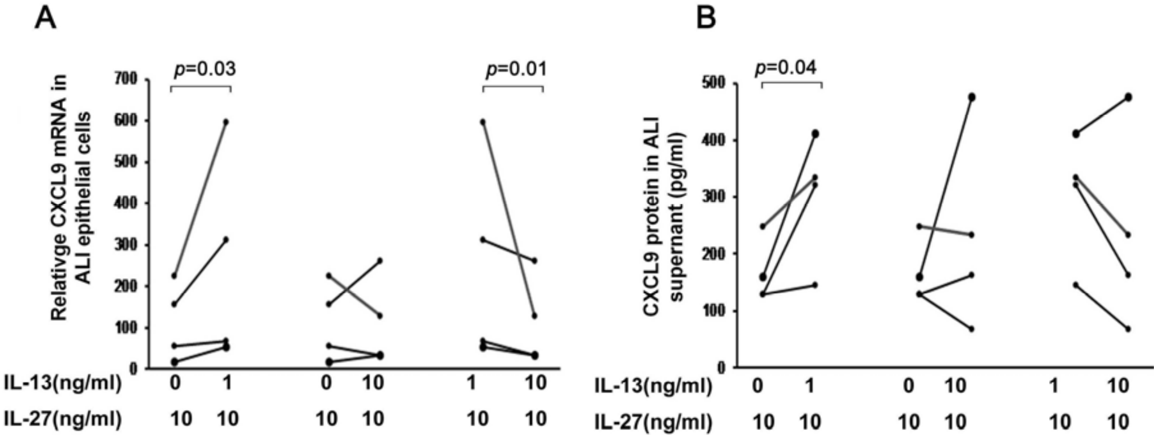
Supple. Fig. 1



Supplemental information

Methods

Subjects

Participants were 18-65 years old and enrolled in the Severe Asthma Research Program (SARP) or the Electrophilic Fatty Acid Derivatives in Asthma study.[1] All studies were approved by the University of Pittsburgh Institutional Review Board and all participants provided informed consent. Subjects were nonsmokers in the last year and all had <5 pack-year smoking history. Severe asthma (SAs) was defined using the American Thoracic Society (ATS) definition.[2] Patients with mild asthma not receiving ICSs (Mild/no ICS group) had a prebronchodilator FEV1 of ≥80% predicted. Patients with mild-to-moderate asthma receiving low to moderate-dose ICSs (Mild-Mod/ICS group) had an FEV1 of greater than 60% predicted[3]. HCs had no history of chronic respiratory disease and normal lung function, but could be atopic. All participants were extensively characterized as previously described, including clinical questionnaires, spirometric measures pre and post bronchodilator, fractional exhaled nitric oxide (FeNO), complete blood counts, allergy skin prick testing and IgE. [3, 4]

Bronchoscopy and sample processing

Epithelial brushings and bronchoalveolar lavage (BAL) cells were obtained bronchscopically from the 4-5th generation airways according to previously published protocols and the SARP manual of procedures.[5, 6] Cells were placed in Qiazol (Qiagen) for extraction of RNA.

Quantitative real-time PCR

Epithelial and BAL cell RNA was extracted in Qiazol (Qiagen, Valencia, CA) and mRNA expression was determined by quantitative real-time PCR (qRT-PCR). Reverse transcription was performed with 1µg of total RNA and random hexamers in a 50µl reaction, according to the manufacturer's protocol (PE Applied Biosystems, Foster City, CA). Primers and probes were purchased from Applied Biosystems (Foster City, CA; Assays on Demand: IL-27 p28, HS00377366_m1; CCL26, Hs00171146_m1; CXCL9, Hs00171065_m1). The probes were labeled with the 5'-reporter dye 6-carboxy fluorescein and the 3'-quencher dye 6-carboxy N, N, N', N' tetramethylrhodamine. VIC-labeled human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe and primers were also obtained from Applied Biosystems (GenBank accession no. NM-002046; part no.4310884E). qRT-PCR was performed on the ABIPrism 7900 sequence detection system (Applied Biosystems) at core facilities at the University of Pittsburgh. The levels of each marker were determined relative to GAPDH using the delta-CT method.

Immunohistochemistry

BAL cell cytospins were fixed in 2% paraformaldehyde. Cytospins were rinsed, blocked and incubated overnight with goat polyclonal anti-human IL-27 antibody (R&D systems, Minneapolis, MN, USA) at a 1:50 dilution. Biotinylated secondary rabbit anti-goat antibody was added and the cytospins incubated with ABC reagent (Vector Laboratories, Burlingame, CA), developed with the chromogen 3-amino-9-ethylcarbazole (AEC),

counterstained with hematoxylin and overlaid with Crystal Mount (Electron Microscopy Sciences, Hatfield, PA). IL-27^{+} cells were counted blindly by two independent observers from 500 or more cells to obtain the percentage of IL-27^{+} cells.

Primary Air Liquid Interface Epithelial Cell Culture and siRNA Transfection

Primary HAEC obtained from bronchoscopic brushings were cultured under Air-liquid interface (ALI) as previously described.[6, 7] From Day 0 of ALI, cells were stimulated with IL-13 (1ng/ml or 10ng/mL) (R&D systems, Minneapolis, MN, USA) or media alone every 48 hours for up to 8 days. IL-27 (10ng/ml, R&D systems, Minneapolis, MN, USA) was added to media at Day 6. Cells were harvested for STAT proteins at 0, 30min, 1 and 6hrs after IL-27 stimulation and for CXCL9 mRNA at 12, 24, and 48hrs after IL-27 stimulation. Lower chamber media was harvested at 12, 24, and 48hrs after IL-27 stimulation for monokine induced by gamma interferon (CXCL9) protein.

siRNA transfection were performed as previously described.[7] 300 μl siRNA mixture (50nM siRNA and 3 μl Mirus transfection reagent) was added to the upper chamber and transferred to the lower chamber 6 hours later. 48 hours later, IL-27 was added to the lower chamber. Cells and supernants were harvest 24 hours after addition of IL-27.

SDS-PAGE and Western Blotting

Expression and activation of signal transducer and activator of transcription (STAT) in cultured epithelial cells were measured by Western blot on 4-12% SDS-PAGE gels using

rabbit polyclonal antibodies for STAT1, p-STAT1, STAT3 and p-STAT3 (1:500; all from Cell Signaling Technology, Danvers, MA, USA). Membranes were incubated with primary antibodies, followed by addition of anti-rabbit HRP-conjugated secondary antibody (1:5000) (ECL™, Little Chalfont Buckinghamshire, UK). Blots were developed using Supersignal West Femto Maximum Sensitivity Substrate (Fisher Scientific, Pittsburgh, PA) and read on a Fuji Film Image Reader LAS-3000.

CXCL9 ELISA

CXCL9 protein was measured in lower ALI supernant by ELISA (detection limit 20 pg/ml) using R&D Systems antibody (Minneapolis, MN) by ELISAtech (Aurora, CO, USA). Undetectable CXCL9 protein was reported as 2 pg/ml.

Statistical Analysis

Data were analyzed for normality, with and without log transformation. Analysis of variance (ANOVA) (for normally distributed data) or Kruskal Wallis variation of ANOVA (for nonparametric data) was utilized to identify an overall level of significance among the groups or conditions. When overall p-value was <0.05, Tukey's (for parametric), Wilcoxon or Chi-Square analysis (categorical data) of individual groups was performed. Bonferroni correction was applied for all intergroup comparisons. Spearman's rho (r_s) was used for correlations of nonparametric data and Pearson correlation for normally distributed data. Pearson's X^2 tests compared categorical values. Analysis of Covariance (ANCOVA) adjusted for age, body mass index and gender to evaluate the association of

asthma severity with IL-27 expression. Blood eosinophils cut-off was ≥300 cells/μl.[4] Median splits defined high vs. low levels of IL-27 mRNA (1.733 IL-27 indexed to GAPDH) and CCL26 mRNA (0.185 CCL26 indexed to GAPDH). Participants were subgrouped by IL-27 and Type-2 signature category into 4 groups, with epithelial CCL26 mRNA used as the biomarker for a Type-2 signature.[8, 9] Groups were defined as Low IL-27 and Low Type-2 (CCL26), Low IL-27 and High Type-2, High IL-27 and Low Type-2 and High IL-27 and High Type-2. For *in vitro* studies, nonparametric signed-rank paired tests compared CXCL9 mRNA/protein in response to scramble or STAT1/3 siRNA. Statistical analysis was performed with JMP SAS software (SAS Institute, Cary, NC), and p-values <0.05 were considered significant except as noted.

References

1. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. Am Rev Respir Dis 1987; 136:225-44.
2. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med 2000; 162:2341-51.
3. Moore WC, Bleecker ER, Curran-Everett D, Erzurum SC, Ameredes BT, Bacharier L, et al. Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. J Allergy Clin Immunol 2007; 119:405-13.
4. Fajt ML, Gelhaus SL, Freeman B, Uvalle CE, Trudeau JB, Holguin F, et al. Prostaglandin D2 pathway upregulation: Relation to asthma severity, control, and TH2 inflammation. J Allergy Clin Immunol 2013; 131:1504-12.e12.
5. Chibana K, Trudeau JB, Mustovich AT, Hu H, Zhao J, Balzar S, et al. IL-13 induced increases in nitrite levels are primarily driven by increases in inducible nitric oxide synthase as compared with effects on arginases in human primary bronchial epithelial cells. Clin Exp Allergy 2008; 38:936-46.
6. Trudeau J, Hu H, Chibana K, Chu HW, Westcott JY, Wenzel SE. Selective downregulation of prostaglandin E2-related pathways by the Th2 cytokine IL-13. J Allergy Clin Immunol 2006; 117:1446-54.
7. Zhao J, Maskrey B, Balzar S, Chibana K, Mustovich A, Hu H, et al. Interleukin-13-induced MUC5AC is regulated by 15-lipoxygenase 1 pathway in human bronchial epithelial cells. Am J Respir Crit Care Med 2009; 179:782-90.
8. Ravensberg AJ, Ricciardolo FL, van Schadewijk A, Rabe KF, Sterk PJ, Hiemstra PS, et al. Eotaxin-2 and eotaxin-3 expression is associated with persistent eosinophilic bronchial inflammation in patients with asthma after allergen challenge. J Allergy Clin Immunol 2005; 115:779-85.
9. Coleman JM, Naik C, Holguin F, Ray A, Ray P, Trudeau JB, et al. Epithelial eotaxin-2 and eotaxin-3 expression: relation to asthma severity, luminal eosinophilia and age at onset. Thorax 2012; 67:1061-6.

Supplemental Table I.

Baseline subject demographic characteristics for IL-27 immunohischemistry staining group (n=51).

Subject group (n)	Healthy Controls (14)	Mild/no ICS (n=9)	Mild-Mod/ICS (n=10)	Severe Asthma (n=18)	Overall Difference (*p*-value)
Demographic					
Age (yr)	24 (23-32)	26 (20-29)	32 (24-51)	49 (32-59)	0.009
Sex (male/female)	9/5	3/6	2/8	7/11	0.03
Race (CA/AA/other)	10/1/3	6/1/2	4/4/2	15/1/2	0.09
BMI (kg/m2)	23 (22-25)	25 (22-32)	28 (26-35)	27 (25-32)	0.004
Exhaled NO (ppb)	21 (16-38)	43 (23-51)	23 (18-29)	42 (32-77)	0.007
Blood eosinophils /µl	100 (100-200)	200 (100-300)	200 (100-300)	200 (100-375)	0.35
Baseline FEV1 (%pred)	99 (94-104)	88 (73-97)	85 (69-104)	61 (49-74)	<0.001
Serum IgE (kU/L)	28 (14-61)	80 (50-648)	133 (39-370)	134 (38-462)	0.004

Definition of abbreviations: CA=Caucasian; AA= African American; BMI= body mass index; IgE= immunoglobulin E; ppb= parts per billion. Categorical analyses were performed using Pearson chi-square tests. Continuous variables analyzed using Kruskal/Wallis tests and presented as medians (25th–75th percentile).

Supplemental Table II.

Subject demographic characteristics for subjects of epithelial cells cultured in ALI system

Subject group (n)	Healthy Controls (4)	Mild/no ICS (n=2)	Mild-Mod/ICS (n=2)	Severe Asthma (n=5)	Overall Difference (*p*-value)
Demographic					
Age (yr)	34 (29-40)	41 (34-48)	32 (22-41)	49 (32-54)	0.37
Sex (male/female)	4/0	0/2	0/2	2/3	0.04
Race(CA /AA/other)	2/0/2	2/0/0	2/0/0	3/1/1	0.41
BMI (kg/m2)	25 (21-25)	29 (25-32)	27 (22-35)	27 (23-32)	0.83
Exhaled NO(ppb)	18 (12-21)	140 (18-263)	34 (5-64)	27 (15-60)	0.007
Blood eosinophils/µl	100 (100-167)	1000 (300-1700)	50 (0-100)	450 (175-575)	0.07
Baseline FEV1 (%pred)	97 (94-104)	85.5 (79-92)	95 (93-97)	63 (41-84)	0.12

Definition of abbreviations: CA=Caucasian; AA= African American; BMI= body mass index; ppb=parts per billion. Categorical analyses were performed using Pearson chi-square tests. Continuous variables analyzed using Kruskal/Wallis tests and presented as medians (25th–75th percentile).

Figure Legends

Supplemental Figure 1. CXCL9 production by primary epithelial cells at IL-27 10 ng/ml stimulation of 24 hours in combination with 1ng/ml IL-13 or 10ng/ml IL-13 for relative mRNA (A) and protein (B) (n=4).