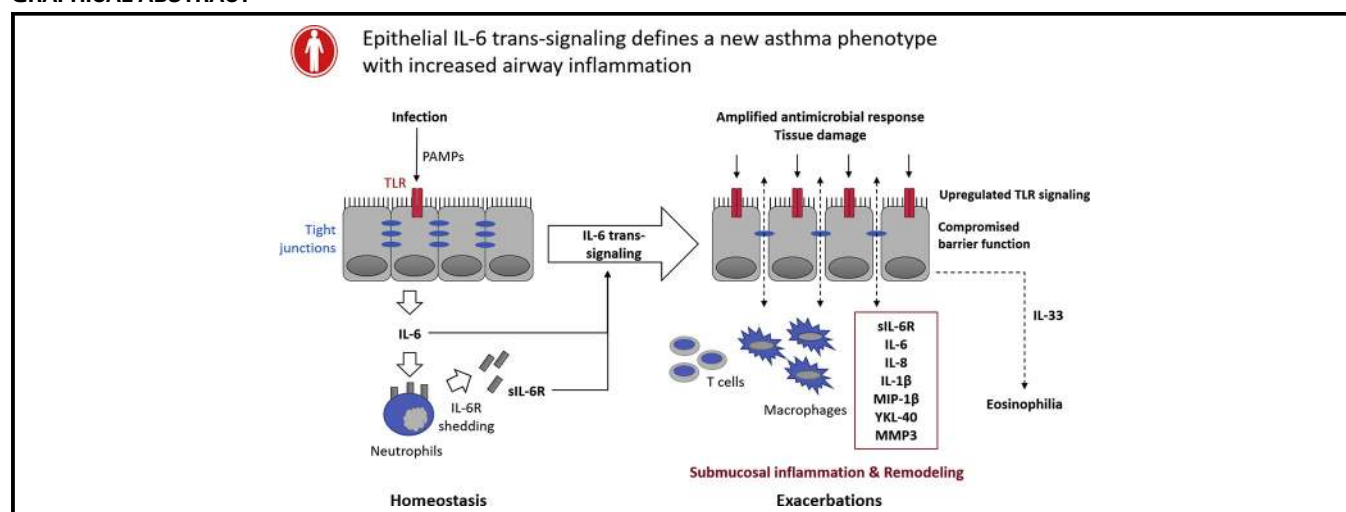# Epithelial IL-6 trans-signaling defines a new asthma phenotype with increased airway inflammation

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## GRAPHICAL ABSTRACT

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**Background:** Although several studies link high levels of IL-6 and soluble IL-6 receptor (sIL-6R) to asthma severity and decreased lung function, the role of IL-6 trans-signaling (IL-6TS) in asthmatic patients is unclear.

**Objective:** We sought to explore the association between epithelial IL-6TS pathway activation and molecular and clinical phenotypes in asthmatic patients.

**Methods:** An IL-6TS gene signature obtained from air-liquid interface cultures of human bronchial epithelial cells stimulated with IL-6 and sIL-6R was used to stratify lung epithelial transcriptomic data (Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes [U-BIOPRED] cohorts) by means of hierarchical clustering. IL-6TS–specific protein markers were used to stratify sputum biomarker data (Wessex cohort). Molecular phenotyping was based on transcriptional profiling of epithelial brushings, pathway analysis, and immunohistochemical analysis of bronchial biopsy specimens.

**Results:** Activation of IL-6TS in air-liquid interface cultures reduced epithelial integrity and induced a specific gene signature enriched in genes associated with airway remodeling. The IL-6TS signature identified a subset of patients with IL-6TS–high asthma with increased epithelial expression of IL-6TS–inducible genes in the absence of systemic inflammation. The IL-6TS–high subset had an overrepresentation of frequent exacerbators, blood eosinophilia, and submucosal infiltration of T cells and macrophages. In bronchial brushings Toll-like receptor pathway genes were upregulated, whereas expression of cell junction genes was reduced. Sputum sIL-6R and IL-6 levels correlated with sputum markers of remodeling and innate immune activation, in particular YKL-40, matrix metalloproteinase 3, macrophage inflammatory protein 1β, IL-8, and IL-1β.

**Conclusions:** Local lung epithelial IL-6TS activation in the absence of type 2 airway inflammation defines a novel subset of asthmatic patients and might drive airway inflammation and epithelial dysfunction in these patients. (J Allergy Clin Immunol 2019;143:577-90.)

Type 2 (T2) inflammation defines one major asthma endotype, but a significant proportion of asthmatic patients do not express airway T2 inflammation markers and do not respond to treatments targeting this pathway.[1] Thus identification of the cellular and molecular disease drivers beyond T2 asthma is required to achieve disease control in these patients.

IL-6 is a pleiotropic cytokine that can be produced by many cell types in response to a wide array of inflammatory stimuli and cytokines.[2] In the classical pathway binding of IL-6 to its membrane-bound receptor (IL-6R) induces recruitment and homodimerization of the signal-transducing receptor glycoprotein 130, which leads to phosphorylation of signal transducer and activator of transcription (STAT) family transcription factors (STAT3, STAT1, or both) by the Janus tyrosine kinase family (Janus kinase [JAK] 1, JAK2, and tyrosine kinase 2). In addition, it causes activation of the mitogen-activated protein kinase and phosphoinositide 3-kinase cascades.[3,4] Classical signaling is

prominent in cells expressing high levels of IL-6R on their surfaces, such as neutrophils, macrophages, and some types of T cells.[5] In contrast, cell types with no or low expression of membrane IL-6R depend on IL-6 trans-signaling (IL-6TS) mediated by the soluble IL-6 receptor (sIL-6R), which associates with glycoprotein 130 after forming a complex with IL-6.[6] sIL-6R is produced as a result of alternative mRNA splicing[7] or through shedding of IL-6R from the cell surface by a disintegrin and metalloprotease and meprin proteases[8,9] in response to various inflammatory signals, such as C-reactive protein, IL-8, CXCL1, bacterial pore-forming toxins, and LPS.[10-13] Neutrophils have been proposed as the main source of sIL-6R generated at sites of inflammation,[11] and a recent study showed that neutrophils might be an important source of sIL-6R in the lungs of asthmatic patients.[14]

Increased levels of IL-6 have been found in serum, sputum, and bronchoalveolar lavage fluid (BALF) of asthmatic patients[15-17] and also in BALF from patients with nonallergic asthma compared with that from patients with allergic asthma, suggesting a role for IL-6 in non-T2 asthma.[18] Supporting this, a recent study conducted in the University of California, San Francisco and Severe Asthma Research Program asthma cohorts identified a strong association between high systemic IL-6 levels and asthma severity, whereas no correlation was found between IL-6 and T2 inflammation biomarkers, including blood and sputum eosinophil counts, blood IgE levels, and fraction of exhaled nitric oxide values.[19] As for IL-6, sIL-6R levels have been found to be significantly increased in serum,[20] BALF,[21] and sputum[22] from asthmatic patients compared with those from control subjects. A role for IL-6 as an asthma disease driver is also emerging from genetic evidence, and a genome-wide association study revealed an association between the single nucleotide polymorphism rs4129267 in intron 8 of the *IL6R* gene and an increased asthma risk.[23]

In this study we identified an IL-6TS–driven patient subset in the Unbiased Biomarkers in Prediction of Respiratory Disease Outcomes (U-BIOPRED) cohorts[24] based on an epithelial IL-6TS–specific gene signature. These patients were characterized by their unique clinical and histopathologic features, including a history of frequent exacerbations and a remarkable increase in submucosal T-cell and macrophage infiltration, and they also

showed markers of impaired epithelial barrier integrity and airway remodeling.

## METHODS
### Primary cell culture and activation

Primary human bronchial epithelial cells (HBECs; Lonza, Basel, Switzerland) were expanded to passage 2 in BEGM Bronchial Epithelial Cell Growth Medium (Lonza). These cells were then seeded on 0.4-μm Corning HTS Transwell 24-well permeable supports (Sigma-Aldrich, St Louis, Mo) coated with PureCol (Advanced BioMatrix, Carlsbad, Calif) and differentiated at the air-liquid interface (ALI) using PneumaCult-Ex and ALI Media (STEMCELL Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's protocol. Once fully differentiated, cells were stimulated for 3 to 48 hours by means of basolateral addition of the human recombinant proteins IL-4 and IL-13 (30 ng/mL; R&D Systems, Minneapolis, Minn) or IL-6 and sIL-6R (10-150 ng/ml; PeproTech, Rocky Hill, NJ) diluted in PneumaCult-ALI medium. JAK1 inhibitor (1 μmol/L)[25] or dimethyl sulfoxide were added apically for 30 minutes in 200 μL of medium that was removed before stimulation. Phosphorylation of STAT3 on tyrosine 705 was quantified with a pSTAT3 detection kit (Meso Scale Discovery, Rockville, Md), according to the manufacturer's instructions.

### Patient cohorts, transcriptomics, and proteomics

The U-BIOPRED study was a cross-sectional observational study using baseline visits of the U-BIOPRED cohorts from 16 clinical centers in 11 countries across Europe. It included steroid-treated adults with asthma classified and treated according to the Global Initiative for Asthma guidelines, as well as healthy control subjects. Definitions for each group of subjects and collection of clinical variables have been published previously by Shaw et al.[24]

The current study usd data from 147 subjects from the U-BIOPRED asthma cohorts, including patients with mild-to-moderate asthma (n = 36), patients with severe asthma (n = 49), smokers with severe asthma (n = 18), and healthy nonsmoking control subjects (n = 44) who underwent fiberoptic bronchoscopy for central airway epithelial cell brushings.[26] Transcriptomic data from brushings were obtained by using a HT HG-U133+ PM microarray platform (Affymetrix Plus 2.0; Affymetrix, Santa Clara, Calif), as previously described.[27] Affymetrix Plus 2.0 microarray data were downloaded from the U-BIOPRED/tranSMART database (August 2016 version). Blood samples were collected,[24] and biomarkers (IL-6 and sIL-6R) were analyzed by using the SomaScan v3 platform (SomaLogic, Boulder, Colo), as previously described.[28]

### Unsupervised hierarchical clustering

Hierarchical clustering of U-BIOPRED gene expression data was performed by using the average linkage and Euclidean metric methods, with each variable normalized to a mean of 0 and variance of 1 by using Qlucore Omics Explorer 3 (Qlucore, Lund, Sweden). Results were visualized as dendrogram heat maps in which the color scale is given as the $\log_2$ fold change with a range from −2.0 (blue) through 0.0 (grey) to +2.0 (red). Data from multiple probes were collapsed to single genes by using the highest value.

### Statistical analysis

Gene expression data were $\log_2$ transformed and analyzed by using general linear model–based statistical tests adjusting for age, sex, and site code with Qlucore Omics Explorer 3.3 (Qlucore). Benjamini-Hochberg multiple correction was used to control for the rate of false-positive results (referred to as q value). Statistical analysis of clinical variables and biomarker data was performed with Kruskal-Wallis tests in Spotfire 7.0.2 (TIBCO Spotfire). The P value for MetaCore (MetaCore; Thomson Reuters, Toronto, Ontario, Canada) pathway analysis and Ingenuity Pathway Analysis (IPA; Qiagen, Hilden, Germany) was calculated by using the right-tailed Fisher exact test. All statistical analyses of *in vitro* data were performed with the 2-tailed unpaired t test. All

data analyses, except analysis of gene expression data, were considered hypothesis based, and significance was reached at a P value of .05 or less. Correlations were tested with Spearman r statistics. Prism 6.0 software (GraphPad Software, La Jolla, Calif) was used for data analysis and graphic representation.

## RESULTS
### IL-6TS induces a specific gene expression profile distinct from the T2 inflammation gene signature in primary bronchial epithelial cells

Stimulation of primary HBECs grown as ALI cultures with either recombinant IL-6 alone (classical IL-6 signaling) or in combination with sIL-6R (IL-6TS) induced robust STAT3 phosphorylation within 30 minutes. The increase in STAT3 phosphorylation was considerably stronger in the presence of sIL-6R, which was added at a concentration (150 ng/mL) comparable with the levels found in sera of asthmatic patients.[20] Moreover, only IL-6TS led to persistent STAT3 phosphorylation after prolonged exposure with daily applications of IL-6 or IL-6/ sIL-6R (Fig 1, *A*). Pretreating cells with a JAK1-selective inhibitor[25] abrogated STAT3 phosphorylation induced by IL-6TS (Fig 1, *B* and *C*).

To identify genes regulated by IL-6TS, we stimulated HBEC ALI cultures for 24 hours with IL-6/sIL-6R, followed by global gene expression analysis using RNA sequencing. In total, 8781 protein-coding genes ($q < 0.05$) were expressed differentially between IL-6/sIL-6R (IL-6TS)–stimulated and unstimulated control cells, whereas 6514 genes were affected by IL-6 alone. All genes regulated by both IL-6 and IL-6/sIL-6R (6041 overlapping genes) had a consistent direction of change, and IL-6/sIL-6R consistently produced a stronger expression of the majority of these genes than IL-6 on its own (Fig 1, *D*, and see Tables E1 and E2 in this article's Online Repository at www.jacionline. org). Expression of selected IL-6TS–inducible genes was validated by using quantitative PCR in 3 additional donors (see Fig E1 in this article's Online Repository at www.jacionline.org).

Several of the genes that were strongly induced by IL-6TS have been associated with airway remodeling in patients with respiratory diseases and include genes for matrix metalloproteinases ([MMPs]; *MMP1*, *MMP3*, and *MMP12*),[29] chitinase-like proteins (*CHI3L1*/YKL-40 and *CHI3L2*/YKL-39),[30] osteopontin (*SPP1*),[31] and *IL-33*.[32] The exact biological role of YKL-40 in asthmatic patients remains unclear, but it correlates consistently with airway obstruction and measures of airway remodeling, such as thickness of the bronchial wall.[30,33] *MMP3* expression has been found to be increased in BALF of patients with severe asthma,[34] and it has been implicated in emphysematous airway remodeling in patients with chronic obstructive pulmonary disease.[35] Specific induction of YKL-40 and MMP3 by IL-6/sIL-6R was confirmed also on protein levels in 6 different HBEC donors (Fig 1, *G*).

As controls, we stimulated the HBEC ALI cultures with a combination of IL-4 and IL-13 to induce a typical T2 inflammatory gene profile, as confirmed by increased expression of T2 signature genes, including *CCL26* and *NOS2*, which was previously reported by Choy et al.[36] The IL-4/IL-13–induced gene signature was distinct from the IL-6TS gene signature, and T2 stimulation of HBEC ALI cultures had no effect or only a weak effect on the majority of IL-6TS–specific genes (Fig 1, *D-F*; see Fig E2, *A*, and Table E3 in this article's Online Repository at
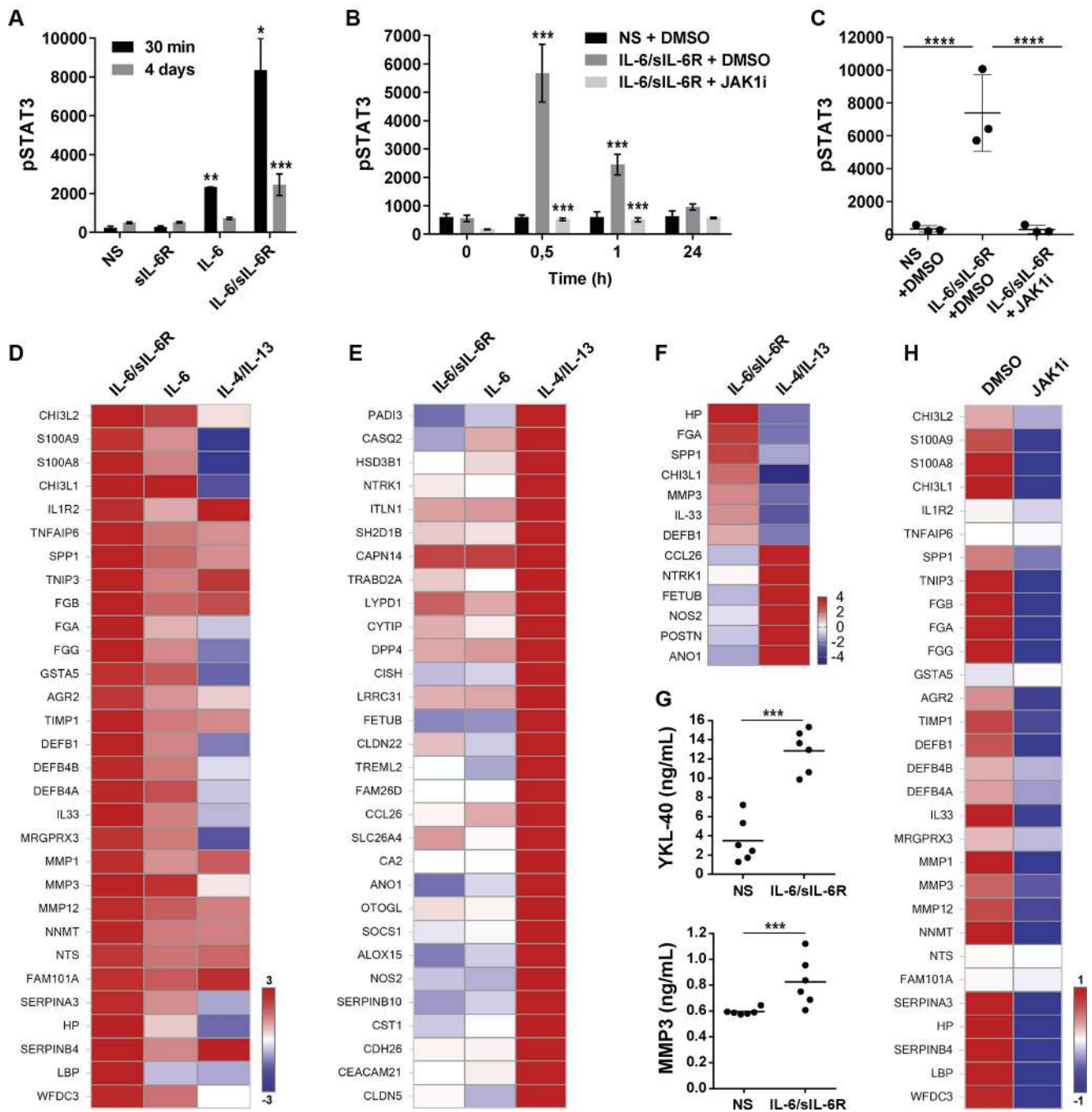
**FIG 1.** IL-6TS induces a specific JAK1-dependent gene expression pattern that is distinct from the T2 inflammation signature in ALI cultures. Primary HBEC ALI cultures were stimulated with IL-6, IL-6/sIL-6R, or IL-4/IL-13. Cells were pretreated (30 minutes) with a JAK1 inhibitor *(JAK1i)* or dimethyl sulfoxide *(DMSO)* control, where indicated. **A-C,** STAT3 phosphorylation. Fig 1, *A* and *B,* Data are representative of 2 independent experiments. Fig 1, *C,* Three different HBEC donors (means and SDs). **D** and **E,** Cells were stimulated for 24 hours, and gene expression was assessed by using RNA sequencing, normalized, and compared with nonstimulated control. The most upregulated genes are shown as heat maps of $\log_2$ fold change. **F,** Expression of indicated genes was analyzed by using quantitative PCR. Mean $\log_2$ fold change values from 3 HBEC donors are shown. **G,** Secreted levels of YKL-40 and MMP3 after 24 hours of stimulation with IL-6 and sIL-6R from 6 HBEC donors. **H,** Cells were stimulated for 24 hours with IL-6/sIL-6R. The effect of IL-6/sIL-6R (vs DMSO, *first column*) and the effect of JAK1i on IL-6/sIL-6R stimulation (IL-6/sIL-6R/JAK1i vs IL-6/sIL-6R/DMSO, *second column*) for the top-induced genes in Fig 1, *D,* is shown as a heat map of $\log_2$ fold change. *$P < .05$, **$P \leq .01$, ***$P \leq .001$, and ****$P \leq .0001$, unpaired *t* test. *NS,* Nonstimulated control.
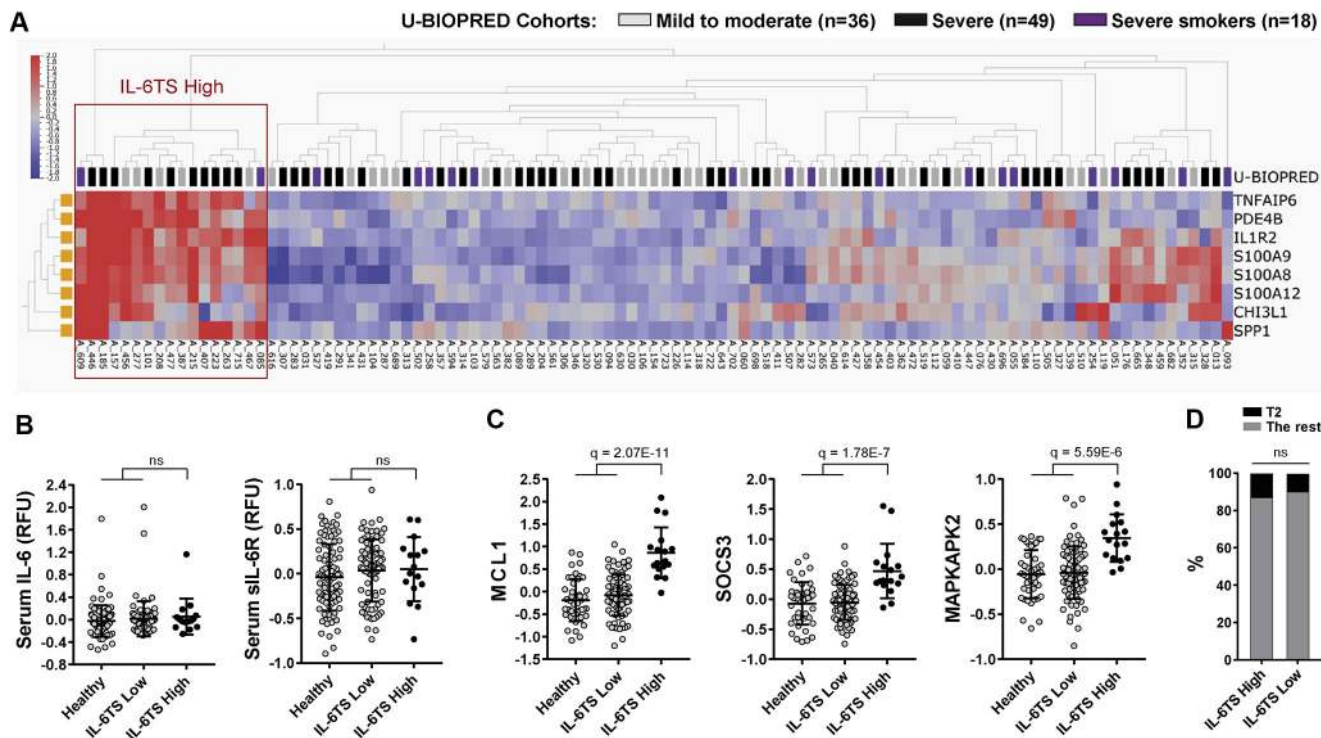
**FIG 2.** The IL-6TS–specific gene signature in bronchial epithelium defines a new IL-6TS–high patient subset within the U-BIOPRED asthma cohorts. **A,** Hierarchical clustering of patients with transcriptomic data derived from bronchial brushings (n = 103) using the IL-6TS–specific gene signature (*TNFAIP6, PDE4B, IL1R2, S100A9, S100A8, S100A12, CHI3L1,* and *SPP1*). High gene expression is denoted in red, and low expression is denoted in blue. The subset of patients with increased IL-6TS gene signature (IL-6TS high) is highlighted *(red box)*. **B,** Serum levels of IL-6 and sIL-6R presented as change in relative fluorescence units *(RFU)*. **C,** Gene expression scores for the main IL-6/JAK/STAT3 pathway genes in bronchial brushings calculated from normalized and zero-centered gene expression values. Protein (Fig 2, *B*) and mRNA (Fig 2, *C*) levels from the IL-6TS–high group (n = 17) were compared with those in the rest of the asthmatic patients (IL-6TS–low group, n = 86) and healthy control subjects (n = 44). Statistically significant differences are indicated (Kruskal-Wallis test for protein levels: $P \le .05$; Benjamini-Hochberg test for gene expression: $q \le 0.05$). **D,** Percentage of subjects with high expression of epithelial T2 inflammation–specific gene signature. The distribution of patients with T2 asthma was not significantly different ($P = .70$, $\chi^2$ test) between the 2 groups. *ns,* Not significant.

www.jacionline.org). Furthermore, IL-6TS–induced gene expression was strongly reduced in the presence of the JAK1 inhibitor, confirming the essential role of the JAK/STAT pathway for downstream IL-6 signaling in lung epithelium (Fig 1, *H*, and see Fig E2, *B*, and Table E4 in this article's Online Repository at www.jacionline.org). Raw and processed data of all RNA sequencing experiments are deposited in the National Center for Biotechnology Information GEO database (accession no. GSE113185).

## IL-6TS–specific gene signature in lung epithelium defines a novel subset of asthmatic patients

To investigate whether the IL-6TS pathway is activated in airway epithelium of patients with asthma, we analyzed the transcriptomic data derived from 103 central airways epithelial brushings from the U-BIOPRED asthma cohorts, including patients with mild-to-moderate (n = 36) and severe (n = 49) asthma and smokers with severe asthma (n = 18). Hierarchical clustering analysis based on the genes most strongly induced by IL-6TS in HBEC ALI cultures identified a set of 8 coclustered genes (*TNFAIP6, PDE4B, IL1R2, S100A9, S100A8, S100A12,*

*CHI3L1,* and *SPP1*) that defined a clear subset of patients (n = 17) with increased expression of these IL-6TS–inducible genes (referred to here as the IL-6TS–high subset; Fig 2, *A*). Unexpectedly, this IL-6TS–high subset did not show increased levels of systemic IL-6 and sIL-6R compared with the remaining asthmatic patients (n = 86; referred to here as the IL-6TS–low subset) or healthy control subjects (n = 95; Fig 2, *B*). The IL-6TS–high subset showed a robust increase in expression of the main IL-6/JAK/STAT3 pathway genes, such as *MCL1, SOCS3,* and *MAPKAPK2*,[37] compared with the IL-6TS–low subset and healthy control subjects (Fig 2, *C*), confirming the connection between the IL-6TS–high subset and IL-6 signaling. Taken together, these results suggest that the identified IL-6TS–high subset is driven by local rather than systemic IL-6 pathway activation.

The same group of patients (apart from smokers) was clustered based on expression of the T2 gene signature (*POSTN, CLCA1,* and *SERPINB2*), which was previously shown to be a hallmark of T2 inflammation,[38] to examine the IL-6TS–high subset in relation to T2 inflammation. There was no enrichment of patients with the epithelial T2 signature in the IL-6TS–high subset (Fig 2, *D*).

**TABLE I.** Clinical characteristics of the IL-6TS–high versus IL-6TS–low subsets from the U-BIOPRED asthma cohorts

| Parameter | IL-6TS-high | IL-6TS-low | P value |
|---|---|---|---|
| Group size | 17 | 86 | |
| Female sex (%) | 41.2 | 53.5 | .35 |
| Body mass index (kg/m$^2$) | 28.3 ± 1.6 | 29.1 ± 0.6 | .70 |
| Age (y) | 43.9 ± 3.9 | 46.2 ± 1.5 | .59 |
| Patients with severe asthma (%) | 70.0 | 64.0 | .6 |
| Smokers with severe asthma (%) | 11.8 | 18.6 | .5 |
| Never smokers (%) | 70.6 | 68.6 | .87 |
| Pack years | 17.8 ± 12.1 | 16.0 ± 3.5 | .78 |
| ICS, high dose (%) | 70.6 | 63.9 | .60 |
| Maintenance OCS (%) | 37.5 (n = 16) | 26.8 (n = 82) | .39 |
| FEV$_1$ (% predicted) | 77.8 ± 5.8 | 80.7 ± 2.3 | .56 |
| Exacerbations (no./previous year) | 3.0 (2.3-4.0 [n = 10]) | 2.0 (1.0-3.0 [n = 52]) | .096 |
| Exacerbations, ≥3/previous year (%) | 70.0 (n = 10) | 36.5 (n = 52) | .049* |
| History of exacerbations (no./year) | 2.5 (0.0-4.0 [n = 16]) | 1 (0.0-2.0) | .13 |
| History of exacerbations, ≥3/y (%) | 50.0 (n = 16) | 23.3 | .028* |
| Positive atopic status (%) | 87.5 (n = 16) | 79.0 (n = 76) | .43 |
| Total IgE (IU/mL) | 131 (43-566) | 105 (40-290) | .48 |
| Serum CRP (μg/mL) | 2 (0.6-5.0) | 1.4 (0.6-3.2) | .64 |
| F$_{ENO}$ (ppb) | 22.7 (19.0-48.8 [n = 16]) | 23.8 (16.0-54.5 [n = 82]) | .98 |
| Blood eosinophils (× 1000/μL) | 0.3 (0.14-0.5) | 0.2 (0.1-0.3) | .050* |
| Blood eosinophils, >300/μL (%) | 47.0 | 15.1 | .0028* |
| Sputum eosinophils (%) | 1.7 (0.8-26.2 [n = 10]) | 0.7 (0-4.6 [n = 33]) | .14 |
| Sputum eosinophils, >3% (%) | 40.0 (n = 10) | 33.3 (n = 33) | .70 |
| Sputum eosinophils, >20% (%) | 40.0 (n = 10) | 6.1 (n = 33) | .007* |
| Blood neutrophils (× 1000/μL) | 4.4 (3.4-7.3) | 4.1 (3.0-5.9) | .46 |
| Sputum neutrophils (%) | 61.9 (47.7-76.5 [n = 10]) | 45.3 (31.9-63.4 [n = 33]) | .13 |
| Sputum neutrophils, >60% (%) | 50.0 (n = 10) | 30.3 (n = 33) | .25 |
| Sputum macrophages (%) | 16.7 (7.2-28.4 [n = 10]) | 44.0 (27.6-63.7 [n = 33]) | .0089* |
| Sputum macrophages (no.) | 125.0 ± 36.9 (n = 10) | 235.3 ± 20.7 (n = 33) | .011* |
| Submucosal CD3$^+$ T cells (cells/mm$^2$) | 70.8 ± 15.0 (n = 17) | 35.7 ± 3.6 (n = 70) | .0008* |
| Submucosal CD4$^+$ T cells (cells/mm$^2$) | 29.3 ± 6.3 (n = 17) | 10.9 ± 1.3 (n = 70) | .00007* |
| Submucosal CD8$^+$ T cells (cells/mm$^2$) | 38.2 ± 6.9 (n = 17) | 17.2 ± 1.9 (n = 70) | .0003* |
| Submucosal CD68$^+$ macrophages (cells/mm$^2$) | 7.0 ± 1.3 (n = 17) | 3.1 ± 0.4 (n = 70) | .0011* |

Data are presented as percentages, means ± SEs, or medians (interquartile ranges [Q1-Q3]). Number of subjects is 17 in the IL-6TS–high subset and 86 in the IL-6TS–low subset, unless stated otherwise. For *P* values, the Kruskal-Wallis test was used for continuous data, and the χ$^2$ test was used for categorized data.

*CRP*, C-reactive protein; *F$_{ENO}$*, fraction of exhaled nitric oxide; *ICS*, inhaled corticosteroids; *ICS, high dose*, 1000 μg or more fluticasone or equivalent; *OCS*, oral corticosteroids.
*Statistically significant (*P* ≤ .05).

## IL-6TS–high patient subset is characterized by frequent exacerbations, eosinophilic airway inflammation, and increased numbers of T cells and macrophages infiltrating the airway submucosa

The IL-6TS–high and IL-6TS–low subsets did not differ in respect to sex, steroid treatment, atopic status, or smoking history. The proportion of patients with 3 or more exacerbations per year was significantly greater in the IL-6TS–high subset compared with the IL-6TS–low subset (Table I and see Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, the number of patients with blood (>300 cells/μL) and sputum (>20%) eosinophilia was significantly increased in the IL-6TS–high subset, whereas the absolute numbers and proportions of sputum macrophages were decreased compared with those in the IL-6TS–low subset. Previous studies have shown IL-6 to be associated with neutrophilic inflammation,[19,39] but even though we observed a trend toward increased sputum neutrophil numbers in the IL-6TS–high subset compared with the IL-6TS–low subset, the difference did not reach statistical significance.

Immunohistochemical analysis of bronchial biopsy specimens showed significantly greater numbers of total immune cells, including mast cells, neutrophils, eosinophils, T cells, and macrophages, in the submucosa of patients with IL-6TS–high versus IL-6TS–low asthma (Fig 3, *A*). This difference was driven mainly by a striking increase in infiltration of CD3$^+$, CD4$^+$, and CD8$^+$ T cells and CD68$^+$ macrophages (*P* = .0008, *P* = .00007, *P* = .0003, and *P* = .001, respectively) but not by granulocytes (Fig 3, *B* and *C*, and Table I).

## Reduced expression of genes related to lung epithelial barrier function in the IL-6TS–high patient subset

We found reduced expression of several genes involved in barrier function in epithelial brushings from the IL-6TS–high subset, with major differences observed for expression of the epithelial cell junction components β-catenin (*CTNNB1*); claudins 1, 8, and 18 (*CLDN1/CLDN8/CLDN18*); and the gene encoding zonula occludens-1 (all *q* < 0.05), whereas there was no difference in expression of occludin (*OCLN*; Fig 4, *A*).

To evaluate the functional effect of IL-6TS on epithelial barrier integrity *in vitro*, we performed prolonged stimulation of HBEC
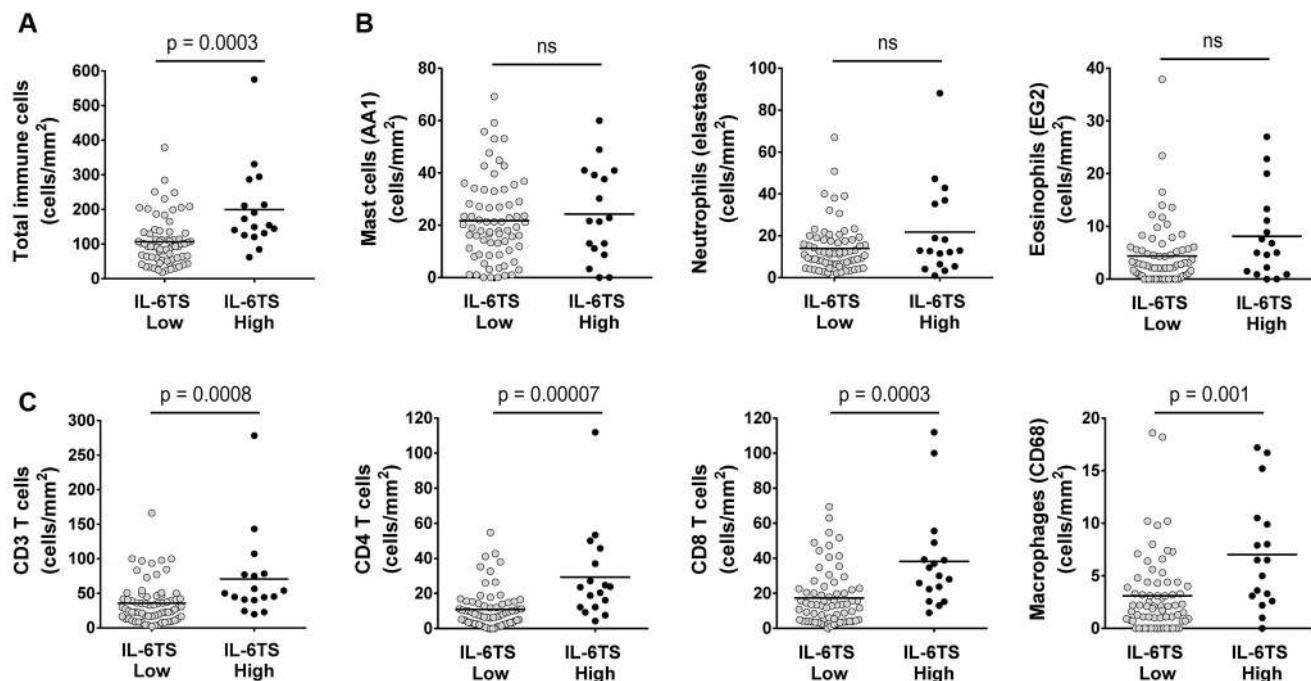
**FIG 3.** The U-BIOPRED IL-6TS–high subset is characterized by significantly higher numbers of T-cell and macrophage infiltration in the submucosa. Immunohistochemical analysis of immune cells in glycol methacrylate resin–embedded biopsy specimens from patients with IL-6TS–high (n = 17) and IL-6TS–low (n = 70) asthma from the U-BIOPRED asthma cohorts. **A,** Total immune cells. **B,** AA1^{+} mast cells, elastase-positive neutrophils, and EG2^{+} eosinophils. **C,** CD3^{+}, CD4^{+} and CD8^{+} T cells and CD68^{+} macrophages. Statistically significant differences ($P \le .05$, Kruskal-Wallis test) are indicated. *ns*, Not significant.

ALI cultures and observed that a combination of IL-6/sIL-6R, but not IL-6 or sIL-6R on their own, resulted in extensive loss of epithelial integrity, as measured based on transepithelial electrical resistance (Fig 4, *B* and *C*). These results suggest that IL-6TS contributes specifically to compromised epithelial integrity as a feature of asthma involving local activation of the IL-6TS pathway.

## Transcriptional profiling links the IL-6TS–high subset with immune cell migration and Toll-like receptor signaling

Separation of the identified IL-6TS–high subset from the rest of the patients was evident also when comparing global transcription profiles (20,233 available mRNAs) in lung epithelial brushings. Remarkably, a total of 4417 transcripts were expressed differentially ($q < 0.05$) in the IL-6TS–high subset compared with the IL-6TS–low subset, and 6491 transcripts were expressed differentially compared with healthy subjects.

Involvement of the differentially expressed transcripts in the IL-6TS–high subset in specific pathophysiologic processes was investigated by using IPA disease function analysis. This showed a striking enrichment of genes involved in activation of cell movement, especially migration and chemotaxis of leukocytes (see Table E5 in this article's Online Repository at www. jacionline.org), an observation well-aligned with the increased submucosal infiltration of immune cells detected in the IL-6TS–high subset (Fig 3). Activation of the same cell migration functions emerged after analysis of the transcripts induced by IL-6/ sIL-6R stimulation of ALI cultures, providing a further

connection between IL-6TS signaling and immune cell infiltration, as observed in the IL-6TS–high subset (see Table E5).

MetaCore pathway analysis identified Toll-like receptor (TLR) signaling as the most strongly activated pathway in the IL-6TS–high subset (see Table E6 in this article's Online Repository at www.jacionline.org), which is in agreement with identification of *TLR2*, *TLR4*, *MYD88*, and *CD14* as upregulated genes in these subjects (Fig 5, *A*). In addition, there was increased expression of *TREM1*, which is known to cooperate with TLR signaling,[40] and several proinflammatory mediators directly induced by TLR2 and TLR4, such as *IL8*, *CCL4* (macrophage inflammatory protein [*MIP*] 1β), *IL-1β*, *IL6*, and *TNF-α* (Fig 5).

## Confirmation of the IL-6TS–high asthma subset in independent replication cohorts

Next, we sought to confirm the existence of an IL-6TS–high subset in 2 independent replication cohorts of asthmatic patients. Hierarchical clustering of transcriptomic data based on the IL-6TS–specific 8-gene signature was done in a cohort including 38 patients with mild-to-moderate or severe asthma (replication cohort 1)[41,42] and in a cohort including 17 patients with severe asthma (replication cohort 2) with epithelial brushing samples from central and/or peripheral airways.[43] This analysis clearly clustered patients from both cohorts into an IL-6TS–high and an IL-6TS–low subsets (see Fig E4, *A* and *B*, in this article's Online Repository at www.jacionline.org). Importantly, the IL-6TS–high subset was again associated with gene upregulation related to TLR signaling, including *TLR2*, *MYD88*, *TREM1*, and *CCL4* (see Fig E4, *C*).
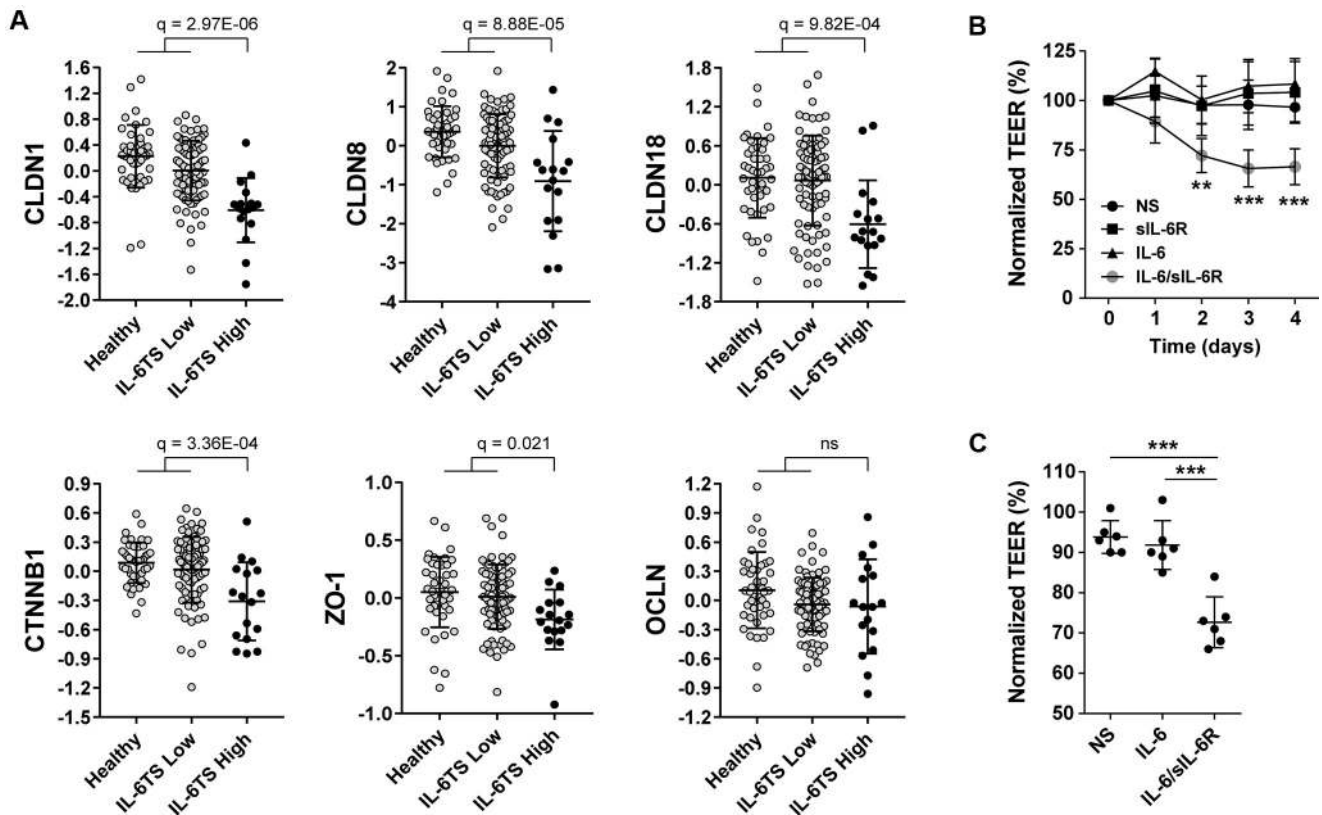
**FIG 4.** Decreased expression of epithelial junction proteins in patients with IL-6TS–high asthma from the U-BIOPRED cohorts. **A,** mRNA levels of epithelial junction components in bronchial brushings from the U-BIO-PRED asthma cohorts. Gene expression from the IL-6TS–high group (n = 17) was compared with that in the rest of the asthmatic patients (IL-6TS–low group, n = 86) and healthy control subjects (n = 44). Gene expression scores, calculated from normalized and zero-centered gene expression values, are shown. Statistically significant differences are indicated. $q \leq 0.05$, Benjamini-Hochberg test. *ns*, Not significant. **B,** Transepithelial electrical resistance *(TEER)* was measured in HBEC ALI cultures after stimulation with sIL-6R (150 ng/mL), IL-6 (150 ng/mL), and IL-6/sIL-6R (both 150 ng/mL). Data are representative of 2 independent experiments. **$P \leq .01$ and ***$P \leq .001$, unpaired *t* test. **C,** Effect of IL-6TS on reduction of epithelial integrity after 48 hours of stimulation with 20 ng/mL IL-6 and 50 ng/mL sIL-6R was confirmed in 6 different HBEC donors. ***$P \leq .001$, unpaired *t* test. *NS*, Nonstimulated control.

## IL-6TS surrogate sputum biomarkers YKL-40, MMP3, IL-8, MIP-1β, and IL-1β define an IL-6TS–high subset in the Wessex Severe Asthma Cohort

A putative IL-6TS–high patient subset was identified by means of hierarchical clustering of asthmatic patients (n = 146; British Thoracic Society [BTS] groups 4 and 5) from the Wessex Severe Asthma Cohort[22] based on levels of 5 sputum biomarkers associated strongly with IL-6TS, namely YKL-40 and MMP3 surrogate biomarkers induced by IL-6TS stimulation of ALI cultures *in vitro* (Fig 1, *F* and *G*) and IL-8, MIP-1β, and IL-1β, which were found to be significantly overexpressed in epithelial brushings of IL-6TS–high patients from the U-BIOPRED cohort (Fig 5, *B*).

The Wessex IL-6TS–high subset (n = 24) was comparable with the U-BIOPRED IL-6TS–high subset in size (around 16% of the asthmatic patients in both cohorts), as well as in clinical characteristics (Fig 6, *A*, and Table II), including significantly increased blood eosinophil numbers (*P* = .0008), higher percentages of patients with blood eosinophilia (>300 cells/μL; *P* = .0004), and a reduced proportion of sputum macrophages

(*P* = .009). In addition, sputum neutrophil counts were increased in the Wessex IL-6TS–high patient subset (*P* = .01).

Importantly, the Wessex IL-6TS–high subset was associated with significantly increased levels of sputum sIL-6R (*P* < .0001) and IL-6 (*P* < .0001), whereas there was no difference in serum C-reactive protein levels (Fig 6, *B-D*), providing evidence for a local IL-6TS–driven inflammation. Also, there was no difference in levels of classical T2 biomarkers, including serum periostin (Fig 6, *E*) or sputum IL-5 and eotaxin (see Fig E5, *E* and *F*, in this article's Online Repository at www.jacionline.org).

## DISCUSSION

Although increased levels of IL-6 and sIL-6R in asthmatic patients have been observed by others,[22,23,44] the molecular understanding of the role of IL-6 signaling in asthmatic airways is limited. Here we generated a gene signature induced by activation of IL-6TS in HBEC ALI cultures and used this signature to identify an asthmatic patient subset in the U-BIOPRED cohorts with signs of lung epithelial IL-6TS pathway activation. We revealed
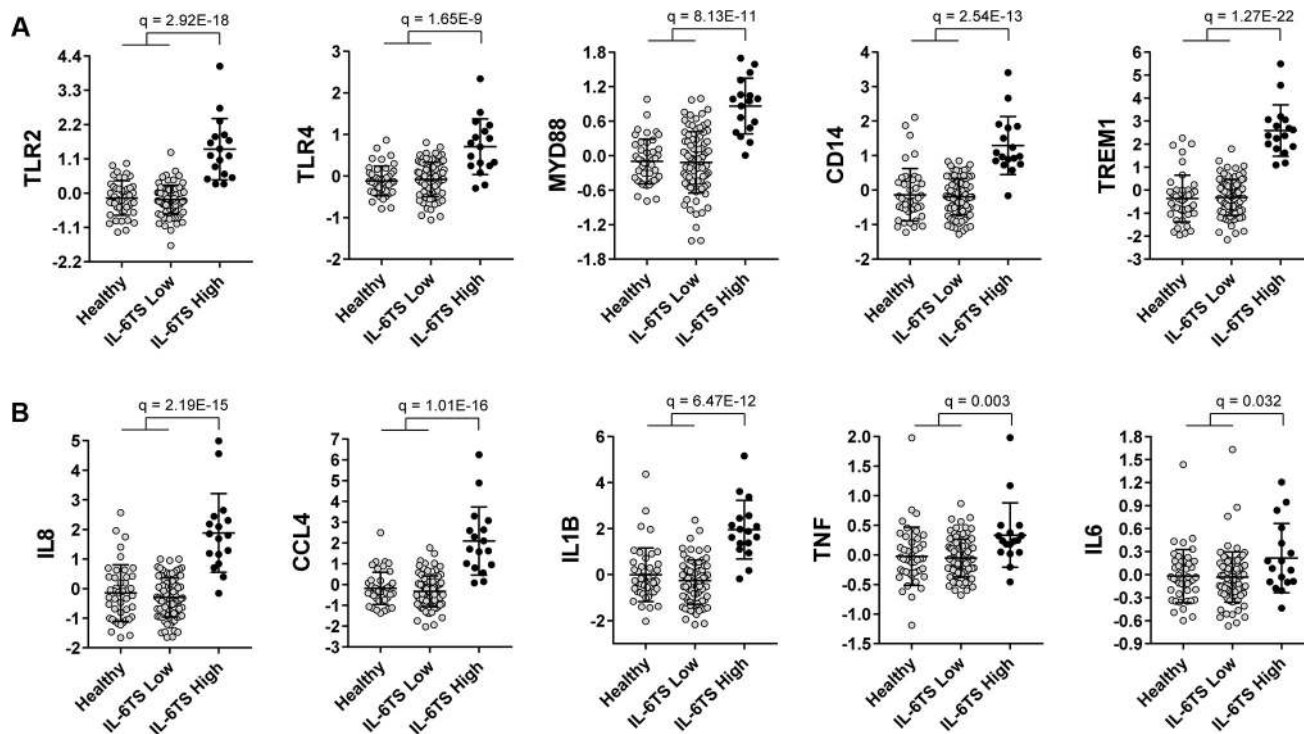
**FIG 5.** TLR pathway genes are upregulated in bronchial brushings from U-BIOPRED patients with IL-6TS–high asthma. mRNA levels of TLR signaling and costimulatory molecules **(A)** and TLR signaling–inducible proinflammatory mediators **(B)** in bronchial brushings from the U-BIOPRED asthma cohorts. Gene expression from the IL-6TS–high group (n = 17) were compared with that of the rest of the asthmatic patients (IL-6TS–low group, n = 86) and healthy control subjects (n = 44). Gene expression scores are calculated from normalized and zero-centered gene expression values. Statistically significant differences are indicated. $q \leq .05$, Benjamini-Hochberg test.

that patients with IL-6TS–driven asthma were characterized by a history of frequent exacerbations and significantly increased submucosal infiltration of T cells and macrophages. Furthermore, they showed reduced expression of genes regulating epithelial barrier function and an increased expression of TLR pathway activation and remodeling genes in their epithelial brushings. Interestingly, the IL-6TS–high asthma subset had increased blood eosinophil counts despite not being enriched with patients with T2 inflammation in the lung epithelium. This suggests that the eosinophilia observed in the IL-6TS–high subset is disconnected from T2 inflammation and results in a direct or indirect consequence of IL-6 signaling in the airways. Our results emphasize that asthmatic patients with eosinophilia need to be further stratified with respect to the biological driver of the eosinophilic lung infiltration, which might not necessarily be T2 inflammation.[38] Although the understanding of the mechanism leading to systemic eosinophilia in the IL-6TS–high subset requires further study, it is tempting to speculate that IL-6TS promotes the observed phenotype by inducing IL-33 expression, which is a potent activator of mature eosinophils,[45] and it can also regulate eosinophil development within the bone marrow[46] and tissue.[47] In support of this hypothesis, *IL33* was one of the genes most strongly induced on stimulation of primary bronchial epithelial cells with IL-6/sIL-6R but not with IL-4/IL-13 (Fig 1, *D* and *F*, and see Fig E1, *B*).

Importantly, despite upregulation of IL-6/JAK/STAT3 pathway genes in epithelial brushings from the IL-6TS–high subset, these patients did not show increased serum IL-6 and sIL-6R levels. This suggests that the IL-6TS–high subset described in this study is distinct from the recently described IL-6–high asthma subset characterized by systemic IL-6 inflammation, metabolic dysfunction, and obesity[19] and instead represents a separate entity with local IL-6TS–driven airway inflammation.

Activation of the IL-6 pathway has been associated previously with neutrophilic and mixed granulocytic airway inflammation in asthmatic patients.[48,49] A connection between IL-6–dependent inflammation and a mixed granulocytic phenotype was observed also in a mouse cockroach-induced asthma model, where specific blocking of IL-6TS reduced accumulation of both eosinophils and neutrophils in BALF.[48] However, although the IL-6TS–high subset described in this study was significantly enriched with highly eosinophilic patients, there were no significant differences in blood or sputum neutrophil counts between the IL-6TS–high and IL-6TS–low subsets. Instead, we observed a striking increase of airway submucosal T-cell populations and macrophages in the IL-6TS–high subset, indicating increased airway inflammation in these patients. In line with this observation, a link between the IL-6TS–high subset and activation of immune cell migration was shown by using IPA of differentially expressed transcripts between the IL-6TS–high and IL-6TS–low subsets. The correlation between IL-6TS and immune cell infiltration is also consistent with previous studies showing that IL-6TS is essential for macrophage and T-cell recruitment to the site of acute inflammation in mice.[11,50] IL-6TS has been proposed to coordinate a temporal
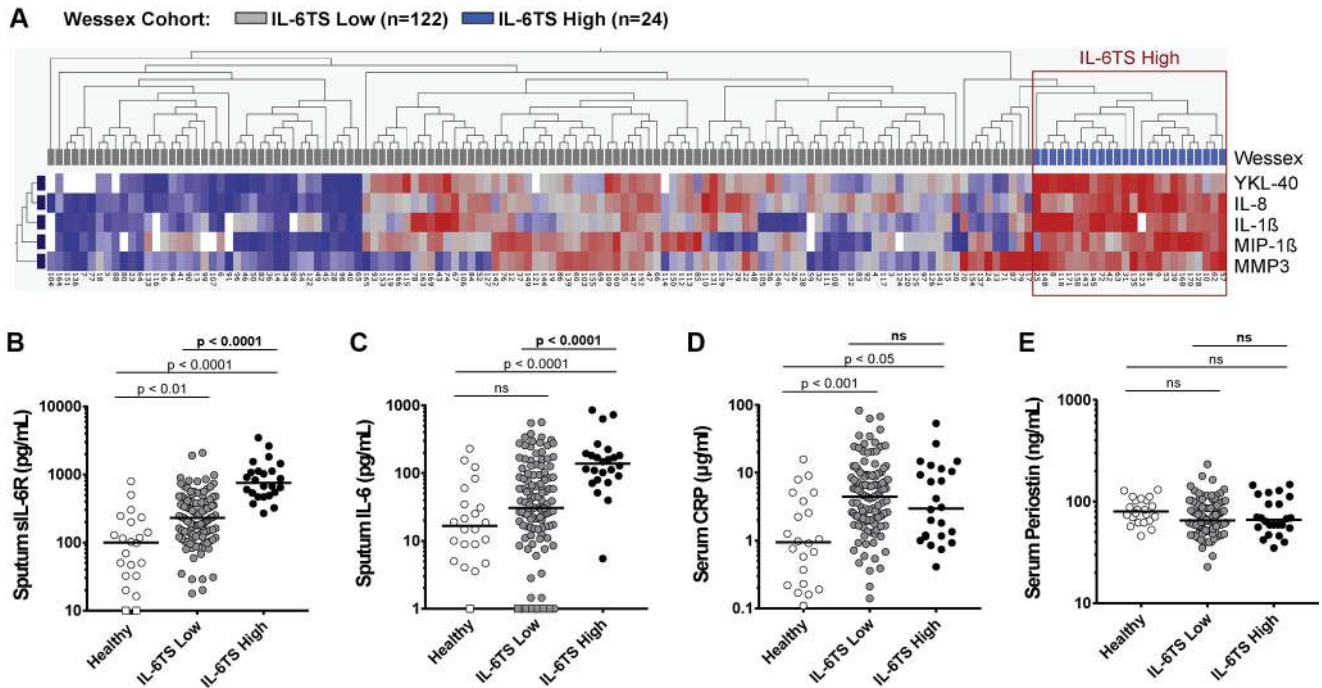
**FIG 6.** Identifying and characterizing the IL-6TS–high subset in the Wessex Asthma Cohort based on IL-6TS surrogate biomarkers in sputum. **A,** Identification of the IL-6TS–high subset in the Wessex Asthma Cohort by hierarchical clustering of patients (BTS groups 4 and 5, n = 146) based on high (red) versus low (blue) expression of the IL-6TS–high surrogate biomarkers YKL-40, IL-8, MIP-1β, IL-1β, and MMP3 in sputum. Subset of patients with increased IL-6TS gene signature (IL-6TS–high group) is highlighted *(red box)*. **B** and **C,** Sputum sIL-6R and IL-6 levels in the Wessex IL-6TS–high subset compared with the IL-6TS–low subset and healthy control subjects. **D** and **E,** Serum C-reactive protein *(CRP)* and periostin levels in the Wessex IL-6TS–high subset compared with the IL-6TS–low subset and healthy control subjects. $Log_{10}$ transformed data are shown. Values of less than the detection range are marked by *squares*. Median values and statistically significant differences are indicated. $P \le .05$, Kruskal-Wallis test. *ns*, Not significant.

switch from initial infiltration of neutrophils to a more sustained population of mononuclear leukocytes by regulating the expression of CXC and CC chemokines,[11] which could potentially explain the absence of a strong neutrophilic inflammation component in the airways of patients in the U-BIOPRED IL-6TS–high subset.

Another key feature of the patients with IL-6TS–high asthma was decreased levels of several cell junction transcripts, with particularly low levels of claudins (1, 8, and 18) and β-catenin. Furthermore, we demonstrated that IL-6TS impairs epithelial integrity *in vitro* in HBEC ALI cultures, providing a direct link between IL-6TS and epithelial dysfunction. Previous reports have shown that dysfunctional cell junctions are closely connected to the severity and progression of asthma and that decreased expression of cell junction proteins, such as zonula occludens-1, claudins, and β-catenin, in asthmatic patients might result in cell junction disruption and decreasing epithelial barrier function.[51-54]

Epithelial barrier defects and reduced cell junction integrity in the intestines are linked to microbial dissemination and inflammation through TLR signaling, and it has been shown that *Haemophilus influenzae* and *Streptococcus pneumoniae* exploit TLR2- and TLR4-mediated downregulation of cell junction components to facilitate translocation across the lung epithelium.[55-57] Notably, TLR signaling was the most significantly activated pathway in the IL-6TS–high subset, as shown by using MetaCore

pathway analysis of differentially expressed transcripts. In addition to the significant upregulation of essential TLR pathway molecules, patients in the IL-6TS–high subset also exhibited significantly increased expression of proinflammatory mediators (IL-8, CCL-4 [MIP-1β], IL-1β, IL-6, and TNF-α) known to be induced in response to TLR2 and TLR4 triggering.[58,59] These results point to hyperactivation of TLR pathways in response to bacterial colonization as one possible cause of the reduced expression of cell junction proteins and the promotion of submucosal inflammation. An important clinical phenotype of the IL-6TS–high subset was the history of frequent exacerbations, which could be related to colonization of the airways, with pathogens such as *H influenzae*[60] triggering an uncontrolled innate inflammatory response.

The potential connection between the IL-6TS–high subset and microbial colonization is supported by a recent publication showing increased sputum bacterial numbers in a subset of patients with asthma and chronic obstructive pulmonary disease, the so-called cluster 2 or IL-1β–high cluster, which is also characterized by significantly increased sputum IL-6 and sIL-6R levels.[44]

In a replication cohort of patients with treatment-resistant severe asthma all receiving high doses of inhaled steroids,[43] the IL-6TS–specific signature was present in nearly half of the patients, suggesting that the IL-6TS–high phenotype correlates

**TABLE II.** Clinical characteristics of the IL-6TS–high versus IL-6TS–low subsets from the Wessex Severe Asthma Cohort

| Parameter | IL-6TS-high | IL-6TS-low | P value |
|---|---|---|---|
| Group size | 24 | 122 | |
| Female sex (%) | 58.3 | 57.4 | .93 |
| Body mass index (kg/m$^2$) | 31.8 ± 0.7 | 30.7 ± 1.4 | .57 |
| Age (y) | 46.5 ± 2.6 | 51.3 ± 1.1 | .09 |
| Current smokers (%) | 8.3 | 8.2 | .98 |
| Never smokers (%) | 50.0 | 48.4 | .59 |
| Pack years | 10.2 ± 3.7 | 9.9 ± 1.3 | .76 |
| ICS dose (equivalent μg of BDP) | 1940 (1600-2000) | 1940 (1600-2060) | .67 |
| Maintenance OCS (%) | 29 | 31 (n = 119) | .79 |
| Prednisolone dose (among OCS users) | 10 (10-10) | 10 (5-15) | 1.00 |
| FEV$_1$ (% predicted) | 66.5 ± 2.1 | 63.9 ± 4.4 | .62 |
| Positive atopic status (%) | 79.2 | 69.7 | .35 |
| Total IgE (IU/mL) | 126 (25-384 [n = 23]) | 190 (68-531 [n = 113]) | .19 |
| F$_{ENO}$ (ppb) | 23 (13.3-42.5 [n = 23]) | 14 (8.3-24.9 [n = 109]) | .023* |
| Blood eosinophils (× 1000/μL)† | 0.51 (0.34-0.91) | 0.21 (0.11-0.49) | .0008* |
| Blood eosinophils, >300/μL (%) | 79.2 | 38.7 | .0004* |
| Sputum eosinophils (%) | 1 (0.33-7.65 [n = 23]) | 1.25 (0.25-8.5 [n = 113]) | .88 |
| Sputum eosinophils >3% (%) | 30.4 (n = 23) | 43.4 (n = 113) | .25 |
| Sputum eosinophils >20% (%) | 8.7 (n = 23) | 14.2 (n = 113) | .48 |
| Blood neutrophils (× 1000/μL)† | 6.07 (3.9-7.5) | 6.12 (4.4-8.1) | .60 |
| Sputum neutrophils (%) | 68.4 (42.8-80.3) | 45.4 (26.1-64.1) | .010* |
| Sputum neutrophils >60% (%) | 62.5 | 31.1 | .004* |
| Sputum macrophages (%) | 18.8 (6.7-36.5) | 36.2 (23.6-56.0) | .009* |

Data are presented as percentage, means ± SEs, or medians (interquartile ranges [Q1-Q3]). Number of subjects is 24 in the IL-6TS–high subset and 122 in the IL-6TS–low subset, unless stated otherwise. For P values, the Kruskal-Wallis test was used for continuous data, and the χ$^2$ test was used for categorized data.

*BDP*, Beclomethasone dipropionate; *F$_{ENO}$*, fraction of exhaled nitric oxide; *ICS*, inhaled corticosteroids; *OCS*, oral corticosteroids.

*Statistically significant (P ≤ .05).

†Statistical tests were performed on transformed data.

with disease severity. In yet another replication cohort consisting of patients with mild-to-moderate and severe asthma,[41,42] the IL-6TS–high subset overlapped with the majority of patients with severe asthma, further strengthening the association of epithelial IL-6TS with asthma severity.

It should be noted that identification of the IL-6TS–high subset in the U-BIOPRED cohort was based on a relatively small number of epithelial brushings with available transcriptomic data. To overcome this limitation, we validated the findings from U-BIOPRED in a larger sputum biomarker data set obtained from the Wessex Severe Asthma Cohort.[22] In this cohort a putative IL-6TS–high subset was identified by using hierarchical clustering of asthmatic patients based on levels of the IL-6TS surrogate biomarkers YKL-40, MMP3, IL-8, MIP-1β, and IL-1β in induced sputum. As in U-BIOPRED, the Wessex IL-6TS–high patients were characterized by blood eosinophilia, without a significant increase in levels of T2 biomarkers. Furthermore, the IL-6TS–high subset was associated with increased sputum neutrophil counts, supporting the connection between IL-6 signaling and neutrophilic inflammation proposed by other studies.[19,39] Finally, the significantly increased sputum levels of sIL-6R and IL-6 directly link the Wessex IL-6TS–high subset to a local IL-6–driven inflammatory response.

The role of local IL-6 signaling as a disease driver in patients with chronic airway disease is supported further by a transgenic mouse model driven by local overexpression of IL-6 in lung tissue. These mice showed emphysema-like airspace enlargement, thickening of the airway walls, subepithelial airway fibrosis, and accumulation of mononuclear immune cells in the peribronchiolar space.[61]

In conclusion, we used an IL-6TS–specific epithelial gene signature to identify and describe a novel asthmatic patient subset with IL-6TS pathway activation in the lung epithelium as a biological driver. We showed that these patients constitute a molecular phenotype characterized by an increased exacerbation rate, T2 inflammation–independent eosinophilia, increased submucosal inflammation, activation of innate signaling pathways, increased markers of airway remodeling, and decreased expression of epithelial junctions components. This novel IL-6TS–high asthma subset appears to be associated with poor asthma control, and our results might provide one further step toward a stratified medicine approach to addressing a significant unmet medical need in patients with severe asthma.

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## Key messages

- A subset of asthmatic patients (IL-6TS–high subset) distinct from T2 asthma shows lung epithelial IL-6TS pathway activation in the absence of systemic IL-6 inflammation.
- The IL-6TS–high subset constitutes a novel asthma phenotype associated with frequent exacerbations, eosinophilia, airway inflammation, remodeling, and impaired epithelial integrity.
- Identification of local airway IL-6TS pathway activation as a potential disease driver might open up a stratified medicine approach to treating asthma.

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