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Anti-apoptotic effects of Z alpha-1 antitrypsin in human bronchial epithelial cells

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Running Title: ZAAT inhibits apoptosis in airway epithelium

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Abstract

Z alpha-1 antitrypsin (AAT) deficiency is a genetic disease, which manifests as early-onset emphysema or liver disease. Although the majority of AAT is produced by the liver, it is also produced, amongst others, by bronchial epithelial cells in the lung. Here, we investigate the effects of ZAAT expression on apoptosis in a human bronchial epithelial cell line (16HBE14o-) and delineate mechanisms involved.

Control, MAAT- or ZAAT-expressing cells were assessed for apoptosis, caspase-3 activity, cell viability, phosphorylation of Bad, NFκB 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 to control cells ($p=0.05$) and was a more potent inhibitor than MAAT. Whilst ZAAT had no effect on activity of Bad, its expression activated NFκB-dependent gene expression above control or MAAT-expressing cells. In 16HBE14o- cells but not HEK293 cells, ZAAT up regulated expression of cIAP-1 an upstream regulator of NFκB. 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.

KEY WORDS

apoptosis, caspase-3, cIAP1, NFκB, Z alpha-1 antitrypsin

Introduction

Alpha-1-antitrypsin (AAT) deficiency is an inherited disorder with the alleles from both parents inherited in an autosomal co-dominant fashion. The disease manifests as early-onset panacinar emphysema or liver disease. The mutant ZAAT protein differs from normal M variant by a single amino acid substitution (Glu 342 $\rightarrow$ 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 AAT. The mutant ZAAT protein occurs in >95% individuals with AAT 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]. The liver however, is not the only source of AAT in the body. AAT 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 AAT is produced locally in the lung [8]. Bronchial epithelial cells may be an important source of this AAT.

It is becoming increasingly evident that the effect of AAT 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]. Others 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 AAT expression in bronchial epithelial cells has been reported to inhibit apoptotic 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 down regulation 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-1α (HIF-1 α). [16]. HIF-1α can be up regulated 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. VEGF [18].

Thus, given the emerging importance of apoptosis in the development of emphysema [13, 14, 19] here we investigate the effect of ZAAT on apoptosis in human bronchial epithelial cells, and compare our findings with the effects of MAAT. We also explore potential new mechanisms involved, specifically the roles of Bad, NFκB and a selection of pro- and anti-apoptotic genes and validate

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 (a gift from D. Gruenert, University of California, and San Francisco, CA) were grown in Eagle's MEM-Glutamax and 10 % FCS. Cells (5 x 10^5) were transiently transfected (Transfast, Promega) with 500 ng pZeoSV2(+) empty vector (Invitrogen), 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 NFκB activity assays cells were co-transfected with 300ng of a *grp78* promoter plasmid (a gift from Amy S. Lee USC/Norris Cancer Center) or an NFκB$_5$ promoter-linked luciferase reporter plasmid. The amount of DNA in each experiment was kept constant by addition of appropriate empty vector DNA. In experiments where fewer or more than 5 x 10^5 cells were used, the amounts of DNA were scaled down or up appropriately. Transfection efficiencies were quantified by luminometry (Victor^2 Wallac) measuring luciferase activity from the co-transfected pRLSV40-control using coelenterazine (Biotium) or by qRTPCR using AAT- and β-actin-specific primers.

TUNEL staining

16HBE14o- cells (1 x 10^5 cells/well) were grown on 4 well chamber slides, transfected and placed under serum free condition for a total of 5 days or left for 24 h then treated with tunicamycin for a further 24 hours. The DeadEnd Colorimetric TUNEL System (Promega) was used to detect apoptotic cells. Stained cells were visualised using a Nikon Coolscope digital microscope.

Cigarette Smoke Extract (CSE) Preparation

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 hour using a Victor^2 Wallac luminometer. 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 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 bioreduction by cells of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS 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 ºC and absorbance at a wavelength of 490nm measured at 1 and 2 hours

Western Immunoblotting

Triplicate wells of 2.5 x 10^6 cells were left untreated, treated with CSE, nicotine (Sigma) or tauroursodeoxycholic acid (TUDCA)(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 phenylmethylsulfonyl fluoride, 3% aprotinin and 1mM 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) or Erk2 (Santa Cruz) antibodies, a goat horseradish peroxidase-conjugated secondary antibody (DakoCytomation) and SuperSignal West Femto chemiluminescent substrate solution (Pierce). Images were recorded on X-ray film and densitometric analysis was performed using the GeneGenius Gel Documentation and Analysis System and Syngene GeneSnap and GeneTools software.

Hypoxia Inducible Factor-1α Activity Assays

Cells (8 x 10^5 cells/well) were transfected with pZAAT, left untransfected or underwent hypoxic challenge (1%O_2/5%CO_2/37ºC) for 24 h. Lysates were prepared in 2 ml of ice-cold PBS/Phosphate Inhibitor Buffer (125mM sodium fluoride, 250mM β-glycerophosphate, 250mM para-nitrophenyl phosphate

(PNPP) and 25mM sodium orthovanadate). HIF-1α activity was determined using the TransAM^{TM} HIF-1 Transcription Factor Assay Kit (Active Motif) according to the manufacturer's protocol.

qRTPCR

RNA was isolated using TRI reagent (Sigma) according to the manufacturers' instructions. Equal quantities of RNA were reverse transcribed into cDNA using the Quantitect Reverse Transcription Kit (Qiagen). The resulting cDNA was the template for quantitative real-time PCR. Oligonucleotide primers were synthesised (MWG Biotech) and quantitative PCR reactions performed in 20 µL containing 2 µL template cDNA, 2x SYBR Green master mix (Roche), and 10 pmoles of each primer (Table 1). Amplification was performed using the LightCycler 480 PCR system (Roche). 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 in Dublin. Two groups of patients were used in this study, 3 patients with known Pi ZZ genotype of AATD (2 male, 1 female, mean age 43.6 years) and 3 healthy volunteers (2 male, 1 female, mean age 45.3 years). This study was approved by the Beaumont Hospital Ethics Committee, and volunteers gave their informed consent in writing. Subjects recruited with AATD had diagnosis

confirmed with serum AAT level less than 11 μM and ZZ phenotype demonstrated by isoelectric focusing. None of the volunteers in the AATD group had any history of allergy, asthma, or other respiratory disease. Normal subjects were healthy non-smoking 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 nebulized salbutamol, and then 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) was introduced via the mouth. Two ml aliquots of 2% lignocaine were introduced via the bronchoscope and applied to the vocal cords, trachea, left and right mainstem 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 (Billerica). Biopsy specimens were fixed in 4% paraformaldehyde, and embedded in glycol methacrylate (GMA) resin.

Immunohistochemistry

Sections (2μm) were cut using an ultramicrotome (Leica), floated onto ammonia water (1:500), placed onto 0.01% poly-l-lysine glass slides (BDH), 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) 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). After washing in TBS, peroxidase was detected with 3,3-diaminobenzidine (DAB) chromogen (DAKO). All sections were counterstained with Mayer's haematoxylin (Sigma). 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.

NFκB- and grp78 promoter-luciferase assays

Cells (5 x 10^5) were co-transfected with SV2 (empty vector), pMAAT or pZAAT and with an $NF\kappa B_5$-luciferase reporter plasmid or a *grp78* promoter-linked luciferase reporter plasmid and pRLSV40 for 24h. Some cells were treated with DMSO (as a vehicle), tunicamycin (1 μg/ml, 24h), or IL-1β (10 ng/ml, 24h) (R&D Systems) 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). Results are expressed as mean +/- S.E.

and were compared by t-test as appropriate. Differences were considered significant when the p value was < 0.05.

RESULTS

Human bronchial epithelial 16HBE14o- cells express AAT.

It has been reported that a variety of human epithelial cells express AAT 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 AAT mRNA expression by 16HBE14o- cells (Figure 1a) and showed up-regulation of AAT gene expression in response to proinflammatory and ER stress stimuli.

Transfection of 16HBE14o- cells with MAAT or ZAAT *trans*genes led to a significant increase in AAT gene expression (Figure 1b). As measured by ELISA, of the total AAT protein produced by SV2-transfected cells, 28% was in the lysates and 72% was secreted into the supernatant (Figure 1c). Compared to SV2-transfected cells expression of MAAT led to a 22% overall increase in AAT protein production (data not shown). In ZAAT-expressing cells versus SV2-transfected cells there was a 16% increase in the amount of AAT detectable in the lysates whereas only a 0.25% increase in AAT was detectable in the supernatants, indicating intracellular retention of the ZAAT protein (Figure 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 (Figure 1d).

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

Next we transiently transfected 16HBE14o- cells with an empty vector, pZeoSV2, or MAAT or ZAAT expression plasmids and evaluated apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) 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. Twenty four hours 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 AAT 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 (L.U.) 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 ± 0.02 % (* $P \leq 0.05$), compared to empty vector transfected cells. This observation is similar to the findings by Petrache *et al.* using rat and mice alveolar cells and endothelial cells [13, 14]. Expression of

ZAAT had an even more pronounced effect on caspase-3 activity causing a decrease in activity by 56.7 ± 0.02 % compared to control cells (* $P \leq 0.05$). There was also a significant difference between the effects induced by MAAT and ZAAT (# $P \leq 0.05$) 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 (Figure 3b).

ZAAT does not exert its anti-apoptotic effects via the phosphorylation of Bad or activation of Hypoxia-inducible factor (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 activation of phosphoinositide 3 kinase (PI3K) [4]. Nicotine can also induce phosphorylation and inactivation of Bad in alveolar cells, enhancing overall cell survival [29]. Here we investigated whether ZAAT regulates inactivation of Bad in bronchial epithelial cells. TUDCA, CSE and nicotine which were used as positive controls [4, 29, 30], led to phosphorylation of Bad compared to untreated cells. Neither ZAAT (Figure 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 up regulates 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 (Figure 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 (Figure 5b). Interestingly expression of ZAAT failed to induce cIAP1 expression in the HEK293 cells, providing a possible 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 NFκB

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

DISCUSSION

AAT is principally a serine protease inhibitor. Other properties of AAT include the ability to inhibit TNFα and MMP in alveolar macrophages in response to thrombin and CSE [31], to impair LPS-induced monocyte activation and to block apoptosis [10-12]. With respect to apoptosis, AAT has been shown to have a direct pro-survival effect in a model of apoptosis-dependent emphysema [13]. Here, we have investigated the pro-survival properties of ZAAT, a misfolded variant of AAT 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 ^{o}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 *et al* [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.

AAT is produced locally in the lung by bronchial epithelial cells, amongst others [5, 8, 23-25]. In non-AAT deficient individuals the AAT that diffuses into the lung from the circulation is the more abundant source of AAT in bronchoalveolar lavage fluid. Whilst it has been shown that AAT 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 likely 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, here we show that expression of cIAP1 is linked to NFκB activation. cIAP1 has previously been shown to activate the non-canonical NFκB pathway via a mechanism involving ubiquitination and proteosomal degradation of NFκB -inducing kinase (NIK) [39]. This group

described how cIAPs can act as ubiquitin E3 ligases promoting NIK ubiquitination and degradation and concomitant activation of NFκB.

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]. Here, using 16HBE14o- bronchial epithelial cells this response is not evident. Others have observed similar anti-caspase activity by MAAT either following ectopic expression or following internalisation of AAT [13, 14]. The reason for this dichotomy is not entirely clear, however, we show here that expression of cIAP1 may have an important role and hypothesize that it may be due 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 NFκB, would have a large impact on the outcome. Our observation that cIAP1 and NFκB are activated by ZAAT in 16HBE14o- cells supports this. Interestingly we also detected increased expression of cIAP1 in ZAAT-expressing cells *in vivo*.

Here we tested 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 down regulation of the expression of pro-apoptotic factors, for example Bcl2, by ZAAT. Our data did reveal roles for cIAP1 and NFκB activation.

Augmentation therapy with AAT is the current treatment for the pulmonary manifestations of AAT deficiency. This approach has the potential not only to redress the protease/antiprotease imbalance and dampen the inflammatory response on the airway surface but also could potentially inhibit apoptosis associated with the development of emphysema by inactivating caspase-3. Here we also observed up regulation 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 studies have identified a unique mechanism of bronchial epithelial cell survival induced by ZAAT involving up regulation of cIAP1 and activation of NFκB.

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REFERENCES

1. McCracken AA, Kruse KB, Brown JL. Molecular basis for defective secretion of the Z variant of human alpha-1-proteinase inhibitor: secretion of variants having altered potential for salt bridge formation between amino acids 290 and 342. *Mol Cell Biol* 1989: 9(4): 1406-1414.

2. Lomas DA, Evans DL, Finch JT, Carrell RW. The mechanism of Z alpha 1-antitrypsin accumulation in the liver. *Nature* 1992: 357(6379): 605-607.

3. Brantly M, Nukiwa T, Crystal RG. Molecular basis of alpha-1-antitrypsin deficiency. *Am J Med* 1988: 84(6A): 13-31.

4. Miller SD, Greene CM, McLean C, Lawless MW, Taggart CC, O'Neill SJ, McElvaney NG. Tauroursodeoxycholic acid inhibits apoptosis induced by Z alpha-1 antitrypsin via inhibition of Bad. *Hepatology* 2007: 46(2): 496-503.

5. Mason DY, Cramer EM, Masse JM, Crystal R, Bassot JM, Breton-Gorius J. Alpha 1-antitrypsin is present within the primary granules of human polymorphonuclear leukocytes. *Am J Pathol* 1991: 139(3): 623-628.

6. Molmenti EP, Perlmutter DH, Rubin DC. Cell-specific expression of alpha 1-antitrypsin in human intestinal epithelium. *J Clin Invest* 1993: 92(4): 2022-2034.

7. Travis J, Shieh BH, Potempa J. The functional role of acute phase plasma proteinase inhibitors. *Tokai J Exp Clin Med* 1988: 13(6): 313-320.

8. 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(5): 1952-1957.

9. 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(47): 39002-39015.

10. Daemen MA, Heemskerk VH, van't Veer C, Denecker G, Wolfs TG, Vandenabeele P, Buurman WA. Functional protection by acute phase proteins alpha(1)-acid glycoprotein and alpha(1)-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. *Circulation* 2000: 102(12): 1420-1426.

11. Ikebe N, Akaike T, Miyamoto Y, Hayashida K, Yoshitake J, Ogawa M, Maeda H. Protective effect of S-nitrosylated alpha(1)-protease inhibitor on hepatic ischemia-reperfusion injury. *J Pharmacol Exp Ther* 2000: 295(3): 904-911.

12. Ikari Y, Mulvihill E, Schwartz SM. alpha 1-Proteinase inhibitor, alpha 1-antichymotrypsin, and alpha 2-macroglobulin are the antiapoptotic factors of vascular smooth muscle cells. *J Biol Chem* 2001: 276(15): 11798-11803.

13. Petrache I, Fijalkowska I, Zhen L, Medler TR, Brown E, Cruz P, Choe KH, Taraseviciene-Stewart L, Scerbavicius R, Shapiro L, Zhang B, Song S, Hicklin D, Voelkel NF, Flotte T, Tuder RM. A novel antiapoptotic role for alpha1-antitrypsin in the prevention of pulmonary emphysema. *Am J Respir Crit Care Med* 2006: 173(11): 1222-1228.

14. Petrache I, Fijalkowska I, Medler TR, Skirball J, Cruz P, Zhen L, Petrache HI, Flotte TR, Tuder RM. alpha-1 antitrypsin inhibits caspase-3 activity, preventing lung endothelial cell apoptosis. *Am J Pathol* 2006: 169(4): 1155-1166.

15. Aldonyte R, Hutchinson ET, Jin B, Brantly M, Block E, Patel J, Zhang J. Endothelial alpha-1-antitrypsin attenuates cigarette smoke induced apoptosis in vitro. *COPD* 2008: 5(3): 153-162.

16. Downward J. How BAD phosphorylation is good for survival. *Nat Cell Biol* 1999: 1(2): E33-35.

17. Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *Am J Physiol* 1996: 271(4 Pt 1): C1172-1180.

18. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 2000: 88(4): 1474-1480.

19. Tuder RM, Petrache I, Elias JA, Voelkel NF, Henson PM. Apoptosis and emphysema: the missing link. *Am J Respir Cell Mol Biol* 2003: 28(5): 551-554.

20. Lawless MW, Greene CM, Mulgrew A, Taggart CC, O'Neill SJ, McElvaney NG. Activation of endoplasmic reticulum-specific stress responses associated with the conformational disease Z alpha 1-antitrypsin deficiency. *J Immunol* 2004: 172(9): 5722-5726.

21. Glader P, Moller S, Lilja J, Wieslander E, Lofdahl CG, von Wachenfeldt K. Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. *Respir Med* 2006: 100(5): 818-827.

22. Wang H, Liu X, Umino T, Skold CM, Zhu Y, Kohyama T, Spurzem JR, Romberger DJ, Rennard SI. Cigarette smoke inhibits human bronchial epithelial cell repair processes. *Am J Respir Cell Mol Biol* 2001: 25(6): 772-779.

23. Cichy J, Potempa J, Travis J. Biosynthesis of alpha1-proteinase inhibitor by human lung-derived epithelial cells. *J Biol Chem* 1997: 272(13): 8250-8255.

24. Venembre P, Boutten A, Seta N, Dehoux MS, Crestani B, Aubier M, Durand G. Secretion of alpha 1-antitrypsin by alveolar epithelial cells. *FEBS Lett* 1994: 346(2-3): 171-174.

25. Hu C, Perlmutter DH. Cell-specific involvement of HNF-1beta in alpha(1)-antitrypsin gene expression in human respiratory epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002: 282(4): L757-765.

26. McColl KS, He H, Zhong H, Whitacre CM, Berger NA, Distelhorst CW. Apoptosis induction by the glucocorticoid hormone dexamethasone and the calcium-ATPase inhibitor thapsigargin involves Bc1-2 regulated caspase activation. *Mol Cell Endocrinol* 1998: 139(1-2): 229-238.

27. Stringer KA, Tobias M, O'Neill HC, Franklin CC. Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis. *Am J Physiol Lung Cell Mol Physiol* 2007: 292(6): L1572-1579.

28. Hakki A, Pennypacker K, Eidizadeh S, Friedman H, Pross S. Nicotine inhibition of apoptosis in murine immune cells. *Exp Biol Med (Maywood)* 2001: 226(10): 947-953.

29. Jin Z, Gao F, Flagg T, Deng X. Nicotine induces multi-site phosphorylation of Bad in association with suppression of apoptosis. *J Biol Chem* 2004: 279(22): 23837-23844.

30. Palozza P, Sheriff A, Serini S, Boninsegna A, Maggiano N, Ranelletti FO, Calviello G, Cittadini A. Lycopene induces apoptosis in immortalized fibroblasts exposed to tobacco smoke condensate through arresting cell cycle and down-regulating cyclin D1, pAKT and pBad. *Apoptosis* 2005: 10(6): 1445-1456.

31. Churg A, Wang RD, Xie C, Wright JL. alpha-1-Antitrypsin ameliorates cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med* 2003: 168(2): 199-207.

32. Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC. Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. *Mol Cell Biol* 1998: 18(12): 7499-7509.

33. Hamanaka RB, Bobrovnikova-Marjon E, Ji X, Liebhaber SA, Diehl JA. PERK-dependent regulation of IAP translation during ER stress. *Oncogene* 2009: 28(6): 910-920.

34. Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, Fairbrother WJ, Vucic D. c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. *J Biol Chem* 2008: 283(36): 24295-24299.

35. Eckelman BP, Salvesen GS. The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *J Biol Chem* 2006: 281(6): 3254-3260.

36. Eckelman BP, Salvesen GS, Scott FL. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep* 2006: 7(10): 988-994.

37. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* 1995: 83(7): 1243-1252.

38. Shu HB, Takeuchi M, Goeddel DV. The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proc Natl Acad Sci U S A* 1996: 93(24): 13973-13978.

39. Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliott LO, Wallweber HJ, Flygare JA, Fairbrother WJ, Deshayes K, Dixit VM, Vucic D. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 2007: 131(4): 669-681.

Table 1. PCR primers

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

Figure Legends

Figure 1. AAT expression in 16HBE14o- cells. (A) 16HBE14o$^-$ cells (1 x 10^5) were left unstimulated or treated with LPS (10 μg/ml) or tunicamycin (1 μg/ml) for 24 h. Total RNA was isolated and the expression of AAT was analysed by qRT-PCR and normalised for β–actin (* vs. untreated cells), (B-D) 16HBE14o$^-$ cells (5 x 10^5) were co-transfected for 24 h with an empty vector (SV2), MAAT or ZAAT expression plasmids and (B) total RNA was isolated and the expression of AAT was analysed by qRT-PCR and normalised for β–actin (* vs. SV2), or (C) supernatants and lysates were assayed for AAT protein expression by ELISA or (D) following co-transfection with a pRLSV40-control luciferase expression vector and a *grp78* promoter-linked luciferase reporter plasmid cells were left untreated, treated with DMSO (as a vehicle) or tunicamycin (1 μg/ml) for 24 h as indicated. *grp78*-promoter activity was quantified by luminometry and normalized to transfection efficiency (* vs. DMSO or SV2). All assays were performed in duplicate three times.

Figure 1

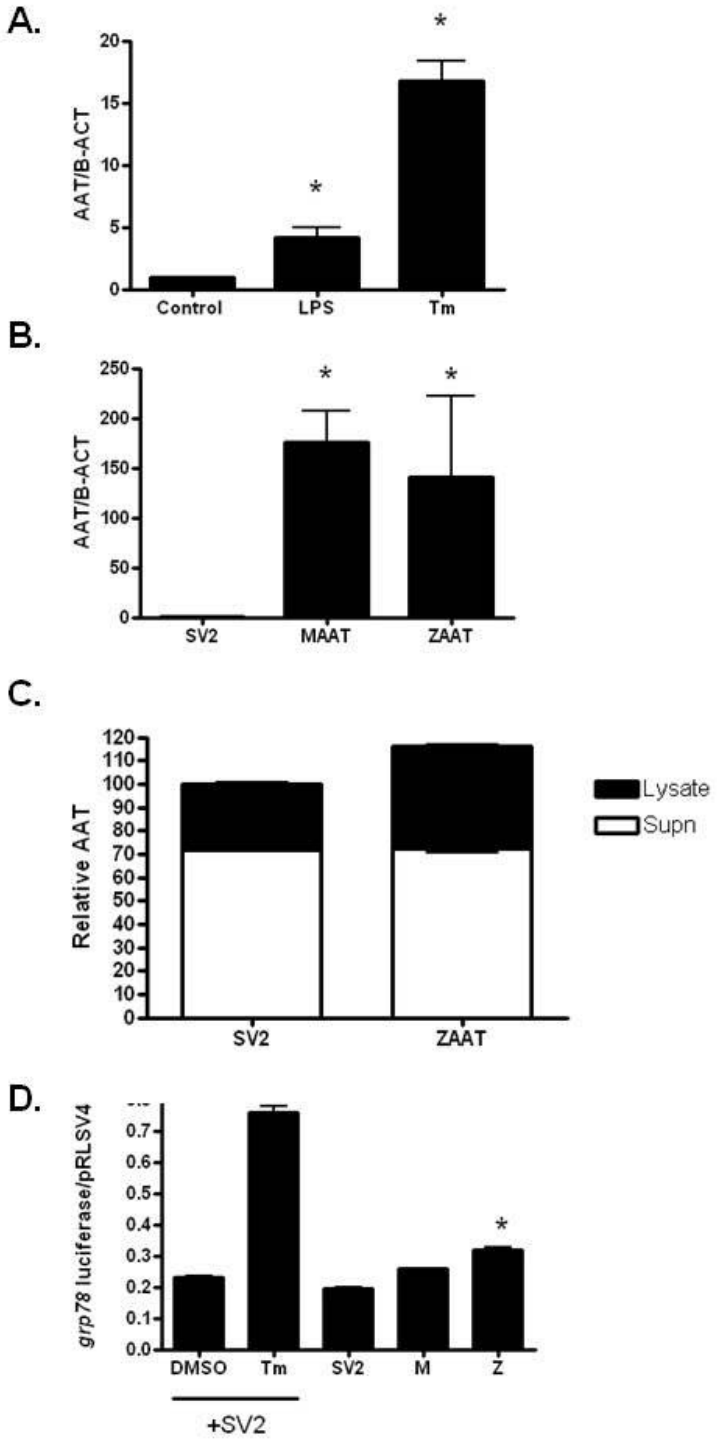

A.
AAT/B-ACT
20
15
10
5
0
Control
LPS
Tm
*
*
B.
AAT/B-ACT
250
200
150
100
50
0
SV2
MAAT
ZAAT
*
*
C.
Relative AAT
120
110
100
90
80
70
60
50
40
30
20
10
0
SV2
ZAAT
Lysate
Supn
D.
grp78 luciferase/pRLSV4
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DMSO
Tm
SV2
M
Z
*
+SV2

Figure 2. Effect of ZAAT expression on apoptosis. 16HBE14o$^-$ cells (1 x 10^5) were (A) DNAse-treated (positive control) or transfected with an empty vector (SV2), MAAT or ZAAT expression plasmids and retained in serum-free medium for 5 days or (B) transfected with an empty vector (SV2), MAAT or ZAAT expression plasmids for 24 h then treated with tunicamycin (1 μg/ml, 24 h) as indicated. Apoptotic cells were detected using TUNEL labelling. Images are representative of three separate experiments

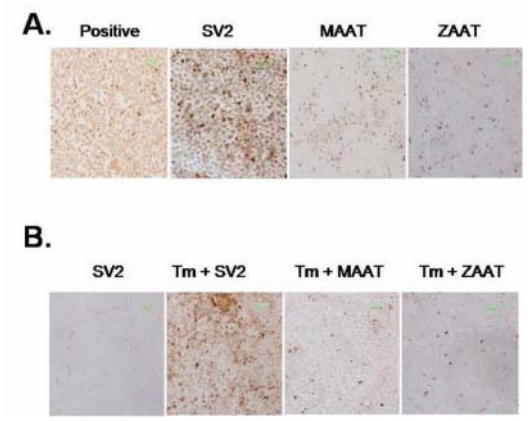
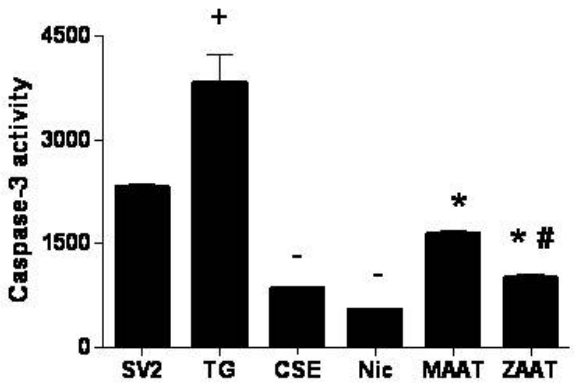


Figure 3. Effect ZAAT on caspase-3 activity and cell viability. (A) 16HBE14o$^-$ cells (5 x 10^5) were co-transfected for 24 h with an empty vector (SV2), MAAT or ZAAT expression plasmids and a pRLSV40-control luciferase expression vector. Cells were left untreated or those transfected with SV2 were treated with thapsigargin (TG, 0.5 μM), CSE (20%) or nicotine (1.5 μM) for 24h and caspase-3 activity was quantified (* vs. SV2, # vs. MAAT). Assays were performed in triplicate (n=3). (B) 16HBE14o$^-$ cells (5 x 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. Results are expressed in absorbance units at a wavelength of 490nm ± S.E.M.. * vs. SFM. Assays were performed in triplicate (n=3).

Figure 3

A.


Caspase-3 activity
4500
3000
1500
0
+
-
-
*
* #
SV2
TG
CSE
Nic
MAAT
ZAAT

B.

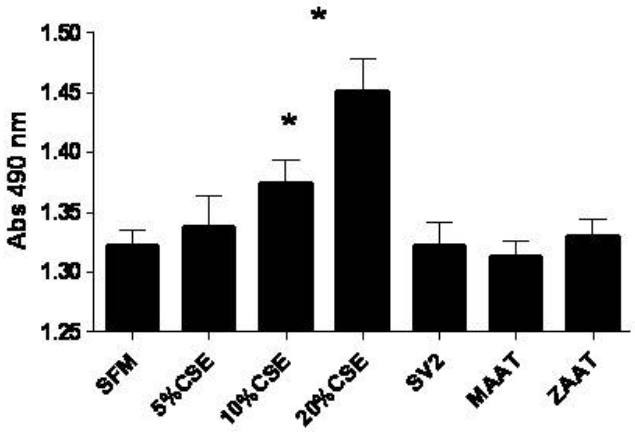

Abs 490 nm
1.50
1.45
1.40
1.35
1.30
1.25
*
*
SFM
5%CSE
10%CSE
20%CSE
SV2
MAAT
ZAAT

Figure 4. Effect of CSE, nicotine, TUDCA and ZAAT on phosphorylation of Bad. 16HBE14o$^-$ cells (2.5 x 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 (* vs. control). Results are representative of three separate experiments.

Figure 4

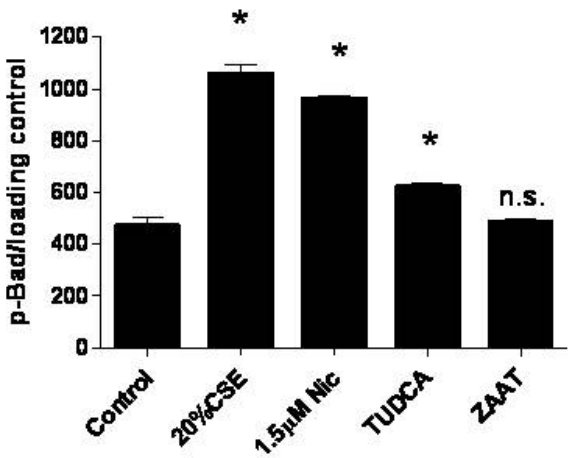

p-Bad/loading control
1200
1000
800
600
400
200
0
*
*
*
n.s.
Control
20%CSE
1.5μM Nic
TUDCA
ZAAT

Figure 5. Effect of ZAAT on anti-apoptotic gene expression in bronchial epithelial cells and HEK293 cells (A) 16HBE14o- cells were transfected with an empty vector (SV2), 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 and 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 (* vs. SV2). (C) Bronchial biopsies from MM (n=3) or ZZ (n=3) homozygous individuals were analysed for cIAP1 expression by immunohistochemistry as indicated. Representative images are shown.

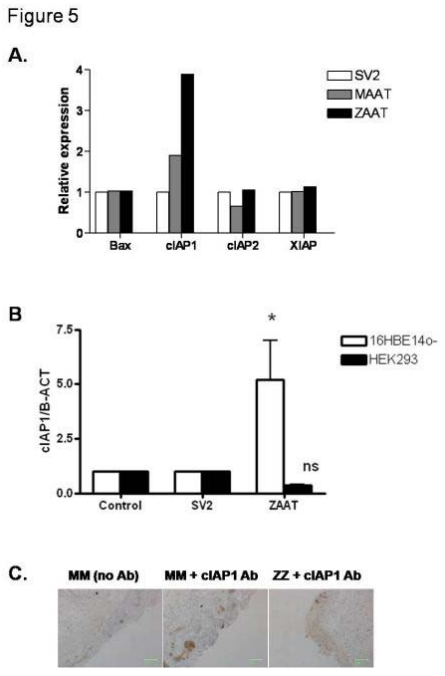


Figure 6. Effects of ZAAT on NFκB activation. 16HBE14o$^-$ cells (5 x 10^5) were co-transfected for 24 h with an empty vector (SV2), MAAT or ZAAT expression plasmids, pRLSV40 (for transfection efficiency) and an NFκB$_5$ promoter-linked luciferase reporter plasmid and left untreated or stimulated with IL-1 (10 ng/ml, 6h). NFκB activity was quantified by luminometry and normalized to transfection efficiency (* vs. SV2). Assays were performed in triplicate a minimum of three times.

Figure 6