Eur Respir J 2003; 22: 207–212
DOI: 10.1183/09031936.03.00098902
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European Respiratory Journal
ISSN 0903-1936

Cyclosporin A, apoptosis of BAL T-cells and expression of Bcl-2 in asthmatics

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Cyclosporin A, apoptosis of BAL T-cells and expression of Bcl-2 in asthmatics. S. Ying, L.N. Khan, Q. Meng, N.C. Barnes, A.B. Kay. ©ERS Journals Ltd 2003.

ABSTRACT: The late asthmatic reaction is characterised by elevated numbers of interleukin-4/interleukin-5/CD4-positive T-helper cells type 2 in bronchoalveolar lavage fluid (BALF). Cyclosporin A (CsA) is known to inhibit T-cell proliferation, induce apoptosis of CD4-positive T-cells and downregulate cytokine gene expression.

It was assessed whether CsA-induced inhibition of the late asthmatic reaction was associated with apoptosis of BALF T-lymphocytes and other cell types, as well as expression of the antiapoptotic protein B-cell leukaemia/lymphoma 2 gene product (Bcl-2). BALF cells were obtained from asthmatics at baseline and 24 h after allergen-inhalation challenge following prior administration of CsA (n=13) or placebo (n=11).

The number of apoptotic CD3-positive T-lymphocytes increased in the CsA but not the placebo group. The numbers of Bcl-2-positive cells were significantly reduced in the CsA but not the placebo group. The majority of Bcl-2-positive cells were CD3-positive T-lymphocytes.

The beneficial effect of cyclosporin A in asthma may be related to its inhibitory effect on the late asthmatic reaction *via* induction of T-cell apoptosis and decreased B-cell leukaemia/lymphoma 2 gene product levels.

Eur Respir J 2003; 22: 207–212.

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Keywords: Apoptosis
asthma
bronchoalveolar lavage
cyclosporin A
cytokine

Received: October 29 2002
Accepted after revision: February 20 2003

This study was supported by the National Asthma Campaign, London, UK.

In asthmatics, allergen inhalation is associated with increases in the numbers of eosinophils, activated T-lymphocytes and interleukin-4 and -5 messenger ribonucleic acid-positive cells in both bronchoalveolar lavage (BAL) fluid (BALF) [1] and bronchial biopsy samples [2]. T-helper cell type 2 cytokines and eosinophil products play pivotal roles in the airway narrowing and remodelling and repair processes characteristic of asthma [3]. Apoptosis (physiological, or programmed cell death) is now widely accepted as a fundamental biological process implicated in events as diverse as embryogenesis, homeostasis, immunological tolerance, resolution of inflammation, normal tissue turnover and various diseases. Recent studies suggest that reduced apoptosis of T-lymphocytes and eosinophils may play a role in asthma pathogenesis [4, 5].

Cyclosporin A (CsA) is a potent immunosuppressive drug that has been used in the management of a number of chronic inflammatory conditions, such as psoriasis, Crohn's disease and rheumatoid arthritis [6]. Although NIZANKOWSKA *et al.* [7] showed that there were no significant changes in lung function or the steroid-sparing effect in CsA-treated asthmatics, placebo-controlled studies in steroid-dependent asthma demonstrated improvement in lung function [8] and decreases in oral corticosteroid requirement [9]. Attenuation of the allergen-induced late asthmatic reaction (LAR) has also been described [10] and shown to be associated with reduced expression of eosinophil growth and chemotactic factor concentrations in BALF [11]. In the present study, the hypothesis that CsA inhibits the LAR by accelerating the apoptosis of CD3-positive cells and that this is associated with decreases in the expression of the antiapoptotic protein B-cell leukaemia/lymphoma 2 gene product (Bcl-2) was tested.

Materials and methods

Patients

In the present study, clinical samples were used from the same patients as described in a previously published report [11]. Thus the subjects had mild atopic asthma (n=24), were sensitive to either grass pollen, house dust mite or cat dander, and were recruited from the London Chest Hospital or the Royal Brompton Hospital, London, UK. Atopy was defined by positive skin-prick tests and asthma was defined as reversible airway obstruction, with a decrease in forced expiratory volume in one second (FEV1) of >20% from baseline in response to allergen. Written informed consent was obtained from all subjects and the study was approved by Royal Brompton & Harefield National Health Service Trust Ethics Committee.

Study design

The study was conducted over 4 weeks, as previously described [11]. Briefly, a full clinical history was taken and examination performed at an initial assessment. The subjects

then underwent allergen challenge. If both an early asthmatic reaction (EAR) and LAR were demonstrated, the subject was selected for randomisation. The EAR and LAR were defined as a decrease of $\geq$20 and $\geq$15% in the FEV1, respectively. After 3 weeks, patients underwent baseline bronchoscopy followed by secondary bronchoscopy 24 h after allergen challenge [11]. In a double-blind manner, each subject received either 500 mg CsA (n=13) or placebo (n=11) at 12 h as well as immediately before allergen challenge [11].

Fibreoptic bronchoscopy and bronchoalveolar lavage

The fibreoptic bronchoscopy procedure was performed before and 24 h after allergen challenge as described previously [11]. The volume of fluid returned from BAL was measured. The BALF was passed through sterile gauze to remove any mucus. Cells were pelleted by centrifugation for 10 min at 300×*g* at 4°C. The cell pellet was washed in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Company Ltd, Poole, UK) and then resuspended in 10 mL of the same medium. A total cell count was performed using a Neubauer haemocytometer and Kimura stain. Cytospins were prepared from the cells as described previously [3].

Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling

Apoptotic cells were determined by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) as described previously [12, 13]. After incubation with streptavidin-conjugated alkaline phosphatase (Amersham, Little Chalfont, UK), the labelling was developed using fast red TR/naphthol AS-MX (Sigma-Aldrich Company Ltd). The cells with distinct red nuclear labelling were defined as TUNEL-positive cells. As a positive control, the permeabilised cytospins were treated with 50 $\mu g \cdot mL^{-1}$ deoxyribonuclease (DNase) I (Sigma-Aldrich Company Ltd) for 10 min at room temperature and then stained using the TUNEL procedure. This resulted in virtually all individual cell nuclei staining with fast red. Negative controls included: 1) performing TUNEL without terminal deoxynucleotidyltransferase, 2) omitting biotin-deoxyuridine triphosphate, and 3) omitting streptavidin-conjugated alkaline phosphatase.

Histological staining and single immunocytochemistry

Eosinophils were identified *via* Congo red staining. The alkaline phosphatase/antialkaline phosphatase (APAAP) technique was used in the enumeration of other cell types in BALF. Monoclonal antibodies used in this study include those directed against human neutrophils (anti-neutrophil elastase; Dako, High Wycombe, UK), macrophages (anti-CD68, Dako) and total T-cells (anti-CD3; Becton Dickinson, Cowley, UK). Expression of Bcl-2 on BALF cells was also analysed using a monoclonal antibody directed against Bcl-2 (anti-human Bcl-2 oncoprotein; Dako). The APAAP technique was performed as described previously [3, 10]. Optimal concentrations of all antibodies used were determined in pilot experiments (anti-neutrophil elastase 0.875 $\mu g \cdot mL^{-1}$, anti-CD68 15.3 $\mu g \cdot mL^{-1}$, anti-CD3 30 $\mu g \cdot mL^{-1}$, and anti-human Bcl-2 oncoprotein 17.5 $\mu g \cdot mL^{-1}$). After washing in tris-(hydroxymethyl)-aminomethane (Tris)-buffered saline (pH 7.2), the slides were incubated with soluble complexes of APAAP (Dako) for a further 30 min and then developed using fast blue (Sigma-Aldrich Company Ltd) as chromogen for signal visualisation.

Positive cells stained a blue colour after development with fast blue. Omission or substitution of the primary antibody with an irrelevant antibody of the same species was used as a negative control. No immunoreactivity was observed in the negative controls.

Double staining

In order to confirm the phenotypes of Bcl-2-positive cells, the cytospins were incubated with mouse anti-Bcl-2 overnight, followed by Texas red-labelled goat antimouse immunoglobulin (Ig)G (ICN pharmaceuticals Ltd, Basingstoke, UK) for 1 h. After washing, cytospins were incubated with mouse IgG (Dako) to saturate any open antigen-binding site on the Texas red-labelled goat antimouse IgG, as recommended by Vector Laboratories, Inc. (Peterborough, UK). Slides were then incubated with anti-CD3 (leucine 4) conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson). IgG1-FITC (Becton Dickinson) was used for controls. The slides were analysed using a fluorescence microscope (Zeiss, Oberkochen, Germany).

Combination of immunocytochemistry and terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling

In order to detect the phenotype of apoptotic cells, a combined immunocytochemistry and TUNEL technique was used as described previously [13]. Briefly, cytospins were first stained immunocytochemically, as described above, and developed with fast blue. This was followed by the TUNEL technique, developed with fast red. After TUNEL, the cytospins were counterstained with methyl green. Using this method, nonapoptotic cell nuclei were stained green, cellular phenotypic markers blue and apoptotic (TUNEL-positive) nuclei red.

Statistical analysis

Immunocytochemical, TUNEL and *in situ* hybridisation slides were counted using an Olympus microscope equipped with an eyepiece graticule (Olympus Corp., NY, USA). The slides were counted in duplicate in a blinded fashion. For each cytospin, $\geq$500 BALF cells were counted. The results are expressed as the percentage of positive cells. The data were analysed by the Wilcoxon matched-pair test for within-group measurements and the Mann-Whitney U-test for between-group measurements. For all tests, a $p<0.05$ was considered significant.

Results

BALF cells obtained during a previous study [11] were used; 13 patients were allocated to CsA and 11 received placebo. Compared to placebo, CsA significantly attenuated the LAR (p=0.048) but not the EAR, as reported previously [11].

Using single immunocytochemical or Congo red staining, it was shown that allergen inhalation induced significant increases in the percentages of elastase-positive neutrophils (p=0.029) and eosinophils (p=0.004) in BALF in the placebo group but not in CsA-treated subjects (table 1). This was associated with a decrease in the percentage of CD68-positive

Table 1. – Effect of cyclosporin A on the percentages of different cell types in bronchoalveolar lavage fluid before and after allergen inhalation challenge

	Placebo			Cyclosporin A			Between-group p-value
	Baseline	Allergen	p-value	Baseline	Allergen	p-value	
Neutrophils %	1.5 (0.6–4.5)	5.1 (0.9–10.9)	0.029	3.7 (0.0–8.7)	6.7 (1.2–21.9)	NS	NS
Eosinophils %	1.3 (0.3–12.3)	5.4 (1.6–13.0)	0.004	8.8 (0.9–34.1)	13.4 (3.0–35.5)	NS	NS
Total T-cells %	5.4 (2.4–9.1)	6.5 (2.8–8.3)	NS	6.7 (2.9–10.5)	6.2 (2.4–8.9)	NS	NS
Macrophages %	85.8 (81.2–93.8)	82.1 (74.6–84.4)	0.004	82.8 (60.0–89.8)	69.1 (37.8–81.4)	0.025	NS
Epithelial cells %	3.0 (2.0–6.3)	3.8 (1.6–7.1)	NS	3.3 (1.2–5.2)	3.0 (1.8–4.0)	NS	NS

Data are presented as median (range). NS: nonsignificant.

macrophages, which presumably reflected a relative shift in the percentage of this cell type (as similar decreases were observed with CsA and placebo). There were no changes before or after allergen challenge in CD3-positive T-cells or cytokeratin-positive epithelial cells in the placebo or CsA group.

Compared to baseline, allergen inhalation induced significant increases in the percentages of total TUNEL-positive apoptotic cells in BALF in both the CsA (p=0.006) and placebo group (p=0.033) (table 2). No significant between-group differences were observed. Apoptotic cells were then phenotyped using a combination of immunocytochemistry (or Congo red staining) and the TUNEL technique. Results are expressed, for each cell type, as a percentage of TUNEL-positive cells (100–500 cells counted) (table 2). In the placebo group, the majority of apoptotic cells were elastase-positive neutrophils, cytokeratin-positive epithelial cells or CD3-positive T-cells. There were only few apoptotic Congo red-positive eosinophils. In the CsA but not the placebo group, there was a significant increase in the percentage of apoptotic CD3-positive T-cells after allergen inhalation (p=0.013) (table 2). After challenge, there was a median of 32.3 TUNEL/CD3-positive cells in the CsA group compared to 3.7 in the placebo arm. The between-group p-value was 0.0019. The individual paired data for CD3/TUNEL-positive T-cells in BALF from the placebo and CsA treatment groups are shown in figure 1. No other significant changes were observed between the CsA and placebo groups for neutrophils, eosinophils, macrophages or epithelial cells (table 2). Of the few apoptotic CD68-positive macrophages identified, some (~2%) were observed engulfing apoptotic cells or apoptotic bodies.

The number of Bcl-2-positive cells (as a percentage of total cells) decreased after allergen challenge in the CsA group (p=0.028) but not in the placebo group (table 3). Similarly, when Bcl-2/CD3-positive T-cells were expressed as a percentage

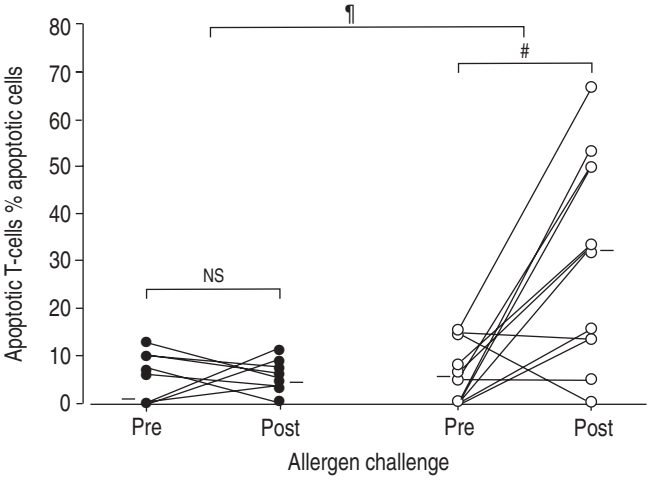

Fig. 1. – Apoptotic T-cells (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling/CD3-positive cells) as a percentage of apoptotic cells in bronchoalveolar lavage fluid from the placebo (●; n=11) and cyclosporin A (○; n=13) groups before (Pre) and after (Post) allergen challenge. Horizontal bars represent medians. NS: nonsignificant. #: p=0.013; ¶: p=0.0019.

of total cells, there was a significant decrease in the CsA group (p=0.028) but not in the placebo group. The individual paired data for Bcl-2/CD3-positive T-cells are shown in figure 2. When Bcl-2-positive cells were expressed as a percentage of TUNEL-positive cells, the numbers were small and there were no significant differences after allergen challenge or between the CsA and placebo group (table 3).

Representative photomicrographs showing CD3/TUNEL-positive cells and Bcl-2/CD3-positive cells are shown in figure 3.

Table 2. – Effect of cyclosporin A on the percentage of apoptotic cells and the percentages of apoptotic cells of different cell types in bronchoalveolar lavage fluid before and after allergen inhalation challenge

	Placebo			Cyclosporin A			Between-group p-value
	Baseline	Allergen	p-value	Baseline	Allergen	p-value	
Total %#	2.6 (0.2–4.5)	3.1 (0.4–10.6)	0.033	2.1 (0.1–6.9)	3.2 (1.6–14.7)	0.006	NS
Neutrophils %¶	50.0 (0.0–84.6)	45.8 (0.0–78.9)	NS	21.9 (0.0–53.3)	35.7 (0.0–67.1)	NS	NS
Eosinophils %¶	0.0 (0.0–8.3)	0.0 (0.0–7.1)	NS	0.0 (0.0–9.3)	1.7 (0.1–15.0)	NS	NS
Total T-cells %¶	0.0 (0.0–30.0)	3.7 (0.0–50.0)	NS	1.7 (0.0–15.0)	32.3 (0.0–66.7)	0.013	0.0019
Macrophages %¶	0.0 (0.0–0.4)	0.0 (0.0–0.7)	NS	0.0 (0.0–0.7)	0.0 (0.0–0.6)	NS	NS
Epithelial cells %¶	18.7 (0.0–25.8)	15.4 (0.0–28.6)	NS	17.5 (0.0–30.0)	16.7 (0.0–30.8)	NS	NS

Data are presented as median (range). NS: nonsignificant. #: as percentage of total cells; ¶: as percentage of apoptotic (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling-positive) cells.

Table 3. – Effect of cyclosporin A on the percentages of B-cell leukaemia/lymphoma 2 gene product (Bcl-2)-positive cells in bronchoalveolar lavage fluid before and after allergen inhalation challenge

	Placebo			Cyclosporin A			Between-group p-value
	Baseline	Allergen	p-value	Baseline	Allergen	p-value	
Bcl-2+ cells %[#]	7.7 (4.9–14.1)	7.3 (4.2–10.0)	NS	7.6 (2.1–15.1)	4.6 (0.6–14.5)	0.028	NS
Bcl-2+ T-cells %[#]	7.7 (4.8–11.9)	6.8 (4.2–10.0)	NS	7.2 (2.1–14.9)	4.3 (0.6–12.3)	0.028	NS
Bcl-2+ cells %[¶]	0.9 (0.0–16.3)	2.1 (0.0–21.5)	NS	3.3 (0.0–14.6)	2.3 (0.0–16.6)	NS	NS

Data are presented as median (range). NS: nonsignificant. [#]: as percentage of total cells; [¶]: as percentage of apoptotic (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling-positive) cells.

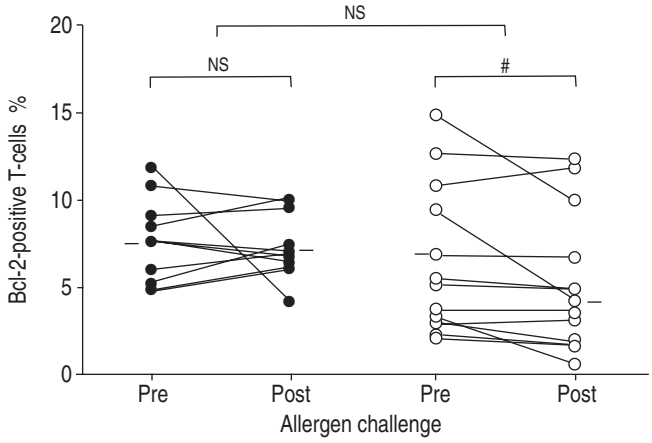

Fig. 2. – B-cell leukaemia/lymphoma 2 gene product (Bcl-2)-positive T-cells (Bcl-2/CD3-positive cells) as a percentage of total cells in bronchoalveolar lavage fluid from the placebo (●; n=11) and cyclosporin A (○; n=13) groups before (Pre) and after (Post) allergen challenge. Horizontal bars represent medians. NS: nonsignificant. [#]: p=0.028.

Discussion

Although the clinical efficacy of CsA has been demonstrated in severe corticosteroid-dependent asthmatics [8, 9], and in allergen challenge models [10,11], its mechanisms of action in the treatment of asthma are still unclear. In the present study, it was found that CsA induced apoptosis of BALF CD3-positive T-lymphocytes (using combined TUNEL/immunocytochemistry) as well as inhibiting Bcl-2 expression.

Although apoptosis may provide a mechanism for the removal of activated T-cells in health, recent studies have suggested that this process may be delayed in the airways of asthmatics [14–18]. For example, apoptosis of lymphocytes was decreased at baseline in asthmatics compared to normal controls and patients with chronic obstructive pulmonary disease (COPD) [14]. Delayed eosinophil apoptosis has also been observed in asthmatics [15]. After effective treatments, such as corticosteroids, increased apoptosis of T-cells and eosinophils was observed in asthmatic subjects [16]. Additionally, T-cells from asthmatics failed to undergo the normal degree of apoptosis following Fas receptor ligation [17], although these cells express the same levels of Fas and Fas ligand as nonasthmatic controls [19]. Taken together, these observations suggested that resistance of T-cells and eosinophils to apoptosis may play a role in maintaining ongoing T-cell activation in asthma.

CsA inhibits the function of calcineurin by binding to the intracellular protein cyclophilin. Calcineurin activates various transcription factors (such as nuclear factor of activated T-cells and nuclear factor-κB) that regulate the expression of key cytokines and signalling proteins [20, 21]. Functionally, CsA inhibits the transcription of numerous cytokines and immunomodulators involved in T-cell activation and proliferation. It has been shown that CsA inhibits T-cell proliferation, induces apoptosis of CD4-positive T-cells and down-regulates cytokine expression in *in vitro* and animal studies [22–24]. In clinical studies in asthma, it has been found that CsA inhibits the LAR, improves lung function, decreases blood and BALF eosinophil numbers, inhibits cytokine/chemokine expression and reduces the requirement for oral corticosteroid in severe disease [7–10, 25]. In the present study, it was found that CsA induced significant apoptosis of CD3-positive T-lymphocytes but did not affect other cell types, including eosinophils and neutrophils (table 2; fig. 1), in the allergen-induced LAR in mild asthma. Similar events may occur in severe asthma, for which benefit was shown after CsA treatment. A definitive study confirming this remains to be performed. To date, there is no evidence that CsA induces neutrophil apoptosis. Although some *in vitro* studies have shown CsA-augmented apoptosis of rat or mouse eosinophils [26, 27], CsA-induced apoptosis of eosinophils was not observed in the present study (table 2). Previous observations show that CsA does not directly induce human eosinophil apoptosis *in vitro* [28]. Furthermore, it has been shown that CsA does not, at pharmacological concentrations, induce apoptosis of macrophages [29, 30]. Thus these observations suggest that the effects of CsA are relatively specific for T-cells. The clinical improvement and marked reduction in total numbers of both lymphocytes and activated T-cells in BALF, as well as bronchial mucosa, were observed up to 6 months after treatment with CsA. However, there were no changes in the numbers of neutrophils and eosinophils [27].

It was also found that numbers of Bcl-2-positive cells were significantly reduced after treatment with CsA (table 3). Bcl-2 can serve as an antiapoptotic protein that inhibits apoptotic cell death induced by a variety of stimuli in several cell types, including T-cells [31, 32]. Although other cell types may also express Bcl-2, the present results showed that the majority of Bcl-2-positive cells were CD3-positive T-cells (table 3; fig. 2). It was previously shown that Bcl-2-positive cell numbers were elevated in asthma compared to normal control subjects and patients with chronic bronchitis, and that expression of Bcl-2 correlated with the severity of asthma [31]. In another study, Bcl-2 served as a marker of infiltrating T-cells, and these were rarely apoptotic in asthma [32]. Furthermore, it has been shown that apoptosis of cells in induced sputum lymphocytes decreased in patients with asthma compared to COPD patients and healthy controls [14]. In contrast, Bcl-2 expression increased in induced sputum lymphocytes from patients with asthma compared with healthy controls and patients with COPD. Taken together, CsA-induced apoptosis of

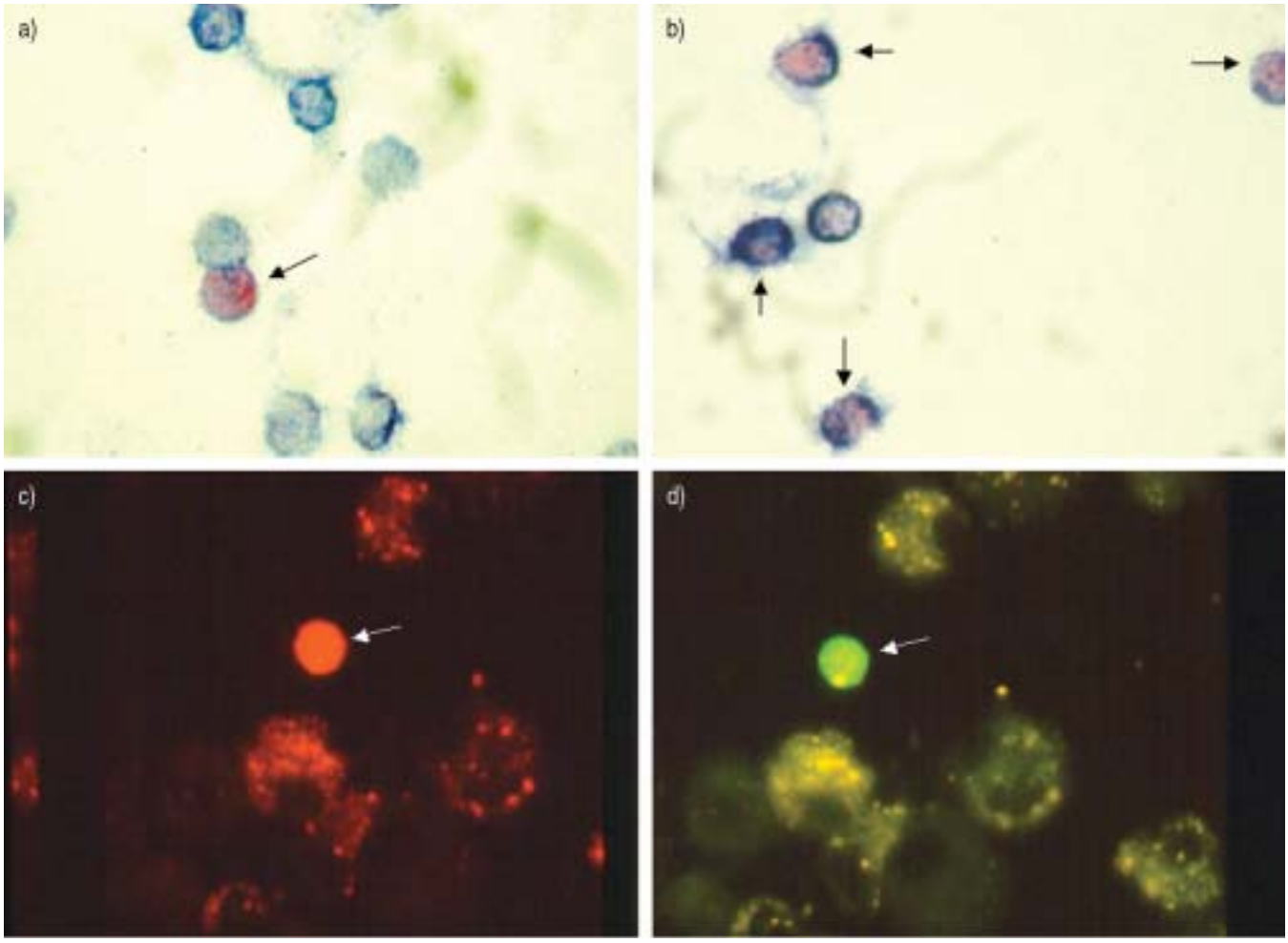

Fig. 3. – Representative apoptotic T-cells (terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL)/CD3-positive cells) from bronchoalveolar lavage fluid (BALF) from patients receiving: a) placebo; and b) cyclosporin A. CD3-positive cells are blue, whereas TUNEL-positive cells are red. Arrows indicate apoptotic T-cells. Double staining showing: c) B-cell leukaemia/lymphoma 2 gene product (Bcl-2)-positive cells (Texas red); and d) T-cells (CD3-positive cells) (fluorescein isothiocyanate (green)). Arrows indicate a Bcl-2-positive T-cell. Autofluorescence of BALF macrophages is also shown.

CD3-positive cells in asthmatics may be associated with inhibition of Bcl-2 expression.

In summary, it has been shown that cyclosporin A enhances apoptosis of CD3-positive cells and reduces B-cell leukaemia/lymphoma 2 gene product expression in bronchoalveolar lavage fluid after allergen inhalation. This further supports the role of the T-lymphocyte as a major effector cell in the late asthmatic reaction.

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