



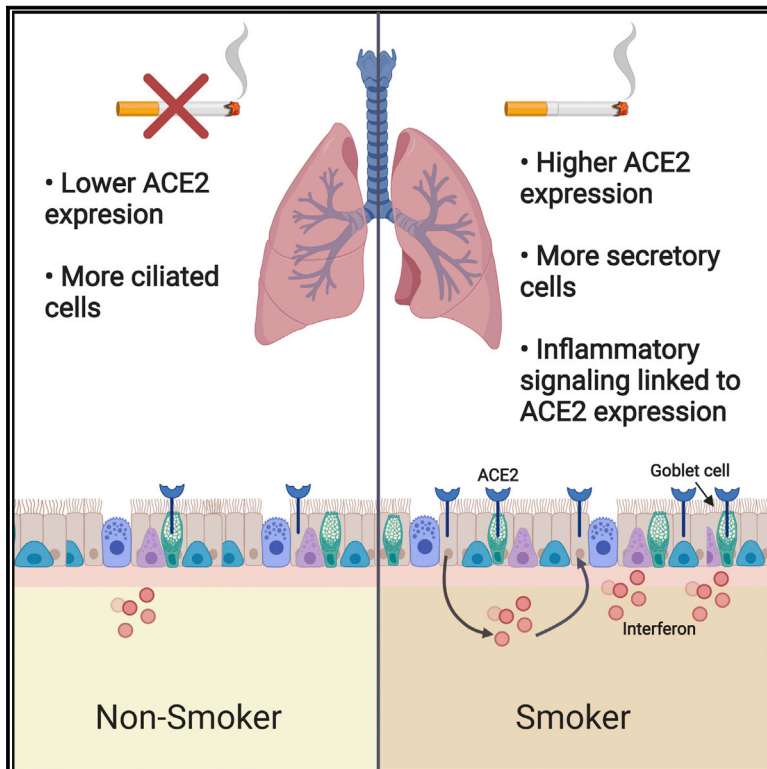
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# Developmental Cell

## Cigarette Smoke Exposure and Inflammatory Signaling Increase the Expression of the SARS-CoV-2 Receptor ACE2 in the Respiratory Tract

### Graphical Abstract



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### In Brief

Smith et al. report that smokers' lungs harbor higher levels of the coronavirus receptor ACE2. They further demonstrate that ACE2 is expressed in a subpopulation of secretory cells that expand in response to smoke exposure. Finally, they establish that ACE2 is an interferon-stimulated gene that is upregulated by viral infections.

### Highlights

- Lung ACE2 levels do not vary by age or sex, but smokers exhibit upregulated ACE2
- ACE2 is expressed in several lung cell types, including the secretory lineage
- Chronic smoking triggers the expansion of ACE2<sup>+</sup> secretory cells
- ACE2 is also upregulated by viral infections and interferon exposure



## Article

# Cigarette Smoke Exposure and Inflammatory Signaling Increase the Expression of the SARS-CoV-2 Receptor ACE2 in the Respiratory Tract

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## SUMMARY

The factors mediating fatal SARS-CoV-2 infections are poorly understood. Here, we show that cigarette smoke causes a dose-dependent upregulation of angiotensin converting enzyme 2 (ACE2), the SARS-CoV-2 receptor, in rodent and human lungs. Using single-cell sequencing data, we demonstrate that ACE2 is expressed in a subset of secretory cells in the respiratory tract. Chronic smoke exposure triggers the expansion of this cell population and a concomitant increase in ACE2 expression. In contrast, quitting smoking decreases the abundance of these secretory cells and reduces ACE2 levels. Finally, we demonstrate that ACE2 expression is responsive to inflammatory signaling and can be upregulated by viral infections or interferon treatment. Taken together, these results may partially explain why smokers are particularly susceptible to severe SARS-CoV-2 infections. Furthermore, our work identifies ACE2 as an interferon-stimulated gene in lung cells, suggesting that SARS-CoV-2 infections could create positive feedback loops that increase ACE2 levels and facilitate viral dissemination.

## INTRODUCTION

In December 2019, a novel respiratory disease emerged in a seafood market in Wuhan, China (Zhu et al., 2020). Genomic sequencing demonstrated that the causative agent was a highly contagious coronavirus, since named SARS-CoV-2 (Wu et al., 2020; Zhou et al., 2020). The disease, called COVID-19, has rapidly spread worldwide, and as of May 2020, more than 4 million people have been infected and more than 300,000 people have died (Our world in data, 2020). No clinically validated treatment or vaccine for COVID-19 is currently available. Thus, understanding the factors that mediate susceptibility to SARS-CoV-2 is crucial for controlling disease transmission.

Molecular analysis has begun to shed light on how SARS-CoV-2 infections occur. Like a related coronavirus that emerged in 2003 (Li et al., 2003), SARS-CoV-2 enters human cells by binding to the extracellular domain of angiotensin converting enzyme 2 (ACE2) (Hoffmann et al., 2020; Zhou et al., 2020). Importantly, ACE2 is both necessary and sufficient for infection by SARS-CoV-2: ACE2-targeting antibodies block viral uptake in permissive cells, while transgenic expression of human ACE2 allows viral entry in non-human cells. ACE2 normally functions in the renin-angiotensin system (RAS) by cleaving the vasoconstrictive hormone angiotensin-II into the vasodilator angiotensin 1–7

(Jiang et al., 2014). Sequestration of ACE2 by coronavirus dysregulates the RAS pathway, contributing to morbidity (Kuba et al., 2005). Additionally, ACE2 levels are capable of influencing disease progression: among a cohort of mice engineered to express human ACE2, mice expressing the highest levels of ACE2 mRNA exhibited the shortest survival time following coronavirus exposure (McCray et al., 2007). Thus, the regulation of ACE2 expression likely has a significant effect on SARS-CoV-2 susceptibility.

Epidemiological studies have identified multiple demographic features that correlate with the severity of clinical COVID-19 cases. While fewer than 5% of SARS-CoV-2 infections are fatal (Ruan, 2020; Wu and McGoogan, 2020), men and elderly patients are particularly at a risk of developing severe disease (Guan et al., 2020; Luo et al., 2020; Rabin, 2020; Remuzzi and Remuzzi, 2020). Additionally, cigarette smokers with COVID-19 are significantly more likely to develop critical illnesses that require aggressive medical intervention (Patanavanich and Glantz, 2020; Zhao et al., 2020a). For instance, in a study of 1,099 patients with laboratory-confirmed COVID-19, 12.3% of current smokers required mechanical ventilation, were admitted to an intensive care unit (ICU), or died, compared with only 4.7% of non-smokers (Guan et al., 2020). The causes underlying these differences in the outcome are at present unknown.



Several clinical features have also been identified that can differentiate between patients with severe and non-severe coronavirus infections. Notably, a dysregulated immune response has been found to be a crucial mediator of COVID-19 mortality (Pedersen and Ho, 2020). Patients who present with elevated levels of inflammatory cytokines are more likely to develop critical illnesses (Chen et al., 2020a; Shi et al., 2020; Yang et al., 2020). These so-called “cytokine storms” cause an increase in vascular permeability that facilitates immune cell efflux into affected tissues, but may also worsen pneumonia (Zhang et al., 2020a). How this immunopathology affects the regulation of the host factors required for coronavirus infections is poorly understood.

## RESULTS

### ACE2 Levels in Mammalian Lungs Are Not Strongly Affected by Age or Sex

In order to study factors that could potentially influence susceptibility to SARS-CoV-2 infection, we investigated the expression of the coronavirus receptor ACE2. We first assessed the expression of ACE2 in a variety of rodent and human tissues (Figure S1; Table S1) (Carithers et al., 2015; GTEx Consortium, 2013; Schaum et al., 2019; Yu et al., 2014). ACE2 was expressed at high levels in mouse, rat, and human kidneys, consistent with its role as a regulator of the RAS pathway. ACE2 was also expressed in rodent and human lungs, the predominant location of coronavirus infections (Wölfel et al., 2020). Interestingly, significant ACE2 expression was also evident in the mouse and human small intestine. Viral RNA has been detected in stool samples from patients with COVID-19 (Xu et al., 2020), and gastrointestinal symptoms have been reported in a subset of affected individuals (Guan et al., 2020), suggesting a potential alternate route for SARS-CoV-2 transmission. However, as SARS-CoV-2 appears to be primarily spread through viral inhalation (Wölfel et al., 2020), we focused our study on factors that affect ACE2 expression in the lung and associated respiratory tissue.

Age and male sex are significant risk factors for severe SARS-CoV-2 infections (Guan et al., 2020; Zhao et al., 2020). We therefore investigated whether either feature was associated with increased ACE2 expression. In four different cohorts of aging mice and one cohort of aging rats, we did not detect a significant age-dependent change in ACE2 expression in the lung (Figures 1A, 1B, and S1D–S1F) (The Tabula Muris Consortium et al., 2020; Jonker et al., 2013; Misra et al., 2007; Soundararajan et al., 2016; Yu et al., 2014). Similarly, ACE2 expression in rodent lungs was not significantly different between sexes (Figures 1C and 1D) (Crowley et al., 2015; Yu et al., 2014). We next assessed the expression of ACE2 in three different human cohorts: (1) lung tissue from the genotype-tissue expression project (GTEx) (Carithers et al., 2015; GTEx Consortium, 2013), (2) whole-lung tissue samples from organ donors (Gruber et al., 2006), and (3) pathologically normal lung tissue from a cohort of patients analyzed as a part of The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research Network et al., 2013). These datasets yielded results that were consistent with our rodent analyses: ACE2 expression was equivalent between men and women and between young (<29 years) and elderly individuals (>70 years) (Figures 1E–1J). In total, these findings suggest that the increased morbidity of men

and older patients with COVID-19 is unlikely to result from inherent differences in the basal level of ACE2 expression in the lung.

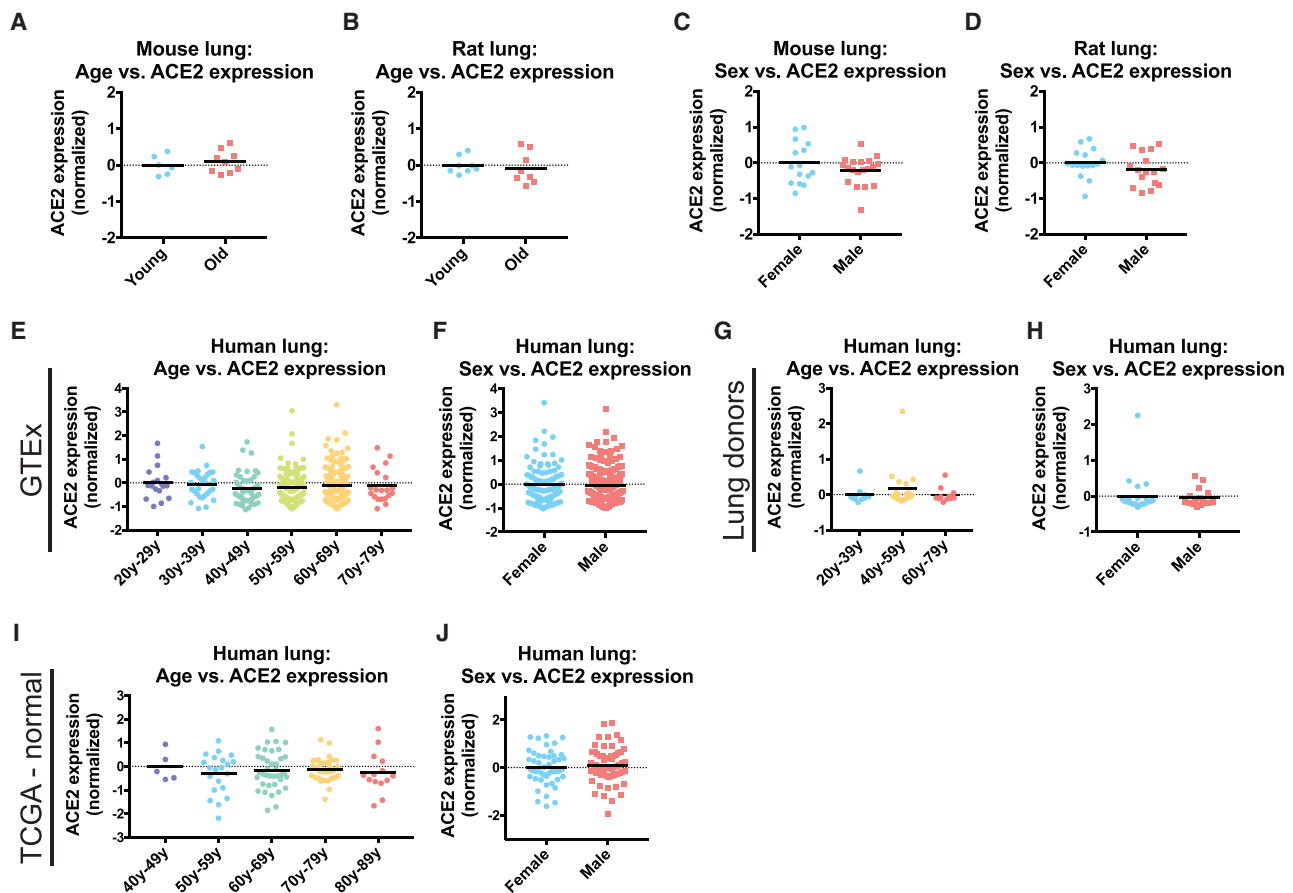
### Cigarette Smoke Increases the Expression of ACE2 in the Mammalian Respiratory Tract

Cigarette smoking is strongly associated with adverse outcomes from COVID-19 (Guan et al., 2020; Hu et al., 2020; Patanavanich and Glantz, 2020; Qi et al., 2020; Wang et al., 2020; Zhang et al., 2020b; Zhao et al., 2020a). To investigate whether smoking could affect ACE2 levels, we first assessed gene expression in mouse lungs. We analyzed a cohort of mice exposed to diluted cigarette smoke for 2, 3, or 4 h per day for 5 months (Gebel et al., 2010). Strikingly, we found a dose-dependent increase in ACE2 expression according to smoke exposure (Figure 2A). Mice exposed to the highest dose of cigarette smoke expressed ~80% more ACE2 in their lungs compared with sham-treated mice. To determine whether this association was present in humans as well, we assessed tissue collected from three cohorts of current smokers and never smokers (Butler et al., 2011; Turetz et al., 2009; Yang et al., 2017). For these analyses, lung epithelial cells were sampled by fiberoptic bronchoscopy from either the trachea, the large airways, or the small airways (Figure 2B). In each cohort, we observed that tissue samples from smokers exhibited ~30%–55% more ACE2 compared with the tissue from non-smokers (Figures 2C–2E). In a combined analysis comprising of data from all three tissue locations, ACE2 was in the top 2% of genes most strongly dysregulated by smoke exposure (Figure 2F).

Next, we sought to determine whether human ACE2 expression showed a dose-dependent relationship with cigarette smoke, as we had observed in mice. To investigate this, we analyzed two human datasets: (1) lung tissue from a cohort of smokers undergoing thoracic surgery for transplantation, lung volume reduction, or nodule resection (Morrow et al., 2017) and (2) pathologically normal lung tissue from TCGA patients (Cancer Genome Atlas Research Network et al., 2013). In both cohorts, lung samples from patients who reported smoking the greatest number of pack years also expressed the highest levels of ACE2 (Figures 2G and 2H). For instance, among smokers undergoing thoracic surgery, patients who had smoked more than 80 pack years exhibited a ~100% increase in ACE2 expression relative to patients who had smoked less than 20 pack years (Figure 2G).

We then investigated whether other demographic features could explain the upregulation of ACE2 in the lungs of smokers. Multivariate linear regression on the thoracic surgery cohort confirmed that smoking history was a significant predictor of ACE2 expression, even when controlling for a patient’s age, sex, race, and body-mass index (Table 1). In the TCGA cohort, pack-year history did not remain significantly associated with ACE2 in a regression with additional clinical variables, which may reflect the very small number of patients who could be included in this analysis (22 patients; data not shown). However, demographic information was available for the tracheal epithelium cohort analyzed above (Figure 2E), and smoking status remained correlated with ACE2 in a regression that included each patient’s age, sex, and race (Table 1).

Finally, we examined the effects of quitting smoking on ACE2 expression. In a cohort of patients comprising either current



**Figure 1. ACE2 Expression in the Lung Is Uncorrelated with Age or Sex**

(A) ACE2 expression in the lungs of young mice (<26 weeks old) and old mice (>78 weeks old).  
 (B) ACE2 expression in the lungs of young rats (6 weeks old) and old rats (104 weeks old).  
 (C) ACE2 expression in the lungs of female mice and male mice.  
 (D) ACE2 expression in the lungs of female rats and male rats.  
 (E) ACE2 expression in lungs from the GTEx cohort by age.  
 (F) ACE2 expression in lungs from the GTEx cohort by sex.  
 (G) ACE2 expression in lungs from a cohort of organ donors by age.  
 (H) ACE2 expression in lungs from a cohort of organ donors by sex.  
 (I) ACE2 expression in pathologically normal lung tissue from patients from TCGA by age.  
 (J) ACE2 expression in pathologically normal lung tissue from patients from TCGA by sex.  
 Each panel displays  $\log_2$ -normalized ACE2 expression relative to a control group. Data analyzed in (A) and (C) were from GSE132040. Data analyzed in (B) and (D) were from GSE53960. Data analyzed in (E) and (F) were from [www.gtexportal.org](http://www.gtexportal.org). Data analyzed in (G) and (H) were from GSE1643. Data analyzed in (I) and (J) were from <https://gdac.broadinstitute.org/>. Additional information on the data sources and sample sizes is included in Table S1.

smokers or former smokers who had refrained from smoking for at least 12 months, quitting smoking was associated with a ~40% decrease in ACE2 expression (Figure 2I). ACE2 was among the top 5% of genes most strongly affected by quitting smoking (Figure 2J). In total, our results demonstrate that exposure to cigarette smoke increases the expression of the coronavirus receptor ACE2 in rodent and human respiratory tissue, and this upregulation is potentially reversible.

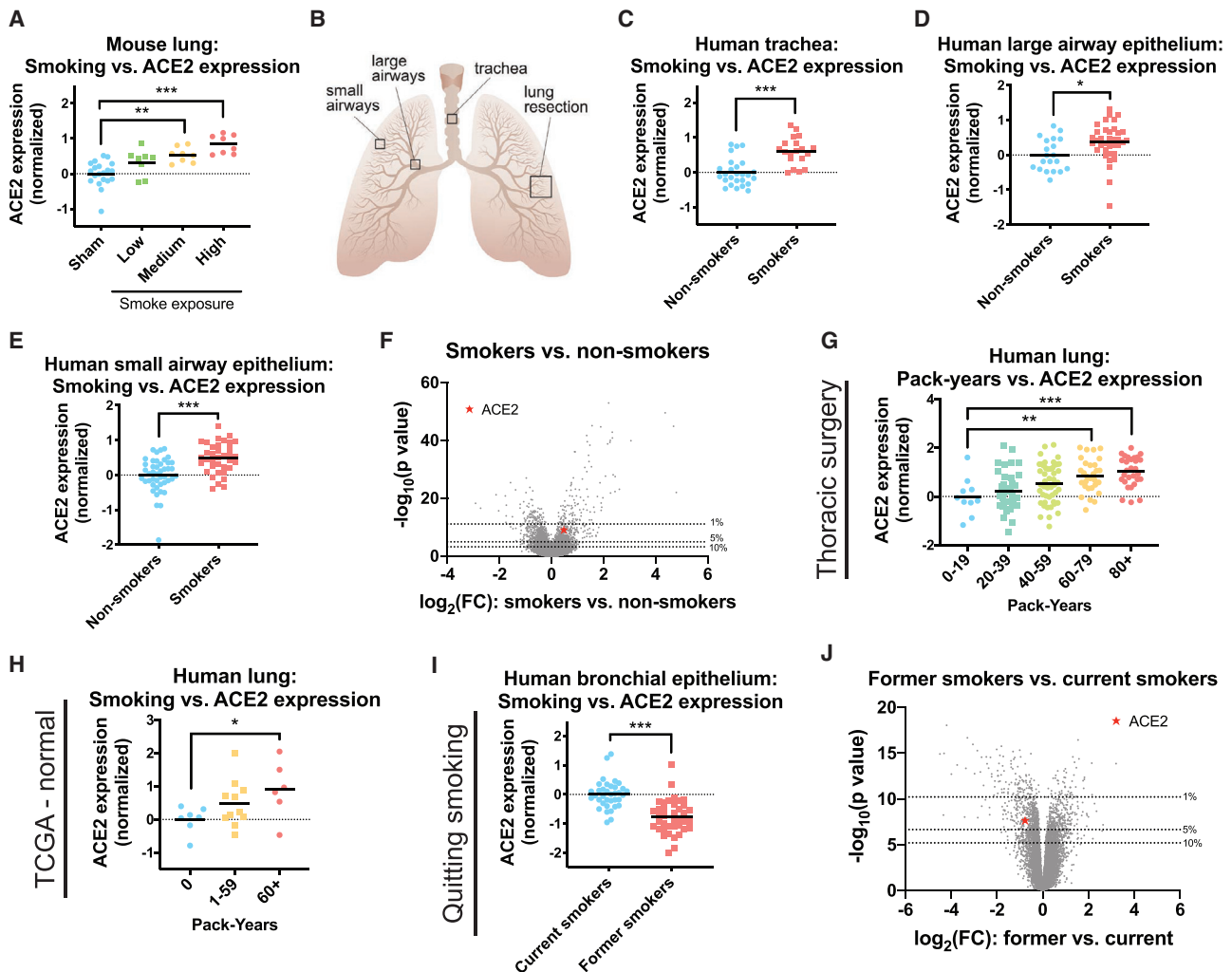
Coronavirus infections are facilitated by a set of host proteases that cleave and activate the viral spike (S) protein (Millet and Whittaker, 2015). SARS-CoV-2 primarily relies on the serine protease TMPRSS2 but can also utilize an alternate pathway involving Cathepsin B/L in TMPRSS2-negative cells (Hoffmann et al., 2020). Interestingly, we observed that Cathepsin B expres-

sion, but not TMPRSS2 or Cathepsin L expression, was consistently increased in mice and humans exposed to cigarette smoke (Figure S2). In a meta-analysis across the trachea, large airways, and small airways, Cathepsin B was in the top 11% of genes dysregulated in the respiratory tract of cigarette smokers (Figure S2D). Thus, smoking can upregulate both the coronavirus receptor as well as a protease that SARS-CoV-2 is capable of using for viral activation.

### ACE2 Is Expressed at High Levels in Secretory Cells in the Lung Epithelium

Mammalian lungs harbor more than 30 distinct cell types representing a variety of epithelial, endothelial, stromal, and immune compartments (Franks et al., 2008). Of note, the upper





**Figure 2. Cigarette Smoke Increases the Expression of ACE2 in Mouse and Human Lungs**

(A) ACE2 expression in the lungs of mice that were sham-treated or that were exposed to diluted cigarette smoke for 2, 3, or 4 h a day (low, medium, and high smoke exposure, respectively).  
 (B) A diagram showing the approximate locations of tissue samples used in this analysis. Tracheal, large airway epithelial, and small airway epithelial specimens were collected by fiberoptic bronchoscopy as described in their respective publications. Lung resections were collected surgically from various locations.  
 (C) ACE2 expression in human tracheal epithelia analyzed according to smoking history.  
 (D) ACE2 expression in human large airway epithelia analyzed according to smoking history.  
 (E) ACE2 expression in human small airway epithelia analyzed according to smoking history.  
 (F) A volcano plot comparing gene expression in the respiratory epithelia of current smokers and never smokers from (C)–(E). The dotted lines indicate various p value thresholds (e.g., genes located above the 10th percentile have a combined p value greater than 90% of the genes included in the meta-analysis). The location of ACE2 is indicated with a red star.  
 (G) ACE2 expression in the lungs of a cohort of patients undergoing thoracic surgery analyzed according to the number of pack years each patient smoked.  
 (H) ACE2 expression in the lungs of TCGA patients analyzed according to the number of pack years each patient smoked.  
 (I) ACE2 expression in respiratory epithelia collected by fiberoptic bronchoscopy among either current smokers or former smokers.  
 (J) A volcano plot comparing gene expression between current smokers and former smokers. The dotted lines indicate various p value thresholds (e.g., genes located above the 10<sup>th</sup> percentile have a combined p value greater than 90% of the genes included in the meta-analysis). The location of ACE2 is indicated with a red star.

Each panel displays log<sub>2</sub>-normalized ACE2 expression relative to a control group. Data analyzed in (A) were from GSE18344. Data analyzed in (C) were from GSE13933. Data analyzed in (D) were from GSE22047. Data analyzed in (E) were from GSE64614. Data analyzed in (G) were from GSE76925. Data analyzed in (H) were from <https://gdac.broadinstitute.org/>. Data analyzed in (I) were from GSE79209. Additional information on the data sources and sample sizes are included in Table S1. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.0005 (Student's t test).

respiratory epithelium comprises mucociliary cells, including goblet cells, club cells, and ciliated cells, that secrete protective fluids and remove inhaled particles from the airways (Whitsett,

2018). The lower respiratory epithelium includes alveolar type 1 cells, which allow gas exchange with the blood, and alveolar type 2 cells, which regulate alveolar fluid balance and can

**Table 1. Patient Characteristics and ACE2 Expression**

		$\beta$ Value	Standard Error	p Value
Thoracic surgery cohort	Age	0.0014	0.0086	0.8700
	BMI	-0.0199	0.0127	0.1193
	Smoking pack years	0.0081	0.0025	0.0015**
	Race: Caucasian = 0; not Caucasian = 1	0.1910	0.1663	0.2527
	Sex: female = 0, male = 1	0.3335	0.1338	0.0138*
Tracheal epithelium cohort	Age	-0.0027	0.0080	0.7364
	Smoking status: never smoker = 0; current smoker = 1	0.5638	0.1269	$7 \times 10^{-5}$ ***
	Race: Caucasian = 0; not Caucasian = 1	0.1252	0.1331	0.3527
	Sex: female = 0; male = 1	0.2538	0.1328	0.0632

Multivariate regression between various patient characteristics, smoking status, and ACE2 expression. The thoracic surgery cohort is from GSE76925 and the tracheal epithelium cohort is from GSE13933. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.0005.

differentiate into type 1 cells following injury (Whitsett and Weaver, 2015).

To gain further insight into coronavirus infections, we profiled multiple single-cell RNA-seq experiments to identify the cell type(s) that express ACE2. We first examined a dataset containing 13,822 cells from normal mouse lungs (Reyffman et al., 2019). We performed unsupervised Leiden clustering to separate the cells into distinct populations and then we assigned cell types to major clusters using established markers (Angelidis et al., 2019; Ardini-Poleske et al., 2017; Plasschaert et al., 2018; Wolf et al., 2018). ACE2 was expressed solely in the EpCAM<sup>+</sup> clusters that comprise the lung epithelium (Kasper et al., 1995), and was not detected in CD45<sup>+</sup> immune cells (Thomas, 1989), PDGFRA<sup>+</sup> mesenchymal cells (Li et al., 2018), or TMEM100<sup>+</sup> endothelial cells (Figures 3A and 3B) (Moon et al., 2010). We therefore focused on localizing ACE2 within the epithelial lineage. We found that ACE2 was present in a cluster of cells that express secretory markers including MUC5AC, GABRP, and SCGB1A1, which we identified as being comprised of closely related goblet and club cells (Figures 3C and 3D) (Angelidis et al., 2019; Guha et al., 2014; Hovenberg et al., 1996; Rawlins et al., 2009). ACE2 expression was also observed in a subset of LAMP3<sup>+</sup> alveolar type 2 cells (Salaun et al., 2004), but was largely absent from RTKN2<sup>+</sup> alveolar type 1 cells (Angelidis et al., 2019).

We then extended our findings to an independent single-cell dataset from human lungs (Reyffman et al., 2019). Consistent with our initial observations, we found that ACE2<sup>+</sup> cells were found almost exclusively in the EPCAM<sup>+</sup> epithelial compartment (Figures 3E and 3F). Within this lineage, we observed ACE2 expression in the LAMP3<sup>+</sup> alveolar type 2 cluster and the MUC5AC<sup>+</sup> goblet/club cell cluster (Figures 3G and 3H). Additionally, in human cells, there was some expression of ACE2 in FOXJ1<sup>+</sup> ciliated cells (Yu et al., 2008). In total, our combined analysis demonstrates that ACE2 is expressed in the mammalian lung epithelium, and is present at particularly high levels in secretory club and goblet cells as well as in alveolar type 2 cells.

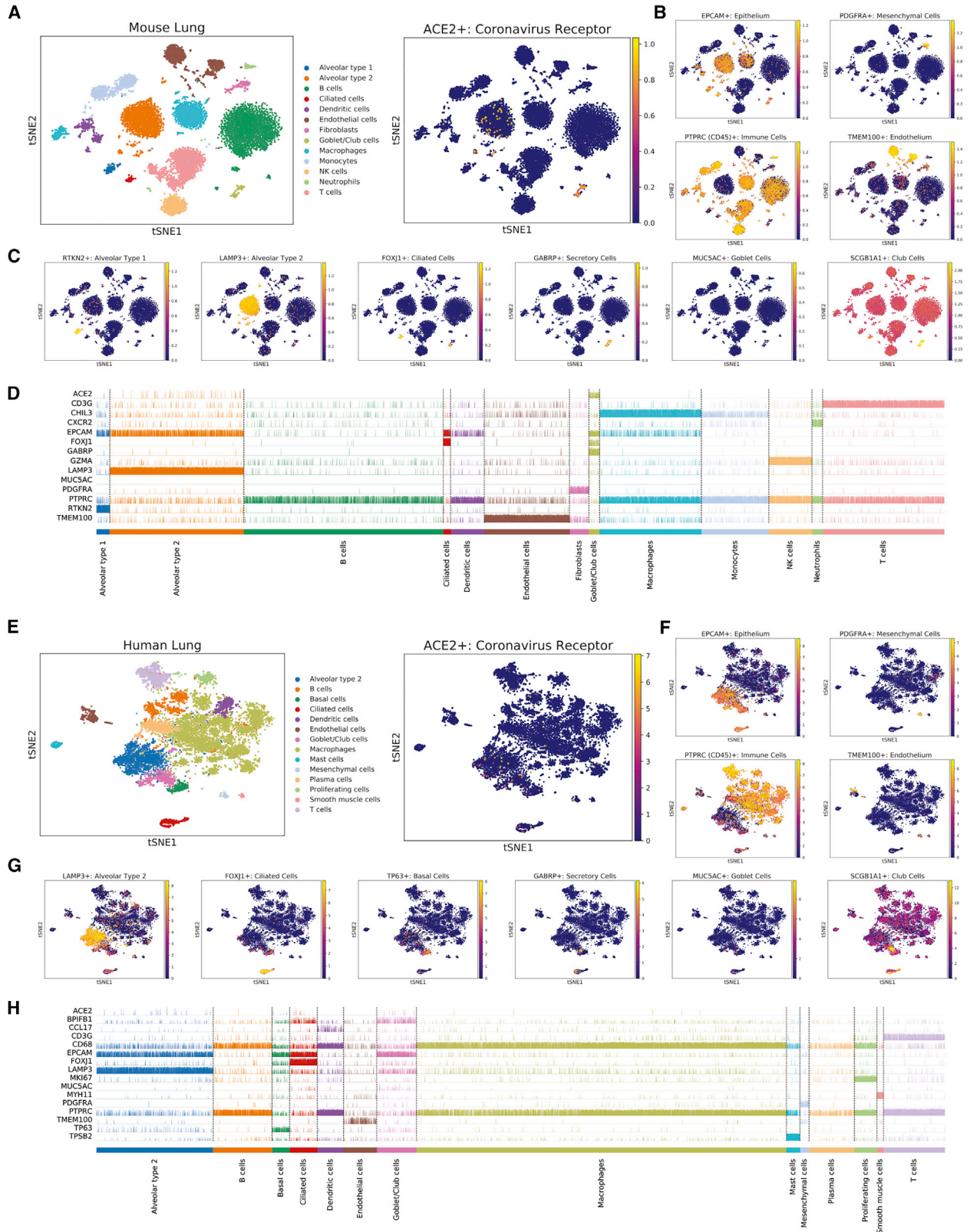
### Cigarette Smoke Triggers an Increase in ACE2<sup>+</sup> Cells by Driving Secretory Cell Expansion

Our findings on ACE2's expression pattern suggested a possible explanation for its upregulation in the lungs of cigarette smokers.

Chronic exposure to cigarette smoke has been reported to induce the expansion of secretory goblet cells, which produce mucous to protect the respiratory tract from inhaled irritants (Cosio et al., 1980; Liu and Di, 2012; Saetta et al., 2000). Thus, the increased expression of ACE2 in smokers' lungs could be a by-product of smoking-induced secretory cell hyperplasia.

To investigate this hypothesis, we examined a dataset of single-cell transcriptomes collected from the tracheas of current smokers and never smokers (Goldfarbmuren et al., 2019). As these cells were derived from the upper airways, they were highly enriched for pseudostratified respiratory epithelial cell types and contained few alveolar cells. Consistent with the initial analysis of this dataset, Leiden clustering revealed a large population of TP63<sup>+</sup> basal cells (the putative stem cell population for the airway epithelium) (Rock et al., 2010), an intermediate KRT8<sup>high</sup> population that showed evidence of ongoing differentiation (Goldfarbmuren et al., 2019), and populations of mature FOXJ1<sup>+</sup> ciliated cells and MUC5AC<sup>+</sup> secretory cells (Figures 4A, 4B, S3A, and S3B). We also identified markers for submucosal gland (SMG) clusters as well as rare epithelial cell types, including CFTR<sup>+</sup> ionocytes (Montoro et al., 2018) and CHGA<sup>+</sup> neuroendocrine cells (Gu et al., 2014). Within this dataset, ACE2 was expressed at the highest levels in the MUC5AC<sup>+</sup> secretory cell cluster, followed by SMG-secretory cells, KRT8<sup>high</sup> intermediate cells, and FOXJ1<sup>+</sup> ciliated cells.

We next identified the transcripts whose expression correlated with ACE2. Across all cells, ACE2 levels were strongly correlated with several mucin genes, including MUC1, MUC4, MUC15, and MUC16, as well as other genes associated with barrier epithelia, including ALCAM (Tomita et al., 2000), CLDN7 (Farkas et al., 2015), and TJP3 (González-Mariscal et al., 2003) (Table S2A). Gene ontology analysis revealed that ACE2-correlated transcripts were enriched for genes involved in secretion, glycosylation, and the response to toxic substances, consistent with the airway epithelium's role as a producer of mucus and a barrier against foreign matter (Figure 4C; Table S2B) (Fahy and Dickey, 2010). ACE2<sup>+</sup> correlates overlapped with but were not identical to goblet/club markers: several genes were widely expressed in both populations (MUC16, CLDN7, S100P), while others were more restricted to single lineages (PIGR, PSCA) (Figure 4D). In general, the ACE2<sup>+</sup> signature was expressed in both secretory



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cells and ciliated cells but not in the basal stem cell compartment.

We next separated the cells harvested from current smokers and never smokers and then analyzed each population separately. Consistent with previous reports (Cosio et al., 1980; Liu and Di, 2012; Saetta et al., 2000), we detected a significant expansion of the secretory cell compartment in smokers' airways, as evidenced by both cluster analysis and by the increase in the number of cells expressing the canonical goblet cell marker MUC5AC (Figures 4E and 4F). FOXJ1<sup>+</sup> ciliated cells decreased in abundance, likely reflecting the fact that smoke exposure inhibits ciliogenesis (Tamashiro et al., 2009). These trends remained apparent when the cells from each patient were examined individually (Figures S4C and S4D). Notably, smoking caused an increase in both the number of ACE2<sup>+</sup> positive cells and in ACE2 expression within ACE2<sup>+</sup> cells (Figures 4G and 4H). This expansion resulted from hyperplasia of the secretory cell compartment: smoke exposure triggered a 2-fold increase in the frequency of MUC5AC<sup>+</sup>/ACE2<sup>+</sup> double-positive cells, while there was no significant change in the frequency of MUC5AC<sup>-</sup>/ACE2<sup>+</sup> cells. In total, these results provide single-cell evidence that the increase in ACE2 expression in smokers' respiratory tracts is caused by the expansion of mucous-secreting goblet cells that co-express ACE2.

To further validate these findings, we derived gene signatures for each of the cell types identified from tracheal single-cell sequencing, and we applied these signatures to analyze the bulk gene expression datasets from smokers and non-smokers that we had previously investigated (Figure 2). The goblet/club cell gene signature was significantly upregulated in current smokers compared with never smokers, while in former smokers these genes were strongly downregulated (Figures 4I and 4J). A signature based on ACE2-correlated transcripts showed a similar expression pattern. In contrast, a ciliated cell transcriptional signature was strongly downregulated among current smokers. As some FOXJ1<sup>+</sup> ciliated cells co-express ACE2, this smoking-dependent suppression of ciliogenesis may partially blunt the increase in ACE2<sup>+</sup> cells caused by smoke-induced goblet cell hyperplasia.

Secretory cell differentiation of lung epithelium can be modeled *in vitro* by culturing cells at an air-liquid interface (ALI) (Jiang et al., 2018; Upadhyay and Palmberg, 2018). Under appropriate conditions, primary respiratory cells growing at an ALI will undergo mucociliary differentiation into a stratified

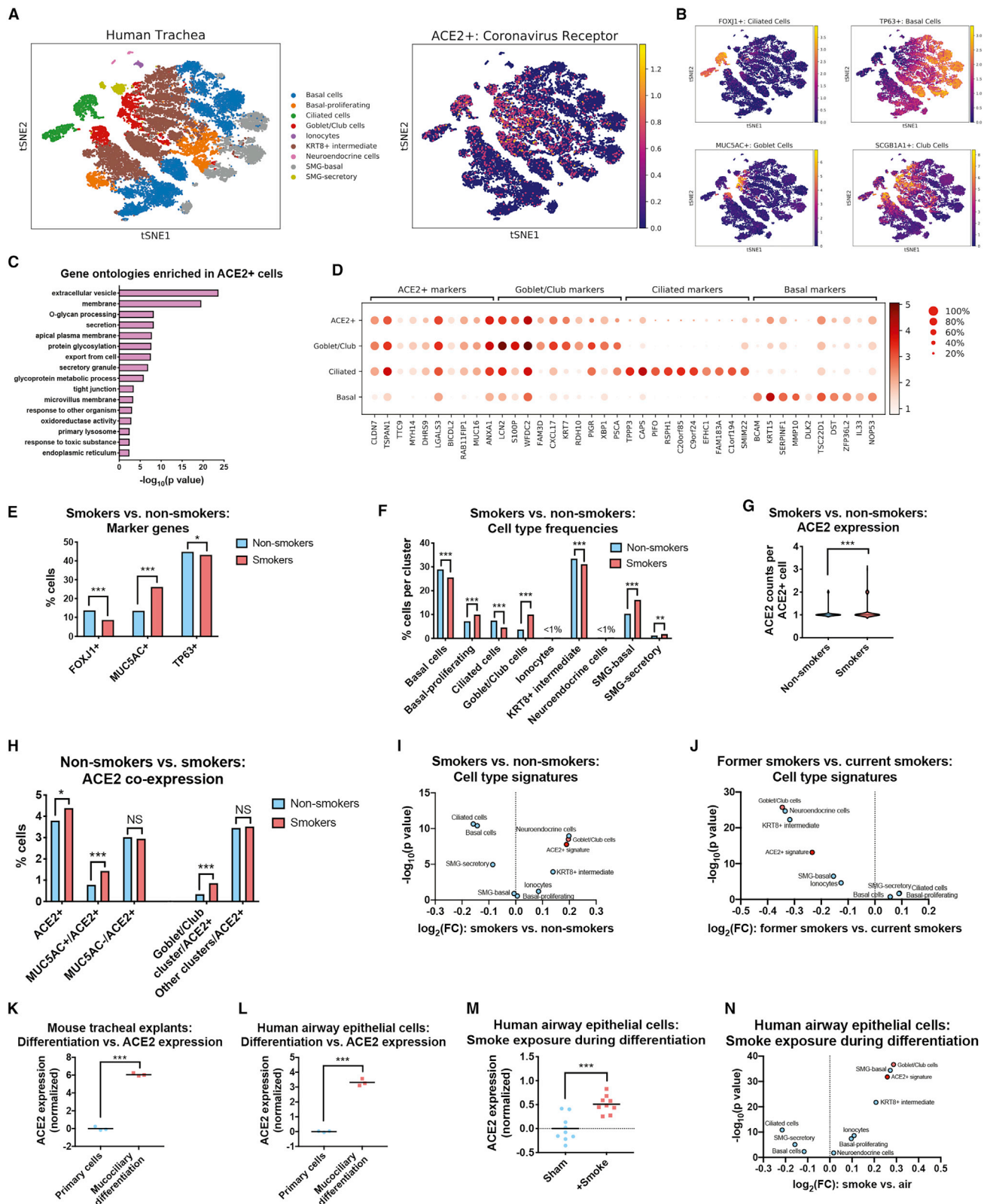
epithelium consisting of ciliated cells, goblet cells, and club cells (Ross et al., 2007). As our single-cell analysis suggested that the coronavirus receptor ACE2 is expressed at higher levels in differentiated secretory and ciliated cells compared with basal stem cells, we investigated whether *in vitro* mucociliary differentiation increases ACE2 expression. Indeed, in mouse tracheal extracts (Nemajerova et al., 2016) and primary human lung cells (Martinez-Anton et al., 2013), mucociliary differentiation resulted in a highly significant upregulation of ACE2 (Figures 4K and 4L). Finally, to investigate the link between smoking, differentiation, and ACE2 expression, we examined data from human bronchial epithelial cells cultured at an ALI in which cells were either exposed to clean air or to diluted cigarette smoke (Gindele et al., 2020). Remarkably, treatment with cigarette smoke during *in vitro* differentiation resulted in a significant upregulation of ACE2 relative to cells that were differentiated in clean air (Figure 4M). Smoke exposure increased ACE2 expression by ~42%, comparable to the increases that we observed between the lungs of non-smokers and smokers (Figure 2). Differentiation in the presence of cigarette smoke similarly resulted in an upregulation of the goblet/club cell transcriptional signature and a downregulation of the ciliated cell transcriptional signature (Figure 4N). In full, our results demonstrate that a subset of lung secretory cells express the coronavirus receptor ACE2, and cigarette smoke promotes the expansion of this cell population.

### ACE2 Is Upregulated in Smoking-Associated Diseases and by Viral Infections

To follow up on these observations, we investigated whether ACE2 expression was affected by other lung diseases and/or carcinogen exposures. Indeed, we observed increased ACE2 expression in multiple cohorts of patients with chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) (Figures S4A–S4D) (Cruz et al., 2019; Kim et al., 2015; McDonough et al., 2019; Pardo et al., 2005). Interestingly, both COPD and IPF are strongly associated with prior cigarette exposure (Baumgartner et al., 1997; Laniado-Laborín, 2009), and COPD in particular has been identified as a risk factor for severe COVID-19 (Lippi and Henry, 2020; Zhao et al., 2020a). However, ACE2 expression was generally not affected by other lung conditions or toxins. We did not observe a significant difference in ACE2 expression in lung samples from a large cohort of patients with asthma or from patients with the lung disease sarcoidosis (Figures S4E and S4F) (Crouser et al., 2009; Voraphani et al.,

### Figure 3. ACE2 Is Expressed in Secretory Club and Goblet Cells Along with Alveolar Type 2 Cells in the Mammalian Lung

- (A) T-SNE clustering of cells from the mouse lung. Cells expressing ACE2 are highlighted in the right panel.
- (B) Cells in the mouse lung that express various lineage markers (TMEM100 for endothelial cells, EPCAM for epithelial cells, PDGFRA for mesenchymal cells, and PTPRC for immune cells) are highlighted.
- (C) Cells expressing markers for various epithelial lineages are highlighted: RTKN2 for alveolar type 1 cells, LAMP3 for alveolar type 2 cells, FOXJ1 for ciliated cells, GABRP for both goblet and club cells, MUC5AC for goblet cells, and SCGB1A for club cells.
- (D) A track plot displaying the expression of ACE2 and several lineage-related genes in different cell populations obtained from Leiden clustering.
- (E) T-SNE clustering of cells from the human lung. Cells expressing ACE2 are highlighted in the right panel.
- (F) Cells in the human lung that express various lineage markers (TMEM100 for endothelial cells, EPCAM for epithelial cells, PDGFRA for mesenchymal cells, and PTPRC for immune cells) are highlighted.
- (G) Cells expressing markers for various epithelial lineages are highlighted: LAMP3 for alveolar type 2 cells, FOXJ1 for ciliated cells, TP63 for basal cells, GABRP for both goblet and club cells, MUC5AC for goblet cells, and SCGB1A1 for club cells.
- (H) A track plot displaying the expression of ACE2 and several lineage-related genes in different cell populations obtained from Leiden clustering.
- The gene expression data used in (A)–(D) are from GSE121611. The gene expression data used in (E)–(H) are from GSE122960. Additional information on the data sources and sample sizes are included in Table S1.



**Figure 4. Cigarette Smoke Causes the Expansion of ACE2<sup>+</sup> Secretory Cells**

(A) T-SNE clustering of the transcriptomes from single cells derived from the airway epithelia of smokers and never smokers. Cells expressing ACE2 are highlighted in the right panel.

(legend continued on next page)

2014). Similarly, ACE2 expression was unaltered in lung tissue from a mouse model of cystic fibrosis and in mice exposed to a variety of carcinogens, including arsenic, ionizing radiation (IR), and 1,3-butadiene (Figures S4G–S4J) (Chappell et al. 2017; Citrin et al., 2013; Haston et al., 2006; Kozul et al., 2009). We conclude that ACE2 upregulation in the lung is tightly associated with a history of cigarette smoking and is not a universal response to pulmonary diseases.

So-called “cytokine storms,” characterized by high levels of circulating inflammatory cytokines, have been identified as a cause of COVID-19-related mortality (Chen et al., 2020a; Pederesen and Ho, 2020; Shi et al., 2020). Cytokine release can be triggered by viral infections, which serve to induce immune cell activation and expansion (Mogensen and Paludan, 2001). Cigarette smoke is also an inflammatory agent, and smokers tend to exhibit an increase in inflammation-related serological markers (Amson et al., 2010; Gan et al., 2005). To investigate a potential link between inflammation and the expression of the host factors required for coronavirus infections, we first examined the levels of ACE2 in published datasets of respiratory epithelial cells challenged with different viruses. We observed a highly significant upregulation of ACE2 in airway cells that were infected with two RNA viruses, influenza and Respiratory Syncytial Virus (Figures 5A and 5B) (Ioannidis et al., 2012). Infection with two coronaviruses, SARS and MERS, that are closely related to the 2019 pandemic strain also caused an increase in ACE2 levels (Figures 5C and 5D) (Menachery et al., 2017; Mitchell et al., 2013). Double-stranded RNA that is produced during viral replication can be detected by host pattern recognition receptors, triggering a strong immune response (Gantier and Williams, 2007). Accordingly, transfection of the dsRNA mimic poly(I:C) into airway epithelial cells was sufficient to induce the upregulation of ACE2 (Figure 5E) (Wagener et al., 2014). These results reveal that inflammatory signals—like those triggered by smoking or by a viral infection—are capable of increasing the expression of ACE2 in the respiratory epithelium.

To further investigate the stimuli that are sufficient to upregulate ACE2, we cultured primary epithelial cells from either the small airways or the trachea in the presence of different com-

pounds. We found that exposure to interferons resulted in a significant upregulation of ACE2 expression (Figures 5F and 5G). IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  treatment increased ACE2 expression in tracheal cells, while only IFN- $\alpha$  and IFN- $\beta$  affected ACE2 in small airway cells. To further verify these results, we re-analyzed a published gene expression dataset of airway epithelial cells exposed to IFN- $\beta$ , and we found a similar increase in ACE2 levels following interferon treatment (Figure 5H) (Rusinova et al., 2013; Shapira et al., 2009). The results identify ACE2 as an interferon-regulated gene and suggest a potential mechanism by which inflammatory stimuli could facilitate SARS-CoV-2 infections.

### ACE2 RNA and Protein Levels Are Tightly Correlated

This paper describes a series of analyses and experiments to identify factors that drive the expression of the coronavirus receptor ACE2. However, these results could potentially be confounded if changes in ACE2 RNA levels fail to affect the steady-state levels of ACE2 protein. While we lack the data to directly test that link in the lungs of smokers, we conducted three additional analyses to investigate whether ACE2 RNA and protein expression are generally correlated. First, we examined a dataset of human cancer cell lines that had been profiled at the transcriptional level by RNA-seq and at the protein level by mass spectrometry (Barretina et al., 2012; Nusinow and Gygi, 2020; Nusinow et al., 2020). Across 53 cell lines, ACE2 RNA and protein levels were strongly correlated ( $r = 0.82$ ,  $p < .0001$ ; Figure S5A). Indeed, ACE2’s RNA-protein correlation coefficient was higher than 95% of human genes that could be reliably detected (Figure S5B). Next, we compared ACE2 RNA levels with ACE2 immunohistochemistry staining in untransformed human tissues. We similarly observed a strong correlation between ACE2 RNA and protein staining: five of the six tissues with the highest levels of RNA expression exhibited strong staining, while none of the 35 other tissues with lower RNA levels exhibited strong staining (Figure S5C) (Uhlén et al., 2015). Lastly, we directly compared ACE2 expression in seven human cell lines by qRT-PCR and by western blotting. We found that cell lines that displayed the highest levels of ACE2 RNA (LoVo, Caco-2, and Calu3) also exhibited the most ACE2 protein, while cell lines

(B) Cells in the human airway that express various lineage markers (FOXP1 for ciliated cells, TP63 for basal cells, MUC5AC for goblet cells, and SCGB1A1 for club cells) are highlighted.

(C) GO terms enriched among ACE2-correlated transcripts.

(D) Dot plots displaying the expression of the top ten differentially expressed marker genes for various airway lineages and for ACE2+ cells.

(E) The fraction of cells expressing the indicated marker genes are displayed. FOXP1 is a marker for ciliated cells, MUC5AC is a marker for goblet cells, and TP63 is a marker for basal cells.

(F) The fractions of cells found in each cell type cluster are displayed.

(G) The number of counts per ACE2+ cell are displayed.

(H) The fraction of ACE2+ cells co-expressing MUC5AC or that are found within the goblet/club cell cluster are displayed.

(I) The 100 top-ranked differentially expressed genes from each cluster in (A) as well as the 100 genes most strongly correlated with ACE2 were used to re-analyze the bulk gene expression data from smokers and non-smokers in Figure 2F. A volcano plot displays the mean expression change of each cell type signature.

(J) The same transcriptional signatures as in Figure 4I were used to re-analyze the data from current smokers and former smokers in Figure 2J. A volcano plot displays the mean expression change of each cell type signature.

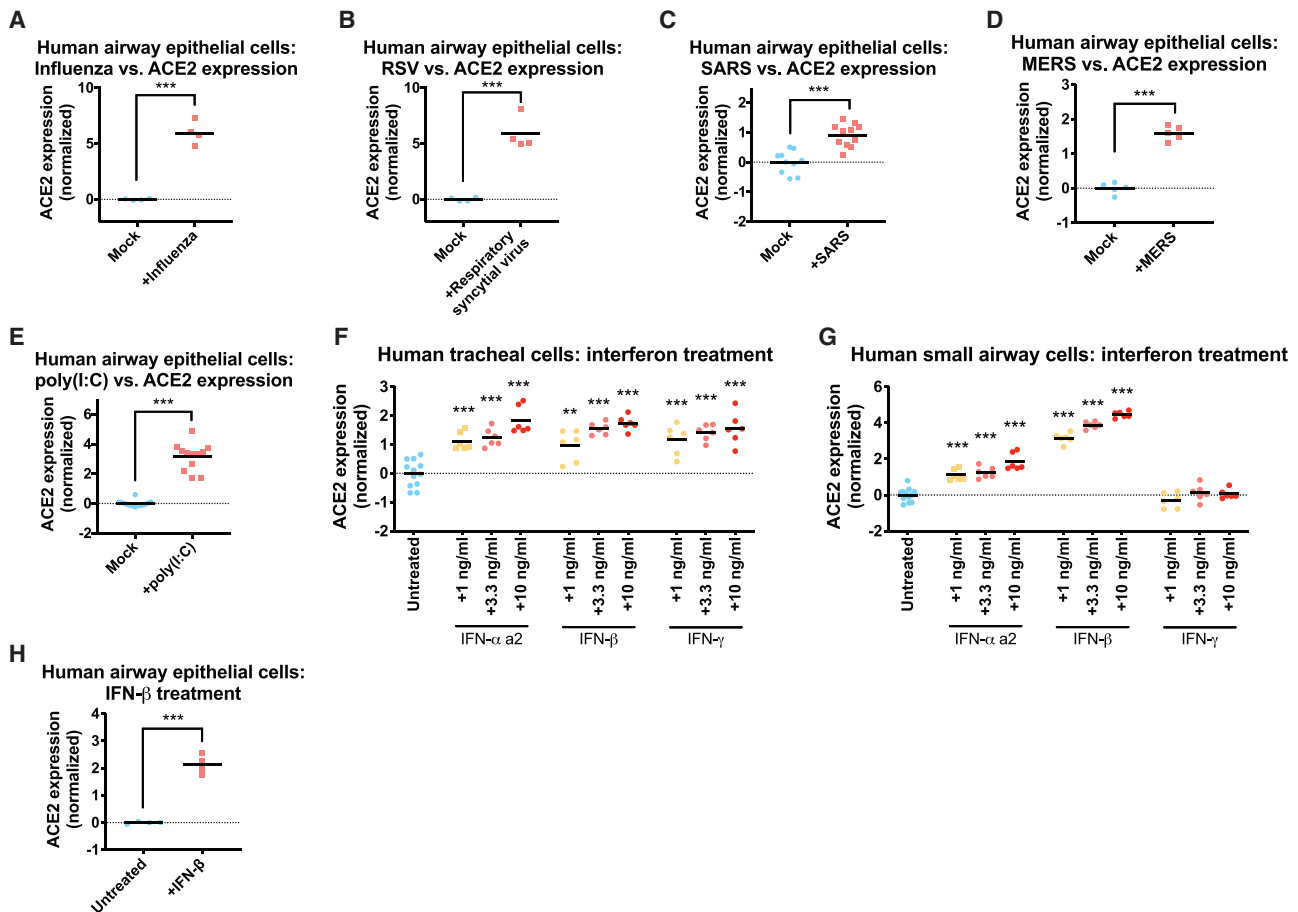
(K) ACE2 expression in mouse tracheal explants undergoing mucociliary differentiation.

(L) ACE2 expression in human airway epithelial cells undergoing mucociliary differentiation.

(M) ACE2 expression in human airway epithelial cells that underwent mucociliary differentiation in the presence of clean air or cigarette smoke.

(N) The same transcriptional signatures as in Figure 4I were used to re-analyze the data from smoke exposure during differentiation from Figure 4M. A volcano plot displaying the mean expression change of each cell type signature is displayed.

Data analyzed in (A)–(H) were from GSE131391. Data analyzed in (K) were from GSE75715. Data analyzed in (L) were from GSE39059. Data analyzed in (M) were from GSE135188. Additional information on the data sources and sample sizes are included in Table S1. In (E), (F), and (H), a chi-square test is applied. In (G), a Mann-Whitney U test is applied. In (K)–(M), a Student’s t test is applied. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ .



**Figure 5. ACE2 Is an Interferon-Stimulated Gene that Is Upregulated by Viral Infections**

(A) ACE2 expression in airway epithelial cells that were infected with influenza.  
 (B) ACE2 expression in airway epithelial cells that were infected with respiratory syncytial virus.  
 (C) ACE2 expression in airway epithelial cells that were infected with SARS.  
 (D) ACE2 expression in airway epithelial cells that were infected with MERS.  
 (E) ACE2 expression in airway epithelial cells that were transfected with the dsRNA mimic poly(I:C).  
 (F) ACE2 expression in human tracheal cells that were cultured in the presence of the indicated cytokine for 24 h.  
 (G) ACE2 expression in human small airway epithelial cells that were cultured in the presence of the indicated cytokine for 24 h.  
 (H) ACE2 expression in airway epithelial cells that were cultured in the presence of IFN- $\beta$ .  
 Each panel displays log<sub>2</sub>-normalized ACE2 expression relative to a control group. Data analyzed in (A) and (B) were from GSE32138. Data analyzed in (C) were from GSE47963. Data analyzed in (D) were from GSE100504. Data analyzed in (E) were from GSE51392. Data analyzed in (H) were from GSE19392. Additional information on the data sources and sample sizes are included in Table S1. \*p < 0.05, \*\*p < 0.005, \*\*\*p < 0.0005 (Student's t test).

with very low levels of ACE2 RNA (HCT116, CAL148, and A2780) had undetectable levels of ACE2 protein (Figures S5D and S5E). Thus, while we cannot rule out the possibility that ACE2 RNA and protein levels are differentially regulated in certain circumstances, our analysis reveals that ACE2 RNA and protein levels are generally correlated.

## DISCUSSION

While SARS-CoV-2 has infected more than 4 million people worldwide, fewer than 5% of COVID-19 cases are fatal (Guan et al., 2020; Our world in data, 2020). Here, we show that cigarette smokers harbor consistently higher levels of the SARS-CoV-2 receptor ACE2 in their respiratory tracts. This upregulation is likely mediated by the expansion of ACE2<sup>+</sup> secretory cells

caused by chronic smoke exposure. Certain inflammatory cytokines also trigger ACE2 upregulation, which could further influence ACE2 expression due to smoking-associated lung inflammation. The overabundance of ACE2 in the lungs of smokers may partially explain why smokers are significantly more likely to develop severe SARS-CoV-2 infections that require aggressive medical interventions (Guan et al., 2020; Patanavanich and Glantz, 2020; Qi et al., 2020; Zhao et al., 2020a). Furthermore, as quitting smoking is associated with a decrease in ACE2 expression, it is possible that giving up cigarettes may reduce susceptibility to deadly COVID-19.

Several contrasting findings exist in the literature on ACE2 and cigarette exposure (Brake et al., 2020; Ferrari et al., 2007, 2008; Leung et al., 2020; Oakes et al., 2018; Yilin et al., 2015; Yue et al., 2018; Cai, 2020). In particular, it has been reported that nicotine



and/or cigarette smoke has the potential to downregulate ACE2 expression in certain tissues or cell types (Ferrari et al., 2007, 2008; Oakes et al., 2018; Yue et al., 2018). In this manuscript, we focused our analysis on factors affecting ACE2 expression in the mammalian lungs and associated respiratory epithelia. We observed a consistent correlation between smoking history and ACE2 expression that was dose-dependent (Figures 2G and 2H), that could be recapitulated in mice (Figure 2A) and *in vitro* (Figure 4M), that was detectable in both bulk and single-cell analyses (Figures 2F, 4G, and 4H), and that remained significant when controlling for other demographic variables (Table 1). Thus, we propose that cigarette smoke causes the upregulation of ACE2 expression in the respiratory tract. However, we recognize that individual components found within cigarettes, like nicotine, may have a different effect on ACE2 than whole smoke, and smoking itself could alter ACE2 levels in non-respiratory organs in different ways (Ferrari et al., 2008).

Several previous studies have examined the localization of ACE2 within the respiratory tract (Ding et al., 2004; Hamming et al., 2004; Jia et al., 2005; Lukassen et al., 2020; Sungnak et al., 2020; Travaglini et al., 2020; Wiener et al., 2007; Wu and Zheng, 2020; Xie et al., 2006; Yilin et al., 2015; Zhao et al., 2020b; Ziegler et al., 2020). Consistent with our results, ACE2 expression has been detected in the respiratory epithelium, including prominent staining in alveolar type 2 cells. However, some prior studies have also documented ACE2 expression in lung endothelial and smooth muscle cells (Hamming et al., 2004; Wiener et al., 2007; Xie et al., 2006), which we did not observe. These findings could represent non-specific staining resulting from the particular antibodies that were used (Bradbury and Plückthun, 2015). Alternately, ACE2 may be expressed in mesenchymal or other lineages within the lung, and our inability to detect it may be a limitation of the single-cell samples that we analyzed. Nonetheless, by examining datasets from both mice and humans, and by including cells collected from bronchial brushing, we demonstrated consistently high levels of ACE2 transcripts in the secretory cells that participate in mucociliary clearance (Whitsett, 2018). As these cells line the upper respiratory tract, they may represent the initial site of coronavirus infections, followed by an eventual spread and migration into the alveoli.

Cigarette smoke has a profound impact on the lungs of chronic smokers. Notably, prolonged smoke exposure triggers secretory cell hyperplasia, thereby increasing the production of mucous in the respiratory tract (Cosio et al., 1980; Liu and Di, 2012; Saetta et al., 2000). We found that this smoking-induced expansion of secretory cells also causes an increase in ACE2 levels, as smoking doubled the number of cells co-expressing ACE2 and MUC5AC. Additionally, consistent with a recent report (Ziegler et al., 2020), we found that ACE2 is an interferon-regulated gene, and is over expressed in lung epithelial cells following viral infection or interferon treatment. Lung damage and inflammation caused by smoking could also contribute to ACE2 upregulation. Additionally, we speculate that the interferon-dependent upregulation of ACE2 could create a positive feedback loop for SARS-CoV-2 infections. That is, interferon secretion following an initial infection could increase ACE2 expression within neighboring cells, thereby rendering those cells susceptible to SARS-CoV-2 and facilitating viral dissemination. Accord-

ingly, clinical interventions to dampen the immune response could benefit patients in part by breaking this positive feedback cycle (Roumier et al., 2020; Zhang et al., 2020a).

The factors that mediate overall susceptibility to SARS-CoV-2 infections are poorly understood. We speculate that the increased expression of ACE2 that we found in the lungs of smokers could partially contribute to the severe cases of COVID-19 that have been observed in this patient population. Chronic smokers may exhibit a number of co-morbidities, including emphysema, atherosclerosis, and immune dysregulation (Office of the Surgeon General (US), and Office on Smoking and Health (US), 2004), that are also likely to affect COVID-19 progression. While the effects of smoking can last for years, smoking cessation causes an improvement in lung function and an overall decrease in disease burden (Office of the Surgeon General (US), and Office on Smoking and Health (US), 2004). Quitting smoking leads to a normalization of respiratory epithelial architecture (Bertram and Rogers, 1981), a decrease in hyperplasia (Lee et al., 2001), and a downregulation of ACE2 levels. Thus, for multiple reasons, smoking cessation could eventually lessen the risks associated with SARS-CoV-2 infections.

### Limitations

Our analysis in this manuscript has a number of important limitations. First, the relationship between age, sex, and ACE2 expression remains controversial. In the literature, ACE2 expression in the lung has been reported to both increase (Chen et al., 2020b; Muus et al., 2020) and decrease (Booeshaghi and Pachter, 2020; Xie et al., 2006) during aging. Across five rodent and three human datasets, we did not observe a significant correlation in either direction between ACE2 levels and age. It remains possible that such a difference could be found using tissue from older or younger individuals. Additionally, it is possible that ACE2 expression within single-cell types correlates with age, but such differences are not prominent enough to detect using bulk microarray and RNA-seq analysis.

In order for SARS-CoV-2 to infect a host cell, the virus must bind to ACE2 protein that is present on the cell surface. Here, we have described the expression of ACE2 transcripts in mammalian lungs, but our work does not guarantee that stimuli affecting the levels of ACE2 mRNA will have the same effect on ACE2 protein. In general, we have documented a strong correlation between ACE2 mRNA and protein levels (Figure S5), but it remains possible that ACE2 protein is differentially regulated in mammalian lungs, or that the localization of ACE2 protein to the cell surface is strictly controlled. Immunohistochemistry using tissue samples from smokers and virus-infected patients will be needed to confirm that these factors also increase the levels of ACE2 protein available to interact with SARS-CoV-2 particles.

Finally, the exact role of ACE2 as a mediator of disease severity remains to be determined. Mice that were engineered to express high levels of human ACE2 succumbed to infections with the SARS coronavirus more quickly than mice that expressed low levels of human ACE2, suggesting that increasing ACE2 enhances viral susceptibility (McCray et al., 2007). At the same time, ACE2-knockout mice are vulnerable to a variety of lung injuries, and ACE2 expression has been reported to play a

protective role in the respiratory tract (Imai et al., 2005; Kuba et al., 2005; Rey-Parra et al., 2012). As ACE2 expression is both necessary and sufficient for SARS-CoV-2 infections (Hoffmann et al., 2020; Zang et al., 2020), it seems highly likely that an expansion of ACE2<sup>+</sup> cells in the lungs will facilitate viral dissemination. However, the exact relationship between ACE2 expression and SARS-CoV-2 uptake remains to be determined, and it is possible that the expression of ACE2 has some beneficial consequences as well. Additional work will be required to determine the precise impact of ACE2 levels on the clinical course of COVID-19.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.devcel.2020.05.012>.

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### AUTHOR CONTRIBUTIONS

Conceptualization: J.C.S. and J.M.S.; Methodology: J.C.S. and J.M.S.; Software: J.C.S.; Formal Analysis: J.C.S. and J.M.S.; Writing – Original Draft: J.M.S.; Writing – Review & Editing: J.C.S. and J.M.S.; Supervision: J.M.S.; Investigation: J.C.S., E.L.S., V.G., M.L.Y., A.V., K.M.J., and J.M.S.

### DECLARATION OF INTERESTS

J.C.S. is a co-founder of Meliora Therapeutics and is an employee of Google, Inc. This work was performed outside of her affiliation with Google and used no proprietary knowledge or materials from Google. J.M.S. has received consulting fees from Ono Pharmaceuticals, is a member of the Advisory Board of Tyra Biosciences, and is a co-founder of Meliora Therapeutics.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Recombinant Anti-ACE2 antibody [ERP4435(2)]	Abcam	ab108252
GAPDH antibody (G-9)	Santa Cruz Biotechnology	sc-365062
Goat Anti-Rabbit IgG H&L (HRP)	Abcam	ab-97051
Goat Anti-Mouse IgG (H + L)-HRP Conjugate	Bio-rad	1706516
<b>Biological Samples</b>		
Primary Small Airway Epithelial Cells	ATCC	ATCC PCS-301-010
Primary Bronchial/Tracheal Epithelial Cells	ATCC	ATCC PCS-300-010
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
IFN- $\gamma$	Stemcell Technologies	78020
IFN- $\alpha$ 2A	Stemcell Technologies	78076
IFN- $\beta$	Stemcell Technologies	78113
<b>Deposited Data</b>		
See <a href="#">Table S1</a>		
<b>Experimental Models: Cell Lines</b>		
HCT116	ATCC	CCL-247
CAL148	DSMZ	ACC 460
A2780	ECACC	93112519
A375	ATCC	CRL-1619
LoVo	ATCC	CCL-229
Caco-2	ATCC	HTB-37
Calu3	ATCC	HTB-55
<b>Oligonucleotides</b>		
ACE2 primer pair 1: CGAAGCCGAAGACCTGTTCTA and GGGCAAGTGTGGACTGTTCC	Integrated DNA Technologies	
ACE2 primer pair 2: CAAGAGCAAACGGTTGAACAC and CCAGAGCCTCTCATTGTAGTCT	Integrated DNA Technologies	
GAPDH primer pair: TGCACCACCAACTGCTTAGC and GGCATGGACTGTGGTCATGAG	Integrated DNA Technologies	
<b>Software and Algorithms</b>		
scanpy	<a href="https://genomebiology.biomedcentral.com/articles/10.1186/s13059-017-1382-0">https://genomebiology.biomedcentral.com/articles/10.1186/s13059-017-1382-0</a>	1.4.6
Pandas	<a href="https://pandas.pydata.org/about/citing.html">https://pandas.pydata.org/about/citing.html</a>	1.0.2
Matplotlib	<a href="https://matplotlib.org/3.1.3/citing.html">https://matplotlib.org/3.1.3/citing.html</a>	3.1.3
multicoreTSNE	<a href="https://github.com/DmitryUlyanov/Multicore-TSNE">https://github.com/DmitryUlyanov/Multicore-TSNE</a>	0.1
Statsmodels	<a href="https://www.statsmodels.org/stable/index.html#citation">https://www.statsmodels.org/stable/index.html#citation</a>	0.11.0

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Dr. Jason Sheltzer ([sheltzer@cshl.edu](mailto:sheltzer@cshl.edu)).

### Materials Availability

This study did not generate any new unique reagents.

### Data and Code Availability

All of the data used in this manuscript are publicly accessible and are described in [Table S1](#). The code used for performing these analyses is available at [github.com/joan-smith/covid19](https://github.com/joan-smith/covid19).

## EXPERIMENTAL MODELS AND SUBJECT DETAILS

### Cell Lines

All cell lines used in this study were acquired from ATCC, except for CAL148 cells that were acquired from DSMZ and A2780 cells that were acquired from ECACC. Primary small airway epithelial cells and primary bronchial/tracheal epithelial cells were cultured in Airway Epithelial Cell Basal Medium (ATCC; Cat. No. PCS-300-030) supplemented with the Bronchial Epithelial Cell Growth Kit (ATCC; Cat. No. PCS-300-040™). HCT116, CAL148, and A375 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco; Cat. No. 11995073) supplemented with 10% fetal bovine serum (FBS)(Corning; Cat. No. 35-010-CV). LoVo and A2780 were cultured in RPMI 1640 (Lonza; Cat. No. 12-115F) supplemented with 10% FBS. Calu-3 was cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC; Cat. No. 30-2003™) supplemented with 10% FBS. Caco-2 was cultured in EMEM supplemented with 20% FBS. All cell lines were grown in a humidified environment at 37°C and 5% CO<sub>2</sub>.

## METHOD DETAILS

### Overall Analysis Strategy

The analysis described in this paper was performed using Python, Excel, and Graphpad Prism. Gene expression data were acquired from the Gene Expression Omnibus (GEO)([Edgar et al., 2002](#)), the GTEx portal ([GTEx Consortium, 2013](#)), the Broad Institute TCGA Firehose ([Broad GDAC Firehose, 2016](#)), the Human Cell Atlas ([Regev et al., 2017](#)), the Human Protein Atlas ([Uhlen et al., 2010](#)), and the Single-Cell Expression Atlas ([Papatheodorou et al., 2020](#)), as described below. For microarray datasets, probeset definitions were downloaded from GEO, and probes mapping to the same gene were collapsed by averaging. For each gene expression comparison, a control population was identified (e.g., young rats, sham-treated mice, non-smokers, etc.), and gene expression values were log<sub>2</sub>-transformed and normalized by subtraction so that the mean expression of a gene of interest in the control population was 0. Graphs of gene expression values were then generated using GraphPad Prism; all data points are displayed and no outliers were excluded from analysis.

### Multivariate Regression and Smoking History

Multivariate regression to investigate the relationship between ACE2 expression and smoking history was performed on the GSE76925 lung tissue dataset and the GSE13933 tracheal epithelium dataset. Regressions were performed in Python using ordinary least squares from the statsmodels package ([Seabold and Perktold, 2010](#)). Results reported include the standard errors ('bse'), betas, and p values.

### Single-Cell Analysis

Single-cell clustering and analysis on the datasets listed in [Table S1](#) was performed in Python using the Scanpy and Multicore-TSNE packages ([Ulyanov, 2020](#); [Wolf et al., 2018](#)). To filter out low-quality cells, only cells in which 500 or more genes were detected were included in this analysis. Before clustering, transcript counts were counts-per-million normalized and log<sub>2</sub> transformed. Highly variable genes were selected using the Seurat approach in Scanpy, and these highly variable genes were used to produce the principal component analysis. A t-SNE projection and unsupervised Leiden clustering were then performed on each dataset using nearest neighbors, as described in the associated code. Parameter selection was performed according to the method described in ([Kobak and Berens, 2019](#)).

In order to label each cluster, a gene ranking analysis was obtained using Scanpy. The 20 most highly-ranked genes from each cluster (as determined by a t-test with overestimated variance) were identified. These genes were then compared against gold-standard marker lists from multiple sources to produce the cluster labels ([Angelidis et al., 2019](#); [Ardini-Poleske et al., 2017](#); [Goldfarbmuren et al., 2019](#); [Plasschaert et al., 2018](#); [Wolf et al., 2018](#)).



### Gene Ontology Analysis

In order to identify the genes whose expression correlates with ACE2, pairwise Pearson correlations coefficients (PCCs) were calculated between ACE2 and every other expressed gene. Genes whose PCC were more than three standard deviations greater than the average gene's PCC were classified as strongly correlated with ACE2. Gene ontology terms enriched in this group were then identified with GProfiler against the background list of non-strongly correlated genes using a Benjamini-Hochberg FDR of 0.05 (Raudvere et al., 2019).

### Volcano Plots

For the meta-analysis of gene expression changes in the small airways, large airways, and trachea, p values from each independent dataset were combined using Fisher's method and fold change values were combined by averaging. To deconvolve the bulk gene expression datasets based on cell type signatures, the 100 highest ranking genes from each cell type cluster (found using a t-test with overestimated variance, as described above) were identified and the 100 genes exhibiting the strongest correlation with ACE2 were identified. Volcano plots were generated using the mean expression of the 100 genes comprising each signature and the p-value of a t-test comparing the signature genes to all genes within a dataset.

### Cytokine Treatments

Early-passage human tracheal epithelial cells and small airways epithelial cells were acquired from ATCC and cultured according to the supplier's recommended method. Cells were grown for 48 hours to approximately 70% confluence, treated with the indicated compound for 24 hours, and then total RNA was harvested.

### RNA Expression Analysis

qRT-PCR was performed as described in (Vasudevan et al., 2020). In short, RNA was extracted using TRIzol (Life Technologies; Cat. No. 15596018) and then purified with a Qiagen RNeasy Mini Kit (Cat. No. 74106). RNA was converted to cDNA using SuperScript IV VILO Master Mix (Thermo Fisher Scientific; Cat. No. 11756500). Quantitative PCR was performed using SYBR Premier Ex Taq (Takara; Cat. No. RR420L) and quantified using the QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). The primers for ACE2 were as follows: (set 1) 5'-CGAAGCCGAAGACCTGTTCTA and 5'-GGCAAGTGTGGACTGTTCC and (set 2) 5'-CAAGAGCAAACGGTTGAACAC and 5'-CCAGAGCCTCTCATTGTAGTCT. GAPDH was assessed as a control using primers 5'-TGCACCACCAACTGCTTAGC and 5'-GGCATGGACTGTGGTCATGAG.

### Western Blotting

Western blotting was performed as described in (Vasudevan et al., 2020). In short, whole cell lysates were harvested and resuspended in RIPA buffer [25 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail (Sigma; Cat. No. 4693159001), and phosphatase inhibitor cocktail (Sigma, Cat. No. 4906845001)]. Protein lysates were loaded onto a 10% SDS-PAGE gel. The Trans-Blot Turbo Transfer System (Bio-Rad) and polyvinylidene difluoride membranes were used for protein transfer. Antibody blocking was done with 5% milk in TBST (19 mM Tris base, NaCl 137 mM, KCl 2.7 mM and 0.1% Tween-20) for 1 hour at room temperature. Primary antibodies used were ACE2 (Abcam; Cat. No. ab108252), used at a 1:1000 dilution in 5% milk, and GAPDH (Santa Cruz Biotechnology; Cat. No. sc-365062), used at a 1:1000 dilution in 5% milk. Blots were incubated overnight at 4°C. Membranes were then washed three times for 10 minutes each before they were incubated in secondary antibodies for an hour at room temperature. For the ACE2 blot, goat anti-rabbit IgG H&L (HRP) (Abcam; Cat. No. ab-97051) was used as a secondary antibody at a 1:20000 dilution in 5% milk. For the GAPDH blot, goat anti-mouse IgG (H + L)-HRP Conjugate (Bio-Rad; Cat. No. 1706516) was used as a secondary antibody at a 1:20000 dilution in 5% milk. Membranes were washed three more times and then developed using ProtoGlow ECI (National Diagnostics; Cat. No. CL-300).

### QUANTIFICATION AND STATISTICAL ANALYSIS

For the qPCR assays in Figures 5F, 5G, and S5D, we performed three biological replicates (for each cytokine) or six biological replicates (for the untreated cells) using two ACE2 primer pairs. Sample sizes of the gene expression datasets that were analyzed are listed in Table S1. Gene expression values in Figures 1, 2, 4K–4M, 5, S1–S3, and S5, and were compared by a two-sided Student's t-test. Gene expression values in Figure 4G were compared by a Mann-Whitney U test. The fractions of cells expressing a particular marker in Figures 4E, 4F, and 4H were compared by a chi-square test. No outliers were excluded from these analyses.