

IFN- γ but not IL-4 T cells of the asthmatic bronchial wall show increased incidence of apoptosis

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Summary

Background Previous observations have established that IFN- γ production is depressed in CD4⁺ T cells from atopic asthmatics compared with non-asthmatics.

Objective The aim of this study was to determine if decreased IFN- γ production could be due to a dissociation between levels of apoptosis within the T cell subsets of the asthmatic bronchial wall.

Methods Twenty asthmatics (10 atopic and 10 non-atopic) and eight non-atopic non-asthmatics underwent bronchoscopy. Cryostat sections of these biopsies were investigated using immunohistological techniques to determine the relative number of CD4/FAS⁺ and CD4/Bcl-2⁺ cells. Detection of IFN- γ ⁺ and IL-4⁺ was combined with TUNEL staining to determine the proportions of the Th1 and Th2 cells undergoing apoptosis.

Results Experiments revealed raised proportions of activated CD4⁺ T cells as assessed by expression of HLA-DR and CD25⁺ expression in the asthmatic samples. Expression of Bcl-2 by the CD4⁺ cell population was significantly reduced in the asthmatic compared with the control group ($P = 0.002$). There was no significant difference in the expression of CD4⁺ Fas-ligand or the number of CD4⁺ undergoing apoptosis in the asthmatic and non-asthmatic groups. However, the IFN- γ ⁺ ($P = 0.04$) but not IL-4⁺ T cells in the asthmatic biopsies had significantly higher proportions of apoptotic cells compared with the control group.

Conclusion The evidence supports the hypothesis that Th1/Th2 imbalance in asthmatic inflammation may be a result of premature apoptosis within the Th1 subset.

Keywords: bronchial biopsy, asthma, apoptosis, Bcl-2, TUNEL, IFN- γ , IL-4

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Introduction

During the last decade significant progress has been made in elucidating the histopathological picture of bronchial asthma [1,2]. The disease is characterized by a chronic inflammation of the bronchial wall dominated by T lymphocytes that cause local eosinophil accumulation and activation *via* the release of specific cytokines [1,3]. A predominance of CD4⁺ T cells as compared with their CD8⁺ counterparts is an invariant feature in the peripheral

blood and bronchial tissue of asthmatics [4–6]. Furthermore, it is now recognized that CD4⁺ T cells may be divided into Th0, Th1 and Th2 subsets based upon their cytokine profiles [7], and the overproduction of the Th2 cytokines (IL-3, IL-4, IL-5, IL-10, IL-13) by allergen specific CD4⁺ T cells is seen as central to the pathogenic process [5,7].

Previous observations from our laboratory have established that CD4⁺ T cells isolated from the peripheral blood of atopic asthmatics produce significantly lower levels of IFN- γ in response to *D. pteronyssinus* compared with atopic non-asthmatics [8]. As IFN- γ is a Th1-derived cytokine this raised the possibility that there was a selective down-regulation of Th1 CD4⁺ T cell activity following

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antigenic stimulation. Such a finding *in vivo* could have profound consequences as Th1 and Th2 subsets interact to regulate each other's functions [9,10].

Programmed cell death, otherwise known as apoptosis, provides a mechanism for the removal of antigen-activated T cells [11] and eosinophils [12], thereby leading to the resolution of the inflammatory response [13]. The accumulation of eosinophils in the asthmatic airway significantly contributes to the persistence of airway inflammation in these patients [14]. Compelling evidence has recently accumulated which attests to a defect in eosinophil apoptosis in asthma. Decreased numbers of eosinophils undergoing apoptosis have been observed in asthmatic subjects when compared with a non-asthmatic control group [15]. In addition, eosinophil expression of the anti-apoptotic gene product Bcl-2 is increased in bronchial biopsy material from asthmatics [16]. Interestingly, corticosteroid administration in asthmatic subjects results in an increase in the proportion of apoptotic eosinophils in tissue biopsy specimens [16] and sputum [12].

The role of T cell apoptosis in asthma has only very recently received any attention. Spinozzi *et al.* recently reported hypoeexpression of FAS mRNA and its receptor FAS ligand, two molecules necessary for activation-induced cell death, by pulmonary T cells isolated from bronchoalveolar lavage fluid (BALF) of atopic asthmatics. Consequently, pulmonary T lymphocytes from asthmatics as opposed to controls did not undergo apoptosis after *in vitro* exposure to IgM anti-Fas [17]. In contrast, Krug *et al.* did not observe any difference in the expression of FAS ligand on BALF T cells from asthmatic and non-asthmatic subjects. There was, however, an increase in FAS-ligand expression in asthmatic, but not in control, subjects following allergen challenge. Interestingly this increase was not accompanied by an increase in the number of T cells undergoing apoptosis [18].

Comparable levels of cell surface Fas expressed by peripheral blood T cells from asthmatic and non-asthmatic subjects have been reported. However, T cells from asthmatics failed to undergo the normal degree of apoptosis following Fas receptor ligation [19].

Data from bronchial biopsy studies have shown no appreciable difference in the T cell expression of Bcl-2, FAS or FAS-ligand [16], or the number of apoptotic T cells [15], between steroid-naïve asthmatics and non-asthmatic control groups. However, no dissection of CD4⁺ T subunits was made.

The aims of this study were first to establish the incidence of T cell apoptosis *in vivo* in the lungs of asthmatics and non-asthmatics, and secondly to determine whether dissociation exists between levels of apoptosis within the T cell subsets of the asthmatic bronchial wall.

Methods

Patient selection

Twenty non-smoking mild to moderate (%FEV1 pred: mean $\pm$ SE; $82 \pm 2.3\%$, range 68–109%) asthmatic subjects participated in the study (mean age 29.4 ± 2.08 , range 15–45 years). All patients were newly diagnosed, therefore all of the subjects were steroid naïve. Furthermore, none of the patients were treated with theophylline, chromoglycate or long-acting β -agonist medications.

A history and examination confirmed asthma, along with a positive histamine bronchial provocation test as previously described [20]. Ten of the subjects were atopic as determined by positivity to one or more of 10 routinely tested allergens by skin prick testing (Der p., cat dander, dog dander, feather, 5 tree mix, grass mix, rye grass mix, milk, wheat and yeast) (Bayer Corporation, Elhart, PA, USA) at the clinic as previously described [8]. Non-atopic subjects ($n = 10$) were defined by negative testing to the above allergens with a normal serum IgE and negative RAST to the same allergens.

Eight subjects with no history of asthma, negative skin prick tests and negative RAST to the same allergens were selected for use as a comparable control group. These subjects were non-smokers, had not previously been treated with corticosteroids and had pulmonary function tests within normal limits.

The presence of a respiratory tract infection in the previous month resulted in exclusion from the study. The ethics committee of the hospital approved the study and all patients gave written informed consent prior to participation.

Bronchoscopy

Endobronchial biopsies were obtained from the second to fourth generation proximal bronchi on the right side under general anaesthesia as previously described [21]. Biopsy material was immediately snap frozen in iso-Pentane (BDH, Poole, UK) cooled to -80°C and then stored in liquid nitrogen. Sections $6\ \mu\text{m}$ in thickness were subsequently cut from the specimens onto poly-L-lysine glass slides (BDH). The integrity and architecture was confirmed by staining with toluidine blue dye. Sections were then air dried for 1 h, fixed in chloroform:acetone 1 : 1 solution for 10 min, and then air-dried for a further 20 min prior to storage at -20°C .

Determination of T cell number

All slides were coded and counted in a blind fashion. The presence or absence of a subepithelial T cell inflammatory infiltrate and the absolute numbers of T cells per unit area

were identified using a cocktail of CD2, CD3, CD5 and CD8 (T mix) monoclonal antibodies (moab) by an indirect immunoperoxidase method as previously described [22]. Endogenous peroxidase activity from eosinophils and mast cells was excluded by the use of negative controls in which the T cell monoclonal antibody layer was omitted. A minimum of five areas in the subepithelial region were analysed and the total number of T cells was determined per unit area ($10^4 \mu\text{m}^2$) using a computerized image analysis system (Seescan, Cambridge, UK).

CD4⁺ : CD8⁺ ratio determination

This was achieved by double immunofluorescence using a mouse anti-human (MAH) CD4⁺ IgG1 (DAKO A/S, Glostrup, Denmark) and a MAH CD8⁺ IgM (Royal Free Hospital, London, UK) moab at dilutions of 1 : 10 and 1 : 5, respectively. The second layer contained a mixture of purified goat anti-mouse IgG (conjugated to tetra ethyl rhodamine isothiocyanate, TRITC) and goat anti-mouse IgM (conjugated to fluorescein isothiocyanate, FITC) (Southern Biotechnology Associates Inc., Birmingham, AL, USA) at dilutions of 1 : 50 and 1 : 25, respectively. Sections were examined using a Zeiss fluorescence microscope with epi-illumination and barrier filters for FITC and TRITC. A minimum of five high-power fields ($\times 40$) were examined (each containing at least 20 cells), and the total number of CD4⁺ and CD8⁺ cells were determined and expressed as a ratio for each subject. Negative controls were achieved by the omission of the first monoclonal antibody layer.

CD4⁺ expression of CD25, HLA-DR and Bcl-2

Activation of CD4⁺ cells as determined by the expression of the CD25⁺ and HLA-DR antigens was investigated by double immunofluorescence [22]. Mouse anti-human CD4 + IgG1 (DAKO A/S) and a moab to either CD25 + IgG2 α (Royal Free Hospital) or HLA-DR IgM (Royal Free Hospital) were applied at dilutions of 1 : 10, 1 : 5 and 1 : 5, respectively. In the case of CD4⁺-CD25⁺ immunofluorescence, the second layer contained goat anti-mouse (GAM) IgG1-TRITC and GAM IgG2 α -FITC (Southern Biotechnology Associates Inc.) at dilutions of 1 : 50 and 1 : 25, respectively, while in the case of CD4⁺-HLA-DR immunofluorescence the second layer contained GAM IgG-FITC and GAM IgM-TRITC (Southern Biotechnology Associates Inc.) at dilutions of 1 : 25 and 1 : 50, respectively.

CD4⁺ expression of the Bcl-2 gene product was also determined by double immunofluorescence as described above with the following modifications. The initial layer applied contained the moabs MAH CD4⁺ IgG2 α (Caltag, Burlingame, CA, USA) and MAH Bcl-2 IgG1 (DAKO A/S) at dilutions of 1 : 5 and 1 : 10, respectively. The second layer

applied contained isotype and subclass matching GAM IgG2 α -FITC and GAM IgG1-TRITC (Southern Biotechnology Associates Inc.) at dilutions of 1 : 25 and 1 : 50, respectively. Negative control was achieved by the omission of the first monoclonal antibody layers in all three experiments.

Sections were examined using a Zeiss fluorescence microscope with epi-illumination and barrier filters for FITC and TRITC. A minimum of five high-power fields ($\times 40$) were examined (each containing at least 20 cells) and the total number of CD4⁺ positive and CD4⁺-Bcl 2⁺ positive cells were counted. The percentage of CD4⁺-Bcl 2⁺ positive cells within the CD4⁺ population was determined as follows: $\text{CD4}^+\text{-Bcl-2}^+/\text{Total CD4}^+ \times 100/1$. The proportions of CD4⁺ cells expressing either CD25⁺ or HLA-DR were determined in an identical fashion.

Direct immunofluorescence combined with biotin-streptavidin fluorescence

To determine the Th1 and Th2 subset proportions within the CD4⁺ group and the proportions of CD4⁺ cells expressing the Fas-ligand, direct immunofluorescence for CD4⁺ was combined with a modified biotin-streptavidin fluorescence staining technique for the relevant cytokines IL-4, IFN- γ or Fas-ligand in separate experiments. Mouse anti-human CD4⁺ IgG1 (DAKO A/S) was applied at a dilution of 1 : 10 followed by application of goat anti-mouse IgG1-TRITC at a dilution of 1 : 50. Mouse anti-human IFN- γ IgG2 α (R & D Systems, Abingdon, UK) moab was applied at 1 : 10 dilution overnight for 18 h at 4 °C. Biotinylated GAM IgG2 α (Southern Biotechnology Associates Inc.) was then applied for 1 h at a 1 : 100 dilution. Tissue-bound moab complexes were highlighted in a pH-dependant enzymatic reaction by the addition of Streptavidin-FITC (Southern Biotechnology Associates Inc.) at a 1 : 25 dilution for 1 h in a humidified dark chamber at 20°C. Sections were viewed under a Zeiss fluorescent microscope.

To determine the Th2 subset proportions a similar procedure was used with the following modifications in order to facilitate the use of anticytokine subclass specific moabs, to produce optimal staining. Mouse anti-human CD4⁺ IgG2 α (Caltag) at a 1 : 5 dilution was applied in the first layer and the corresponding isotype and subclass specific GAM-IgG2 α -FITC at a 1 : 25 dilution applied in the second layer. Mouse anti-human IL-4 IgG1 (Pharmingen, San Diego, CA, USA) moab was applied overnight at a 1 : 10 dilution and subclass-corresponding biotinylated GAM IgG1 was subsequently applied for 1 h. Finally the tissue bound moab complex was highlighted by the addition of Streptavidin-TRITC (Southern Biotechnology Associates Inc.) at a dilution of 1 : 50.

To determine the proportions of Fas-ligand positive CD4⁺ cells this procedure was repeated with the replacement of

Fas-ligand specific MAH IgG1 (Pharmingen) at a dilution of 1 : 50 instead of MAH IL-4 IgG1 as above.

Negative control was achieved in all three of these experiments by the omission of the first monoclonal antibody layer.

A minimum of five high-power fields ($\times 40$) were examined (containing at least 20 cells), and the total number of CD4⁺ positive staining cells and CD4⁺/cytokine positive cells and CD4⁺/Fas-ligand positive cells were counted. The percentage of the subset (Th1 or Th2) within the CD4⁺ population was determined as follows: CD4⁺-cytokine+/Total CD4⁺ $\times 100/1$. The percentage of Fas-ligand positive cells within the CD4⁺ population was determined also as follows CD4⁺-Fas-ligand+/Total CD4⁺ $\times 100/1$.

Incidence of apoptosis in the CD4⁺ group

Following application of MAH CD4⁺ IgG1 (see above), 50 μ L of TUNEL-terminal deoxynucleotidyl (dUTP) nick end labelling (Boehringer Mannheim, East Sussex, UK) solution was applied to each test specimen. The reaction mixture contained 50 μ L terminal deoxynucleotidyl transferase from calf thymus and 450 μ L fluorochrome conjugated nucleotide dUTP. In the negative control the reaction mixture was omitted. All sections were incubated for 1 h in darkness at 37 °C. Sections were mounted in Citifluor (Citifluor Ltd, Canterbury, UK) solution and read under a Zeiss fluorescent microscope. The percentage of CD4⁺ cells which were also TUNEL positive were determined as follows: CD4⁺-TUNEL+ cells/Total CD4⁺ cells $\times 100/1$.

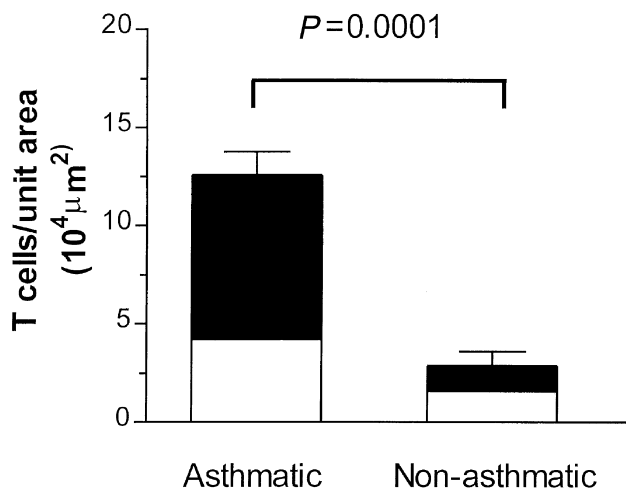


Fig. 1. T cell concentration per unit area ($10^4 \mu\text{m}^2$) in the subepithelial regions (mean $\pm$ SEM) of bronchial biopsies from asthmatic ($n = 20$) and non-asthmatic ($n = 8$) groups. Proportions of CD4⁺ and CD8⁺ cells represented by black and white bars respectively.

Incidence of apoptosis within the Th1 and Th2 subsets

The percentages of Th1 and Th2 CD4⁺ subsets (as assessed by IFN- γ and IL4⁺ cells) undergoing apoptosis were determined by a combination of biotin-streptavidin-fluorescence for cytokine with the TUNEL fluorescein procedure (see above). The percentage of cytokine positive cells which were also TUNEL positive were determined as follows: cytokine+-TUNEL+ cells/Total cytokine+ cells $\times 100/1$.

Statistical analysis

Calculations of geometric mean PC₂₀ values were performed on log-transformed data. Results are expressed as medians and 25–75th percentiles and the Mann–Whitney *U*-test was used for unpaired comparisons. Normally distributed data are expressed as mean $\pm$ SE and group means were compared by

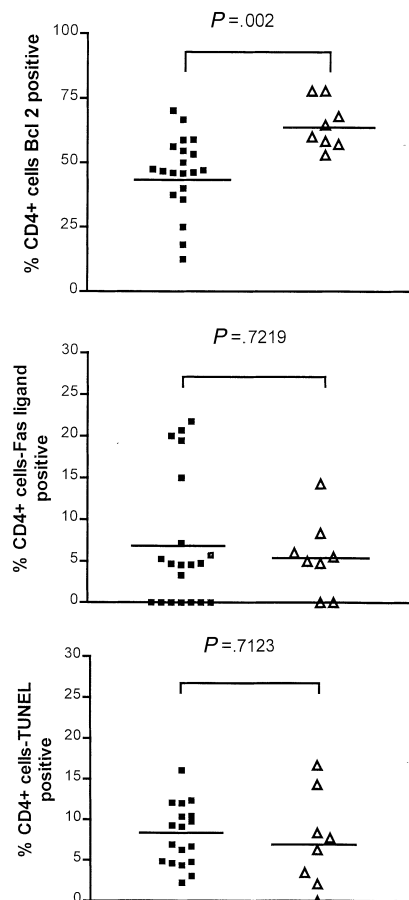


Fig. 2. Scattergram representations of CD4⁺ cells expressing Bcl-2 (panel a), FAS-ligand (panel b) and TUNEL positive (panel c) CD4⁺ cells in the subepithelial region of bronchial biopsies from asthmatic (closed squares) ($n = 20$) and non-asthmatic (open triangles) ($n = 8$) groups. Median represented by line.

Fig. 3. Percentages of IFN- γ (squares) and IL-4 (triangles) cells (median represented by line) within the CD4⁺ population as measured by double immunofluorescence in the superepithelial region of bronchial biopsies from asthmatic ($n = 20$) and non-asthmatic ($n = 8$) groups.

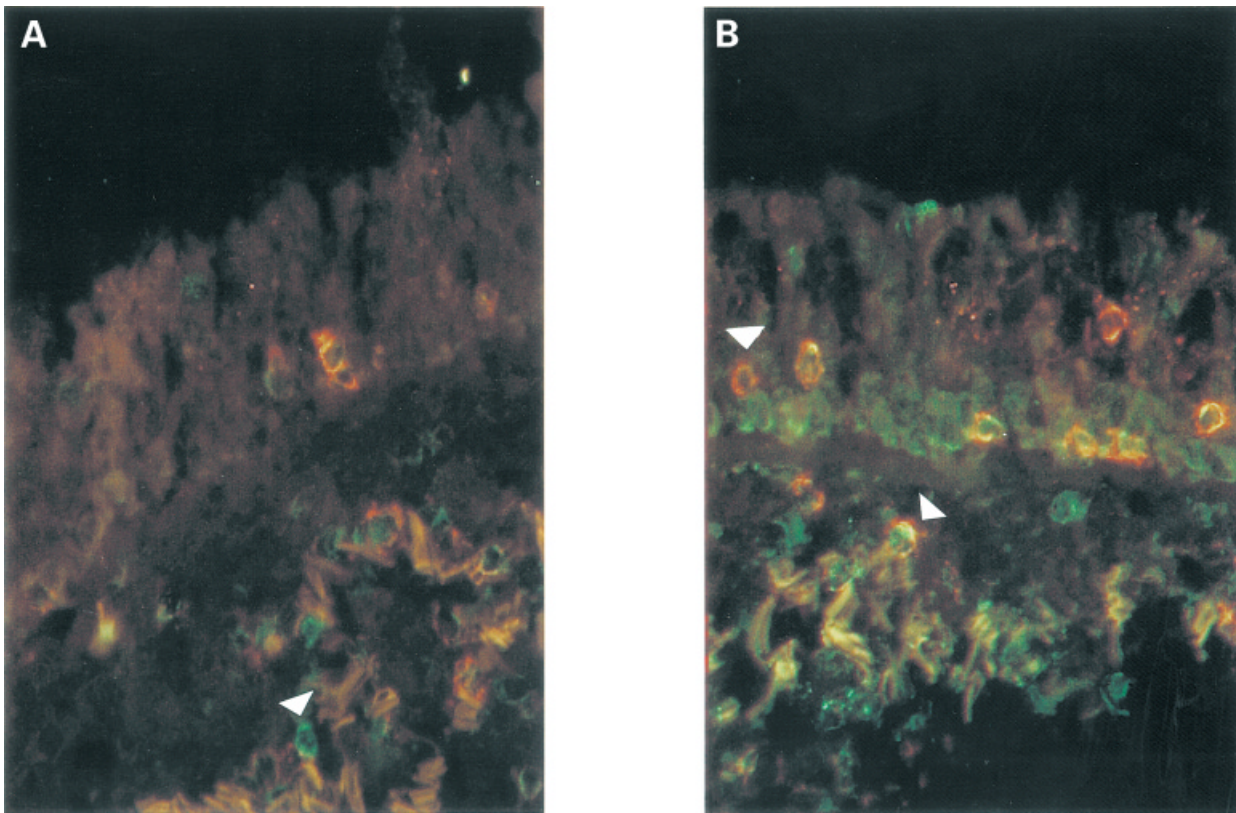
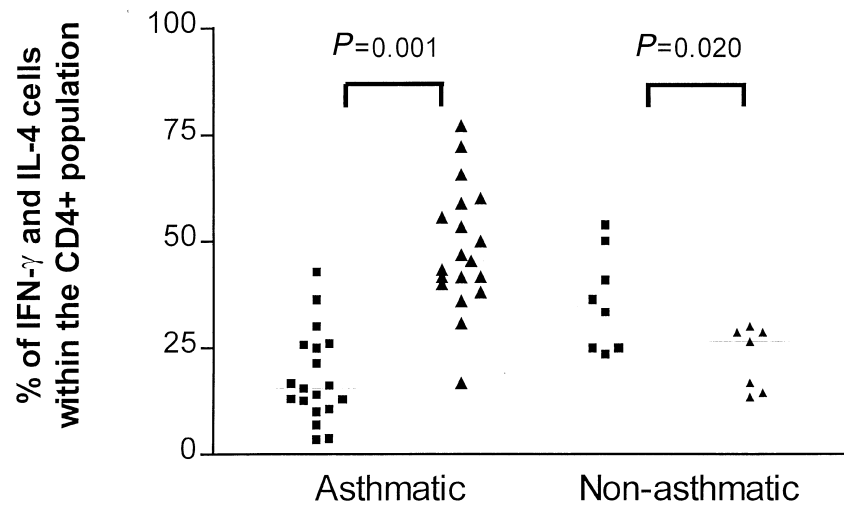


Fig. 4. Panel a: bronchial tissue from a non-asthmatic subject stained for IFN- γ ⁺ cells (stained red) and TUNEL positive cells (stained green). The arrow indicates an IFN- γ negative TUNEL positive cell. Panel b: bronchial tissue from an asthmatic subject stained for IFN- γ ⁺ cells (stained red) and TUNEL positive cells (stained green). The superior arrow indicates an IFN- γ positive TUNEL negative cell. The inferior arrow indicates a cell positive for both IFN- γ and TUNEL.

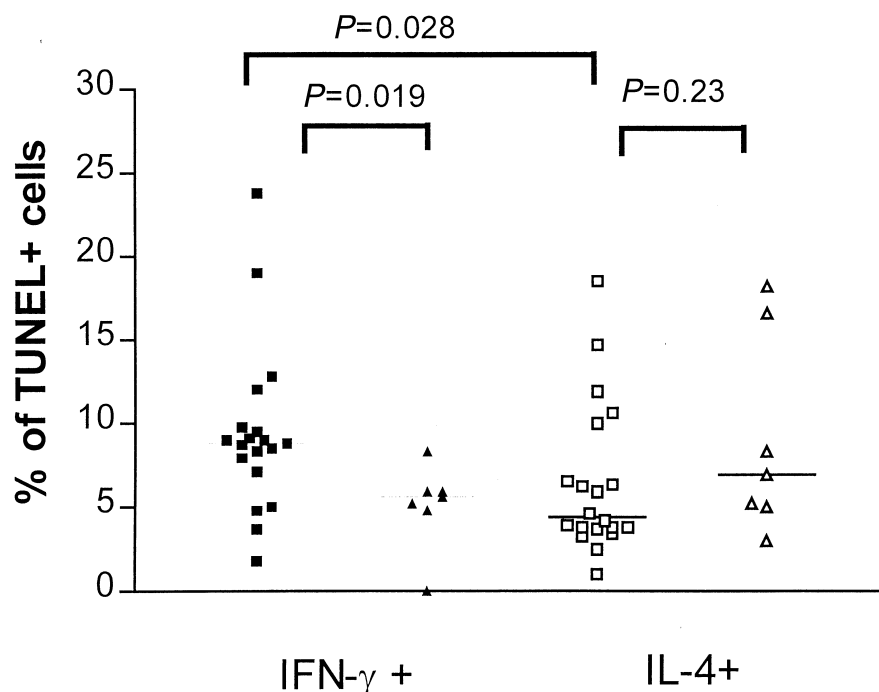


Fig. 5. Percentages of CD4⁺/IFN- γ and IL-4 subsets undergoing apoptosis. There is a significant difference in the number of IFN- γ ⁺ cells (filled squares), compared with IL-4⁺ cells (open squares) undergoing apoptosis in the asthmatic group ($n = 20$). The incidence of IFN- γ ⁺ apoptosis in the asthmatic (filled squares) compared with the normal controls (filled triangles) is also significantly increased.

using Student's *t*-test and considered significant if $P \geq 0.05$. The statistical calculations were performed with the use of a validated statistical software package for personal computers (SigmaStat 2.0, Jandel Scientific, Chicago, IL, USA).

Results

All asthmatic bronchial biopsies contained a subepithelial inflammatory infiltrate that was not present in the control biopsies. The asthmatic biopsies contained a significantly greater number of T cells per unit area as compared with the T cells counted from a similar area from the control group (12.6 ± 1.2 vs. 2.8 ± 0.7 ; $P = 0.0001$). There was a predominance of CD4⁺ over CD8⁺ cells in the inflammatory infiltrate of the asthmatic biopsies (8.3 ± 1.2 : 4.3 ± 0.7 ; $P = 0.0355$) (Fig. 1). In contrast there was a larger proportion of

CD8⁺ cells in the non-asthmatic group but this difference was not significant ($P > 0.05$).

Asthmatic CD4⁺ lymphocytes expressed significantly higher levels of HLA-DR and CD25⁺ (markers of activation) than their non-asthmatic counterparts ($54.3 \pm 4.6\%$ vs. $16.0 \pm 3.7\%$; $P = 0.0002$) and ($10.60 \pm 1.7\%$ vs. $2.6 \pm 1.4\%$; $P = 0.0199$), respectively.

Expression of the anti-apoptotic gene product Bcl-2 by the CD4⁺ population was significantly reduced in the asthmatic group compared with the control group (median 25th–75th percentiles; 46.8 (39–55) vs. 62.3 (58–73) ($P = 0.002$) (Fig. 2a). There was no significant difference in the expression of CD4⁺ Fas ligand, 5.25 (2–7) vs. 4.62 (0–11) (Fig. 2b), or the number of CD4⁺ TUNEL positive cells, 7.99 (4.8–10.4) vs. 6.97 (2.8–16.7) (Fig. 2c) between the asthmatic and non-asthmatic groups.

There was a significant predominance of the IL-4 over the IFN- γ CD4⁺ group in the asthmatic subjects; 45.5 (40–58)

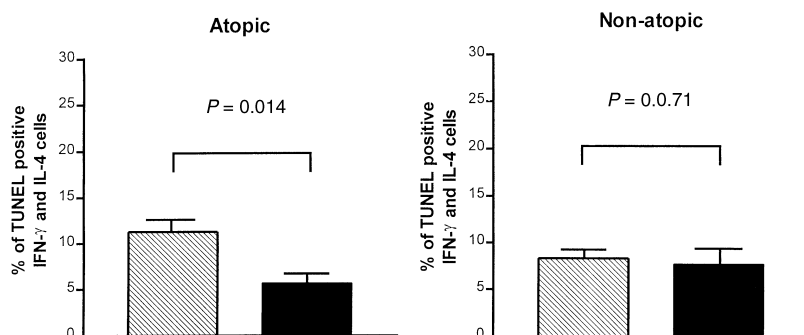


Fig. 6. Comparison of percentages of apoptotic IFN- γ ⁺ (hatched) and IL-4⁺ (black) cells within the atopic asthmatic ($n = 10$) and non-atopic asthmatic ($n = 10$) populations. There was a statistically significant increase ($P \leq 0.05$) in the number of IFN- γ ⁺ cells undergoing apoptosis in the atopic as compared with the non-atopic asthmatic group.

vs. 15.4 (11–26) ($P = 0.0001$). However, the reverse was seen in the control group ($P = 0.020$) (Fig. 3).

Comparing the incidence of apoptosis within the IFN- γ and IL-4 T cells of the asthmatic group, there was a significant increase in the incidence of apoptosis in the IFN- γ cells; 8.8 (7–10) vs. 4.4 (4–8) ($P = 0.028$) (Fig. 5). Interestingly, upon sub-analysis it was found that increased IFN- γ apoptosis was a feature of atopic asthma ($P = 0.014$), but not of non-atopic asthma ($P = 0.71$) (Fig. 6).

The incidence of apoptosis in IL-4⁺ cells was not significantly different between the asthmatic and non-asthmatic patient groups ($P = 0.23$) (Fig. 5). However, the incidence of apoptosis within the IFN- γ ⁺ cells (Fig. 4) was significantly greater in the asthmatic compared with the non-asthmatic group; 8.8 (7–10) vs. 5.6 (5–6) ($P = 0.019$) (Fig. 5).

In order to rule out a non-CD4⁺ source of IFN- γ and IL-4 their expression by the CD8⁺ population was examined and found to be $13.40 \pm 1.6\%$ and $12.80 \pm 1.1\%$ ($n = 20$), respectively. Furthermore, CD8⁺ cells represented less than 2% of TUNEL positive cells. The contribution of Th0 cells to Th1 and Th2 TUNEL positive cells was investigated by staining of sequential sections from the same patients for IL-4/TUNEL and IFN- γ /TUNEL, respectively, and by comparison of the incidence of double staining the Th0 population was calculated to be $3.2 \pm 0.9\%$.

Discussion

It is now widely accepted that CD4⁺ Th2-based lymphocytic activity plays a central role in the genesis and maintenance of the chronic inflammatory reaction in the bronchioles, which results in the signs and symptoms of asthma [4,5,23]. The present investigation provides novel evidence to support the role of T cell apoptosis as central to the CD4⁺ T cell dysregulation in asthma. Expression of the anti-apoptotic protein Bcl-2 was down-regulated on CD4⁺ T cells from bronchial biopsies obtained from asthmatic subjects, potentially rendering these cells more susceptible to apoptosis. Further, a significant increase in the number of CD4⁺ Th1 cells undergoing apoptosis in the asthmatic population as compared with normal controls was observed.

These observations extend previous *in vitro* findings from our laboratory [8], that there is a selective loss of the Th1 CD4⁺ subset upon antigenic stimulation of asthmatic peripheral blood T lymphocytes. The data also lend strong support to the hypothesis that an aberration in T cell apoptosis may be an important mechanistic feature of bronchial asthma.

There have been a handful of reports on the expression of FAS and FAS-ligand on BALF [17,18], peripheral blood [19] and bronchial biopsy [15,16] T cells from asthmatic

subjects. The data are grossly conflicting, with some studies reporting an increase [17], and others a decrease or comparable level [16,18,19], of expression of these markers of apoptosis. However, despite varying levels of FAS/FAS-ligand expression, the majority of these studies report a defect in the T cell apoptotic response. In the present study, we observed no difference in the level of expression of FASL on CD4⁺ cells between the asthmatic and control groups. Krug *et al.* has reported similar findings with respect to FASL expression on peripheral blood and BAL CD4⁺ (as distinct from CD3⁺ cells) obtained from asthmatic subjects [18]. Interestingly, we could document a reduced expression of the anti-apoptotic molecule Bcl-2 on CD4⁺ cells from asthmatics as compared with non-asthmatic subjects. Druilhe *et al.* observed no difference in the proportion of T cells expressing Bcl-2 in bronchial biopsies from steroid-treated/steroid-untreated asthmatics and controls [16]. One explanation of this disparity could be that in the present study CD4⁺ cells were differentially examined for Bcl-2 expression, while Druilhe *et al.*'s observations relate to the entire CD3⁺ population.

Thus far, investigations have been restricted to T cell apoptosis and the constituent subgroups have not been examined. Unlike in the human system, a clear functional dichotomy in CD4⁺ T cells is more evident in the murine model. This facilitates a more detailed investigation of T cell apoptosis. Recent studies have provided some evidence of differential expression of FAS-ligand between Th1 and Th2 subsets. Some Th2 clones [24] and Th2 cell lines [25] lack the expression of FAS-ligand and FAS-mediated cytotoxicity. Watanabe *et al.* have demonstrated that FAS ligand is equally expressed on Th1 and Th2 cell lines taken from ovalbumin-specific TCR transgenic mice, and that both cell lines equally undergo FAS-dependant and FAS-independent activation-induced death [26]. In contrast, Zang *et al.* have demonstrated that Th1 and Th2 cell lines cultured from TCR transgenic mice have a preferential loss of the Th1 subset upon re-stimulation with antigen via a FAS/FAS-ligand dependant AICD mechanism, which was only partially seen in the Th2 subset [27]. This unequal susceptibility to death is apparently an intrinsic property of the subsets, since there is also a selective loss of the Th1 population in mixed cultures of Th1 and Th2 cells [27].

The findings presented here have extended those initial observations in the murine model to the *in vivo* situation in man. The data strongly support the existence of an unequal incidence of apoptotic death within the CD4⁺ T cell subsets. Furthermore, it is a selective increase in the incidence of apoptosis in the TH1 subset, which is evident in the bronchial wall of the asthmatic patient. The incidence of Th1 and Th2 apoptosis in bronchial biopsies from non-asthmatic subjects does not differ significantly, thereby

excluding an inherent susceptibility of the Th1 subset to premature apoptotic death at this site.

Within the asthmatic population the incidence of Th1 apoptosis was greater than that seen in the Th2 subset in atopic asthmatics. Interestingly, CD4⁺ FASL expression was substantially higher (borderline significance) in the atopic vs. the non-atopic asthmatics. Jayaraman *et al.* observed FAS resistance (failure to undergo a normal degree of apoptosis after FAS receptor ligation) in CD4⁺ peripheral blood T cells from non-atopic asthmatics [19]. This raises the possibility of a defect either in the expression or functional activity of FASL in asthma. The possibility that this defect is atopy independent is a question that requires further investigation.

Differentiation of the subsets Th1 and Th2 was achieved on the basis of cytokine production in combination with TUNEL technology. Due to technical difficulties it was not possible to combine these markers with an anti-CD4⁺ monoclonal. We did, however, circumvent the possible confounding factors of cytokine production and TUNEL positivity by CD8⁺ cells by measuring these parameters in the CD8⁺ population. The contribution of the CD8⁺ cells to IL-4 and IFN- γ production was less than 15% and only 2% of CD8⁺ cells were TUNEL positive, thereby validating our approach to this complex problem.

The precise mechanism of this defect in apoptosis in the CD4⁺ T cell population in the asthmatic remains obscure. Zhang *et al.* observed no difference in the expression of the anti-apoptotic product Bcl-2 between Th1 and Th2 subsets [27]. In the present investigation, expression of Bcl-2 on CD4⁺ T cells in asthmatic bronchial biopsies was significantly lower than in normal control biopsies. Unfortunately, it was not possible to elucidate if this decrease was consistent amongst the subsets or if in fact there existed a selective decrease in the expression of Bcl-2 on the Th1 cells.

The results presented here represent the first *in vivo* finding of an unequal rate of apoptosis within the CD4⁺ T cell population in the inflammatory infiltrate of the bronchial wall of asthmatics. Furthermore, the selective death of the Th1 population provides a likely explanation for the skewed Th2 response so characteristic of this disease.

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