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 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 FEV₁, 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 FEV₁. 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-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).

Bulb 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...
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.

**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.
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 smokers compared with ex-/never-smokers or upregulated after smoking cessation.
### Table 1. Baseline Characteristics of the Datasets

<table>
<thead>
<tr>
<th>Study Description</th>
<th>Sample Types</th>
<th>Smoking Status</th>
<th>n (%)</th>
<th>Age, yr</th>
<th>Male Sex</th>
<th>Pack-Years</th>
<th>FEV₁% Predicted</th>
<th>FEV₁/FVC</th>
<th>Platform</th>
<th>Profiling Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk RNA-seq/microarray studies</td>
<td>Bronchial biopsies</td>
<td>Current smokers</td>
<td>16 (100%)</td>
<td>54 ± 6.6</td>
<td>9 (56.3%)</td>
<td>32.3 ± 13.7</td>
<td>82.1 ± 23.3</td>
<td>70.6 ± 14.3</td>
<td>Bulk RNA-seq</td>
<td>Illumina HiSeq 2500</td>
</tr>
<tr>
<td>STOP Smoking RNA-seq (longitudinal) (20)</td>
<td>Ex-smokers</td>
<td></td>
<td>33 (41.8%)</td>
<td>64.4 ± 7.4</td>
<td>31 (93.9%)</td>
<td>45.0 ± 25.6</td>
<td>54.0 ± 12.0</td>
<td>47.9 ± 9.0</td>
<td>Microarray, bulk RNA-seq</td>
<td>ST microArray, Illumina HiSeq 2500</td>
</tr>
<tr>
<td>GLUCOLD Microarray and RNA-seq (21, 22)</td>
<td>Bronchial biopsies</td>
<td>Current smokers</td>
<td>46 (58.2%)</td>
<td>58.4 ± 7.7</td>
<td>35 (76.1%)</td>
<td>44.5 ± 17.1</td>
<td>56.8 ± 10.2</td>
<td>50.6 ± 8.7</td>
<td>Microarray</td>
<td>Affymetrix HG 1.0</td>
</tr>
<tr>
<td></td>
<td>Ex-smokers</td>
<td></td>
<td>33 (41.8%)</td>
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<td>45.0 ± 25.6</td>
<td>54.0 ± 12.0</td>
<td>47.9 ± 9.0</td>
<td>Bulk RNA-seq</td>
<td>ST microArray, Illumina HiSeq 2500</td>
</tr>
<tr>
<td>COPD Microarray (GSE37147) (23)</td>
<td>Bronchial brushings</td>
<td>Current smokers</td>
<td>37 (48.1%)</td>
<td>63.3 ± 10.9</td>
<td>49 (63.6%)</td>
<td>44.3 ± 20.8</td>
<td>NA</td>
<td>NA</td>
<td>Bulk RNA-seq</td>
<td>Illumina HiSeq 2500</td>
</tr>
<tr>
<td></td>
<td>Ex-smokers</td>
<td></td>
<td>151 (64%)</td>
<td>66.9 ± 8.5</td>
<td>104 (68.8%)</td>
<td>41.3 ± 27.3</td>
<td>NA</td>
<td>NA</td>
<td>Bulk RNA-seq</td>
<td>Illumina HiSeq 2500</td>
</tr>
<tr>
<td></td>
<td>Never-smokers</td>
<td></td>
<td>8 (3.4%)</td>
<td>66.9 ± 8.5</td>
<td>5 (62.5%)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single-cell RNA-seq studies</td>
<td>Bronchial brushings</td>
<td>Current smokers</td>
<td>6 (50%)</td>
<td>42.7 ± 9.7</td>
<td>3 (50%)</td>
<td>15.3 ± 7.9</td>
<td>N/A</td>
<td>N/A</td>
<td>Single-cell RNA-seq</td>
<td>Illumina HiSeq 2500</td>
</tr>
<tr>
<td>Smokers in vivo scRNA-seq (GSE131391) (26)</td>
<td></td>
<td></td>
<td>6 (50%)</td>
<td>29.5 ± 7.6</td>
<td>3 (50%)</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
<td>Single-cell RNA-seq</td>
<td>BD Rhapsody Cell Capture System</td>
</tr>
<tr>
<td>COPD ALI in vitro scRNA-seq (27)</td>
<td>Primary bronchial airway epithelial cells</td>
<td>Ex-smokers</td>
<td>2 (28.6%)</td>
<td>80.5 ± 6.4</td>
<td>0</td>
<td>62 ± 12.7</td>
<td>605 ± 9.2</td>
<td>125 ± 12.0</td>
<td>Single-cell RNA-seq</td>
<td>BD Rhapsody Cell Capture System</td>
</tr>
<tr>
<td></td>
<td>from healthy never-smokers and COPD ex-current smokers</td>
<td>Never-smokers</td>
<td>3 (42.6%)</td>
<td>69 ± 12.2</td>
<td>1 (33.3%)</td>
<td>0</td>
<td>90 ± 18.3</td>
<td>74.3 ± 5.1</td>
<td>Single-cell RNA-seq</td>
<td>Cell Ranger v2.1.1 (10X Genomics)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 (50%)</td>
<td>56.7 ± 5.5</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Single-cell RNA-seq</td>
<td>Cell Ranger v2.1.1 (10X Genomics)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 (50%)</td>
<td>49 ± 15.7</td>
<td>1 (33.3%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Single-cell RNA-seq</td>
<td>Cell Ranger v2.1.1 (10X Genomics)</td>
</tr>
</tbody>
</table>

**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).
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 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.
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 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.
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) 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.

**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.
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

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**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.
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 \((4 \text{ d})\) and a long term \((28 \text{ d})\). 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 6.

**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 that histology of 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 pathophysiology; 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


