Z α_1 -Antitrypsin Confers a Proinflammatory Phenotype That Contributes to Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: Severe α_1 -antitrypsin deficiency caused by the Z variant (Glu342Lys; ZZ-AT) is a well-known genetic cause for emphysema. Although severe lack of antiprotease protection is the critical etiologic factor for ZZ-AT-associated chronic obstructive pulmonary disease (COPD), some reports have suggested enhanced lung inflammation as a factor in ZZ-AT homozygotes.

Objectives: To provide molecular characterization of inflammation in ZZ-AT.

Methods: Inflammatory cell and cytokine profile (nuclear factor- κ B, IL-6, tumor necrosis factor- α), intracellular polymerization of Z-AT, and endoplasmic reticulum (ER) stress markers (protein kinase RNA-like ER kinase, activator transcription factor 4) were assessed in transgenic mice and transfected cells in response to cigarette smoke, and in explanted lungs from ZZ and MM individuals with severe COPD.

Measurements and Main Results: Compared with M-AT, transgenic Z-AT mice lungs exposed to cigarette smoke had

higher levels of pulmonary cytokines, neutrophils, and macrophages and an exaggerated ER stress. Similarly, the ER overload response was greater in lungs from ZZ-AT homozygotes with COPD, and was particularly found in pulmonary epithelial cells. Cigarette smoke increased intracellular Z-AT polymers, ER overload response, and proinflammatory cytokine release in Z-AT-expressing pulmonary epithelial cells, which could be prevented with an inhibitor of polymerization, an antioxidant, and an inhibitor of protein kinase RNA-like ER kinase.

Conclusions: We show here that aggregation of intracellular mutant Z-AT invokes a specific deleterious cellular inflammatory phenotype in COPD. Oxidant-induced intracellular polymerization of Z-AT in epithelial cells causes ER stress, and promotes excess cytokine and cellular inflammation. This pathway is likely to contribute to the development of COPD in ZZ-AT homozygotes, and therefore merits further investigation.

Keywords: α_1 -antitrypsin; oxidation; polymerization; COPD; ER stress

α_1 -Antitrypsin (AT) is an important inhibitor of neutrophil elastase in the lung. It is primarily synthesized and secreted by hepatocytes from where it diffuses into alveoli, but is also synthesized by macrophages and monocytes, pulmonary

epithelial cells, neutrophils, and endothelial cells (1, 2). The normal variant is termed M-AT according to its isoelectric point. Severe deficiency of AT is usually caused by homozygosity for the Z mutation (Glu342Lys, Z-AT). The prevalence of

ZZ-AT is 1 in 2,000 in northern European populations, and 1 in 4,455 individuals in the United States (3, 4). The misfolded Z-AT protein polymerizes within hepatocytes, and when the degradative processes are overwhelmed, intrahepatic aggregates of

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At A Glance Commentary

Scientific Knowledge on the

Subject: Homozygosity for the Z variant of antitrypsin (Glu342Lys; Z-AT) results in severe plasma deficiency and is the only known genetic cause for emphysema. The principal accepted mechanism of ZZ-AT-related lung disease has been that of proteinase excess, which leads to accelerated alveolar destruction. There is no known cure for ZZ-related emphysema; therefore, it remains important to understand the mechanism of disease. Some reports have suggested excessive pulmonary inflammation in ZZ-AT individuals; however, there has been no explanation for these proposals to date.

What This Study Adds to the

Field: We have demonstrated that oxidant-mediated intracellular aggregation of Z-AT in pulmonary epithelial cells incites a deleterious proinflammatory phenotype in the lungs. Our data increase knowledge of the mechanism of lung disease in ZZ homozygotes by revealing a novel pathway of lung injury, in addition to antiproteinase deficiency. This pathway is likely to contribute to the accelerated health decline observed in ZZ-AT homozygotes with emphysema, and offers opportunities to maximize the benefit of augmentation therapy.

Z-AT protein contribute to the development of neonatal hepatitis, liver cirrhosis, and hepatoma in a subset of ZZ-AT homozygotes (5–9). The consequential secretory defect leads to severe plasma deficiency, predisposing ZZ-AT individuals to early onset emphysema.

The association of severe AT deficiency with emphysema underpins the antiproteinase-proteinase hypothesis of the pathogenesis of emphysema (10–12). Severe AT deficiency is responsible for 1–3% of all cases of chronic obstructive pulmonary disease (COPD) (1, 2). However, it is recognized that there is variability in the expression of the lung disease. For example; only subsets of ZZ-AT homozygotes

experience a rapid decline in lung function. COPD can develop as early as the fourth decade of life in ZZ-AT individuals who smoke, compared with fifth to sixth decade in nonsmokers (3, 13–15). There is no cure for the lung disease, which can progress inexorably, resulting in severe disability and death with only some selected cases receiving lung transplantation. It therefore remains important to understand the molecular mechanisms of disease. Some reports have suggested the presence of excess lung inflammation in Z-AT individuals with COPD compared with individuals with normal (MM)-AT-related COPD. Specifically, ZZ-AT individuals with COPD have increased lung neutrophils (16–18), increased leukotriene B₄, and IL-8 (19). However, the cause of this intensified inflammation in ZZ-AT individuals has not been fully elucidated.

In this study, we have characterized the inflammatory processes related to Z-AT to aid understanding of the mechanism of dysregulated inflammation in ZZ-AT homozygotes.

Methods

See the online supplement for information regarding methods.

Animal Model

Acute cigarette smoke exposure of transgenic mice and analysis of bronchoalveolar lavage fluid and lungs. All experimental protocols were approved by the Home Office, United Kingdom. Cigarette smoke (CS) exposure of heterozygous transgenic for human M-AT (M-AT mice) (n = 8) and human Z-AT (Z-AT mice) (n = 8) was performed for 5 days (20–22). Aliquots of bronchoalveolar lavage fluid (BALF) were frozen directly with and without the addition of proteinase inhibitors. BALF and lungs (lung perfusion and homogenization) were assessed for free and intracellular neutrophil elastase, AT concentration and conformations (polymeric/oxidized/oxidized-polymers), lung injury (total BALF protein and wet:dry ratio of lungs) (18, 20, 23), murine tumor necrosis factor (TNF)- α , IL-6, and CCL2/Mouse JE (respective DuoSets; R&D Systems Minneapolis, MN) were performed. Murine TNF- α , IL-6, JE, protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK), activator

transcription factor (ATF) 4, ATF6, and glyceraldehyde phosphate dehydrogenase (GAPDH) were analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blot. Nuclear factor-kappa B (NF- κ B) and activator protein 1 (AP-1) were assessed (24).

Human Tissue

Gene expression analysis of emphysematous explanted lungs of MM-AT and ZZ-AT individuals. Tissue sections from lung explants of ex-smokers with Global Initiative for Chronic Obstructive Lung Disease stage IV MM-AT COPD (23 individual cases; mean age, 56.7 ± 4.5 yr) and ZZ-AT COPD (16 individual cases; mean age, 44.7 ± 5.3 yr) were stained (Hemalum and the cell Cut Plus system); cells were harvested (laser-assisted microdissection); and gene expression, NF- κ B (n = 6 each), IL-6 (n = 10 and n = 9, respectively), CCL2 (monocyte chemotactic protein [MCP-1]; n = 3 each), PERK (n = 20 and n = 5, respectively), and ATF4 (n = 20 and n = 13, respectively) were analyzed by real-time PCR (25, 26).

Immunolocalization of PERK, ATF4, and macrophages. Immunolocalization for human PERK and ATF4 was analyzed on lung tissue from ex-smokers with Global Initiative for Chronic Obstructive Lung Disease stage IV COPD individuals with MM-AT COPD (n = 4; mean age, 52.3 ± 3.8 yr) and ZZ-AT COPD (n = 4; mean age, 43.3 ± 5.7 yr), and three control samples (n = 3; age, 34.5 ± 3.7 yr) observed by light microscopy (Nikon Eclipse E600, Tokyo, Japan) (18). Macrophages were identified by immunohistochemistry: CD14 (monocytes), CD68 (mature tissue macrophages), and MAC387 (recently blood derived macrophages) (27). For quantitative comparison of the number and phenotype of infiltrating cells, positive cells were counted in five high power fields.

Cell Model

Analysis of the effect of CS extract. Transgenic human M-AT and Z-AT cells were generated using human alveolar epithelial (A549) cells and primary normal human bronchial epithelial (NHBE) cells were evaluated (see Figure E1 in the online supplement) (24). In this model, Z-AT accumulates in the ER (see page E8) (24). A549-M-AT and A549-Z-AT cells incubated with 12.5% CS

extract (20) had no evidence of cytotoxicity (see Table E1) or apoptosis (see Figure E2). ELISA, RT-PCR, or Western blot was used to analyze supernatant, cell lysates, and inclusion bodies from 24 hours for conformations of human AT, PERK, ATF4, ATF6, regulator of G-protein signaling protein 16 protein (RGS16), calnexin and GAPDH, TNF- α , IL-6, MCP-1, and NF- κ B (20, 24).

Inhibitor studies. A549-M-AT/Z-AT cells and primary NHBE-Z-AT cells were preincubated with either inhibitor of polymerization: 4M (20 μ g) (24, 28), the antioxidant *N*-acetylcysteine (NAC; 10 mM) (20) (dose was selected after assessment of inhibition of glutathione) (see Figure E3) or PERK inhibitor I (GSK2606414; 2 μ M) (29) (see Figure E4) for 0.5–1 hour followed by incubation with CS extract (12.5%) for a further 24 hours. Supernatant and cells were collected for mRNA or protein analysis.

Statistical Analysis

All statistical analysis was performed using SigmaStat and SPSS software (version

12.0.1, for Windows; SPSS Inc., Chicago, IL). A *P* value less than 0.05 was considered statistically significant.

Results

Effect of CS on NF- κ B and AP-1 Activity in Z-AT and M-AT Mice

NF- κ B activity was induced in both M-AT and Z-AT mice after CS exposure (Table 1). However, NF- κ B activity was higher in CS-exposed Z-AT mice lungs compared with CS-exposed M-AT mice lungs (*P* < 0.001). AP-1 was increased after CS to similar extent in both Z-AT and M-AT mice (*P* = 0.109) (Table 1).

Effect of CS Exposure on TNF- α , IL-6, and JE in Murine Lungs

Bronchoalveolar lavage. TNF- α concentrations were significantly elevated in both CS-exposed and control Z-AT BALF compared with their respective M-AT BALF samples (*P* < 0.001 for both)

(Table 1). CS induced significantly increased IL-6 concentrations and JE (mouse equivalent of MCP-1) in BALF of Z-AT compared with CS-M-AT mice (*P* = 0.001 and *P* = 0.002, respectively) (Table 1).

Lung homogenates. Lung TNF- α mRNA (381 bp) was significantly elevated after CS exposure in Z-AT mice compared with CS-M-AT mice (*P* < 0.001) (Figure 1A). CS exposure up-regulated IL-6 mRNA (413 bp) to a greater extent in Z-AT mice compared with M-AT mice (*P* < 0.001) (Figure 1B). Further analysis confirmed excess TNF- α , IL-6, and JE protein in CS-Z-AT lungs (see Table E2). CS had no effect on JE mRNA at 24 hours (Figure 1C).

Pulmonary Inflammatory Cell Profile in Z-AT and M-AT Mice

Acute CS exposure of Z-AT transgenic mice resulted in a greater influx of total cells, neutrophils, and macrophages compared with CS-exposed M-AT mice (*P* < 0.001 for all) (Table 1). The total number of macrophage and neutrophil

Table 1: Acute CS Exposure Induces Exaggerated Inflammatory Response *In Vivo*, Pulmonary Infiltration of Inflammatory Cells, and Lung Injury in Z-AT Mice

	M-AT		<i>P</i> Value: Non-CS vs. CS	Z-AT		<i>P</i> Value: Non-CS vs. CS	<i>P</i> Value	
	Non-CS	CS		Non-CS	CS		Non-CS M-AT vs. Non-CS-Z-AT	CS M-AT vs. CS Z-AT
Transcriptional activity in mice lungs, O.D. at 450 nm, mean $\pm$ SEM								
NF- κ B	0.28 $\pm$ 0.05	0.71 $\pm$ 0.07*	<0.001	0.37 $\pm$ 0.05†	1.06 $\pm$ 0.105*‡	<0.001	0.025	<0.001
AP-1	0.24 $\pm$ 0.06	0.52 $\pm$ 0.1*	<0.001	0.26 $\pm$ 0.05	0.69 $\pm$ 0.1*	<0.001	0.183	0.109
Mediators in mice BALF, pg/ml, median (IQR)								
TNF- α	15.1 (16.15–13.1)	243.1 (284.5–223)*	<0.001	40.5 (50.2–35.6)†	425 (486.5–362)*‡	<0.001	<0.001	<0.001
IL-6	12.5 (13.9–11.2)	254.5 (299.7–202.6)*	<0.001	12.6 (14.9–11.6)	357.7 (406.7–340.7)*‡	<0.001	1.000	0.001
JE (homolog of human MCP-1)	13.6 (18.6–11.7)	35.8 (40.7–30.2)*	<0.001	15 (17.25–13.5)	70.8 (107–53.8)*‡	<0.001	0.248	0.002
Differential cell profile in BALF, $\times 10^4$, mean $\pm$ SEM								
Total cells	9.97 $\pm$ 1.62	15.08 $\pm$ 0.97*	<0.001	12.94 $\pm$ 1.1†	18.71 $\pm$ 1.29*‡	<0.001	0.048	<0.001
MACs	9.38 $\pm$ 1.54	14.49 $\pm$ 1.06*	<0.001	12.53 $\pm$ 1.05†	17.87 $\pm$ 1.29*‡	<0.001	0.032	<0.001
Neutrophils	0.09 $\pm$ 0.08	0.63 $\pm$ 0.07*	<0.001	0.15 $\pm$ 0.09	0.88 $\pm$ 0.05*‡	<0.001	0.563	<0.001
Lymphocytes	0.024 $\pm$ 0.01	0.002 $\pm$ 0.001*	<0.001	0.034 $\pm$ 0.01	0*‡	<0.001	0.271	<0.001
NE as a measure of pulmonary neutrophils, ng/ml, median $\pm$ IQR								
Free NE	0 (0–0)	184.5 (242.1–160.2)*	<0.001	0 (0–0)	413.4 (482.5–331.8)*‡	<0.001	1.000	<0.001
Intracellular NE	0 (0–0)	291.5 (320.4–242.5)*	<0.001	0 (0–0)	576.3 (713–513.9)*‡	<0.001	1.000	<0.001
Mice lung injury, mean $\pm$ SEM								
Total protein in BALF, μ g/ml	307.4 $\pm$ 32.54	558.13 $\pm$ 38.4*	<0.001	339.5 $\pm$ 14.3	715.6 $\pm$ 46.9*‡	<0.001	0.382	0.022
Wet:dry ratio of lungs	1.94 $\pm$ 0.22	6.3 $\pm$ 0.49*	<0.001	2.4 $\pm$ 0.15	8.8 $\pm$ 0.4*‡	<0.001	0.083	0.001

Definition of abbreviations: AP = activator protein; AT = antitrypsin; BALF = bronchoalveolar lavage; CS = cigarette smoke; IQR = interquartile range; M-AT = normal variant AT; MCP = monocyte chemotactic protein; NE = neutrophil elastase; NF- κ B = nuclear factor kappa B; TNF = tumor necrosis factor; Z-AT = Z mutation AT.

*Non-CS vs. CS.

†Non-CS M-AT vs. non-CS-Z-AT.

‡CS M-AT vs. CS Z-AT.

Figure 1. Acute cigarette smoke (CS) exposure up-regulates inflammatory mediator mRNA *in vivo* in Z variant of antitrypsin (Z-AT) mice lungs. Acute CS exposure significantly up-regulated expression of *tumor necrosis factor* (*TNF*)- α mRNA ($P = 0.006$ and $P < 0.001$, respectively) (A) and *IL-6* mRNA ($P = 0.003$ and $P < 0.001$, respectively) (B) in lungs of normal variant AT (M-AT) and Z-AT mice compared with their respective non-CS-exposed controls. CS-exposed Z-AT mice lungs had significantly elevated *TNF*- α and *IL-6* mRNA compared with CS-exposed M-AT mice ($P = 0.003$ and $P < 0.001$, respectively). At 24 hours *JE* mRNA was unaffected by CS exposure in Z-AT or M-AT mice lungs (C). Negative control was mastermix (final reaction volume compensated with water) and positive control was LPS (20 ng) treated J774.1 cells. Band density was determined using the ImageJ program and expressed relative to murine GAPDH mRNA. Results presented are from analysis of bronchoalveolar lavage fluid and lung homogenates (lungs) from $n = 8$ mice per group, and of three independent experiments ($n = 3$). *Non-CS-exposed versus CS exposed, **M-AT mice versus Z-AT mice. Values are expressed as mean $\pm$ SEM. GAPDH = glyceraldehyde phosphate dehydrogenase.

numbers was significantly greater in CS-Z-AT mice compared with CS-M-AT mice (Table 1). Acute CS exposure also resulted in a significant increase in free neutrophil elastase in BALF of Z-AT

mice compared with M-AT mice ($P < 0.001$) (Table 1). Acute CS exposure resulted in a greater neutrophilia (neutrophil elastase from lysed neutrophils) in CS-Z-AT lung tissue

compared with CS-M-AT lung tissue ($P < 0.001$) (Table 1). Lymphocyte BALF numbers were reduced similarly after CS exposure in both M-AT and Z-AT mice (Table 1).

Figure 2. Z variant of antitrypsin (Z-AT) mice have an enhanced endoplasmic reticulum (ER) stress following acute cigarette smoke (CS) exposure. Acute CS exposure significantly increased ER stress markers; protein kinase RNA-like ER kinase (PERK) (A and B) and activator transcription factor (ATF) 4 (C and D) in Z-AT mice compared with normal variant AT (M-AT). (A and B) Baseline expression of *PERK* mRNA was higher in unstimulated Z-AT controls compared with M-AT controls ($P = 0.026$). CS exposure significantly up-regulated *PERK* mRNA in both M-AT and Z-AT mice lungs compared with non-CS exposed M-AT and Z-AT controls ($P = 0.004$ and $P = 0.044$, respectively). (A) CS-exposed Z-AT mice had significantly greater *PERK* mRNA than CS-exposed M-AT mice ($P = 0.010$). (B) There was significant up-regulation of PERK protein product (140 kD) in non-CS-exposed and CS-exposed Z-AT mice compared with their

Figure 2. (Continued). respective M-AT mice ($P = 0.015$ and $P = 0.012$, respectively). (C and D) Baseline expression of *ATF4* mRNA was higher in Z-AT mice lungs ($P = 0.006$) (C). CS exposure induced significant up-regulation of *ATF4* mRNA in Z-AT mice lungs compared with the non-CS-exposed Z-AT mice lungs ($P = 0.038$). *ATF4* mRNA was not affected by CS exposure in M-AT mice lungs compared with non-CS-exposed M-AT mice lungs ($P = 0.345$). (D) There was significant up-regulation of *ATF4* protein product (38 kD) in non-CS-exposed and CS-exposed Z-AT mice compared with their respective M-AT mice ($P = 0.013$ and $P = 0.021$, respectively). (E and F) Baseline expression of *ATF6* mRNA and protein was unaffected in control M-AT and Z-AT mice. CS exposure significantly up-regulated expression of *ATF6* mRNA and protein in both M-AT and Z-AT compared with their respective non-CS-exposed controls; $P < 0.001$ for all. There was no difference in the induction of *ATF6* between CS-M and CS-Z mice (E and F). Thapsigargin (0.5 $\mu\text{g/ml}$) was used to induce ER stress and thus provide a positive control for *ATF4*, *PERK*, and *ATF6* (39). Band density was determined using the ImageJ program and expressed relative to murine GAPDH mRNA (A, C, and E) or murine GAPDH protein (B, D, and F). Results are presented for $n = 8$ mice per group and of three independent experiments ($n = 3$). Values are expressed as mean $\pm$ SEM. *Non-CS-exposed versus CS exposed, **M-AT mice versus Z-AT mice. GAPDH = glyceraldehyde phosphate dehydrogenase.

ER Stress Genes

Baseline mRNA and protein expression of *PERK* was higher in Z-AT controls compared with M-AT controls ($P = 0.026$ and $P =$

0.015, respectively) (Figures 2A and 2B). After CS exposure there was significantly greater up-regulation of *PERK* mRNA and *PERK* protein in Z-AT mice lungs compared

with M-AT mice lungs ($P = 0.010$ and $P = 0.012$, respectively).

Baseline lung expression of *ATF4* mRNA and *ATF4* protein was higher in Z-AT mice

Table 2: Gene Expression of NF- κ B and Inflammatory Mediators in Emphysematous Lungs

	Homozygotes with COPD		P Value: MM-AT vs. ZZ-AT
	MM-AT	ZZ-AT	
Affimetrix value transcriptional activity, median (IQR)			
NF- κ B	17.43 (19.32–13.64)	21.67 (22.4–21.21)*	0.002
Relative gene expression of mediators, median (IQR)			
IL-6	1.41 (2.884–0.52)	14.2 (28.18–5.60)*	0.005
CCL2 (MCP-1)	580 (864–272)	3,903 (5,626–2,178)	0.100
Relative gene expression of ER overload response markers, median (IQR)			
PERK	0.095 (0.150–0.075)	1.19 (1.218–0.185)*	0.007
ATF4	5.13 (7.85–4.24)	48.9 (92–14)*	<0.001

Definition of abbreviations: AT = antitrypsin; ATF = activator transcription factor; COPD = chronic obstructive pulmonary disease; ER = endoplasmic reticulum; IQR = interquartile range; MCP = monocyte chemotactic protein; NF- κ B = nuclear factor kappa B; PERK = protein kinase RNA-like endoplasmic reticulum kinase.

*MM-AT vs. ZZ-AT.

Figure 3. Expression of endoplasmic reticulum (ER) stress markers in emphysematous lungs. (A) Immunolocalization of protein kinase RNA-like ER kinase (PERK) and activator transcription factor (ATF) 4 in frozen lung sections from MM-AT and ZZ-AT emphysematous lung tissue and controls. PERK (arrows, top panels) and ATF4 (arrows, middle panels) are localized to the cytoplasm of respiratory epithelial cells with staining significantly more intense and frequent in ZZ-AT as compared with MM-AT and control lungs. Representative images ($n = 3-4$). Original magnification $\times 40$. (B–D) Immunohistochemistry localization of macrophage phenotypes in MM-AT and ZZ-AT chronic obstructive pulmonary disease (COPD). (B) Sections of lung stained with the monocyte marker CD14 demonstrate a significantly increased number of monocytes in ZZ-AT when compared with MM-AT (arrows, top panels) ($P < 0.001$) (graph). (C) Sections of lung stained for matured and activated macrophage marker the panmacrophage marker CD68 show significantly increased number of macrophages in ZZ-AT when compared with MM-AT (arrows, top) ($P < 0.001$) (graph). (D) Sections of lung stained for MAC387, a marker of blood-derived macrophages, showing localization to cells present within the parenchymal wall and within the lumen of vessels. Significantly more cells were positive for MAC387 in ZZ-AT when compared with MM-AT (arrows, top) ($P < 0.001$) (graph). (B–D) Although MM-AT COPD lung sections had

Figure 3. (Continued). significantly more monocytes, macrophages, and recently blood-derived macrophages compared with normals ($P < 0.001$ for all). ZZ-AT COPD demonstrated significantly increased numbers of all of these macrophages compared with normals or MM-AT COPD lungs ($P < 0.001$ for all). Note the presence of cells within the alveolar wall and alveolar spaces (arrows). Images are representative of $n = 10$ in each group and are at a magnification of $\times 400$. Values are expressed as mean ($\pm$ SEM). *Normal versus MM-AT or ZZ-AT and **MM-AT versus ZZ-AT.

Figure 4. Cigarette smoke (CS) extract induces nuclear factor (NF)-κB activity in Z variant of antitrypsin (Z-AT) cells. CS extract (12.5%) exposed A549-Z-AT cells had significantly greater NF-κB activity than A549-normal variant AT (M-AT) cells (Z-AT cells vs. M-AT cells at 24 h; $P < 0.001$). Positive control for NF-κB was Jurkat cells. Results presented are from three independent experiments ($n = 3$). Values are expressed as mean $\pm$ SEM. (A) *CS extract M-AT cells versus CS extract Z-AT cells.

compared with M-AT mice ($P = 0.006$ and $P = 0.013$, respectively) (Figures 2C and 2D). There was significant up-regulation of pulmonary *ATF4* mRNA and ATF4 protein in Z-AT mice unlike CS-M-AT mice ($P = 0.021$ and $P = 0.009$, respectively).

Baseline mRNA and protein expression of ATF6 was similar between M and Z transgenic mice controls. ATF6 was significantly induced ($P < 0.001$ for mRNA

and protein) by CS exposure. However, there was no difference in the degree of *ATF6* mRNA or protein induction between M-AT and Z-AT mice ($P = 0.815$ and $P = 0.923$, respectively) (Figures 2E and 2F).

Lung Injury after Acute CS Exposure

Acute CS exposure induced more pronounced lung injury in Z-AT compared with M-AT mice lungs as measured by total

protein in BALF and wet:dry ratio of the lungs ($P = 0.022$ and $P = 0.001$, respectively) (Table 1).

Pulmonary Expression of NF-κB, IL-6, and MCP-1 in Individuals with COPD

Analysis of nuclear protein demonstrated a significantly increased pulmonary NF-κB in the lungs of ZZ-AT COPD compared with MM-AT COPD (1.24-fold increase in ZZ-AT; $P = 0.002$) as established by the fold-difference relative to GAPDH (Table 2). Lung tissue of ZZ-related COPD had a significantly higher IL-6 expression compared with MM-related COPD (10.12-fold increase; $P = 0.005$) (Table 2). Moreover, lung tissue of ZZ-related COPD had a trend toward higher MCP-1 expression compared with MM-related COPD (Table 2).

Pulmonary Expression of ER Stress Genes in Individuals with COPD

There was significantly increased pulmonary expression of ER overload response genes: *PERK* (12.54-fold increase; $P < 0.001$) and *ATF4* in ZZ-related COPD (9.5-fold increase; $P < 0.001$) compared with MM-related COPD (Table 2).

Immunohistochemical Localization for PERK and ATF4 in Individuals with COPD

Immunostaining demonstrated positive staining predominantly within cytoplasm

Table 3: CS Extract Induces Exaggerated Inflammation, Polymeric AT, and Oxidized-Polymeric AT in Z-AT Cells

	A549-M-AT Cells		P Value: Non-CS Extract vs. CS Extract*	A549-Z-AT Cells		P Value		
	Non-CS Extract	CS Extract		Non-CS Extract	CS Extract	Non-CS Extract vs. CS Extract	Non-CS Extract M-AT vs. Non-CS Extract Z-AT	CS Extract M-AT vs. CS Extract Z-AT
Mediators (pg/ml) in supernatant, mean $\pm$ SEM								
TNF-α	14.63 $\pm$ 7.14	40.43 $\pm$ 5.1*	<0.001	30.54 $\pm$ 5.2†	212 $\pm$ 20.71*‡	<0.001	0.039	<0.001
IL-6	12.53 $\pm$ 5.0	96.3 $\pm$ 12.11*	<0.001	20.57 $\pm$ 12	421.37 $\pm$ 20.82*‡	<0.001	0.146	<0.001
MCP-1	1,120 $\pm$ 350	5,329 $\pm$ 706*	<0.001	7,124 $\pm$ 610†	23,564 $\pm$ 1,852*‡	<0.001	<0.001	<0.001
Polymeric Z-AT (ng/ml) at 24 h in cell-model, median (IQR)								
Supernatant	0 (0–0)	0 (0–0)	1.000	0 (0–0)	2,240 (2,360–1,980)*‡	<0.001	1.000	<0.001
Inclusion	0 (0–0)	0 (0–0)	1.000	1,970 (2,220–1,710)†	3,720 (4,710–3,380)*‡	<0.001	<0.001	<0.001
Oxidized-polymeric Z-AT (ng/ml) at 24 h in cell-model, median (IQR)								
Supernatant	0 (0–0)	0 (0–0)	1.000	0 (0–0)	1,910 (2,160–1,750)*‡	<0.001	1.000	<0.001
Inclusion	0 (0–0)	0 (0–0)	1.000	0 (0–0)	3,000 (3,750–2,840)*‡	<0.001	1.000	<0.001

Definition of abbreviations: AT = antitrypsin; CS = cigarette smoke; IQR = interquartile range; M-AT = normal variant AT; MCP = monocyte chemotactic protein; TNF = tumor necrosis factor; Z-AT = Z mutation AT.

*Non-CS extract vs. CS extract.

†Non-CS extract M-AT vs. non-CS extract Z-AT.

‡CS extract M-AT vs. CS extract Z-AT.

Figure 5. Cigarette smoke (CS) extract induces the endoplasmic reticulum (ER) stress in Z variant of antitrypsin (Z-AT) cells. Representative Western blot analysis of whole cell extract on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (A) A polyclonal anti–protein kinase RNA-like ER kinase (PERK) antibody detected significant up-regulated PERK protein (125 kD) in CS extract A549–Z-AT cells compared with their respective non-CS extract Z-AT cells ($P < 0.001$). (B–D) Polyclonal antibodies against activator transcription factor (ATF) 4 protein (38 kD), regulator of G-protein signaling protein 16 protein (RGS16) (predicted at 23 kD and detected at 29 kD) and the ER chaperone calnexin (predicted at 90 kD and detected at 75 kD), respectively, in CS extract A549–Z-AT cells compared with their respective non-CS extract Z-AT cells ($P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively). (E) A polyclonal anti-ATF6 antibody detected ATF6 protein (85 kD) in CS extract exposed both A549–normal variant AT (M-AT) and A549–Z-AT cells

Figure 5. (Continued). compared with their respective non-CS extract A549-Z-AT cells ($P < 0.001$ for both). There was no difference between non-CS extract exposed A549-M-AT and A549-Z-AT controls ($P = 0.795$) or CS extract exposed A549-M-AT and A549-Z-AT cells ($P = 0.912$). Positive control, the ER stress-inducing control agent thapsigargin, treated HeLa cell RNA for ATF4, PERK, and ATF6. Untreated MCF-7 cells constitutively express RGS16 and calnexin (24) ($n = 3$). Band density was determined using ImageJ program and expressed relative to human GAPDH protein. Values are expressed as mean $\pm$ SEM. *Non-CS extract versus CS extract and **M-AT cells versus Z-AT cells. GAPDH = glyceraldehyde phosphate dehydrogenase.

of alveolar epithelial cells for both PERK (Figure 3A, *top*, left to right) and ATF4 (Figure 3A, *middle*, left to right) in all MM-AT and ZZ-AT COPD cases. However, in keeping with the gene expression analysis data, the staining intensity and frequency seemed to be greater in ZZ-AT compared with MM-AT tissue sections for both PERK and ATF4. Some weak staining for ATF4 was noted in control tissues; however, the intensity and frequency was markedly lower compared with COPD tissue.

The most intense staining was seen in alveolar epithelial cells, although weak immunostaining was also noted in some alveolar macrophages of both ZZ-AT and MM-AT COPD tissue sections. Some staining was also present on the apical edge of the bronchiole epithelial cells, but this was more difficult to characterize because only a few bronchioles were present in the sections.

Macrophage Numbers in ZZ-AT and MM-AT COPD Lungs

Immunohistochemistry localization of macrophage phenotypes in MM-AT and ZZ-AT COPD revealed that there were excess monocytes (CD14) (Figure 3B), macrophages (CD68) (Figure 3C), and blood-derived macrophages (MAC387) (Figure 3D) in ZZ-AT COPD lungs compared with MM-AT lungs, showing localization to cells present within the parenchymal wall and within the vessel lumen.

Effect of CS Extract on NF- κ B and AP-1 Activity in Z-AT and M-AT Alveolar Cells

CS extract (12.5%) significantly increased NF- κ B activity at 24 hours in A549-Z-AT cells compared with A549-M-AT cells ($P < 0.001$). CS extract induced NF- κ B activity in A549-M-AT cells at 24 hours was comparable with vehicle A549 cells ($P = 0.715$) (Figure 4).

There were no differences in the up-regulation of AP-1 between A549-Z-AT and A549-M-AT cells (see Figure E5).

CS Extract Induces Excess Inflammation in Z-AT Cells

Baseline TNF- α secretion was higher in A549-Z-AT cells compared with their respective control M-AT cells (2.1-fold increase; $P = 0.039$) (Table 3). CS extract increased TNF- α secretion in A549-Z-AT to a greater extent compared with A549-M-AT cells (5.2-fold increase; $P < 0.001$). CS extract increased production of IL-6 (4.5-fold) and MCP-1 (4.42-fold) to a much greater extent in A549-Z-AT compared with A549-M-AT cells ($P < 0.001$ for both) (Table 3).

CS Extract Induces Excess ER Stress in Z-AT Cells

Western blot demonstrated up-regulation of PERK (125 kD), ATF4 (38 kD), RGS16

(29 kD), and calnexin (75 kD) proteins in control A549-Z-AT cells compared with A549-M-AT control ($P < 0.001$ for all) (Figures 5A–5D). CS extract resulted in a further increase in PERK, ATF4, RGS16, and calnexin proteins in A549-Z-AT cells compared with A549-M-AT cells ($P < 0.001$, $P = 0.012$, $P = 0.001$, and $P < 0.001$, respectively).

After CS exposure expression of ATF6 protein (85 kD) was significantly up-regulated in both M-AT and Z-AT mice compared with their respective non-CS-exposed mice ($P < 0.001$ for both) (Figure 5E). However, the level of ATF6 protein expression in CS-M-AT mice was comparable with CS-Z-AT mice ($P = 0.912$).

CS Extract Induces Formation of Intracellular Oxidized Polymers of Z-AT

There was significant retention of polymeric Z-AT as inclusion bodies in Z-AT cells (Table 3). Exposure to CS extract caused a significant increase in inclusion body polymeric Z-AT compared with control Z-AT cells ($P < 0.001$). These data were confirmed on Western blot (Figure 6). Polymeric AT was not detected in A549-M-AT cells (Table 3).

More detailed analysis found that the inclusion body Z-AT induced by CS extract were oxidized polymers of Z-AT (oxidized-polymeric Z-AT) (Table 3, Figure 6). Monomeric oxidized AT alone

was detected in the CS extract-exposed M-AT cell supernatant (Figure 6).

Effect of a Polymerization Inhibitor and an Antioxidant on Z-AT-associated Cellular Activity

Inhibitor of polymerization. Treatment with the inhibitor of polymerization, 4M, prevented CS extract-induced formation of oxidized-polymeric Z-AT in inclusions of A549-Z-AT cells ($P < 0.001$) (Table 4). The residual Z-AT monomer remaining was oxidized (*see* Figure E6). Treatment with 4M significantly reduced up-regulation of *PERK*, *ATF4*, *RGS16*, and *calnexin* mRNA in control and CS

Polyclonal anti-human AT antibody

Monoclonal anti-Ox-AT antibody

Figure 6. Cigarette smoke (CS) extract induces formation of intracellular polymeric Z variant of antitrypsin (Z-AT) in Z-AT cells. (Top) Representative Western blot on 7.5% nondenaturing polyacrylamide gel electrophoresis using antihuman AT. Monomeric AT was detected in non-CS extract or CS extract-exposed A549-normal variant AT (M-AT) cell supernatant, and in the supernatant of non-CS extract A549-Z-AT cells. Monomeric Z-AT and polymeric Z-AT were detected in inclusion bodies of non-CS extract A549-Z-AT cells and in the supernatant and inclusion bodies of CS extract A549-Z-AT cells. (Bottom) Representative Western blot using a monoclonal antioxidized AT antibody. This detected monomeric oxidized AT in CS extract A549-M-AT cell supernatant, and both oxidized AT and oxidized-polymeric Z-AT in the supernatant and inclusion bodies of CS extract A549-Z-AT cells. (Top and bottom) Protein loading was equalized for 150 ng of total AT per lane. Oxidized AT and oxidized-polymeric AT were prepared by oxidizing plasma purified native AT or polymer AT, respectively, using *N*-chlorosuccinimide oxidizing agent.

Table 4: The Effect of Inhibition of Polymerization and an Antioxidant in Z-AT Cells

	A549-Z-AT Cells			P Value: CS Extract vs. CS Extract + 4M	A549-Z-AT Cells: CS Extract + NAC	P Value	
	Non-CS Extract	CS Extract	CS Extract + 4M			CS Extract vs. CS Extract + NAC	CS Extract + 4M vs. CS Extract + NAC
Inclusion oxidized-polymeric Z-AT at 24 h, ng/ml, median (IQR)	0 (0–0)	2,971 (3,910–2,660)	0 (0–0)*	<0.001	0 (0–0) [†]	<0.001	1.000
NF-κB activity, O.D. at 450 nm, mean ± SEM	0.354 ± 0.1	1.053 ± 0.105	0.654 ± 0.107*	<0.001	0.384 ± 0.105 ^{††}	<0.001	0.043
Mediators in supernatant, pg/ml, mean ± SEM							
TNF-α	39.54 ± 15	212 ± 23.7	93.23 ± 20.4*	<0.001	29.8 ± 10 ^{††}	<0.001	0.021
IL-6	18.67 ± 20.7	421.37 ± 20.82	151.7 ± 15.4*	<0.001	45.7 ± 22.85 ^{††}	<0.001	0.028
MCP-1	975 ± 530	19,758 ± 2,559	10,356 ± 1,750*	<0.001	2,234 ± 556 ^{††}	<0.001	<0.001

Definition of abbreviations: AT = antitrypsin; CS = cigarette smoke; IQR = interquartile range; MCP = monocyte chemotactic protein; NAC = N-acetylcysteine; NF-κB = nuclear factor kappa B; TNF = tumor necrosis factor; Z-AT = Z mutation AT.

*CS extract vs. CS extract + 4M.

[†]CS extract vs. CS extract + NAC.

^{††}CS extract + 4M vs. CS extract + NAC.

extract-treated A549-Z-AT cells ($P < 0.001$ for all) (Figures 7A–7D).

Treatment with 4M had no effect on the CS extract-induced up-regulation of *ATF6* ($P = 0.925$) in A549-Z-AT cells (Figure 7E). Treatment with 4M also inhibited the increased NF-κB, TNF-α, IL-6, and MCP-1 in CS extract-exposed A549-Z-AT cells ($P < 0.001$ for all) (Table 4).

Oxidant inhibition. Treatment with NAC prevented the ER accumulation of oxidized-polymeric Z-AT in A549-Z-AT cells ($P < 0.001$) (Table 4). CS extract-associated up-regulation of *PERK*, *ATF4*, *RGS16*, and *calnexin* mRNA in A549-Z-AT cells was significantly attenuated by NAC ($P < 0.001$ for all) (Figures 7A–7D).

Treatment with NAC significantly reduced CS extract-induced up-regulation of *ATF6* ($P < 0.001$) in A549-Z-AT cells (Figure 7E). NAC also significantly reduced CS extract-induced NF-κB, production of TNF-α, IL-6, and MCP-1 in A549-Z-AT cells ($P < 0.001$ for all) (Table 4).

Effect of NAC Compared with 4M

The accumulation of oxidized-polymeric Z-AT in inclusion bodies was completely abrogated by both 4M and NAC (Table 4). Similarly, 4M and NAC were equipotent with respect to preventing *PERK*, *ATF4*, *RGS16*, and *calnexin* activation in A549-Z-AT cells exposed to CS extract ($P = 0.724$, $P = 0.913$, $P = 0.819$, and

$P = 0.956$, respectively) (Figures 7A–7D). Although 4M had no effect on *ATF6* expression ($P = 0.952$), NAC was a potent inhibitor of *ATF6* expression in Z-AT cells (Figure 7E). NAC was also a more potent inhibitor of the Z-AT-associated exaggerated inflammatory response than 4M (NF-κB, $P = 0.043$; TNF-α, $P = 0.021$; IL-6, $P = 0.028$; MCP-1, $P < 0.001$) (Table 4).

The Effect of PERK Inhibitor I on Cellular Activation in Z-AT Cells

The *PERK* inhibitor I had no effect on formation of polymeric Z-AT (see Table E3). This inhibitor, however, prevented the activation of *ATF4*, *RGS16*, and *calnexin*

Table 5: The Effect of *PERK* Inhibitor I on Inflammatory Response in Z-AT Cells

	A549-Z-AT Cells			P Value: CS Extract + DMSO vs. CS Extract + <i>PERK</i> Inhibitor I (GSK2606414)
	Non-CS Extract	CS Extract + DMSO	CS Extract + <i>PERK</i> Inhibitor I (GSK2606414)	
NF-κB activity, O.D. at 450 nm, mean ± SEM	0.307 ± 0.09	1.124 ± 0.15	0.579 ± 0.103*	<0.001
Mediators in supernatant, pg/ml, mean ± SEM				
TNF-α	33.56 ± 15.5	219.5 ± 20.7	96.8 ± 16.5*	0.013
IL-6	13.5 ± 5.6	314.6 ± 35.7	154.2 ± 16.5*	0.015
MCP-1	1,550 ± 752	21,285 ± 3,050	11,452 ± 1,621*	<0.001

Definition of abbreviations: AT = antitrypsin; CS = cigarette smoke; DMSO = dimethyl sulfoxide; IQR = interquartile range; MCP = monocyte chemotactic protein; NF-κB = nuclear factor kappa B; *PERK* = protein kinase RNA-like endoplasmic reticulum kinase; TNF = tumor necrosis factor; Z-AT = Z mutation AT.

*CS extract + DMSO vs. CS extract + *PERK* inhibitor I.

Figure 7. The effect of inhibition of polymerization and an antioxidant. (A–D) Cigarette smoke (CS) extract significantly up-regulated expression of human protein kinase RNA-like endoplasmic reticulum (ER) kinase (*PERK*), activator transcription factor (*ATF*) 4, regulator of G-protein signaling protein 16 (*RGS16*), and *calnexin* mRNA in A549–Z variant of antitrypsin (Z-AT) cells ($P < 0.001$ for all). Inhibitor of polymerization (4M, 20 μ g) and *N*-acetylcysteine (NAC, 10 mM) independently significantly inhibited CS extract-induced human *PERK*, *ATF4*, *RGS16*, and *calnexin* mRNA ($P < 0.001$ for all). (E) CS extract-induced up-regulation of *ATF6* mRNA in A549–Z-AT cells was unaffected by 4M ($P = 0.925$). However, the antioxidant NAC significantly reduced CS extract-induced *ATF6* expression in A549–Z-AT cells ($P < 0.001$). Positive control, the ER stress-inducing control agent thapsigargin treated HeLa cell

Figure 7. (Continued). RNA for PERK, ATF4, and ATF6. Untreated MCF-7 cells constitutively express RGS16 and calnexin. Band density was determined using ImageJ program and expressed relative to human GAPDH mRNA. $n = 3$. Values are expressed as mean $\pm$ SEM. *Non-CS extract normal variant AT (M-AT) cells versus non-CS extract Z-AT cells, **non-CS extract Z-AT cells versus CS extract Z-AT cells, and ***CS extract Z-AT cells versus CS extract 4M or + *N*-acetylcysteine. GAPDH = glyceraldehyde phosphate dehydrogenase.

protein ($P = 0.021$, $P < 0.001$, and $P < 0.001$, respectively) (Figures 8A–8C). PERK inhibitor I had no effect in the CS extract-induced expression of ATF6 protein, which was comparable with CS extract-exposed A549-Z-AT cells ($P = 0.935$) (Figure 8D). PERK inhibitor I also significantly reduced NF- κ B activation ($P < 0.001$) and TNF- α

($P = 0.013$), IL-6 ($P = 0.015$), and MCP-1 ($P < 0.001$) in CS-exposed A549-Z-AT cells (Table 5).

Assessment in Primary Lung Epithelial Cells

Primary NHBE cells transfected with the human Z-AT gene (primary NHBE-Z-AT cells) when exposed to CS extract were

also found to accumulate oxidized-polymeric Z-AT in inclusion bodies ($P < 0.001$) (Table 6). This was associated with significant up-regulation of PERK, ATF4, RGS16, and calnexin proteins ($P < 0.001$, $P < 0.001$, $P = 0.042$, and $P = 0.036$, respectively) (Figures 9A–9D). CS extract-induced accumulation of oxidized-polymeric Z-AT in inclusion bodies of

Table 6: The Effect of Inhibition of Polymerization and an Antioxidant in Primary NHBE-Z-AT Cells

	Primary NHBE-Z-AT Cells		<i>P</i> Value: Non-CS Extract vs. CS Extract	Primary NHBE-Z-AT Cells: CS Extract + 4M	<i>P</i> Value: CS Extract vs. CS Extract + 4M	Primary NHBE-Z-AT Cells: CS Extract + NAC	<i>P</i> Value	
	Non-CS Extract	CS Extract					CS Extract vs. CS Extract + NAC	CS Extract + 4M vs. CS Extract + NAC
Inclusion oxidized-polymeric Z-AT at 24 h, ng/ml, median (IQR)	0 (0–0)	2,924 (2,993–2,581) *	<0.001	0 (0–0) [†]	<0.001	0 (0–0) [‡]	<0.001	1.000
NF- κ B activity, O.D. at 450 nm, mean $\pm$ SEM	0.375 $\pm$ 0.1	1.245 $\pm$ 0.2*	<0.001	0.708 $\pm$ 0.1 [†]	<0.001	0.297 $\pm$ 0.04 ^{‡§}	<0.001	0.004
Mediators in supernatant, pg/ml, mean $\pm$ SEM								
TNF- α	36 $\pm$ 10	198 $\pm$ 20*	<0.001	80 $\pm$ 10 [†]	<0.001	32 $\pm$ 13 ^{‡§}	<0.001	0.041
IL-6	21 $\pm$ 5	32 $\pm$ 7.3*	<0.001	160 $\pm$ 20 [†]	<0.001	32 $\pm$ 10 ^{‡§}	<0.001	0.009
MCP-1	4,381 $\pm$ 900	19,220 $\pm$ 310*	<0.001	12,542 $\pm$ 1,712 [†]	<0.001	6,483 $\pm$ 1,810 ^{‡§}	0.031	<0.001

Definition of abbreviations: AT = antitrypsin; CS = cigarette smoke; IQR = interquartile range; MCP = monocyte chemotactic protein; NF- κ B = nuclear factor kappa B; NAC = *N*-acetylcysteine; NHBE = normal human bronchial epithelial; PERK = protein kinase RNA-like endoplasmic reticulum kinase; TNF = tumor necrosis factor; Z-AT = Z mutation AT.

*Non-CS extract vs. CS extract.

[†]CS extract vs. CS extract + 4M.

[‡]CS extract vs. CS extract + NAC.

[§]CS extract + 4M vs. CS extract + NAC.

Figure 8. The effect of protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) inhibitor I on ER stress in Z variant of antitrypsin (Z-AT) cells. (A–C) Pretreatment of A549-Z-AT cells with the PERK inhibitor I (2 μ g) prevented cigarette smoke (CS) extract-induced up-regulated expression of (A) activator transcription factor (ATF) 4 protein (38 kD) ($P = 0.021$), which was comparable with non-CS extract A549-Z-AT cells ($P = 0.439$), (B) regulator of G-protein signaling protein 16 protein (RGS16) (29 kD) ($P < 0.001$), and (C) calnexin protein (75 kD) ($P < 0.001$). (D) Pretreatment of A549-Z-AT cells with the PERK inhibitor I did not prevent CS extract-induced up-regulated expression of ATF6 protein (85 kD) ($P = 0.935$). Vehicle DMSO (0.03%) had no effect compared with CS extract. Band density was determined using the ImageJ program and expressed relative to human GAPDH protein. Positive control, the ER stress-inducing control agent thapsigargin treated HeLa cell RNA for PERK, ATF4, and ATF6. Untreated MCF-7 cells constitutively express RGS16 and calnexin. $n = 3$. Values are expressed as mean $\pm$ SEM. *CS extract versus PERK inhibitor I + CS extract. DMSO = dimethyl sulfoxide; GAPDH = glyceraldehyde phosphate dehydrogenase.

Figure 9. *N*-Acetylcysteine (NAC) inhibited cigarette smoke (CS) extract induced endoplasmic reticulum (ER) overload response in primary normal human bronchial epithelial cells transfected with human Z variant of antitrypsin (Z-AT) gene. (A–D) In primary normal human bronchial epithelial (primary NHBE) cells transfected with human Z-AT (primary NHBE-Z-AT cells) CS extract significantly induced up-regulation of (A) protein kinase RNA-like ER kinase (PERK) protein (125 kD), (B) activator transcription factor (ATF) 4 protein (38 kD), (C) regulator of G-protein signaling protein 16 protein (RGS16) (29 kD), and (D) calnexin protein (75 kD) ($P < 0.001$, $P < 0.001$, $P = 0.042$, and $P = 0.036$, respectively). Inhibitor of polymerization (4M, 20 μ g) and NAC (10 mM) independently significantly inhibited CS extract-induced human PERK, ATF4, RGS16, and calnexin proteins ($P < 0.001$ for all). (E) CS extract induced up-regulation of ATF6 protein (85 kD) in primary NHBE-Z-AT cells was unaffected by 4M ($P = 0.789$). However, the antioxidant NAC significantly reduced CS extract-induced ATF6 expression in NHBE-Z-AT cells ($P < 0.001$). Band density was determined using the ImageJ program and expressed relative to human GAPDH protein. Positive control, the ER stress inducing control agent thapsigargin treated HeLa cell RNA for ATF4, PERK, and ATF6. Untreated MCF-7 cells constitutively express RGS16 and calnexin. $n = 3$. Values are expressed as media. *Non-CS extract primary NHBE cells versus non-CS extract primary NHBE-Z-AT cells, **non-CS extract primary NHBE-Z-AT cells versus CS extract primary NHBE-Z-AT cells, and ***CS extract primary NHBE-Z-AT cells versus CS extract primary NHBE-Z-AT cells + *N*-acetylcysteine or 4M. GAPDH = glyceraldehyde phosphate dehydrogenase.

Figure 9. (Continued).

primary NHBE-Z-AT cells was also associated with significantly increased NF- κ B activity ($P < 0.001$) and increased production of TNF- α , IL-6, and MCP-1 ($P < 0.001$ for all) (Table 6). Expression of ATF6 protein was also increased in CS extract-exposed NHBE-Z-AT cells (Figure 9E).

Treatment with 4M and NAC independently abrogated CS extract-induced ER accumulation of oxidized-polymeric Z-AT in inclusions ($P < 0.001$ for both). 4M and NAC also inhibited the inflammatory and ER stress response in primary

NHBE-Z-AT cells: PERK, ATF4, RGS16, calnexin, NF- κ B, TNF- α , IL-6, MCP-1 activity (NAC, $P < 0.001$ for all; for 4M, PERK, ATF4, RGS16, calnexin, NF- κ B, TNF- α , IL-6, $P < 0.001$ for all; and $P = 0.031$ for MCP-1) (Table 6, Figures 9A–9D). 4M had no effect in the CS extract-induced expression of ATF6 protein, which was comparable with CS extract-exposed primary NHBE-Z-AT cells ($P = 0.798$) (Figure 9E). However, the antioxidant NAC significantly reduced CS extract-induced expression of ATF6 protein ($P < 0.001$) (Figure 9E).

Effect of PERK Inhibitor I on Cellular Activation in Primary NHBE-Z-AT Cells

PERK inhibitor I had no effect on formation of polymeric Z-AT in primary NHBE-Z-AT cells (see Table E3). The PERK inhibitor I prevented the activation of ATF4 ($P = 0.007$), RGS16, and calnexin protein ($P < 0.001$ for both) (Figures 10A–10C). PERK inhibitor I had no effect in the CS extract-induced expression of ATF6 protein, which was comparable with CS extract-exposed primary NHBE-Z-AT cells ($P = 0.916$) (Figure 10D). PERK inhibitor I

Table 7: The Effect of PERK Inhibitor I on Inflammatory Response in Primary NHBE-Z-AT Cells

	Primary NHBE-Z-AT Cells			<i>P</i> Value: CS Extract + DMSO vs. CS Extract + PERK Inhibitor I (GSK2606414)
	Non-CS Extract	CS Extract + DMSO	CS Extract + PERK Inhibitor I (GSK2606414)	
NF- κ B activity, O.D. at 450 nm, mean $\pm$ SEM	0.395 $\pm$ 0.04	1.113 $\pm$ 0.08	0.675 $\pm$ 0.05*	0.016
Mediators in supernatant, pg/ml, mean $\pm$ SEM				
TNF- α	18 $\pm$ 13	190 $\pm$ 18	770 $\pm$ 17*	0.005
IL-6	120 $\pm$ 6	323 $\pm$ 12	146 $\pm$ 9*	<0.001
MCP-1	980 $\pm$ 630	18,360 $\pm$ 3,660	10,370 $\pm$ 1,012*	0.018

Definition of abbreviations: AT = antitrypsin; CS = cigarette smoke; DMSO = dimethyl sulfoxide; IQR = interquartile range; MCP = monocyte chemoattractant protein; NF- κ B = nuclear factor kappa B; NAC = *N*-acetylcysteine; NHBE = normal human bronchial epithelial; PERK = protein kinase RNA-like endoplasmic reticulum kinase; TNF = tumor necrosis factor; Z-AT = Z mutation AT.

*CS extract + DMSO vs. CS extract + PERK inhibitor I (GSK2606414).

Figure 10. The effect of protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) inhibitor I on ER overload response in primary normal human bronchial epithelial (NHBE)-Z variant of antitrypsin (Z-AT) cells. (A–C) Pretreatment of primary NHBE-Z-AT cells with the PERK inhibitor I (2 μ g) prevented cigarette smoke (CS)-extract induced up-regulated expression of (A) activator transcription factor (ATF) 4 protein (38 kD) ($P = 0.007$), which was comparable with non-CS extract NHBE-Z-AT cells ($P = 0.617$), (B) regulator of G-protein signaling protein 16 (RGS16) (29 kD) ($P < 0.001$), and (C) calnexin protein (75 kD) ($P < 0.001$). (D) Pretreatment of NHBE-Z-AT cells with the PERK inhibitor I did not prevent CS extract-induced up-regulated expression of ATF6 protein (85 kD) ($P = 0.916$). Band density was determined using the ImageJ program and expressed relative to human GAPDH protein. Positive control, the ER stress-inducing control agent thapsigargin treated HeLa cell RNA for ATF4, PERK, and ATF6. Untreated MCF-7 cells constitutively express RGS16 and calnexin. $n = 3$. Values are expressed as mean $\pm$ SEM. *CS extract versus PERK inhibitor I + CS extract. DMSO = dimethyl sulfoxide; GAPDH = glyceraldehyde phosphate dehydrogenase.

Figure 11. Schematic diagram showing the mechanisms contributing to the development of lung disease of ZZ-AT homozygotes. The *left side* of the diagram details our new findings; the *right side* (separated by the *dashed line*) shows what is already established in the study of α_1 -antitrypsin (AT) deficiency. Severe deficiency of AT is the main predisposing factor to emphysema. In addition, there is baseline intracellular polymerization and aggregation in alveolar epithelial cells, which activates endoplasmic reticulum (ER) stress. Oxidants from cigarette smoke (CS) and inflammatory cells potentiate this process by accelerating the formation of oxidized-polymeric Z-AT, which in turn activates protein kinase RNA-like ER kinase (PERK)-dependent nuclear factor (NF)- κ B production of inflammatory mediators contributing to lung damage. Confirmation of this pathway is provided by the interruption of this process by (1) targeting the structural differences in Z-AT by directly preventing polymerization (with 4M), (2) by inhibiting oxidant-mediated acceleration of polymerization with an antioxidant (*N*-acetylcysteine), and (3) inhibiting PERK with the PERK inhibitor I (GSK2606414). CS-induced ER overload response markers; PERK, activator transcription factor (ATF) 4, and regulator of G-protein signaling protein 16 (RGS16), and the ER chaperone calnexin could be inhibited below the expression level of non-CS exposed Z-AT cells independently by both the inhibitor of Z-AT polymerization, 4M or an antioxidant, *N*-acetylcysteine (NAC). Diagram also shows the previously reported effect of oxidants on inducing polymerization of extracellular Z-AT in plasma and lung, further resulting in reduced inhibitory activity of Z-AT and an effect on neutrophil chemotaxis (17, 20).

also significantly reduced NF- κ B ($P = 0.016$), production of TNF- α ($P = 0.005$), IL-6 ($P < 0.001$), and MCP-1 ($P = 0.018$) in CS extracted-exposed primary NHBE-Z-AT cells (Table 7).

Discussion

Despite the uniformly severe plasma deficiency found in ZZ-AT, there

is significant variability in the expression of the lung disease (i.e., nonsmokers with ZZ-AT deficiency can develop emphysema or can be asymptomatic and the risk of developing disease is greatly increased by smoking and environmental pollutants) (13–15). This suggests the importance of additional factors that influence and/or mediate disease expression. Some reports have indicated excess of pulmonary inflammation in

ZZ-AT, but this remains poorly characterized. To explore these issues, we first exposed transgenic mice for Z-AT and M-AT to CS.

TNF- α , IL-6, and CCL2 have been implicated in the pathobiology of COPD (12, 30–32). In response to CS, Z-AT mice produced higher amounts of pulmonary TNF- α and IL-6 relative to M-AT. Although CS had no effect on CCL2 (*JE*) mRNA at 24 hours in murine lung

homogenates, JE BALF protein concentrations were significantly elevated. This dichotomy is likely to be caused by the fact that JE is an early response gene (33), and as such RNA expression of JE is most likely to have normalized by 24 hours.

This excess of lung inflammation also correlated with greater lung injury in Z-AT compared with M-AT mice. As a matter of fact, the finding of increased lung macrophages and neutrophils in our model supports previous data in a different background strain of mice (20). The observation that acute exposure to CS depleted lymphocytes from BALF in M-AT and Z-AT mice is unexplained yet is in agreement with previous findings published (34).

We particularly wanted to test the relevance of these findings in human lung tissue. However, the scarcity of appropriate tissue samples and the fact that the inflammatory mediators are subject to multiple influences, such as duration and intensity of tobacco smoke exposure and environmental contaminants, makes analysis of human lung tissue in COPD challenging. Nevertheless, there was a clear trend toward increased inflammatory cytokines and macrophages consisting of monocytes, macrophages, and recently infiltrating monocytes and macrophages in ZZ-AT with advanced COPD compared with MM-AT COPD.

This finding is supported by previous reports of excess neutrophils in ZZ-AT lungs (16–18) and thus our human data, although not alone confirmatory, do provide clinical support for our murine data. Collectively, our data strongly point to dysregulated inflammation in ZZ-AT in response to CS and in established COPD.

Our findings show that when compared with M-AT, Z-AT mice exposed to acute CS had excessive ER stress, displaying up-regulation of PERK, ATF4, and NF- κ B. Our further studies using ZZ-AT COPD lung clearly demonstrated that Z-AT lung tissue has an excess of ER stress proteins, PERK and ATF4 localized predominantly in the alveolar epithelial cells. Interestingly, the distribution of PERK and ATF4 was similar to that of polymeric Z-AT in lung tissue (18). Therefore, we hypothesized that polymeric Z-AT may contribute to the inflammatory burden in ZZ-AT by inducing ER stress in epithelial cells.

To probe this hypothesis, we assessed the properties of Z-AT expressing

pulmonary epithelial cells. These cells when exposed to CS were found to produce greater inflammatory cytokines compared with M-AT cells. It is well established that Z-AT protein accumulates in the ER of hepatocytes, which stimulates the ER overload response including NF- κ B and RGS16 (9, 35–38). This form of ER stress is particularly specific for ZZ-AT (9, 35–39), but until now has not been studied in relation to lung disease. Unprovoked Z-AT lung epithelial cells had increased expression of ER overload response genes *PERK*, *ATF4*, *RGS16*, and *NF- κ B* and the ER chaperone *calnexin* compared with M-AT cells, which increased still further in response to CS exposure. PERK and ATF4 are also involved in the unfolded protein response (UPR), hence we also measured another part of the UPR, ATF6, which is not involved in the ER overload response. ATF6 was increased to an equivalent degree in CS treated in M-AT and Z-AT cells. In CS-treated Z-AT cells it is likely to be caused by the induction of UPR by CS (40). Hence, the finding of specific elevation of ER overload proteins in CS Z-AT cells and the lack of increase in ATF6 in CS-treated Z-AT cells in comparison with CS-treated M-AT cells suggests that the ER overload response is activated in Z-AT cells compared with M-AT cells. After CS exposure there was significant intracellular accumulation of polymeric Z-AT. The 4M peptide directly interferes with deviant intermolecular linkage between the reactive loop of one Z-AT molecule and β -sheet A of another Z-AT molecule to inhibit Z-AT polymerization in cells (24, 28). Inhibiting Z-AT polymerization markedly reduced the ER overload response (RGS16) and expression of the ER chaperone calnexin, but not the UPR (ATF6). Inhibiting Z-AT polymerization also markedly reduced CS-exposed induced proinflammatory cytokine production, thus providing direct evidence for the role of intracellular polymers in the proinflammatory phenotype of Z-AT lung epithelial cells. The interaction between oxidants and the formation of intracellular polymer aggregates has not been studied to date. After CS exposure there was significant intracellular accumulation of oxidized-polymeric Z-AT, contrasting with the significantly lesser amount of nonoxidized polymers in unstimulated Z-AT cells. Furthermore, the antioxidant NAC was able to inhibit intracellular oxidized polymers and in so-doing reduce

the ER overload response and proinflammatory cytokine production. PERK is a key sensor of ER stress (39, 41). PERK inhibitor I prevented the activation of ATF4, RGS16, and calnexin protein and inflammatory cytokines after CS exposure in Z-AT cells, thus confirming the pathway by which intracellular polymers exert their proinflammatory effect. In comparison with 4M and PERK inhibitor I, NAC was a more potent inhibitor of the CS-induced exaggerated inflammatory response in Z-AT cells. This is likely to be caused by the ability of NAC to inhibit proinflammatory effects of oxidants from CS independent and additional to its ability to inhibit CS-induced Z-AT aggregation (Figure 11) (32, 42).

Our findings suggest that although there is spontaneous formation of polymers in Z-AT epithelial cells that activates ER stress and the inflammatory response, oxidative stress was able to greatly accelerate this intracellular process by a “second hit” (i.e., a gene-environment interaction). In keeping with other published data, it is likely that oxidative stress generated from free radicals released from activated phagocytes would have a similar effect (20, 43, 44). We speculate that this proinflammatory process may also occur in other cells, such as pancreatic, endothelial, and monocytes, and also contribute to panniculitis and systemic vasculitis associated with ZZ-AT (45, 46). The effects described are directly related to the accumulation of aggregated protein; as such the rate of clearance by processes, such as autophagy and nonproteasomal degradation, could modify the phenotype (47–49). Indeed, a significant interplay seems to exist between Z-AT protein and oxidative stress in Z-AT-related COPD. Interestingly, the outcome of this interaction varies depending on the compartment in which polymerization occurs, i.e., extracellular polymers result in reduced antielastase function and neutrophil chemotaxis (17, 20), whereas the polymerization in alveolar cells results in ER stress and proinflammatory cytokine production. The combined effect of these processes would be to exacerbate emphysema development (Figure 11). We speculate that in light of the greater extent of cellular activation in ZZ-AT COPD lungs in comparison with the low concentrations of extracellular polymers, the intracellular effects of polymers identified in this study

are likely to be of greater clinical significance.

Our findings in human tissue revealed that most staining of PERK and ATF4 was predominantly in the alveolar epithelial cells. However, there was some staining for PERK and ATF4 in macrophages, which is also likely to contribute to excess inflammation in ZZ-AT-related COPD (50). COPD is usually the result of repeated acute CS exposure, which results in chronic disease. Our data in humans exposed to chronic CS resulting in emphysema do provide some support that the acute smoking model replicates some of the features of chronic smoke

exposure. However, in this study, we have not directly explored the effect of chronic CS exposure and oxidative stress or the interaction with the UPR and other mediators induced by CS-induced oxidation, such as NF-erythroid 2 related factor 2 and histone deacetylases (51, 52). These are important areas for future research.

In conclusion, our data show for the first time that oxidants accelerate self-aggregation and accumulation of Z-AT in lung epithelial cells. In so doing, this aggregation activates the ER overload response and PERK-dependent NF- κ B-mediated exaggerated cytokine-driven

inflammation. Our findings reveal an additional pathway of cellular injury in ZZ-AT individuals, thus providing support for the inflammatory nature of lung disease in these individuals. Furthermore, our findings support further *in vitro* studies with inhibitors of polymerization and antioxidants before considering studies *in vivo* as an adjunct to AT augmentation therapy. ■

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References

- Stoller JK, Aboussouan LS. A review of α 1-antitrypsin deficiency. *Am J Respir Crit Care Med* 2012;185:246–259.
- Eriksson S. Studies in α 1-antitrypsin deficiency. *Acta Med Scand Suppl* 1965;432:1–85.
- Blanco I, de Serres FJ, Fernandez-Bustillo E, Lara B, Miravittles M. Estimated numbers and prevalence of P1'S and P1'Z alleles of alpha1-antitrypsin deficiency in European countries. *Eur Respir J* 2006;27:77–84.
- de Serres FJ, Blanco I, Fernández-Bustillo E. Genetic epidemiology of alpha-1 antitrypsin deficiency in North America and Australia/New Zealand: Australia, Canada, New Zealand and the United States of America. *Clin Genet* 2003;64:382–397.
- Lomas DA, Evans DL, Finch JT, Carrell RW. The mechanism of Z alpha 1-antitrypsin accumulation in the liver. *Nature* 1992;357:605–607.
- Alam S, Mahadeva R. Cellular consequences of Z alpha-1 antitrypsin accumulation and prospects for therapy. *EMJ Hepatol* 2013;1:44–51.
- Teckman JH. Liver disease in alpha-1 antitrypsin deficiency: current understanding and future therapy. *COPD* 2013;10:35–43.
- Perlmutter DH. Liver injury in alpha1-antitrypsin deficiency: an aggregated protein induces mitochondrial injury. *J Clin Invest* 2002;110:1579–1583.
- Perlmutter DH. Pathogenesis of chronic liver injury and hepatocellular carcinoma in alpha-1-antitrypsin deficiency. *Pediatr Res* 2006;60:233–238.
- Laurell CB, Eriksson S. The electrophoretic alpha-1-globulin pattern of serum in alpha-1 antitrypsin deficiency. *Scand J Clin Lab Invest* 1963;15:132–140.
- Snider GL, Lucey EC, Stone PJ. Pitfalls in antiprotease therapy of emphysema. *Am J Respir Crit Care Med* 1994;150:S131–S137.
- Stockley RA. Cellular and biochemical mechanisms in chronic obstructive pulmonary disease. In: Calverley P, Pride N, editors. *Chronic obstructive pulmonary disease*. London: Chapman and Hall; 1995. pp. 93–133.
- Silverman EK, Palmer LJ, Mosley JD, Barth M, Senter JM, Brown A, Drazen JM, Kwiatkowski DJ, Chapman HA, Campbell EJ, et al. Genomewide linkage analysis of quantitative spirometric phenotypes in severe early-onset chronic obstructive pulmonary disease. *Am J Hum Genet* 2002;70:1229–1239.
- Seersholm N, Kok-Jensen A, Dirksen A. Decline in FEV1 among patients with severe hereditary alpha 1-antitrypsin deficiency type P1Z. *Am J Respir Crit Care Med* 1995;152:1922–1925.
- DeMeo DL, Campbell EJ, Brantly ML, Barker AF, Eden E, McElvaney NG, Rennard SI, Stocks JM, Stoller JK, Strange C, et al. Heritability of lung function in severe alpha-1 antitrypsin deficiency. *Hum Hered* 2009;67:38–45.
- Morrison HM, Kramps JA, Burnett D, Stockley RA. Lung lavage fluid from patients with alpha 1-proteinase inhibitor deficiency or chronic obstructive bronchitis: anti-elastase function and cell profile. *Clin Sci (Lond)* 1987;72:373–381.
- Mulgrew AT, Taggart CC, Lawless MW, Greene CM, Brantly ML, O'Neill SJ, McElvaney NG. Z alpha1-antitrypsin polymerizes in the lung and acts as a neutrophil chemoattractant. *Chest* 2004;125:1952–1957.
- Mahadeva R, Atkinson C, Li Z, Stewart S, Janciauskiene S, Kelley DG, Parmar J, Pitman R, Shapiro SD, Lomas DA. Polymers of Z alpha1-antitrypsin co-localize with neutrophils in emphysematous alveoli and are chemotactic *in vivo*. *Am J Pathol* 2005;166:377–386.
- Hill AT, Bayley DL, Campbell EJ, Hill SL, Stockley RA. Airways inflammation in chronic bronchitis: the effects of smoking and alpha1-antitrypsin deficiency. *Eur Respir J* 2000;15:886–890.
- Alam S, Li Z, Janciauskiene S, Mahadeva R. Oxidation of Z α 1-antitrypsin by cigarette smoke induces polymerization: a novel mechanism of early-onset emphysema. *Am J Respir Cell Mol Biol* 2011;45:261–269.
- Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277:2002–2004.
- Ali R, Perfumo S, della Rocca C, Amicone L, Pozzi L, McCullagh P, Millward-Sadler H, Edwards Y, Povey S, Tripodi M. Evaluation of a transgenic mouse model for alpha-1-antitrypsin (AAT) related liver disease. *Ann Hum Genet* 1994;58:305–320.
- Li Z, Alam S, Wang J, Sandstrom CS, Janciauskiene S, Mahadeva R. Oxidized alpha1-antitrypsin stimulates the release of monocyte chemoattractant protein-1 from lung epithelial cells: potential role in emphysema. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L388–L400.
- Alam S, Wang J, Janciauskiene S, Mahadeva R. Preventing and reversing the cellular consequences of Z alpha-1 antitrypsin accumulation by targeting s4A. *J Hepatol* 2012;57:116–124.
- Theophile K, Jonigk D, Kreipe H, Bock O. Amplification of mRNA from laser-microdissected single or clustered cells in formalin-fixed and paraffin-embedded tissues for application in quantitative real-time PCR. *Diagn Mol Pathol* 2008;17:101–106.
- Jonigk D, Golpon H, Bockmeyer CL, Maegel L, Hoepfer MM, Gottlieb J, Nickel N, Hussein K, Maus U, Lehmann U, et al. Plexiform lesions in pulmonary arterial hypertension composition, architecture, and microenvironment. *Am J Pathol* 2011;179:167–179.
- Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005;5:953–964.
- Chang YP, Mahadeva R, Chang WS, Lin SC, Chu YH. Small-molecule peptides inhibit Z alpha1-antitrypsin polymerization. *J Cell Mol Med* 2009;13:2304–2316.
- Harding HP, Zyryanova AF, Ron D. Uncoupling proteostasis and development *in vitro* with a small molecule inhibitor of the pancreatic endoplasmic reticulum kinase, PERK. *J Biol Chem* 2012;287:44338–44344.
- Reynolds PR, Cosio MG, Hoidal JR. Cigarette smoke-induced EGR-1 upregulates proinflammatory cytokines in pulmonary epithelial cells. *Am J Respir Cell Mol Biol* 2006;35:314–319.

31. Rahman I, van Schadewijk AA, Hiemstra PS, Stolk J, van Krieken JH, MacNee W, de Boer WI. Localization of gamma-glutamylcysteine synthetase messenger RNA expression in lungs of smokers and patients with chronic obstructive pulmonary disease. *Free Radic Biol Med* 2000;28:920–925.
32. Yoshida T, Tuder RM. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol Rev* 2007;87:1047–1082.
33. Ajuebor MN, Flower RJ, Hannon R, Christie M, Bowers K, Verity A, Perretti M. Endogenous monocyte chemoattractant protein-1 recruits monocytes in the zymosan peritonitis model. *J Leukoc Biol* 1998;63:108–116.
34. Vlahos R, Bozinovski S, Jones JE, Powell J, Gras J, Lilja A, Hansen MJ, Gualano RC, Irving L, Anderson GP. Differential protease, innate immunity, and NF-kappaB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L931–L945.
35. Hidvegi T, Schmidt BZ, Hale P, Perlmutter DH. Accumulation of mutant alpha1-antitrypsin Z in the endoplasmic reticulum activates caspases-4 and -12, NFkappaB, and BAP31 but not the unfolded protein response. *J Biol Chem* 2005;280:39002–39015.
36. Hidvegi T, Mirnics K, Hale P, Ewing M, Beckett C, Perlmutter DH. Regulator of G Signaling 16 is a marker for the distinct endoplasmic reticulum stress state associated with aggregated mutant alpha1-antitrypsin Z in the classical form of alpha1-antitrypsin deficiency. *J Biol Chem* 2007;282:27769–27780.
37. Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol* 2011;54:795–809.
38. Kelly E, Greene CM, Carroll TP, McElvaney NG, O'Neill SJ. Selenoprotein S/SEPS1 modifies endoplasmic reticulum stress in Z variant alpha1-antitrypsin deficiency. *J Biol Chem* 2009;284:16891–16897.
39. Pahl HL, Baeuerle PA. A novel signal transduction pathway from the endoplasmic reticulum to the nucleus is mediated by transcription factor NF- κ B. *EMBO J* 1995;14:2580–2588.
40. Jorgensen E, Stinson A, Shan L, Yang J, Gietl D, Albino AP. Cigarette smoke induces endoplasmic reticulum stress and the unfolded protein response in normal and malignant human lung cells. *BMC Cancer* 2008;8:229.
41. Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008;454:455–462.
42. Rahman I, MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax* 1998;53:601–612.
43. Matheson NR, Wong PS, Travis J. Enzymatic inactivation of human alpha-1-proteinase inhibitor by neutrophil myeloperoxidase. *Biochem Biophys Res Commun* 1979;88:402–409.
44. Matheson NR, Travis J. Differential effects of oxidizing agents on human plasma alpha 1-proteinase inhibitor and human neutrophil myeloperoxidase. *Biochemistry* 1985;24:1941–1945.
45. Warter J, Storck D, Grosshans E, Metais P, Kuntz JL, Klumpp T. Weber-Christian syndrome associated with an alpha-1 antitrypsin deficiency. Familial investigation [in French]. *Ann Med Interne (Paris)* 1972;123:877–882.
46. Savage JA, Chang L, Cook L, Burdon J, Daskalakis M, Doery J. Alpha 1-antitrypsin deficiency and anti-proteinase 3 antibodies in anti-neutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis. *Clin Exp Immunol* 1995;100:194–197.
47. Wu Y, Whitman I, Molmenti E, Moore K, Hippenmeyer P, Perlmutter DH. A lag in intracellular degradation of mutant alpha 1-antitrypsin correlates with the liver disease phenotype in homozygous PiZZ alpha 1-antitrypsin deficiency. *Proc Natl Acad Sci USA* 1994;91:9014–9018.
48. Hidvegi T, Ewing M, Hale P, Dippold C, Beckett C, Kemp C, Maurice N, Mukherjee A, Goldbach C, Watkins S, et al. An autophagy-enhancing drug promotes degradation of mutant alpha1-antitrypsin Z and reduces hepatic fibrosis. *Science* 2010;329:229–232.
49. Pan S, Huang L, McPherson J, Muzny D, Rouhani F, Brantly M, Gibbs R, Sifers RN. Single nucleotide polymorphism-mediated translational suppression of endoplasmic reticulum mannosidase I modifies the onset of end-stage liver disease in alpha1-antitrypsin deficiency. *Hepatology* 2009;50:275–281.
50. Carroll TP, Greene CM, O'Connor CA, Nolan AM, O'Neill SJ, McElvaney NG. Evidence for unfolded protein response activation in monocytes from individuals with alpha-1 antitrypsin deficiency. *J Immunol* 2010;184:4538–4546.
51. Kosmider B, Messier EM, Chu HW, Mason RJ. Human alveolar epithelial cell injury induced by cigarette smoke. *PLoS ONE* 2011;6:e26059.
52. Foronjy R, D'Armiento J. The effect of cigarette smoke-derived oxidants on the inflammatory response of the lung. *Clin Appl Immunol Rev* 2006;6:53–72.