Eur Respir J 2010; 35: 1155–1163
DOI: 10.1183/09031936.00191908

Anti-apoptotic effects of Z α_1-antitrypsin in human bronchial epithelial cells

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ABSTRACT: **α_1-antitrypsin (α_1-AT) deficiency is a genetic disease which manifests as early-onset emphysema or liver disease. Although the majority of α_1-AT is produced by the liver, it is also produced by bronchial epithelial cells, amongst others, in the lung. Herein, we investigate the effects of mutant Z α_1-AT (ZAAT) expression on apoptosis in a human bronchial epithelial cell line (16HBE14o-) and delineate the mechanisms involved.**

Control, M variant α_1-AT (MAAT)- or ZAAT-expressing cells were assessed for apoptosis, caspase-3 activity, cell viability, phosphorylation of Bad, nuclear factor (NF)-κB activation and induced expression of a selection of pro- and anti-apoptotic genes.

Expression of ZAAT in 16HBE14o- cells, like MAAT, inhibited basal and agonist-induced apoptosis. ZAAT expression also inhibited caspase-3 activity by 57% compared with control cells ($p=0.05$) and was a more potent inhibitor than MAAT. Whilst ZAAT had no effect on the activity of Bad, its expression activated NF-κB-dependent gene expression above control or MAAT-expressing cells. In 16HBE14o- cells but not HEK293 cells, ZAAT upregulated expression of cIAP-1, an upstream regulator of NF-κB. cIAP1 expression was increased in ZAAT *versus* MAAT bronchial biopsies.

The data suggest a novel mechanism by which ZAAT may promote human bronchial epithelial cell survival.

KEYWORDS: **Apoptosis, caspase-3, cIAP1, nuclear factor-κB, Z α_1-antitrypsin**

In α_1-antitrypsin (α_1-AT) deficiency, an inherited disorder, the alleles from both parents are inherited in an autosomal co-dominant fashion. The disease manifests as early-onset panacinar emphysema or liver disease. The mutant Z α_1-AT protein (ZAAT) differs from the normal M variant α_1-AT (MAAT) by a single amino acid substitution (Glu 342 → Lys) [1] leading to an alteration in the secondary structure of ZAAT, which in turn leads to aberrant protein folding and the accumulation of misfolded ZAAT in the endoplasmic reticulum (ER) of hepatocytes [2]. This prevents adequate secretion of protein and results in lower than normal plasma concentrations of α_1-AT. The mutant ZAAT protein occurs in >95% individuals with α_1-AT deficiency [3].

We have previously shown that the accumulation of ZAAT in HEK293 cells, used as a model of ZAAT liver disease, leads to ER stress-induced apoptosis [4]. However, the liver is not the only source of α_1-AT in the body. α_1-AT is also produced by macrophages, monocytes and intestinal epithelial cells [5–7]. Our studies have detected ZAAT in bronchoalveolar lavage fluid from a protease inhibitor (Pi) ZZ individual following liver transplantation, also indicating that α_1-AT is produced locally in the lung [8]. Bronchial epithelial cells may be an important source of this α_1-AT.

It is becoming increasingly evident that the effect of α_1-AT on apoptosis can be cell type-specific. Whilst ZAAT-induced ER stress can activate caspases and apoptotic events in a fibroblast model system [4] and *in vivo* in rat hepatocytes expressing ZAAT [9], MAAT has been shown to have anti-apoptotic effects in other cell types [10–12]. PETRACHE *et al.* [13] showed that MAAT can inhibit apoptosis in alveolar epithelial cells following transduction of a MAAT-expressing adeno-associated virus in a mouse model of apoptosis-dependent emphysema. The mechanism by which MAAT mediates this effect is *via* direct inhibition of caspase-3 binding to its substrate [14]. Other studies have reported anti-apoptotic effects of MAAT in porcine pulmonary endothelial cells [15]. The effect of ZAAT on caspase-3 activation and apoptosis in airway epithelial cells has not been investigated.

Given that α_1-AT expression in bronchial epithelial cells has been reported to inhibit apoptotic

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Received:
Dec 19 2008
Accepted after revision:
Sept 29 2009
First published online:
Oct 19 2009

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

cell death, additional mechanisms by which this occurs, particularly in the context of expression of ZAAT, are deserving of further investigation. For example can ZAAT promote cell survival *via* downregulation of the expression of pro-apoptotic factors? Alternatively, is the expression of certain anti-apoptotic factors increased by ZAAT? Other possible mechanisms include the status of the anti-apoptotic factor Bad and the transcription factor hypoxia inducible factor (HIF)-1α [16]. HIF-1α can be upregulated and activated by a variety of stimuli including hypoxia [17], growth factors and cytokines leading to the transcriptional induction of genes involved in cell proliferation and viability, *e.g.* vascular endothelial growth factor [18].

Thus, given the emerging importance of apoptosis in the development of emphysema [13, 14, 19] we investigated the effect of ZAAT on apoptosis in human bronchial epithelial cells, and compared our findings with the effects of MAAT. We also explored potential new mechanisms that are involved, specifically the roles of Bad, nuclear factor (NF)-κB and a selection of pro- and anti-apoptotic genes and validated our *in vitro* observations *in vivo* by using bronchial biopsies from ZAAT-deficient individuals.

MATERIALS AND METHODS

Cell culture, treatments and transfections

Human bronchial epithelial (16HBE14o-) cells (personal gift: D. Gruenert; University of California, San Francisco, CA USA) were grown in Eagle's MEM-glutamax and 10% fetal calf serum (FCS). Cells (5×10^5) were transiently transfected (Transfast; Promega, Madision, WI, USA) with 500 ng pZeoSV2(+) empty vector (Invitrogen, Paisley, UK), pMAAT or pZAAT (the same vector containing a normal MAAT or mutant ZAAT) [20] and, in some experiments, 200 ng pRLSV40-control luciferase expression plasmid (Promega). For *grp78* promoter and NF-κB activity assays cells were co-transfected with 300 ng of a *grp78* promoter plasmid (personal gift; A.S. Lee; USC/Norris Cancer Center, Los Angeles, CA, USA) or an NF-κB_5 promoter-linked luciferase reporter plasmid. The amount of DNA in each experiment was kept constant by the addition of appropriate empty vector DNA. In experiments where fewer or more than 5×10^5 cells were used, the amounts of DNA were scaled down or up appropriately. Transfection efficiencies were quantified by luminometry (Victor^2 1420 Multilabel counter; PerkinElmer, Waltham, MA, USA) measuring luciferase activity from the co-transfected pRLSV40-control using coelenterazine (Biotium) or by qRTPCR using α_1-AT - and β-actin-specific primers.

TUNEL staining

16HBE14o- cells (1×10^5 cells·well^{-1}) were grown on four-well chamber slides, transfected and placed under serum free conditions for a total of 5 days or left for 24 h then treated with tunicamycin for a further 24 h. The DeadEnd Colorimetric TUNEL System (Promega) was used to detect apoptotic cells. Stained cells were visualised using a Nikon Coolscope II digital microscope (Nikon Corporation, Kingston upon Thames, UK).

Cigarette smoke extract preparation

Cigarette smoke extract (CSE) preparation was modified from previously published methods [21, 22]. Briefly, the smoke from one research grade cigarette (1 mg nicotine, 10 mg tar), with its filter removed, was bubbled through 25 mL of serum free medium (SFM). The resulting suspension was then filtered through a 2-μm pore filter. This sterile solution (100% CSE) was further diluted as appropriate and used within 30 min of preparation.

Caspase-3 activity assays

Caspase-3 activity was measured using the luminescent substrate Z-DEVD-aminoluciferin (Caspase-Glo 3/7 assay; Promega). Cells were lysed in caspase-glo reagent as recommended and luminescence (substrate turnover) of triplicate samples was determined at times 0 and 1 h using a Victor^2 luminometer (PerkinElmer). Caspase activities were calculated as Δ luminescence units per μg protein. Protein quantification was determined using the method of Bradford. Values were further corrected for transfection efficiency as appropriate.

Cell proliferation assays

Cell proliferation was measured using the CellTiter 96 AQ_{ueous} one solution cell proliferation assay (Promega) with the protocol adapted for a 12-well format. This assay is a colorimetric method for determining the number of viable cells. It is based on the bio-reduction by cells of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to a coloured formazan product. Aliquots containing 200 μL reagent were added to each well containing 1 mL of SFM. Cells were incubated at 37°C and absorbance at a wavelength of 490 nm was measured at 1 and 2 h.

Western immunoblotting

Triplicate wells of 2.5×10^6 cells were left untreated, were treated with CSE, nicotine (Sigma Aldrich, St Louis, MO, USA) or tauroursodeoxycholic acid (TUDCA; Sigma Aldrich), or were transfected as indicated. After 24 h, cells were lysed in lysis buffer (1% igepal CA-640, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate (SDS) supplemented with 0.1 mg·mL^{-1} phenylmethylsulfonyl fluoride, 3% aprotinin and 1 mM sodium orthovanadate). Samples (20 μg) were separated by electrophoresis on 15% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Immunoreactive proteins were detected using p-Bad Ser136 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or Erk2 (Santa Cruz Biotechnology) antibodies, a goat horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark) and SuperSignal West Femto chemiluminescent substrate solution (Pierce, Rockford, IL, USA). Images were recorded on X-ray film and densitometric analysis was performed using the G: box Chemi XL gel documentation analysis system (Syngene, Cambridge, UK).

HIF-1α activity assays

Cells (8×10^5 cells·well^{-1}) were transfected with pZAAT and either left untransfected or underwent hypoxic challenge ($1\%O_2/5\%CO_2/37°C$) for 24 h. Lysates were prepared in 2 mL of ice-cold PBS/phosphate inhibitor buffer (125 mM sodium fluoride, 250 mM β-glycerophosphate, 250 mM para-nitrophenyl phosphate and 25 mM sodium orthovanadate). HIF-1α activity was determined using the TransAM™ HIF-1 transcription factor assay kit (Active Motif, Rixensart, Belgium) according to the manufacturer's protocol.

qRTPCR

RNA was isolated using TRI reagent (Sigma Aldrich) according to the manufacturers' instructions. Equal quantities of RNA were reverse transcribed into cDNA using the quantitect reverse transcription kit (Qiagen, Crawley, UK). The resulting cDNA was the template for quantitative real-time PCR. Oligonucleotide primers were synthesised (Eurofins MWG, Ebersberg, Germany) and quantitative PCR reactions were performed in a 20-μL volume containing 2-μL template cDNA, 2x SYBR Green master mix (Roche Diagnostics, Mannheim, Germany), and 10 pmoles of each primer (table 1). Amplification was performed using the LightCycler 480 PCR system (Roche Diagnostics). Quantity values for gene expression were generated by the relative quantification ($2^{-\Delta\Delta CT}$) method where fluorescence generated by each sample was normalised to the β-actin product for each gene of interest.

Subject recruitment

Subjects were recruited from the respiratory outpatient clinic in Beaumont Hospital (Dublin, Ireland). Two groups of patients were used in this study, three patients with known Pi ZZ genotype of α_1-AT deficiency (two male, one female, mean age 43.6 yrs) and three healthy volunteers (two male, one female, mean age 45.3 yrs). This study was approved by the Beaumont Hospital Ethics Committee, and volunteers gave their informed consent in writing. Subjects recruited with α_1-AT deficiency had the diagnosis confirmed by a serum α_1-AT level <11 μM and ZZ phenotype demonstrated by isoelectric focusing. None of the volunteers in the α_1-AT deficiency group had any history of allergy, asthma or other respiratory disease. Normal subjects were healthy nonsmoking volunteers with no history of lung disease, allergy or asthma, and had no respiratory symptoms.

Bronchial biopsy

Endobronchial biopsies were performed on recruited patients. Briefly, subjects received pre-treatment with 2.5 mg of nebulised salbutamol followed by intravenous fentanyl 50 μg and midazolam 2–10 mg until conscious sedation was achieved. Xylocaine was sprayed at the posterior pharynx and a flexible fibreoptic bronchoscope (Olympus BF Type XT20; Olympus, Southend on Sea, UK) was introduced *via* the mouth. 2-mL aliquots of 2% lignocaine were introduced *via* the bronchoscope and applied to the vocal cords, trachea, and left and right main stem bronchi. Endobronchial biopsies were then obtained from the subcarinae of the second to fourth generation bronchi of the right upper, right middle and right lower lobe bronchi using a BARD precisor pulmonary coated disposable biopsy forceps. Biopsy specimens were fixed in 4% paraformaldehyde and embedded in glycol methacrylate resin.

TABLE 1 PCR primers

Gene	Product size bp	Primer sequence 5′-3′	Temp °C
Bcl-2	113	Forward: TCCGCATCAGGAAGGCTAGA	59.4
		Reverse: AGGACCAGGCCTCCAAGCT	61
Bax	191	Forward: GGGTGGTTGGGTGAGACTC	61.6
		Reverse: AGACACGTAAGGAAAACGCATTA	60.2
cIAP1	139	Forward: CTGGGCCTAGATGCAGTTCAG	62.2
		Reverse: ACGGCTCATAAGTCACAAAAGTC	60.8
cIAP2	122	Forward: GTTTCAGGTCTGTCACTGGAAG	60.5
		Reverse: TGGCATACTACCAGATGACCA	60
XIAP	157	Forward: GCAGGTTGGGTGTACGATGT	62.2
		Reverse: GCTGCCACAGTAGGACTCG	62.1
AAT	91	Forward: ATGCTGCCCAGAAGACAGATA	60.7
		Reverse: CTGAAGGCGAACTCAGCCA	62
β-actin	150	Forward: GGACTTCGAGCAAGAGATGG	59.4
		Reverse: AGGAAGGAAGGCTGGAAGAG	59.4

Immunohistochemistry

Sections (2 μm) were cut using an ultramicrotome (Leica, Solms, Germany), floated on to ammonia water (1:500), placed onto 0.01% poly-L-lysine glass slides (BDH Laboratory Supplies, Poole, UK), and dried at room temperature for 1 h. The sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase. Nonspecific antibody binding was blocked using Tris-buffered saline (TBS) with 10% FCS for 30 min followed by incubation with cIAP-1 antibody (R&D Systems Inc., Minneapolis, MN, USA) overnight. After washing in TBS, biotinylated universal secondary antibody was applied for 2 h, followed by the streptavidin-biotin horseradish peroxidase complex for 2 h (Vectastain Universal Elite kit; Vector LaboratoriesUK, Peterborough, UK). After washing in TBS, peroxidase was detected with 3,3-diaminobenzidine chromogen (DakoCytomation). All sections were counterstained with Mayer's haematoxylin (Sigma Aldrich). Isotype-matched antibody controls were negative in all cases. Slides were coded and examined under a light microscope (Nikon Coolscope). Images were captured using a high-definition digital camera attached to the microscope.

NF-κB and grp78 promoter-luciferase assays

Cells (5×10^5) were co-transfected with SV2 (empty vector), pMAAT or pZAAT and with an NF-κB_5-luciferase reporter plasmid or a *grp78* promoter-linked luciferase reporter plasmid and pRLSV40 for 24 h. Some cells were treated with dimethyl sulfoxide (as a vehicle), tunicamycin (1 μg·mL^{-1}, 24 h) or interleukin (IL)-1β (10 ng·mL^{-1}, 24 h) (R&D Systems Inc.) as indicated. Relative luciferase production was quantified by luminometry.

Statistical analysis

Data were analysed with the GraphPad Prism 4.0 software package (GraphPad Software, San Diego, CA, USA). Results are expressed as mean ± SE and were compared by paired or unpaired t-tests as appropriate. Differences were considered significant when the p-value was <0.05.

RESULTS

Human bronchial epithelial 16HBE14o- cells express α_1-AT

It has been reported that a variety of human epithelial cells express α_1-AT, including Calu-3, A549 and H441 [23–25]. We determined whether the 16HBE14o- human bronchial epithelial cell line belongs to this category. Using qRTPCR we detected α_1-AT mRNA expression by 16HBE14o- cells (fig. 1a) and showed upregulation of the α_1-AT gene expression in response to pro-inflammatory and ER stress stimuli.

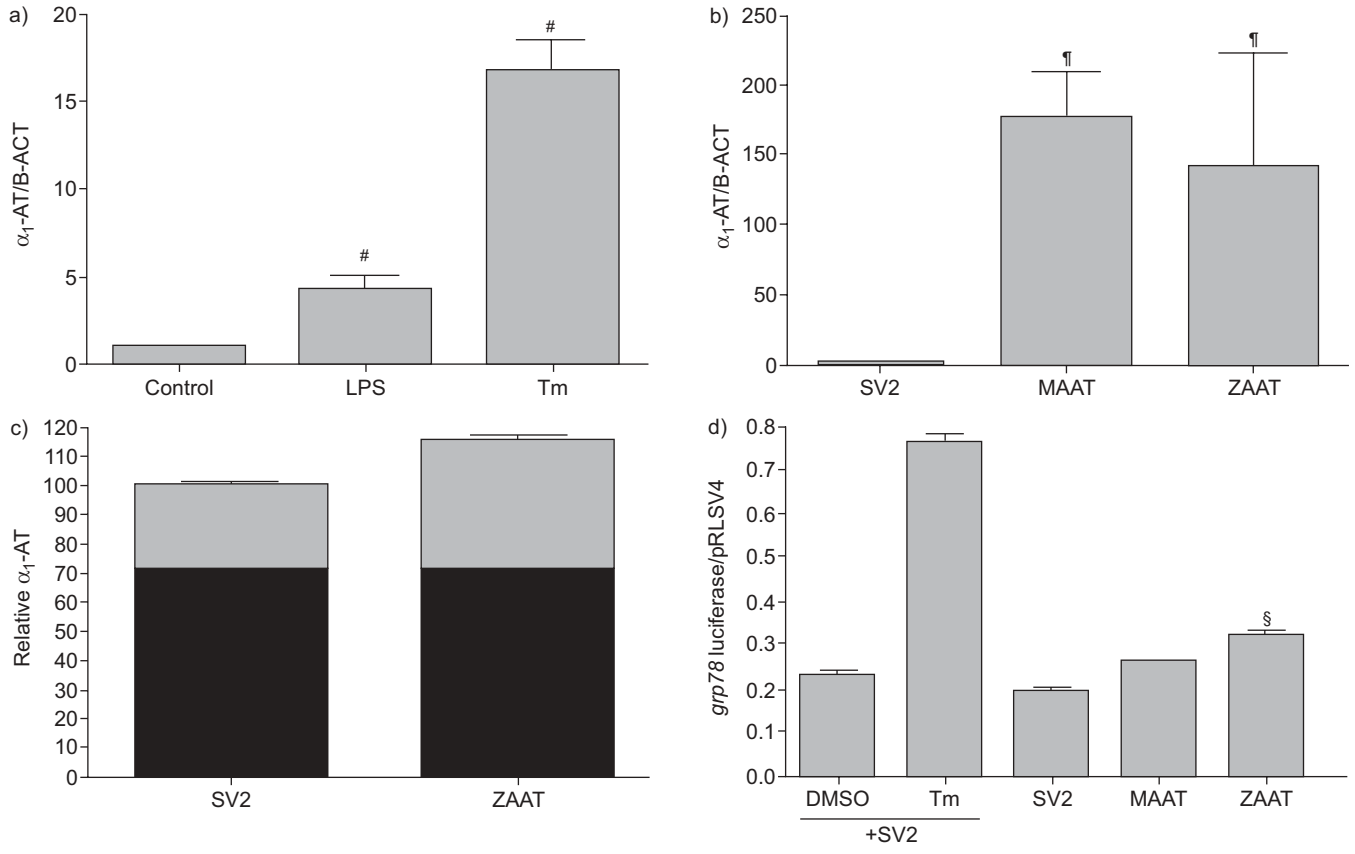

FIGURE 1. α_1-antitrypsin (α_1-AT) expression in 16HBE14o- cells. a) 16HBE14o- cells (1×10^5) were left unstimulated or treated with lipopolysaccharide (LPS; 10 $\mu g \cdot mL^{-1}$) or tunicamycin (Tm; 1 $\mu g \cdot mL^{-1}$) for 24 h. Total RNA was isolated and the expression of α_1-AT was analysed by qRT-PCR and normalised for β-actin. b–d) 16HBE14o- cells (5×10^5) were co-transfected for 24 h with an empty vector (SV2), M variant α_1-AT (MAAT) or mutant Z α_1-AT (ZAAT) expression plasmids. b) Total RNA was isolated and the expression of α_1-AT was analysed by qRT-PCR and normalised for β-actin. c) Supernatants (■) and lysates (■) were assayed for α_1-AT protein expression by ELISA. d) following co-transfection with a pRLSV40-control luciferase expression vector and a *grp78* promoter-linked luciferase reporter plasmid cells were left untreated or treated with dimethyl sulfoxide (DMSO; as a vehicle) or Tm (1 $\mu g \cdot mL^{-1}$) for 24 h as indicated. *grp78*-promoter activity was quantified by luminometry and normalised to transfection efficiency. All assays were performed in duplicate three times. B-ACT: β-actin. #: *versus* untreated cells; ¶: *versus* SV2; §: *versus* DMSO or SV2.

Transfection of 16HBE14o- cells with MAAT or ZAAT transgenes led to a significant increase in α_1-AT gene expression (fig. 1b). Of the total α_1-AT protein produced by SV2-transfected cells, 28% was in the lysates and 72% was secreted into the supernatant (fig. 1c) as measured by ELISA. Compared with SV2-transfected cells, expression of MAAT led to a 22% overall increase in α_1-AT protein production (data not shown). In ZAAT-expressing cells *versus* SV2-transfected cells there was a 16% increase in the amount of α_1-AT detectable in the lysates whereas only a 0.25% increase in α_1-AT was detectable in the supernatants, indicating intracellular retention of the ZAAT protein (fig. 1c). Treatment of 16HBE14o- cells with the ER stress agonist tunicamycin caused a significant increase in expression of the ER stress-responsive gene *grp78*. Similarly cells over expressing ZAAT, but not MAAT, also showed evidence of ER stress with *grp78* promoter activity being significantly increased (fig. 1d).

ZAAT, like MAAT, inhibits apoptosis in 16HBE14o- cells

We then transiently transfected 16HBE14o- cells with an empty vector, pZeoSV2, or MAAT or ZAAT expression plasmids and evaluated apoptosis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) staining 5 days post-transfection. Figure 2a shows that expression of ZAAT, like MAAT, inhibits basal apoptosis due to serum starvation in the cells. We also evaluated the effect of ZAAT on agonist-induced apoptosis. 24 h post-transfection cells were left untreated or stimulated with the ER agonist tunicamycin. Figure 2b shows that ZAAT is as effective as MAAT at inhibiting tunicamycin-induced apoptosis. For this experiment we also used qRTPCR to confirm that the cells were transfected efficiently. Cells transfected with MAAT or ZAAT expression plasmids showed up to a 300-fold increase in α_1-AT mRNA.

ZAAT inhibits caspase-3 activity in human bronchial epithelial cells

Caspase-3 is a key caspase involved in the apoptotic response. A range of pro-apoptotic stimuli, including the ER stress agonist thapsigargin, can activate caspase-3 [26]. Other factors, such as CSE and nicotine, can inhibit basal or agonist-induced caspase-3 activation [27, 28]. MAAT is known to inhibit caspase-3 activity [13, 14]. We evaluated the effect of ZAAT *versus* MAAT on caspase-3 activity per light unit, *i.e.* normalised for transfection efficiency. Figure 3a shows that expression of MAAT significantly decreased caspase-3 activity in human bronchial epithelial cells by $29.3 \pm 0.02\%$ ($p \leqslant 0.05$),

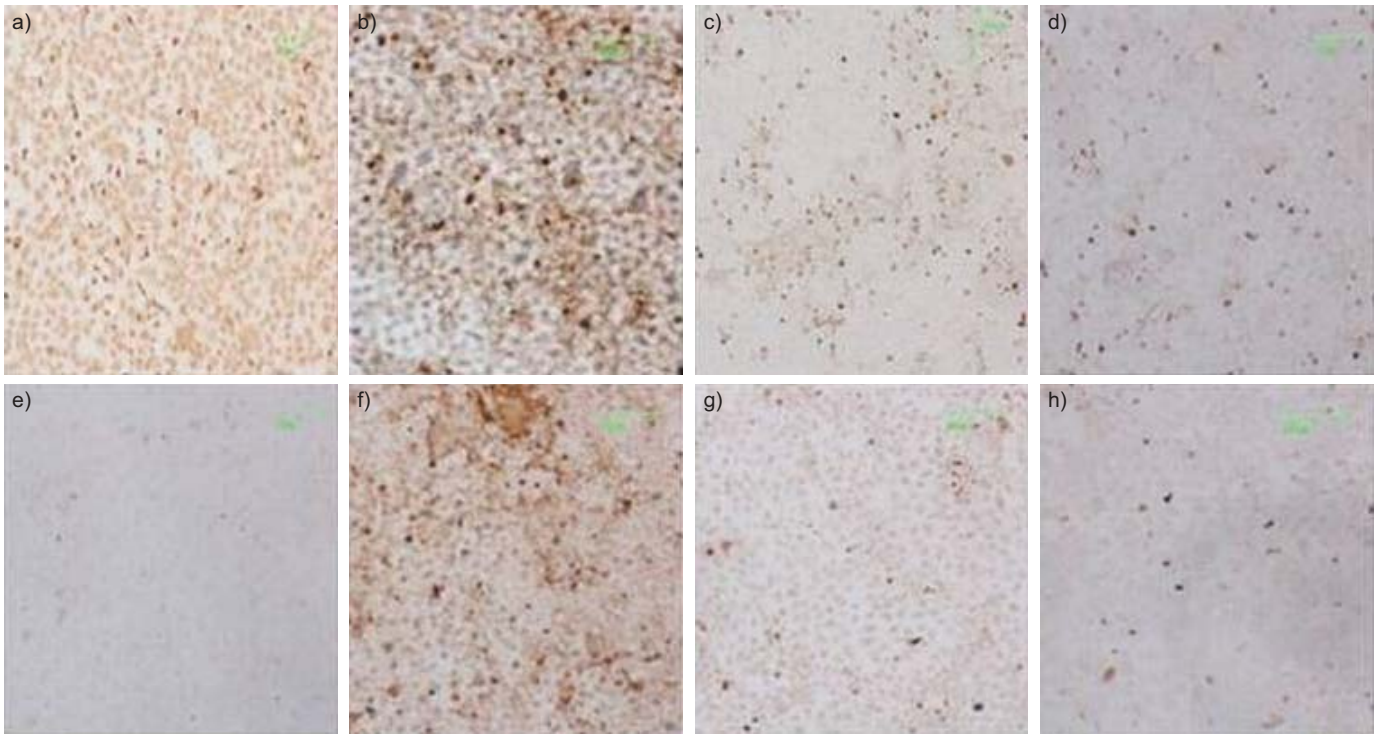

FIGURE 2. Effect of mutant Z α_1-antitrypsin (ZAAT) expression on apoptosis. a–d) 16HBE14o- cells (1×10^5) were a) DNAse-treated (positive control) or transfected with b) an empty vector (SV2), c) M variant α_1-AT (MAAT) or d) ZAAT expression plasmids and retained in serum-free medium for 5 days. e–h) 16HBE14o- cells (1×10^5) were transfected with e) an empty vector (SV2) or f) SV2, g) MAAT or h) ZAAT expression plasmids for 24 h then treated with tunicamycin (1 $\mu g \cdot mL^{-1}$). Apoptotic cells were detected using TUNEL labelling. Images are representative of three separate experiments.

compared with empty vector transfected cells. This observation is similar to the findings by PETRACHE and co-workers [13, 14] using rat and mice alveolar cells and endothelial cells. Expression of ZAAT had an even more pronounced effect on caspase-3 activity causing a decrease in activity by $56.7 \pm 0.02\%$ compared with control cells ($p \leqslant 0.05$). There was also a significant difference between the effects induced by MAAT and ZAAT ($p \leqslant 0.05$) indicating that ZAAT inhibits caspase-3 activity more strongly than MAAT in these cells. Thapsigargin (an ER stress agonist) and CSE/nicotine were used as positive (+) and negative (-) controls, respectively.

We also determined whether inhibition of caspase-3 activity and apoptosis led to an increase in cell proliferation by ZAAT. Unlike CSE which dose-dependently increased cell proliferation in 16HBE14o- cells, neither ZAAT nor MAAT had any effect on proliferation (fig. 3b).

ZAAT does not exert anti-apoptotic effects via *the phosphorylation of Bad or activation of HIF-1α*

Bad is a pro-apoptotic factor. Phosphorylation of Bad leads to its inactivation and inhibits apoptosis. Previously we demonstrated that treatment of HEK293 cells with TUDCA leads to phosphorylation and inactivation of Bad leading to increased cell survival. The mechanism by which this occurred involved the activation of phosphoinositide-3 kinase (PI3K) [4]. Nicotine can also induce phosphorylation and inactivation of Bad in alveolar cells, enhancing overall cell survival [29]. We investigated whether ZAAT regulates inactivation of Bad in bronchial epithelial cells. TUDCA, CSE and nicotine were used as positive controls [4, 29, 30] and led to phosphorylation of Bad compared with untreated cells. Neither ZAAT (fig. 4) nor MAAT (data not shown) had any effect on phosphorylation of Bad.

We also determined whether the transcription factor HIF-1α was activated by ZAAT as a possible anti-apoptotic mechanism. Compared to hypoxia-treated cells, expression of ZAAT had no effect on HIF-1α activity (data not shown). These data indicate that ZAAT does not utilise PI3K/Bad or HIF-1α signalling to inhibit apoptosis in human bronchial epithelial cells.

ZAAT upregulates cIAP1 gene expression in 16HBE14o- cells and in vivo *in bronchial epithelium*

Using qRTPCR we next assessed the effect of ZAAT on the expression of a selection of pro- and anti-apoptotic genes. Expression of the pro-apoptotic Bcl2 was unaffected by over expression of ZAAT (data not shown). Compared to empty vector-transfected cells, ZAAT induced 4-fold increase in the expression of the cellular inhibitor of apoptosis, cIAP1 (fig. 5a). MAAT expression led to less than a 2-fold increase. There were no changes in the expression of the anti-apoptotic factors Bax, cIAP2 or XIAP.

In order to reconcile the differences in our observations regarding the effects of ZAAT on apoptosis in 16HBE14o- and HEK293 cells [4] *i.e.* anti-apoptotic in 16HBE14o- cells and pro-apoptotic in HEK293 cells, we investigated the expression of cIAP1 in both cell lines in response to over expression of ZAAT (fig. 5b). Interestingly expression of ZAAT failed to induce cIAP1 expression in the HEK293 cells, providing a possible

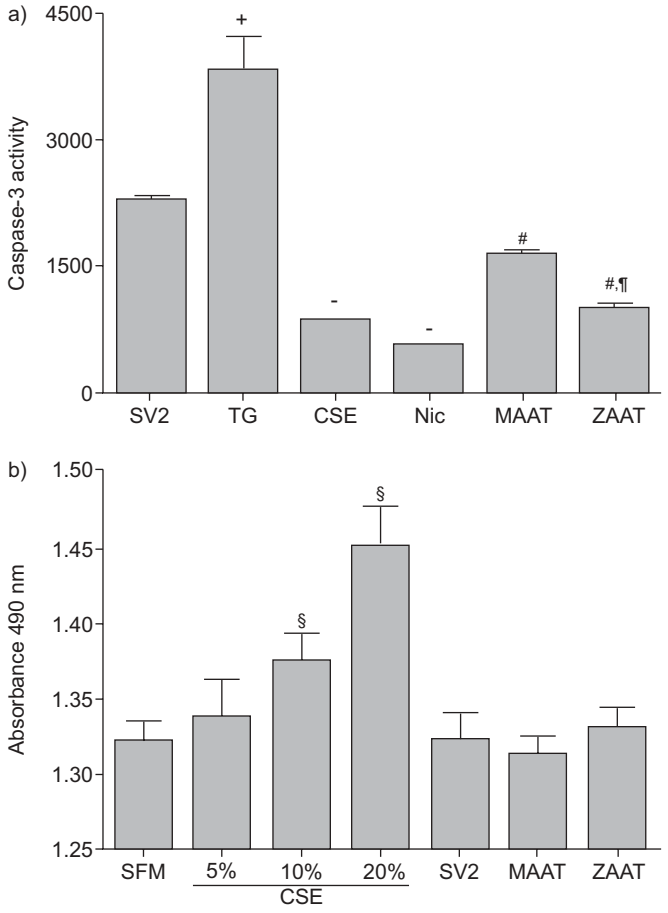

FIGURE 3. Effect of mutant Z α_1-antitrypsin (ZAAT) on caspase-3 activity and cell viability. a) 16HBE14o- cells (5×10^5) were co-transfected for 24 h with an empty vector (SV2), M variant α_1-AT (MAAT) or ZAAT expression plasmids and a pRLSV40-control luciferase expression vector. Cells were left untreated or those transfected with SV2 were treated with thapsigargin (TG; 0.5 μM), cigarette smoke extract (CSE; 20%) or nicotine (Nic; 1.5 μM) for 24 h and caspase-3 activity was quantified. b) 16HBE14o- cells (5×10^5) were left untreated or stimulated with CSE (as indicated for 24 h) or co-transfected with an empty vector (SV2), MAAT or ZAAT expression plasmids. Cell viability was quantified by reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Results are expressed in absorbance units at a wavelength of 490 nm ± SEM. Assays were performed in triplicate (n=3). +: positive control; -: negative control. #: *versus* SV2; ¶: *versus* MAAT; §: *versus* serum free medium (SFM).

explanation for the different apoptotic responses displayed by both cell lines in response to over expression of ZAAT.

We also evaluated whether our *in vitro* observations were replicated *in vivo* by using bronchial biopsies taken from MM and ZZ homozygous individuals and carrying out immunohistochemistry for cIAP1. Figure 5c shows that the bronchial epithelial cells in a MM biopsy stain only faintly for cIAP1. In contrast, cIAP1 expression is clearly detectable and is largely compartmentalised to the bronchial epithelial cells in a ZZ biopsy.

ZAAT expression activates NF-κB

cIAP1 is an upstream regulator of the cytoprotective transcription factor NF-κB. Having shown that ZAAT over expression

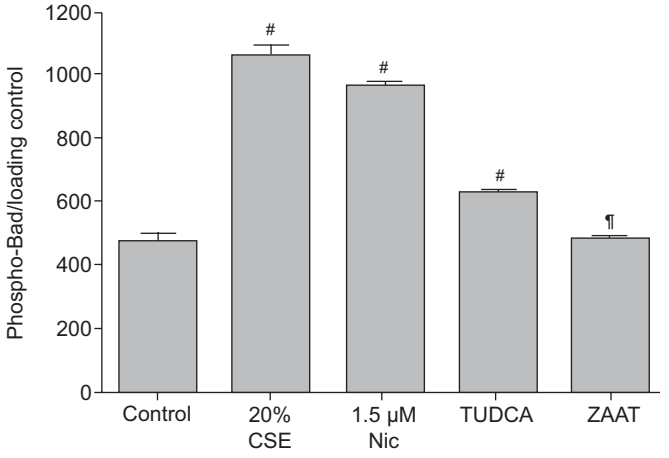

FIGURE 4. Effect of cigarette smoke extract (CSE), nicotine (Nic), tauroursodeoxycholic acid (TUDCA) and mutant Z α_1-antitrypsin (ZAAT) on phosphorylation of Bad. 16HBE14o- cells (2.5×10^6) were left untreated or were treated with 20% CSE, 1.5 μM nicotine or 300 μM TUDCA (24 h), or were transected with a ZAAT expression vector for 48 h. Protein extracts were prepared and equal amounts analysed for phospho-Bad and total protein by western blot analysis. The histogram shows densitometric analysis of the pBad/loading control ratios. Results are representative of three separate experiments. #: *versus* control; ¶: nonsignificant.

in 16HBE14o- cells can induce cIAP1 expression we investigated whether it could also lead to a concomitant increase in NF-κB activity. Figure 6 shows that ZAAT potently activates NF-κB in these cells. Expression of MAAT also activated NF-κB but the effect was less pronounced and is in keeping with the lower induction of cIAP1 by MAAT (fig. 5a). IL-1β was used as a positive control.

DISCUSSION

α_1-AT is principally a serine protease inhibitor. Other properties of α_1-AT include the ability to inhibit tumour necrosis factor (TNF)-α and matrix metalloproteinase in alveolar macrophages in response to thrombin and CSE [31], to impair lipopolysaccharide-induced monocyte activation and to block apoptosis [10–12]. With respect to apoptosis, α_1-AT has been shown to have a direct pro-survival effect in a model of apoptosis-dependent emphysema [13]. We investigated the pro-survival properties of ZAAT, a misfolded variant of α_1-AT associated with heritable emphysema.

Our data show that ZAAT, like MAAT, can inhibit caspase-3 activity in human bronchial epithelial cells. Interestingly, even taking into account potential differences due to transient transfection, the effect of ZAAT expression on caspase-3 activity was more pronounced than that of MAAT. It has been reported that misfolded polymeric forms of MAAT, induced by heating to 60°C for 2 h, cannot be internalised by lung endothelial cells and, thus, are unable to block caspase-3, nor can they directly inhibit caspase-3 *in vitro* [13]. Notwithstanding the differences between our ectopic expression studies and the protein transduction approach of PETRACHE and co-workers [13, 14] our data indicates that the ability of AAT to inhibit caspase-3 activity, *per se*, is not linked to misfolding due to the Glu342Lys Z mutation.

α_1-AT is produced locally in the lung by bronchial epithelial cells, amongst others [5, 8, 23–25]. In non-α_1-AT deficient

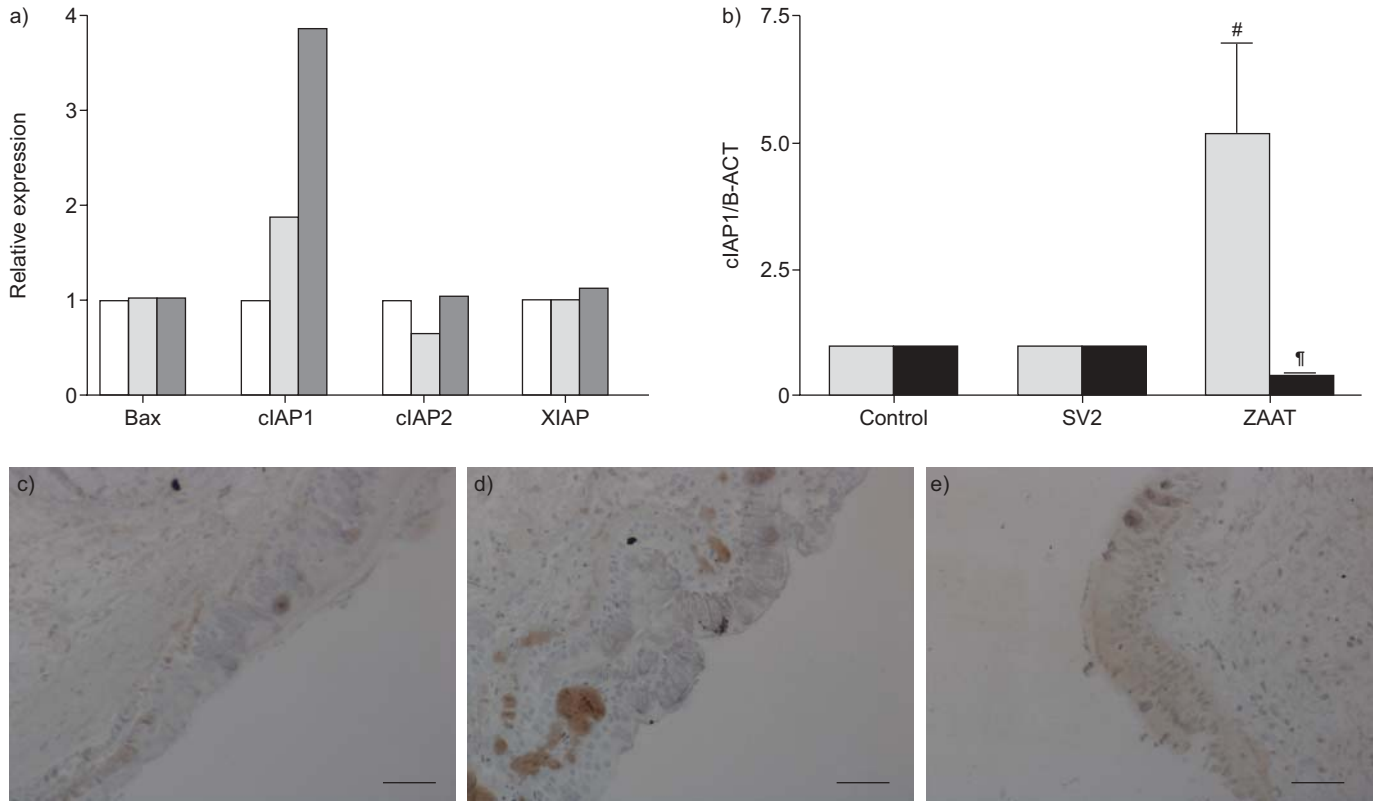

FIGURE 5. Effect of mutant Z α_1-antitrypsin (ZAAT) on anti-apoptotic gene expression in bronchial epithelial cells and HEK293 cells. a) 16HBE14o- cells were transfected with an empty vector (SV2; □), M variant α_1-AT (MAAT) (■) or ZAAT (■) expression plasmids for 48 h. Total RNA was isolated and the expression of Bax, cIAP1, cIAP2 and XIAP was analysed by qRT-PCR and normalised to β-actin. Assays were performed in duplicate twice. b) 16HBE14o- (■) or HEK293 (■) cells were left untransfected or transfected with an empty vector (SV2) or ZAAT expression plasmid for 48 h. Total RNA was isolated and the expression of cIAP1 was analysed by qRT-PCR and normalised for β-actin. Assays were performed in duplicate three times. B-ACT: β-actin. #: *versus* SV2; ¶: nonsignificant. c–e) Bronchial biopsies from MM (n=3) or ZZ (n=3) homozygous individuals were analysed for cIAP1 expression by immunohistochemistry. c) MM no antibody, d) MM+cIAP1 antibody and e) ZZ+cIAP1 antibody. Representative images are shown. Scale bars=50 μm.

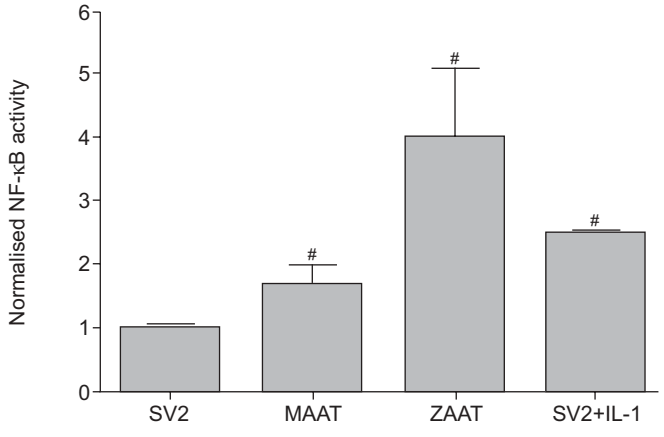

FIGURE 6. Effects of mutant Z α_1-antitrypsin (ZAAT) on nuclear factor (NF)-κB activation. 16HBE14o- cells (5×10^5) were co-transfected for 24 h with an empty vector (SV2), M variant α_1-AT (MAAT) or ZAAT expression plasmids, pRLSV40 (for transfection efficiency) and an NF-κB_5 promoter-linked luciferase reporter plasmid and left untreated or stimulated with interleukin (IL)-1 (10 ng·mL^{-1}, 6 h). NF-κB activity was quantified by luminometry and normalised to transfection efficiency. Assays were performed in triplicate a minimum of three times. #: *versus* SV2.

individuals, the α_1-AT that diffuses into the lung from the circulation is the more abundant source of α_1-AT in bronchoalveolar lavage fluid. Whilst it has been shown that α_1-AT can internalise into cells to directly inhibit caspase-3 [13] in an individual with ZAAT deficiency, although the potential to inhibit caspase-3 is still evident, the levels of ZAAT on the epithelial surface are probably inadequate to execute this event effectively and it is unlikely that polymerised ZAAT can be internalised by airway epithelial cells. Instead the ZAAT produced by bronchial epithelial cells themselves may have *cis*-acting effects that engender cell survival *in vivo*. Our immunohistochemistry studies evaluating cIAP1 expression support this. Thus, although ZAAT deficiency is associated with extensive alveolar epithelial cell apoptosis, bronchial cells in the ZAAT deficient lung may be protected from apoptotic death due to their expression of ZAAT.

Exposure of cells to ER stress leads to transcriptional induction of the inhibitor of apoptosis family of proteins *via* activation of the PKR-like ER kinase (PERK) [32]. In turn, PERK activity has been shown to inhibit ER stress-induced apoptosis by the induction of cellular inhibitor of apoptosis (cIAP1 and cIAP2) proteins [33]. The IAP family enhances cell survival in response to diverse stimuli [34]. Over expression of cIAP1 in

PERK$^{-/-}$ murine embryonic fibroblasts during ER stress has been shown to delay the early onset of ER stress-induced caspase activation and apoptosis observed in these cells [33]. Previously it was thought that IAPs can directly inhibit caspases; however, only XIAP exhibits strong binding to caspases [35, 36]. Instead there are at least two potential mechanisms by which IAPs achieve their anti-apoptotic effects. One is by suppression of TNF receptor type I signalling [37, 38]. However, we have shown that expression of cIAP1 is linked to NF-κB activation. cIAP1 has previously been shown to activate the non-canonical NF-κB pathway *via* a mechanism involving ubiquitination and proteosomal degradation of NF-κB inducing kinase (NIK) [39]. This group described how cIAPs can act as ubiquitin E3 ligases promoting NIK ubiquitination and degradation and concomitant activation of NF-κB.

An interesting phenomenon that we have observed in our studies is the cell type-specific properties of ZAAT with respect to caspase activation or inhibition. In HEK293 cells, expression of ZAAT strongly induces activation of caspase-3 leading to apoptosis in response to ER stress [4]. We found that using 16HBE14o- bronchial epithelial cells this response is not evident. Other studies have observed similar anti-caspase activity by MAAT either following ectopic expression or following internalisation of α_1-AT [13, 14]. The reason for this dichotomy is not entirely clear; however, we have shown that expression of cIAP1 may have an important role and hypothesise that it may be due to the ability of different cell types to respond to ER stress. In the course of the ER stress response, cells are forced to undergo apoptosis and the ability of different cell types to regulate cell survival, possibly *via* activation of cytoprotective NF-κB, would have a large impact on the outcome. Our observation that cIAP1 and NF-κB are activated by ZAAT in 16HBE14o- cells supports this theory. Interestingly we also detected increased expression of cIAP1 in ZAAT-expressing cells *in vivo*.

We investigated whether ZAAT may be exerting its anti-apoptotic effect *via* Bad or HIF-1α; however, there was no evidence to support this. We also investigated other potential mechanisms by which ZAAT may be exerting unique anti-apoptotic effects. We found no involvement of Bax, cIAP2 or XIAP, nor did we see any downregulation of the expression of pro-apoptotic factors, for example Bcl2, by ZAAT. Our data did reveal roles for cIAP1 and NF-κB activation.

Augmentation therapy with α_1-AT is the current treatment for the pulmonary manifestations of α_1-AT deficiency. This approach has the potential not only to redress the protease/antiprotease imbalance and dampen the inflammatory response on the airway surface but could also potentially inhibit apoptosis associated with the development of emphysema by inactivating caspase-3. We also observed upregulation of cIAP1 in response to over expression of MAAT, albeit less strongly than that induced by ZAAT. This suggests that the potential anti-apoptotic effects of augmentation therapy may be mediated, in part, *via* cIAP1.

Overall our results have identified a unique mechanism of bronchial epithelial cell survival induced by ZAAT involving upregulation of cIAP1 and activation of NF-κB.

SUPPORT STATEMENT

This study was funded by the Health Research Board of Ireland (Dublin, Ireland) and the Alpha One Foundation (Miami, FL, USA).

STATEMENT OF INTEREST

A statement of interest for T.P. Carroll can be found at www.erj.ersjournals.com/misc/statements.dtl

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