

ORIGINAL ARTICLE

T cells and eosinophils in bronchial smooth muscle cell death in asthma

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Summary

Background Bronchial smooth muscle cells (SMC) proliferate, express adhesion molecules, secrete cytokines and thus efficiently contribute to the pathogenesis of asthma.**Objective** The aim of the study was to investigate whether, and by which mechanism, T cells and eosinophils can cause death of airway SMC.**Methods** The T cell- and eosinophil-induced cell death was analysed in primary human bronchial SMC cultures as well as in bronchial biopsy specimens from non-asthmatic and asthmatic individuals.**Results** Bronchial SMC death showed characteristic morphological features of apoptosis in 3–6 days cultures with inflammatory cytokines (IFN- γ , TNF- α), soluble death ligands [sFasL, TNF-related apoptosis-inducing ligand (TRAIL)] and activated T-helper type 1 (Th1) and Th2 cell supernatants. The recombinant eosinophil cationic protein induced SMC necrosis within 1 h. Resting SMC expressed the death receptors TNFR1, TNFR2, Fas, TRAILR1, TRAILR2 and membrane FasL as a death-inducing ligand. IFN- γ and TNF- α up-regulated TNFR1, TNFR2, Fas and membrane FasL on SMC. TNF- α up-regulated TRAILR1 and TRAILR2; sFasL up-regulated TNFR2. The intracellular caspase-3 activation in SMC was significantly increased by IFN- γ , sFasL, TRAIL, Th1 and Th2 cell supernatants. Increased expression of TRAIL in asthmatics, but not in non-asthmatic individuals was demonstrated *in situ*. The apoptosis receptors TRAILR1 and TRAILR2 were expressed in SMC and epithelial cells both in healthy and asthmatic biopsies. Prominent apoptosis of SMC was observed in fatal asthma, but not intermittent asthma biopsies.**Conclusion** The demonstration of bronchial SMC death both by apoptosis and necrosis indicates the essential role of T cells and eosinophils in the bronchial tissue injury particularly in the severe asthma.**Keywords** apoptosis, asthma, IFN- γ , smooth muscle, TNF- α *Submitted 11 July 2008; revised 27 November 2008; accepted 5 January 2009*

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Introduction

Persistent airway inflammation and irreversible structural changes of the bronchial wall, defined as airway remodelling, are crucial processes in asthma development [1]. An altered expression and/or function of a large variety of pro-inflammatory cytokines, growth factors and their receptors, as well as a dysregulation in the differentiation, proliferation and apoptosis of multiple cell types results in epithelial damage, subepithelial basement membrane

thickness, subepithelial fibrosis, leucocyte infiltrates with predominant eosinophils and T cells, as well as mucous gland and smooth muscle cells (SMC) hypertrophy, hyperplasia and dysfunction [1]. The increase in SMC content can explain the mechanical consequences of airway remodelling, such as airway luminal narrowing and permanent reduction of the airway calibre [1].

Certain cytokines, most notably specific T-helper type 2 (Th2) cytokines and IL-1 β , have been shown to directly regulate airway smooth muscle responsiveness [2]. Growing evidence has been provided that T cells play a role in chronic asthma [3]. T cells are well-documented

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inflammatory cells in asthma, infiltrating bronchial mucosa [2] as well as airway smooth muscle layer [4]. Large numbers of activated eosinophils are present in the mucosa of asthmatic patients and they are thought to be the central effector cells in asthma [4]. The numbers of activated T cells and eosinophils detected in the bronchial mucosa correlate with disease severity [4]. Bidirectional stimulatory cross-talk has been demonstrated between activated T cells and airway SMCs, a process that results in the induction of asthmatic changes in airway SMC responsiveness [5]. In addition, eosinophils can be the source of pro-inflammatory cytokines including GM-CSF, IL-4, IL-5, IL-6, IL-8 and TNF- α [6]. Recent studies have highlighted the relationship between T cells and eosinophils and their effector functions, leading to epithelial cell apoptosis in allergic diseases such as asthma and atopic dermatitis as key tissue injury mechanisms [7, 8]. These studies demonstrate that IFN- γ , TNF- α and Fas-ligand play essential roles in the allergic inflammation in eczema/spongiosis formation in atopic eczema and bronchial epithelial shedding in asthma. Eosinophils can also induce direct damage to cells of the inflammatory environment by granule products, such as eosinophil cationic protein (ECP), major basic protein (MBP) and eosinophil peroxidase (EPO) [6]. The threshold for IFN- γ response of epithelial cells is relatively low, so that a Th2-like cell, or a Th0 cell, which secretes small quantities of IFN- γ and TNF- α can also induce epithelial cell apoptosis.

The TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis of many transformed but also non-transformed cells. In addition, TRAIL receptor (TRAILR) activation has been reported to trigger non-apoptotic signalling pathways. Five different receptors have been identified to interact with TRAIL. The TRAILR1/death receptor 4 (DR4) and TRAILR2/DR5 are the genuine death receptors, while the TRAILR3/DcR1, TRAILR4/DcR2 and osteoprotegerin function as decoy receptors, which do not transmit apoptotic signals [9, 10]. Triggering of Fas, TNFR1, TRAILR1 and TRAILR2 by specific ligands leads to the recruitment of the Fas-associated death domain (FADD) to the membrane. FADD recruits procaspase-8 leading to the activation of effector caspases, such as caspase-3, followed by the DNA fragmentation and cell death [11].

The aim of this study was to investigate whether, and by which mechanism, T cells and eosinophils, present in the SMC tissue or in its nearest proximity, can cause death of airway SMC. Here we show that bronchial SMCs are an important target of the inflammatory attack by T cells and eosinophils. The excessive apoptosis may play a role in remodelling, mainly in the change of tissue localization of SMCs. The consequent turnover of the SMCs may finally account for morphological changes and functional contraction/relaxation disorder in bronchial wall, particularly in severe forms of asthma.

Material and methods

Subjects

A total of 10 asthmatic patients (seven severe, three intermittent, age range 42–60 years) and six control subjects (age range 40–63 years) were investigated. Standard pulmonary function testing was performed in all subjects. Asthma was diagnosed by a pneumologist and was confirmed by an improvement in forced expiratory volume in 1 s (FEV₁) of 12%, with an absolute increase of ≥ 200 mL from baseline, after administration of a β -adrenergic agonist or by a $\geq 20\%$ improvement in FEV₁ after a steroid trial. Severe asthma was defined according to criteria adapted from an American Thoracic Society workshop on refractory asthma [12]. The patients were all treated with 1600 μ g/day beclomethasone or equivalent and long-acting bronchodilators and were all non-smokers (smoking history ≤ 10 pack-year). All patients were symptomatic and had experienced at least one severe exacerbation during the past year or were dependent on maintenance therapy with oral prednisone. The study was approved by the Local Medical Ethics Committee and all patients gave informed consent. Biopsy samples from non-asthmatic (control subjects) individuals were from non-involved area of four lung adenocarcinoma patients and two patients suffering from chronic cough, who did not show any features of atopy and asthma. Biopsy specimens from two fatal asthma cases were also examined. The histology sections of these two patients were obtained from the ongoing research project of the University of Sao Paulo, Brazil, and the anonymized tissue slides were kindly provided by Dr Thais Mauad.

Procedures

Bronchoscopy was performed according to standard procedures. A total of four to six biopsy specimens per patient were taken at the carina of segmental bronchi using cup forceps. Biopsy specimens were placed in Tris-buffered saline solution and immediately transported to the laboratory for processing. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were then sectioned (5 μ m thickness) and placed on glass slides coated with poly-L-lysine (0.1%) and baked overnight at 37 °C. Results are shown as means $\pm$ SD. Wilcoxon's rank sum test and Mann-Whitney *U*-test were used for statistical evaluation.

Reagents

Human recombinant IFN- γ and IL-5 were purchased (Peprotech EC Ltd, London, UK); GM-CSF was a gift (Novartis Pharma, Basel, Switzerland). Ethidium bromide was purchased (Sigma Chemical Co., St Louis, MO, USA).

The following antibodies (Abs) were purchased: anti-FasL-PE, anti-TRAIL 1 and 2-FITC (Alexis Corp., San Diego, CA, USA); anti-Fas-FITC (Immunotech Coulter Company, Mar-seilles, France); anti-TNFR1-PE and anti-TNFR2-FITC (R&D Systems Europe Ltd, Abingdon, UK); anti-active caspase-3 – PE (BD Pharmingen, Basel, Switzerland). Iso-type Control IgG1-FITC/PE monoclonal antibody (mAb) was from Beckman Coulter. Human recombinant soluble FasL, TRAIL, TNF- α , and TWEAK were from Alexis Corp., propidium iodide (PI) was purchased from Fluka (Sigma-Aldrich Co., Buchs, Switzerland), annexin V-FITC from BD Pharmingen. The following cytokines and Abs were used in T cells cultures: IL-2 (Roche, Basel, Switzerland); anti-IL-12 and IL-12 (BD Pharmingen); IL-4 and anti-IL-4 (Novartis Pharma). Anti-CD2 (clones 6G4 and 4B2) and anti-CD28 were purchased from Sanquin Blood Supply Foundation, Amsterdam, the Netherlands. Anti-CD3 has been produced from clone CRL 8001 obtained from ATCC (Manassas, VA, USA). Ionomycin, phorbol myristate acetate, phytohaema-glutinin and brefeldin were purchased from Sigma Chemi-cal Co. Anti-TRAIL mAb were from R&D Systems Europe Ltd, anti-TRAILR1 Ab and anti-TRAILR2 Abs were from Abcam Ltd (Cambridge, UK).

Cell cultures

Primary (passage 3 and 4) normal bronchial SMC were purchased (Clonetics–BioWhittaker, Cambrex Company, San Diego, CA, USA). Cells were grown in growth medium (basal SMC growth medium – SMCGM), supplemented with human recombinant epidermal growth factor 0.5 ng/mL, insulin 5 μ g/mL, human recombinant fibroblast growth factor 2 ng/mL, gentamicin 5 μ g/mL, amphotericin B 5 μ g/mL, 5% fetal bovine serum, in humidified atmo-sphere containing 5% CO₂, at 37 °C. Bronchial SMCs were seeded into 24- or 48-well culture plates (30 000 and 15 000/well, respectively) in supplemented SMC growth medium and incubated for 2 days to form a monolayer at the bottom of plates. Afterwards different stimulants were added to the SMC cultures in the growth phase of the cell cycle and incubated for various time-points between 1 h for ECP and up to 6 days (other stimulants). To determine single concentrations of the stimulants, several dose response experiments were performed, and optimal con-centrations were determined and used in further experi-ments. Similarly, the SMC dose response analysis to different ECP concentrations was also performed. Cells were harvested by trypsinization (trypsin/EDTA solution, neutralized by trypsin neutralizing solution, Clonetics–BioWhittaker).

Flow cytometry

Bronchial SMCs were incubated with appropriate FITC- or PE-labelled mAb for 45 min at 4 °C (receptors staining) or

15 min at room temperature for annexin V and PI staining and then fixed with 4% paraformaldehyde in PBS (pH 7.4). For anti-active caspase-3 intracellular staining cells were fixed and permeabilized according to the manufacturer's protocol (Cytofix/Cytoperm solution, Becton Dickinson, Franklin Lakes, NJ, USA) and incubated with anti-active caspase-3 mAb for 30 min at room temperature. Negative control cells were prepared in a similar fashion with isotype control IgG1-FITC/PE mAb. Fluorescence inten-sity was measured by flow cytometry (EPICS XL-MCL flow cytometer, Beckmann Coulter Int. S.A., Nyon, Swit-zerland). 2000–5000 events were counted in the gated region. The already apoptotic cells were not more than 20% of all cells. They were gated out to stain the receptors.

T cell differentiation and supernatant generation

Th1 and Th2 cells were obtained by differentiation of purified cord blood CD45RA⁺ T cells as described in detail before [13]. For Th1 and Th2 differentiation we use 100 000 naïve cells in 500 μ L completed growth medium (cRPMI 1640), in 48 well/plate. Cytokine production by Th1 and Th2 revealed distinct profiles. 10⁶/mL Th1 cells secreted 7.8 ng/mL IFN- γ 0.1 ng/mL IL-4, <0.1 ng/mL IL-5, <0.2 ng/mL IL-13, 37.4 pg/mL TRAIL, 3.3 ng/mL TNF- α ; Th2 cells secreted 3.3 ng/mL IL-4, 5.8 ng/mL IL-5, 7.6 ng/mL IL-13, <0.1 ng/mL IFN- γ , 0.4 ng/mL TNF- α and 4.8 pg/mL TRAIL, 48 h after anti-CD2, anti-CD3 and anti-CD28 mAb stimulation. In the experiments, freshly prepared undiluted supernatants were used. In some experiments, T cells were isolated from peripheral venous blood by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation as described before [13]. Undi-luted, freshly prepared supernatants from unstimulated (resting) as well as stimulated (48 h with anti-CD2, anti-CD3 and anti-CD28 mAb) T cells were collected and used. In the control SMC group cRPMI was used.

Cloning, expression and purification of human eosinophil cationic protein

Eosinophils were isolated from peripheral blood of hyper-eosinophilia patients mainly with atopic dermatitis and hypereosinophilic syndrome patients. Messenger ribonu-cleic acid from 10⁷ purified eosinophils was isolated using the Oligotex Direct mRNA Midi Kit (Qiagen AG, Basel, Switzerland) followed by oligo-dT primed reverse tran-scription. This cDNA was used to amplify a fragment containing the open reading frame encoding mature ECP by PCR using the following primers: 5'-primer 5'-CG GGA TCC AGA CCC CCA CAG TTT ACG AGG G and 3'-primer 5' GGG AAG CTT TTA GAT GGT GGT ATC CAG GTG AAC TGG. The PCR product was gel purified and restricted with *Bam*HI and *Hind*III incorporated into the amplification primers (underlined sequences). Restricted fragments were

gel purified and ligated into *Bam*HI/*Hind*III-restricted pQE30 (Qiagen AG) expression vector to produce hexahistidine-tagged recombinant protein. The ligation mixture was transformed into *Escherichia coli* strain M15 by electroporation [14] and the nucleotide sequence was confirmed to code the reported amino acid sequence of human ECP [15]. Correct constructs were inoculated into 2×YT broth and induced with 2 mmol/L isopropyl-β-D-thiogalactoside at an OD₅₉₅ of 0.7 to produce recombinant ECP. The protein was found in insoluble inclusion bodies, which were solubilized in guanidinium hydrochloride and one-step purified over Ni²⁺-NTA metal affinity chromatography resin (Qiagen AG) under denaturing conditions. The eluted protein was dialysed against water containing 0.05% glacial acetic acid and 5 mmol/L EDTA. The protein was devoid of relevant contaminants as determined by SDS-PAGE.

Staining of TRAIL, TRAILR1 and TRAILR2 in bronchial sections

The tissue sections were obtained from biopsy tissues of severe asthmatic patients (diagnosed according to the GINA criteria, treated with inhaled steroids and β-2 agonists), approved by Institutional Review Board. Paraffin tissue sections were deparaffinized in HistoClear II (Chemie Brunschwig AG, Basel, Switzerland) and hydrated through graded alcohol series. A high-temperature antigen unmasking technique was used to retrieve antigens (Vector Laboratories Inc., Burlingame, CA, USA). Endogenous peroxidase activity was quenched by incubating tissue sections in 0.3% hydrogen peroxide. Slides were washed and blocked with 2.5% normal horse blocking serum. Appropriate primary and isotype control Ab were applied. In case of TRAIL staining, incubation with Impress reagent (Vector Laboratories Inc.) was used before incubation with peroxidase-specific substrate solution, chromogen diaminobenzidine (DAB). TRAILR1 and TRAILR2 stainings were performed with EnVision+System-HRP kit (DakoCytomation, Glostrup, Denmark). Monoclonal anti-smooth muscle α-actin (Sigma Chemical Co.) specific for the single isoform α-actin was used for α-actin staining. Slides were counterstained with haematoxylin and mounted with permanent mounting media (DakoCytomation, Glostrup, Denmark).

Real-time (RT) PCR for TRAIL, TRAILR1 and TRAILR2

Total RNA was isolated from SMCs using the RNeasy mini kit (Qiagen AG) according to the manufacturer's protocol. The RNA was eluted in 60 mL of RNase-free water and subjected for reverse transcription. Approximately 0.25–0.5 µg total RNA (12 µL) was transcribed using Taq Man Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's guidelines. RT-PCR

reaction was performed using Taq Man Universal PCR Master Mix (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol, using the following primers to amplify specific gene products: TRAIL forward primer, 5'-CCC AAT GAC GAA GAG AGT ATG AAC A-3', reverse primer, 5'-CTC AAA ATC ATC TTT CTA ACG AGC TGA-3', probe, FAM-CTG CTG GCA AGT CAA GTG GCA ACT CC-TAMRA; TRAILR1 forward primer, 5'-TGT ACG CCC TGG AGT GAC AT-3', reverse primer, 5'-CAC CAA CAG CAA CGG AAC AA-3', probe, FAM-TGT CCA CAA AGA ATC AGG CAA TGG ACA TAA T-TAMRA; TRAILR2 forward primer, 5'-CAC TCA CTG GAA TGA CCT CCT TT-3', reverse primer, 5'-GTG CAG GGA CTT AGC TCC ACT T-3', probe, FAM-TCA CAC CTG GTG CAG CGC AAG CAG-TAMRA. Amplification of human 18S rRNA, probe dye VIC-TAMRA (Applied Biosystems, Foster City, CA, USA), was used as the housekeeping gene control for each experimental sample, which remained stable under different conditions of stimulation. Relative quantification was performed in ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The quantification of the amplicons in each well was determined according to the comparative C_t (threshold cycle number) method as described [16].

Demonstration of bronchial SMC viability and apoptosis in cultures and tissues

SMCs viability was evaluated by ethidium bromide (50 µM) uptake and flow cytometry. Annexin V, a phosphatidylserine binding protein, was used to detect apoptotic cells [7, 17]. Hoechst staining was performed as described [7]. SMCs cultures were evaluated with an inverted microscope equipped with interference contrast and UV light (Axiovert 405M; Carl Zeiss AG, Feldbach, Switzerland). Apoptotic cells were identified *in situ* with terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), according to the manufacturer's manual (MEBSTATIN apoptosis Kit II, MBL Co. Ltd, Nagoya, Japan) as described [8]. For active caspase-3 *in situ* staining, paraffin embedded sections were used. Endogenous peroxidase activity was blocked with methanol/H₂O₂ 0.3% for 10 min. After antigen retrieval with 0.01 M citrate (pH 6.0), the slides were incubated for 60 min with primary Ab against the cleaved active caspase-3 (Asp 175) (Cell Signalling, Basel, Switzerland), diluted in PBS containing 1% bovine serum albumin at room temperature and subsequently incubated with the second Ab-rabbit Envision-HRP (DakoCytomation, Zug, Switzerland). The slides were then developed with NovaRed substrate kit for peroxidase SK-4800 (Vector, Servion, Switzerland). Neutralizing experiments to block the effects of a certain apoptosis-inducing ligand were performed as described previously [7, 8].

Results

Induction of bronchial SMC death by activated Th1 and Th2 cells, IFN- γ , sFasL, TNF- α and TRAIL

To investigate whether SMC are particularly affected in the process of an active injury and regeneration of resident tissue cells in the asthmatic lung we analysed cellular and molecular mechanisms of SMC death. Stimulation with IFN- γ sFasL, TNF- α and TRAIL induced death of SMC after 3–6 days of culture ($P < 0.001$ day 3–6, except for TNF- α at days 3 and 4) (Fig. 1a). The combination of IFN- γ and TNF- α escalated the process compared with either cytokines alone. Similarly, supernatants from *in vitro* differentiated Th1 and Th2 cells induced bronchial SMC death ($P < 0.001$, days 5 and 6). Although both effector T cell subsets killed bronchial SMC, supernatants from Th1 cells appeared to be more potent ($P < 0.01$) (Fig. 1b). Cytokine production by Th1 and Th2 revealed distinct profiles. $10^6/\text{mL}$ Th1 cells secreted 7.8 ng/mL IFN- γ , 0.1 ng/mL IL-4, <0.1 ng/mL IL-5, <0.2 ng/mL IL-13, 37.4 pg/mL TRAIL, 3.3 ng/mL TNF- α ; Th2 cells secreted 3.3 ng/mL IL-4, 5.8 ng/mL IL-5, 7.6 ng/mL IL-13, <0.1 ng/mL IFN- γ , 0.4 ng/mL TNF- α and 4.8 pg/mL TRAIL, 48 h after anti-CD2, anti-CD3 and anti-CD28 mAb stimulation. Th1 cells produced higher quantities of IFN- γ and TNF- α

compared with Th2 cells. Although it was relatively high in Th1 cells, TRAIL was produced both by Th1 and Th2 cells. Because of the multiple factors that may play a role in SMC apoptosis our efforts to neutralize single cytokines did not lead to a significant change in SMC viability *in vitro*. Resting T cell supernatants, which do not contain any detectable amounts of Th1 and Th2 cytokines, did not induce SMC death. Annexin V staining of SMC membrane phosphatidylserine 5 days after stimulation with IFN- γ TNF- α sFasL and activated T cell supernatant showed that the SMC cell death occurs *via* apoptosis (Fig. 1c).

Regulation of death receptors expression on bronchial SMC

Analysis of death receptors and ligands in primary human bronchial SMC demonstrated that unstimulated SMC expressed high amounts of TNFR1, Fas, TRAILR1, TRAILR2 and membrane FasL and detectable TNFR2 (Fig. 2). IFN- γ and TNF- α appear to be the effector molecules increasing the bronchial SMCs susceptibility to apoptosis. They both up-regulated TNFR1 and TNFR2 as well as the membrane FasL expression on SMC 48 h after stimulation (Fig. 2a). TRAIL stimulation significantly up-regulated TNFR2 without affecting TNFR1, Fas and FasL (Fig. 2b). The apoptosis-inducing TRAIL receptors TRAILR1 and

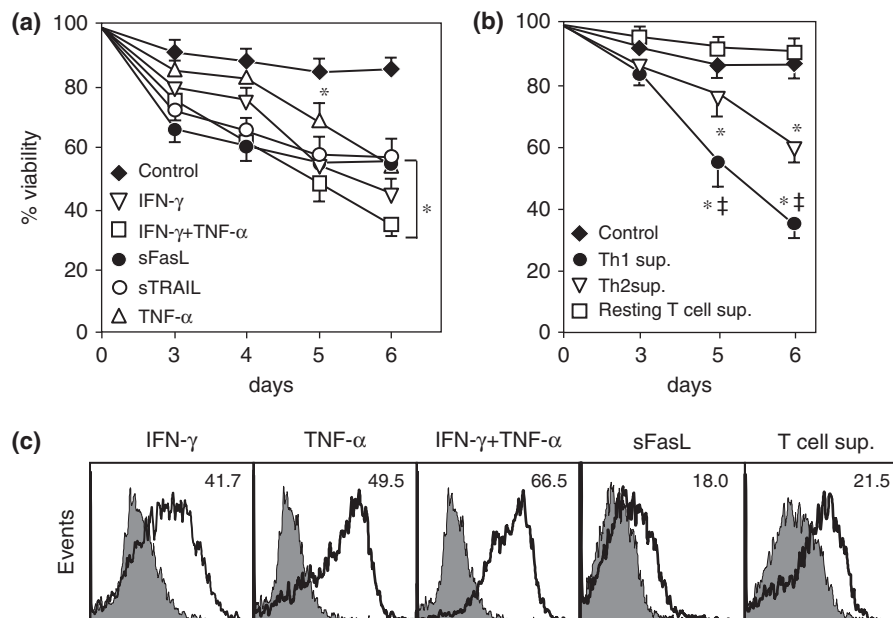


Fig. 1. Induction of bronchial smooth muscle cell (SMC) death by T cells and cytokines. Bronchial SMC were stimulated with IFN- γ , TNF- α , sFasL and TNF-related apoptosis-inducing ligand (TRAIL) (all at 10 ng/mL). The control group was not stimulated. The SMCs viability was assessed by ethidium bromide (50 μM) exclusion and flow cytometry after 3–6 days of culture (a). SMCs were stimulated with supernatants obtained from resting T cells (US T cell sup.) and anti-CD2, CD3, CD28 mAb-activated Th1 or Th2 cells. The control group was stimulated with cRPMI. Percentage of viable cells was determined by ethidium bromide exclusion. Results represent four experiments, performed in duplicates. * $P < 0.001$ (compared with the control), $^{\dagger}P < 0.01$ (the difference between Th1 and Th2 supernatants). Asterisks cover all samples for days 3–5 (b). Annexin V staining of SMC membrane phosphatidylserine shows the apoptotic features of SM cell death 5 days after stimulation (% of positive cells in comparison with the control group – grey histograms). Supernatant was obtained from anti-CD2, CD3, CD28 mAb-activated T cells. Results are representative of one out of three experiments with different SMC lines. * $P < 0.001$ (c).

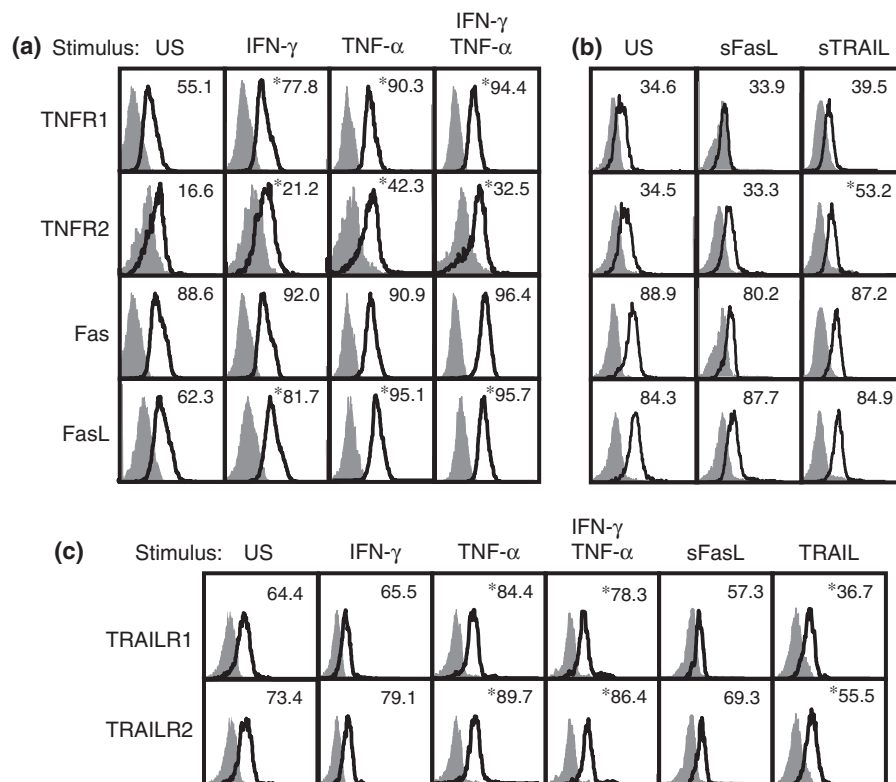


Fig. 2. Death receptor expression on smooth muscle cells (SMCs). SMCs cultured in the absence (us) or presence of IFN- γ , TNF- α , both cytokines (a), sFasL or TNF-related apoptosis-inducing ligand (TRAIL) (all at 10 ng/mL) (b). The percentage of SMC positively stained for TNFR1, TNFR2, Fas, FasL after 48 h of culture was demonstrated by flow cytometry. SMCs were stimulated with the above reagents and the percentage of TRAILR1 and TRAILR2 expressing cells is investigated (c). Filled curve shows isotype control Ab. Results are representative of one out of three separate experiments each section, performed in duplicates, with different SMC lines. * $P < 0.001$.

TRAILR2 were present on the surface of unstimulated SMC. TRAILR1 and TRAILR2 were significantly up-regulated by TNF- α and down-regulated by TRAIL stimulation (Fig. 2c). These results demonstrate significant expression and regulation of TNF- α and TRAIL receptors on bronchial SMCs, whereas already highly expressed Fas in resting SMC was not affected by these stimuli.

Comparison of necrosis and apoptosis of SMC induced by eosinophils and T cells

To estimate the role of the eosinophils in asthma, the influence of human recombinant ECP on bronchial SMC death was also investigated. ECP induced cell death of bronchial SMC as assessed by ethidium bromide exclusion within hours, in a concentration dependant manner (Fig. 3a). The SMC viability was significantly decreased after 1 h of stimulation with 20 μ g/mL ECP. In contrast, the effects of TNF- α , IFN- γ and TRAIL stimulation became visible after 3–4 days. The coupling of Fas signalling to the death pathway activates the caspase enzyme cascade, ultimately resulting in apoptosis [18]. Caspase-3 activity in SMCs was assessed by staining with anti-active caspase-3 mAb and flow cytometry measurements after 2 days of stimulation.

The bronchial SMC death by ECP did not show features of apoptotic death assessed by caspase-3 activation. For comparison, stimulation of SMC with IFN- γ , sFasL, TRAIL, Th1 or Th2 cell supernatants was investigated. A kinetics of analysis was performed to optimize the time-point. The data collected after 48 h demonstrate that caspase-3 activation becomes visible in T cell supernatant and IFN- γ , FasL and TRAIL stimulations ($P < 0.01$) (Fig. 3b). ECP-stimulated SMC did not show the morphological features of apoptosis; the SMC became rather big with neither nuclear condensation nor fragmentation (Fig. 3c). For comparison TNF- α - and IFN- γ -stimulated samples showed typical round and shrunken cells, nuclear condensation and fragmentation (Fig. 3c). These data demonstrate that depending on the severity of eosinophil infiltration and the amount of released active factors, eosinophils possess the capacity to induce rapid bronchial SMC death by necrosis within hours (by secreted ECP) or contribute to their apoptosis (by secreted TNF- α).

TRAIL, TRAILR1 and TRAILR2 expression in asthma

To investigate whether TRAIL plays a role in the inflammatory and structural changes, the expression of TRAIL,

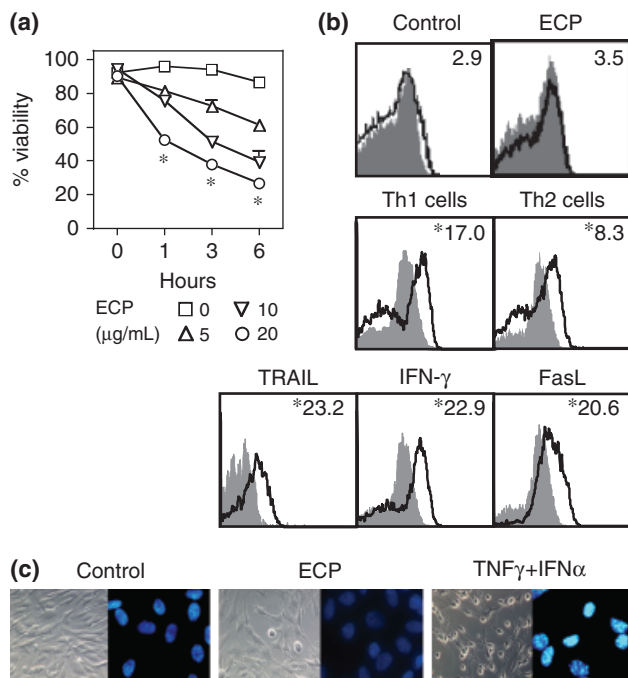


Fig. 3. Eosinophil cationic protein (ECP) induces necrosis of bronchial smooth muscle cell (SMC). Bronchial SMC were stimulated with human recombinant ECP and the cell viability was assessed by ethidium bromide exclusion (50 µM) and flow cytometry after 1, 3, and 6 h (a), expression of intracytoplasmic active caspase-3 after 48 h of stimulation was investigated by ECP (1 µmol/L) and for comparison Th1 cell, Th2 cell supernatants, TNF-related apoptosis-inducing ligand (TRAIL), IFN-γ and FasL (10 ng/mL each) stimulation. No increase in active caspase-3 was investigated after 48 h of stimulation by ECP (b). Phase-contrast microscopy and HOECHST staining ($\times 200$) do not demonstrate condensed and fragmented nuclei after ECP stimulation. In contrast, typical apoptotic features are demonstrated in TNF-α and IFN-γ-stimulated cultures (c). (b) and (c) were performed after 48 h of cultures. Results represent one out of three experiments, performed in duplicates. * $P < 0.001$.

TRAILR1, TRAILR2 was determined in the sections of the proximal conducting airways from severe asthmatic patients and healthy control subjects. High expression of TRAIL was demonstrated in the smooth muscle layer only in asthmatic subjects (Fig. 4a) but not in non-asthmatic controls (Fig. 4b). In severe asthmatic patients visible morphological changes that include aberrant hypertrophic and hyperplastic SMC islets were shown. The SMC tissue sections were positive for TRAILR2 in both asthmatic and non-asthmatic subjects (Figs 4a and b). As demonstrated in the cultured SMC, IFN-γ stimulation significantly induced expression of TRAIL mRNA, but not TRAILR1 and TRAILR2 mRNA (Fig. 4c), TNF-α stimulus showed a weak up-regulation of TRAIL mRNA, approximately ninefold less than induced by IFN-γ and did not influence TRAILR1 and TRAILR2 expression. IFN-γ does not bind to a death receptor and cannot induce apoptosis alone. Taken together with Fig. 2, SMC apoptosis induced by IFN-γ is apparently due to the up-regulation of TNFR1, TRAIL and FasL. Neutralizing experiments to block the

effects of a certain apoptosis-inducing ligand were performed as previously demonstrated in epithelial cells [7]. Because of the involvement of all three pathways, a very limited effect of a single apoptosis factor neutralization was observed.

SMC apoptosis in severe asthma

In vivo relevance of the findings was investigated in bronchial biopsy specimens. Airway SMC layer from the proximal conducting airways was examined for histologic changes and characteristic features of SMC apoptosis. The mucosa of severe asthmatic subjects in a representative central bronchus showed marked damage of the epithelial layer with exfoliation of epithelial cells (Fig. 5a). Significantly expressed active caspase-3 was also shown in bronchial SMC and epithelial cells (Fig. 5a). This was parallel with epithelial shedding. Biopsies from several clinical severity stages of asthma patients were investigated. PI was used to locate the bronchial SMC nuclei. TUNEL staining demonstrated double stranded DNA breaks in the nuclei of SMC in lung biopsies of particularly fatal asthma patients *in vivo* (Fig. 5b). There was no evidence of bronchial SMC apoptosis in intermittent asthma biopsies. PI and TUNEL staining always overlapped in the analysed sections, suggesting that the *in vivo* occurring bronchial SMC death is an apoptotic rather than a necrotic process.

Discussion

The present study shows that bronchial SMC appear as an essential target of the inflammatory attack by T cells and eosinophils. The process of cell death triggered in the environment of chronic inflammation can become an important pathogenic mechanism of the abnormal cell turnover and impaired tissue regeneration. This can lead to morphological and functional disorder of the tissue and organ [1]. Bronchial SMC apoptosis has been demonstrated in cultures and *in situ* as the mechanism of essential tissue injury in especially severe forms of asthma.

The crucial role of infiltrating T cells and eosinophils has already been suggested in pathogenic mechanisms responsible for breakdown of airway tissue and damage of structural cells such as bronchial epithelial cells and SMC [2, 19, 20]. A morphologic study of the bronchial mucosa from patients with asthma demonstrated 54.5% lymphocytes, 22.1% eosinophils, 4.9% neutrophils, and 4.6% mast cells in the inflammatory infiltrate [21]. T cells may contribute to airway inflammation by activating and prolonging the life span of eosinophils *via* IL-5, IL-3, and GM-CSF [22]. They can also directly induce apoptosis of epithelial cells due to IFN-γ secretion, and also facilitate the TNF-α-mediated apoptosis [7]. In the present study, we

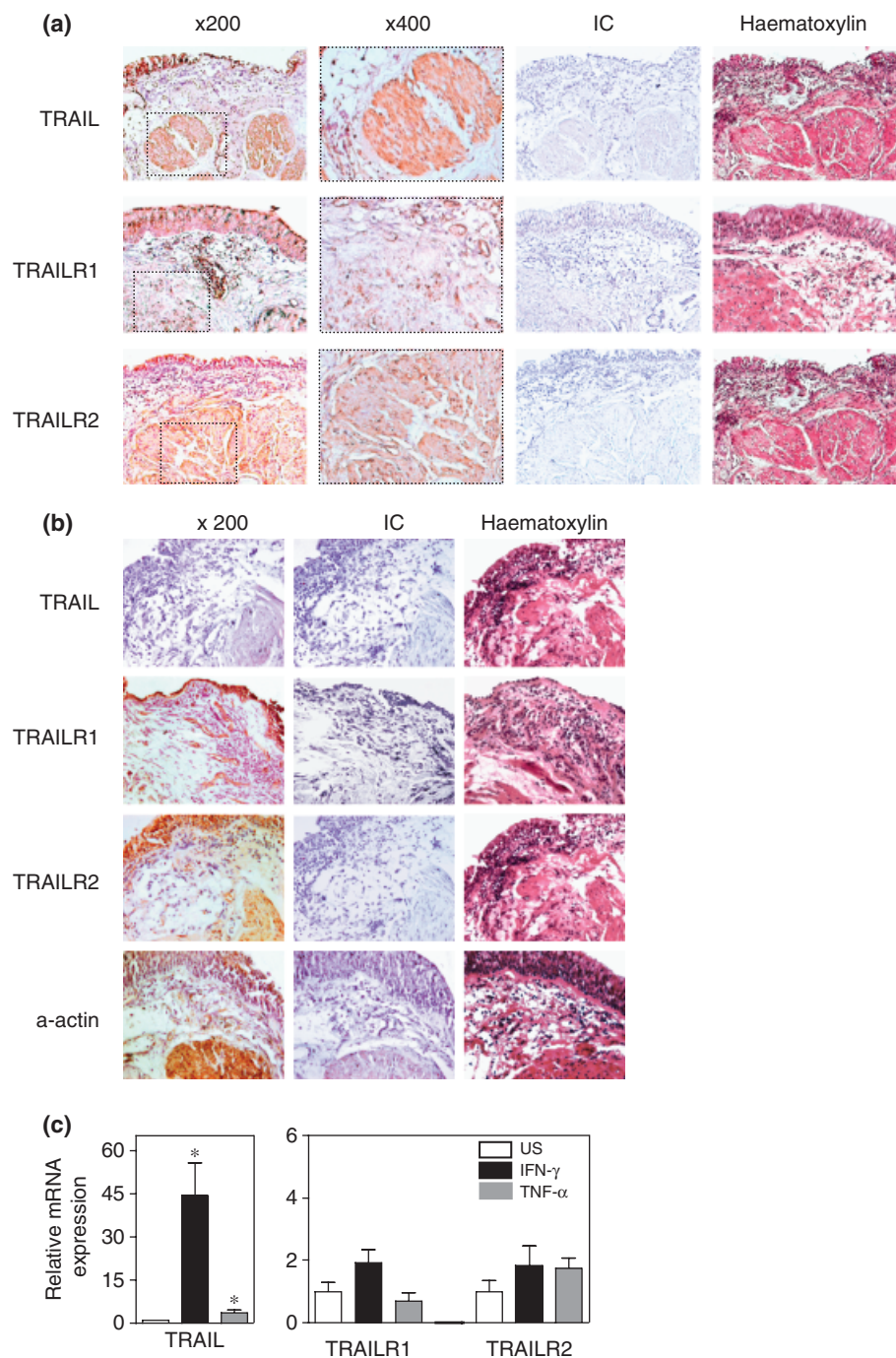


Fig. 4. Asthmatic tissue TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptor (TRAILR) expression. High TRAIL, TRAILR1 and TRAILR2 expression is shown *in situ* in the smooth muscle cell (SMC) layer of severe asthma. Three severe asthma patients were analysed. Second column shows $\times 400$ magnification of the indicated area in the left column (a). Non-asthmatic (control) tissue TRAIL and TRAILR expression. Biopsies from four non-asthmatic control subjects were analysed. IC, isotype control antibody. α -actin staining was used in sequential sections to demonstrate SMC (b). Healthy SMC cell culture TRAIL and TRAILR expression. IFN- γ and TNF- α significantly induce the TRAIL, but not TRAILR1 and TRAILR2 mRNA expression after 8 h in cultured SMC. * $P < 0.001$ (c).

show *in vitro* that IFN- γ , TNF- α and TRAIL are capable of rendering bronchial SMC susceptible to apoptosis. The cooperation of TNF- α , TRAIL and FasL in inducing apoptosis of Jurkat T cells has also been described [23].

In this study we show that IFN- γ and TNF- α up-regulate TNFR1, TNFR2, as well as the membrane FasL on SMC, whereas TNF- α up-regulates also TRAILR1 and TRAILR2. Fas and TRAIL were shown to be expressed by SMC tissue

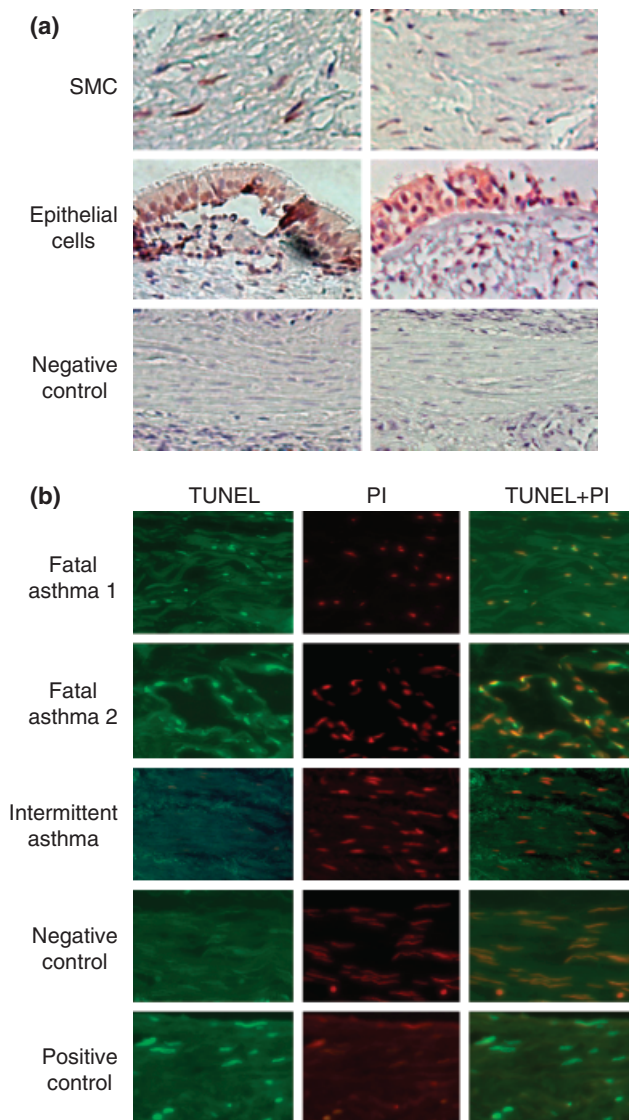


Fig. 5. Demonstration of active caspase-3 *in situ* in bronchial smooth muscle cell (SMC) and epithelial cells in two severe asthma patients (a). TUNEL and propidium iodide (PI) staining of two fatal cases of asthma and one representative of three intermittent asthma patients (b). DNase I treated slides served as positive control. Non-asthmatic bronchial mucosa biopsies were used as negative control.

in vivo and on the surface of cultured human airway myocytes *in vitro* [20]. Although not related to asthma, the cooperation of three death pathways have been demonstrated for vascular SMC (VSMC). TNF- α -treated VSMC have been shown to undergo apoptosis upon Fas cross-linking [24]. In addition, death pathway triggered by TRAIL-producing CD4⁺ T cells has been demonstrated as a direct mechanism of VSMC apoptosis in atherosclerosis [25].

T cells are well documented to contact airway smooth muscle by directly infiltrating the muscle layer of the airways [2] as well as by acting in its proximity – the bronchial submucosa [21]. Although Th2 cells have been

mostly focused as cells responsible for the development of allergic diseases, Th1 cells may contribute to chronicity and effector phase in allergic diseases [26]. In humans, there is not a straight bias for type 1 and type 2 differentiated T cell subsets in disease. In addition to typical Th2 cytokines (like IL-5 and IL-13), IFN- γ , TNF- α and TRAIL have also been elevated in cultures of bronchoalveolar lavage fluid (BALF) cells from asthma patients [27]. During asthma exacerbations, viral infections may contribute to the IFN- γ increases in the airways [28]. In this study, Th1 cell supernatants induced stronger apoptotic cell death of SMC than Th2 cell supernatants. Because of multiple factors, that may play a role in SMC apoptosis, our efforts for neutralization of IFN- γ , TNF- α and TRAIL did not lead to significant change in SMC viability. SMCs exhibited a relatively low threshold in response to IFN- γ , in the range of 1–10 ng/mL. Significant Th2 cell-induced SMC apoptosis can be attributed to the effects of TRAIL, which is also secreted by these cells.

Although there are similarities, bronchial epithelial cells, skin keratinocytes and bronchial SMC do not utilize exactly the same predominant apoptosis pathway in response to allergic inflammation. Epidermal keratinocytes do not express FasL in the course of eczematous dermatitis and up-regulation of Fas mainly by IFN- γ renders the cells susceptible to apoptosis. The death signal is provided by FasL expressed by activated skin-infiltrating T cells [8]. In contrast, the TNFR-related apoptotic pathway is predominant in bronchial epithelial cells. TNF- α which can be released by activated eosinophils, macrophages, mast cells and stimulated epithelial cells, up-regulates both Fas and FasL as well as its own receptors TNFR1 and TNFR2 on bronchial epithelial cells [7]. Bronchial SMCs utilize both apoptosis pathways. Bronchial SMCs highly expressed TNFR1, Fas, FasL, TRAILR1 and TRAILR2 on IFN- γ and TNF- α stimulation. Thus, control of SMC number through Fas, TNFR or TRAILR ligation may be a general mechanism for regulating the architecture of smooth muscles in the inflammatory lungs.

However not seen directly in SMC, large numbers of activated eosinophils are present in its proximity – in the mucosa of asthmatic patients and they are thought to be the central effector cells in asthma [28, 29]. IFN- γ -activated eosinophils can trigger bronchial epithelial cell apoptosis [7] as well as bronchial tissue injury due to released cytotoxic proteins including ECP, eosinophil-derived neurotoxin, EPO and MBP [6]. Our data suggest that eosinophils may possess the capacity to promote also bronchial SMC apoptosis by secreted TNF- α , as well as induce SMC necrosis within hours, by secreted ECP, ECP damages cells by a colloid-osmotic process, introducing non-ion selective pores in both cellular and synthetic membranes [30]. However, to induce toxic epithelial cell death, doses far higher than those measured in the lysates of eosinophils were required, obviously *in vivo* release and

production in cultures may not be fully comparable to each other [30].

The stressed epithelium which can secrete TNF- α may contribute to the structural remodelling of the airways, including thickening of the basement membrane and induction of myofibroblasts [31]. Bronchial SMC constitute an autocrine environment, which activated in the atopic (IgE mediated) sensitized state or by specific respiratory viruses induces the autologous expression of specific cell surface proteins (e.g. CD23, ICAM-1, etc.) and secretion of specific pro-inflammatory cytokines [5]. As it was shown before in cultured SMCs, airway SMC receive strong anti-apoptotic survival signals from a system of extra-cellular matrix proteins, like fibronectin, laminin and collagens I and IV [32]. However, the exact rate of smooth muscle turnover within airways is not known, the proliferative index and turnover of airway SMC appears to be very low even in the presence of substantial airway inflammation [33]. On the other hand, even low intensity of apoptosis may have a significant impact on SMC accumulation within intact asthmatic airways. The role of TNF- α in severe asthma has been revealed by demonstration of high concentrations of TNF- α in BALF from patients with more severe asthma [34]. Furthermore, a 10-week clinical trial of treatment with a soluble TNF- α receptor was associated with a significant decrease in bronchial hyperresponsiveness and the improvement in both asthma-related quality-of-life score and lung ventilation parameters [35].

Although not much is known of its role in atopic disease, increased TRAIL expression was demonstrated by several inflammatory cells under *ex vivo* and *in vivo* conditions in atopic dermatitis [36]. Also in the airways TRAIL can be expressed and released by inflammatory cells infiltrating the bronchial mucosa and by the airway wall structural cells (fibroblasts, epithelial, endothelial and SMC) as well [37]. The increased levels of TRAIL are observed in BALF from asthmatic patients [37]. This study demonstrates increased expression of TRAIL, TRAILR1 and TRAILR2 in SMC of severe asthma patients. Although both TRAIL receptors were expressed in non-asthmatic patients, the expression of TRAIL was missing in these individuals, suggesting an important role of this cytokine in the pathogenesis of asthma. It has previously been shown that non-neoplastic cells like osteoblasts are a target for TRAIL-mediated death induced by myeloma cells. On the other hand, osteoblasts express osteoprotegerin – a factor which protects myeloma cells from TRAIL-mediated apoptosis [10]. The expression of TRAIL and its receptors is up-regulated by IFN- γ . It has been shown that TRAIL and FasL are expressed in distinct patterns in Th1 and Th2 cells upon activation through the TCR [38]. Th2 cells seem to be more resistant to either TRAIL or FasL-induced apoptosis compared with Th1 cells. Thus, increased secretion of TRAIL by SMC after IFN- γ stimulation

may contribute to control of Th1-dependent inflammatory events. Taken together, these findings suggest that TRAIL plays a significant role in inflammation and tissue remodelling in asthma.

In conclusion, the demonstration of bronchial SMC death both by apoptosis and necrosis particularly *in vivo* in fatal asthma, indicates the essential role of T cells and eosinophils in the asthmatic bronchial tissue injury and induction of bronchi pathology in asthma.

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