

# Cytokine mRNA expression in patients with mild allergic asthma following low dose or cumulative dose allergen provocation

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## Summary

**Background** Allergen provocation is a very useful way to study the inflammatory response in asthmatic patients. Although cumulative dose regimens are most often applied, another provocation model with repeated inhalations of low doses of allergens has recently come into use.

**Objective** We were interested to compare these two allergen provocation models. To evaluate the inflammation induced by either model, we examined the mRNA expression of several cytokines that are implicated in the orchestration of the inflammatory response observed in asthma.

**Methods** Interleukin (IL)-4, IL-5, IL-13 and interferon (IFN)- $\gamma$  mRNA expression was analysed in bronchoalveolar lavage (BAL) cells and peripheral blood (PB) CD4<sup>+</sup> and PB CD8<sup>+</sup> T cells following any of the two provocation regimens. IL-4 and IFN- $\gamma$  mRNA expression was analysed by a competitive reverse transcriptase-polymerase chain reaction (RT-PCR) method, while IL-5 and IL-13 were analysed semiquantitatively, before and after allergen provocation with either model.

**Results** After low dose provocations none of the cell populations studied showed a clear change in the pattern of IL-4 or IFN- $\gamma$  gene expression. In contrast, after cumulative dose provocation there was a clear tendency towards an increased IL-4 mRNA expression in BAL cells, correlating with a significant increase in IL-4 mRNA in PB CD4<sup>+</sup> as well as in CD8<sup>+</sup> T cells ( $P = 0.005$  and  $P = 0.04$ , respectively). Regardless of the allergen provocation method used, in PB IL-4 mRNA was preferentially expressed by CD4<sup>+</sup> cells while IFN- $\gamma$  was expressed more by CD8<sup>+</sup> cells. IL-5 transcripts increased after low dose provocations in PB CD4<sup>+</sup> T cells in six of eight patients, while after cumulative dose provocation IL-5 mRNA increased in BAL cells in six out of nine patients but decreased especially in PB CD8<sup>+</sup> T cells in six out of eleven patients, suggesting an accumulation of IL-5 expressing cells to the lungs.

**Conclusion** Thus, the cumulative dose provocation regimen can induce a more pronounced Th2-like immune response in asthmatic patients than the low dose provocation model.

**Keywords:** asthma, cumulative dose provocation method, cytokines, low dose provocation method

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## Introduction

Asthma is characterized by the infiltration of the bronchial mucosa with activated eosinophils and T lymphocytes as well as other inflammatory cells [1,2]. T cells have been suggested to control the acute and chronic inflammation in the airways through their ability to express and secrete cytokines. The array of cytokines is regarded as an important modulator of the immune system and is thought to play a key role in the pathophysiological changes observed in asthma. Especially the Th2-like cytokines interleukin (IL)-4, IL-5 and IL-13 directly or indirectly have been implicated in the inflammatory features and disease severity of asthma [3]. IL-4 and IL-13 are required for the switch to IgE production by activated B cells and IL-5 selectively promotes eosinophil differentiation, activation and survival [4–6]. In addition IFN- $\gamma$  down-regulates IL-4 production and its levels are thought to be unaltered in asthmatic patients [4,5].

Allergens are one of the most important inducers of bronchial hyper-responsiveness (BHR) and the most important factor involved in the development of atopic asthma [7]. Different allergen provocation methods are used to induce an inflammatory response in atopic asthmatic patients, allowing studies of pathophysiological events that occur during an allergic reaction. One method is a cumulative dose allergen provocation, that consists of an inhaled allergen challenge that provokes an acute asthmatic reaction [8]. There are also low dose allergen provocation models that consist of repeated inhalations of low doses of allergen, to simulate a natural antigen exposure. In our study we used a model where patients, through inhalation, were exposed for seven days to a dose corresponding to 10% of the allergen dose causing a 20% fall in forced expiratory volume in one second (FEV<sub>1</sub>) (PD<sub>20</sub>). This regimen has been shown to provoke a subclinical airway inflammation with an increase in responsiveness to methacholine [9–11].

The aim of the present study was to compare two different allergen provocation methods, the cumulative and the low dose model. The study was focused on the expression of cytokine transcripts before and after allergen provocation, in bronchoalveolar lavage (BAL) cells as well as peripheral blood (PB) CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, to evaluate the inflammatory response induced by the two allergen provocation models.

## Methods

### Subjects

All patients had a history of mild allergic seasonal asthma and were sensitive to pollen and/or to animal dander with

positive skin prick test and positive radioallergoabsorbent test (RAST, Pharmacia, Uppsala, Sweden). The patients allergic to animal dander did not have pets of their own and were asked to avoid animal contact during the study period. All patients were in a stable phase of the disease when the study started and all patients were free from symptoms of airway infection for at least four weeks prior to the study. Two different allergen provocation methods were used: low dose and cumulative dose allergen provocation. Both studies were performed outside the pollen season. Eight subjects (seven females and one male, age 24–41 years, mean 30 years) participated in the low-dose allergen provocation study, with median FEV<sub>1</sub> values of 93% of the predicted value and median total IgE values of 79 kU/L before allergen provocation (for details, see [9]). In the cumulative dose allergen provocation, 11 subjects were included. There were nine females and two males (age 25–32 years, mean 29 years) and had median FEV<sub>1</sub> values of 97% of the predicted value and median total IgE values of 99 kU/L before allergen provocation. No medication was taken except for occasional  $\beta$ -2 agonist. All subjects gave their informed consent and approval was obtained from the local Ethics Committee of Karolinska Hospital.

### Study design

All bronchial provocations were performed by using a dosimeter controlled jet nebulizer Spira Electro 2 (Respiratory Care Center, Hameenlinna, Finland). To determine the non-specific bronchial responsiveness, methacholine challenge was performed and the PD<sub>20</sub> value was obtained as previously described [9]. The low dose provocation procedure has been extensively described by our group [9]. Briefly, methacholine challenge was performed at the beginning of the trial and repeated one day after the final allergen dose as well as three weeks after the low dose allergen provocations were completed. A BAL was performed 2–3 weeks before starting the allergen provocations. Allergen doses of birch or grass pollen (Aquagen, ALK, Copenhagen, Denmark) were selected according to previously determined PD<sub>20</sub>. Inhalations of doses of allergen corresponding to 10% of PD<sub>20</sub> were administered in the mornings, and at the same time daily during seven weekdays, as described in [9]. The same dose was given every day and varied from 8.4 SQ to 113 SQ between the individuals. FEV<sub>1</sub> was measured before and 15 min after each allergen inhalation. A second BAL was performed one day after the last allergen provocation. Peripheral blood was drawn in connection to bronchoscopies, as well as on days 3 and 5 of allergen provocations and 3 weeks after ending the provocations.

The cumulative dose provocation procedure has been described elsewhere [8]. Briefly, the provocation trial was started by performing bronchoscopy with BAL. After

2–3 weeks, patients underwent an allergen provocation. Challenges were started by the inhalation of diluent. Half-log increments in the cumulative dose of allergen were inhaled every 15 min until the FEV<sub>1</sub> value fell by at least 20% from postdiluent baseline. A second bronchoscopy with BAL was performed the day after the allergen provocation. Methacholine challenges were performed the day before the allergen provocation as well as the day after the second bronchoscopy. Peripheral blood was drawn in connection with the bronchoscopies, as well as before and after the allergen provocation and before the last methacholine challenge.

#### *BAL and lymphocyte separation*

Bronchoscopy with BAL was performed using a flexible fibre optic bronchoscope (Olympus Optical Co. Ltd, Tokyo, Japan) as previously described [12]. In brief, the patients were premedicated with Morphine-Scopolamine and administered inhaled  $\beta$ -2 agonist 10 min before the bronchoscopy. The right middle lobe was instilled with five aliquots of 50 mL sterile buffered saline and the fluid gently aspirated after each aliquot, collected in a sterile siliconized bottle and kept on ice. The lavage fluid was filtered through a single layer of dacron net (type AP32, Millipore, Bedford, Ireland) and centrifuged at 400 *g* for 10 min at 4 °C. The cell pellet was resuspended in RPMI 1640 (Sigma-Aldrich Co. St Louis, MO, USA), counted in a Bürker chamber and the viability assessed by cellular exclusion of trypan blue. Cytospin preparation was performed by centrifugation of  $6 \times 10^4$  cells at 500 *g* for 3 min (Cytospin 2, Shandon Southern Instruments, Runcorn, UK). One slide was stained in May-Grünwald Giemsa for evaluation of cell differential count, and another in toluidine blue for assessment of metachromatic cell count.

BAL cells were separated in two different fractions using anti-CD2 coated magnetic beads (Dynabeads, Dynal, Oslo, Norway), according to the manufacturer's instructions. The lymphocyte-depleted (macrophage-enriched) cell fraction was used in a previous study [13], while the present study included the lymphocyte enriched (from 8.8 to 30.5%) BAL cell fractions. These BAL cells included 30.5% lymphocytes; the remaining cells were mostly alveolar macrophages ( $\approx$  65%) and granulocytes and mast cells (< 5%).

Peripheral blood mononuclear cells were separated from heparinized blood by Ficoll-Hypaque (Pharmacia). Lymphocytes from peripheral blood were separated into CD4<sup>+</sup> and CD8<sup>+</sup> T cells by positive selection using CD4 and CD8 coated magnetic beads (Dynabeads, Dynal), according to the manufacturer's instructions. CD4<sup>+</sup> and CD8<sup>+</sup> isolated fractions contained more than 95% positive cells as assessed by flow cytometry.

#### *RNA extraction, cDNA synthesis and reverse transcriptase PCR*

Total cellular RNA was extracted from  $1\text{--}2 \times 10^6$  cells with RNeasy (Qiagen, Crawley, UK) according to the manufacturer's instructions. The same number of cells were taken before and after provocation in each individual subject. The RNA was quantified by spectrophotometry at 260 nm and stored at  $-70$  °C. The integrity of the RNA sample was assessed by electrophoresis in a 1.6% agarose gel (BRL, Gaithersburg, MD, USA) with 0.2  $\mu$ g/mL ethidium bromide. RNA was denatured at 90 °C for 5 min and chilled on ice. First strand cDNA synthesis was performed in a final volume of 20  $\mu$ L containing: 10  $\mu$ L of denatured RNA (1–5  $\mu$ g), 4  $\mu$ L buffer (5x, BRL), 1.5  $\mu$ L dithiothreitol (100 mM, BRL), 2  $\mu$ L dNTP (dATP, dCTP, dGTP and dTTP, 5 mM each, (Pharmacia), 0.5  $\mu$ L RNasin (40 U/ $\mu$ L, Promega, Madison, WI, USA), 1.0  $\mu$ L random hexamer primers pd(N)6 (1 mM, Pharmacia) and 1.0  $\mu$ L reverse transcriptase from murine Moloney leukaemia virus (200 U/ $\mu$ L, BRL). The samples were incubated at 40 °C for 45 min and then heated for 5 min at 95 °C.

Competitive RT-PCR was performed to measure the relative expression of mRNA for IL-4, IFN- $\gamma$  and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Competitors or MIMICs (Clontech, Palo Alto, CA, USA) were internal standards corresponding to a non-homologous cDNA that shared the same primer pair as the target mRNA but yielded a PCR product of a different size. Thus, the two PCR products could easily be separated by gel electrophoresis after amplification. The expression of IL-5 and IL-13 transcripts were semiquantitatively calculated using Southern blot and <sup>32</sup>P-labelled specific probes, according to standard protocols. The PCR was performed in a 20  $\mu$ L reaction mixture containing 2  $\mu$ L of target cDNA, 2  $\mu$ L of competitor (when used), 0.25  $\mu$ M primers, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.025 U/ $\mu$ L Taq polymerase (Sigma) using a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA). For each sample, five dilutions of the competitor corresponding to three-fold dilution series were run. Prior to PCR, samples were tested for expression of G3PDH using competitive PCR. This procedure compensates for differences in the amounts and quality of the RNA extracted, and the efficiency of the reverse transcription in each sample. The levels of G3PDH were not constant in all samples but using competitive PCR the relative amounts could be determined in each. The samples were then diluted accordingly to obtain equal concentrations of cDNA. The expression of each cytokine was calculated in relation to G3PDH expression. PCR was conducted for 35 (G3PDH) or 39 (cytokines) cycles under the following conditions: denaturation at 94 (G3PDH) or

| Cytokine         | Primer Sequence<br>5'-3'          | PCR Product<br>Size (bp) | Table 1. Cytokine list and<br>oligonucleotide sequences for primers and<br>probes |
|------------------|-----------------------------------|--------------------------|-----------------------------------------------------------------------------------|
| IL-4 s           | CGGCAACTTTGACCACGGACACAAGTGCGATA  | 344                      |                                                                                   |
| IL-4 as          | ACGTACTCTGGTTGGCTTCCTTCACAGGACAG  |                          |                                                                                   |
| IL-5 s           | GCTTCTGCATTTGAGTTTGCTAGCT         | 293                      |                                                                                   |
| IL-5 as          | TGGCCGTCAATGTATTTCTTTATTAAG       |                          |                                                                                   |
| IL-5 probe       | TCAGGGAATAGGCACACT                |                          |                                                                                   |
| IL-13 s          | CTCCTCAATCCTCTCCTGTT              | 441                      |                                                                                   |
| IL-13 as         | TTTCGCGAGGGACGGTTCAAC             |                          |                                                                                   |
| IL-13 probe      | TCTGGTTCTCGGTGATGT                |                          |                                                                                   |
| IFN- $\gamma$ s  | GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC  | 427                      |                                                                                   |
| IFN- $\gamma$ as | CTCCTTTTTCGCTTCCCCTGTTTtagctgctgg |                          |                                                                                   |
| G3PDH s          | TGAAGGTCGGAGTCAACGGATTTGGT        | 983                      |                                                                                   |
| G3PDH as         | CATGTGGGCCATGAGGTCCACCAC          |                          |                                                                                   |
| G3PDH probe      | CACGGAAGGCCATGCCAGTGAGCTTCCCCGT   |                          |                                                                                   |

95 °C (cytokines), annealing at 60 (G3PDH) or 62 °C (cytokines), and extension at 72 °C. Specific primers for IL-4, IL-5, IFN- $\gamma$  and G3PDH were purchased from Clontech. The sequences of the IL-13 primers were as previously described [14]. Primer sequences are listed in Table 1. Phytohaemagglutinin-stimulated mononuclear cells expressed all cytokines tested for and thus served as positive control to ascertain that each primer yielded a PCR product of the correct size (data not shown). The PCR products were visualized on a 1.6% agarose gel containing 0.2  $\mu$ g/mL ethidium bromide, and analysed using a DC120 Zoom Digital Camera connected to ID Image Analysis Software (Kodak, Eastman Kodak Co. Rochester, NY, USA).

**PCR experiments for IL-4 and IFN- $\gamma$ :** Calculation of the ratio target : competitor for each point of a dilution series of competitor was performed [15]. These values were plotted against the concentration of competitor for this three-fold dilution series and the concentration corresponding to the equivalence point gave the concentration of the target. This point was determined for IL-4 and IFN- $\gamma$  as well as for G3PDH and a molar ratio between each cytokine and the housekeeping gene was obtained. Concentrations of IL-4 and IFN- $\gamma$  mRNA were expressed as moles of each cytokine per moles of G3PDH (molar ratio). Undetectable mRNA levels were set up at molar ratio =  $10^{-5}$ , corresponding to cytokine concentrations below  $10^{-6}$  attomol/ $\mu$ L.

**Qualitative PCR experiments for IL-5 and IL-13:** Radioactive products were scanned and analysed in a Phosphor-imager (Molecular Dynamics, CA, USA). The relative mRNA levels were expressed as the ratio of the net

intensity detected in a given cytokine band divided by that of its respective G3PDH band.

#### Statistical analysis

The Mann–Whitney *U*-test and the Wilcoxon matched pair test were used for intergroup and intragroup analysis respectively. A *P*-value less than 0.05 was considered statistically significant.

## Results

#### Clinical parameters

After seven weekdays of repeated low dose allergen provocations, a subclinical allergic inflammatory reaction was observed in six out of eight asthmatic subjects, as determined by increased airway methacholine reactivity, increased BAL fluid total cell and eosinophil counts (in 7 out of 8 patients) and increased serum eosinophilic cationic protein (ECP) levels (in all patients). Detailed data are presented in Lensmar *et al.* [9]. The cumulative allergen provocation also induced an increased airway methacholine reactivity (PD<sub>20</sub> decreased from 696 (mean value) to 221  $\mu$ g; *P* < 0.01) and significantly increased BAL cell concentrations (from 111 (mean value) to  $199 \times 10^6$  cells/L; *P* < 0.01). Moreover, there was a significant increase of eosinophil granulocyte numbers in BAL fluid (from 0.5 to 2.8%; *P* < 0.01) as well as in PB (from 2.7 to 5.3%; *P* = 0.01) and an increase in serum ECP values (from 14.1 to 22.0  $\mu$ g/L; *P* = 0.05).

**Table 2.** Expression of IL-5 and IL-13 mRNA from BAL fluid and PB T cells before and after allergen provocations

| Provocation method | IL-5   |       |        |       |        |       | IL-13  |       |        |       |        |       |
|--------------------|--------|-------|--------|-------|--------|-------|--------|-------|--------|-------|--------|-------|
|                    | BAL    |       | PB-CD4 |       | PB-CD8 |       | BAL    |       | PB-CD4 |       | PB-CD8 |       |
|                    | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After |
| Patient 1          | –      | –     | ++     | +++   | –      | +     | –      | –     | ++     | +     | +      | ++    |
| 2                  | –      | –     | +      | +++   | ++     | –     | –      | –     | ND     | ND    | –      | –     |
| 3                  | –      | –     | +      | ++    | +      | ++    | –      | –     | –      | ++    | –      | –     |
| 4                  | –      | –     | +++    | +     | ND     | ND    | –      | –     | –      | –     | ND     | ND    |
| 5                  | +++    | –     | ++     | –     | ++     | –     | ++++   | ++++  | +      | –     | +      | ++    |
| 6                  | –      | –     | –      | +     | ++     | +++   | +++    | –     | –      | –     | –      | –     |
| 7                  | +++    | +++   | –      | +     | –      | +     | –      | +++   | –      | –     | –      | –     |
| 8                  | –      | –     | +      | +++   | +++    | ++    | –      | –     | ND     | ND    | –      | –     |
| Cumulative dose    |        |       |        |       |        |       |        |       |        |       |        |       |
| Patient 1          | ND     | ND    | –      | –     | +      | ++    | ND     | ND    | –      | –     | –      | –     |
| 2                  | –      | +     | –      | –     | +      | +     | –      | –     | –      | –     | –      | ++++  |
| 3                  | –      | ++    | –      | –     | +++    | +     | –      | –     | –      | –     | +++    | –     |
| 4                  | +      | ++    | +++    | –     | –      | +     | ++++   | +++   | –      | –     | –      | –     |
| 5                  | ++++   | –     | –      | –     | ++     | +     | –      | –     | +++    | –     | –      | –     |
| 6                  | ++++   | +++   | +      | ++    | ++     | +     | +++    | ++++  | –      | +++   | –      | –     |
| 7                  | –      | +++   | ++     | –     | ++     | +     | –      | ++++  | –      | ++++  | –      | ++++  |
| 8                  | +      | +     | ++     | –     | –      | ++    | +++    | ++++  | –      | –     | –      | –     |
| 9                  | +      | ++    | –      | +     | –      | –     | –      | ++++  | +++    | ++++  | –      | –     |
| 10                 | +      | +++   | ++     | +     | ++     | +     | +++    | ++++  | +++    | ++++  | –      | ++++  |
| 11                 | ND     | ND    | –      | –     | ++     | +     | ND     | ND    | –      | ++++  | –      | –     |

mRNA relative values obtained from Southern blot analysis. Ratio between cytokine/G3PDH is given as arbitrary values: > 0.6 (+++), 0.4–0.5 (+++), 0.2–0.3 (++), 0.1 (+), undetected (–).

ND, not determined.

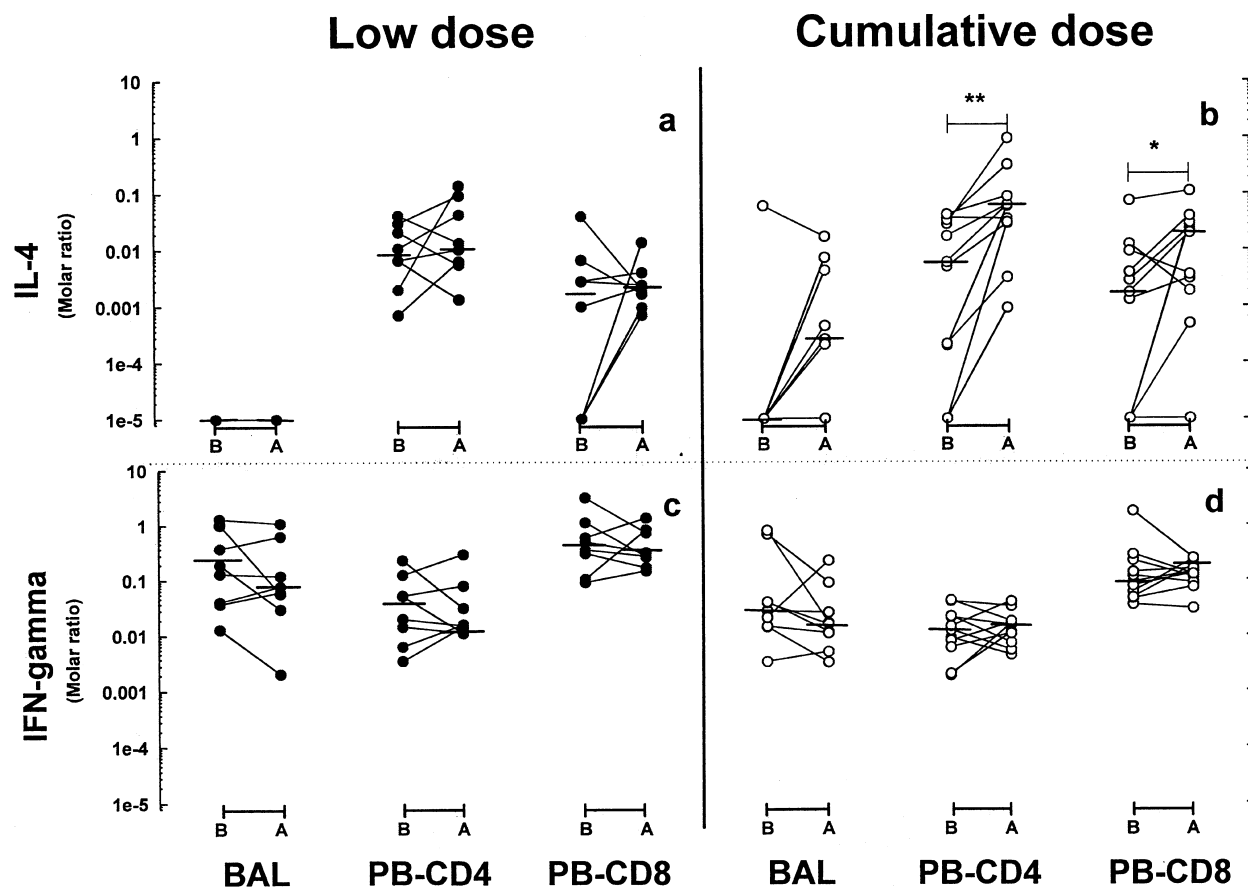


**Fig. 1.** Competitive PCR analysis of IL-4 and IFN- $\gamma$  mRNA in BAL fluid and PB cells obtained from mild asthmatic patients before and after allergen low dose or cumulative dose provocation methods. Data are presented as ratio of cytokine/G3PDH mRNA (molar ratio). Undetectable mRNA levels were set up at molar ratio =  $10^{-5}$ , corresponding to cytokine concentrations below  $10^{-6}$  attomol/ $\mu$ L. Median values are indicated. B = before and A = after allergen provocations. \* $P < 0.05$  and \*\* $P < 0.01$ .

#### Expression of IL-4 and IFN- $\gamma$ mRNA

Interleukin-4 and IFN- $\gamma$  mRNA levels in samples obtained before and after provocations were analysed by competitive RT-PCR. After low dose allergen provocation, no significant changes in the expression of IL-4 or IFN- $\gamma$  mRNA in either BAL fluid cells, PB CD4 $^{+}$  or CD8 $^{+}$  T cells were observed (Figs 1a and c). IL-4 mRNA levels were undetectable in BAL cells before as well as after low dose provocation.

In contrast to low dose provocations, cumulative dose allergen provocation induced a significant increase in IL-4 mRNA expression in both PB CD4 $^{+}$  ( $P = 0.005$ ) and CD8 $^{+}$  T cells ( $P = 0.04$ ) (Fig. 1b). In BAL cells, eight out of nine patients did not express IL-4 before cumulative dose provocation but following allergen provocation the transcripts were clearly detected in six out of nine patients. Interestingly, the only patient with high levels of IL-4 mRNA in BAL cells before challenge also had unusually

high serum ECP values (83  $\mu$ g/L). As in low dose provocation, no significant changes in IFN- $\gamma$  transcripts were observed after cumulative dose provocation (Fig. 1d).

When the ratio between the expression of IL-4 over IFN- $\gamma$  mRNA was calculated, an increase after cumulative dose but not after low dose provocation was found (Fig. 2).

Before provocation and independently of the provocation method used there was a tendency of IL-4 mRNA to be preferentially expressed by PB CD4 $^{+}$  compared to CD8 $^{+}$  T cells. This difference became statistically significant after provocation (Figs 3a and b). IFN- $\gamma$  transcripts were on the other hand significantly more expressed by PB CD8 $^{+}$  than by CD4 $^{+}$  T cells before as well as after provocations (Figs 3c and d).

#### Expression of IL-5 and IL-13 mRNA

There was a tendency to an increased IL-5 mRNA

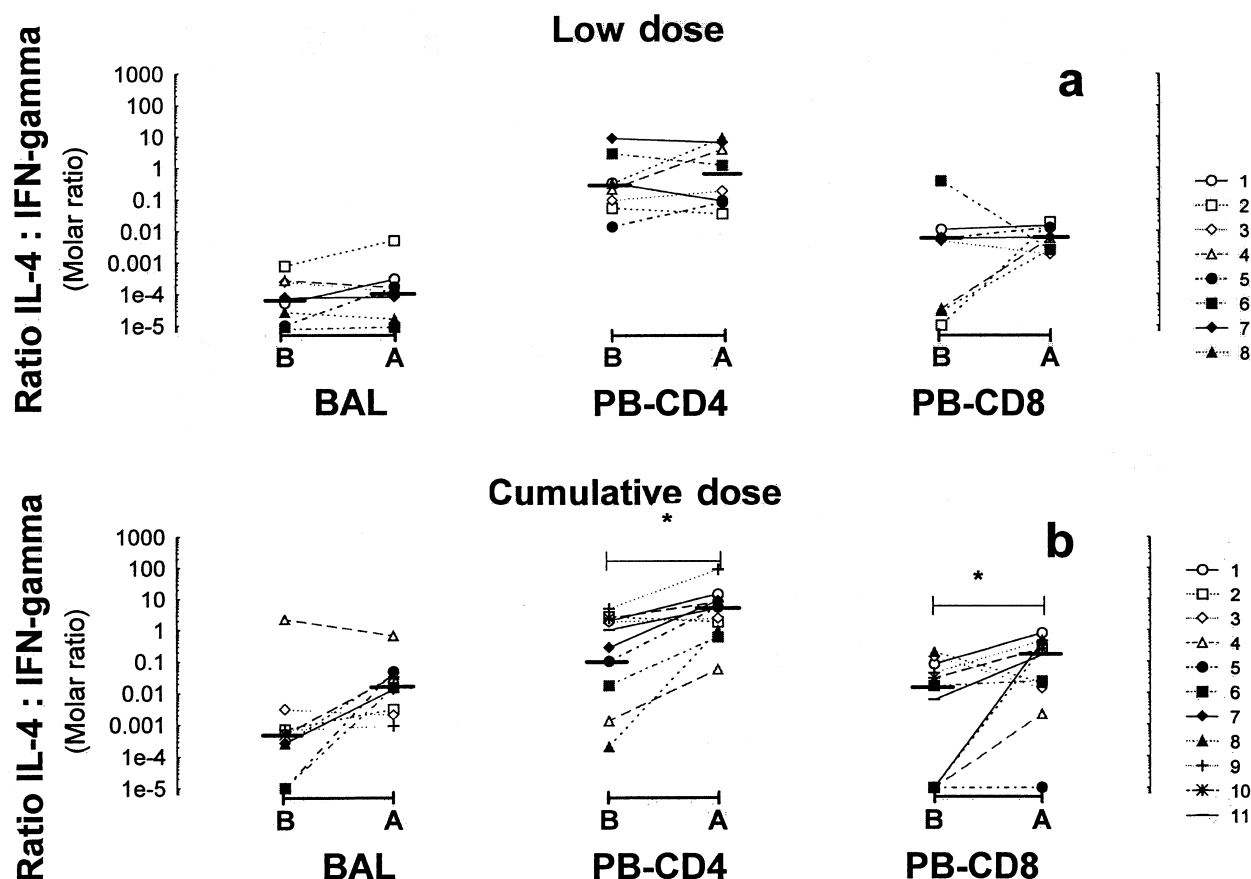


**Fig. 2.** Competitive PCR analysis of the IL-4/IFN- $\gamma$  mRNA ratio in BAL fluid and PB cells obtained from mild asthmatic patients before and after allergen low dose or cumulative dose provocation methods. Data are presented as a ratio between the IL-4 and IFN- $\gamma$  mRNA molar levels. Undetectable mRNA levels were set up at molar ratio =  $10^{-5}$ , corresponding to cytokine concentrations below  $10^{-6}$  attomol/ $\mu$ L. Median values are indicated. B = before and A = after allergen provocations. \* $P < 0.05$ .

expression in PB CD4<sup>+</sup> T cells in six out of eight patients (6/8 $\uparrow$ ) and decreased IL-5 in two out of eight patients (2/8 $\downarrow$ ) after low dose provocation, but there were no clear patterns in BAL cells or PB CD8<sup>+</sup> T cells. Following cumulative dose provocation, there was instead a tendency to a decreased IL-5 expression in both PB CD4<sup>+</sup> and PB CD8<sup>+</sup> T cells (CD4: 4/11 $\downarrow$ , 2/11 $\downarrow$ ; CD8: 6/11 $\downarrow$ , 3/11 $\downarrow$ ) and a concomitant increase of IL-5 expression in BAL cells (6/9 $\uparrow$ , 2/9 $\downarrow$ ) (Table 2).

IL-13 expression did not show significant changes after either low dose or cumulative dose provocation. However, a tendency to an increased expression after cumulative dose was observed in BAL cells (5/9 $\uparrow$ , 1/9 $\downarrow$ ) as well as in PB CD4<sup>+</sup> (5/11 $\uparrow$ , 1/11 $\downarrow$ ) and CD8<sup>+</sup> T cells (3/11 $\uparrow$ , 1/11 $\downarrow$ ) (Table 2).

## Discussion

In this work we have compared two different methods to

induce an inflammatory reaction in asthmatic subjects. One method is a classic allergen challenge, which causes an immediate reaction as determined by a 20% fall in FEV1, and in our study, is accompanied by a significant increase in serum ECP values, in blood and BAL eosinophils as well as an increase in BAL macrophages and mast cells [16]. The low dose provocation model used in this study is characterized by an increased bronchial hyper-reactivity, without distinct clinical symptoms but with a subclinical inflammation as determined by an increase in BAL fluid eosinophils and serum ECP values [9]. Following low dose allergen provocation, we have previously noted signs of alterations of BAL cell phenotypes [9], but no signs of activation of peripheral blood cells [17]. The difference between the two models in their capacity to induce an acute inflammatory response in asthmatic patients may be reflected by the cellular production of cytokines that will drive the immune response towards a Th2-type of response.

The cumulative dose provocation model has been more

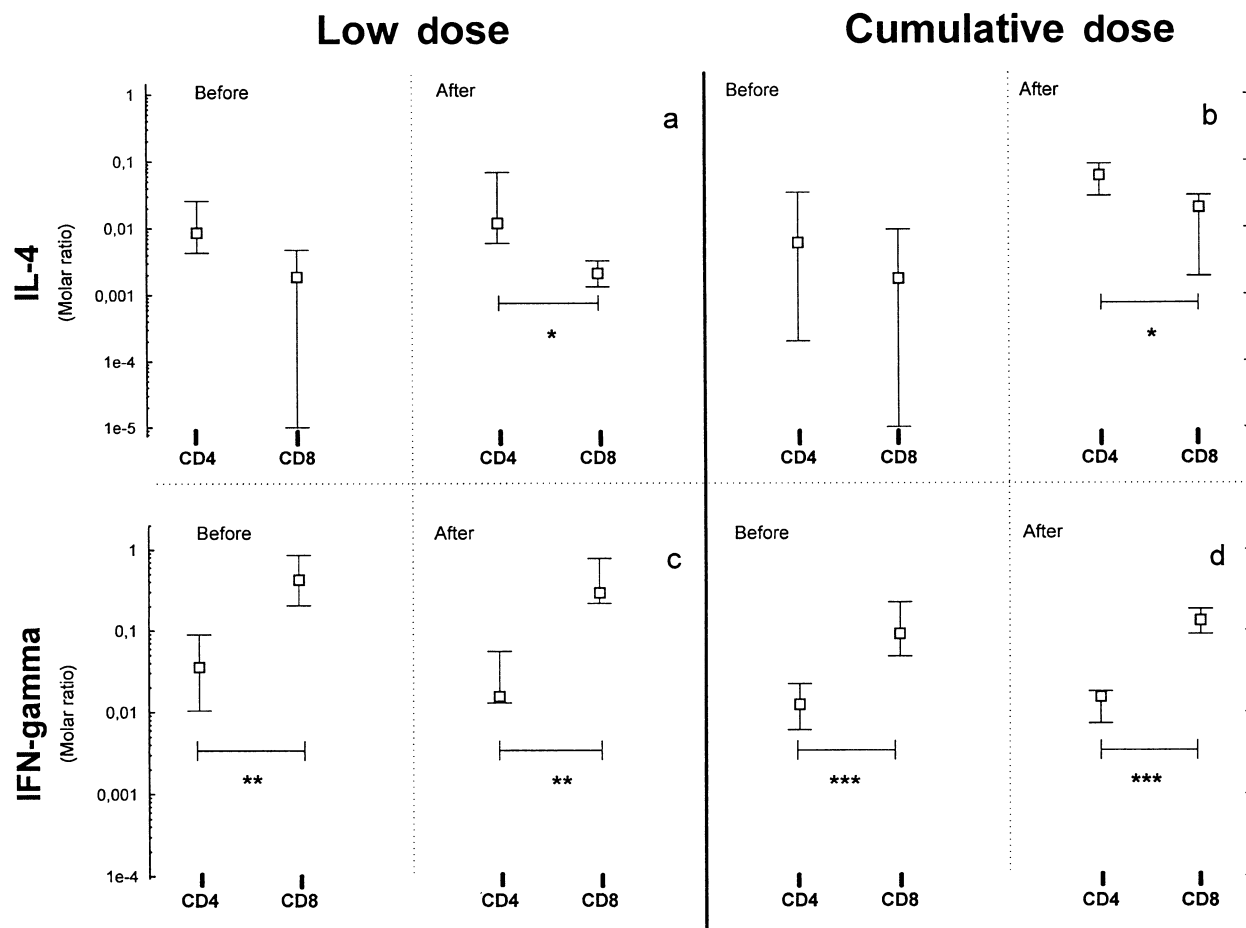


**Fig. 3.** Comparison of IL-4 and IFN- $\gamma$  mRNA expression between PB CD4<sup>+</sup> and CD8<sup>+</sup> T cells before and after allergen low dose and cumulative dose provocation methods. Data are presented as ratio of cytokine/G3PDH mRNA (molar ratio). Median, min and max values are indicated. CD4 = PB CD4<sup>+</sup> T cells and CD8 = PB CD8<sup>+</sup> T cells. \* $P < 0.05$ , \*\* $P < 0.005$  and \*\*\* $P = 0.0001$ .

frequently used, and activation markers and cytokines are readily detected in cells from BAL fluid, peripheral blood and bronchial biopsies following this provocation model [16,18,19]. These reports are in agreement with our present findings where increased IL-4 mRNA expression after cumulative allergen provocation in BAL fluid cells and peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells was observed. We identified one patient with an unusually distinct IL-4 mRNA expression even before allergen provocation, correlating with a highly elevated s-ECP level. This relationship suggested to us that this patient might have been exposed to an unidentified allergen and responded with an allergic reaction, and moreover shows the great sensitivity of the technique used in this report to identify and quantify IL-4 mRNA. In addition, we found that IL-5, a cytokine expressed primarily by T lymphocytes, showed a tendency to be up-regulated in BAL cells while down-regulated in both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes after cumulative allergen provocation. Thus, IL-5-producing T

cells might selectively migrate to the site of inflammation, i.e. to the lungs, following allergen inhalation [20]. These results seem to be in discrepancy with another study where an increase of the absolute number of IL-5-producing blood lymphocytes was reported [18], even though another method (flow cytometry) was used in that study. However, it is plausible that low dose allergen provocation recruits IL-5-producing T cells from lymphoid organs to the PB and cumulative dose provocation may lead to a further recruitment from the blood to the lungs. The pronounced increase of BAL eosinophils after cumulative allergen provocation could also contribute to an increased IL-5 expression in BAL. Finally, following the cumulative allergen provocation, we could also detect a tendency towards an increased IL-13 production in both BAL cells and PB cells, while IFN- $\gamma$  did not show any changed pattern of expression. Altogether, a clear Th-2 immune response seems to be induced in atopic asthmatic patients through the cumulative allergen provocation.

The present study is the first to analyse IL-4 and IFN- $\gamma$  mRNA expression in a quantitative way in paired cells from BAL fluid and PB after low dose provocation, a method suggested to mimic a natural allergen exposure. Previous reports using this method did not show any change in the PB cells surface activation markers [17] while an allergen specific Th2 response *in vitro* was still induced [21]. We have recently reported on an altered phenotype in BAL cells [9] following low dose allergen provocation, and others have shown an increased IL-5 secretion in sputum after low dose provocations [22]. In this report, we did not detect any specific changes in cytokine expression in BAL or PB cells after low dose allergen provocations, apart from a tendency to an increase in IL-5 mRNA expression in PB CD4<sup>+</sup> T cells. Th2 cytokine producing cells localized at other sites, such as in the lung interstitium, may here contribute to the airway inflammation that was recorded following this low dose allergen provocation [9].

T cell mediated immune responses are dependent on which cytokine predominates during the course of that response. In asthma, a common observation appears to be an increased expression of Th2 cytokines, with an elevated production of IL-4, IL-5 and IL-13 [23]. Our results from cumulative dose provocation corroborate this observation. Moreover, we demonstrated that in addition to CD4<sup>+</sup> T cells, PB CD8<sup>+</sup> T cells were also able to produce IL-4 mRNA and subsequently increase its expression after cumulative dose provocation. However, CD4<sup>+</sup> T cells were the cells that produced the greater amount of IL-4 mRNA. This observation applied also to PB lymphocytes in the low dose provocation group. Previous reports showed that CD8<sup>+</sup> T cells from atopic asthmatic patients contained significantly more IL-4 than those of non-atopic controls [24,25]. It would therefore be interesting also to analyse CD8<sup>+</sup> BAL cells separately for cytokine expression, especially since we previously reported the accumulation of CD8<sup>+</sup> T cell expansions in the lung after cumulative dose provocation [26]. We conclude that PB CD8<sup>+</sup> T cells can express significant amounts of IL-4 in addition to their expression of high levels of IFN- $\gamma$  mRNA.

In this study, we have shown that the cumulative allergen provocation regimen, in contrast to the low dose allergen provocation model used in this study, is capable of inducing a distinct Th2-type cytokine pattern in BAL cells as well as in CD4<sup>+</sup> and CD8<sup>+</sup> peripheral blood T cells. The cumulative allergen provocation model therefore seems better suited for functional studies of the immune response to specific allergens. However, the low dose provocation model could still be of importance to analyse early inflammatory events preceding cytokine production by T lymphocytes.

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