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## The Anti-inflammatory Effect of Alpha-1 Antitrypsin in Rhinovirus-infected Human Airway Epithelial Cells

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### Abstract

**Objective**—Excessive airway inflammation is seen in chronic obstructive pulmonary disease (COPD) patients experiencing acute exacerbations, which are often associated with human rhinovirus (HRV) infection. Alpha-1 antitrypsin (A1AT) has anti-inflammatory function in endothelial cells and monocytes, but its anti-inflammatory effect has not been investigated in COPD airway epithelial cells. We determined A1AT's anti-inflammatory function in COPD airway epithelial cells and the underlying mechanisms such as the role of caspase-1.

**Methods**—Brushed bronchial epithelial cells from COPD and normal subjects were cultured at air-liquid interface and treated with A1AT or bovine serum albumin (BSA, control) two hours prior to whole cigarette smoke (WCS) or air exposure, followed by HRV-16 infection. After 24 hours of viral infection, cell supernatants were collected for measuring IL-8, and cells were examined for caspase-1. The *in vivo* anti-inflammatory function of A1AT was determined by infecting mice intranasally with HRV-1B followed by aerosolized A1AT or BSA.

**Results**—A1AT significantly reduced WCS and HRV-16-induced IL-8 production in normal and COPD airway epithelial cells. COPD cells are less sensitive to A1AT's anti-inflammatory effect than normal cells. A1AT exerted the anti-inflammatory function in part via reducing caspase-1 in normal cells, but not in COPD cells. In mice, A1AT significantly reduced HRV-1B induced lung neutrophilic inflammation.

**Conclusions**—A1AT exerts an anti-inflammatory effect in cigarette smoke-exposed and HRV-infected human airway epithelial cells, which may be related to its inhibitory effect on caspase-1 activity.

### Keywords

Alpha-1 antitrypsin; COPD; Airway epithelial cell; Rhinovirus; Cigarette smoke

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### Conflict of Interest

The authors report no conflicts of interest in this work.

## Introduction

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States, and the prevalence of COPD has been steadily increasing [1]. One of the major unmet needs in COPD healthcare is the lack of effective treatment for patients experiencing acute exacerbations (AECOPD), which poses the highest mortality risk to patients. Respiratory viral (e.g., rhinovirus) infections and cigarette smoke significantly contribute to excessive airway inflammation, a salient feature of AECOPD pathobiology [2–4]. However, there is no effective treatment to attenuate or eliminate virus-mediated damage (e.g., inflammation) to the airways.

The primary inflamed site during AECOPD associated with human rhinovirus (HRV) infection is the airway [5] as airway epithelial cells represent the major site of HRV infection [6,7]. Alpha-1 antitrypsin (A1AT) is a serine protease inhibitor mainly produced in the liver, but also found in epithelial cells and macrophages. A1AT is widely recognized for its critical role to maintain lung tissue structure and homeostasis. Although previous studies have demonstrated that A1AT possesses anti-inflammatory functions in human endothelial cells and monocytes [8,9], its therapeutic effect on respiratory viral infection, particularly in COPD airway epithelial cells have not been investigated. The primary goal of our current study is to elucidate A1AT's anti-inflammatory function in rhinovirus-infected airway epithelial cells from subjects with or without COPD. Our secondary goal is to explore the underlying mechanisms of A1AT's anti-inflammatory properties against HRV infection in airway epithelial cells. Previous studies have shown that caspase-1, a key component of inflammasomes, is involved in pro-inflammatory responses (e.g., release of IL-1 $\beta$ ) to bacteria and viruses [10,11]. Interestingly, caspase-1 activity is increased in HRV-infected human airway epithelial cells [2], as well as in bacteria-infected lung macrophages [12]. Importantly, IL-1 $\beta$  has been shown to promote IL-8 production in human airway epithelial cells during HRV infection [2]. Therefore, we hypothesized that inhibition of caspase-1 activation by A1AT is one of the major mechanisms whereby A1AT exerts its anti-inflammatory function in human airway epithelial cells during viral infection in a cigarette smoke exposure setting.

## Methods

### Ethic statement

The collection and the use of bronchial epithelial cells were approved by Institutional Review Board (IRB) under protocol # HS-2271 and HS-2598 at National Jewish Health, Denver, Colorado, USA, and all subjects provided written informed consent. All the animal procedures were covered under a protocol (Reference# AS2792-04-17) approved by Institutional Animal Care and Use Committee (IACUC) of National Jewish Health, Denver, Colorado, USA.

### Bronchoscopy and brushed bronchial epithelial cell collection

Bronchoscopy and endobronchial epithelial brushings were performed on 12 human subjects (COPD patients, n=6; normal healthy subjects, n=6). The criteria for COPD diagnosis and

the patient characteristics were described in our previous publication [13]. Bronchial brushings were performed with a single sheathed cytology brush (#CF-001, Medical Engineering Laboratory, Durham, NC) as previously described [13,14].

### **Propagation of HRV-16 and HRV-1B**

HRV-16 and HRV-1B (American Type Culture Collection, Manassas, VA) were propagated in H1-Hela cells (CRL-1958, ATCC) and purified as previously described [15]. Viral titer quantification was carried out by tissue culture infective dose per ml (TCID<sub>50</sub>/ml) in our cell culture experiments [16], and PFU/ml in our mouse model [15].

### **Whole cigarette smoke (WCS) exposure and HRV-16 infection in well-differentiated primary human bronchial epithelial cells**

Brushed bronchial epithelial cells were cultured in 60 mm collagen-coated dishes in bronchial epithelial cell growth medium (BEGM) with supplements (Lonza, Walkersville, MD), and incubated at 37°C with 5% CO<sub>2</sub> until confluence. The cells were then trypsinized and reseeded onto collagen-coated transwell inserts (4 × 10<sup>4</sup> cells/insert) in 12-well plates as previously described [15]. After approximately 7 days, cells were shifted to air-liquid interface (ALI) for an additional 10 days to induce mucociliary differentiation. On day 10 of ALI, cells were treated with A1AT (Grifols Inc., NC) or bovine serum albumin (BSA, control for A1AT, Sigma-Aldrich) at 1 mg/ml for 2 hours at both apical and basolateral sides. After A1AT treatment, cells on the apical side were exposed to air (control) or WCS using a research cigarette 3R4F (University of Kentucky, Lexington, KY, USA) for 10 minutes in a cell culture smoking chamber (British American Tobacco Science, South Hampton, UK) as we described before [17,18]. To mimic the diffusion of smoke passing through the epithelial cells, cigarette smoke extract collected during the WCS exposure was added to the basolateral side of the transwell to expose cells for the duration of the experiment. Immediately after WCS exposure, cells were infected at the apical side with HRV-16 at 5 × 10<sup>4</sup> TCID<sub>50</sub>/transwell. Cells were then cultured for additional 24 hours. Basolateral supernatants were collected to measure IL-8 levels by ELISA, and cells were harvested in Pierce™ RIPA buffer for Western blot analysis of caspase-1.

### **Treatment of a caspase-1 inhibitor in well-differentiated primary human bronchial epithelial cells**

To determine if caspase-1 inhibitor reduces IL-8 production induced by WCS and HRV-16, cells on day 10 of ALI culture were treated at the apical surface with a selective caspase-1 inhibitor Ac-YVAD-CHO (100 μM, Enzo life sciences, Farmingdale, NY) in the presence or absence of WCS and HRV-16. Twenty-four hours after infection, basolateral supernatants were collected to measure IL-8 protein levels, and cells were harvested for caspase-1 Western blot. In a preliminary experiment, we confirmed the inhibitory effect of Ac-YVAD-CHO on caspase-1 by incubating it with recombinant caspase-1 protein (Enzo life sciences) at 1 unit for up to 90 minutes.

## Western blot analysis

Cells were lysed in Pierce™ RIPA Buffer with protease inhibitors (1:100) (Fisher Scientific, Waltham, MA). Equal amount of proteins was separated on a 15% SDS-PAGE gel, transferred onto the Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA), blocked with 5% BSA in TBS-T, and probed with a rabbit anti-caspase-1 p20 (Cell Signaling Technology, Danvers, MA) or a mouse anti-GAPDH (Santa Cruz Biotech Inc., Santa Cruz, CA) antibody. Blots were then incubated with appropriate HRP-linked secondary antibodies and Pierce® ECL Western blotting substrate (Fisher Scientific, Waltham, MA). Densitometry was performed using the TotalLab 1D analysis software (Fotodyne, Hartland, WI). The ratio of caspase-1 p20/GAPDH was used to indicate the level of caspase-1 activation.

## ELISA

Protein levels of human IL-8, or mouse KC were determined by using a human IL-8 or a mouse KC DuoSet ELISA kit (R&D Systems, Minneapolis, MN) as per manufacturer's instruction.

## A mouse model of HRV-1B infection with A1AT treatment

A murine model of lung rhinovirus infection was used to reveal the *in vivo* anti-inflammatory function of A1AT. Female wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and housed in our biological resource center at National Jewish Health under pathogen-free conditions, and tested to establish that they were virus and *M. pulmonis* free. We chose to use the female mice because: (1) female mice are easy to work for effective delivery of viruses and A1AT; (2) in the US, the number of male (20%) smokers is close to the number of female (15%) smokers; and (3) recent studies have suggested that female smokers have an increased risk of developing COPD compared with male smokers [19,20]. HRV-1B ( $1 \times 10^7$  PFU/mice in 50  $\mu$ l PBS) or PBS control was delivered intranasally to mice, and A1AT or BSA was delivered to mice 2 hours after viral infection by aerosolization as described previously [9,22]. Mice were sacrificed after 24 hours of infection to determine the effect of A1AT on virus-mediated acute lung inflammation and viral load. Mouse lungs were lavaged with 1 ml of sterile saline, and bronchoalveolar lavage (BAL) fluid was collected for leukocyte quantification and measurement of chemokine KC. BAL cell cytopins were stained with a Diff-Quick Kit (IMEB INC., San Marcos, CA, USA), and leukocyte differentials were determined as described previously [21].

## Statistical analysis

Data are presented as means  $\pm$  SEM. One-way analysis of variance (ANOVA) was used for multiple comparisons and a Tukey's post hoc test was applied where appropriate. Student's *t* test was used when only two groups were compared. A *p* value  $<0.05$  was considered significant.

## Results

### **Airway epithelial cells from COPD patients produce higher levels of IL-8 than those from normal subjects**

COPD airways are characterized by excessive airway inflammation. IL-8 level is used as a pro-inflammatory marker to indicate whether the COPD cells are more pro-inflammatory than the normal cells. As shown in Figure 1, after 24 hours of air exposure and PBS treatment, IL-8 levels in COPD cells were significantly higher than normal cells, indicating a higher baseline level of inflammation in airway epithelial of COPD patients.

### **Whole cigarette smoke (WCS) and human rhinovirus 16 (HRV-16) increase IL-8 production in airway epithelial cells from COPD patients and normal subjects**

Although the pro-inflammatory effects of WCS exposure and HRV infection have been previously evaluated in human airway epithelial cell lines, their effects in primary airway epithelial cells particularly from both COPD patients have not been examined. After 24 hours of HRV-16 infection in airway epithelial cells with or without WCS, the change of IL-8 production was determined. We used the change of IL-8 to indicate pro-inflammatory effect of HRV-16 or WCS as the baseline (air + PBS) IL-8 data varied greatly among COPD subjects. Compared to the air control, HRV-16 or WCS significantly increased IL-8 levels in both COPD (Figure 2A) and normal (Figure 2B) airway epithelial cells. The combination of WCS and HRV-16 did not further increase IL-8 production in COPD cells. Although the combination of WCS and HRV-16 trended to further increase IL-8, but the change was not statistically significant.

### **Alpha-1 antitrypsin (A1AT) exerts less anti-inflammatory effect in COPD airway epithelial cells exposed to WCS and HRV-16**

When cells were exposed to WCS and infected with HRV-16, A1AT significantly reduced IL-8 level in both normal and COPD subjects (Figure 3). We then compared the potency of A1AT's anti-inflammatory effect between normal and COPD cells. In cells treated with both WCS and HRV-16, A1AT trended to have a greater inhibitory effect on IL-8 production in normal cells than in COPD cells, but the difference did not reach the level of  $p$  value  $<0.05$ . Despite this, IL-8 reduction by A1AT in WCS-exposed COPD cells was up to 5000 pg/ml, which is within the therapeutic range of salmeterol/fluticasone [22], one of the most common therapies in COPD. In air-exposed cells, no significant differences of A1AT's anti-inflammatory effect were observed between normal and COPD cells.

### **A1AT reduces caspase-1 activity in normal, but not in COPD airway epithelial cells**

Although A1AT exerts an anti-inflammatory effect in human airway epithelial cells, the underlying mechanisms remain unclear. To explore the potential mechanism of IL-8 reduction in human airway epithelial cells by A1AT, caspase-1 p20 was examined by Western blot. As the activation (cleavage) of pro-caspase-1 generates the active tetrameric caspase-1 p20 and p10 fragments, caspase-1 p20 has been used to indicate caspase-1 activation [23]. In normal (Figures 4A and 4B) but not in COPD cells (Figures 4C and 4D), WCS alone and the combination of WCS and HRV-16 significantly increased the level of

caspase-1 p20. Interestingly, HRV-16 infection alone did not alter caspase-1 activity. Furthermore, A1AT treatment significantly reduced the level of caspase-1 p20 in WCS-exposed normal cells, but not in COPD cells (Figure 4).

### IL-8 regulation by caspase-1 in human airway epithelial cells

A selective caspase-1 inhibitor Ac-YVAD-CHO (YVAD) was used to determine whether activation of caspase-1 promotes IL-8 production in normal human airway epithelial cells. We used normal cells in this experiment because in our WCS and HRV-16 cell culture model, we did not find significant changes of caspase-1 p20 levels under various treatments in COPD cells. As shown in Figure 5, at the baseline (DMSO+PBS), YVAD did not alter the IL-8 level. However IL-8 induction by WCS and/or HRV-16 was significantly reduced by YVAD. Collectively, our data suggested the involvement of caspase-1 in IL-8 production in WCS-exposed airway epithelial cells.

### A1AT attenuates rhinovirus-induced lung inflammation in mice

In our above human airway epithelial cell culture models, we have shown that A1AT has the anti-inflammatory function in airway epithelial cells from normal subjects, but not from COPD patients. We thus sought to determine whether A1AT inhibits the *in vivo* proinflammatory response to rhinovirus by utilizing a mouse model of HRV-1B infection without WCS exposure. In control (PBS-treated) mice, A1AT did not alter the number of leukocytes (Figure 6A) including neutrophils (Figure 6B) in BAL fluid. In contrast, in HRV-1B-infected mice, A1AT treatment significantly reduced the levels of BAL leukocytes including neutrophils, nearly back to the normal level. The levels of KC (murine IL-8 homolog), a chemoattractant for neutrophils, were consistent with the neutrophil data (Figure 6C).

## Discussion

In the current study, we have for the first time revealed the anti-inflammatory effect of alpha-1 antitrypsin (A1AT) in primary human brushed airway epithelial cells exposed to cigarette smoke and/or rhinovirus infection. A1AT appears to be more effective in reducing IL-8 production in normal cells than COPD cells. Mechanistically, A1AT exerts the anti-inflammatory effect in part through reducing caspase-1 activation in normal cells, but not in COPD cells.

Respiratory viral (e.g., HRV-16) infections are associated with excessive airway inflammation, one of the most common etiologies of AECOPD [24–27]. Previous studies have focused on investigating the anti-inflammatory properties of A1AT in endothelial cells and monocytes. As a result, A1AT has been considered as a novel anti-inflammatory agent in these cell types. However, it is unclear whether A1AT exerts similar anti-inflammatory function in primary airway epithelial cells exposed to cigarette smoke and viral infections, a condition mimicking the airway milieu during AECOPD. We found that when well-differentiated airway epithelial cells are exposed to HRV-16 infection, WCS, or combination of both, IL-8 production was significantly increased. Importantly, we demonstrated that A1AT attenuates the inflammation induced by WCS and/or HRV-16 infection. Together, our



data suggest that enhanced IL-8 production by viral infection and WCS was inhibited by A1AT treatment.

Current literatures suggested that COPD patients are more resistant to drug treatments during exacerbations due to the nature of multi-drug resistant bacteria (e.g., *pseudomonas aeruginosa*) and excessive inflammation [28,29]. Moreover, the classical anti-inflammatory therapy such as corticosteroids is not very effective in COPD patients [30,31]. Therefore, novel therapies or repurposing the existing medications such as A1AT would be much needed to improve the treatment of COPD, particularly during pathogen (e.g., viruses)-mediated AECOPD. In the current study, we have found that A1AT reduces IL-8 production in COPD cells exposed to WCS and HRV-16. Interestingly, A1AT's inhibitory effects on IL-8 are less potent in COPD cells than normal cells, suggesting again the insensitive nature of COPD cells to a new class of anti-inflammatory candidate. The weaker anti-inflammatory effect of A1AT in COPD cells suggests the need of a combinational therapy of A1AT with other anti-inflammatory drugs to more effectively reduce lung inflammation during AECOPD.

How A1AT regulates airway epithelial IL-8 production is unclear. We attempted to uncover a potential mechanism underlying the anti-inflammatory function of A1AT in airway epithelial cells. We centered our mechanistic study on caspase-1, a key pro-inflammatory component in the inflammasome pathway, which has been shown to regulate inflammatory responses in airway epithelial cell lines [32–34]. By using a potent and selective caspase-1 inhibitor, we found that the caspase-1 inhibitor directly inhibited IL-8 production by WCS and HRV-16. Collectively, inhibition of caspase-1 activation may in part contribute to A1AT's anti-inflammatory function. In contrast, the caspase-1 pathway does not appear to be involved in the pro-inflammatory response to WCS and HRV-16 infection in COPD airway epithelial cells, suggesting the involvement of other inflammatory pathways. Since caspase-1 activity is associated with IL-1 $\beta$  production in macrophages, it is important to determine if caspase-1 is involved in airway epithelial cell IL-1 $\beta$  production. We measured IL-1 $\beta$  protein levels in supernatants of cultured airway epithelial cells by ELISA, but found that IL-1 $\beta$  protein levels were too low to reach the detectable level, which is consistent with a previous airway epithelial study [35]. Given the fact that rhinovirus infection and cigarette smoke activate nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway and subsequently promote inflammation [36,37], we speculate that, A1AT reduces rhinovirus and cigarette smoke induced IL-8 by inhibiting airway epithelial NF- $\kappa$ B activation. How A1AT exerts the anti-inflammatory function remains to be explored. Although our data and others [38] suggest that A1AT may reduce caspase-1 levels or activation, it is also possible that A1AT reduces inflammation which in turn decreases caspase-1.

Although our current study was focused on the *in vitro* effect of A1AT, we also tested whether A1AT exerts an *in vivo* anti-inflammatory effect against rhinovirus infection. In line with our findings in normal human airway epithelial cell cultures, we found that A1AT *in vivo* reduced lung inflammation (e.g., neutrophils and KC) during rhinovirus infection, but it did not significantly change the viral load (data not shown). This may be explained by the fact that the anti-viral pathway and pro-inflammatory pathway could be differently regulated *in vivo* as leukocytes such as macrophages are also involved in the anti-viral response. Thus,

our data suggest that A1AT has an inhibitory effect on lung neutrophilic inflammation, but not viral load. We realized the need to detect activated caspase-1 in mouse lung tissue. Although we tried Western blot of caspase-1 p20 in the lung tissue, for unknown reasons we could not clearly see the caspase-1 p20 band. Other caspase-1 p20 antibodies will be considered to determine if activated caspase-1 p20 can be identified in lung homogenates or lung tissue sections.

We realized the multifunctional nature of A1AT. Therefore, we cannot exclude whether A1AT modulates anti-viral signaling pathways, and subsequently regulates the pro-inflammatory response, which will be considered in our future studies. One limitation for our current study is the relatively small sample size (n=6 for normal controls and n=6 for COPD patients). We will collect more brushed airway epithelial cells from both groups of subjects to expand the current study. Another limitation is that our current animal model only focused on the effects of A1AT during rhinovirus infection, future studies are warranted to test the *in vivo* anti-inflammatory effect of A1AT in the context of cigarette smoke exposure. Lastly, we measured the cytokines (e.g., IL-8 and KC) involved in neutrophil recruitment and activation as a marker for inflammation in our cell culture and animal models, other cytokines such as TNF- $\alpha$  should be measured to look at the broader impact of A1AT on inflammation.

One intriguing finding in the current study is that combination of HRV and WCS did not further increase IL-8, particularly in COPD airway epithelial cells. In a clinical study of rhinovirus-associated COPD exacerbations [39], the absolute IL-8 levels in induced sputum samples were actually lower in COPD patients with viral infection than in those without viral infection. However, the change (exacerbation minus baseline) of IL-8 levels appears to be greater in patients with rhinovirus infection. Notably, IL-8 levels significantly varied in sputum samples, resulting in no significant differences of IL-8 between COPD patients at exacerbation with or without rhinovirus infection. Why COPD cells in our current study are less responsive to WCS and/or HRV with regard to IL-8 induction remains unclear. We speculate that since the baseline IL-8 level is higher in COPD cells than normal cells, there may be a stronger negative immune regulatory mechanism in COPD cells that make them less responsive to WCS and/or HRV infection. Alternatively, this may be explained by the varying effect of different components in cigarette smoke. For example, while oxidants may increase IL-8, nicotine may inhibit IL-8 production in response to HRV infection. It is also intriguing that caspase-1 p20 was detectable in baseline (non-stimulated) airway epithelial cells as it is generally considered as a marker of activated inflammasomes. Although the exact reason is unknown, we speculate that baseline caspase-1 p20 may be involved in cell proliferation and/or differentiation as a previous study has shown caspase-1 p20 expression in non-treated primary bronchial epithelial cells [40].

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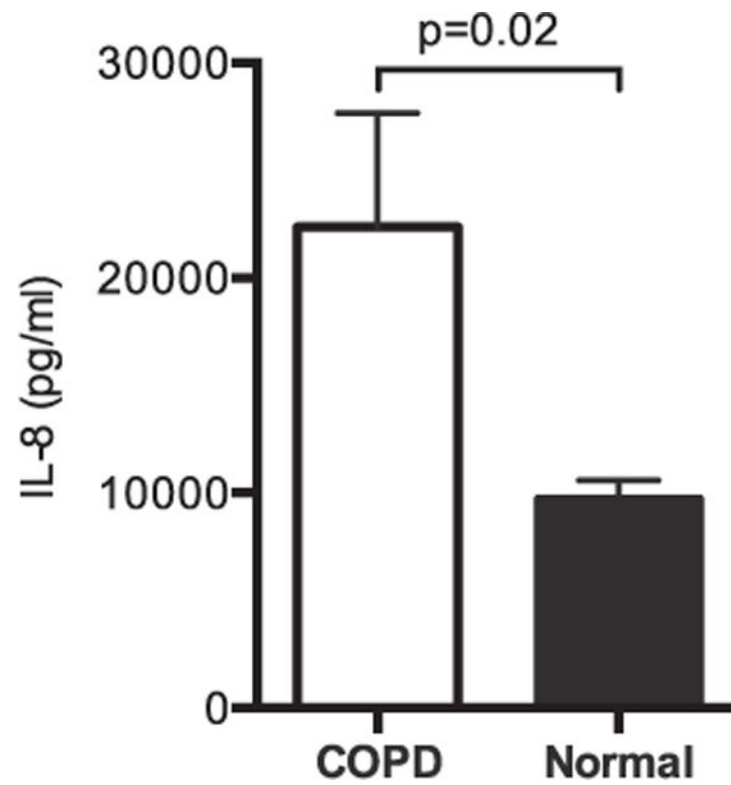


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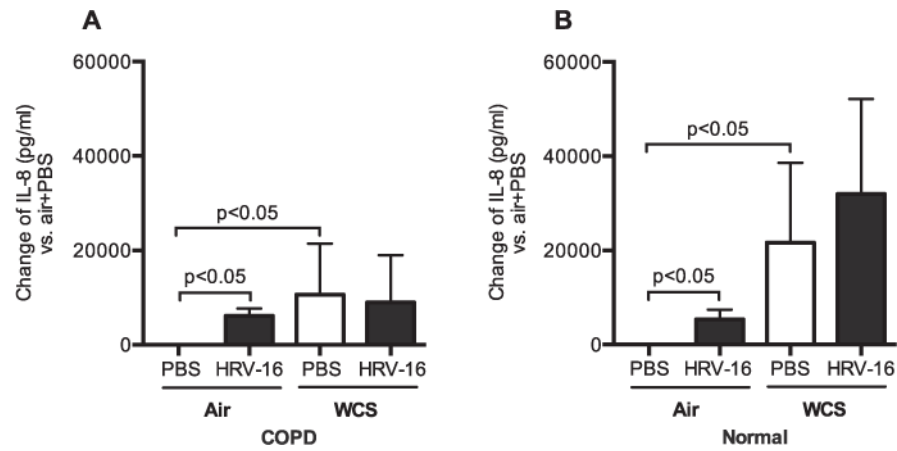
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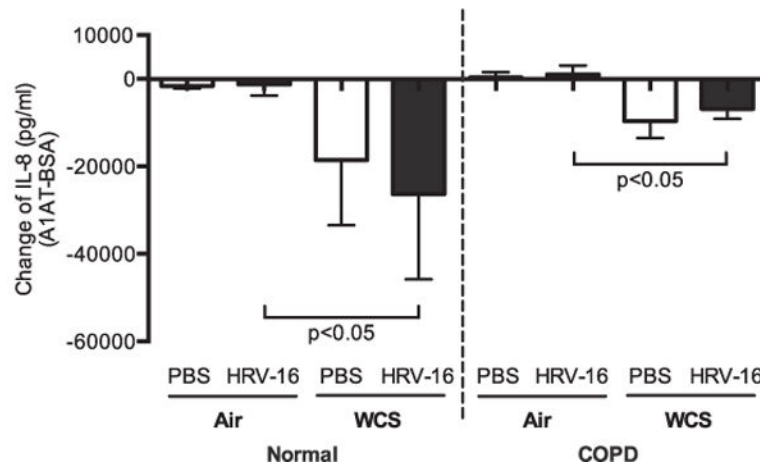


**Figure 1.**

Increased IL-8 production in cultured COPD brushed airway epithelial cells. Brushed airway epithelial cells from COPD patients (n=6) and normal subjects (n=6) were cultured under air-liquid interface (ALI) condition for 10 days. After 24 hours of air exposure, IL-8 was measured by ELISA. Data are expressed as means  $\pm$  SEM.

**Figure 2.**

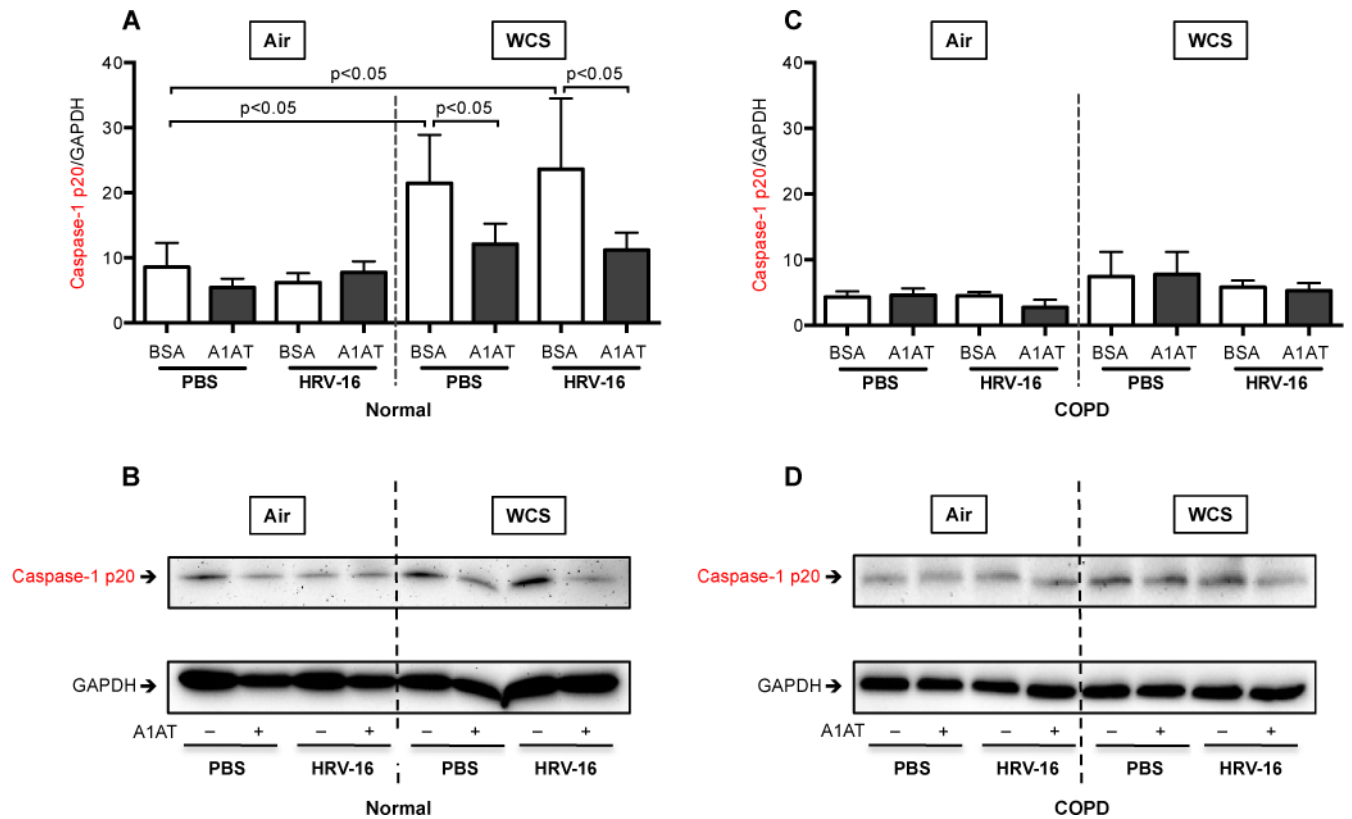
Whole cigarette smoke (WCS) and human rhinovirus 16 (HRV-16) increase IL-8 production in COPD (A) and normal (B) airway epithelial cells. COPD (n=6) and normal (n=6) airway epithelial cells were smoked and infected as described in the Methods section. The changes of IL-8 levels after HRV-16 infection and/or WCS exposure over the control (air + PBS treatment) were compared. Data are expressed as means  $\pm$  SEM.



**Figure 3.**

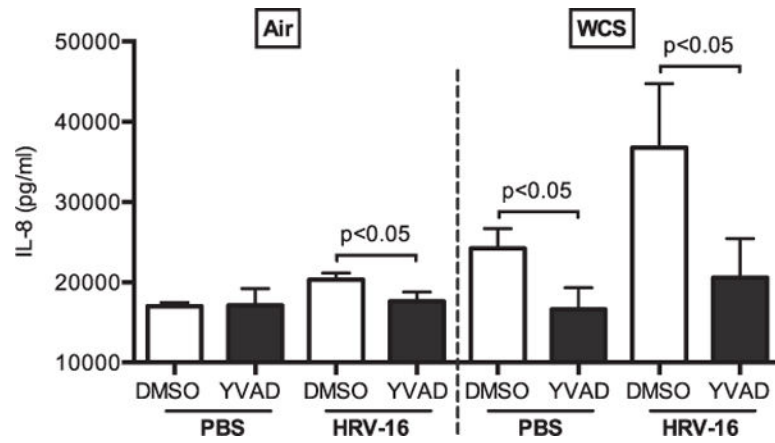
Alpha-1 antitrypsin (A1AT) reduces IL-8 in whole cigarette smoke (WCS)-exposed and/or HRV-16-infected normal and COPD airway epithelial cells. A1AT or BSA at 1 mg/ml was added to cells, followed by WCS or air exposure, and then with HRV-16 infection or PBS treatment for 24 hours. A1AT's anti-inflammatory effect was indicated by the change (A1AT – BSA) of IL-8 levels at indicated conditions. Data are expressed as means  $\pm$  SEM.





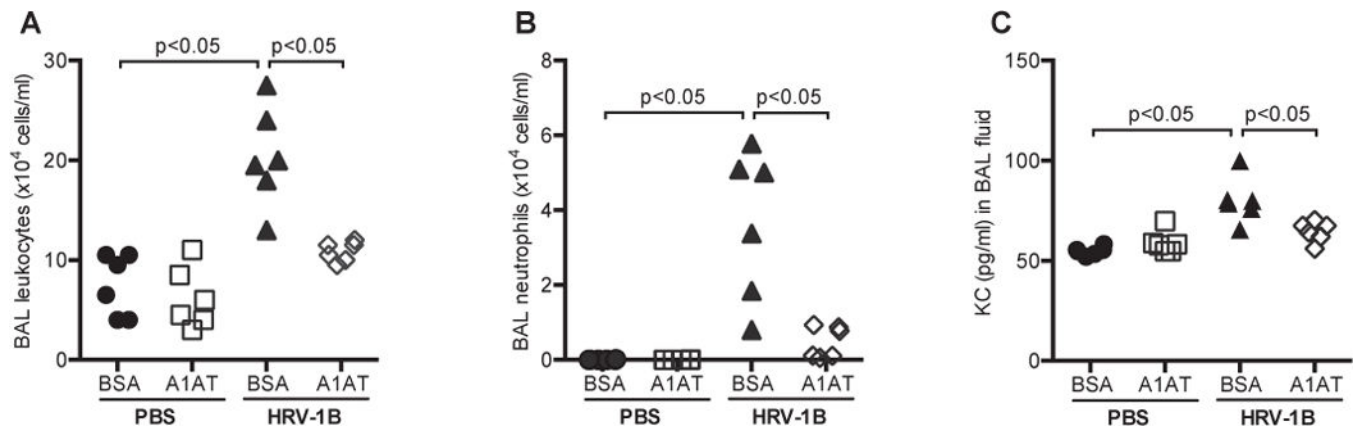
**Figure 4.**

A1AT reduces caspase-1 p20 levels in airway epithelial cells from normal subjects but not COPD patients. Cultured airway epithelial cells from normal subjects (n=6) and COPD patients (n=6) were treated as indicated, and processed for caspase-1 p20 Western blot as described in the Methods section. (A,B) – Quantitative analysis of caspase-1 p20 expression normalized by GAPDH and representative Western blot images of caspase-1 p20 from normal subjects. (C,D) – Quantitative analysis of caspase-1 p20 expression normalized by GAPDH and representative Western blot images of caspase-1 p20 from COPD patients. The vertical dotted blue lines separate the air group from the WCS group. Data are expressed as means ± SEM.



**Figure 5.**

Caspase-1 inhibitor YVAD inhibits IL-8 production following HRV-16 and WCS treatment. Airway epithelial cells from normal subjects (n=6) were treated as indicated and basolateral supernatants were harvested for measuring IL-8 protein. Absolute IL-8 levels were compared among various treatments. Data are expressed as means  $\pm$  SEM.



**Figure 6.**

A1AT reduces HRV-1B-induced lung inflammation in mice. Wild-type C57BL/6 mice were treated as described in the Methods section. Bronchoalveolar lavage (BAL) fluid was collected for quantifying leukocytes (A), neutrophils (B) and KC (C). N=6 mice per group.