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Serine Protease Imbalance in the Small Airways and Development of Centrilobular Emphysema in COPD

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A.S.: designing the overall project and specific experiments, analyzing and interpreting data, and editing the draft.

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Abstract (248/250 words)

Epithelial dysfunction in the small airways may cause the development of emphysema in chronic obstructive pulmonary disease. CCAAT/enhancer binding protein-α (C/EBPα), a transcription factor, is required for lung maturation during development and is also important for lung homeostasis after birth, including the maintenance of serine protease/antiprotease balance in the bronchiolar epithelium. This study aimed to show the roles of C/EBPα in the distal airway during chronic cigarette smoke (CS) exposure in mice and in the small airways in smokers.

In a model of chronic smoke exposure using epithelial cell-specific C/EBPα-knockout mice, significant pathological phenotypes, such as higher protease activity, impaired ciliated cell regeneration, epithelial cell barrier dysfunction via reduced Zo-1, and decreased alveolar attachments, were found in C/EBPα-knockout mice compared to control mice. We found that Spink5 gene (encoding Lympho-epithelial Kazal-type-related inhibitor (LEKTI), an anti-serine protease) expression in the small airways is a key regulator of protease activity in this model. Finally, we showed that daily antiprotease treatment counteracted the phenotypes of C/EBPα-knockout mice.

In human studies, CEBPA expression in the lung was downregulated in patients with emphysema, and 6 smokers with centrilobular emphysema (CLE) showed a significant reduction in LEKTI in the small airways compared to 22 non-CLE smokers.

LEKTI downregulation in the small airways was associated with disease development during murine small airway injury and CLE in humans, suggesting that LEKTI might be a key factor linking small airway injury to the development of emphysema. LEKTI replacement may be a therapeutic option in smoke-related lung disease.
Keywords
C/EBPα, LEKTI, Spink5, small airway, alveolar attachments, centrilobular emphysema, COPD
Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide. Cigarette smoking is a major risk factor for COPD, but the disease may continue to progress even after smoking cessation and while using standard medications such as bronchodilators (1).

COPD is pathologically characterized by emphysema and small airway disease (1, 2), and emphysema is further classified into 3 major subtypes: centrilobular emphysema (CLE), panlobular emphysema (PLE), and paraseptal emphysema (PSE) (3, 4). CLE is of clinical interest, as this subtype is the most prevalent in CS-induced COPD and is closely associated with clinical outcomes (4-6). While the pathogenesis of CLE is not fully understood, recent reports have suggested that CLE and small airway disease may share common disease processes. Indeed, the destruction of the alveolar septum attached to the outer walls of small airways, termed alveolar attachments, is linked to the wall remodeling and luminal narrowing of these airways in CLE (7-9). Therefore, exploring the mechanism that drives the destruction of alveolar attachments in the small airways could help discover a novel therapeutic target for CLE.

CCAAT/enhancer binding protein-α (C/EBPα) is a critical transcription factor for alveolar type 2 (AT2) cell maturation during lung development (10), and incomplete deletion of C/EBPα using surfactant protein-C promoter mice induces COPD phenotypes in the lung (11). C/EBPα also encourages pulmonary cytoprotection during hyperoxic stress (12), promotes bronchial epithelial cell regeneration after naphthalene-induced injury (by regulating serine protease activity) (13), and suppresses lung cancer development (14, 15). Additionally, a gene signature of the distal airway epithelium includes the CEBPA gene in humans (16). Therefore, C/EBPα could maintain the homeostasis of the distal airway during CS exposure and might be involved in small airway dysfunction in COPD.
While protease/antiprotease imbalance is a cause of alveolar destruction in emphysema (17-19), little is known about its role in the small airways in humans. In murine airways, protease/antiprotease imbalance causes epithelial barrier dysfunction in vitro (20-22), and the regulation of serine protease/antiprotease balance by C/EBPα is essential for regeneration of ciliated cells after injury (13). These results led us to hypothesize that serine protease/antiprotease imbalance in the distal airways during chronic CS exposure could cause epithelial dysfunction and destruction of alveolar attachments, leading to the development of emphysema.

This study aimed to evaluate whether the absence of C/EBPα expression in lung epithelial cells could exacerbate serine protease/antiprotease imbalance in the distal airways and thereby impair epithelial cell regeneration and induce barrier dysfunction and destruction of alveolar attachments in CS-exposed mice. In addition, we aimed to test whether the murine findings could be relevant to the pathogenesis of human CLE by using surgically resected lung tissues obtained from smokers with and without CLE.
MATERIAL AND METHODS

Animal studies

The Animal Research Committee of Kyoto University approved the study (ID: MedKyo 18295).

Transgenic mice

All transgenic mice were maintained on a mixed C57/BL6 and FVB/N genetic background.

Triple-transgenic Scgb1a1-rtTA\textsuperscript{tg}/Line 2)/(tetO)\textsuperscript{7}CMV-Cre\textsuperscript{tg}/Cebpa\textsuperscript{flox/flox} mice were bred with double-transgenic (tetO)\textsuperscript{7}CMV-Cre\textsuperscript{tg}/Cebpa\textsuperscript{flox/flox} mice (13) to generate Scgb1a1-rtTA\textsuperscript{tg}/Line 2)/(tetO)\textsuperscript{7}CMV-Cre\textsuperscript{tg}/Cebpa\textsuperscript{flox/flox} mice (here termed C/EBPα\textsuperscript{Δ/Δ}) and (tetO)\textsuperscript{7}CMV-Cre\textsuperscript{tg}/Cebpa\textsuperscript{flox/flox} mice (here termed control).

To evaluate lung epithelial cell differentiation, Scgb1a1-rtTA\textsuperscript{tg}/(tetO)\textsuperscript{7}CMV-Cre\textsuperscript{tg}/Cebpa\textsuperscript{flox/flox} mice were bred with ROSA26-H2B-EGFP mice (23) to generate triple-transgenic Scgb1a1-rtTA\textsuperscript{tg}/(tetO)\textsuperscript{7}CMV-Cre\textsuperscript{tg}/ROSA26-H2B mice (here termed H2B-control) and quadruple-transgenic Scgb1a1-rtTA\textsuperscript{tg}/(tetO)\textsuperscript{7}CMV-Cre\textsuperscript{tg}/ROSA26-H2B/Cebpa\textsuperscript{flox/flox} mice (here termed H2B-C/EBPα\textsuperscript{Δ/Δ}). These mice were given chow containing doxycycline (600 ppm) for 3 weeks to activate Cre-mediated recombination at 5 to 9 weeks of age.

Chronic CS exposure and antiprotease treatment

Nine- to 12-week-old male mice were placed in Plexiglas boxes and exposed to air or mainstream CS for 1 hour daily (10 cigarettes daily), 5 days a week, for up to 6 months.

To evaluate the potential of anti-serine proteases, a serine protease inhibitor (bovine pancreatic trypsin inhibitor: BPTI) was intratracheally administered to CS-exposed H2B-C/EBPα\textsuperscript{Δ/Δ} mice after daily CS exposure for 6 months. The online supplement provides further information (also see Fig E1).
Analyses of proteases/antiproteases and related molecular factors

Lung sections were obtained from air/CS-exposed control and C/EBPαΔ/Δ mice to evaluate local gene expression in the distal airways. Tissues from the distal airways (100 µm in length from the bronchioalveolar duct junction, BADJ) were collected for microarray analysis using laser-capture microdissection (LCM) from chronic (3 months) air/CS-exposed mice. The protease activity in bronchoalveolar lavage fluid (BALF) was measured using protease substrates (see the online supplement).

Morphometry

Airspace enlargement was evaluated using the mean linear intercept (MLI). The percentage of abnormal alveolar attachment (%AA) to the outer wall of distal airways <2 mm in diameter was quantified for murine samples according to a published method (24-26) (see the online supplement).

Human studies

Lung specimens were obtained from former smokers who underwent lung lobectomy for tumor resection at Kyoto University Hospital. The Ethics Committee of Kyoto University (G155 and G0620) approved the study. All participants provided written informed consent before surgery. The details of the protocols for tissue processing, visual assessment of preoperative contrast-enhanced computed tomography (CT) images, mRNA analysis, and immunostaining are described in the online supplement.

Statistical analysis

All statistical analyses were performed using JMP 11 software (SAS Institute Inc., Cary, NC, USA). Data are presented as the mean ± the standard error (SE). Detailed statistical methods are described in the online supplement and figure legends. P <0.05 was considered significant.
Results

Murine studies

**Scgb1a1 lineage-positive progenitor ciliated cells increased after chronic CS exposure in control mice but not in C/EBPαΔΔ mice**

Airway epithelial cell composition was evaluated after exposing control mice to air or CS for 6 months. The number of club (Scgb1a1-positive) and ciliated (FoxJ1-positive) cells in the distal airways was not altered by CS exposure in control mice (Fig 1A-B). We also exposed the lineage tracing model mice (H2B-control), in which the eGFP signal turns on in both club cells and AT2 cells after doxycycline administration, to CS for 6 months. The number of eGFP-positive ciliated cells in the distal airways was greater in CS-exposed mice than in air-exposed control mice (Fig 1C), suggesting that Scgb1a1-positive cells differentiated into ciliated cells in a compensatory manner during chronic CS exposure.

In contrast, the number of ciliated cells in the distal airways was lower in CS-exposed C/EBPαΔΔ mice than in CS-exposed control mice (Fig 1B). Furthermore, the number of eGFP-positive regenerated ciliated cells in the distal airways was also lower in CS-exposed H2B-C/EBPαΔΔ mice than in CS-exposed H2B-control mice (Fig 1C), suggesting that the deletion of C/EBPα impaired ciliated cell regeneration after chronic CS exposure.

On electron microscopy (Fig 1D), compared to air-exposed C/EBPαΔΔ mice, CS-exposed C/EBPαΔΔ mice had shortened cilia, impaired ciliated cell contiguity and flattened cells in the distal airways; these effects were consistent with the airway pathology in human COPD (27). The remodeling and mucous cell hyperplasia in the distal airways did not differ among the groups (data not shown). In the proximal conducting airways, CS exposure did not decrease the number of ciliated cells, even in C/EBPαΔΔ mice (Fig E2).

**Deletion of C/EBPα in the murine lung enhanced protease activity during chronic CS exposure**
To explore the associations between the deletion of C/EBPα and the enhancement of serine protease activity in CS-exposed mice, BALF protease activity was measured using a BODIPY® TR-X dye-labeled casein substrate.

Caseinolytic activity, which presumably represented total protease activity, was increased significantly in the BALF of C/EBPαΔ/Δ mice during chronic CS exposure (Fig 2A and Table E2, n = 6/group). Additionally, chymotrypsin-like activity, which is known as a subtype of serine protease activity, tended to be increased by CS exposure in C/EBPαΔ/Δ mice (P = 0.07), but this tendency was not observed in control mice (Table E2). Furthermore, MMP-like activity was significantly increased by CS exposure in control mice, but there was no significant difference between air-exposed and CS-exposed C/EBPαΔ/Δ mice. These results suggested that the protease activity in the distal lung of CS-exposed C/EBPαΔ/Δ mice was mainly determined by serine proteases rather than MMPs.

**Gene expression of proteases and antiproteases in the distal airways during chronic CS exposure in mice**

To obtain information about the microenvironment in the distal airways, 500 pg of total RNA was extracted from laser-dissected tissues of each mouse (100 µm in length from the BADJ), and a pico-microarray was performed (n = 3/group, 3 months of air/CS exposure in control/C/EBPαΔ/Δ mice).

Table 1 shows a gene list of proteases and antiproteases that were differentially expressed between air-exposed and CS-exposed control mice (P <0.05 and log2 fold change value ≥1.00). The gene expression levels of serine proteases, including *Kallikrein-related peptides* (*Klk13, Klkb1, Klk10, and Klk8*) and cathepsin *S (Ctss)*, were significantly higher in CS-exposed control mice than in air-exposed control mice, and *Klk13* and *Ctss* were upregulated by chronic CS exposure in C/EBPαΔ/Δ mice.
Regarding anti-serine proteases, *Slpi*, *Serpind1*, *Serpina3n*, *Spink5*, and *Spint3* were significantly upregulated after CS exposure in control mice. *Slpi* and *Serpina3n* were upregulated by CS exposure regardless of the presence of C/EBPα, whereas *Spink5* and *SerpinD1* were significantly downregulated in CS-exposed C/EBPαΔ/Δ mice. These results suggested that the deletion of C/EBPα induced serine protease/anti-serine protease imbalance in the distal airways during chronic CS exposure.

**Deletion of C/EBPα deteriorated the airway epithelial cell barrier during chronic CS exposure**

Since airway epithelial barrier dysfunction is a pathologic characteristic of COPD (22, 28-30), the zonula occludens-1 (Zo-1) protein, an adhesion molecule in airway epithelial cells, was evaluated using immunostaining and immunoblotting in murine lungs. Zo-1-positive regions in the distal airways were found at similar levels in chronic air-exposed control and C/EBPαΔ/Δ mice (Fig 2B). In contrast, Zo-1-positive regions were decreased by CS exposure in C/EBPαΔ/Δ mice, and the protein level of Zo-1 in the lung was significantly lower in CS-exposed C/EBPαΔ/Δ mice than in air-exposed C/EBPαΔ/Δ mice or CS-exposed control mice, suggesting that the deletion of C/EBPα might dysregulate the airway barrier during chronic CS exposure.

Because epidermal growth factor receptor (EGFR) activation degrades tight junction proteins in airway epithelial cells in COPD (22), EGFR and Tyr-1068-phosphorylated EGFR (pEGFR) protein levels were also evaluated in murine lungs using immunoblotting. The amount of pEGFR was significantly higher in C/EBPαΔ/Δ mice than in control mice, irrespective of CS exposure, but EGFR levels did not differ between the two groups (Fig 2C), indicating that the deletion of C/EBPα consistently induced the phosphorylation of EGFR, even in unstressed conditions.

To examine whether the deletion of C/EBPα also affected the lung parenchyma along
with the distal airways during CS exposure, distal lung inflammation and alveolar epithelial barrier function were evaluated. The number of alveolar macrophages, but not neutrophils, in BALF was significantly greater in CS-exposed C/EBPαΔ/Δ mice than in CS-exposed control mice (Table E3). Regarding lung injury, C/EBPαΔ/Δ mice showed insufficient secretion of phosphatidylcholine and greater protein leakage in BALF during chronic CS exposure (Table E3), suggesting that the deletion of C/EBPα was associated with alveolar epithelial barrier function.

**Deletion of C/EBPα increased abnormal attachments during chronic CS exposure**

Airspace enlargement, as assessed by the MLI, tended to be greater in CS-exposed control mice than in air-exposed control mice (P = 0.11). In C/EBPαΔ/Δ mice, the MLI increased significantly during 6 months of exposure to CS; however, there was no difference between 6 months air-exposed and CS-exposed C/EBPαΔ/Δ mice (P = 0.39), suggesting that the deletion of C/EBPα might induce airspace enlargement, even in unstressed conditions (Fig 3A).

In contrast, the percentage of abnormal alveolar attachment (%AA) increased in CS-exposed C/EBPαΔ/Δ mice compared to that in CS-exposed control and air-exposed C/EBPαΔ/Δ mice (Fig 3B), suggesting that the deletion of C/EBPα augmented the CS-induced destruction of alveolar attachments.

**In vivo daily treatment with BPTI in CS-exposed C/EBPαΔ/Δ mice restored ciliated cell regeneration, reduced broken alveolar attachments, and maintained the airway epithelial barrier**

To explore the effect of serine protease imbalance on the pathological changes in vivo, a serine protease inhibitor, bovine pancreatic trypsin inhibitor (BPTI), or vehicle, was intratracheally administered to H2B-C/EBPαΔ/Δ mice daily for 6 months. Compared to the vehicle treatment, BPTI treatment increased the number of GFP-positive ciliated cells (Fig
and decreased the %AA (Fig 4B) in the distal airways in CS-exposed H2B-C/EBPαΔ/Δ mice. Additionally, BPTI treatment tended to increase Zo-1 protein expression in the lungs (P = 0.07) (Fig 4C).

These results suggested that regulation of serine protease activity in the distal airways might contribute to the maintenance of epithelial cell regeneration, intact alveolar attachments, and barrier function during chronic CS exposure.

Human studies

Patient demographics

To examine whether the findings from the murine studies were relevant to the pathogenesis of CLE in humans, CEBPA and serine proteases/antiproteases were quantified in the small airways of lung tissues obtained from 28 smokers. Based on a visual assessment of chest CT images (Fig E3), all smokers were divided into 3 groups: the CLE-dominant (n = 6), PSE-dominant (n = 10), and nonemphysema groups (n = 12). There were no PLE-dominant cases. Table E4 summarizes the demographics of these groups.

Immunohistochemistry and mRNA expression analysis of CEBPA, serine proteases and antiproteases

In the immunostaining of human lung sections, C/EBPα was identified in both airway and alveolar epithelial cells, which was consistent with the distribution of C/EBPα in the murine lung (Fig E4 and E5). In the mRNA expression analysis of lung homogenates, CEBPA expression was significantly lower in the PSE group (P <0.05) and tended to be lower in the CLE group (P = 0.08) than in the nonemphysema group (Fig E6A). CEBPA gene expression was significantly associated with SPINK5 expression but not with expression of KLK13 and SERPIND1 (Fig E6B), which were identified as the main regulators of serine protease/antiprotease balance in murine distal airways (Table 1).
In immunostaining (Fig 5), Lympho-epithelial Kazal-type-related inhibitor (LEKTI), which is known as an anti-serine protease that is regulated by *SPINK5*, *SERPIND1*, and *KLK13*, was localized mainly in the small airways. Semiquantitative scoring of each type of staining showed that the LEKTI score, but not the SERPIND1 or KLK13 score, was lower in the CLE group than in the nonemphysema and PSE groups. The LEKTI score tended to be associated with FEV₁/FVC (*P* = 0.07) (Table E5).
In this study, we showed that deletion of C/EBPα impaired ciliated cell regeneration and caused epithelial cell barrier dysfunction and destruction of alveolar attachments in the small airways of chronic CS-exposed mice. These molecular and pathological changes were caused by excessive activity of serine proteases in the lung and improved after treatment with an anti-serine protease. Additionally, CEBPA expression was downregulated in human lung tissues from emphysema patients, and LEKTI, a downstream target of C/EBPα, was decreased in the small airways of smokers with CT-defined CLE compared to those of individuals without emphysema. These results suggest that serine protease/antiprotease imbalance in the small airways might be involved in the pathogenesis of CLE and could be a potent therapeutic target for COPD.

Earlier studies have proposed the hypothesis that protease/antiprotease imbalance is associated with the pathogenesis of COPD (1, 31, 32), and it is well known that neutrophil elastase and MMPs contribute to the development of emphysema in murine models (33-36). However, the pathological role of proteases and antiproteases in the small airways remains unclear. Therefore, the present results are important, as they provide the first evidence that serine protease/antiprotease imbalance in the murine distal airways induces the loss of ciliated cells, impairs epithelial integrity, and destroys alveolar attachments, all of which are the main features of small airway disease in human COPD (25, 27).

In microarray data, the local gene expression of two serine proteases, Klk13 and Ctss, and two antiproteases, Slpi and Serpina3n, were similarly increased in the distal airways of control and C/EBPαΔ/Δ mice during chronic CS exposure. In contrast, chronic CS exposure increased the expression of two anti-serine proteases, Spink5 and Serpind1, in control mice but not in C/EBPαΔ/Δ mice. These results suggest that insufficient expression of Spink5 and
Serpind1 could cause protease/antiprotease imbalance in the distal airways of C/EBPαΔ/Δ mice.

In the human small airways, the immunostaining score of LEKTI was lower in the CLE group than in the nonemphysema and PSE groups. LEKTI is an anti-serine protease that is regulated by SPINK5 and works as a multidomain serine protease inhibitor (37-39). Deficiency of LEKTI is involved in skin diseases, such as Netherton’s syndrome, that are associated with respiratory comorbidity (40-42), and SPINK5 gene mutations are associated with asthma phenotypes (43-45). The present data build on these earlier reports by suggesting that emphysema and COPD are also associated with the abnormal expression of LEKTI, especially in the small airways.

The expression of EGF and EGFR in the bronchiolar epithelium was increased in COPD (46), and activation of the EGFR pathway could impair ciliogenesis in bronchial epithelial cells (47). Therefore, we evaluated EGFR in CS-exposed C/EBPαΔ/Δ mice and found that the lung expression of pEGFR was higher in C/EBPαΔ/Δ mice than in control mice. Interestingly, although there was no difference in the increase in pEGFR between CS-exposed and air-exposed C/EBPαΔ/Δ mice, loss of ciliated cells in the distal airways and bronchiolar epithelial barrier dysfunction were found in only CS-exposed C/EBPαΔ/Δ mice. Because reactive oxygen species (ROS) exacerbate epithelial dysfunction (22), we speculate that pEGFR overexpression might lead to insufficient regeneration of ciliated cells during chronic CS exposure, and pEGFR overexpression along with ROS production might induce epithelial cell barrier dysfunction, a loss of cell-cell interactions and an increase in proteolytic activity, which might induce the destruction of alveolar attachments (Fig 6). Therefore, conditional deletion of C/EBPα in the CS exposure model might represent a more relevant and novel system to reproduce the regenerative dysfunction of small airway epithelial cells that occurs in COPD.
Morphometric analysis showed that the extent of airspace enlargement did not differ between air-exposed and CS-exposed C/EBPαΔ/Δ mice, and C/EBPαΔ/Δ mice had the potential to develop emphysema even without CS exposure. This result may have occurred because the alveolar type 1 cell fragility in C/EBPαΔ/Δ mice might affect the structure of the lung parenchyma more strongly than CS exposure (see online supplement III and Fig E7 for further discussion).

Although LEKTI might also be a downstream target of C/EBPα in humans and the LEKTI staining score in small airways was significantly lower than that of the other types only in CLE (Fig 5), CEBPA expression in whole lung tissues was lower in both PSE and CLE patients than in nonemphysema patients (Fig E6A). Since C/EBPα is expressed in bronchial epithelial cells, alveolar type 2 cells and alveolar macrophages and PSE is characterized as alveolar damage without bronchial remodeling (48-50), CEBPA-expressing cells might be decreased in PSE.

This study has some limitations. First, nonsmokers and patients with severe emphysema were not included. However, the present data clearly showed that the LEKTI score was lower in CLE patients than in smokers without emphysema or in those with PSE, suggesting that targeting the serine protease/antiprotease imbalance in the early stage of CLE is a reasonable approach to prevent further disease progression. Second, we did not analyze local gene expression in the human small airways as we did in the murine experiments.

In conclusion, deletion of C/EBPα suppresses anti-serine protease activity during chronic CS exposure, resulting in upregulation of serine protease activity in the small airways and subsequent epithelial barrier dysfunction and alveolar attachment loss in mice. Insufficient expression of LEKTI was also found in the small airways of human CLE
patients. The augmentation of LEKTI might be a novel therapeutic strategy to prevent the
development and progression of CLE.
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Table 1. Differences in Protease and Antiprotease Gene Expression in the Distal Airways

<table>
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<tr>
<th>Category</th>
<th>Gene Symbol (Assay ID)</th>
<th>Log₂ FC (CS-exposed control - Air-exposed control)</th>
<th>Log₂ FC (Air-exposed C/EBPαΔ/Δ - Air-exposed control)</th>
<th>Log₂ FC (CS-exposed C/EBPαΔ/Δ - Air-exposed C/EBPαΔ/Δ)</th>
<th>Log₂ FC (CS-exposed C/EBPαΔ/Δ - CS-exposed control)</th>
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<td>Serine protease</td>
<td>Klk13 (0700000770)</td>
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<td>-1.99</td>
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<td>n.s.</td>
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<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>Serine protease</td>
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<td>n.s.</td>
<td>n.s.</td>
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<td>n.s.</td>
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<td>1.87</td>
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</table>
Definitions of abbreviations: FC = fold change; n.s.: not significant.

Control and C/EBPαΔ/Δ mice were exposed to CS or air for three months, and terminal bronchioles were isolated using laser microdissection (LMD) (four mice/group). Three mice/group were selected for microarray analysis based on the RNA quality and total airway number. Genotype- and time-dependent mRNA expression levels were assessed by ANOVA, using P <0.05 and a log2 fold change value greater than 1.00 as thresholds for expression.

The protease and antiprotease genes that had significantly different expression in air-exposed and CS-exposed control mice are listed.

Among matrix metalloproteinases (MMPs), the expression levels of Mmp12 and Mmp9 were significantly higher in CS-exposed control mice than in air-exposed control mice. Among serine proteases, the expression levels of Kallikrein-related peptides (Klk13, Klkb1, Klk10, and Klk8) and cathepsin S (Ctss) were significantly higher in CS-exposed control mice than in air-exposed control mice.

These gene expression levels were not significantly different between air-exposed control and air-exposed C/EBPαΔ/Δ mice, except for Klkb1 upregulation, which was observed in C/EBPαΔ/Δ mice. Additionally, CS-exposed C/EBPαΔ/Δ mice showed higher expression of Klk13, Klkb1, Klk10, Mmp12 and Ctss than did air-exposed control mice.
For antiprotease gene expression levels, the levels of *Slpi*, *Serpind1*, *Serpina3n*, *Spink5*, *Spint3* and *Timp1* were significantly upregulated after CS exposure in the control mice. Among these genes, *Spink5*, *Serpind1*, *Spint3*, and *Timp1* were not upregulated during CS exposure in C/EBPαΔ/Δ mice, which was inconsistent with the results obtained for the control. Furthermore, the *Serpind1*, *Spink5* and *Timp1* gene expression levels were significantly lower in CS-exposed C/EBPαΔ/Δ mice than in CS-exposed control mice.
C/EBPa\textsuperscript{Δ/Δ} mice exhibited impaired regeneration of ciliated cells, and CS-exposed C/EBPa\textsuperscript{Δ/Δ} mice had fewer ciliated cells during chronic CS exposure.

Mice were exposed to air or cigarette smoke (CS) for 6 months, and the left lungs were removed and fixed. Lung sections were immunostained with Scgb1a1 (A), FoxJ1 (B) and Scgb1a1 (magenta)/acetylated tubulin (yellow)/green fluorescent protein (GFP) (green)/4',6-diamidino-2-phenylindole (DAPI) (blue) (D) antibodies.

The numbers of club cells (A) and ciliated cells (B) in the distal airways were counted in a 100-µm area from the bronchioalveolar duct junction (BADJ) in each mouse (n = 6/group) (scale bar = 50 µm). The bar chart indicates the mean and the standard error of the mean (SEM). (A) The number of club cells was not different among the groups. (B) After CS exposure, the number of ciliated cells significantly decreased in C/EBPa\textsuperscript{Δ/Δ} mice, and CS-exposed C/EBPa\textsuperscript{Δ/Δ} mice showed fewer ciliated cells than did control mice.

(C) Scgb1a1-rtTA/(tetO)\textsuperscript{7}CMV-Cre/H2B-eGFP (H2B-control) and Scgb1a1-rtTA/(tetO)\textsuperscript{7}CMV-Cre/H2B-eGFP/ Cebp\textsuperscript{a-flox/flox} (H2B-C/EBPa\textsuperscript{Δ/Δ}) mice were exposed to air or CS for 6 months after doxycycline administration. In these models, histone 2B-GFP was originally expressed on club cells and alveolar type 2 (AT2) cells after doxycycline administration, and ciliated cells that differentiated from progenitor cells after doxycycline administration maintained GFP expression. Almost all club cells expressed GFP after doxycycline administration, while ciliated cells generated before doxycycline administration did not express GFP (white arrows). GFP-positive ciliated cells (green arrows) represent cells that differentiated from club cells after doxycycline administration (scale bar = 50 µm). The dot plot indicates the number of GFP-positive ciliated cells in each mouse. In control mice, the number of GFP-positive ciliated cells in the distal airways increased during
chronic CS exposure. However, in C/EBPαΔ/Δ mice, the number of GFP-positive ciliated cells was lower than that in the control mice, even during air exposure.

(D) Scanning electron microscopy images of the distal airways. In control mice, chronic CS exposure shortened the cilia, while the area covered with cilia did not change. In C/EBPαΔ/Δ mice, CS exposure resulted in not only the shortening of cilia but also the disruption of ciliated cell continuity and cell flattening (scale bar = 10 µm).

The data were analyzed using one-way ANOVA followed by the post-hoc Tukey-Kramer test. * indicates P <0.05 compared to the control mice exposed to the same conditions. † indicates P <0.05 compared to the same genotype exposed to air.

**Figure 2:**

Caseinolytic activity in the C/EBPαΔ/Δ murine lung was enhanced by chronic CS exposure, and CS-exposed C/EBPαΔ/Δ mice showed disruption of the airway epithelial cell barrier.

Total protease activity in the supernatant of bronchoalveolar lavage fluid (BALF) was measured using BODIPY® TR-X dye-labeled casein (A). The airway epithelial barrier was evaluated by immunostaining for the zonula occludens-1 (Zo-1) protein (red), which is representative of tight junction proteins (B), and Z-stack images from every 0.43 µm were synthesized to create an image representing 13.3 µm in total (blue indicates DAPI). Zo-1 protein expression in murine lungs was quantitatively assessed by immunoblotting. The epidermal growth factor receptor (EGFR) protein expression in murine lungs was also quantitatively assessed by immunoblotting (C).

(A) The fluorescence intensity in BALF was measured quantitatively by a fluorescence microplate reader and converted to protease activity using trypsin as a standard (n = 6/group). The bar chart indicates the mean and the standard error of the
mean (SEM). BALF caseinolytic activity was significantly increased during CS exposure in C/EBPαΔ/Δ mice, and CS-exposed C/EBPαΔ/Δ mice showed higher BALF caseinolytic activity than did CS-exposed control mice. Details of the quantitative data are also shown in Table E2.

The data were analyzed using one-way ANOVA followed by the post-hoc Tukey-Kramer test. * indicates P <0.05 compared to the control mice exposed to the same conditions. † indicates P <0.05 compared to the same genotype exposed to air.

(B) A Zo-1-positive area was clearly observed on the apical sides of the bronchial epithelial cell layer in control mice and air-exposed C/EBPαΔ/Δ mice, while CS-exposed C/EBPαΔ/Δ mice showed a reduction in the Zo-1-positive area compared to that in the other groups (scale bar = 10 µm). The protein level of Zo-1 in the murine lung was measured quantitatively by immunoblotting. The Zo-1 protein expression level relative to beta-actin in each mouse was calculated (Zo-1/β-actin), and the dot plot indicates the ratio of Zo-1/β-actin in each mouse to the mean value of Zo-1/β-actin at 6 mo. in air-exposed control mice (n = 3/group). CS-exposed C/EBPαΔ/Δ mice had lower Zo-1 expression than air-exposed C/EBPαΔ/Δ mice and CS-exposed control mice.

The data were analyzed using one-way ANOVA followed by the post-hoc Tukey-Kramer test. ‡ indicates P <0.05 compared to the control mice exposed to the same conditions. § indicates P <0.05 compared to the same genotype exposed to air.

(D) The dot plot shows the protein expression level relative to that of beta-actin in each mouse (n = 3/group). The EGFR protein expression levels were not significantly different among the groups, but the levels of Tyr-1068 phosphorylated EGFR (pEGFR) protein were significantly higher in C/EBPαΔ/Δ mice than in control mice, even under unstressed conditions.

The data were analyzed using one-way ANOVA followed by the post-hoc Tukey-
Kramer test. ‡ indicates P <0.05 compared to the control mice exposed to the same conditions.

**Figure 3:**

*Deletion of C/EBPα increased abnormal alveolar attachments but did not deteriorate air space enlargement during CS exposure.*

The left lungs of nine- to twelve-week-old mice (here termed 0 mo. air) and mice exposed to or CS for 6 months (6 mo. air and 6 mo. CS) were removed and fixed. The lung sections were subjected to Diff-Quik staining (upper images), and microscopic images of the alveoli were digitalized (lower images) and morphometrically analyzed.

(A) Airspace enlargement was assessed with the mean linear intercept length (MLI) (n = 6/group). C/EBPαΔ/Δ mice showed a significant increase in the MLI after 6 months CS-exposure. In addition, there was no significant difference in the MLI between 6 mo. air-exposed and CS-exposed mice.

(B) The percentage of abnormal alveolar attachment (%AA) was calculated as the ratio of the number of broken alveolar attachments (*red arrows* in the images) to the number of total alveolar attachments (n = 6/group).

The bar chart indicates the mean and the SEM. There was no significant difference between control and air-exposed C/EBPαΔ/Δ mice. On the other hand, CS-exposed C/EBPαΔ/Δ mice had a significantly higher %AA than did the mice in the other groups.

The data were analyzed using one-way ANOVA followed by the post-hoc Tukey-Kramer test. * indicates P <0.05 compared to the same genotype exposed to 0 months of air. † indicates P <0.05 compared to the same genotype exposed to 6 months of air. ‡ indicates P <0.05 compared to the control mice exposed to the same conditions.
**Figure 4:**

**Daily treatment of C/EBPαΔ/Δ mice with a serine protease inhibitor during chronic CS exposure restored ciliated cell regeneration and the epithelial barrier and reduced abnormal alveolar attachments.**

Scgb1a1-rtTA/(tetO)\textsuperscript{7}CMV-Cre/H2B-eGFP/ Cebp\textsuperscript{Δ/Δ}floxflox (H2B-Cebp\textsuperscript{Δ/Δ}) mice were administered normal saline (here termed 6 mo. Vehicle Tx) or a serine protease inhibitor (BPTI: 0.2 µg/mouse) (here termed 6 mo. BPTI Tx) daily one hour after CS exposure for 6 months. Ciliated cell regeneration was evaluated by immunostaining for Scgb1a1 (magenta)/tubulin (yellow)/GFP (green)/DAPI (blue) (A). The %AA was calculated in the same manner described above (see Fig 3 and the Materials and methods) (B). The airway epithelial barrier was evaluated by immunostaining for the zonula occludens-1 (Zo-1) protein (red) (C), and the Z-stack images from every 0.43 µm were synthesized to create an image representing 13.3 µm in total (blue indicates DAPI), with the Zo-1 protein expression in murine lungs quantitatively assessed by immunoblotting.

(A) The green arrow indicates GFP-positive ciliated cells that differentiated from Scgb1a1-positive cells after doxycycline administration, and the white arrow indicates GFP-negative ciliated cells that existed before doxycycline administration (scale bar = 50 µm).

The dot plot indicates the number of GFP-positive ciliated cells or total ciliated cells (GFP-positive ciliated cells + GFP-negative ciliated cells) in each mouse.

The number of GFP-positive ciliated cells in the distal airway was significantly higher at 6 mo. BPTI Tx than in 6 mo. Vehicle Tx, and the number of total ciliated cells was also increased by BPTI treatment (n = 3/group).

(B) The %AA was lower in 6 mo. BPTI Tx mice than in 6 mo. Vehicle Tx mice (n = 3/group, scale bar = 50 µm).
In H2B-C/EBPαΔ/Δ mice, BPTI treatment tended to increase Zo-1 protein expression in the lung. The Zo-1 protein expression level was calculated relative to beta-actin in each mouse (Zo-1/β-actin), and the dot plot indicates the ratio of Zo-1/β-actin in each mouse to the mean value of Zo-1/β-actin in 6 mo. Vehicle Tx mice (n = 3/group).

Data were analyzed using Student’s t test (* indicates P <0.05).

Figure 5: Ex-smokers with centrilobular emphysema showed weaker LEKTI staining than ex-smokers with no emphysema or with paraseptal emphysema.

Definitions of abbreviations: paraseptal emphysema = PSE; centrilobular emphysema = CLE; Lympho-epithelial Kazal-type-related inhibitor = LEKTI; Serpin family D member 1 = SERPIND1.

Representative immunostaining images of the small airways in nonemphysema, PSE, and CLE patients (the lower left frame in each image shows a high-power field; scale bar: solid line = 100 µm, dashed line = 25 µm).

LEKTI and Kallikrein13 were detected primarily on airway epithelial cells and were barely detected in alveoli or interstitial regions. SERPIND1 was stained on airway epithelial cells, vascular endothelial cells and smooth muscle layers.

The levels of immunostaining were semiquantitatively scored (n; nonemphysema = 12, PSE = 10, and CLE = 6), and CLE patients showed a lower staining score for LEKTI in the small airways than did ex-smokers without emphysema or patients with PSE. The dot plot indicates the immunostaining score, and the bar indicates the mean and SE. On the other hand, there was no association between the emphysema type and SERPIND1 or Kallikrein13 staining scores.
The data were analyzed using one-way ANOVA followed by the post-hoc Tukey-Kramer test (* indicates P < 0.05).

**Figure 6:**

The suggested mechanisms by which C/EBPα deficiency in Scgb1a1-positive cells resulted in small airway injury in mice

Definitions of abbreviations: ROS = reactive oxygen species

From the results of the present study, C/EBPα deficiency in Scgb1a1-positive cells led to insufficient anti-serine protease expression, resulting in serine protease imbalance in the distal airways during chronic CS exposure. The serine protease imbalance accelerated the phosphorylation of EGFR, and excessive pEGFR expression combined with ROS might have inhibited ciliated cell regeneration after CS-induced bronchial epithelial injury and decreased tight junction proteins, i.e., the epithelial barrier. Additionally, the enhanced proteolytic activity might have weakened alveolar attachments, resulting in an increase in abnormal alveolar attachments. These pathological changes in the distal airways were counteracted by BPTI treatment after daily CS exposure.
Figure 1.

A. Scgb1a1 immunostaining

6 mo. Air

Scgb1a1 cells (100 μm)

6 mo. CS

B. FoxJ1 immunostaining

6 mo. Air

FoxJ1 cells (100 μm)

6 mo. CS

C. Scgb1a1, Tubulin, GFP, DAPI

6 mo. Air

6 mo. CS

D. Distal airway

H2B-Control

H2B-C/EBPαΔ/Δ

6 mo. Air

6 mo. CS
**Figure 2.**

A. Total protease activity

![Graph showing caseolytic activity in Control and C/EBPαΔ/Δ over 6 months of Air and CS exposure.](image)

B. 2D image and Z-stack visualization

<table>
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<tr>
<th></th>
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C. Western blot analysis

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<tr>
<td>C/EBPα</td>
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</tbody>
</table>

**Legend:**
- Control
- C/EBPαΔ/Δ

**Graphs:**
- Caseolytic activity (μU USP units/ml/g)
- β-actin kD
- Zo-1/β-actin
- pEGFR/β-actin
- EGFR/β-actin

**Notes:**
- * indicates significant difference at p < 0.05
- † indicates significant difference at p < 0.01
- ‡ indicates significant difference at p < 0.001
- § indicates trend towards significant difference
- ‡ indicates significant difference at p < 0.001
- ▲ indicates significant difference at p < 0.01
- ▲ indicates significant difference at p < 0.001
- ▲ ▲ indicates very significant difference at p < 0.001
- ▲ ▲ ▲ indicates extremely significant difference at p < 0.001
Figure 3.

A. Digitalized images showing the effect of chronic smoke exposure (CS) on the mean linear intercept (µm) and abnormal alveolar attachment (%). The images are categorized by treatment groups: 6 mo. Air and 6 mo. CS for both Control (C/EBPαΔΔ) and C/EBPαΔΔ conditions.

B. Abnormal alveolar attachment (%) for 6 mo. Air and 6 mo. CS conditions in Control (C/EBPαΔΔ) and C/EBPαΔΔ groups. The graphs indicate statistical significance (*, †) for different treatment groups.
Figure 4.

**A**

6 mo. CS-exposed H2B-C/EBPαΔ/Δ

- CC10 Tubulin
- GFP DAPI

6 mo. Vehicle Tx  |  6 mo. BPTI Tx

**B**

6 mo. CS-exposed H2B-C/EBPαΔ/Δ

- Abnormal alveolar attachment (%)

6 mo. Vehicle Tx  |  6 mo. BPTI Tx

**C**

6 mo. CS-exposed H2B-C/EBPαΔ/Δ

- Z-stack
- Zo1 DAPI

6 mo. Vehicle Tx  |  6 mo. BPTI Tx

- Zo1/β-actin in lung (% 6 mo. Vehicle Tx)
  - p = 0.07
Figure 5.

LEKTI (SPINK5)
Nonemphysema
PSE-dominant
CLE-dominant

SERPIND1
Nonemphysema
PSE
CLE

Kallikrein13
Nonemphysema
PSE
CLE

Staining score
High
Low

Nonemphysema
PSE
CLE

*
Figure 6.

- Deficiency of C/EBPα
- Anti-serine protease expression
- Serine protease/antiprotease imbalance
- Small airway injury
- ROS
- Inflammation
- Distal airway
- Ciliated cell differentiation
- Tight junction protein
- Alveolar attachment
- BPTI treatment
- Restored disease phenotypes
- Scgb1a1^+ cells
- Ciliated cells