ORIGINAL ARTICLE

IL-33 Expression Is Lower in Current Smokers at both Transcriptomic and Protein Levels

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Abstract

Rationale: IL-33 is a proinflammatory cytokine thought to play a role in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD). A recent clinical trial using an anti–IL-33 antibody showed a reduction in exacerbation and improved lung function in ex-smokers but not current smokers with COPD.

Objectives: This study aimed to understand the effects of smoking status on IL-33.

Methods: We investigated the association of smoking status with the level of gene expression of *IL-33* in the airways in eight independent transcriptomic studies of lung airways. Additionally, we performed Western blot analysis and immunohistochemistry for IL-33 in lung tissue to assess protein levels.

Measurements and Main Results: Across the bulk RNA-sequencing datasets, *IL-33* gene expression and its signaling pathway were significantly lower in current versus former or never-smokers and increased upon smoking cessation ($P < 0.05$). Single-cell sequencing showed that *IL-33* is predominantly expressed in resting basal epithelial cells and decreases during the differentiation process triggered by smoke exposure. We also found a higher transitioning of this cellular subpopulation into a more differentiated cell type during chronic smoking, potentially driving the reduction of *IL-33*. Protein analysis demonstrated lower IL-33 levels in lung tissue from current versus former smokers with COPD and a lower proportion of IL-33–positive basal cells in current versus ex-smoking controls.

Conclusions: We provide strong evidence that cigarette smoke leads to an overall reduction in IL-33 expression in transcriptomic and protein level, and this may be due to the decrease in resting basal cells. Together, these findings may explain the clinical observation that a recent antibody-based anti–IL-33 treatment is more effective in former than current smokers with COPD.

Keywords: IL-33; gene expression; cigarette smoke; basal cell; COPD

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Chronic obstructive pulmonary disease (COPD) is a highly prevalent disease characterized by progressive airflow limitation, shortness of breath, cough, and sputum production and is associated with a significant health care burden (1). Despite receiving standard-of-care treatments, including bronchodilators and antiinflammatory therapies, a subset of patients with COPD continues to experience exacerbations and accelerated decline in lung function (2). An enormous unmet need exists to identify novel disease-modifying therapies for COPD.

IL-33 is a member of the IL-1 family, which plays an essential role in innate and adaptive immunity. It is a proinflammatory cytokine, or alarmin, localized in the nuclei of various cell types, including airway epithelial cells, endothelial cells, and fibroblasts, which send signals to these cell types to produce inflammatory cytokines in response to infection and tissue injury (3, 4). IL-33 is rapidly released into the extracellular space following cellular damage, e.g., by exposure to viruses and cigarette smoke or air pollutants (5, 6). As a ligand, IL-33 binds to its cognate receptor, ST2 (suppression of tumorigenicity 2), also known as IL1RL1. Subsequently, it engages the IL-1RAcP (IL-1 receptor accessory protein) as a coreceptor to initiate signaling and activation of downstream inflammatory pathways (6). As such, IL-33 can activate type 2 immune cells expressing high levels of ST2, e.g., Th2 cells, ILC2s (type 2 innate lymphoid cells), mast cells, basophils, and eosinophils (5, 7). Over the years, *IL-33* gene expression has been associated with the pathogenesis of lung diseases, and human gene expression studies demonstrated a clear link between *IL-33* and the risk for asthma and COPD (8–10). Several studies have shown that smoking, the major risk factor for COPD, induces inflammation and influences *IL-33* expression (11–14). Moreover, lung homogenates from patients with COPD reveal that higher *IL-33* gene expression correlates with severe airflow obstruction (15).

IL-33 has been a target of therapeutic development in asthma and COPD (8, 9). In mouse studies, neutralizing IL-33 with an anti–IL-33 monoclonal antibody, itepekimab, improved airway inflammation and tissue remodeling in a house dust mite–induced model of asthma (16). In patients with moderate to severe asthma, blockade of the IL-33 pathway with itepekimab resulted in *1*) a significantly reduced proportion of patients with asthma exacerbation and *2*) improved lung function relative to placebo (8). In another proof-of-concept clinical trial including patients with moderate to severe COPD, itepekimab led to a numerical but nonsignificant decrease in the annualized rate of exacerbations and significantly improved prebronchodilator FEV_1 by 60 ml (9). The benefit was highest in the subgroup of ex-smokers, with a statistically significant 42% reduction of exacerbations and 90-ml improvement in FEV_1. In contrast, in current smokers, there was no benefit at all in those end points. These findings indicate that anti–IL-33 treatment is most effective in ex-smokers, although the mechanism behind this observation remains unclear (9, 17).

In this study, we investigated the influence of smoking status on the expression of *IL-33* and its receptors, *IL1RL1* and *IL1RAP*, in bronchial biopsies and bronchial brushings to identify cellular mechanisms of differential responses to anti–IL-33 therapies between current and ex-smokers. Moreover, we assessed IL-33 protein levels as well as their splice variants in lung tissue. Some of the results of these studies have been previously reported in the form of abstracts (18, 19).

Methods

We investigated the expression of *IL-33* and its receptors in eight different studies of lung airway. In five of these studies, we looked at the average gene expression (bulk RNA-sequencing [RNA-seq] or microarray), which looks at the average expression of the genes per participant. In the other three studies, we looked at the gene expression at a single-cell level (single-cell RNA-seq [scRNA-seq]), meaning investigating the expression of IL-33 and related receptors at an individual cell level for each participant or sample. A brief description of the studies is given below (Figure 1, Table 1, and Table E1 in the online supplement).

Bulk RNA-Seq/Microarray Studies

The Stop Smoking Study (STOP) used RNA-seq to investigate the effects of smoking on gene expression in a longitudinal setting (20). The study collected bronchial biopsy specimens from participants ($N = 16$) before and after smoking cessation. The study aimed to identify gene expression changes that occur as a result of smoking cessation.

The Groningen and Leiden Universities Study of Corticosteroids in Obstructive Lung Disease (GLUCOLD) study used gene expression profiles from bronchial biopsies to investigate the effects of smoking on COPD (21, 22). The study collected biopsies from current ($n = 33$) and ex-smokers ($n = 46$) with moderate to severe COPD at a baseline time point. It is important to note that 95% of participants were steroid-naive and 5% had no steroid treatment in the previous 6 months. The study analyses were conducted using microarray, with a subset also conducted with RNA-seq.

The COPD microarray study used a cross-sectional design to investigate gene expression changes in current ($n = 30$) and

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the Subject: IL-33 gene expression has been associated with the pathogenesis of lung diseases. Human gene-expression studies have demonstrated a link between IL-33 and the risks of asthma and chronic obstructive pulmonary disease (COPD). Several studies have shown that smoking, the major risk factor for COPD, induces inflammation and influences IL-33 expression. IL-33 protein expression has been shown to be specific to the basal cells in the airways. A previous clinical trial using an anti–IL-33 antibody showed a reduction in exacerbations and improved lung function in ex-smokers but not current smokers with COPD.

What This Study Adds to the Field: We investigated the association between IL-33 levels and smoking status and whether this was related to changes in cellular composition in the airways. IL-33 gene expression and protein levels were found to be lower in current smokers than in never-/ex-smokers. IL-33 expression was found only in a subset of basal cells known as resting basal cells and was lost quickly after differentiation to suprabasal cells. The abundance of resting basal cells was found to be lower during smoke exposure, whereas suprabasal cell levels were higher *in vitro* and *in vivo*. The present study provides evidence that the clinical observation of greater efficacy of anti–IL-33 treatment in ex-smokers versus current smokers with COPD may be due to the inherently lower levels of IL-33 expression in current smokers.

ex-smokers ($n = 57$) with COPD (23). The study collected large-airway bronchial brush samples and used Affymetrix HG 1.0 ST arrays to acquire gene expression profiles. The study aimed to identify specific genes and pathways that are altered in COPD as a result of smoking.

The Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects (NORM) study aimed to compare gene expression profiles of never-smokers ($n = 40$) and current smokers ($n = 37$) (24). The study collected bronchial brushing and biopsy specimens from healthy never- and current smokers and aimed to identify genes and pathways that are specifically affected by smoking in healthy individuals.

The Cancer Research UK Papworth Hospital (CRUKPAP) study recruited donors, including never-smokers ($n = 8$), ex-smokers ($n = 151$), and current smokers ($n = 77$) being investigated for suspicion of lung cancer (25). The study collected bronchial brushing samples and used short-read total RNA-seq to investigate gene expression changes in relation to cigarette smoking status.

scRNA-Seq Studies

We investigated one *in vivo* and two *in vitro* scRNA-seq studies to understand the transcriptomic dynamics of IL-33 in response to smoke exposure. The *in vivo* study used scRNA-seq on the bronchial brushing samples of healthy current smokers ($n = 6$) and never-smokers ($n = 6$) (26). The COPD ALI *in vitro* study used scRNA-seq of airway epithelial cell cultures from bronchial brushings of former smokers with COPD ($n = 2$), current smokers ($n = 2$), and healthy never-smokers ($n = 3$) (27). The Smoke ALI *in vitro* study used scRNA-seq data from healthy ($n = 3$) and COPD ($n = 3$) small airway epithelial cells that were treated with acute (4 d) and chronic (28 d) smoke exposure (28).

Gene Expression Analysis

Differential gene expression analysis was performed on bulk RNA-seq and microarray datasets using edgeR (version 3.32.1) and limma (version 3.46.0) packages, respectively (26, 29–31). These analyses were corrected for age and sex. Pathway analysis, cellular deconvolution, and expression quantitative trait loci (eQTL) analysis were also performed, as detailed in the online supplement. scRNA-seq datasets were processed and analyzed using the Seurat (version 4.0.1) package. Trajectory analyses were performed using the Monocle (version 2.18.0) package.

Protein Quantification of IL-33

IL-33 was measured using Western blot and immunohistochemistry as outlined in the online supplement.

Statistical Analysis

The Wilcoxon signed-rank test was used to statistically analyze paired samples from longitudinal studies, whereas the Mann-Whitney nonparametric test was used for unpaired samples from the cross-sectional studies. The Bonferroni method was used to correct for multiple testing, and an adjusted P value less than 0.05 was considered significant for all gene expression results. For one-way ANOVAs, Dunnett correction for multiple testing was conducted. Correlation analyses incorporated the nonparametric Spearman correlation test.

Results

IL-33 Gene Expression Increases Following Smoking Cessation and Is Lower Overall in Current Smokers Compared with ex- and Never-Smokers

We investigated the association of smoking with the gene expression of *IL-33* and its receptors, *IL1RL1* and *IL1RAP*, in one longitudinal and four cross-sectional studies. Initially, *IL-33*, *IL1RL1*, and *IL1RAP* gene expression was quantified in the STOP study with bronchial biopsies of COPD patients and healthy controls collected before and 12 months after smoking cessation ($n = 16$). *IL-33* gene expression significantly increased after smoking cessation ($P = 0.003$; Figure 2A) regardless of disease status, and a similar but nonsignificant trend was found for *IL1RL1* ($P = 0.057$; Figure 2B), whereas no difference was observed for *IL1RAP* ($P = 0.261$; Figure E1A). In four independent cross-sectional studies of bronchial biopsies (Figures 2C–2F) and brushes, we replicated these findings, showing significantly higher *IL-33* gene expression in former and never-smokers versus current smokers ($P < 0.05$). Trends toward, or significantly, lower levels in current smokers were observed in these studies for *IL1RL1* (Figures 2G–2J), whereas results were inconsistent for *IL1RAP* (*see* Figures E1A–E1E). Similar results were found in a microarray dataset of small airways, showing that the association between the gene expression of IL-33 and smoking status extends to small airways, where disease-driving pathology in COPD develops (Figure E2) (32).

In addition, we investigated whether acute smoke exposure shows a similar effect on the gene expression profile of *IL-33*. To this end, we assessed two additional studies: *1*) a longitudinal dataset of bronchial brushings

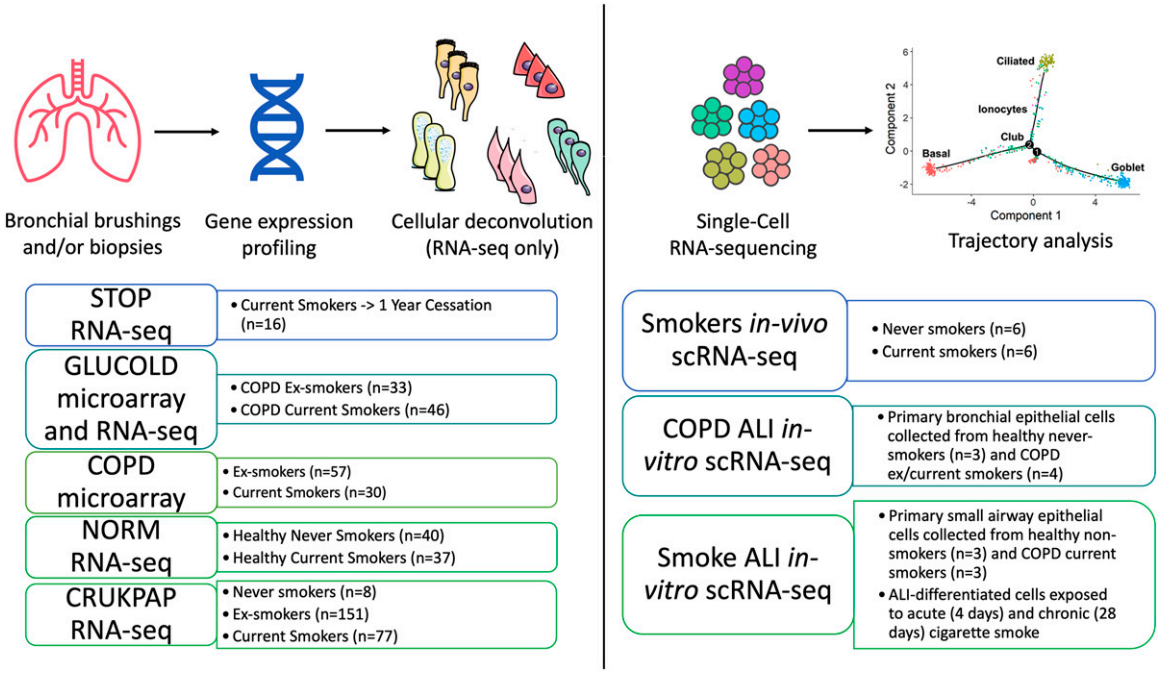

Figure 1. Flow chart of the present study methods. One longitudinal and four cross-sectional *in vivo* studies were analyzed to assess the gene expression profile of *IL-33* and IL-33 pathway related genes. Cellular deconvolution was performed on the studies in which bulk RNA sequencing was performed, as the method is not suitable for microarray study. One *in vivo* and two *in vitro* single-cell RNA sequencing studies were analyzed to assess the *IL-33* expression at the single-cell level and cell trajectory (20–26, 27, 28). ALI = air-liquid interface; COPD = chronic obstructive pulmonary disease; CRUKPAP = Cancer Research UK Papworth Hospital; GLUCOLD = Groningen and Leiden Universities Study of Corticosteroids in Obstructive Lung Disease; NORM = Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects; STOP = Stop Smoking Study.

collected before and 24 hours after smoking three cigarettes in "party smokers" who refrained from smoking for >2 days before the baseline visit ($n = 63$); and *2*) a differentiated *in vitro* model of primary bronchial epithelial cells treated with whole cigarette smoke extract (33, 34). In both acute smoke exposure studies, there were no significant changes in IL-33 gene expression in response to smoking (Figures E3A and E3B).

Influence of Genetics on *IL-33* Gene Expression

To investigate whether an association between genetics and *IL-33* gene expression exists, we performed an eQTL analysis (35). This analysis can assess whether a genetic variant or SNP has any interaction with nearby or distantly located genes, in this case *IL-33*. We focused on one of the top asthma SNPs (rs992969), which was previously shown to influence *IL-33* gene expression (36–40). We performed eQTL in the CRUKPAP study, excluding the never-smokers as a result of a small sample size ($n = 8$). Although there was no significant interaction with smoking in the eQTL analysis, we found that the GG and AG variants in the rs992969 SNP had significantly lower *IL-33* gene expression in current smokers compared with ex-smokers (*see* Figure E3C). However, there was no difference between current and former smokers who carried the AA allele. Overall, our results suggest that, even though the rs992969 SNP is associated with *IL-33* gene expression in the airway, smoking status potentially has a more substantial effect on this relationship.

Splicing Variants of *IL1RL1* Are Not Influenced by Smoking

There are two main splice variants of IL1RL1: a full-length membrane-bound variant and a truncated soluble variant that acts as a decoy receptor (41). An analysis was performed to assess whether the decoy receptor is expressed instead of the membrane-bound variant, which would indicate a false-positive finding. In two studies in which the raw RNA-seq data were available, the expressions of these two variants were investigated separately. We found that the membrane to IL1RL1 (soluble and membrane common region) was not significantly different in current smokers compared with ex- or never-smokers (Figures E4A and E4D). Furthermore, the membrane-bound and IL1RL1 (soluble and membrane common region) had similar expression profiles between current and ex-/never-smokers, indicating that these variants are likely coexpressed (*see* Figures E4B, E4C, E4E, and E4F). Thus, it indicates that the splicing does not play a role in the association between the gene expression of IL1RL1 and smoking status.

Association between IL-33 Activation Pathway and Smoking

To explore the potential association between the IL-33 pathway and smoking, we used a gene signature derived from multiple cell types (human basophils, ILC2, regulatory T cells, and endothelial cells) treated with IL-33 *in vitro* (42). This IL-33 activation pathway signature was investigated in the same bulk RNA-seq studies. Similar to the gene expression, the IL-33 activation pathway signature was significantly lower in current smokers compared with ex-/never-smokers or upregulated after smoking cessation (Figures 3A–3E). Together, this analysis showed consistently lower activation of the IL-33 signaling pathway in current

Table 1. Baseline Characteristics of the Datasets

Study	Sample Types	Smoking Status	*n* (%)	Age, yr	Male Sex	Pack-Years	FEV_1% Predicted	FEV_1/FVC	Platform	Profiling Assay
Bulk RNA-seq/microarray studies										
STOP Smoking RNA-seq (longitudinal) (20)	Bronchial biopsies	Current smokers	16 (100%)	54 ± 6.6	9 (56.3%)	32.3 ± 13.7	82.1 ± 23.3	70.6 ± 14.3	Bulk RNA-seq	Illumina HiSeq 2500
GLUCOLD Microarray and RNA-seq (21, 22)	Bronchial biopsies	Current smokers	46 (58.2%)	58.4 ± 7.7	35 (76.1%)	44.5 ± 17.1	56.8 ± 10.2	50.6 ± 8.7	Microarray, bulk RNA-seq	Affymetrix HG 1.0 ST microArray, Illumina HiSeq 2500
		Ex-smokers	33 (41.8%)	64.4 ± 7.4	31 (93.9%)	45.0 ± 25.6	54.0 ± 12.0	47.9 ± 9.0		
COPD Microarray (GSE37147) (23)	Bronchial brushings	Current smokers	30 (34.5%)	63.2 ± 6.68	16 (53.3%)	47.5 ± 13.6	57.6 ± 16.4	56.9 ± 11.7	Microarray	Affymetrix HG 1.0 ST microArray
		Ex-smokers	57 (65.5%)	66.1 ± 5.6	36 (63.2%)	52.9 ± 28.1	61.6 ± 12.1	60.3 ± 7.5		
NORM RNA-seq (24)	Bronchial biopsies and brushings	Current smokers	37 (48.1%)	41.6 ± 15.2	22 (59.5%)	18.8 ± 15.1	99.3 ± 9.2	78.2 ± 6.1	Bulk RNA-seq	Illumina HiSeq 2500
		Non-Smokers	40 (51.9%)	38.5 ± 18.9	20 (50%)	0	101.5 ± 11.9	80.7 ± 6.8		
CRUKPAP RNA-seq (25)	Bronchial brushings	Current smokers	77 (32.6%)	63.3 ± 10.9	49 (63.6%)	44.3 ± 20.8	NA	NA	Bulk RNA-seq	Illumina HiSeq 2500
		Ex-smokers	151 (64%)	69.3 ± 9.1	104 (68.9%)	41.3 ± 27.3	NA	NA		
		Never-smokers	8 (3.4%)	66.9 ± 8.5	5 (62.5%)	0	NA	NA		
Single-cell RNA-seq studies										
Smokers *in vivo* scRNA-seq (GSE131391) (26)	Bronchial brushings	Current smokers	6 (50%)	42.7 ± 9.7	3 (50%)	15.3 ± 7.9	N/A	82.7 ± 5.5	Single-cell RNA-seq	Illumina HiSeq 2500
		Never-smokers	6 (50%)	29.5 ± 7.6	3 (50%)	0	N/A	82.6 ± 3.7		
COPD ALI *in vitro* scRNA-seq (27)	Primary bronchial airway epithelial cells from healthy never-smokers and COPD ex-/current smokers	Current smokers	2 (28.6%)	72.5 ± 7.8	2 (66.7%)	55 ± 4.2	66.5 ± 7.8	15.5 ± 4.9	Single-cell RNA-seq	BD Rhapsody Express Cell Capture System
		Ex-smokers	2 (28.6%)	80.5 ± 6.4	0	62 ± 12.7	60.5 ± 9.2	12.5 ± 12.0		
		Never-smokers	3 (42.6%)	69 ± 12.2	1 (33.3%)	0	90 ± 18.3	74.3 ± 5.1		
Smoke ALI CS *in vitro* scRNA-seq (28)	Primary small-airway epithelial cells from healthy never-smokers and COPD donors	Current smokers	3 (50%)	56.7 ± 5.5	0	NA	NA	NA	Single-cell RNA-seq	Cell Ranger v2.1.1 (10X Genomics)
		Never smokers	3 (50%)	49 ± 15.7	1 (33.3%)	NA	NA	NA		

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; CRUKPAP = Cancer Research UK Papworth Hospital; GLUCOLD = Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease; NORM = Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects; RNA-seq = RNA sequencing; STOP = Stop Smoking Study.
Data presented as mean ± SD where applicable.

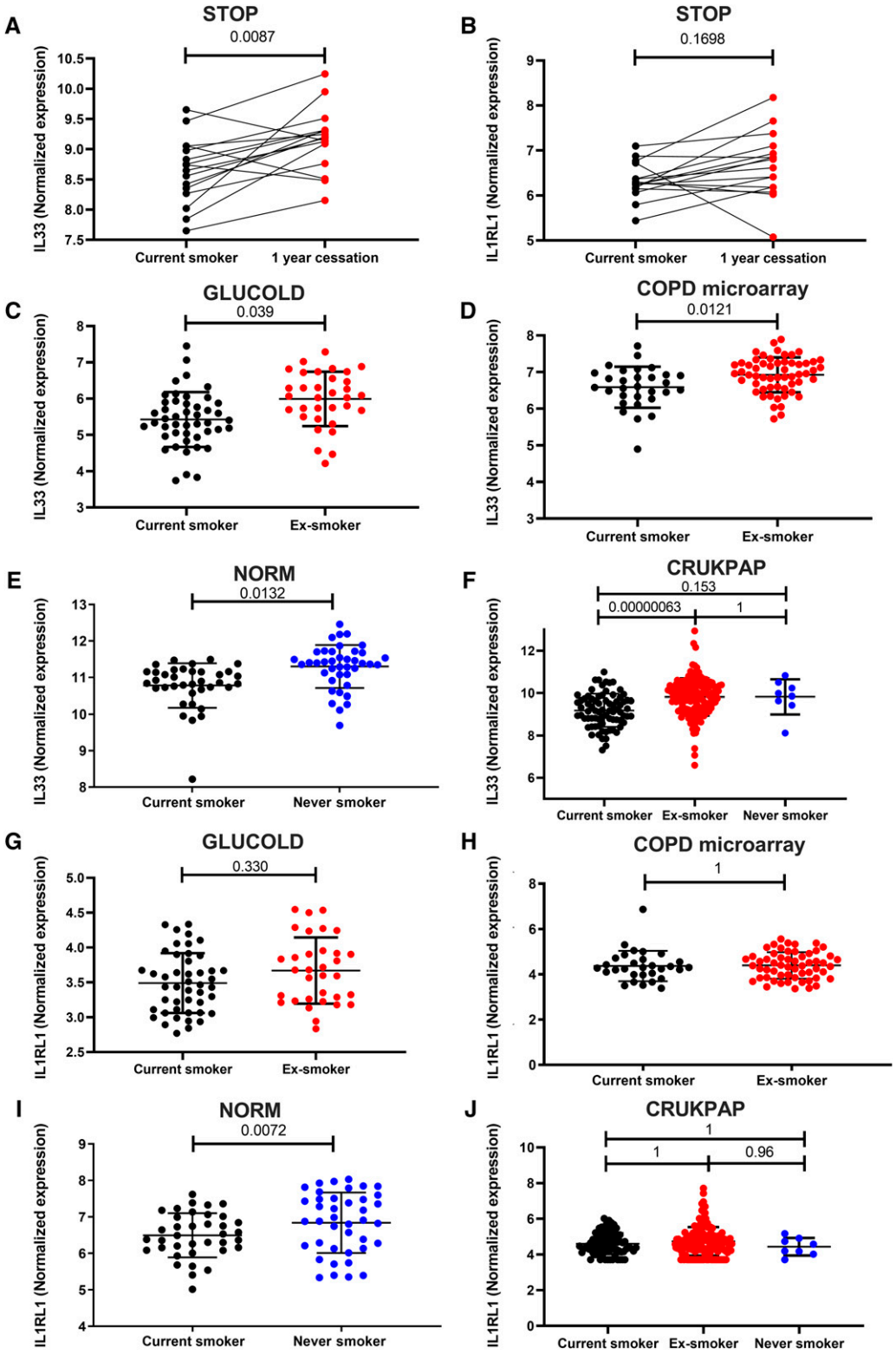

Figure 2. Expression of *IL-33* and *IL1RL1* in bronchial datasets. (*A*) *IL-33* and (*B*) *IL1RL1* expression levels from the STOP study of bronchial biopsies ($N = 16$) before and 12 months after smoking cessation. (*C*) *IL-33* and (*G*) *IL1RL1* expression levels of bronchial biopsies from the GLUCOLD (microarray) study of ex-smokers ($n = 33$) and current smokers ($n = 46$) with chronic obstructive pulmonary disease (COPD). (*D*) *IL-33* and (*H*) *IL1RL1* expression levels of bronchial brushing samples from the COPD microarray study of ex-smokers ($n = 57$) and current smokers ($n = 30$) with COPD (GSE37147). (*E*) *IL-33* and (*I*) *IL1RL1* expression of bronchial biopsies from the NORM study of never-smokers ($n = 40$) and healthy current smokers ($n = 37$). (*F*) *IL-33* and (*J*) *IL1RL1* expression of bronchial biopsies from the CRUKPAP study of never-smokers ($n = 8$),

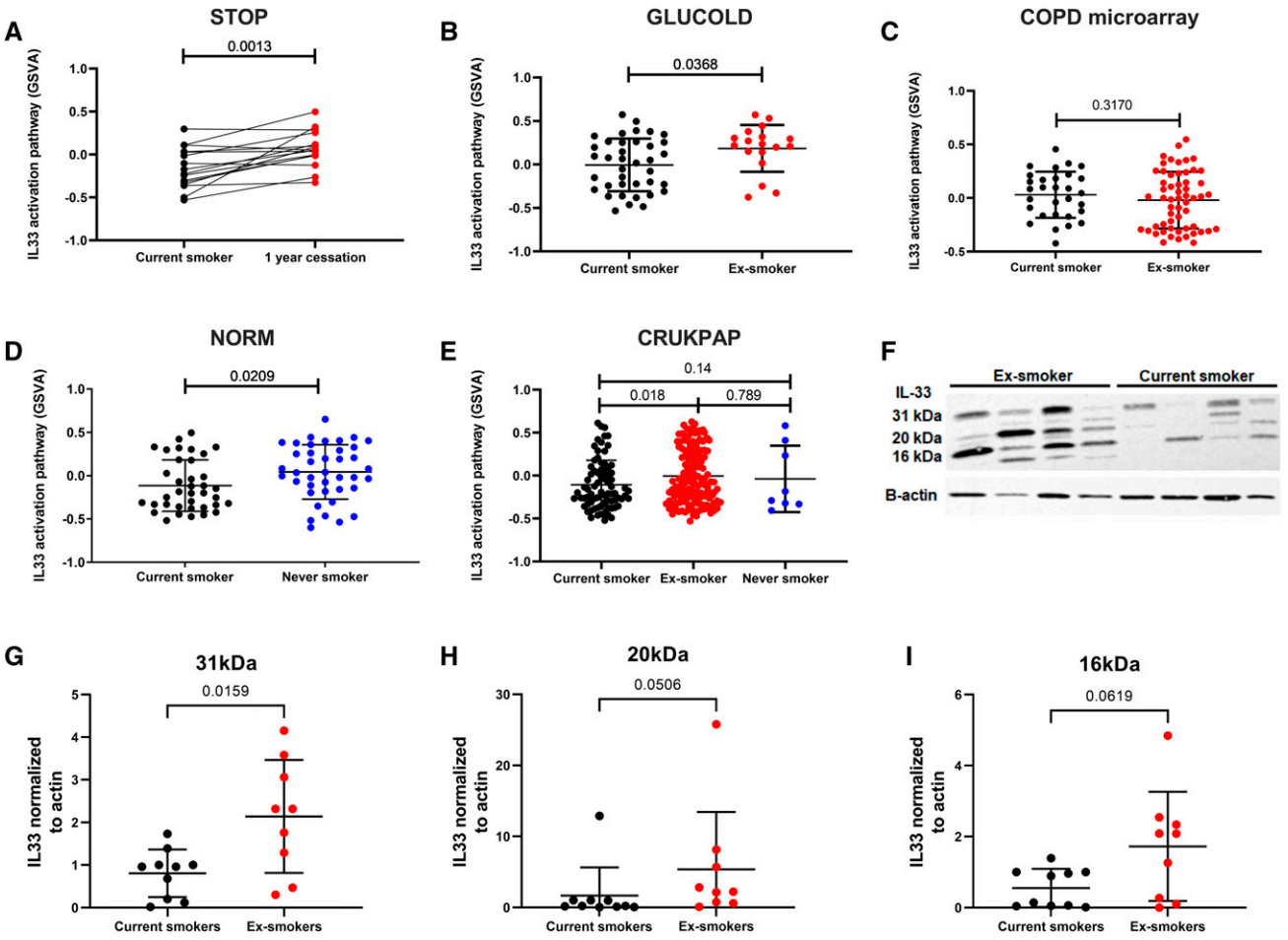

Figure 3. The influence of smoking status on the IL-33 pathway and protein levels. IL-33 activation pathway analysis from (*A*) the STOP study of bronchial biopsies ($n = 16$) during current smoking activity and 1 year after cessation, (*B*) the GLUCOLD (RNA-Seq) study of ex-smokers ($n = 18$) and current smokers ($n = 38$) with chronic obstructive pulmonary disease (COPD), (*C*) the COPD microarray study of bronchial brushing samples from ex-smokers ($n = 57$) and current smokers ($n = 30$), (*D*) the NORM study of bronchial biopsies from healthy never-smokers ($n = 40$) and current smokers ($n = 37$), and (*E*) the CRUKPAP study of bronchial biopsies from never-smokers ($n = 8$), ex-smokers ($n = 151$), and current smokers ($n = 77$). (*F*) Western blot of whole tissue lysates from current ($n = 10$) and former ($n = 9$) smokers with COPD for IL-33 and β-actin. Quantification of the (*G*) 31-kD (full-length protein), (*H*) 20-kD (processed active form), and (*I*) 16-kD (small IL-33 isoform) bands corrected by β-actin. *P* values were acquired from Wilcoxon signed rank tests for paired analyses and Mann-Whitney tests for unpaired analyses. Error bars represent the SD. CRUKPAP = Cancer Research UK Papworth Hospital; GLUCOLD = Groningen and Leiden Universities Study of Corticosteroids in Obstructive Lung Disease; NORM = Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects; STOP = Stop Smoking Study.

smokers compared with never- and ex-smokers regardless of the disease status, which is likely associated with lower IL-33 levels in the airways.

Protein Levels of IL-33 Are Lower in Current Smokers

Next, we investigated protein levels of IL-33 in lung homogenates of current and former smokers with COPD. Western blot staining identified three bands (31 kD, 20 kD, and 16 kD) that correspond to known sizes of IL-33 protein (Figure 3F). Here, we observed that the 31 kD corresponding to the full-length protein was significantly lower in current smokers compared with former smokers, matching the transcriptomic results ($P < 0.05$; Figure 3G). Trends for lower levels in current smokers were observed for the 20-kD band (Figure 3H), likely associated with the processed active form of IL-33 and the 16-kD band corresponding to the smaller IL-33 splice variant (NP_001186570.1) (Figure 3I) (43).

High Gene Expression of *IL-33* in Basal Cells

Next, we investigated *IL-33* and *IL1RL1* gene expression in major cell types from scRNA-seq data of the Human Lung Cell Atlas. A

Figure 2. (*Continued*). ex-smokers ($n = 151$), and current smokers ($n = 77$). All analyses were corrected for age and sex. The numbers shown are *P* values taken from the results of differential expression analyses conducted in edgeR, corrected for age and sex. Error bars represent the SD. *P* values shown are false discovery rate–corrected for the number of genes tested. CRUKPAP = Cancer Research UK Papworth Hospital; GLUCOLD = Groningen and Leiden Universities study of Corticosteroids in Obstructive Lung Disease; NORM = Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects; STOP = Stop Smoking Study.

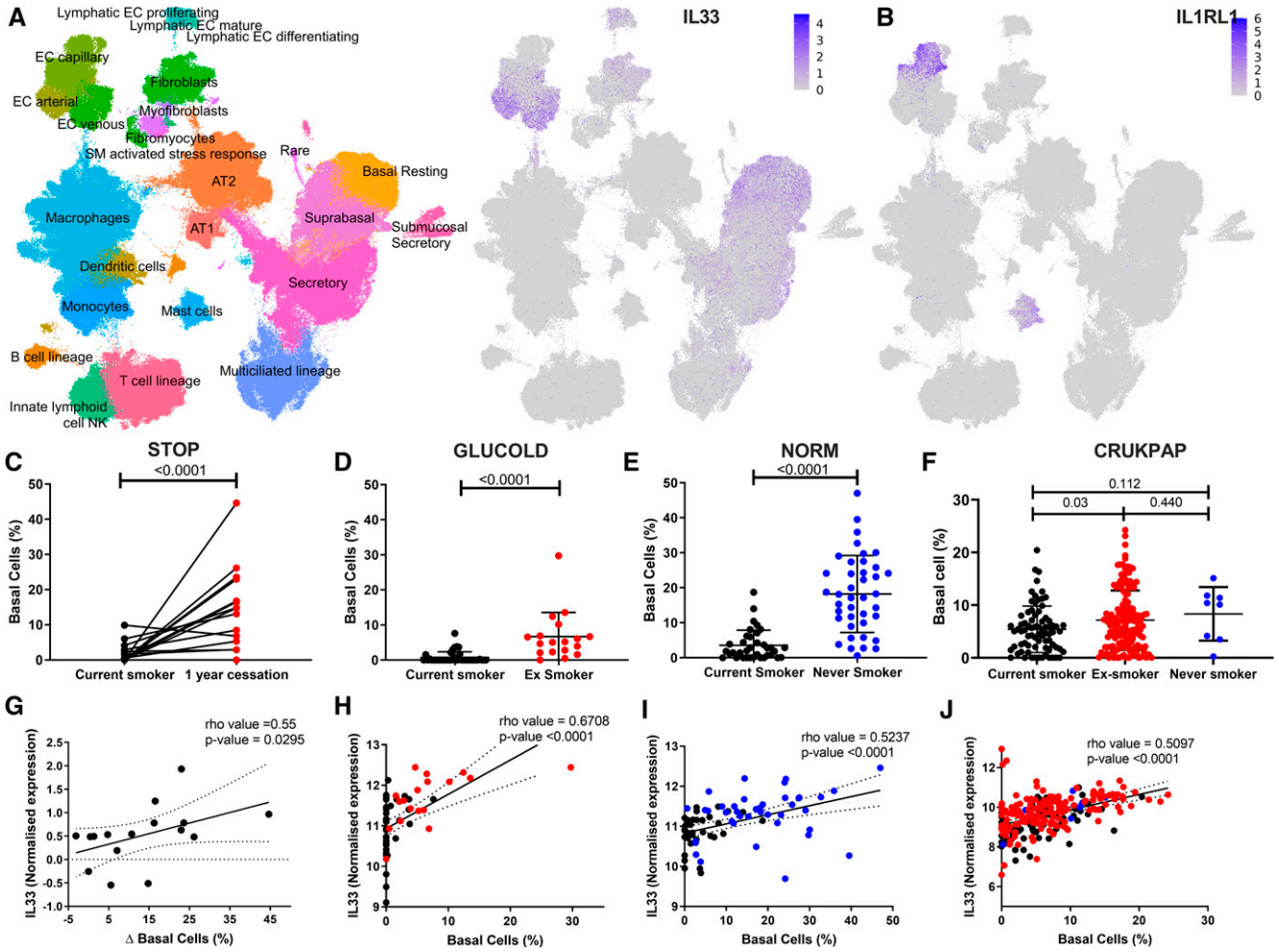

Figure 4. High expression of *IL-33* in basal cells. (*A* and *B*) Uniform Manifold Approximation and Projection (UMAP) of *IL-33* and *IL1RL1* expression of samples taken from the nasal, airways and lungs from the Human Lung Cell Atlas V1.0 Predicted basal cell percentages based on cellular deconvolution from (*C*) the STOP study of bronchial biopsy RNA sequencing data before and after 1 year of smoking cessation ($n = 16$), (*D*) GLUCOLD (RNA-Seq) study of bronchial biopsies of ex-smokers ($n = 18$) and current smokers ($n = 38$) with chronic obstructive pulmonary disease, (*E*) NORM study of never-smokers ($n = 40$) and respiratorily healthy current smokers ($n = 37$), and (*F*) CRUKPAP study of current smokers ($n = 77$), former smokers ($n = 151$), and never-smokers ($n = 8$). *P* values were obtained by Mann-Whitney nonparametric unpaired *t* test. (*G*) Correlation between change in basal cell percentage and change in *IL-33* expression from the STOP study comparing before versus after smoking cessation. Correlation between basal cell percentage and *IL-33* expression levels in bronchial biopsies from (*H*) the GLUCOLD (RNA-Seq) study of current smokers ($n = 46$) and former smokers ($n = 33$), (*I*) the NORM study of never-smokers ($n = 40$) and respiratorily healthy current smokers ($n = 37$), and (*J*) the CRUKPAP study of never-smokers ($n = 8$), ex-smokers ($n = 151$), and current smokers ($n = 77$). *P* values were obtained by nonparametric Spearman correlation analysis. A significant difference was noted at $P < 0.05$. Error bars represent the SD. CRUKPAP = Cancer Research UK Papworth Hospital; EC = endothelial cell; GLUCOLD = Groningen and Leiden Universities Study of Corticosteroids in Obstructive Lung Disease; NORM = Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects; STOP = Stop Smoking Study.

Uniform Manifold Approximation and Projection (UMAP) and violin plot show that the majority of *IL-33*–expressing cells are resting basal cells and endothelial cells (Figure 4A and Figure E5A). Although *IL1RL1* was found to be expressed mainly in mast cells, mirroring a previous report (44), it was also present in the endothelial aerocyte capillary cells (Figure 4B and *see* Figure E5B), whereas IL1RAP was mainly in monocytes (*see* Figure E5C). Based on this finding, we conducted cellular deconvolution to examine the association between smoking status and cellular composition. This method can be performed in the four bulk RNA-seq datasets. Interestingly, basal cells significantly increased in smokers after smoking cessation or were lower in proportion in current smokers compared with ex-/never-smokers (Figures 4C–4F). A significant positive correlation between basal cell proportions and IL-33 gene expression was found for all four studies (Figures 4G–4J).

IL-33 Gene Expression Is Decreased in Smokers, Potentially Because of Phenotypic Changes in Basal Cells

It should be noted that cell deconvolution results are prediction, not a precise representation of cell distribution *in vivo*.

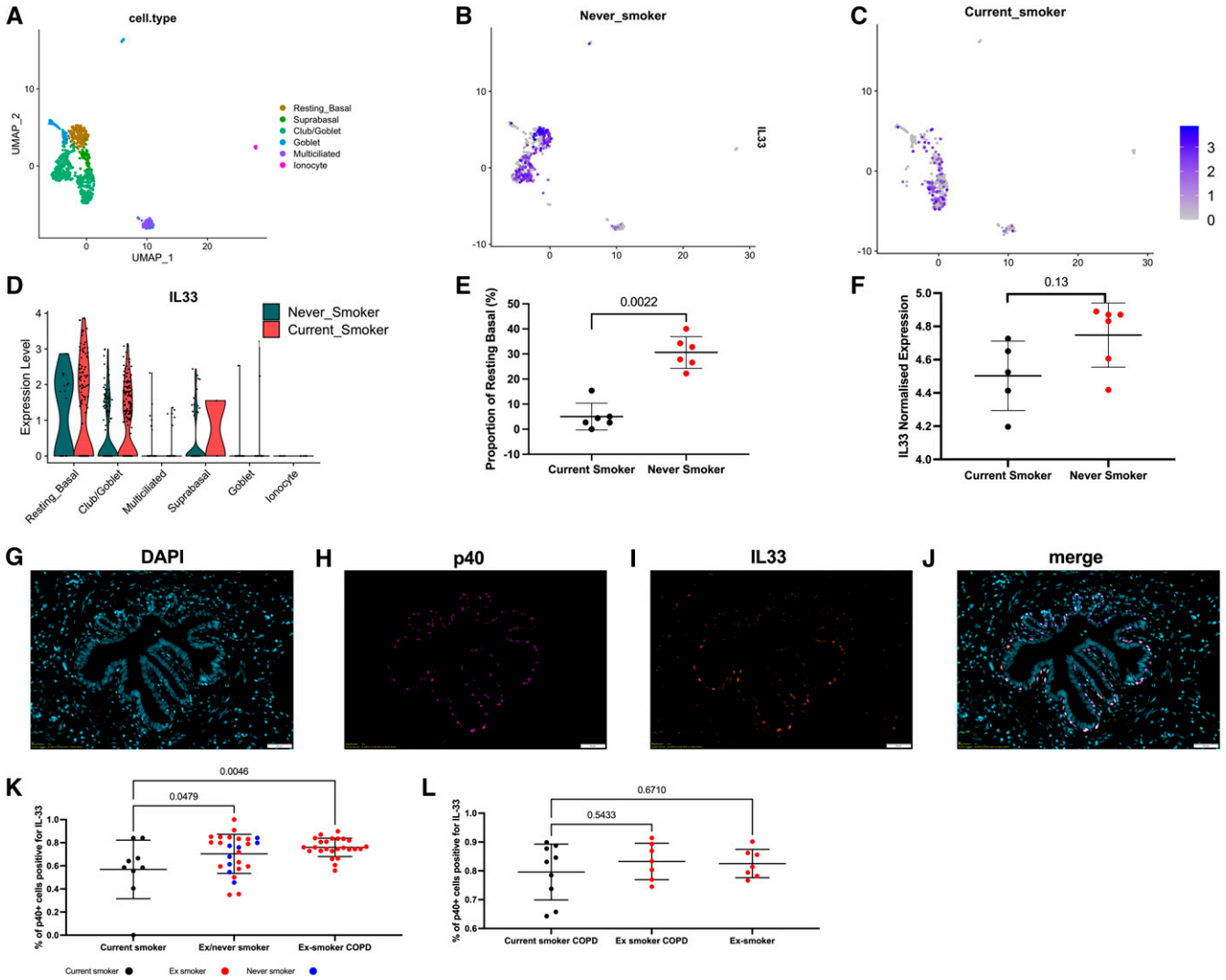

Figure 5. Expression of *IL-33* in current smokers compared with never-smokers in single-cell RNA sequencing and at the protein level. (*A*) Uniform Manifold Approximation and Projection (UMAP) of cell type clusters of bronchial brushing samples from never-smokers ($n = 6$) and current smokers ($n = 6$) in the single-cell RNA sequencing dataset (GSE131391). UMAP of *IL-33* expression in different cell clusters of (*B*) never-smokers and (*C*) current smokers. (*D*) Violin plots of *IL-33* expression across cell types separated based on smoking status. (*E*) Proportion of resting basal cells (%) in never-smokers and current smokers. (*F*) Expression of *IL-33* in the resting basal cell population. Mann-Whitney nonparametric unpaired *t* test was performed to assess significance. Error bars represent the SD. Immunofluorescent staining for IL-33 and p40 were performed in lung tissue sections. Representative images of (*G*) DAPI, (*H*) p40 (polyclonal rabbit, anti-human, ab 167612, 1:100), (*I*) IL-33 antibody (monoclonal mouse, anti-human, ALX-804–840-C100, 1:800), and (*J*) merged lung tissue section. (*K*) Staining quantification of the percentage of IL-33–positive p40$^+$ cells in current smokers ($n = 9$), ex-/never-smokers ($n = 26$), and ex-smokers with chronic obstructive pulmonary disease (COPD) ($n = 25$). (*L*) Secondary cohort staining quantification of the percentage of p40$^+$ cells positive for IL-33 in current smokers with COPD ($n = 9$), ex-smokers with COPD ($n = 7$), and ex-smokers without COPD ($n = 7$). One-way ANOVA was conducted with a Dunnett *P* value correction for multiple testing. Error bars represent the SD.

scRNA-seq analyses are deemed more robust than cellular deconvolution of bulk RNA-seq data. They represent variations at the individual cell level, which may be overlooked by bulk analyses. Therefore, to verify the predicted relationship between lower *IL-33* gene expression and lower (predicted) basal cells in smokers, we directly analyzed a single-cell sequencing dataset of airway brushes from healthy smokers ($n = 6$) and never-smokers ($n = 6$) (26). The single-cell mapping shows that, in current smokers, *IL-33* was predominantly expressed in resting basal cells, followed by club/goblet and suprabasal cells (Figures 5A–5D). Together, these results indicate that *IL-33* gene expression may be specific to a subtype of basal cells. In resting basal cells, the percentage of cells is significantly lower in current smokers compared with never-smokers (Figure 5E). If we solely focus on resting basal cells, we observe a downward trend in the *IL-33* gene expression between smokers and never-smokers ($P = 0.13$; Figure 5F).

To validate the finding that current smoking is associated with lower levels of IL-33–positive basal cells (resting basal cells), we conducted a double staining for the general basal cell marker p40 and IL-33 in

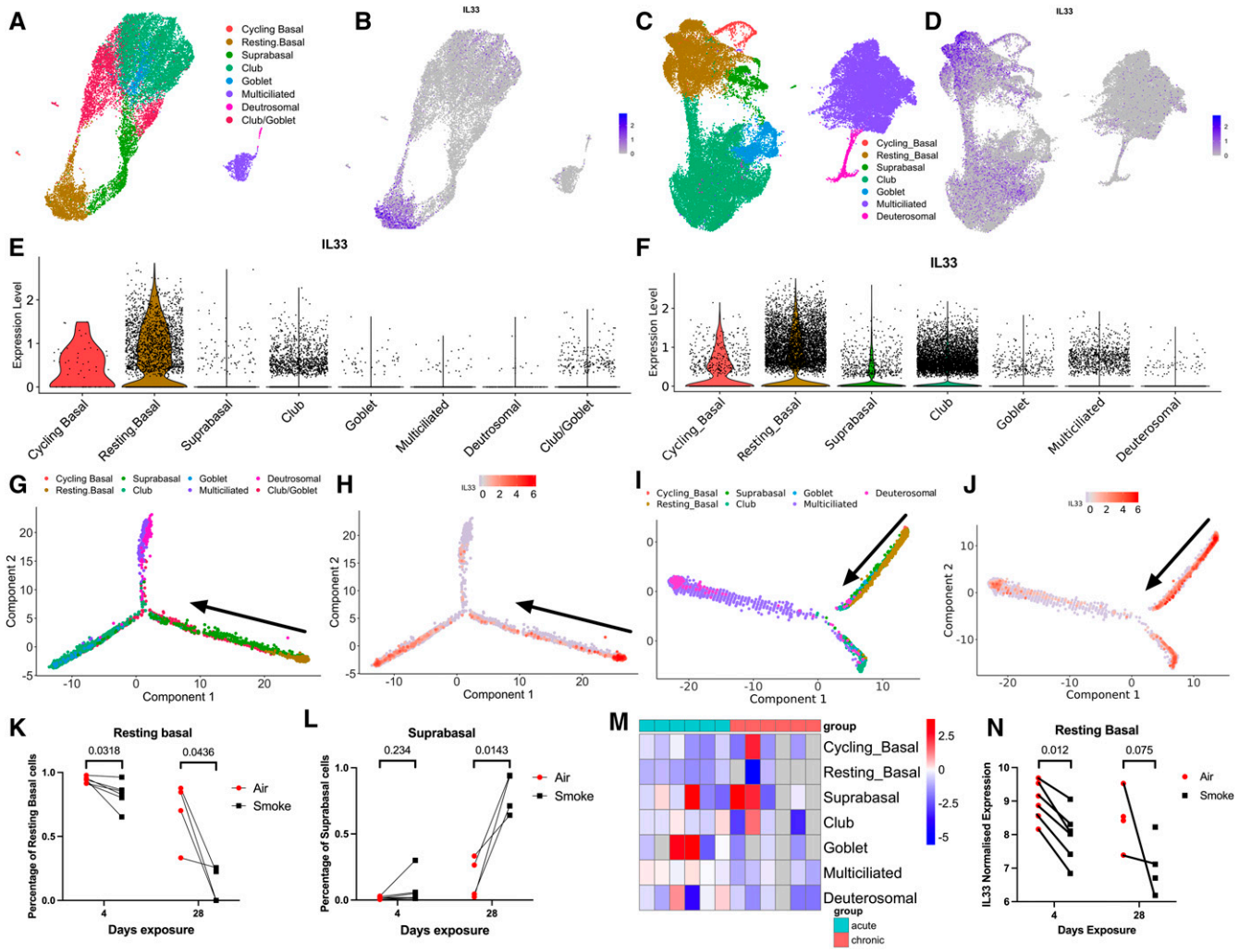

Figure 6. Expression of *IL-33* during basal cell differentiation in single-cell RNA sequencing *in vitro* studies. Two *in vitro* single-cell RNA sequencing studies of airway epithelial cells differentiated at an air–liquid interface were analyzed. The first was performed on the bronchial epithelial cells collected from healthy never-smokers ($n = 3$) and former and current smokers with chronic obstructive pulmonary disease (COPD) ($n = 4$), and the second was performed on small airway epithelial cells collected from healthy never-smokers ($n = 3$) and current smokers with COPD ($n = 3$). Uniform Manifold Approximation and Projection (UMAP) and *IL-33* expression of the first (*A* and *B*) and the second (*C* and *D*) study. Violin plot of the expression of *IL-33* is shown for the first (*E*) and the second (*F*) single-cell study. The cell trajectories of different cellular subpopulations along with *IL-33* expression for the first (*G* and *H*) and the second (*I* and *J*) *in vitro* study. The arrow represents the direction of the cell trajectory. Percentages of (*K*) resting basal cells and (*L*) suprabasal cells from the second single-cell study whereby the airway epithelial cells were exposed to acute (4 d) and chronic (28 d) cigarette smoke. (*M*) Delta heat map of the *IL-33* expression in smoke exposure compared with air shown for different cell types. The color scale indicates the change in expression of *IL-33* in smoke exposure compared with air. There are gray blocks in the heat map that are missing values, as there was a missing value for smoke or air for the respective sample. (*N*) A dot plot representing the *IL-33* expression in resting basal cells for acute (4 d) and chronic (28 d) exposure in the second single-cell study.

lung tissue derived from current and ex-/never-smokers and calculated the proportion of IL-33–positive cells within the p40-positive basal cells in the airways (Figures 5G–5L). We found a significantly lower proportion of IL-33–positive p40 cells in non-COPD current smokers compared with ex-smokers with COPD and non-COPD never-/ex-smokers (false discovery rate <0.05; Figure 5K). In a secondary cohort of current and former smokers with COPD and ex-smoker controls, we found no significant difference between the groups, although a similar pattern was observed with lower proportions in current smokers with COPD (Figure 5L).

IL-33 Gene Expression Is Decreased During Basal Cell Differentiation

Because previous findings demonstrated that *IL-33* expression varies between specific basal cell subtypes (45), we next investigated the gene expression of *IL-33* upon differentiation of basal cells. For this, we used scRNA-seq data of airway epithelial cell cultures differentiated in an air–liquid interface. These air–liquid interface cell culture data reflect a more controlled system in which basal cells differentiate into secretory (goblet and club) cells, ciliated cells, and other less abundant luminal cell types. This allows for the distinction between basal cell subtypes (resting basal and suprabasal cells), which

may be more abundant in air–liquid interface–cultured epithelial cells than in biopsies/brushings (46). To this effect, we analyzed two scRNA-seq datasets; the first study was performed in primary bronchial epithelial cells collected from healthy subjects ($n = 3$) and patients with COPD ($n = 4$; Figures 6A and 6B), and the second study was performed in primary small airway epithelium collected from current smokers ($n = 3$) and never-smokers ($n = 3$; Figures 6C and 6D). Both studies were differentiated in the air–liquid interface, with the latter study including exposure to cigarette smoke for a short term (4 d) and a long term (28 d). From the violin plots (Figures 6E and 6F) and the trajectory analysis (Figures 6G–6J), we can observe that *IL-33* gene expression was predominantly observed in resting and cycling basal cells, with a rapid decrease in gene expression as these cells transition into suprabasal cells. Together, these analyses show that *IL-33* gene expression is specific to resting and cycling subsets of basal cells.

Smoke Exposure Is Associated with a Decrease in Resting Basal Cells and an Overall Reduction in *IL-33* Gene Expression *in vitro* and *in vivo*

Finally, we investigated the influence of smoke exposure on the latter scRNA-seq study in which air–liquid interface–differentiated small airway epithelium was exposed to cigarette smoke for 4 days and 28 days. The percentage of resting basal cells decreased significantly in both time periods, as suprabasal cells had increased significantly on day 28 of exposure ($P < 0.05$; Figures 6K and 6L). We also found that the *IL-33* gene expression was significantly less in resting basal cells at 4 days after smoke exposure (Figures 6M and 6N). These results provide strong evidence that cigarette smoke leads to an overall reduction in IL-33 expression at the transcriptomic and protein levels, which may be due to the decrease in resting basal cells. An overview of all results is presented in Figure S6.

Discussion

This study demonstrates that *IL-33* gene expression is lower in the airways of current smokers compared with former smokers and never-smokers and increases upon smoking cessation, which is supported by the protein data. We further show that *IL-33* is predominantly expressed in resting basal epithelial cells, which occur in lower proportions compared with more differentiated airway epithelial cell types in the airways of current smokers. We provide evidence that the lower gene expression of *IL-33* in current smokers can largely be attributed to a reduction in the proportion of basal cells, perhaps due to increased differentiation toward squamous and goblet cell subtypes. This is further supported by the protein analysis, in which we confirmed the lower proportion of IL-33–positive basal cells in current smokers and the decrease in total IL-33 protein levels in current smokers. In current smokers, our findings suggest that a distinct inflammatory phenotype is present that is not IL-33–driven. Together, these results may help explain the recent clinical observation that anti–IL-33 treatment is more efficacious in ex-smokers with COPD than in current smokers.

We show that *IL-33* gene expression and protein levels are lower with current smoking and increases following smoking cessation. We did not find an acute effect of smoking on *IL-33* gene expression in epithelial cells collected by bronchial brushings 24 hours later, suggesting that the effects of smoking occur only with chronic exposure. Our Western blot protein findings are in line with prior *in vivo* human data, showing lower IL-33 protein levels in nasal lavage and BAL in current versus former smokers (11, 12). However, these previous protein studies need to be viewed with caution, as Pace and colleagues (12) employed the R&D Systems ELISA assay, which was recently highlighted to lack specificity and sensitivity in detecting IL-33 (47), and the immunoassay method by Gómez and colleagues (11) has not been subjected to validation tests. This lack of specificity of IL-33 antibodies is not as much an issue with Western blot, as we were able to focus on bands that correspond to known sizes of IL-33 variants. In contrast, *in vitro* studies on human bronchial epithelial cell cultures and murine cells found *IL-33* gene expression to increase after whole cigarette smoke exposure for 24 hours and for three separate 1-hour periods per day for four consecutive days, respectively (13, 14). A possible cause for this discrepancy is that, in a closed *in vitro* system, cells are exposed to cigarette smoke in an acute setting and before or after cell differentiation. Hence, proinflammatory responses such as the release of IL-33 may take place before the shifting of cell types occurs.

Recently, an anti-IL1RL1 agent, astegolimab, was used to treat asthma and COPD in separate and independent trials. The trials showed that the anti-IL1RL1 treatment reduced the rate of exacerbations in participants with asthma (48); however, in COPD, it did not significantly reduce exacerbations compared with placebo (49). Larger ongoing clinical studies with anti-IL-33 or anti-IL1RL1 agents will provide more conclusive data on the respective role of IL-33 and its receptor in COPD.

We show that changes in *IL-33* gene expression may be related to changes in airway epithelial cell composition. IL-33 was found to be predominantly expressed in resting basal cells, corresponding with previous studies at the transcriptional and protein levels (45, 50, 51). A previous study by Byers and colleagues on the airways of patients with COPD found though histology that IL-33 is localized to a subset of basal epithelial cells but is absent in more differentiated ciliated and secretory epithelial cell types. This matches the histology findings in the present study. Additionally, IL-33 partially overlapped with the conventional basal cell biomarkers KRT5 and TP63, indicating that it is not expressed in all basal epithelial cells, but likely only in a distinct proportion (45). This finding is supported by our single-cell analyses of differentiated airway epithelial cells grown, in which we particularly found *IL-33* gene expression in resting and cycling basal cells, further suggesting that the lower levels of *IL-33* gene expression in current smokers is, to a large extent, explained by a reduction in a specific basal cell subtype (45).

We were able to show that resting basal cells decrease during smoke exposure *in vivo* and *in vitro*. A recent meta-analysis of lung single-cell data found an overall reduction of basal cells in current smokers compared with nonsmokers, supporting these findings (52). Of interest, histological staining of KRT5, a basal cell marker in a previous study, showed a trend toward a decrease in absolute numbers of basal cells in current smokers versus never-smokers (26). The lower number of basal cells may result from cell death of basal cells in response to inflammation or from their transition into different cell types in association with their progenitor role (53, 54). Based on our trajectory analyses and current literature (45), the latter is the more plausible.

Endothelial cells were also found to have high levels of *IL-33*; however, they

comprise a very small proportion of the cells from the bronchial biopsy samples. Despite this, they may still play an important role in the release of IL-33, especially into the bloodstream.

During smoking cessation, we observed an increase of the basal cell population returning the reservoir of *IL-33* in the airways. However, return of this *IL-33* reservoir was paired with the emphysema and cellular death associated with COPD, which *1*) continues after smoking cessation, *2*) results in the release of this trapped *IL-33*, and *3*) likely leads to IL-33 driven inflammation, which would be absent from asymptomatic former smokers as a result of the lack of continued cellular damage. Interestingly, our IL-33 pathway findings suggest that there is a shift of the main inflammation profiles following smoking cessation in COPD from smoking-induced oxidative stress to a more IL-33–driven inflammation. This finding may lead to a paradigm shift in our understanding of COPD pathology; however, further studies need to be conducted to validate this theory.

There are several limitations to our study. First, the *in vivo* single-cell data analyzed in the present study came from healthy individuals. Future bronchial epithelial data from the Human Cell Atlas should include subjects with different lung diseases because the presence of the disease may greatly affect cellular activity and phenotypes. It is also important for further studies to investigate the mechanisms of regulation and effects of *IL-33* using single-cell data in a longitudinal setting.

In conclusion, we show that *IL-33* expression, at the gene and protein levels, is lower in current smokers than in former smokers. Lower expression of *IL-33* is likely due to the differentiation of resting basal cells toward a more differentiated subtype (i.e., suprabasal), as *IL-33* is predominantly expressed in the least differentiated epithelial cells. Our findings help explain the clinical observation of greater efficacy of a recent antibody-based anti–IL-33 treatment in ex-smokers compared with current smokers with COPD, as *IL-33* expression appears to be inherently lower in current smokers. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Celli BR, Wedzicha JA. Update on clinical aspects of chronic obstructive pulmonary disease. *N Engl J Med* 2019;381:1257–1266.
2. Barnes PJ, Burney PG, Silverman EK, Celli BR, Vestbo J, Wedzicha JA, *et al.* Chronic obstructive pulmonary disease. *Nat Rev Dis Primers* 2015; 1:15076.
3. Zhao J, Zhao Y. Interleukin-33 and its receptor in pulmonary inflammatory diseases. *Crit Rev Immunol* 2015;35:451–461.
4. Le H, Kim W, Kim J, Cho HR, Kwon B. Interleukin-33: a mediator of inflammation targeting hematopoietic stem and progenitor cells and their progenies. *Front Immunol* 2013;4:104.
5. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, *et al.* IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;23:479–490.
6. Liew FY. Cigarette smoke resets the alarmin IL-33 in COPD. *Immunity* 2015;42:401–403.
7. Gabryelska A, Kuna P, Antczak A, Białasiewicz P, Panek M. IL-33 mediated inflammation in chronic respiratory diseases-understanding the role of the member of IL-1 superfamily. *Front Immunol* 2019;10:692.
8. Wechsler ME, Ruddy MK, Pavord ID, Israel E, Rabe KF, Ford LB, *et al.* Efficacy and safety of itepekimab in patients with moderate-to-severe asthma. *N Engl J Med* 2021;385:1656–1668.
9. Rabe KF, Celli BR, Wechsler ME, Abdulai RM, Luo X, Boomsma MM, *et al.* Safety and efficacy of itepekimab in patients with moderate-to-severe COPD: a genetic association study and randomised, double-blind, phase 2a trial. *Lancet Respir Med* 2021;9: 1288–1298.
10. Abonia JP, Blanchard C, Butz BB, Rainey HF, Collins MH, Stringer K, *et al.* Involvement of mast cells in eosinophilic esophagitis. *J Allergy Clin Immunol* 2010;126:140–149.
11. Gómez RM, Croce VH, Zernotti ME, Muiño JC. Active smoking effect in allergic rhinitis. *World Allergy Organ J* 2021;14:100504.
12. Pace E, Di Sano C, Sciarrino S, Scafidi V, Ferraro M, Chiappara G, *et al.* Cigarette smoke alters IL-33 expression and release in airway epithelial cells. *Biochim Biophys Acta* 2014;1842:1630–1637.
13. Qiu C, Li Y, Li M, Li M, Liu X, McSharry C, *et al.* Anti-interleukin-33 inhibits cigarette smoke-induced lung inflammation in mice. *Immunology* 2013;138:76–82.
14. Xia J, Zhao J, Shang J, Li M, Zeng Z, Zhao J, *et al.* Increased IL-33 expression in chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol* 2015;308:L619–L627.
15. Kearley J, Silver JS, Sanden C, Liu Z, Berlin AA, White N, *et al.* Cigarette smoke silences innate lymphoid cell function and facilitates an exacerbated type I interleukin-33-dependent response to infection. *Immunity* 2015;42:566–579.
16. Allinne J, Scott G, Lim WK, Birchard D, Erjefält JS, Sandén C, *et al.* IL-33 blockade affects mediators of persistence and exacerbation in a model of chronic airway inflammation. *J Allergy Clin Immunol* 2019;144: 1624–1637.e10.
17. Rabe KF, Celli BR, Wechsler ME, Abdulai R, Luo X, Boomsma MM, *et al.* Efficacy and safety of itepekimab in patients with moderate to severe COPD. *Lancet Respir Med* 2021;9:1288–1298.
18. Faiz A, Boedijono F, Timens W, Nawijn M, Hansbro P, Mahbub R, *et al.* The regulation of IL33 following smoking cessation [abstract]. *Eur Respir J* 2022;60:3314.
19. Faiz A, Mahbub R, Boedijono FS, Timens W, Nawijn M, Hansbro P, *et al.* Using single cell sequencing to understand treatment response to anti-IL33 treatment in chronic obstructive pulmonary disease (COPD) [abstract]. *Am J Respir Crit Care Med* 2023;207:A4414.
20. Willemse BW, ten Hacken NH, Rutgers B, Lesman-Leegte IG, Postma DS, Timens W. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J* 2005; 26:835–845.
21. Faiz A, Steiling K, Roffel MP, Postma DS, Spira A, Lenburg ME, *et al.* Effect of long-term corticosteroid treatment on microRNA and gene-expression profiles in COPD. *Eur Respir J* 2019;53:1801202.
22. van den Berge M, Steiling K, Timens W, Hiemstra PS, Sterk PJ, Heijink IH, *et al.* Airway gene expression in COPD is dynamic with inhaled corticosteroid treatment and reflects biological pathways associated with disease activity. *Thorax* 2014;69:14–23.
23. Steiling K, van den Berge M, Hijazi K, Florido R, Campbell J, Liu G, *et al.* A dynamic bronchial airway gene expression signature of chronic obstructive pulmonary disease and lung function impairment. *Am J Respir Crit Care Med* 2013;187:933–942.
24. Vermeulen CJ, Xu CJ, Vonk JM, Ten Hacken NHT, Timens W, Heijink IH, *et al.* Differential DNA methylation in bronchial biopsies between persistent asthma and asthma in remission. *Eur Respir J* 2020;55: 1901280.
25. Aliee H, Massip F, Qi C, Stella de Biase M, van Nijnatten J, Kersten ETG, *et al.* Determinants of expression of SARS-CoV-2 entry-related genes in upper and lower airways. *Allergy* 2022;77:690–694.
26. Duclos GE, Teixeira VH, Autissier P, Gesthalter YB, Reinders-Luinge MA, Terrano R, *et al.* Characterizing smoking-induced transcriptional heterogeneity in the human bronchial epithelium at single-cell resolution. *Sci Adv* 2019;5:eaaw3413.
27. Johansen MD, Mahbub RM, Idrees S, Nguyen DH, Miemczyk S, Pathinayake P, *et al.* Increased SARS-CoV-2 infection, protease, and inflammatory responses in chronic obstructive pulmonary disease

primary bronchial epithelial cells defined with single-cell RNA sequencing. *Am J Respir Crit Care Med* 2022;206:712–729.
28. Wohnhaas CT, Gindele JA, Kiechle T, Shen Y, Leparc GG, Stierstorfer B, *et al.* Cigarette smoke specifically affects small airway epithelial cell populations and triggers the expansion of inflammatory and squamous differentiation associated basal cells. *Int J Mol Sci* 2021; 22:7646.
29. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-seq experiments with respect to biological variation. *Nucleic Acids Res* 2012;40:4288–4297.
30. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010;26:139–140.
31. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43:e47.
32. Ammous Z, Hackett NR, Butler MW, Raman T, Dolgalev I, O'Connor TP, *et al.* Variability in small airway epithelial gene expression among normal smokers. *Chest* 2008;133:1344–1353.
33. Billatos E, Faiz A, Gesthalter Y, LeClerc A, Alekseyev YO, Xiao X, *et al.* Impact of acute exposure to cigarette smoke on airway gene expression. *Physiol Genomics* 2018;50:705–713.
34. van der Does AM, Mahbub RM, Ninaber DK, Rathnayake SNH, Timens W, van den Berge M, *et al.* Early transcriptional responses of bronchial epithelial cells to whole cigarette smoke mirror those of in-vivo exposed human bronchial mucosa. *Respir Res* 2022;23: 227.
35. Shabalin AA. Matrix eQTL: ultra fast eQTL analysis via large matrix operations. *Bioinformatics* 2012;28:1353–1358.
36. Aneas I, Decker DC, Howard CL, Sobreira DR, Sakabe NJ, Blaine KM, *et al.* Asthma-associated variants induce IL33 differential expression through a novel regulatory region. *Nat. Commun* 2021;12:6115.
37. Grotenboer NS, Ketelaar ME, Koppelman GH, Nawijn MC. Decoding asthma: translating genetic variation in IL33 and IL1RL1 into disease pathophysiology. *J Allergy Clin Immunol* 2013;131:856–865.
38. Imkamp K, Berg M, Vermeulen C, Heijink I, Guryev V, Koppelman G, *et al.* Comparison of gene expression profiles from nasal and bronchial brushes. *Eur Respir J* 2017;50:OA2910.
39. Ketelaar ME, Portelli MA, Dijk FN, Shrine N, Faiz A, Vermeulen CJ, *et al.* Phenotypic and functional translation of IL33 genetics in asthma. *J Allergy Clin Immunol* 2021;147:144–157.
40. Li X, Hastie AT, Hawkins GA, Moore WC, Ampleford EJ, Milosevic J, *et al.* eQTL of bronchial epithelial cells and bronchial alveolar lavage deciphers GWAS-identified asthma genes. *Allergy* 2015;70:1309–1318.
41. Gordon ED, Palandra J, Wesolowska-Andersen A, Ringel L, Rios CL, Lachowicz-Scroggins ME, *et al. IL1RL1* asthma risk variants regulate airway type 2 inflammation. *JCI Insight* 2016;1:e87871.
42. Badi YE, Salcman B, Taylor A, Rana B, Kermani NZ, Riley JH, *et al.* IL1RAP expression and the enrichment of IL-33 activation signatures in severe neutrophilic asthma. *Allergy* 2023;78:156–167.
43. Talabot-Ayer D, Lamacchia C, Gabay C, Palmer G. Interleukin-33 is biologically active independently of caspase-1 cleavage. *J Biol Chem* 2009;284:19420–19426.
44. Faiz A, Pavlidis S, Kuo C-H, Rowe A, Hiemstra PS, Timens W, *et al.* Th2 high and mast cell gene signatures are associated with corticosteroid sensitivity in COPD. *Thorax* 2023;78:335–343.
45. Byers DE, Alexander-Brett J, Patel AC, Agapov E, Dang-Vu G, Jin X, *et al.* Long-term IL-33-producing epithelial progenitor cells in chronic obstructive lung disease. *J Clin Invest* 2013;123:3967–3982.
46. Dvorak A, Tilley AE, Shaykhiev R, Wang R, Crystal RG. Do airway epithelium air-liquid cultures represent the in vivo airway epithelium transcriptome? *Am J Respir Cell Mol Biol* 2011;44:465–473.
47. Ketelaar ME, Nawijn MC, Shaw DE, Koppelman GH, Sayers I. The challenge of measuring IL-33 in serum using commercial ELISA: lessons from asthma. *Clin Exp Allergy* 2016;46:884–887.
48. Kelsen SG, Agache IO, Soong W, Israel E, Chupp GL, Cheung DS, *et al.* Astegolimab (anti-ST2) efficacy and safety in adults with severe asthma: a randomized clinical trial. *J Allergy Clin Immunol* 2021;148: 790–798.
49. Yousuf AJ, Mohammed S, Carr L, Yavari Ramsheh M, Micieli C, Mistry V, *et al.* Astegolimab, an anti-ST2, in chronic obstructive pulmonary disease (COPD-ST2OP): a phase 2a, placebo-controlled trial. *Lancet Respir Med* 2022;10:469–477.
50. Goldfarbmuren KC, Jackson ND, Sajuthi SP, Dyjack N, Li KS, Rios CL, *et al.* Dissecting the cellular specificity of smoking effects and reconstructing lineages in the human airway epithelium. *Nat Commun* 2020;11:2485.
51. Osei ET, B Mostaço-Guidolin L, Hsieh A, Warner SM, Al-Fouadi M, Wang M, *et al.* Epithelial-interleukin-1 inhibits collagen formation by airway fibroblasts: implications for asthma. *Sci Rep* 2020;10:8721.
52. Nakayama J, Yamamoto Y. Single-cell meta-analysis of cigarette smoking lung atlas. bioRxiv; 2021.
53. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am J Pathol* 2004;164:577–588.
54. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, *et al.* Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci USA* 2009;106:12771–12775.