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Altered Apoptosis in BAL Lymphocytes after Allergen Exposure of Atopic Asthmatic Subjects

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ABSTRACT

The increased number of lymphocytes in airways during an asthmatic response is believed to be the result of increased recruitment of these cells. However, it is possible that a decreased apoptotic rate could also contribute to the increased number. Herein we investigate whether allergen airway provocation influences the apoptotic phenotype of lung and peripheral blood lymphocytes (PBL) in subjects with atopic asthma.

Bronchoalveolar lavage (BAL) fluid lymphocytes and PBL from twelve asthmatic subjects previously challenged with allergen (n=7) or saline (n=5) were exposed to the apoptotic stimulus tributyltin (TBT) *in vitro* and assayed for apoptosis. Airway allergen provocation resulted in decreased sensitivity of BAL lymphocytes to TBT-induced apoptosis, with 42.2% (range 33.9-62.5%) apoptotic cells before challenge versus 23.5% (range 15.3-42.4%) after challenge (p=0.023), while PBL were unaffected. The increased apoptosis resistance correlated with higher numbers of Bcl-2 expressing lymphocytes. Interestingly, baseline caspase-3-like activity was significantly elevated in viable BAL fluid lymphocytes compared to viable PBL and was unaltered by allergen exposure. In conclusion, allergen inhalation renders BAL fluid lymphocytes more resistant to apoptosis while PBL were not influenced at all, indicating that the apoptotic phenotype of airway lymphocytes may play a role in asthmatic inflammation.

INTRODUCTION

Allergic asthma is a chronic disease in which allergen-induced inflammatory processes in the airways contribute to the development of symptoms and may eventually lead to remodelling of the airway tissue. Inflammation at the sites of target organs is a pathological feature of the disease process and among the various types of cells involved in tissue infiltration and damage are T lymphocytes, i.e. one of the main effector cells priming the local ongoing allergic immune response (1). These cells are involved in the local recognition of allergens, acting through secreting Th2-cytokines and thereby determining IgE synthesis and the mucosal recruitment of other inflammatory cells such as neutrophils and eosinophils (2, 3). Although our knowledge of the initiation of the allergic response has rapidly expanded during recent years, little is known about how the inflammatory response develops into a chronic inflammation.

Previous studies have demonstrated that reduced apoptosis of T cells may play a role in asthma pathogenesis (4, 5). Apoptosis of lymphocytes was decreased at baseline levels in asthmatic subjects compared to normal controls and patients with obstructive pulmonary disease (6). Additionally, T cells from asthmatic subjects fail to undergo the normal degree of apoptosis following Fas receptor ligation, and $CD45RO^{+}$ T cells in the inner airway wall of patients with mild and server asthma were less apoptotic compared to controls (7, 8). The observed insufficient T cell apoptosis may interfere with clonal deletion and maintenance of tolerance, resulting in T cell accumulation and contributing to the chronic inflammation of asthma. Thus previous data indicate alterations in the apoptotic phenotype of cells involved in asthmatic inflammatory processes (4, 6, 7). However, changes in apoptosis sensitivity following an allergen challenge are still elusive .

Apoptotic cell death results from the activation of an internally encoded suicide program which is induced by a variety of intrinsic and extrinsic signals and is one important tool in the regulation of the immune system. The amount of reactive T-cells in an immune response can be controlled by a process called "activation induced cell death" (AICD), which mainly involves the activation of death receptors such as Fas (9). However, apoptosis of activated T-cells can also be induced independently of death receptor signalling by the induction of the mitochondrial pathway, which is activated by signals such as cytokine withdrawal or the presence of reactive oxygen species (ROS) (10-12). Death receptor mediated apoptosis involves the recruitment of the initiator caspase-8 while the initiation of the mitochondrial pathway leads to the cytoplasmic release of pro-apoptotic factors from the mitochondrion and the subsequent activation of caspase-9 (13, 14). The caspases belong to a family of cysteine proteases which play a central role in the transduction of apoptotic signals either by cleaving and activating downstream caspases or by processing vital proteins which will lead to the death of the cell. Until recently Caspase-3 has, been considered to be an exclusively pro-apoptotic protein whose activation has been believed to be the 'point of no return' in the apoptotic pathway. However, new evidence has emerged suggesting an additional role for caspase-3 together with caspase-8 in differentiation and proliferation of activated T cells (15-21).

We have previously demonstrated high caspase-3-like activity in non-apoptotic BAL fluid lymphocytes recovered from sarcoidosis patients (22). These lymphocytes were also shown to be highly resistant to mitochondrial-mediated apoptosis induced by tributyltin (TBT) (22). In the present study allergen inhalation challenge of individuals with asthma was used to delineate the processes of apoptosis occurring in the inflammatory phase of asthma and since little is known about mitochondrial induced apoptosis of airway lymphocytes in asthma we were particularly interested to investigate the impact of this pathway in these cells.

To this end we used TBT, a mitochondrial toxin that specifically triggers the mitochondrial pathway by loss of mitochondrial membrane potential and cytochrome C release (23-25).

The BAL fluid lymphocytes from asthmatic subjects provoked with allergen were more resistant to mitochondrial-mediated apoptosis while no change in apoptosis sensitivity was evident in BAL fluid lymphocytes obtained from subjects challenged with saline. The decreased apoptosis sensitivity in allergen-exposed BAL lymphocytes was associated with a higher proportion of Bcl-2 expressing lymphocytes. Furthermore, BAL fluid lymphocytes from all subjects had an elevated caspase-3-like activity compared to PBL, and airway exposure to allergen did not increase the activity further. These data indicate that airway inflammation in asthma is associated with a reduced apoptosis-susceptibility of lung lymphocytes, which may lead to an enhanced survival of BAL fluid lymphocytes and consequently prolonged inflammation.

MATERIALS AND METHODS

Subjects

Twelve atopic patients, 3 male and 9 female, aged 22-46 years (median 27) with a history of mild allergic asthma participated in the study. All were non-smokers except one who was an ex-smoker. All subjects in the study population had previously documented reactions following allergen broncho-provocation. The patients allergic to animals did not have pets of their own and were asked to avoid animal contact during the study period. Their asthma was stable and controlled solely with β2-agonists taken as required. As an inclusion criterion in the study the forced expiratory volume in 1s (FEV_1) was required to be more than 70% of predicted. Prior to the study bronchial hyper-responsiveness to methacholine with a PD_{20} of <2530 ug (provocative dose causing a 20% fall in FEV_1) was demonstrated. The methacholine provocation test was performed as previously described (26). Patient characteristics and clinical data before and following allergen provocation are presented in Table I. The study was approved by the Ethical Committee at the Karolinska University Hospital, and all subjects gave their written informed consent.

Study design

The study was performed out of the pollen season. Patients were blinded and administered either saline or an allergen (Table I) for which they had a positive history and RAST result. The allergen extracts used were standardized and freeze-dried (Aquagen, ALK, Copenhagen, Denmark). Peripheral blood and bronchoalveolar lavage (BAL) samples were obtained 2-3 weeks before (baseline) and one day following the allergen/saline challenge. The type of allergen used for challenge, cumulative allergen dose, peak drop during the EAR and the PD_{20} values are presented in Table 1. All bronchial provocations were performed using a dosimeter-controlled jet nebulizer (Spira Electro 2, Respiratory Care Center, Hameenlinna,

Finland). In brief, the allergen challenge was initiated by inhalation of diluent. Provided that the FEV_1 did not change more than 10%, allergen was inhaled every 15 minutes with half-log increments of the cumulated dose until the FEV_1 dropped 20% or more from the post-diluent baseline value (27). The occurrence of any late asthmatic reactions (LAR) were examined by measurements of peak expiratory flow (PEF) rates every waking hour during the first 24 hours following challenge. The patients were instructed to make additional recordings if any airway symptoms occurred.

Bronchoalveolar lavage (BAL), differential cell count, separation and handling of cells.

BAL was performed as previously described (28, 29). In brief, under local anaesthesia the flexible fibreoptic bronchoscope (Olympus BF type 1TR; Olympus Optical Co Ltd, Tokyo, Japan) was wedged into a middle lob bronchus and sterile phosphate buffered saline (PBS) solution at 37^oC was instilled in five aliquots of 50ml. After each instillation the fluid was gently aspirated and collected in a siliconised plastic bottle kept on ice. The BAL fluid was strained through a Dracon net (Millipore, Cork, Ireland), centrifuged at 400g for 10 minutes at 4^0C, and the cell pellet was resuspended in RPMI 1640 medium (Sigma chemicals, St Louis, USA). Cells were counted using a Bürker chamber and cell viability was determined by trypan blue exclusion. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, washed three times in PBS and diluted in RPMI 1640. For differential cell counts, cytospins were prepared by cytocentrifugation (Cytospin 2; Shandon, Runcorn, Chesire, UK) of 60,000 cells per slide at 20g for 3 minutes. In order to determine cell differentials, cytospin-slides were stained with May-Grünwald-Giemsa and 400 cells were counted microscopically. Mast cells were stained using 2 parts toluidine blue 0.5%, pH0.5 and 1 part 0.2% hematoxylin solution and the number of mast cells from 10 vision fields at a magnification of X16 were counted

microscopically. Both BAL fluid cells and PBMC was kept on ice until the initiation of the experiments.

Induction and detection of cell death

BAL cells and PBMC were centrifuged at 400g for 10 minutes at 4^0C and resuspended in complete medium at a concentration of $1x10^6$cells/ml, CM (RPMI-1640 containing 5% FCS, Gibco, BRL, Life Technologies, UK), 2mM L-Glutamine, penicillin (100U/ml) and streptomycin (0.1mg/ml*)*. Apoptosis was induced by exposure of the cells to 2µM tributyltin chloride, TBT (Sigma Aldrich, Germany) for 60 minutes at 37^oC in a humidified air chamber with 5% CO_2. Dead cells were detected using FITC-labelled Annexin V and Propidium Iodide (PI) (Annexin V/PI) (Pharmingen, San Diego, USA) staining. Briefly, BAL cells and PBMC were resuspended in annexin-binding buffer containing Annexin V-FITC and PI (100цg/ml) and incubated for 15min at room temperature. The samples were analysed using a flow cytometer (FACSCalibur, Becton Dickinson, USA). BAL lymphocytes and Peripheral blood lymphocytes (PBL) were identified and gated by forward and side light scattering properties. Ten thousand events were analysed and dot plots used to determine the percentage of apoptotic cells showing annexin V-FITC (FL-1 channel) binding; a minority of these cells were also permeabile to PI (FL-3 channel). 'Apoptotic cells' in the result section refers to lymphocytes stained with annexin V, with or without PI-staining, including mainly cells in early apoptosis (annexin V positive/PI-negative; encompassing the vast majority of the cells) but also cells in late apoptosis (annexin V positive/PI-positive; minority).

Detection of caspase-3 like activity in intact cells

Caspase-3-like activity in intact BAL and PBL was measured using the cell-permeable substrate PhiPhilux-G2D2 (OncoImmunin, Inc., Kensington, MD, USA), according to the

manufacturers recommendations. Briefly, cells were washed, resuspended in 50µl substrate (10µM) and incubated for 1 hour at 37°C in humidified air chamber with 5% CO_2 followed by immediate flow cytometric analysis (FL-2 channel). Caspase-3-like activity was expressed as mean fluorescence intensity from viable lymphocytes which were identified and gated by forward and side light scattering properties.

Immunocytochemistry double staining

Cytospins were allowed to reach room temperature and the cells were fixed in paraformaledyde 4% for 15 minutes. Antigen unmasking was performed by boiling the cytospins in 10mM citrate buffer for 15 minutes and then allowed cooling for 45 minutes at room temperature. Cells were permeabilised using ice-cold methanol for 10 minutes at –20^{o}C. To avoid non-specific staining the cells smears were incubated in blocking buffer containing PBS, 5% horse serum and 0.1% bovine serum albumin for 1 hour at room temperature or overnight at 4^{o}C. The cell smears were incubated with goat anti-CD33 (Santa Cruz Biotechnology, Santa Cruz, California) diluted 1:100 in blocking buffer overnight at 4^{o}C. Cell smears were washed 3x5 minutes in TBS in the second wash with 0.1% TWEEN 20 added. Alkaline phosphatase-conjugated rabbit anti-goat (Sigma) were diluted 1:100 in TBS containing 5% horse serum and 0.1% bovine serum albumin was then added for 1 hour at room temperature. The cell smears were washed 3x5 minutes in TBS with 0.1% TWEEN 20 added in the second wash before staining using the alkaline substrate Fast Red for 10 minutes. The slides were washed in running water for 10 minutes, incubated in PBS for another 10 minutes followed by a second primary antibody. The antibodies used were either mouse anti-human-BCL-2 1:50 (clone 124, Dako Cytomation,Glostrup, Denmark) and PCNA 1:500 (clone PC10, Calbiochem, Darmstadt, Germany) or mouse IgG as isotype control (Dako Cytomation, Glostrup, Denmark) which were diluted in blocking buffer . The cell smears were

incubated with the second primary antibody overnight at 4^{o}C. Endogenous peroxide activity was quenched by incubating the slides with 0.3% H_2O_2 in PBS for 30 minutes. Following incubation with secondary antibody, biotinylated horse anti-goat IgG (Vector Laboratories, Inc, Burlingame, USA) diluted in blocking buffer 1:100 for 1 hour the cell smears were incubated with avidin-conjugated peroxidase substrate Vectasin elite ABC kit (Vector Laboratories, Inc, Burlingame, USA) for 90 minutes. DAB (3,3´-Diaminobenzidine) (Dako Cytomation, Glostrup, Denmark) was used for colour development. The exposure time was 8 minutes for Bcl-2 staining, 5 minutes for the PCNA antibody and 10 minutes for the IgG isotype control antibody. The stained cytospins were counted in a blinded manner three times.

Statistics

Results are presented as median values with minimum and maximum values as range. Comparisons of data retrieved before and following provocation were made using Wilcoxons matched pairs analysis. P-value <0.05 was considered significant.

RESULTS

Clinical parameters

All patients provoked with allergen, but not those receiving saline, had an early asthmatic reaction (EAR) defined as a 20% or more decrease in FEV_1 within the first 3 hours post-challenge. One of these patients also had a late asthmatic reaction (LAR), defined as >15% decrease in FEV_1 during 3-24 hours post-challenge (Table 1).

BAL and blood cell data

BAL fluid recovery and cell viability did not differ in samples retrieved at baseline and after allergen or saline challenge. Although an increased BAL cell concentrations was recorded in 10/12 patients, i.e. in both allergen- and saline-exposed individuals compared to baseline, there was only a statistically significant ($p<0.05$) increase determined in those patients challenged with saline (Table 2). The differential count of BAL cells revealed a significant increase in percent eosinophils following allergen challenge ($p<0.05$). However, both allergen and saline seemed to influence the amount of eosinophils in the lungs, since 3/5 patients challenged with saline and 5/7 patients challenged with allergen had an increase in the proportion of BAL eosinophils compared to baseline levels. A tendency of a relative increase in BAL fluid lymphocytes was observed in patients challenged with allergen. Here, the differential count showed an increase of lymphocytes in 6/7 patients challenged with allergen compared to only 1/5 challenged with saline (Table 2). The proportion T cells were 86% (range 75%-91%) among the BAL lymphocytes and 74% (range 61%-86%) in PBL. The differential cell counts of whole blood revealed no change in the cellular composition at baseline and after allergen or saline challenge (data not included).

Determination of apoptosis in BAL fluid cells and PBL at baseline and following allergen/saline challenge

Paired BAL fluid lymphocytes and PBL from all individuals were tested for their apoptosis sensitivity at baseline and post-challenge with either allergen or saline. Susceptibility to apoptosis induced by the mithochondrial pathway was investigated by stimulation with 2μM tributyltin (TBT) *in vitro*, an agent that specifically induces mitochondrial permeability transition, loss of mitochondrial membrane potential, cytochrome C release and apoptosis (24) (25). Apoptotic cells were identified using annexin-V staining and FACS analysis. The rate of spontaneous apoptosis among BAL fluid lymphocytes and PBL was not altered by airway allergen or saline exposure (Table 3 and 4).

TBT exposure of BAL fluid lymphocytes from patients in the allergen provocation group (n=7) resulted in 42.2 % (range 33.9-62.5%) apoptotic BAL fluid lymphocytes at baseline. After airway allergen challenge the same treatment of the BAL fluid cells resulted in a significant decrease in apoptotic lymphocytes (p=0.023), since only 23.5% (range 15.3-42.4%) of the lymphocytes became apoptotic (Table 3 and Fig. 1a). This phenomenon was not recorded in the group of patients which inhaled saline (n=5). In this group TBT induced apoptosis in 37.8% (range 26.6-41.5%) of the BAL fluid lymphocytes obtained at baseline and in 36.8% (range 14.5-48.5%) (n.s) of the lymphocytes retrieved 24hours post-saline challenge (Table 3 and Fig. 1b).

Inhalation of allergen or saline did not significantly alter the sensitivity of PBL to TBT-induced apoptosis. In the allergen provoked group 35.7% (21.4-69.9%) of the control lymphocytes were apoptotic upon treatment with 2μM TBT while the same treatment 24hours after challenge induced apoptosis in 47.0% (11.8-80.06%) (n.s) of the lymphocytes (Table 4 and Fig. 2a). In the group of patients inhaling saline, TBT-exposure of PBL induced apoptosis

in 51.4% (28.6-75.1%) of the lymphocytes at baseline while the same treatment 24hours after challenge triggered apoptosis in 40.5% (24.6-75.8%) of the PBL (n.s) (Table 4 and Fig. 2b).

Analysis of Bcl-2 and PCNA in BAL lymphocytes.

BAL fluid lymphocytes (cytospins) from three subjects at baseline and post-allergen challenge, respectively, were stained for the expression of the anti-apoptotic protein Bcl-2 and for PCNA (proliferation marker) together with an antibody against CD3. Allergen exposure increased the proportion of Bcl-2 expressing BAL fluid lymphocytes, the majority being T-cells, from 54% (range 47%-63%) to 70% (range 59%-77%) in the paired samples, i.e. at baseline and after allergen challenge from the same subject (Fig 3a). Data from unpaired samples from three additional patients, at baseline (patient 9), following allergen exposure (patient 10) and after saline challenge (patient 12) was in line with the above results (55% Bcl-2 expressing BAL fluid lymphocytes in patient 9, 71 % in patient 10 and 49 % in patient 12, respectively). One representative experiment of BAL fluid cells from patient 11 stained for Bcl-2 at baseline and 24 hours after challenge is also presented (Fig 3b,c).

All BAL fluid lymphocytes analysed were PCNA negative at baseline, following saline and allergen challenge, indicating no proliferative activity among these cells (data not included).

Detection of caspase-3 activity in BAL fluid lymphocytes and PBL at baseline and after airway allergen challenge

To evaluate whether the pro-apoptotic enzyme caspase-3 was activated in BAL fluid lymphocytes and PBL, cells were loaded with a fluorigenic caspase-3 substrate and the production of the cleavage product was monitored using flow cytometry. BAL fluid lymphocytes possessed significantly higher endogenous caspase-3 activity than did PBL

(p=0.002) (Figure 4). However, allergen provocation did not significantly alter the basal caspase-3 levels in either BAL fluid lymphocytes or PBL (Figure 5a and 5b).

DISCUSSION

In the present study we report that BAL fluid lymphocytes from asthmatic subjects exposed to allergen were significantly more resistant to mitochondrial-mediated apoptosis compared to non-allergen exposed BAL lymphocytes from the same subjects. This decreased sensitivity to apoptosis was accompanied by an increase in the proportion of BAL fluid lymphocytes expressing the Bcl-2 protein. However, airway challenge with saline did not alter the sensitivity of BAL fluid lymphocytes to TBT-induced apoptosis and neither did allergen or saline provocation influence the sensitivity of PBL to mitochondrial-mediated apoptosis. These data suggest that airway provocation with allergens triggers an inflammatory response that results in the alteration of the apoptotic phenotype of lung lymphocytes but not of peripheral blood lymphocytes. The observed discrepancy in apoptosis-sensitivity between BAL and PBL following allergen challenge may reflect the different activation status of the cells in these two compartments; it is known that airway allergen challenge can cause an accumulation of activated allergen-specific lymphocytes in the airways (2, 30, 31). The present results concord with our previous studies of apoptosis in lymphocytes activated *in vitro* (32) and in BAL cells from patients with sarcoidosis (22).

The major proportion of lung lymphocytes are T cells (33), which in asthma are characterised by a Th2-phenotype (1). T cells play a role in controlling chronic inflammation in asthma (34) and the number of T cells infiltrating the bronchial airways is significantly increased in this disease (2, 31).

It is accepted that the increased numbers of mucosal T cells is the result of an increased recruitment of these cells within the airways (3, 38, 39). However, it is also likely that the number of T cells in the airways of asthmatic patients depends upon an increased survival of these cells. This hypothesis supports several studies in which it has been reported that cells engaged in the inflammatory asthmatic process are less apoptotic.

The potential role of the Fas receptor pathway in asthma has been intensively studied during recent years, albeit with contradictory results. Some studies have demonstrated a decreased expression of Fas receptor or Fas ligand (6) in asthmatic subjects while other studies have reported an increased expression of Fas receptor and Fas ligand in asthmatic subjects (7, 40). Another study showed that T cells from asthmatic subjects failed to undergo apotosis following Fas receptor ligation, although these cells express the same levels of Fas and Fas ligand as in non-asthmatic subjects (41).

In the present study we were particularly interested to study the sensitivity of lymphocytes to mitochondrial-mediated apoptosis in asthma, since this pathway has been suggested to play a substantial role in down-regulation of an immune response (10). To this end we used TBT, a mitochondrial toxin that specifically triggers the mitochondrial pathway by loss of mitochondrial membrane potential and cytochrome C release (23-25).

The Bcl-2 family consists of both proteins that protect from mithochondrial-mediated apoptosis, such as Bcl-2 and Bcl-xL, and proteins that promote this apoptotic pathway, i.e. Bax, Bak, Bid and Bad (42). The Bcl-2 protein is mainly localized in the mitochondria membrane where it blocks the release of cytochrome C and consequently caspase activation and apoptosis (42-44). In the present study we observed an increase in resistance to mitochondrial-mediated apoptosis in BAL fluid lymphocytes following airway allergen challenge and this was associated with an increase in the proportion of Bcl-2 expressing lymphocytes. These data agree with several previous reports; in bronchial biopsies taken from asthmatic subjects most T cells do not appear to be apoptotic and express Bcl-2, suggesting that these cells may have the ability to live longer in the inflamed airways (4). This was also confirmed by results obtained using an allergen-induced cutaneous late-phase response model (45). Moreover, a recent study reported that cyclosporin treatment of asthmatic subjects prior to airway allergen challenge resulted in an increased apoptosis and reduced Bcl-2 expression

among BAL fluid lymphocytes (46). In addition, Bcl-2 expression was increased in sputum mononuclear cells from asthmatic patients compared to patients with COPD and healthy controls (6)

Sensitised individuals with allergic asthma develop an early asthmatic reaction (EAR) 10-20 minutes after airway allergen challenge which resolves after approximately 1 hour .

The bronchial constriction, increased vascular permeability and mucus production during EAR is caused by rapidly metabolised mediators such as histamine, prostaglandins and leukotrienes which are released by IgE cross-linked mast cells. Some individuals develop a late asthmatic reaction (LAR) 3-4 hours after challenge, which can persist for 24 hours or more. LAR is caused by the induced synthesis and release of inflammatory mediators including leukotrienes, chemokines and cytokines which recruits other leukocytes such as eosinophils and T-lymphocytes to the bronchial mucosa.(35). In the present study, airway allergen challenge induced EAR in all exposed subjects although only one out of these seven individuals developed LAR. However, allergen exposure resulted in a significantly rise in the proportion of BAL fluid eosinophils although an increase in lung eosinophils was also seen in three out of five subjects receiving saline. Interestingly, in six out of seven subjects, inhalation of airway allergen resulted in a non significant increase in BAL fluid lymphocytes and this tendency was not found among the saline exposed individuals suggesting a enhanced recruitment and/or survival of these cells after an allergen exposure which do not induce LAR (36, 37). The increased apoptosis resistance of BAL fluid lymphocytes observed in our study may reflect a cellular event that allergen, present in the normal milieu of the asthmatic individual, induces in airway lymphocytes. A decreased apoptosis sensitivity among these lymphocytes could contribute to the development of a sub-clinical inflammation in the bronchial mucosa resulting in thickening of the basement membrane and remodelling of the airways.

Several lines of evidence suggest additional non-apoptotic functions for traditionally pro-apoptotic caspases. Active caspase-3 participates in the differentiation of such diverse cell types as neurons, muscle cells, monocytes and erythroblasts (15-18). Moreover, the proliferative machinery of T cells appears to require active caspase-8 and caspase-3, and the activation of caspase-3 has been proposed to be essential for cell cycle progression in B cells (19, 47-49). Although it is rather established that caspases do possess a non-apoptotic role in activated T cells it is not clear what initiates this activity and what are the caspase-3 substrates. However, in a recently published article the authors suggest that the transcription factors NFATc2 and NFAT, which are crucial for cytokine production and activation of T cells, are substrates of caspase-3 (50). BAL fluid lymphocytes seem to have higher caspase-3-like activity than do peripheral lymphocytes, probably due to the fact that BAL fluid cells are more highly activated than are peripheral lymphocytes. This was further confirmed in the present study, in which viable BAL fluid lymphocytes from asthmatic subjects had a significant higher caspase-3-like activity than viable PBL from the same subjects. However, no PCNA expression was detected in BAL fluid lymphocytes from the six subjects tested, suggesting that the elevated caspase-3 activity observed was not associated with proliferation in these cells.

It is still unclear how cells with high caspase-3 activity could be rescued from the proteolytic degradation machinery that is normally active in an apoptotic cell. There must be tight regulation of the apoptotic machinery in activated cells in order to prevent caspase-3 from completing its commitment to degrade vital proteins. How such protection is achieved is not known as yet, but compartmentalisation, conformational changes of the target proteins or protection via chaperones are possible mechanisms.

In summary, we have demonstrated an alteration in the apoptotic machinery of BAL fluid lymphocytes recovered from asthmatic subjects following airway allergen. This altered

apoptotic phenotype may not be due to changes in the receptor inducible pathway but are definitely related to the mitochondrial apoptotic pathway. We also recorded a significantly higher baseline level of caspase-3 activity in BAL fluid lymphocytes from these asthmatic subjects compared to PBL from the same subjects. These data indicate that airway inflammation in asthma is associated with a reduced apoptosis susceptibility, which may lead to an enhanced survival of lymphocytes in the bronchial mucosa and consequently prolonged inflammation.

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FIGURE LEGENDS

Figure 1: Apoptosis susceptibility of BAL fluid lymphocytes at baseline and after allergen or saline challenge.

BAL fluid cells were exposed to 2μM TBT for 60 minutes followed by annexin-V staining and FACS analysis. Data depict the change in percent apoptotic cells after TBT treatment compared to control, at baseline and after airway allergen **(a)** or saline **(b)** challenge.

Figure 2: Apoptosis susceptibility of PBL at baseline and after allergen or saline challenge. Influence of TBT on the induction of apoptosis in PBL of asthmatic subjects provoked by allergen **(a)** or saline **(b)**. Data represent the change in percent apoptotic cells after TBT treatment, before and after allergen challenge.

Figure 3: Expression of Bcl-2 in BAL fluid lymphocytes. BAL fluid cells on cytospins were double-stained using immunocytochemistry for Bcl-2, brown (DAB) and for CD3, red (Fast Red). Paired samples, at baseline and 24hours after allergen challenge, are indicated with lines. Data is expressed as percent Bcl-2 positive lymphocytes **(a)** Immunocytochemistry of BAL fluid cells expressing Bcl-2 from one representative experiment. Squares in the upper right corner are BAL fluid cells stained against CD3 (Fast Red) and with IgG isotype matched control for Bcl-2. Black arrows indicate Bcl-2 positive T celsl. Baseline **(b)** and 24hours after challenge **(c).**

Figure 4: Baseline caspase-3-like activity in BAL fluid lymphocytes and PBL from asthmatic subjects. Cells were loaded with the cell-permeable substrate PhiPhilux (10μM) to detect caspase-3-like activity. Data is expressed as mean fluorescence intensity (MFI), and the lines indicate median values.

Figure 5: **Caspase-3-like activity in BAL fluid lymphocytes and PBL at baseline and after allergen challenge.** Caspase-3-like activity in BAL fluid lymphocytes **(a)** and PBL **(b)** at baseline and after allergen challenge. Data is expressed as mean fluorescence intensity (MFI).

Table 1. Patient characteristics, clinical parameters and broncho provocation data.

Patient	Age	Sex	Methacoline PD_{20} (µg)	Total IgE[a] (ku/L)	RAST[b]	Allergen	FEV_1 Prior to challenge.	Max. drop in FEV_1 (%)	Allergen PD_{20}[c] (SQ)[d]	Response [e]
1	22	F	2200	59	4	Timothy	97	31	130	EAR
2	34	F	290	120	-	Diluent	98	7	-	-
3	46	M	155	500	-	Diluent	73	3	-	-
4	22	M	400	26	2	Cat	83	22	1800	EAR
5	27	F	120	400	2	Cat	96	25	450	EAR
6	24	F	21	150	-	Diluent	94	7	-	-
7	31	F	265	140	4	Birch	96	21	200	EAR
8	32	F	64	37	3	Birch	77	41	320	EAR
9	27	M	1210	53	-	Diluent	112	4	-	-
10	27	F	1050	190	4	Timothy	105	26	450	EAR
11	28	F	23	290	3	Timothy	97	24	160	EAR/LAR
12	28	F	88	120	-	Diluent	85	17	-	-
Geo. Mean			201						337	
Median	27		210	130	3		96		320	
Range	22-46		21-2200	26-500	2-4		73-112		130-1800	

a. Normal range = 1.6-122 kU/*l*

b. RAST, Radio allergosorbent test (measurement of allergen-specific IgE), 0 = negative; 1-6 = increasingly positive

c. PD_{20}, Provocative dose causing a 20% reduction in forced expiratory volume in 1s (FEV_1)

d. SQ units, Arbitrary units related to the concentration of major allergen.

e. EAR, Early asthmatic reaction, >20% drop in FEV_1 during 0-3hours after challenge; LAR, Late asthmatic reaction, >15% drop in FEV_1 during 3-24 hours after challenge

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Table 2. Bronchoalveolar lavage fluid cell count data, before and after diluent or allergen exposure respectively.

	Diluent (n=5)		Allergen (n=7)	
	Before	After	Before	After
Viability %	94.5 (82.5-99.0)	94.0 (86.0-98.7)	93.6 (83.0-95.0)	94.5 (87.7-100.0)
Cell concentration ($x10^6$/L)	71.0 (68.8-90.3)	194.4 (97.8-393.0)*	114.7 (44.6-131.2)	154.3 (82.4-216.1)
Total cell count ($x10^6$)	12.5 (11.5-16.7)	52.5 (13.5-86.7)	18.3 (8.7-25.0)	24.0 (15.0-47.10)
Macrophages %	91.6 (81.8-95.8	91.6 (76.4-98.0)	90.6 (70.6-96.6)	81.4 (46.0-88.4)
Total macrophage count ($x10^6$)	12.0 (9.8-14.9)	51.4 (12.4-81)	15.6 (8.2-29.5)	17.9 (12.6-41.6)
Lymphocytes %	8.0 (2.2-13.4)	4.8 (1.2-8.2)	8.4 (2.2-28.0)	12.0 (8.4-20.6)
Total lymphoycte count ($x10^6$)	1.2 (0.3-1.6)	2.7 (0.9-3.5)	1.1 (0.4-5.1)	3.8 (1.5-4.3)
Neutrophiles %	0.2 (0.0-4.4)	0.8 (0.0-1.4)	1.4 (0.4-1.6)	1.2 (0.2-4.1)
Total netrophile count ($x10^6$)	0.02 (0.00-0.53)	0.2 (0.0-0.4)	0.2 (0.1-0.4)	0.3 (0.1-1.0)
Eosinophils %	0.4 (0.0-2.8)	2.5 (0.0-18.8)	0.2 (0.0-2.4)	2.4 (0.0-42.2)*
Total eosinophile count ($x10^6$)	0.05 (0.00-0.47)	2.2 (0.0-13.7)	0.04 (0.0-0.2)	0.7 (0.0-16.5)
Basophils %	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.2)	0.0 (0.0-0.6)
Total basophile count ($x10^6$)	0.0 (0.0-0.0)	0.0 (0.0-0.09	0.0 (0.0-0.04)	0.0 (0.0-0.2)
MAST cells [a]	0.0 (0.0-2.0)	2.0 (0.0-5.0)	0.0 (0.0-4.0)	4.0 (1.0-9.0)

a. Number in 10 visual fields, *X16 magnification.

* $P< 0.05$

Table 3. Apoptosis-susceptibility of BAL fluid lymphocytes in asthmatic subjects before and after allergen or saline inhalation. Data depicts percentage of Annexin-V positive BAL fluid cells.

Challenge	*Patient No.*	*Before challenge*		*After challenge*	
		- TBT	+ TBT	- TBT	+ TBT
Allergen	1	15.9	43.3	19.0	25.8
	4	11.0	38.6	8.5	20.5
	5	10.4	52.2	5.8	23.6
	7	9.2	62.5	5.1	42.4
	8	9.4	33.9	12.3	23.5
	10	10.6	42.2	7.6	18.2
	11	8.4	34.8	6.5	15.3
	Median	**10.4**	**42.2**	**7.6**	**23.5**
Saline	2	18.4	37.1	4.4	48.5
	3	6.6	41.5	9.7	43.2
	6	12.4	37.8	7.9	14.5
	9	9.6	26.6	9.1	32.3
	12	12.5	38.2	16.1	36.8
	Median	**12.4**	**37.8**	**9.1**	**36.8**

Table 4. Apoptosis-susceptibility of PBL from asthmatic subjects before and after allergen or saline inhalation. Data show percentage of annexin-V positive PBL.

Challenge	*Patient No.*	*Before challenge*		*After challenge*	
		- TBT	+ TBT	- TBT	+ TBT
Allergen	1	1.4	35.7	3.0	47.0
	4	3.3	35.5	8.1	47.0
	5	2.8	25.2	4.9	24.2
	7	1.6	58.6	2.3	11.3
	8	2.4	21.4	6.2	25.7
	10	3.5	54.9	5.6	80.0
	11	2.6	69.9	2.7	62.9
	Median	**2.6**	**35.7**	**4.9**	**47.0**
Saline	2	2.6	51.4	3.9	40.5
	3	1.9	46.7	6.8	51.8
	6	3.3	75.1	8.1	32.6
	9	4.0	28.6	3.9	24.6
	12	2.5	56.4	3.9	75.8
	Median	**2.6**	**51.4**	**3.9**	**40.5**

Figure 1a

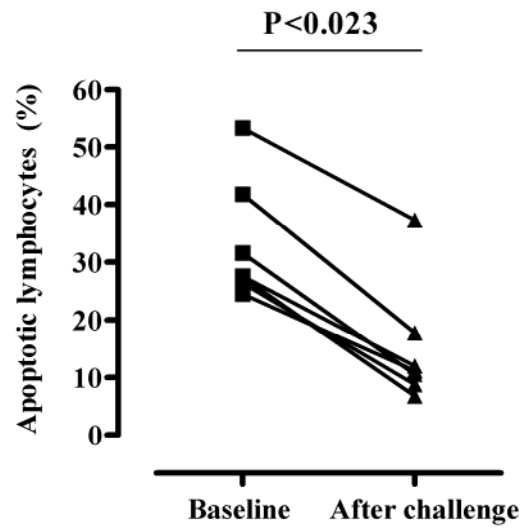

Figure 1b

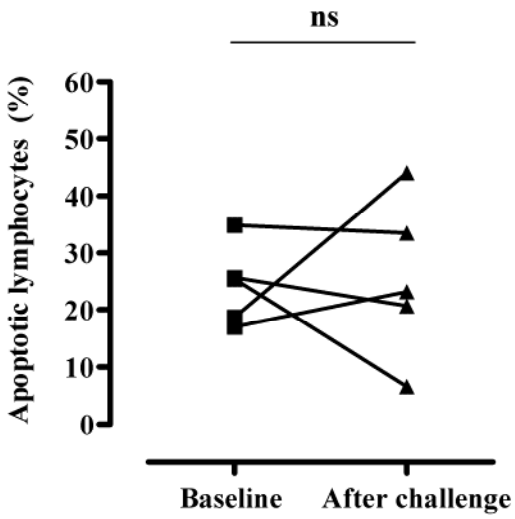

Figure 2a

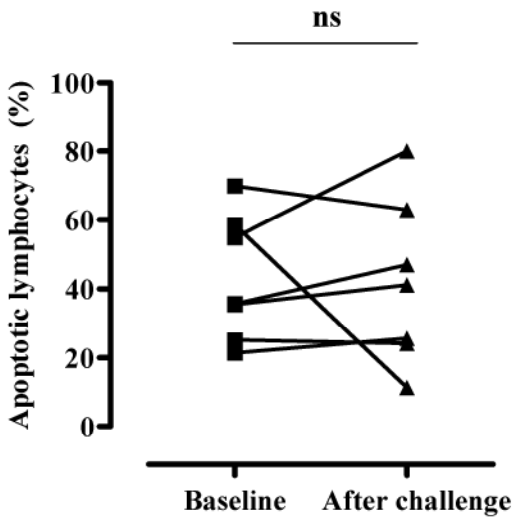

Figure 2b

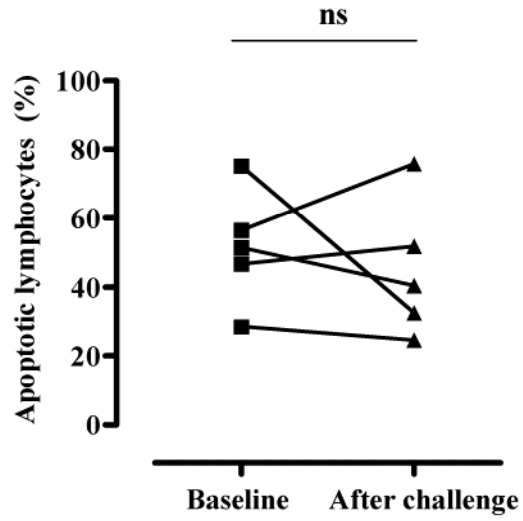

Figure 3a

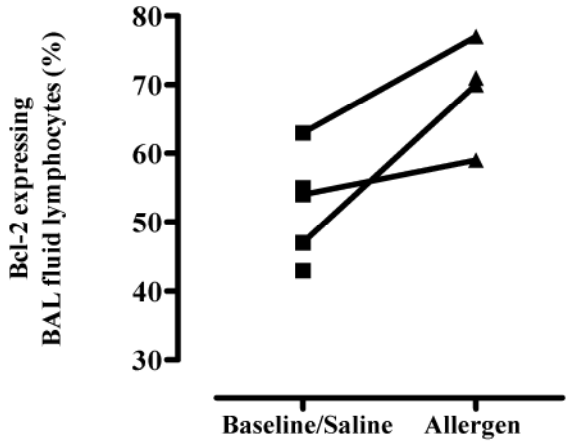

Figure 3b

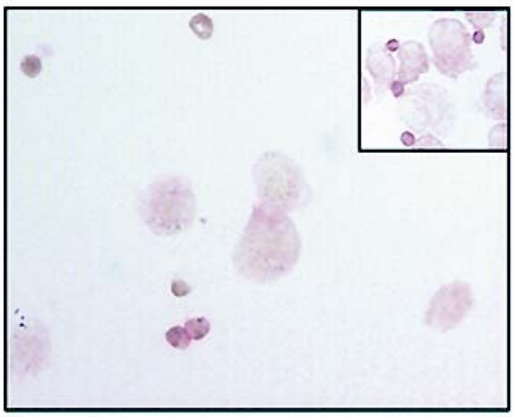

Figure 3c

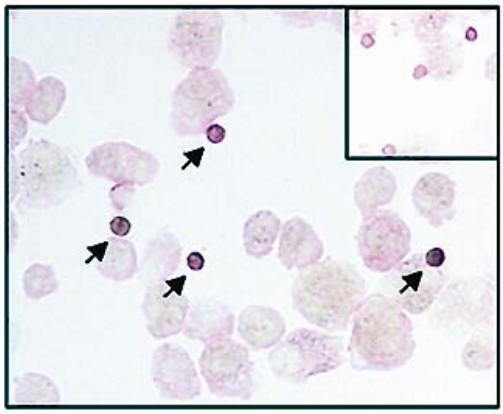

Figure 4

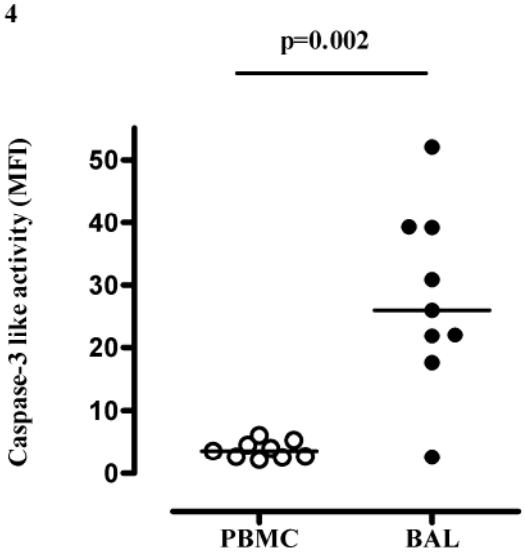

Figure 5a

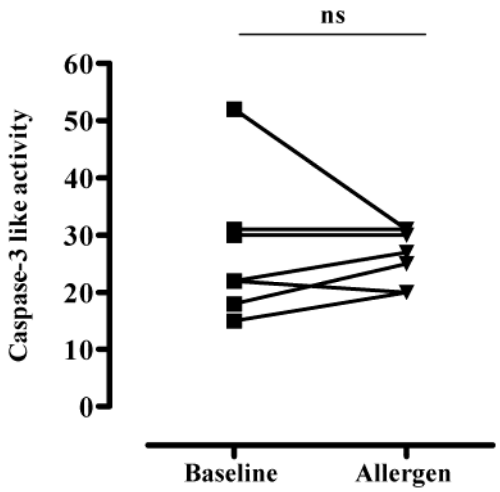

Figure 5b