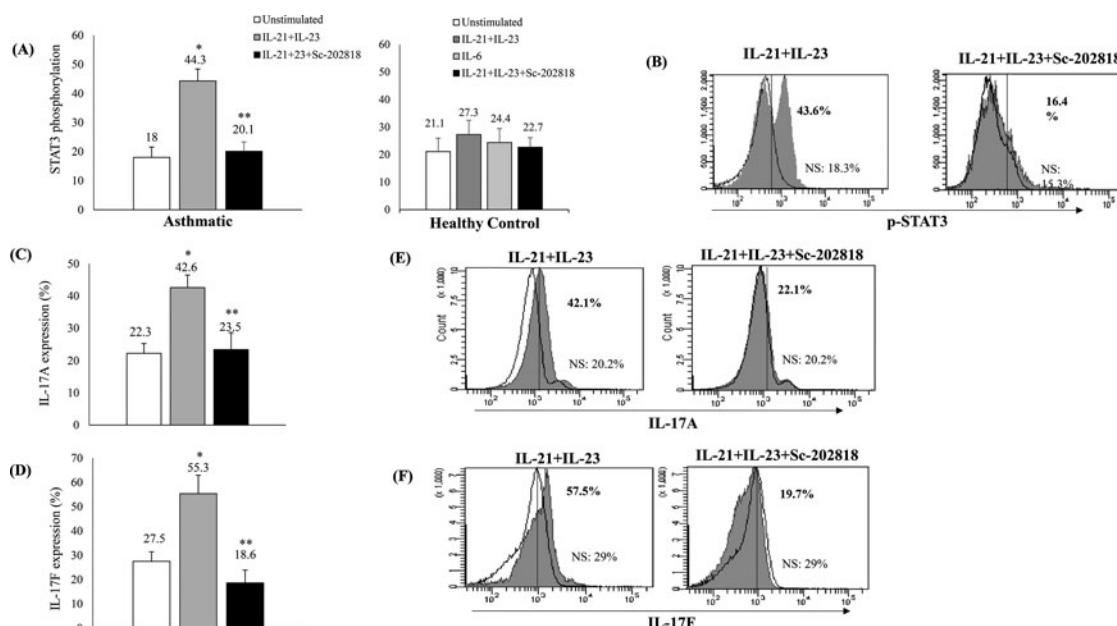


Figure 5. Activation of STAT3 pathway is required for neutrophil-derived IL-17 production. The level of STAT3 phosphorylation was determined in asthmatic and healthy neutrophils following stimulation with IL-21 and IL-23 cytokines was determined using phosflow assay. (A) STAT3 phosphorylation is significantly upregulated in asthmatic, but not healthy, neutrophils following cytokine stimulation. This increase in phosphorylation was inhibited when stimulating neutrophils in the presence of Stat3 Sc-202818 inhibitor. (B) Representative histograms of the increase in STAT3 phosphorylation in asthmatic neutrophils following cytokine stimulation in the presence or absence of Stat3 Sc-202818 inhibitor. (C&D) Asthmatic neutrophils were stimulated in the presence or absence of Sc-202818 and the frequency of IL-17A (C) and IL-17F (D) was determined using a flow cytometer. Representative histograms of IL-17A (E) and IL-17F (F) expression following inhibition of STAT3 phosphorylation are shown. Data are expressed as means $\pm$ SEM ($n = 28$). * $p \leq 0.05$ stimulated (without inhibitor) vs non-stimulated cells; ** $p \leq 0.05$ stimulated with inhibitor vs stimulated without inhibitor cells.

healthy neutrophils with IL-21 + 23 or IL-6 cytokines upregulated pSTAT3 levels but not to significant levels. IL-21 + IL23-induced phosphorylation was also inhibited using Sc-202818 (Figure 5A). Representative histogram is shown in Figure 5B reflecting the percent of asthmatic cells with pSTAT3 following stimulation with IL-21 + IL-23 in the presence or absence of Sc-202818.

To determine whether STAT3 activation is essential for IL-21 and IL-23 induced IL-17A and IL-17F expression in neutrophils, cells were starved and stimulated with IL-21 + IL-23 cytokines in the presence or absence of Sc-202818 inhibitor. The frequency of cells expressing IL-17A and IL-17F were then determined by flow cytometry. A substantial increase in IL-17A positive cells (NS: 22.3% vs IL-21 + IL-23: 42.6%, $p = 0.011$) was observed when neutrophils were stimulated with IL-21 + IL-23 cytokines in the absence of the inhibitor (Figure 5C). A similar result was observed for IL-17F (NS: 27.5% vs IL-21 + IL-23: 55.3%, $p = 0.009$) (Figure 5D). However, when STAT3 phosphorylation was inhibited using Sc-202818, a significant decrease in cells positive for IL-17A (NS: 22.3% vs IL-21 + IL-23: 23.5%, $p = 0.020$) and IL-17F (NS: 27.5% vs IL-21 + IL-23: 18.6%, $p = 0.003$) was observed, indicating that STAT3 phosphorylation is required for IL-21 and IL-23 induced expression of IL-17 in neutrophils (Figure 5C and D). Representative histogram is shown reflecting the

percent of asthmatic cells with IL-17A (Figure 5E) and IL-17F (Figure 5F) expression following stimulation with IL-21 + IL-23 in the presence or absence of Sc-202818.

Discussion

Although IL-17-producing peripheral blood CD177+ neutrophils were recently reported to increase in allergic asthmatic subjects [44], the mediators and the mechanism(s) involved are not well characterized. In this report, we show that human peripheral blood neutrophils isolated from asthmatics produce IL-17A and IL-17F upon stimulation with Th-17 regulatory cytokines, IL-21, IL-23, IL-6 alone, as well as the IL-21 + IL-23 combination with higher efficacy compared to neutrophils isolated from healthy controls. This suggests that asthmatic neutrophils are primed or polarized to better respond to IL-21 and IL-23 cytokines stimulation. IL-6 cytokine, which is known to enhance cellular expression of IL-21R and IL-23R [45], was shown to be upregulated in peripheral blood of asthmatic patients [46]. IL-6 may then act on asthmatic neutrophils to upregulate the levels of IL-21R and IL-23R which may contribute to their enhanced response to cytokines stimulation. In fact, we observed a slight increase of these receptors in neutrophils isolated from asthmatics compared to those of healthy subjects

but not to a significant level (Figure 1B). Alternatively, Taylor et al. reported that human peripheral blood neutrophils produce IL-17A upon stimulation with IL-6 and IL-23 cytokines via a mechanism completely dependent on RAR-related orphan receptor gamma ($ROR\gamma$)t [30]. Similarly, $ROR\gamma$ t expression was shown to be required for $Gr-1^+$ neutrophils derived IL-17 cytokine production [47]. Interestingly, STAT3, the main regulator of IL-21, IL-23, and IL-6 signaling [28,48–50] and hence IL-17 production in various cell types [28,51] including neutrophils (Figure 5), is known to have a critical role in regulating the expression of $ROR\gamma$ t [50]. The observed cytokine induced increase in STAT3 phosphorylation in asthmatic neutrophils could hence induce $ROR\gamma$ t expression which results in upregulation of IL-17 production.

Stimulating asthmatic neutrophils with a combination of cytokines had an additive effect in terms of IL-17 production compared to stimulating with only one cytokine. Moreover, IL-17F was produced at significantly higher levels compared to IL-17A following stimulation with almost each of the Th-17 regulatory cytokines (Figure 1). This could be due to an alternative regulatory mechanism involving other STAT factor members such as STAT1 or STAT5, known to be activated upon stimulation with the Th-17 regulatory cytokines [28,51]. Interestingly, Yang et al. suggested that under conditions of low IL-2 but high IL-6, STAT3 signals dominate and IL-17A and IL-17F are produced [52]. However, under high IL-2 and high IL-6 conditions, STAT5 represses IL-17A transcription while STAT3 sustains the expression of IL-17F as the replacement of STAT3 by STAT5 at the IL-17F locus is prevented [52,53]. IL-6 is known to be upregulated in patients with asthma [54], while IL-2 was shown to be upregulated in exhaled breath condensates of non-allergic severe asthmatic patients [55]. Hence in patients with atopic asthma, such as the ones involved in this study, STAT3 signals may dominate and drive the production of both IL-17A and IL-17F cytokines. Moreover, STAT3 was moderately increased in control neutrophils following stimulation with IL-21/IL-23 but not to a significant level (Figure 5A). Sc-202818 also inhibited IL-21 + IL-23-induced STAT3 phosphorylation confirming cytokine triggering of this pathway in healthy neutrophils. However, this increase in STAT3 phosphorylation was not sufficient to induce significant IL-17 production (Figure 2). The difference in the expression levels of IL-21/23 receptors on asthmatic versus healthy cells does not explain the differential increase in STAT3 phosphorylation between asthmatic and healthy neutrophils (Figure 1B). One possible explanation is that, in inflammatory conditions such as in asthma, STAT3- $ROR\gamma$ t pathway could be primed to efficiently respond to IL-21/IL-23 stimulation, contrary to the case in normal conditions, and to the level that

induces IL-17 production. Alternatively, besides STAT3, other mediators/pathways may co-regulate IL-17 production in inflammatory condition, and which could be absent in normal condition. This possibility should not be excluded and requires further investigations.

Accumulating evidence has confirmed a critical role for IL-17 cytokine in enhancing tissue inflammation during autoimmune and various inflammatory disorders in addition to its role in protection against bacterial and fungi infections [56,57]. Patients with genetic mutations causing impaired IL-17 responses, due to defects in IL-17 production or signaling, exhibited enhanced susceptibility to mucocutaneous candidiasis [58–62]. Although Th-17 cells are considered the main source of IL-17, other cells have been shown to significantly produce IL-17 including $\gamma\delta$ T cells, natural killer cells (NK cells), and B cells [41,63]. Neutrophils have also been reported to produce IL-17 in human peripheral blood, in psoriatic lesions [30,64] and in several mouse models of auto-immune and infectious inflammation [65–70].

In addition to upregulating IL-17R on neutrophils [30], IL-6 and IL-23 could upregulate these receptors on lung structural cells during airway inflammation. Activation of IL-17R on epithelial cells and fibroblasts triggers their production and release of proinflammatory and chemotactic cytokines that mediate the recruitment of neutrophils to inflamed lung tissues [56]. The upregulated lung tissue levels of IL-21, IL-23, and IL-6 during asthma and Chronic Obstructive Pulmonary Disease (COPD) would trigger infiltrated neutrophils to themselves produce IL-17. This environment of high IL-17 levels in lung tissue stimulates neutrophils to produce highly reactive oxygen radicals (ROS) [71–73] that would exacerbate the inflammatory response via their cytotoxic and tissue-destructive activity. This may contribute to the persistence of airway tissue remodeling during chronic inflammatory lung disease. Therefore, drug interference with IL-21, IL-23, and IL-6 effect on neutrophils may help to reduce lung tissue remodeling especially in severe disease conditions, where novel approaches are critically needed.

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Declaration of interest

The author(s) declare that they have no competing interests.

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Authors' contributions

RH carried out the real-time PCR experiments and FACS analysis experiments, conceived of the study, lead efforts on its design, and coordinated and finalized the manuscript draft. AS carried out phosflow experiments. AJ performed the western analysis and densitometry measures. AVT and AA participated in the design of the study and performed the statistical analysis. SM contributed in recruiting patients to the study. All authors read and approved the final manuscript.

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