

Immuno-regulatory cytokines in asthma: IL-15 and IL-13 in induced sputum

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Summary

Background The importance of Th2-type lymphocyte function in asthmatic airway inflammation is well recognized, but less is known about the factors which regulate the function of these lymphocytes in asthma. The macrophage-derived cytokine, interleukin (IL)-15 has a number of T cell regulatory properties which might be of relevance to asthma and its treatment.

Objective The aims were to identify and quantify the T cell regulatory cytokine IL-15 in induced sputum samples from asthmatic patients, in comparison with IL-13, and to relate the levels of these cytokines to treatment with inhaled steroids.

Methods Induced sputum was collected from 16 asthmatics (eight steroid and eight non-steroid treated) and eight normal controls. IL-15 and IL-13 levels were measured by enzyme-linked immunoassay (ELISA) in sputum. IL-15 levels were also measured in sputum cell culture supernatants and localized to specific sputum cells by immunocytochemistry.

Results IL-15 levels were increased and IL-13 levels were decreased in sputum fluid from steroid-treated compared with non-steroid-treated asthmatics. IL-15 was localized specifically to macrophages and the proportion of these cells expressing IL-15 correlated with sputum fluid IL-15 and IL-15 levels in cell culture supernatants, and all were higher in the steroid-treated asthmatics.

Conclusion IL-15 and IL-13 production appears to be reciprocally regulated by steroid therapy in asthma patients. The steroid-associated increase in IL-15 may regulate a fundamental shift away from an inflammatory Th2-type environment in asthma and may be an essential component of the cytokine modulation underlying the therapeutic benefit of corticosteroids in this condition.

Keywords: asthma, IL-15, sputum-derived macrophage, induced sputum

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Introduction

Atopic asthma is associated with high serum IgE antibodies and local lung eosinophilia as a consequence of the function of several T cell cytokines, especially interleukin (IL)-4, IL-5 and IL-13 [1–4]. Studies of bronchoalveolar lavage and biopsies from asthmatic airways have shown

that these cytokines, derived predominantly from Th2-type T helper lymphocytes, play a critical role in the genesis of the allergic cascade which leads to persistent airway inflammation [2–5]. Thus, regulating these Th2 CD4⁺ T cells may provide a therapeutic target.

T cell regulatory cytokines include macrophage-derived IL-15. This cytokine has been described in chronic inflammatory conditions including sarcoidosis, rheumatoid arthritis, inflammatory bowel disease and chronic active hepatitis [6–9], but has not yet been studied in asthma.

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IL-15 induces the proliferation and migration of T cells [10,11], maturation and isotype switching of B cells [12], enhances NK cell cytotoxicity and cytokine production [13], and modifies macrophage pro-inflammatory cytokine secretion [14]. IL-15 shares many properties with IL-2 via common signalling through interaction with the β - and γ -chains of the IL-2 receptor but has a unique α -chain [10]. In addition IL-15 is recognized by distinct receptors on mast cells [15]. Thus IL-15 has the potential to regulate various stages of the immune and inflammatory response associated with asthma.

Induced sputum has proved to be a useful, non-invasive method of sampling airway cells and secretions allowing the identification of local pro-inflammatory factors *ex-vivo*. The aims of the present study were to identify and quantify IL-15 in sputum samples from asthmatic patients and controls and compare this with IL-13 levels, and to examine the cellular distribution of IL-15 expression. We found that sputum fluid IL-15, sputum cell IL-15 production *in vitro* and expression of IL-15 *in vivo* by sputum-derived macrophages was significantly increased in asthmatic patients treated with steroids, but not in β_2 -agonist-treated asthmatic subjects compared with normal control subjects. This is in contrast with IL-13, which is reduced in steroid treated asthmatics but increased in non-steroid-treated asthmatics. Together, these data provide the first evidence demonstrating the presence of steroid-responsive IL-15 in asthmatic airways and suggests that regulating the balance of cytokine activities may be of clinical importance in asthma.

Materials and methods

Subjects

Sixteen asthmatic patients and eight normal subjects who were non-smokers were studied. Asthma was defined according to the American Thoracic Society definition [16]. Asthmatic patients gave no history within the preceding 6 weeks of respiratory infection, antibiotics or oral corticosteroid use or exacerbations of asthma. One group of asthmatic subjects received treatment as required with inhaled β_2 agonists alone. The other asthma group received regular inhaled corticosteroids for at least 3 months, β_2 agonists as required and no other asthma drugs in addition. Normal subjects were asymptomatic and gave no history of asthma or other allergic disease. Atopy was assessed by either positive skin prick testing with common allergens or raised total IgE level (> 120 international units/mL). All control subjects were non-atopic and all asthma patients tested were atopic except for one β_2 agonist-treated asthmatic who had an IgE level of 117 IU/mL.

The study was approved by the West Ethics Committee,

West Glasgow Hospitals University NHS Trust. All volunteers gave informed consent.

Sputum induction

Sputum induction was performed following assessment of reversibility to salbutamol, using a modification of the method described by Pin *et al.* [17]. Briefly, following salbutamol, sputum induction was commenced using hypertonic (3%) saline administered via an ultrasonic nebuliser (Sonix 2000, Medix Ltd, Harlow, UK) over a period of 20 min. The subjects were encouraged to expectorate at any time throughout the procedure, and in addition inhalation was stopped every 5 min to allow expectoration, and to allow spirometry to be performed. The sample was collected in a sterile container and transferred to the laboratory on ice as soon as possible, and in all cases in less than 2 h.

Sputum cell preparation

All samples were processed without the laboratory staff being aware of the clinical information relating to the individual subject. Sputum samples were transferred to a Petri dish and the volume and macroscopic characteristics of the whole sample recorded. Sputum plugs were separated from contaminating saliva using sterile forceps. The plugs were placed in a pre-weighed tube and the weight recorded. The sputum was then mixed 1.3 w/v with freshly prepared 0.1% dithiothreitol solution made by mixing dithiothreitol powder (Sigma Chemical Co., Poole, UK) with Dulbecco's MEM medium (Gibco BRL, Paisley, UK) plus 10% fetal calf serum (FCS). This was vortexed briefly and incubated at 37 °C for 15 min with constant agitation. The suspension was filtered through monofilament filter cloth (Cadish Precision Meshes Ltd, London, UK) to remove mucus and then centrifuged at 300 g for 10 min. The cell pellet was washed four times in Dulbecco's MEM medium before analysis. The supernatants were further centrifuged at 600 g for 10 min to remove debris and stored at -70 °C for future analysis.

Total cell counts were determined with a Neubauer haemocytometer using Kimura staining and trypan blue exclusion to determine cell viability; dead cells and epithelial cells were excluded. The cell suspension was then mixed with Dulbecco's MEM medium to obtain a count of 1.0×10^6 cells/mL and cell smear preparations were made using a cytocentrifuge at 270 g for 5 min (Shandon, Pittsburgh, PA, USA). These cytospin slides were fixed using 50% alcohol/acetone (v/v) and then stained with May-Grunwald-Giemsa stain. Differential cell counts were performed by counting 300 cells and expressed as percentages. In general there was less than 20%

squamous epithelial cell contamination and the viability was greater than 85%. Samples that had total cell count less than 0.5×10^6 cells or viability lower than 70% were insufficient for analysis and were excluded. Samples from five normal controls, two non-steroid-treated asthmatics and one steroid-treated asthmatic were excluded on these criteria.

Sputum cell culture

Sputum cells were cultured in 96-well flat-bottom micro-culture plates (Nunc, Roskilde, Denmark) at 2×10^5 cells/well at 37 °C in 5% CO₂ for 48 h in the presence or absence of a macrophage activator, staphylococcal enterotoxin B (SEB) (Sigma). Supernatant was collected and assayed for cytokine content.

Measurement of IL-15 and IL-13

The IL-15 concentration in sputum fluid and culture supernatants were determined by ELISA using paired antibodies (MAB647, BAM247: R & D System, Abingdon, UK) and developed with TMB peroxidase substrate (Insight Biotechnology Ltd, Wembley, Middlesex, UK) according to the manufacturer's recommendations. The sensitivity of the assay was 3 pg/mL. IL-13 in sputum fluid was similarly determined using paired antibodies (MAB213, BAF213: R & D System). The sensitivity of the assay was 70 pg/mL.

Immuno-cytochemistry of IL-15 expression

IL-15 localization on sputum cells cytopreparation smears was performed as previously described [7,8]. Freshly isolated sputum cells were cytocentrifuged at 270 g for 5 min (Cytospin-3, Shandon), fixed with alcohol/acetone 50% (v/v) and stored at -70 °C until analysis. Cultured sputum cells were stimulated with SEB at 1 µg/mL for 48 h, washed and cytospin slides were prepared as above. Slides were rehydrated in tris buffered saline (TBS) and incubated with blocking buffer (ABC kit,

Vector Laboratories Ltd, Peterborough, UK). IL-15 was localized using mouse anti-human IL-15 antiserum (R & D System). Specificity of staining was demonstrated by pre-incubation with recombinant human IL-15 (gift of Dr D. Cosman, Immunex Corp., Seattle, USA) which neutralized binding, or the equivalent concentration of mouse IgG₁ as control (DAKO, Ely, UK). Bound antibody was detected with biotinylated anti-mouse IgG, washed in TBS buffer, and incubated with an alkaline phosphatase complex (Vector Laboratories Ltd) for 30 min in the dark. Endogenous alkaline phosphatase activity was abolished by pre-incubation with levamisole solution (Vector Laboratories Ltd). Immuno-reactive cells were visualized by the addition of alkaline phosphatase substrate (Vector Laboratories Ltd) and lightly counterstained with Harris haematoxylin (BDH, Poole, England). All incubations were conducted at room temperature (18–22°C).

Statistical analysis

Results are expressed as mean and SEM unless otherwise specified. Non-parametric statistical analysis was used to compare cell counts, immunocytochemistry and cytokine concentrations (Mann–Whitney *U*-test) using a software package (Minitab Statistical Software; Mininc, State College, PA, USA). Significance was accepted at the 95% level.

Results

Patient details and pulmonary function

Table 1 presents the age, gender and spirometry (forced expiratory volume in 1 second; FEV₁ and percentage predicted FEV₁) in three study groups: normal healthy volunteers and asthma patients treated with β₂ agonists alone (non-steroid treated) or with steroids. The steroid treated asthmatics are older than controls ($P < 0.03$) and have a reduced pulmonary function ($P < 0.046$). There

Table 1. Subject characteristics ($n = 8$ in each group, mean $\pm$ SEM)

Subjects	Age (yr)	Sex M/F	Baseline FEV ₁ (L)	FEV ₁ % predicted	Inhaled corticosteroid (µg daily*)
Controls	39 (3)	2/6	3.51 (0.3)	109 (6)	–
Asthma					
No inhaled steroids	40 (6)	5/3	3.37 (0.6)	94 (5)	–
Inhaled steroids	47 (4)	3/5	2.79 (0.3)	91 (5)	450 (298)

*Beclomethasone dipropionate or equivalent. Steroid dose, median and interquartile range 400, 200–700.

Table 2. Differential leucocyte profile

	Asthma patients		Controls
	Non-steroid-treated	Steroid-treated	
Cells/mL 1×10^6	$3.1 \pm 0.7^{**}$	1.8 ± 0.2	1.4 ± 0.3
Total cell yield 1×10^6	$7.6 \pm 2.8^*$	$4.9 \pm 0.9^\dagger$	2.0 ± 0.6
Viability (%)	87.8 ± 2.5	88.9 ± 1.7	86 ± 2
Macrophage (%)	61.5 ± 4.6	59 ± 2.4	54.6 ± 2.9
Neutrophil (%)	27.9 ± 4.3	29.8 ± 3.6	28.8 ± 3.6
Epithelial cells (%)	7.4 ± 1.2	7.6 ± 1.4	12.4 ± 2.5
Lymphocyte (%)	3.3 ± 1.1	3.3 ± 0.7	5.5 ± 1.5
Eosinophil (%)	0.13 ± 0.13	1 ± 0.57	0.0 ± 0.0

Total and differential white cell profiles obtained from induced sputum samples from asthma patients and controls ($n = 8$ in each category, mean $\pm$ SEM). Significantly different from controls (* $P < 0.004$, ** $P < 0.03$, $^\dagger P < 0.02$).

were no significant differences in age or pulmonary function between the two asthmatic groups.

Sputum cells

The total cell number and cell distribution in the induced sputum of asthma patients and controls are shown in Table 2. The total cell yields from both the non-steroid-treated and steroid-treated asthma patients were significantly greater than normal controls ($P < 0.004$ and $P < 0.02$, respectively), and the cell concentration was significantly greater than normal in the non-steroid treated asthmatic group ($P < 0.03$). The proportions of the different cell types were similar in the three study groups, but the absolute counts of macrophages and neutrophils were significantly higher in both the asthmatic groups than controls ($P < 0.004$ and $P < 0.005$ for non-steroid-treated, and $P < 0.03$ and $P < 0.02$ for steroid-treated, respectively), and the lymphocyte counts were significantly greater in the steroid-treated group ($P < 0.04$) compared with controls.

IL-15 and IL-13 in induced sputum

Induced sputum was tested for the presence of secreted IL-15 and IL-13 protein (Fig. 1). The results demonstrated that IL-15 levels were significantly higher in sputum from steroid-treated asthmatics (mean $\pm$ SEM, 6.86 ± 1.8 pg/mL) than non-steroid-treated asthmatics (undetectable) and controls (mean $\pm$ SEM, 0.76 ± 0.44 pg/mL) ($P < 0.01$ and $P < 0.02$, respectively). In contrast, IL-13 levels were significantly higher in non-steroid-treated asthmatics (mean $\pm$ SEM, 553 ± 168 pg/mL) than steroid-treated

asthmatics (undetectable) and controls (mean $\pm$ SEM, 176 ± 46 pg/mL) ($P < 0.04$ and $P < 0.02$, respectively).

IL-15 in sputum cell culture supernatants

The production of IL-15 by sputum cells in culture was tested. Sputum cells were stimulated in medium alone or in the presence of the macrophage activator SEB ($1 \mu\text{g/mL}$). A time course analysis demonstrated that the production of SEB-stimulated IL-15 increased up to 96 h in cultured cells from steroid-treated asthmatic patients, but not from non-steroid-treated asthmatics (Fig. 2a). Cells from normal control subjects showed a modest elevation after 48 h and the viability of cells was found to decrease after 48 h, therefore this time-point was used optimally for monitoring IL-15 production.

The production of IL-15 by the sputum cells *in vitro* is shown in Fig. 2(b). Levels of IL-15 were significantly higher in supernatants from cells of steroid-treated patients (mean $\pm$ SEM, 6.03 ± 1.66 pg/mL) compared with non-steroid-treated asthmatics (undetectable) and controls (mean $\pm$ SEM, 0.69 ± 0.34 pg/mL) ($P < 0.01$ and $P < 0.03$, respectively).

There was a highly significant correlation between the culture supernatant IL-15 and the sputum fluid IL-15 levels ($n = 24$, $r = 0.77$, $P < 0.001$).

IL-15 localization in sputum cells by immunocytochemistry

The cellular distribution of IL-15 synthesis was visualized by immunocytochemistry staining of cytospin preparations of sputum cells (Fig. 3). This demonstrated specific immuno-localization of IL-15 to macrophages. However, not all macrophages stained for IL-15; the proportion of

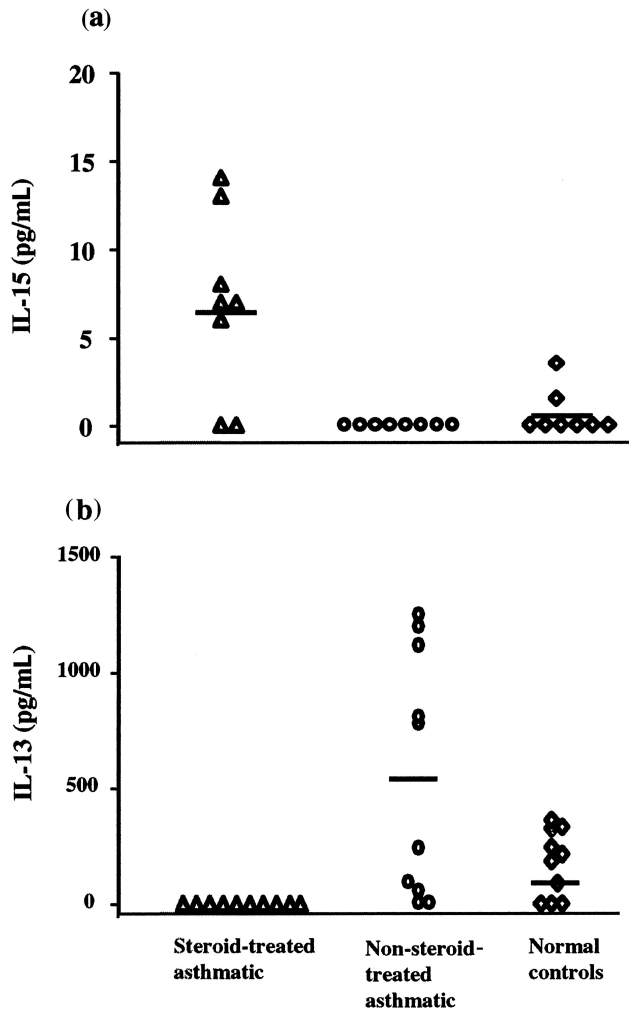


Fig. 1. IL-15 (a) and IL-13 (b) levels in the fluid phase of induced sputum of steroid-treated and non-steroid-treated asthmatics and normal controls ($n = 8$).

IL-15⁺ macrophages from freshly isolated sputum cells or from sputum cells after culture with SEB is shown on Fig. 4. Freshly isolated sputum cells from steroid-treated patients showed a significantly increased proportion of macrophages staining positive for IL-15 (mean $\pm$ SEM, $56.5 \pm 9.3\%$) compared with those from either non-steroid-treated patients (mean $\pm$ SEM, $24.8 \pm 4.2\%$) or normal controls (mean $\pm$ SEM, $22 \pm 5.9\%$) ($P < 0.005$ and $P < 0.01$, respectively).

Cultured sputum cells from steroid-treated asthmatics also demonstrated a significantly increased proportion of macrophages staining for IL-15 (mean $\pm$ SEM, $62 \pm 5.1\%$) than non-steroid-treated patients (mean $\pm$ SEM, $21.4 \pm 3.3\%$) or normal controls (mean $\pm$ SEM, $27.4 \pm 5.1\%$) ($P < 0.012$ and $P < 0.012$, respectively).

The proportion of macrophages staining for IL-15 was

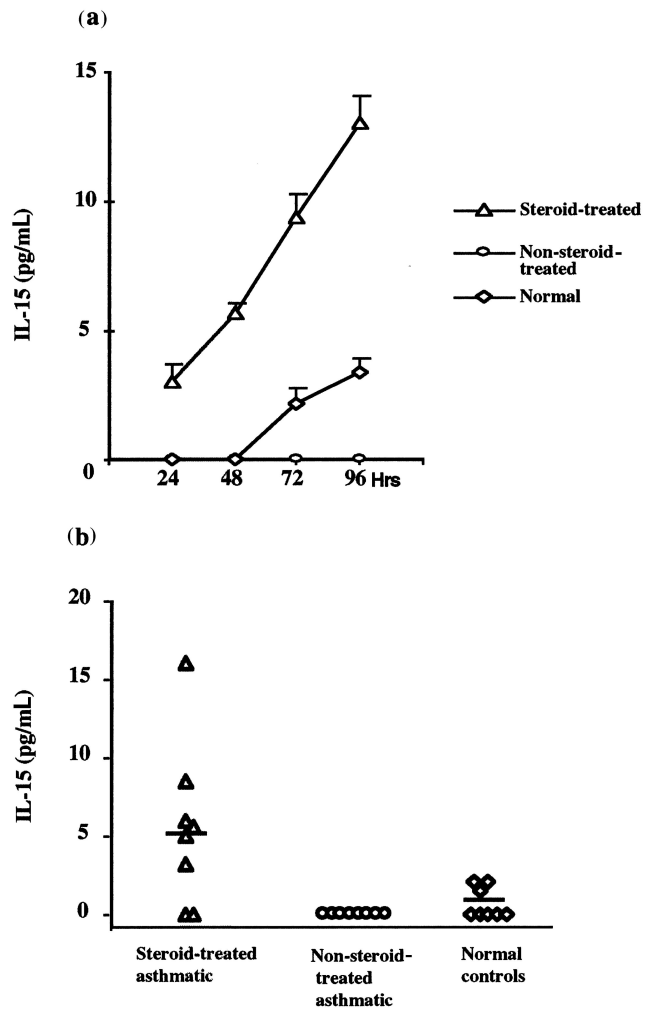


Fig. 2. IL-15 levels in the supernatant of culture of cells isolated from induced sputum stimulated with the macrophage activator SEB. (a) Time course of IL-15 production (one patient in each group, measurements done in triplicate, vertical bars = SEM). (b) Levels of IL-15 in different subject groups ($n = 8$).

significantly associated with the sputum fluid IL-15 level ($n = 24$, $r = 0.69$, $P < 0.002$) and with the IL-15 levels in culture ($n = 24$, $r = 0.65$, $P < 0.01$)

Discussion

Asthma is an inflammatory airways disease in which cytokines derived from Th2 lymphocytes have a predominant role [1,3–5,18]. The regulation of these Th2 cytokines may therefore be of importance in attenuating the disease process. Cytokines derived from Th1 lymphocytes such as interferon (IFN)- γ , can down-regulate the Th2 response in experimental asthma [19]. In lymphocytes, a relative decrease in the production of IFN- γ compared with IL-4

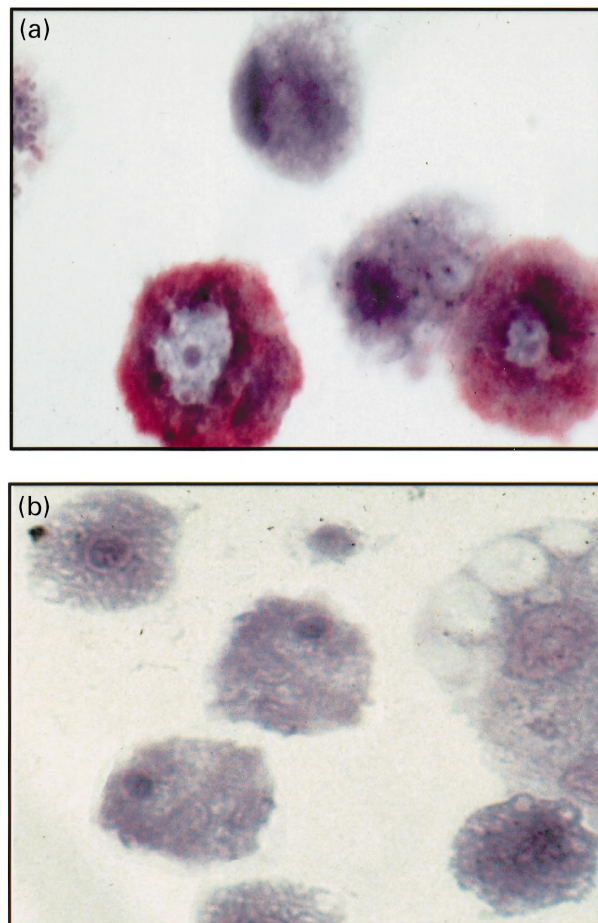


Fig. 3. Immuno-cytochemistry of IL-15 in sputum cells. Freshly isolated sputum cells from a steroid-treated asthmatic were stained with either antihuman IL-15 (a) or normal murine IgG1 control (b). IL-15 positive stained cells appear red. Staining was observed only on macrophages.

leads to increased IgE production and increased airway hyper-responsiveness [20]. This is supported by clinical studies in neonates where the ability to produce IFN- γ was found to be reduced in those who subsequently developed atopy [21].

The balance between Th1/Th2 lymphocyte responses may also be achieved at a more fundamental level by the monocyte/macrophage-derived T cell regulatory cytokines such as IL-15 and IL-12 [22–24]. In the present paper we present the first evidence that IL-15 may have an immunoregulatory role in asthma. We report the identification and measurement of IL-15 in airway fluid and cells using induced sputum. IL-15 levels were significantly raised in both the fluid phase of induced sputum and in the culture supernatants of cells derived from induced sputum from asthma patients on corticosteroid therapy, but were undetectable in sputum samples from asthma patients with no steroid treatment. This profile is in contrast to that of IL-13, a cytokine which has a confirmed role in asthma airway inflammation [25], levels of which were significantly raised in asthmatic patients treated only with β_2 agonist but were undetectable in sputum fluid from steroid-treated asthmatics.

This steroid-dependent reciprocal relationship between IL-15 and IL-13 is similar to that reported between another macrophage-derived T cell regulatory cytokine IL-12 and IL-13 [22]. In this paper higher than normal levels of the expression of mRNA for IL-13 were detected in bronchial biopsies of asthmatics, which decreased after steroid treatment. In contrast, IL-12 expression was lower than in normal controls but increased with steroid treatment to levels comparable with normal. In a similar study, the expression of mRNA for IFN- γ was increased following steroid therapy, coincidental with reduction of expression of mRNA for IL-4 and IL-5 [26]. At the protein translation level, there was increased secretion of IL-12 and IFN- γ

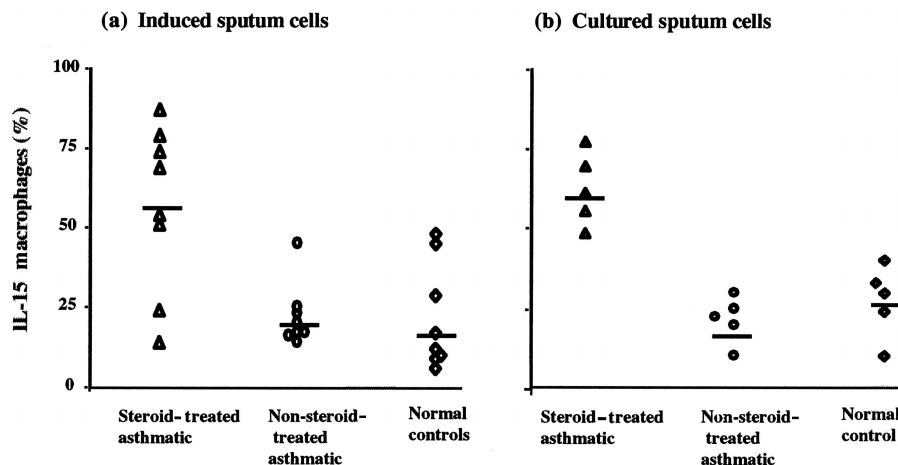


Fig. 4. The proportion of alveolar macrophages stained by immunocytochemistry for the presence of IL-15. (a) Cells isolated from induced sputum and (b) cells isolated from induced sputum after 48 h *in vitro* culture with the macrophage activator, SEB.

following steroid therapy [27]. It appears that increased IL-4, IL-15 and IL-13 are associated with an allergic Th2 inflammatory environment in the lung and that increased IFN- γ , IL-12 and IL-15 are associated with steroid therapy and resolution of the allergic inflammation.

IL-12 and IFN- γ secretion by macrophages can be stimulated with IL-15 [23], and IL-15 has also been shown to stimulate the release of IFN- γ from lymphocytes [24]. By modulating these cytokines therefore IL-15 may be an important upstream regulator of Th2 suppression. However, excess IL-15 production may also be associated with pathology. IL-15 has been described in other lung diseases such as sarcoidosis, hypersensitivity pneumonitis, cryptogenic fibrosing alveolitis and tuberculosis [6,8]. The contribution of IL-15 to the pathogenesis of these diseases is unresolved but appears to promote a shift in the normal Th1/Th2 balance towards excess Th1 with associated cell-mediated immune pathology.

Sputum IL-15 appeared to be derived locally from macrophages but not all macrophage stained positively for IL-15. Sputum cells from normal control subjects had a mean of 22% positive staining, possibly a reflection of macrophages heterogeneity, and this was similar to the proportion in non-steroid-treated asthmatics. However, the proportion of macrophages staining positive for IL-15 was significantly associated with the sputum fluid IL-15 levels and the IL-15 levels in culture and all were significantly increased in the steroid-treated asthmatics.

The basis for this steroid-dependent up-regulation of IL-15 production is at present unclear. Our main aim was to detect IL-15 in sputum and relate this to clinical presentation. The regulation of this cytokine may be addressed using longitudinal studies. At the molecular level, IL-15 mRNA is constitutively expressed in macrophages but the regulation of translation and secretion of IL-15 is unknown [28]. Our observations that IL-15 production is increased in steroid-treated asthmatics suggests either direct regulation by steroid response elements or indirect regulation by the action of steroid-sensitive proteins which normally repress IL-15 production.

In conclusion, this study shows an increase in IL-15 levels in the induced sputum and supernatants from cultured sputum cells in steroid-treated asthmatics, but not in non-steroid-treated asthmatic or control subjects. This increase in IL-15 levels is associated with a decrease in IL-13 levels. IL-15 may therefore play an important role in the regulation of the inflammatory response in asthma, perhaps by induction of IL-12 and IFN- γ which promote a Th1-type environment in the lung. Corticosteroid treatment may enhance this by facilitating its production and release. This supports the suggestion that the clinical effects of glucocorticoids are at least partially mediated through the modulation of cytokine production. Further studies are

necessary to elucidate the exact mechanisms of this and determine its therapeutic relevance.

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