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Alpha-1 Antitrypsin Augmentation Therapy Corrects Accelerated Neutrophil Apoptosis in Deficient Individuals

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Alpha-1 antitrypsin (AAT) deficiency (AATD) is characterized by neutrophil-driven lung destruction and early emphysema in a low AAT, and high neutrophil elastase environment in the lungs of affected individuals. In this study, we examined peripheral blood neutrophil apoptosis and showed it to be accelerated in individuals with AATD by a mechanism involving endoplasmic reticulum stress and aberrant TNF-α signaling. We reveal that neutrophil apoptosis in individuals homozygous for the Z allele (PiZZ) is increased nearly 2-fold compared with healthy controls and is associated with activation of the external death pathway. We demonstrate that in AATD, misfolded AAT protein accumulates in the endoplasmic reticulum of neutrophils, leading to endoplasmic reticulum stress and the expression of proapoptotic signals, including TNF-α, resulting in increased apoptosis and defective bacterial killing. In addition, treatment of AATD individuals with AAT augmentation therapy decreased neutrophil ADAM-17 activity and apoptosis in vivo and increased bacterial killing by treated cells. In summary, this study demonstrates that AAT can regulate neutrophil apoptosis by a previously unidentified and novel mechanism and highlights the role of AAT augmentation therapy in ameliorating inflammation in AATD. The Journal of Immunology, 2014, 193: 000–000.
with decreased antimicrobial defenses (26), incomplete microbial clearance, and sustained inflammation (27, 28). AAT has been shown to possess antiapoptotic properties and prevents apoptosis in alveolar cells (29), β cells (30, 31), and transplanted pancreatic islet cells (32). In line with these studies, we hypothesized that neutrophil apoptosis is altered in AATD, contributing to early-onset emphysema and declining lung function, which characterizes this disease (33, 34). This contention is supported by the observation that AAT augmentation therapy leads to reduced exacerbation and lung infections (16). The results of the current study provide a mechanism by which AAT augmentation therapy impacts on the apoptotic rate of circulating neutrophils, potentially reducing the risk of bacterial infection in exacerbations of AATD.

**Materials and Methods**

**Chemicals and reagents**

All chemicals and reagents used in this study were of the highest purity, endotoxin free, and purchased from Sigma-Aldrich (Dublin, Ireland), unless otherwise indicated.

**Study population**

Ethical approval was obtained from Beaumont Hospital institutional review board, and written informed consent was obtained from all study participants. Five distinct patient groups were included in this study, as follows: three test groups and one healthy and one disease control (Table I). In the 8 wk prior to obtaining blood samples, all patients in all five patient groups had no respiratory symptoms, were exacerbation free, and did not require antibiotics or steroids for any reason.

**ZZ phenotype AATD individuals (ZZ-AATD) without obstructive lung disease and not receiving augmentation therapy were recruited from the Irish Alpha-1 Antitrypsin Deficiency Registry (n = 15, mean age 39.29 ± 17.57 y); FEV$_1$ was 103.9 ± 1.3% predicted.

Healthy control volunteers (n = 15, mean age [31.4 ± 9.1 y]), had a FEV$_1$ of 103.3 ± 9.4% predicted and showed no evidence of any inflammatory or lung disease. This group was defined as having a MM phenotype by isoelectric focusing with serum AAT concentrations within the normal range (25-50 μM).

**ZZ-AATD patients with COPD, not on augmentation therapy but possessing a FEV$_1$ within the range of 30-75% predicted (n = 20, mean age 53.7 ± 9.6 y, FEV$_1$ 47.0 ± 14.0% predicted), were included in an analysis of plasma samples for TNF-α and TNFR1.

**COPD patients with a MM phenotype attending a COPD specialist clinic (FEV$_1$ range of 30-70%) were used as a disease control in analysis of FEV$_1$ 54.4 ± 9.6 y, FEV$_1$ 47.0 ± 14.0% predicted).**

**ZZ-AATD patients with COPD on augmentation therapy (n = 20, mean age 66.9 ± 10.37 y, and mean FEV$_1$ 54.4 ± 12.6%).**

**ZZ-AATD patients with COPD on augmentation therapy (n = 5, mean age 56.2 ± 7.6, FEV$_1$ 43.8 ± 3.6% predicted) were receiving weekly i.v. infusions of plasma-purified AAT from CSL Behring (Zemaira), given at a dosage of 60 mg/kg body weight. A patient with ZZ-AATD and co-inherited X-linked chronic granulomatous disease (gp91 PHOX mutant) who subsequently required a bone marrow transplant for rejection (n = 1, mean age 39.29 ± 17.57 y); FEV$_1$ was 103.9 ± 1.3% predicted.**

**ZZ-AATD with and coninherited X-linked chronic granulomatous disease (gp91 PHOX mutation) who subsequently required a bone marrow transplant for recurrent childhood infections (FEV$_1$ 71%) is included in a single identified experiment. In addition, one patient with a diagnosis of Null/Null (Q80b) AATD (FEV$_1$ 57%) was included in the same identified experiment.**

**AAT**

Commercially available plasma-derived human AAT (Athens Research & Technology) was used in experiments involving MM and ZZ neutrophils. To ensure the removal of endotoxins from all AAT preparations, Detoxi-Gel Affinity Pak columns were used according to instructions from the manufacturer (Pierce). Batches of purified AAT were tested for endotoxin contamination with a chromogenic Limulus amebocyte lysate kit (Lonza), and all preparations had endotoxin <0.05 enzyme Units/ml.

**Plasma isolation**

Phlebotomy was carried out on all study participants using Sarstedt Monovette tubes containing lithium-heparin. Plasma was immediately isolated by centrifugation of the blood (500 × g, 5 min room temperature), which was then aliquoted and stored at −80°C until required.
Flow cytometry analysis

In experiments to determine apoptosis, nonfixed neutrophils were used to quantify phosphatidylserine externalization using FITC-conjugated annexin V and dual stained with PE-conjugated propidium iodide to exclude necrotic cells. All staining was carried out immediately prior to flow cytometry analysis. The Annexin V-FTIC ApoTox Kit (BioVision) was used as per the manufacturer’s instructions. Gating and quadrant strategies are shown in Supplemental Fig. 1. Percentage of neutrophil apoptosis denoted in Results refers to annexin V-positive/propidium iodide-negative cells. In other experiments, isolated neutrophils were fixed with 4% (w/v) paraformaldehyde for 30 min. Cells were then washed and blocked with 2% (w/v) BSA, followed by incubation with primary detection Abs at a concentration of 1 µg/ml cells for 30 min. Conjugated primary Abs consisted of monoclonal anti-TNF-A FITC-conjugated IgGl (R&D Systems) and a FITC-labeled goat polyclonal anti-AAT (Abcam). Other unconjugated primary Abs included monoclonal anti-TNFRI (R&D Systems), followed by a FITC-labeled secondary Ab. All control samples were exposed to irrelevant nonspecific isotype control IgG or secondary labeled Ab alone. Cells were washed in PBS, and fluorescence was counted using a FACSscan (BD Biosciences). For all flow cytometry, a total of 10,000 events was collected, and each experiment was carried out in triplicate. BD CellQuest Pro software was used to analyze all cytometric data and is expressed as percentage of 10,000 cells stained for annexin V staining and in median fluorescent units (MFU) for other experiments.

Confocal immunofluorescence

Isolated neutrophils were fixed with 4% (w/v) paraformaldehyde, cell membranes were disrupted using 0.2% Triton X-100 in TBS, and non-specific binding was blocked using 4% (w/v) BSA in PBS. To determine colocalization, cells were incubated with 1 µg/ml FITC-labeled goat polyclonal anti-AAT (Abcam) and 1 µg/ml mouse anti-KDEL (Enzo Life Sciences, Exeter, U.K.), followed by rhodamine-labeled anti-mouse secondary conjugate (Abcam) to visualize anti-KDEL Ab. Neutrophils were mounted employing Vectashield mounting medium with DAPI for nuclear staining (Vectorshield Lab). The controls for this experiment included cells alone, nonspecific isotype control IgG (Santa Cruz Biotechnology), and cells exposed to secondary Ab only. All immunofluorescence slides were viewed, and images were acquired using a Zeiss LSM 710 confocal immunofluorescence microscope (Zeiss). Images were captured at original magnification ×63 with excitation wavelengths for FITC, rhodamine, and DAPI of 488, 543, and 364 nm, respectively. The images represented are 2.5D reconstructions using Zen software (2011 edition; Zeiss) and are representative of three independent experiments for three clinically stable nonobstructed ZZ-AATD patients.

ADAM-17 activity assay

Neutrophil ADAM-17 activity was determined by incubating isolated neutrophils with assay buffer and the fluorogenic peptide substrate MCAProLeuAlaGlnAlaValDPAArgSerSerArgNH for 0.5, and 10 min at room temperature, according to the manufacturer’s instructions (R&D Systems). ADAM-17 enzymatic activity is presented as a percentage of the positive control, recombinant human ADAM-17 (rhADAM-17; R&D Systems), which has a sp. act. of 500 pmol/min/µg.

Neutrophil bacterial killing assay

Pseudomonas aeruginosa (PA01) was cultured overnight in Luria–Bertani (LB) broth. Neutrophils were isolated from blood and cultured for 6 h ± AAT (27.5 µM) or thapsigargin (Thaps; 1 µM). After culturing, the neutrophils were washed three times with PBS containing 5 mM glucose. For the killing assay, bacteria and neutrophils (1 × 10^5) were mixed at a ratio of 1:1 in a temperature-controlled string chamber set at 37°C. Aliquots were removed at 0 and 15 min into ice-cold LB broth. Samples were then diluted and plated in triplicate on LB agar plates and incubated overnight at 37°C. CFUs were counted, and percentage of survival was quantified by setting the number of CFU at 0 min as 100% survival.

Statistical analyses

The data were analyzed with the GraphPad Prism version 4.03 for Windows (GraphPad Software). Results are expressed as means ± SEM and compared by Student unpaired t test, Student paired t tests, or ANOVA, followed by post hoc Tukey or Bonferroni tests, where appropriate. Differences were considered significant when p < 0.05.

Results

Accumulation of AAT in the ER of ZZ-AATD neutrophils is associated with increased ER stress markers

We have previously identified ER stress in monocytes of individuals with ZZ-AATD as a result of ER accumulation of Z-AAT (8), and therefore, we set out to determine whether this phenomenon was occurring in ZZ-AATD neutrophils. First, the protein expression of the ER stress-associated transcription factor ATF6 and the chaperone protein GRP 78 in whole-cell lysates isolated from neutrophils of individuals with ZZ-AATD (n = 3) was compared with healthy control neutrophils from MM individuals (n = 3) (Fig. 1A, 1B). Western blot results show that both the active form of ATF6 (cleaved ATF6) and GRP 78 protein expression are increased in ZZ neutrophils compared with MM neutrophils (p = 0.01 and p = 0.02, respectively). Second, we examined nuclear fractions for protein expression of phosphorylated JNK, and results revealed significantly increased levels of this proapoptotic transcription factor in ZZ neutrophils compared with MM control neutrophils (Fig. 1C) (p = 0.03, n = 5). Third, the protein expression of phosphorylated JNK was determined in whole-cell lysates, and significantly increased levels of this cellular stress marker were found in ZZ-AATD neutrophils (p = 0.05, n = 5; Fig. 1D). Finally, the intracellular accumulation of AAT in the ER of ZZ neutrophils was determined using confocal microscopy (Fig. 1E; n = 3). We demonstrate that AAT protein is localized to the ER of ZZ neutrophils using a fluorescent Ab to AAT and an ER-specific marker that recognizes the KDEL motif present in ER-resident chaperones GRP 78 and GRP 94. Collectively, these results indicate that AAT protein accumulates specifically within the ER, leading to the expression of ER stress markers and, importantly, the proapoptotic associated proteins CHOP and JNK, in neutrophils of individuals with ZZ-AATD.

Induction of ER stress leads to increased mRNA expression of proapoptotic transcription factors and increased TNF-α mRNA expression and cytokine release, and results in increased neutrophil apoptosis

To investigate the effects of induced ER stress on the physiological functions of neutrophils, we treated MM phenotype neutrophils with increasing concentrations of Thaps, a known inducer of ER stress and the unfolded protein response (8). Significantly, incubation of MM neutrophils with Thaps in a dose-dependent manner caused early apoptosis as determined by increased annexin V staining (Supplemental Fig. 2, Fig. 2A) (n = 3, p = 0.001). The effect of increasing doses of Thaps on the mRNA expression of the transcription factor ATF6 (Fig. 2B) and its related chaperone protein GRP 78 (Fig. 2C) was investigated by real-time RT-PCR, and results revealed a dose-dependent induction of these ER stress-associated mRNAs (n = 3, p = 0.02, p = 0.02, respectively). Significantly, the mRNA expression of the proapoptotic transcription factor CHOP in neutrophils also demonstrated a dose-dependent increase in response to Thaps treatment (Fig. 2D; n = 3, p = 0.03). Moreover, Thaps-treated MM neutrophils illustrate an increase in the proapoptotic cytokine TNF-α mRNA expression (Fig. 2E; n = 3, p = 0.02) and increased release of TNF-α protein (Fig. 2F), as measured by ELISA of harvested culture supernatants (n = 3, p = 0.03). In summary, the above results indicate that induction of ER stress in MM neutrophils leads to increased apoptosis and increased expression of the proapoptotic cytokine TNF-α at the gene and protein level.

ZZ-AATD is associated with increased neutrophil apoptosis

In light of results indicating increased ER stress markers observed in ZZ-AATD neutrophils coupled with increased apoptosis and release of the proapoptotic cytokine TNF-α in response to induced
ER stress, subsequent studies investigated neutrophil apoptosis in AATD. To this end, the apoptotic rate of peripheral neutrophils was determined for patients with ZZ-AATD without obstructive lung disease compared with healthy control MM cells. To eliminate the effect of inflammation on neutrophil apoptosis, an AATD patient group was selected that possessed the ZZ mutation but without having obstructive lung disease or other inflammatory conditions. Plasma levels of AAT were measured by nephelometry for all the ZZ-AATD and MM control individuals and are represented in Fig. 3A. Results demonstrate a marked decrease in AAT levels in the ZZ-AATD (0.2027 ± 0.02636 g/L or 3.89 ± 0.05 M) patients compared with MM controls (1.543 ± 0.1077 g/L or 29.67 ± 0.0001 M, p < 0.0001, n = 11).

Flow cytometry was performed to determine apoptosis employing dual staining of cultured neutrophils with annexin V and propidium iodide, the latter allowing for the exclusion of necrotic cells. The representative density plots (Fig. 3B, 3C) and the resulting dot plot of annexin V–positive/propidium iodide–negative staining indicating early apoptosis (Fig. 3D) demonstrate that there is a significant (1.8-fold) increase in apoptosis in ZZ neutrophils (17.54 ± 1.846%) after 6-h incubation compared with that of MM neutrophils (9.959 ± 0.9397%) (n = 11, p = 0.002). This observed increase in apoptotic rate of ZZ neutrophils was confirmed by Western blot analysis of whole-cell lysates of cultured neutrophils. Results demonstrated increased protein expression of the cleaved p17 subunit of the executioner caspase, caspase-3 (Fig. 3E; n = 3, p = 0.03). In addition, results revealed the activation of the external death pathway with increased protein expression of the active cleaved p18 subunit of caspase-8 (Fig. 3F; n = 3, p = 0.0006). Taken together, results indicate an overall 1.8-fold increase in apoptosis of ZZ-AATD neutrophils, which is confirmed by caspase-3 cleavage, and furthermore suggest that the
observed increase in apoptosis was associated with activation of the external death pathway.

Increased activity of the sheddase ADAM-17 in AATD is associated with increased TNF-α release from the cell surface of neutrophils

The previous experiments have demonstrated that ER stress leads to increased release of the proapoptotic cytokine TNF-α in vitro, and that caspase-8 cleavage, which can be activated by the binding of TNF-α with its cognate receptor TNFR1, is increased in ZZ-AATD neutrophils. Moreover, we have previously shown that plasma-purified AAT can inhibit the activity of recombinant ADAM-17 (TACE) (5). Therefore, subsequent experiments were designed to determine whether ADAM-17 enzymatic activity was elevated in ZZ-AATD, leading to increased cleavage of membrane-bound TNF (mTNF)-α to its soluble form (soluble TNF [sTNF]-α). In Fig. 4A, the activity of ADAM-17 was measured on freshly isolated neutrophils and was found to be significantly increased in ZZ (168.9 ± 13.46% rhADAM-17) compared with MM neutrophils (136.0 ± 7.819% rhADAM-17), a finding that persists, as elevated activity was measured after incubation of neutrophils for 6 h (Fig. 4B). Moreover, analysis of cell surface TNF-α protein expression on freshly isolated neutrophils (Fig. 4C) demonstrated that TNF-α expression is significantly increased on ZZ neutrophils (1.388 ± 0.1338 MFU) compared with MM neutrophils (1.045 ± 0.04552 MFU, n = 3), and this result may indicate cleavage by ADAM-17 activity. Subsequently, TNF-α release from ZZ neutrophils compared with healthy control MM neutrophils was determined using a sandwich ELISA. TNF-α levels in supernatants of ZZ (39.25 ± 13.55 pg/ml) compared with MM neutrophils (6.801 ± 5.048 pg/ml) demonstrated a 6-fold increase after 6-h incubation (Fig. 4E; p = 0.04, n = 6). Taken together, these results show that TNF-α release and membrane expression are increased in ZZ-AATD and that TNF-α is removed from the cell surface by increased ADAM-17 activity. These observations can be explained by two linked mechanisms supported by recent literature, as follows: first, ER stress can induce TNF-α release (40), and second, increased ADAM-17 activity leads to increased cleavage of mTNF-α to sTNF-α from neutrophils (41). The net effect of these two processes is increased sTNF-α release.

Neutrophil ADAM-17 activity is regulated by membrane-bound AAT

We have previously described how serum AAT binds to the neutrophil membrane to impact on chemotaxis, and furthermore, that in vivo the majority of AAT on the membrane is of serum rather than cell origin (5). These earlier findings led us to design experiments to better understand the effect of AAT on ADAM-17 activity. To illustrate this mechanism, we examined two exceptional clinical cases of AATD that demonstrate this membrane-located phenomenon (Table I). The first is an individual with AATD (ZZ) and co-inherited X-linked chronic granulomatous disease who subsequently required a bone marrow transplant for recurrent childhood infections. This individual has the unique characteristics of producing ZZ AAT protein from the liver (plasma level 0.17 g/L or 3.27 μM) while possessing neutrophils of the MM phenotype. The second individual was diagnosed with Null/Null (Q0bolton) AATD, and therefore, plasma levels of AAT were undetectable by nephelometry.
When we examined the level of membrane-bound AAT (Fig. 5A) on neutrophils of the bone marrow transplant individual (5.707 ± 0.037 MFU), results revealed decreased levels compared with that seen in the MM control (15.49 ± 0.046 MFU), but that were comparable to ZZ-AATD neutrophil levels (4.307 ± 0.033 MFU). Moreover, the Null/Null individual had markedly reduced membrane-bound AAT levels (1.827 ± 0.01 MFU) compared with MM control and ZZ levels.

When the ADAM-17 activity of neutrophils from the same individuals was determined, results revealed neutrophils from the ZZ-AATD individual with the bone marrow transplant had increased ADAM-17 activity (225.6 ± 47.42%) compared with MM control neutrophils (136.0 ± 7.819%, p = 0.003), but that were comparable to ZZ-AATD activity levels (168.9 ± 13.46%, p = NS) (Fig. 5B). The Null/Null individual had increased levels of neutrophil ADAM-17 activity (212.8 ± 32.50%, p = 0.006) compared with the MM control, but comparable to that observed in ZZ-AATD. Overall, the results of this experiment indicate that ADAM-17 activity on the surface of neutrophils in all patient groups appears to be inversely related to membrane-bound AAT levels. Indeed, the authors have previously shown that polymerized AAT does not effectively inhibit ADAM-17 activity (5), which may explain why the two individuals who possess Z-AAT on their cell surface have similar ADAM-17 activity to the Null/Null individual despite having higher measured membrane-bound Z-AAT.

Treatment of neutrophils with plasma-purified human AAT in vitro is associated with reduced neutrophil ADAM-17 activity and normalization of cell apoptosis

To examine the effect of AAT on neutrophil ADAM-17 activity and apoptosis, neutrophils were cultured with or without human AAT (hAAT) for 6 h, and subsequently, ADAM-17 activity and apoptosis were determined by fluorimetric assay and flow cytometry, respectively. The representative bar graph of annexin V staining (Supplemental Fig. 3, Fig. 6A) demonstrated that there was a significant difference in apoptosis in both ZZ and MM neutrophils after 6-h incubation (p = 0.03, n = 3 subjects per group, Student t test). (F) Western blot and densitometry bar graphs of active cleaved caspase-8 (p18 subunit) in whole-cell lysates of MM and ZZ neutrophils after 6-h incubation (p = 0.0006, n = 3 subjects per group, Student t test). Results in bar graphs (E) and (F) represent the mean ± SEM.

FIGURE 3. ZZ-AATD is associated with increased neutrophil apoptosis. (A) Dot plot of plasma AAT levels measured by nephelometry for ZZ patients and healthy controls (MM) in g/L (p < 0.0001, n = 11 subjects per group, Student t test). Flow cytometry density plots showing annexin V (x-axis) and propidium iodide (y-axis) staining of (B) MM and (C) ZZ neutrophils cultured for 6 h. The right lower quadrant represents annexin V-positive/propidium iodide-negative staining, indicating early apoptosis that is increased significantly in ZZ neutrophils compared with MM. The right upper quadrant represents both high annexin V and propidium iodide staining, indicating late apoptosis, and the left upper quadrant represents low annexin V and high propidium iodide staining, indicating necrosis. The left lower quadrant indicates viable cells. (D) Dot plot of annexin V-positive/propidium iodide-negative staining of MM compared with ZZ neutrophils, representing the results of 11 independent experiments shown in (B) and (C) (p = 0.002, n = 11 subjects per group, Student t test, horizontal line represents the mean of values). (E) Western blot and densitometry bar graphs of active cleaved caspase-3 (p17 subunit) in whole-cell lysates of MM and ZZ neutrophils after 6-h incubation (p = 0.03, n = 3 subjects per group, Student t test). (F) Western blot and densitometry bar graphs of active cleaved caspase-8 (p18 subunit) in whole-cell lysates of MM and ZZ neutrophils after 6-h incubation (p = 0.0006, n = 3 subjects per group, Student t test). Results in bar graphs (E) and (F) represent the mean ± SEM.
and ZZ neutrophils with and without the addition of a physiological relevant concentration of hAAT (27.5 μM). Results are expressed as a percentage of rhADAM-17 activity and reveal that there is a significant reduction in ADAM-17 activity after the treatment of MM (136.0 ± 7.819 versus 116.3 ± 8.067% activity, p = 0.03) and ZZ (168.9 ± 13.46 versus 104.7 ± 13.25% activity, p = 0.02) neutrophils with 27.5 μM hAAT (n = 6). In experiments designed to examine the dose of hAAT required to affect apoptosis, it was observed that concentrations as low as 3 μM significantly reduced the percentage of TNF-α–induced MM cell apoptosis (Supplemental Fig. 4A). Moreover, 3 μM AAT also reduced the percentage of apoptotic MM and ZZ neutrophils post 6-h incubation (Fig. 6C). To confirm the involvement of TNF-α autocrine signaling in induction of neutrophil apoptosis in the latter experiment (40), MM neutrophils were cocultured in the presence of a TNF-α blocking Ab. After 6-h incubation, TNF-α blockade had a partial, but significant effect on apoptosis. The levels of apoptotic cells treated with either 6.25 or 25 ng TNF-α blocking Ab were reduced compared with isotype control Ab–treated cells (p = 0.02 and p = 0.002, respectively) (Fig. 6D). In summary, apoptotic rates observed in ZZ and MM neutrophils are reduced in response to treatment with hAAT by ~50%. This reduction in apoptosis is associated with reduced ADAM-17 activity in both cell types.

AAT augmentation therapy reduces neutrophil ADAM-17 activity and TNF-α signaling, resulting in decreased apoptosis

A recent publication has shown AAT to be an inhibitor of TNF-α signaling by preventing its interaction with its cognate receptors TNFR1 and TNFR2 (4). Therefore, in an attempt to understand the effect of AAT on neutrophil apoptosis in vivo, we examined neutrophil ADAM-17 activity, TNF-α signaling through caspase-8...
cleavage, and apoptosis by caspase-3 cleavage and annexin V staining in ZZ-AATD patients receiving weekly pulse therapy. Relative ADAM-17 activity was found to decrease postaugmentation therapy from 183.9 ± 22.4% rhADAM-17 at day 0 to 133.3 ± 11.4% rhADAM-17 at day 2 postaugmentation therapy (p = 0.04, n = 5) (Fig. 7A). Western blot analysis of whole-cell lysates from isolated neutrophils also revealed that augmentation therapy significantly reduced caspase-8 cleavage (Fig. 7B) (n = 5, p = 0.01), which in turn led to decreased caspase-3 cleavage (Fig. 7C; n = 5, p = 0.01). Annexin V staining for apoptotic cells showed a significant decrease in apoptosis in ZZ-AATD neutrophils postaugmentation therapy (p = 0.0102, n = 3), resulting in a similar level to MM cells (p = NS, n = 3) (Fig. 7D). There appeared to be no effect of augmentation therapy on the ER stress markers, as the chaperone protein GRP 78 determined by Western blotting remained unchanged after augmentation therapy (Fig. 7E).

It is well recognized that ADAM-17 mediates both the shedding of TNF-α from its pro to soluble form and the proteolytic cleavage of the 28-kDa ectodomain of TNFR1 to release its soluble form (sTNFR1) (42, 43). Given the increased activity of neutrophil ADAM-17 observed in AATD, we hypothesized that plasma levels of both TNF-α and TNFR1 would be elevated in affected individuals. In contrast, there is no significant alteration in plasma levels of TNF-α in ZZ-AATD individuals either receiving or not receiving AAT augmentation therapy compared with healthy controls or FEV1-matched COPD (MM) controls (Supplemental Fig. 4B). This finding is consistent with other reports (12) and may in part be due to TNF-α’s short t1/2 in circulation, which is only minutes (44), renal excretion, and/or binding to its soluble receptors or target neutrophils (45). In contrast, sTNFR1 levels are markedly increased in AATD plasma (1089 ± 47.94, n = 20) compared with healthy (600.0 ± 48.49, n = 15) and FEV1-/matched COPD (MM) controls (918.8 ± 54.00, n = 20) (p < 0.0001, p = 0.02, respectively) (Fig. 7F). Furthermore, long-term treatment of ZZ-AATD individuals with AAT augmentation therapy significantly reduces sTNFR1 levels (781.5 ± 95.38, p = 0.003, n = 10). Plasma levels of sTNFR1 are recognized as a marker of TNF-α activation, and increased levels have been found in patients with COPD (46, 47).

Collectively, these results demonstrate that AAT augmentation therapy leads to reduced ADAM-17 activity and engagement of the external death receptor pathway through caspase-8 cleavage, which in turn results in diminished neutrophil apoptosis in individuals with AATD. Furthermore, AAT augmentation does not affect ER stress markers in vivo, which suggests that the predominant mechanism of normalized neutrophil apoptosis is due to altered TNF-α signaling at the membrane level. Moreover, these results demonstrate a significant activation of the TNF-α system in ZZ-AATD individuals. Importantly, this phenomenon appears to be ameliorated by AAT augmentation therapy. However, it is important to point out that these results may reflect an as yet to be described indirect rather than direct effect of AAT on neutrophil apoptosis, which may lead to the downstream decrease of ADAM-17 activity and TNF-α signaling.

**Defective bacterial killing by AATD neutrophils**

The consequence of accelerated neutrophil apoptosis in ZZ-AATD was evaluated at the level of neutrophil-mediated bacterial killing, as an association between accelerated apoptosis and increased infections has been reported (26). Both MM and ZZ-AATD neutrophils were incubated in the presence of 27.5 μM P. aeruginosa to induce ER stress demonstrated a reduced ability to kill P. aeruginosa with 38 ± 2% bacterial killing recorded for MM cells compared with 30 ± 2% by ZZ cells (p = 0.01). MM neutrophils that were exposed to Thaps (1 μM) to induce ER stress demonstrated a reduced ability to kill P. aeruginosa, with similar levels of killing observed for ZZ cells (p = 0.06). Culturing of MM and ZZ-AATD neutrophils with physiological levels of AAT (27.5 μM) prior to carrying out the killing assays resulted in a significant reduction in bacterial survival (54 ± 2% and 57 ± 3% survival, respectively) when compared with cells not exposed to AAT (p = 0.006 and p = 0.0002, respectively). The presence of AAT resulted in a similar level of bacterial killing between MM and ZZ-AATD neutrophils (p = 0.37). Of major importance, neutrophils isolated from AATD individuals 2 d post-AAT augmentation therapy showed enhanced killing ability and successfully reduced bacterial survival to ~60%, similar to bacterial survival observed for healthy MM control cells (Fig. 8B) (p = 0.003). Overall, these results indicate that the observed accelerated rate of apoptosis in ZZ-AATD can negatively impact on the ability of these cells to kill bacteria, and that in vitro and in vivo AAT can restore ZZ-AATD neutrophil-killing capacity to that of control MM cells.

**Discussion**

Emerging evidence suggests that the development of emphysema in patients with ZZ-AATD is not only due to the disruption of the protease–antiprotease balance, but is in fact caused by the absence of the important anti-inflammatory effects of AAT (48). Key to the successful resolution of inflammation is the tightly controlled process of neutrophil apoptosis. It is recognized that ER stress can cause reduced cellular adaption to stress and increased apoptosis (49, 50), which together can lead to the reduction of normal cell function. A study examining the role of macrophage in tuberculosis infection demonstrated that infection can induce ER
stress, leading to apoptosis and reduced mycobacteria killing (51). For the first time, to our knowledge, this study demonstrates AAT’s ability to regulate this process and elucidates the mechanism by which its deficiency leads to accelerated neutrophil apoptosis, resulting in decreased bacterial killing in ZZ-AATD. In patients with ZZ-AATD, the misfolded Z-AAT protein accumulates within the ER of neutrophils, leading to the expression of ER stress proteins, the proapoptotic transcription factor CHOP, and the proapoptotic cytokine TNF-α. In tandem on the cell surface, increased mTNF-α is cleaved to its soluble form by the uninhibited activity of the sheddase ADAM-17, a process that can be modulated by AAT. In combination, these two linked signaling pathways cause increased expression of TNF-α on the cell surface (mTNF-α) and released locally (sTNF-α), which have been previously shown to exert cytotoxic effects on neutrophils at a cell-to-cell and paracrine level, respectively (52). These observations in association with the finding of overexpression of TNF-α soluble receptor TNFR1 in patients with ZZ-AATD support our hypothesis that neutrophil apoptosis is accelerated in ZZ-AATD and furthermore describe a mechanism involving abnormal TNF-α signaling. Moreover, to our knowledge, this study shows for the first time that in vivo AAT augmentation therapy can attenuate ADAM-17 activity, leading to decreased activation of the external death receptor pathway, as demonstrated by reduced caspase-8 cleavage and results in the regulation of neutrophil apoptosis.

Although many investigators agree that mutations of the AAT protein can lead to the formation of polymers that are retained within the ER of hepatocytes in ZZ-AATD (3, 53), much debate exists regarding the presence of the classical unfolded protein response (UPR), a cellular stress response that leads to attenuation of protein translation and enhancement of ER protein folding in AATD (54, 55). Rather, opinion is now moving toward the concept that retention of ordered polymers of AAT within the ER of hepatocytes may predispose cells to ER stress and fully activate the UPR as misfolded protein levels increase (2). Of interest, whereas these findings are well recognized in primary hepatic and various transfected cell line models, the characteristics of ER stress in immune cells from ZZ-AATD patients are less well studied. Therefore, the finding that accumulation of AAT within the ER of monocytes has been shown to cause ER stress and UPR in resting and stimulated cells of ZZ-AATD individuals is of importance (8). This later study prompted us to investigate whether a similar stress response occurred in the short-lived professional phagocyte, the neutrophil. Indeed, we have found that retention of the misfolded AAT protein within the ER of ZZ-AATD neutrophils leads to the activation of the ER stress sensor ATF 6 and the consequent increase of the chaperone protein GRP 78. Interestingly, ER accumulation of Z-AAT polymers also associated with the increased expression of the proapoptotic transcription factor CHOP and the activation of the cellular stress response kinase, JNK, which together suggested that the ER stress in ZZ-AATD neutrophils may be associated with early apoptosis.

Indeed, when ER stress was induced using Thaps, the results revealed an increase in the level of CHOP and proapoptotic cytokine TNF-α, and resulted in increased apoptosis in a dose-dependent manner. In support of our findings, it is now well recognized that ER stress can cause apoptosis if ER dysfunction is prolonged or severe (56, 57), and importantly, investigators have found in a cell line model that ER stress can induce TNF-α expression, which can act in an autocrine fashion to amplify ER-initiated apoptotic signaling (40). The observation of proapoptotic signaling in ZZ-AATD neutrophils led us to examine the apoptotic rate of ZZ-AATD neutrophils from clinically stable patients without obstructive lung disease on spirometry compared with MM control neutrophils. Altered neutrophil apoptosis is known to occur in a wide variety of inflammatory diseases such as vasculitis and cystic fibrosis, but a growing number of authors believe that neutrophil dysfunction is due to factors intrinsic to the neutrophil rather than simply secondary to extracellular inflammatory signals (24, 58, 59). Accelerated neutrophil apoptosis has been previously associated with rheumatoid arthritis (60), chronic renal failure (61), and HIV (62), and within sputum neutrophils of COPD patients (63), and is well recognized in the peripheral neutrophils of individuals with systemic lupus erythematosus (64). Moreover, accelerated neutrophil apoptosis is known to cause increased infections (26) and deregulated macrophage clearance of neutrophils (65), and, importantly, it is associated with worse disease progression and outcomes for patients...
In fact, the contribution of defective efferocytosis of apoptotic cells in chronic lung disease has been highlighted in a recent review (67). The results of this study reveal that apoptosis was significantly accelerated in ZZ-AATD neutrophils compared with MM control, a finding that has never been previously documented. This observation is supported by results demonstrating increased CD16b membrane shedding in neutrophils from ZZ-AATD individuals, a marker of the onset of apoptosis specific to neutrophils (5). Although a number of investigators have shown that AAT possess antiapoptotic properties in a number of cell types (29–31), none have examined the effect of AAT on neutrophil apoptosis and its consequences in AATD. In addition, important work on the mechanism by which AAT inhibits apoptosis has focused on cells that are capable of internalizing AAT, such as airway endothelial cells (68), and is thought to involve the direct inactivation of caspase-3, the primary cytoplasmic executor protein of cellular apoptosis (69). Importantly, neutrophils have been shown to localize 80% of AAT to the membrane, and a lesser amount to the secretory vesicles where interaction with the cystolic cysteine–aspartic proteases or caspases would be unlikely (5). This observation together with our finding of membrane-bound AAT’s ability to regulate ADAM-17 activity and results from the ZZ-AATD bone marrow transplant recipient (Fig. 5) supports the hypothesis that the mechanism of AAT’s antiapoptotic ability lies at the outer plasma membrane level in neutrophils. It is important to note that, although this study found that AAT protein can delay neutrophil apoptosis in vitro and AAT augmentation therapy can correct the accelerated neutrophil apoptosis found in AATD, delaying apoptosis in non-AAT–deficient neutrophils using AAT augmentation has, as of yet, no proven clinical benefit. In animal studies of diabetes and pancreatic islet cell transplant, AAT therapy has shown some promise in ameliorating inflammation and accelerated apoptosis (30–32). Caution must be exercised, however, to differentiate between therapeutic interventions that aim to correct abnormal/accelerated apoptosis and those that may delay normal apoptosis, potentially inducing a proinflammatory response.

AAT’s relationship with TNF-α is now well recognized as significant and complex (4, 12–14), and this study further elucidates this intricate association. TNF-α is a pleiotropic cytokine, and in neutrophils binding with its cognate receptors, TNFR1 and/or TNFR2 lead to a number of diverse cellular events such as cell priming, NF-κB, and MAPK activation and degranulation, but it is best recognized for its effects on the induction of cell death (70–72), where divergent effects are seen depending on TNF-α dose (71, 73). Although many authors have shown that TNFRI stimulation leads to activation of caspase-8, which in turn leads to the induction of apoptosis through effector caspasess such as caspase-3,
Geering et al. (74) have found that in neutrophils, proapoptotic pathways are not initiated by caspase-8. Although our model suggests that caspase-8 is activated in individuals with ZZ-AATD, this may in part be due to TNFR1 stimulation or an interaction of ER stress and caspase-8, inducing cell death that has previously been reported in a number of cell types (75–77). Interestingly, TNF-\(\alpha\) has also been found to induce the UPR in a reactive oxygen species–dependent manner and leads to cell death in a fibrosarcoma cell line (78), a finding that may have relevance to our observation that ER stress markers are increased in ZZ-AATD. Indeed, it might be interesting to speculate that autocrine priming of the neutrophil by TNF-\(\alpha\) in ZZ-AATD may be the second hit that some investigators believe is needed to cause ER stress in an otherwise hypersensitive cell (2). Interestingly, we have found that levels of mTNF-\(\alpha\) are increased on the cell surface of freshly isolated neutrophils from individuals with ZZ-AATD, a finding that is supported by recently published work by Bergin et al. (4), showing that AAT can inhibit the interaction of TNF-\(\alpha\) and its cognate receptor (4). In the setting of AAT deficiency, TNF-\(\alpha\) may bind to its receptor in increased amounts, and therefore is found in relative abundance on the surface of neutrophils in AATD. Essential to the release of sTNF-\(\alpha\) from the cell surface of neutrophils

**FIGURE 7.** Neutrophil apoptosis is decreased, and levels of TNF-\(\alpha\) are reduced after AAT augmentation therapy in vivo. (A) The enzymatic activity of ADAM-17 in neutrophils isolated from patients receiving AAT augmentation therapy was determined pre or day 0 (D0) and post or day 2 (D2) after infusion and was found to be reduced after treatment (\(p = 0.04, n = 5\) subjects per group, Student \(t\) test). (B) Cleavage of caspase-8 (\(p = 0.01, n = 5\) subjects per group, Student \(t\) test) and (C) caspase-3 (\(p = 0.01, n = 5\) subjects per group, Student \(t\) test) markers of TNF-\(\alpha\) signaling and apoptosis, respectively, assessed by Western blotting of whole-cell lysates of neutrophils, was found to be reduced postaugmentation therapy. (D) Apoptosis assessed by annexin V staining by flow cytometry of MM neutrophils and paired ZZ-AATD cells on D0 pretherapy and D2 postaugmentation therapy (\(p = 0.01, n = 3\) subjects per group, ANOVA). (E) There was no effect of augmentation therapy on the expression of the ER stress chaperone protein GRP 78, as assessed by Western blotting (\(n = 5\) subjects per group, Student \(t\) test). Results in (B)–(E) represent the mean ± SEM. (F) Represents the results of an ELISA for TNFR. Plasma samples were collected from ZZ-AATD patients either receiving weekly treatment with pooled 60 mg/kg AAT (AAT Aug Tx, \(n = 10\) subjects) or not on treatment (ZZ-AATD, \(n = 20\) subjects), healthy control individuals (HC, \(n = 15\) subjects), or from patients with COPD (MM) phenotype (\(n = 20\) subjects). All patient groups except the healthy controls had a FEV\(_1\) range of 30–70% of predicted and had no exacerbation in the previous 8 wk. In (F) there were significant differences in the mean plasma TNFR1 levels of ZZ-AATD compared with AAT Aug Tx patients \((p = 0.003, n = 20, n = 10\) subjects per group, Student \(t\) test), ZZ-AATD compared with HC \((p < 0.0001, n = 20, n = 15\) subjects per group, Student \(t\) test), and ZZ-AATD compared with COPD (MM) \((p = 0.02, n = 20\) subjects per group, Student \(t\) test). The results in (F) represent experiments performed in triplicate, and the mean is represented by a horizontal line in each group.
is the activity of ADAM-17, which has been shown to increase after cell activation (79), and induction of apoptosis (80–82). Our observation that ADAM-17 activity is increased in freshly isolated, nonactivated neutrophils before the onset of significant apoptosis has never been described before. Moreover, the finding of decreased ADAM-17 activity and normalization of apoptosis in vivo after augmentation therapy greatly supports our hypothesis of the ability of AAT to modulate neutrophil apoptosis in ZZ-AATD by modulating ADAM-17 activity. Indeed, others have found that ADAM-17 activity is increased in cultured neutrophils at 6 h in Fas-induced apoptosis compared with nontreated neutrophils (83), a finding we have replicated in non-

Figure 8. Neutrophil killing of bacteria is reduced in AATD. MM and ZZ-AATD neutrophils (1 × 10^7) were cultured for 6 h ± AAT (27.5 μM) or in the presence of Thaps (1 μM). Post culturing neutrophil killing of P. aeruginosa was assessed by incubating bacteria and neutrophils at ratio of 1:1 in a temperature-controlled stirring chamber at 37°C. Aliquots were removed at 0 and 16 min into ice-cold LB broth. Serial dilutions were plated on LB agar plates in triplicate, and percentage of survival was determined. Results indicated that ZZ-AATD neutrophils killed significantly less bacteria compared with MM cells (p = 0.01, n = 3 subjects per group, Student t test). The survival of P. aeruginosa incubated with ZZ-AATD cells was not significantly different from that observed with MM cells cultured in Thaps (p = 0.06, n = 3 subjects per group, Student t test). Furthermore, the presence of AAT increased killing of P. aeruginosa by both MM and ZZ-AATD cells compared with AAT-untreated cells (p = 0.006 and p = 0.0002, respectively, n = 3 subjects per group, Student t test). (B) On day 2 postaugmentation therapy (D2), there was significantly increased bacterial killing by ZZ-AATD neutrophils compared with ZZ-AATD cells on day 0 pretherapy (p = 0.003). No significant difference (NS) was observed between MM controls and ZZ-AATD D2 (n = 3 subjects per group, ANOVA).

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References


