In vitro and in vivo modulation of human T lymphocytes from allergic asthmatic subjects
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Production of IL-16 by T lymphocytes from bronchoalveolar lavage fluid of patients with allergic asthma

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Chapter 5

Abstract

IL-16 is an inflammatory mediator which is able to attract CD4+ T-lymphocytes, monocytes and eosinophils into the airways of asthmatic patients. Its release from cells in the airways is induced by histamine. T lymphocytes are an important source of IL-16.

We have tested the hypothesis that IL-16 is released into bronchoalveolar lavage (BAL) fluid at early time-points after contact of local cells with allergen. Furthermore we have characterised airway derived T lymphocytes as a source of IL-16 and compared their IL-16 release with that of other cytokines.

Fifteen allergic asthmatics and 9 non-allergic healthy controls participated in this study. IL-16 was measured by ELISA in BAL fluid obtained before, and at 5 min and 4 h after Segmental Allergen Challenge (SAC), in in vitro cultures of polyclonal BAL fluid cells, and in in vitro cultures of T-cell clones derived from BAL fluid cells. Bioactivity of IL-16 was assessed in migration studies.

After SAC, IL-16 was increased in BAL fluid at 5 min and 4 h (p = 0.02) in allergic asthmatic subjects only. Short-term in vitro cultures of freshly isolated BAL fluid cells of asthmatic subjects released significantly higher levels of IL-16 than those of healthy subjects (p < 0.03). CD8+ and CD4+ T-cell clones released similar levels of antigenic IL-16. For CD8+ T cells only, a significant correlation was found between antigenic IL-16 and bioactive IL-16 released (rho 0.96; p < 0.003).

In vivo allergen challenge of the airways induced rapid (5 min and 4 h) increases in IL-16 in asthmatic patients. Airway-derived CD8+ T cells of allergic asthmatic subjects showed early in vitro release of bioactive IL-16. The results suggest an important role for CD8+ T cells in the inflammatory reactions in the airways of asthma patients.

Introduction

Patients with asthma have a chronic airway inflammation characterised by a hypersecretion of mucus and an increased plasma protein leakage into the airways, and the presence of increased numbers of activated inflammatory cells in the airway mucosa. Activated mast cells, eosinophilic granulocytes and T lymphocytes are most prominent [1]. It is supposed that T lymphocytes play an important regulatory role in this inflammation [2,3]. It is important to know therefore the factors that attract the T cells into the airways and that may activate them. Among these interleukin-16 (IL-16) has been recognised as a key cytokine.
IL-16 was first described in 1982 when it was named Lymphocyte Chemoattractant Factor [4]. IL-16 occurs as homotetramers composed of 14-17 kDa chains. The tetrameric structure appeared to be an absolute requirement for IL-16 bioactivity [5]. Proforms of IL-16 (80 kDa and 60 kDa) may serve as functional precursors [6]. The pro-IL-16 was shown to be subject to proteolytic processing when incubated with cell lysates from CD8⁺ cells [7]. The receptor for IL-16 is the CD4 molecule. IL-16 functions as a chemoattractant for CD4⁺ T-lymphocytes, monocytes and eosinophils [8]. In addition, it may further activate cells, induce cell adhesion and protect cells against antigen-induced cell death [8,9].

The first identified source of IL-16 was the CD8⁺ T lymphocyte [4]. In the airways the epithelial cells are recognised now as an important source as well [10]. In the CD8⁺ cells IL-16 is constitutively synthesized and stored as bioactive protein. IL-16 is released from these cells in response to histamine [11]. In this way IL-16 may play an important role in the sequence of events leading from activation of airway-tissue mast cells to infiltration of inflammatory leukocytes into the airways, characteristic for allergic asthma. CD4⁺ T lymphocytes contain constitutive IL-16 mRNA and the precursor protein but no preformed bioactive protein [12]. Bioactive IL-16 was found in bronchoalveolar lavage (BAL) fluid of asthmatic subjects 6 h and 24h after allergen challenge [10,13,14] and at 6h after histamine challenge [15]. No IL-16 bioactivity was found in BAL fluid from challenged normal subjects or from atopic non-asthmatic subjects.

Since there may be an immediate release of IL-16 from cells in the airways upon contact with histamine we have tested the hypothesis that IL-16 is released into BAL fluid at early time-points after contact of local cells with inhalant allergen. We chose to study the time-point of 5 min after allergen challenge for some patients and the time-point of 4 h after allergen challenge in a larger group. Both time-points precede the development of the late phase allergic reaction and the infiltration of inflammatory cells which may occur after allergen inhalation [16].

Furthermore, we wanted to study CD4⁺ and CD8⁺ T lymphocytes from the BAL fluid as the major non-epithelial cellular source of IL-16 in the airways [10,14]. Earlier, IL-16 release studies were performed with T lymphocytes from the peripheral blood [11,12]. In our studies we have compared the production of IL-16 with the production of other cytokines relevant for T-cell reactions in asthma, namely IFN-γ, IL-4 and IL-5. As study material we took short-term cell cultures with total BAL fluid cell populations, and cell cultures with CD8⁺ and CD4⁺ T-lymphocyte clones derived from BAL fluid T cells. We have studied both antigenic IL-16 as detected by ELISA, and bioactive IL-16 as detected by migration studies [17,18].
Chapter 5

Material and Methods

Subjects
Twenty-four volunteers (15 allergic asthmatics and 9 non-allergic healthy controls) were selected for the study. See for patient characteristics Table 1. No significant differences were observed in baseline characteristics between the asthmatic and the control group with respect to patient demographics and pulmonary functions. Asthma was defined according to the American Thoracic Society (ATS) criteria and included a history of recurrent episodes of wheezing, chest tightness and dyspnoea [19]. Asthma severity ranged from Grade I (episodic) to Grade III (moderate persistent), according to the staging as proposed in the Global Initiative for Asthma [20]. None had experienced an exacerbation of asthma during at least 2 months before the study. None of the subjects used anti-inflammatory medication, in particular they did not use corticosteroids for at least 6 weeks prior to the study. Inhaled bronchodilators were withheld at least 8 h before all investigations. All asthmatic subjects had a positive RAST and a positive skin prick test for house dust mite or grass pollen allergens.

The control subjects had no past or present history of wheezing, chest tightness, recurrent episodes of reversible airway obstruction, or allergy. They were RAST and skin prick test negative for a standard package of allergens tested.

None of the subjects had experienced recent airway infections. The study was approved by the Internal Review Board of the Academic Medical Center, Amsterdam, and was performed after written informed consent had been obtained. Patient 1 participated only in the study on BAL fluid T-cell clones; all other patients from the AA group participated in the allergen challenge study.

Segmental Allergen Provocation - study design
The study design was as described previously [21]. All subjects completed a respiratory and allergy questionnaire and were tested for IgE allergy [21]. Baseline spirometry and histamine provocation tests were performed. Intracutaneous dose response series with house dust mite were performed to determine the allergen dose for the allergen provocation procedure [21] (Table 1).
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Age (Yr)</th>
<th>Sex</th>
<th>FEV₁ (%pred)</th>
<th>PC₂₀hist (mg/ml)</th>
<th>Skin threshold (ng/ml)</th>
</tr>
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<td></td>
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<tr>
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<tr>
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<td>F</td>
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<td>0 *</td>
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<tr>
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<td>F</td>
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<td>4.5</td>
<td>60</td>
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<tr>
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<td>25</td>
<td>M</td>
<td>92</td>
<td>1.1</td>
<td>6</td>
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<tr>
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<td>F</td>
<td>95</td>
<td>1.7</td>
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<td>6</td>
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<td>22</td>
<td>F</td>
<td>67</td>
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<td>&gt;32</td>
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<tr>
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<td>6F/3M</td>
<td>110</td>
<td>&gt;30</td>
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ND, not determined. * subject showed a fall in FEV₁ of 20% when breathing PBS only.

Lung function tests
FEV₁ and IVC were measured with a dry rolling-seal spirometer (Sensor Medics BV, Bilthoven, the Netherlands) according to standardized guidelines [21]. Bronchial reactivity to histamine was determined by a two-minute tidal-breathing method [21]. The histamine threshold (PC₂₀histamine) was defined as the interpolated concentration of histamine that caused a fall in FEV₁ of 20% of the baseline value.

Fiberoptic bronchoscopy and Segmental Allergen Challenge
BAL was performed as previously described [21]. Transcutaneous oxygen saturation was monitored throughout the bronchoscopy. After premedication with atropine and codeine
and after local anaesthesia with lidocaine, a flexible fiberoptic video-bronchoscope (Olympus p200) was wedged into a (sub)segment of the lingula. Seven successive 20 ml aliquots of sterile, prewarmed NaCl (154 mM) were instilled and each aspirated immediately with low suction. The aliquots four to seven were combined and used for the experiments described here. A baseline BAL was performed of the lingula and a postchallenge BAL at 5 min and/or at 4 h of the right middle lobe segment. After the control lavage, allergen was administered in (both the lateral segment and the medial segment of) the right middle lobe. When lavage was performed at 5 min, the bronchoscope was retracted above the carina for 5 min. Next, lavage was performed of one of the challenged segments. Four h after challenge, bronchoscopy was repeated and the (remaining) allergen challenged segment of the right middle lobe was lavaged. Subjects were observed for at least 3 h after the last bronchoscopy.

**Cell differentials**

Cells from BAL fluid were separated by centrifugation at 500 x g for 10 min at 4°C. Cells were resuspended in phosphate-buffered saline (140 mM NaCl and 10 mM sodium phosphate, pH 7.4) containing 0.5% (wt/vol) bovine serum albumin (Boseral DEM, Organon Teknika b.v., Boxtel, The Netherlands; PBA). The number of erythrocytes was counted with Daecie suspension (1.2% (wt/vol) trisodium citrate \( \cdot 2 \) \( \text{H}_2\text{O} \) (Merck, Munich, Germany) + 0.4% formaldehyde 37% (Merck)) in a Fuchs-Rosenthal counting chamber. All pools contained less than \( 1 \times 10^5 \) erythrocytes/ml, indicating no significant bleeding during bronchoscopy. Total cell number was determined by counting manually in a Buerker counting chamber. Cells were cytocentrifuged at 500 rpm for 2 min in a Shandon Cytocentrifuge (model cytospin 2) and stained with Romanovsky (DiffQuick) and Jenner Giemsa. For differential cell counts, a total of 1,000 cells were enumerated with the investigator being blinded for subject identity and lavage time-point. Epithelial cells, macrophages, lymphocytes, neutrophils and eosinophils were identified.

**Generation of clonal T cells**

CD4\(^+\) and CD8\(^+\) T-cell clones were generated from (baseline) BAL fluid T cells of two allergic asthmatic (AA) patients (patient numbers 1 and 2, Table 1). CD4\(^+\) and CD8\(^+\) fluorescent-labeled T cells were sorted by FACS Star Plus (Becton Dickinson (BD), San Jose, CA) and seeded at 1 cell per well by an Automated Cell Deposition Unit device (BD) [22]. The cells were cultured in a medium containing phytohaemagglutinin-A (PHA; Murex Diagnostics Ltd., MO, USA), irradiated peripheral blood mononuclear cells (PBMC) and IL-
Interleukin-16 in asthma

2 (20 U/ml) [22]. Anti-CD4 and anti-CD8 FITC and PE-labeled MoAb were from BD. The phenotype (CD4 and CD8) of the resulting clones was checked by flow cytometry again. The cloning efficiency in patient 1 and 2 was 5.9% and 31%, respectively, for the BALF CD4+ T lymphocytes, and 6.4% and 15%, respectively, for BAL fluid CD8+ T lymphocytes. Control procedures with PBMC performed in parallel experiments yielded a cloning efficiency of 52% and 63%. The numbers of BAL fluid T-lymphocyte clones used for experiments were 15 and 21 CD4+, and 20 and 26 CD8+ T-lymphocyte clones from patient 1 and 2, respectively.

**Cell cultures**

For the study on the production of cytokines total (polyclonal) BAL fluid cells and clonal cells were collected by centrifugation and resuspended in medium consisting of Iscove’s Modified Dulbecco’s Medium, 10% heat-inactivated pooled human serum (BioWhittaker, Walkersville, MD), 2 x 10^{-5} M beta-mercaptoethanol (Merck, Munich, Germany), 2 mM NAP-pyruvate (Merck), penicillin (100 U/ml, Gist-Brocades, Delft, The Netherlands) and streptomycin (0.1 g/l, Gibco) and seeded at a concentration of 100,000 cells/well to culture wells of 96-well roundbottom plates (Costar, Cambridge, MA). They were incubated either with medium alone or stimulated with one of the following stimuli: histamine 10^{-4} M, PHA 5 μg/ml, anti-CD3 (1:10,000 ascites, 16A9; gift from dr. R.A.W. van Lier) or phorbol myristate acetate (PMA; Sigma, St. Louis, MO, USA; 1 ng/ml) + ionomycin (Sigma; 1 μg/ml). Supernatants were obtained at different time-points by centrifugation of the plates after which the medium was carefully removed by pipetting. Supernatants from at least three wells were pooled and stored at -20°C until use. BAL fluid cells were always freshly used. Clonal cells were expanded till sufficient cells were present for phenotyping and cytokine production. Cytokine production experiments with T-cell clones were performed with cells that had not been previously frozen.

**Bronchoalveolar lavage fluid**

The cell-free supernatant was stored at -80°C. BAL fluid was concentrated prior to cytokine determination using centriprep 10 concentrators (cut off membrane of 10 kD; Amicon, Beverly, USA) according to the manufacturer’s protocol. Albumin was measured in BAL fluid by immunoturbidimetric assay before and after concentration (Cobas Bio analyzer; Roche Diagnostics Inc) [21] and used to determine the concentration factor of BAL fluid.
Chapter 5

Migration assay
To measure bioactive IL-16 cell migration was performed as previously described [12,13,15]. To obtain information on IL-16 dependent migration, the migration was performed in the absence and presence of anti-IL-16 antibodies (clone 17.1 at a final concentration of 5 to 10 μg/ml), and the net migration was by calculated by subtraction.

ELISA of IL-16, IFN-γ, IL-4 and IL-5
High binding ELISA plates (Costar) were coated for at least 48 h at 4°C with anti-IL-16 mAb 17.1 at a concentration of 5 µg/ml. At the day of assay plates were washed with PBS containing 0.02% Tween-20 (Merck). To prevent aspecific binding, plates were incubated with PBS containing 0.02% Tween-20 and 0.1% Gelatine (Merck) for 30 min at room temperature. Plates were then incubated with sample material, rIL-16 standard and positive control samples for 2 h at 37°C. Samples were in HPE buffer (CLB, Amsterdam, the Netherlands) + 1% Normal Mouse Serum (NMS). After this incubation the plates were washed and incubated for 2 h at 37°C with the detecting biotin-labeled anti-IL-16 mAb 6, also diluted in HPE buffer + 1% NMS. Next, plates were washed and incubated with streptavidin-poly-HRP (CLB; 1/10,000 v/v) in PBS containing 2% (v/v) cow’s milk, and subsequently developed with tetramethylbenzidine (Sigma) and H₂O₂ (Merck). Lower limit of detection was 15 pg/ml.

Levels of IFN-γ and IL-5 were measured by ELISA as described earlier [23]. IL-4 was measured by ELISA (CLB). Lower limits of detection were 62.5 pg IFN-γ/ml, 2 pg IL-5/ml and 1.95 pg IL-4/ml.

Statistical analysis
The Wilcoxon matched pairs signed rank test was used to evaluate differences between paired groups. The Mann-Whitney U test was used to evaluate differences between the AA and C group. Spearman's rank correlation was used to assess relations between variables. Two-sided probability values less than 0.05 were considered significant.
Results

Levels of IL-16, IFN-γ, IL-4 and IL-5 in concentrated BAL fluid before and after Segmental Allergen Challenge

The recovery of BAL fluid before and after segmental allergen challenge (SAC) were similar. In asthma patients before: 64% ± 9.7% (mean ± sd); after: 65% ± 9%, and there was no difference between asthma patients and controls. Before SAC no differences in IL-16 levels in BAL fluid were detected between the AA group (median 22.0 pg/ml and range 2.7 to 107.5 pg/ml) and the C group (9.6 pg/ml (≤1-51.0)). In the AA patients 10-15 (Table 1) BAL was performed at 5 min after SAC. In 3 cases the IL-16 levels were already increased at 5 min (from mean 47 pg/ml at baseline to 262 pg/ml at 5 min). At 4 h after SAC, IL-16 levels in BAL fluid were raised in the (total) AA group (p = 0.02) but not in the C group (Figure 1). Thus, in several asthma patients SAC resulted in an almost immediate increase in IL-16 levels in BAL fluid. In some healthy subjects there was a large increase of IL-16 after SAC. The rise of IL-16 in AA tended to be higher than that in controls (p = 0.07).

Before allergen challenge IFN-γ was detected in BAL fluid of 3 out of 8 asthma patients (range 93-263 fg/ml) and 1 of 8 control subjects (2543 fg/ml). IL-4 was detected in BAL fluid of 8 of 15 asthma patients (range 1-794 fg/ml) and 4 of 8 control subjects (range 50-362 fg/ml). IL-5 was detected in 12 of 15 asthma patients (range 20-953 fg/ml), which was significantly different from that in controls (detected in 2 of 8 control subjects; range 53-152 fg/ml; p = 0.03). No significant changes were found due to SAC at 4 h.

Figure 1. IL-16 in BAL fluid before and 4 h after Segmental Allergen Challenge measured by ELISA. There was a significant increase of IL-16 in asthma patients (p = 0.02). The increases of IL-16 in asthma tended to be higher than those in controls (p = 0.07).
Production of IL-16, IFN-γ, IL-4 and IL-5 by unseparated (polyclonal) BAL fluid cells

Cells from BAL fluid obtained before allergen challenge were cultured in vitro to obtain information whether they could serve as a potential source of IL-16. The cell differentials showed no significant differences between the patients with allergic asthma and non-allergic healthy subjects (Table 2). The AA patients tended to have higher numbers of eosinophils and epithelial cells (p = 0.06; MWU) than C subjects.

Table 2. Cell differentials of cultured BAL fluid cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Allergic asthma group</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 15</td>
<td>n = 4</td>
</tr>
<tr>
<td>Total cells</td>
<td>129 (67-268) *</td>
<td>138 (115-312)</td>
</tr>
<tr>
<td>Mac</td>
<td>118 (46-172)</td>
<td>126 (106-296)</td>
</tr>
<tr>
<td>Lym</td>
<td>6.9 (0-35.9)</td>
<td>10.7 (7.4-13.7)</td>
</tr>
<tr>
<td>Eo</td>
<td>1.2 (0-39.4)</td>
<td>0.5 (0.3-0.9)</td>
</tr>
<tr>
<td>Neu</td>
<td>2.2 (0.5-90.0)</td>
<td>1.3 (0.7-2.1)</td>
</tr>
<tr>
<td>Epi</td>
<td>4.2 (0-79.3)</td>
<td>0.3 (0-0.5)</td>
</tr>
</tbody>
</table>

Mac, alveolar macrophages; Lym, lymphocytes; Eo, eosinophils; Neu, neutrophils; Epi, epithelial cells. * 10⁶ cells/L, median and range.

The release of IL-16 by unstimulated BAL fluid cells of AA subjects was significantly higher than that from C subjects (Figure 2; p < 0.03; MWU). The addition of a stimulus (either histamine or PMA + ionomycin) did not significantly increase IL-16 release neither for AA nor for C cultures (data not shown).

The release of IFN-γ by unstimulated BAL fluid cells of AA subjects was detectable in only 5 out of 15 AA subjects (2 showing levels of > 200 pg IFN-γ/ml; Figure 2). In C subjects, very low amounts of IFN-γ were detected in 4 out of 5 subjects. IFN-γ production was not significantly different between the two groups. PMA + ionomycin significantly enhanced IFN-γ production in the AA group to 1.5 ng/ml (< 0.0625-8.6) (p < 0.001). Also in the C group PMA + ionomycin stimulated IFN-γ production in all cultures (0.6 ng/ml (0.3-5.8)).
Figure 2. IL-16 release (pg/ml, filled symbols) and IFN-γ release (open symbols) at 24 h by unstimulated BAL cell cultures of allergic asthmatic (AA, squares) and control (C, circles) subjects.

IL-4 was not detectable in unstimulated cultures, neither from AA nor from C subjects. PMA + ionomycin increased IL-4 production in the AA group to 11 pg/ml (<1.95-254), whereas in the C group IL-4 was not enhanced. The difference in IL-4 production between the AA and C group did not reach statistical significance.

IL-5 was not detectable in unstimulated cultures, neither from AA nor from C subjects. PMA + ionomycin increased IL-5 production in the AA group to 59 pg/ml (<2-1740) and in the C group to 108 pg/ml (29 and 187, respectively). There was no statistically significant difference between the AA and C group.

Production of IL-16, IFN-γ, IL-4 and IL-5 by clonal T cells

The production of IL-16 by T-cell clones was studied to obtain information on a homogeneous T-cell population as a source of IL-16. T-cell clones were stimulated with either histamine or coated anti-CD3. After 4 h of culture, supernatants were collected and the volume was restored with culture medium (including the stimulus where indicated). At 24 h, supernatants were collected again. Forty-three out of 47 clones did produce IL-16, at 4 h and/or 24 h. At 4h of culture, both CD4⁺ and CD8⁺ T-cell clones produced similar amounts of IL-16, either unstimulated or stimulated (Figure 3a). At 24h, CD8⁺ T-cells showed significantly increased levels of IL-16 after anti-CD3 stimulation (213 pg/ml (<15-801)) as compared to unstimulated cells (< 15 pg/ml (<15 - 403)) or stimulation with histamine (<15 pg/ml (<15-399); Figure 3b). For the CD4⁺ T-cells there were no significant differences between the different modes of stimulation (median <15 pg/ml for unstimulated, histamine stimulated and anti-CD3 stimulated cells).
**Figure 3.** A: IL-16 release (ng/ml) after 4h culture of CD4+ and CD8+ T-lymphocyte clones. Stimulation was by histamine or coated anti-CD3. Squares: unstimulated (CD4, n = 16; CD8, n = 21); triangles: histamine 10^4 M (CD4, n = 21; CD8, n = 26); circles: anti-CD3 10^4 (CD4, n = 21; CD8, n = 26). No significant differences were detected between groups. The numbers in the figure indicate the number of clones not producing detectable IL-16.

B: as A but now after 24 h culture. Anti-CD3 stimulated CD8+ clones released higher levels of IL-16 than unstimulated clones (p<0.05) and than histamine-stimulated clones (p<0.01).

All CD4+ and CD8+ T-cell clones from another patient produced IL-16 when stimulated by histamine for 24 hours (Fig. 4a). The IL-16 release upon PHA stimulation was similar and it was significantly correlated with IL-16 release upon histamine stimulation (Spearman rho 0.63, p<0.0001).

The production of IFN-γ, IL-4 and IL-5 were measured in comparison. Histamine did not induce production of these cytokines, whereas anti-CD3 or PHA did induce their production. The IFN-γ production by CD8+ T-cell clones from this patient showed a tendency to be higher than that by CD4+ clones from the same patient (anti-CD3 stimulation for 24 h; Fig. 4b). CD4+ T cell clones produced more IL-4 than CD8+ T-cell clones (p<0.02). Together this resulted in a significantly higher ratio of IFN-γ production/IL-4 production by CD8+ T-cell clones (93.5 ± 103; mean ± sd) than by CD4+ T-cell clones (14.9 ± 48) (p<0.0001). The IL-5 production was not significantly different between CD4+ and CD8+ T cell clones.
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Figure 4. A: IL-16 release (ng/ml) after 24 h culture of CD4⁺ and CD8⁺ T lymphocyte clones generated from BAL fluid T cells of a second patient. Filled symbols: stimulation by histamine 10⁻⁴ M; open symbols: stimulation by PHA 5 μg/ml. B: Release of IFN-γ, IL-4 and IL-5 (ng/ml) after 24 h culture of CD4⁺ and CD8⁺ T lymphocyte clones. Stimulation was by anti-CD3. Circles: IFN-γ, squares: IL-4; triangles: IL-5. Median levels and lower limit of detection are shown. Release of IL-4 by CD4⁺ clones was higher than by CD8⁺ clones (p<0.02).

IL-16 bioactivity

A selection of samples from clonal T cells was analyzed for bioactive IL-16 by migration assay. The results are depicted in Table 3. CD4⁺ T-cells did not release bioactive IL-16 at 4 h. At 24 h one sample of anti-CD3 stimulated CD4⁺ cells did display biological activity. Unstimulated CD8⁺ cells released low amounts of IL-16 protein which hampered the detection of biological activity. Both after histamine and anti-CD3 stimulation the CD8⁺ cells released higher amounts of biologically active IL-16. There was a close agreement between antigenic IL-16 and bioactive IL-16 in the supernatant from CD8⁺ T-cells (rho 0.96; p<0.003). For the supernatants from CD4⁺ T-cells there was no such correlation.
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Table 3. IL-16 production by T lymphocyte clones; comparison of antigenic IL-16 levels (in pg/ml) as detected by ELISA and IL-16 bioactivity (in %) as detected by migration assay

<table>
<thead>
<tr>
<th>No.</th>
<th>Type of cell</th>
<th>Stimulus</th>
<th>Time-point (h)</th>
<th>IL-16⁺ (pg/ml)</th>
<th>Migration⁺ (%)</th>
<th>Migration⁺ + anti-IL-16 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD4</td>
<td>None</td>
<td>4</td>
<td>&lt;</td>
<td>120 ± 5</td>
<td>115 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>CD4</td>
<td>None</td>
<td>4</td>
<td>&lt;</td>
<td>110 ± 7</td>
<td>108 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>CD4</td>
<td>None</td>
<td>24</td>
<td>1176</td>
<td>127 ± 6</td>
<td>113 ± 7</td>
</tr>
<tr>
<td>4</td>
<td>CD4</td>
<td>None</td>
<td>24</td>
<td>169</td>
<td>119 ± 10</td>
<td>109 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>CD4</td>
<td>Histamine</td>
<td>4</td>
<td>&lt;</td>
<td>115 ± 8</td>
<td>111 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>CD4</td>
<td>Histamine</td>
<td>4</td>
<td>546</td>
<td>106 ± 7</td>
<td>119 ± 6</td>
</tr>
<tr>
<td>7</td>
<td>CD4</td>
<td>Histamine</td>
<td>24</td>
<td>742</td>
<td>117 ± 5</td>
<td>121 ± 9</td>
</tr>
<tr>
<td>8</td>
<td>CD4</td>
<td>aCD3</td>
<td>4</td>
<td>193</td>
<td>136 ± 8</td>
<td>121 ± 5</td>
</tr>
<tr>
<td>9</td>
<td>CD4</td>
<td>aCD3</td>
<td>24</td>
<td>124</td>
<td>196 ± 9</td>
<td>137 ± 8</td>
</tr>
<tr>
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<td>CD8</td>
<td>None</td>
<td>4</td>
<td>&lt;</td>
<td>102 ± 3</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>11</td>
<td>CD8</td>
<td>None</td>
<td>24</td>
<td>&lt;</td>
<td>109 ± 7</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>12</td>
<td>CD8</td>
<td>None</td>
<td>24</td>
<td>168</td>
<td>110 ± 7</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>13</td>
<td>CD8</td>
<td>Histamine</td>
<td>4</td>
<td>87</td>
<td>161 ± 8</td>
<td>108 ± 7</td>
</tr>
<tr>
<td>14</td>
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<td>Histamine</td>
<td>4</td>
<td>613</td>
<td>212 ± 12</td>
<td>115 ± 7</td>
</tr>
<tr>
<td>15</td>
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<td>aCD3</td>
<td>4</td>
<td>603</td>
<td>186 ± 11</td>
<td>107 ± 9</td>
</tr>
<tr>
<td>16</td>
<td>CD8</td>
<td>aCD3</td>
<td>24</td>
<td>309</td>
<td>196 ± 11</td>
<td>122 ± 9</td>
</tr>
</tbody>
</table>

⁺, antigenic IL-16 as measured by ELISA; †, total migration induced by cell culture supernatants, †, migration in the presence of anti-IL-16 antibodies. The difference between the two latter columns gives the biologically active IL-16.

Discussion

We have found that subjects with stable asthma have similar baseline levels of antigenic IL-16 in concentrated BAL fluid as healthy control subjects. The level of IL-16 was raised in BAL fluid of allergic subjects (and not in control subjects) at 5 min and 4 h after segmental allergen challenge. Cruikshank and coworkers [13] found an increase of bioactive IL-16 at 6 h after allergen provocation. Also, after histamine challenge of the airways of patients with allergic asthma they found increased levels of IL-16 at 6h after challenge in comparison to 15 min after challenge [15]. IL-16 was not measured before challenge, so it was not clear whether there had been a rise of IL-16 as early as 15 min after the challenge. Here we show that already at 5 min and 4 h after allergen challenge the levels of IL-16 can be higher than before the challenge. This demonstrates that IL-16 may
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Interleukin-16 actively participate in the very early reactions that occur after allergen deposition in the airways. There was a rise of IL-16 in some of the healthy subjects as well. Possibly this was related to the very high doses of allergen instilled in the lungs of the controls.

The possible sources of IL-16 release in vivo in the airways may be T lymphocytes, bronchial epithelial cells, mast cells and eosinophils [8,10,24]. The T lymphocytes may impose the antigen-specific event in the IL-16 local release [3]. Therefore, we have focussed in this study on airway derived T lymphocytes as a possible source of IL-16. In cultures with total BAL fluid cells, IL-16 was released by cells from subjects with allergic asthma and less so by control subjects. This was the case for both unstimulated and histamine-stimulated cultures. From staining of cytospin preparations we know that these cell cultures contained mainly alveolar macrophages, lymphocytes, eosinophils, neutrophils, less than 0.6% monocytes and less than 0.9% epithelial cells. No mast cells and basophils could be detected. So, probably lymphocytes are the main source of IL-16 in these cultures; the eosinophils might add slightly.

In contrast to other cytokines measured in the same supernatants (IFN-γ, IL-4 and IL-5), IL-16 release was already high without stimulation and further stimulation hardly altered the levels of IL-16 released. Most probably the lymphocytes from the BAL fluid of asthmatic subjects were already activated in vivo and released IL-16 subsequently in vitro. Such an activation state may underlie the rapid release of IL-16 in vivo after allergen contact. However, it did not result in increased baseline levels of IL-16 in BAL fluid of asthmatic patients.

To obtain more information on T lymphocytes as a possible source of IL-16 we studied IL-16 production by BAL fluid derived CD4* and CD8* T-lymphocyte clones [17,18]. Antigenic IL-16 was produced in similar amounts by both CD8* and CD4* T lymphocyte clones obtained from BAL fluid from 2 allergic asthmatic subjects. After in vitro stimulation more than 90% of the clones produced IL-16, which is in line with the detection of intracellular IL-16 in in vitro stimulated BAL fluid T cells [14]. Biologically active IL-16 was detected in 4 h supernatant samples of the CD8* T-cells but not in those of the CD4* T-cells. This confirms earlier reports that only CD8* T-cells constitutively contain biologically active IL-16, and not CD4* T-cells [11]. Both, CD8* and CD4* T cells were able to produce bioactive IL-16 at 24 h after stimulation with anti-CD3, though antigenic IL-16 and bioactive IL-16 correlated in the supernatants from CD8* T cells but not in the supernatants from CD4* T cells. Interestingly, the CD8* T lymphocytes derived from the airways were able to release (bioactive) IL-16 already within 4 h after stimulation. Thus, CD8* T lymphocytes could be implicated in the early events after allergen challenge. After
being stimulated by histamine they may release IL-16 thereby enhancing the allergic inflammation by attracting CD4⁺ T-lymphocytes, monocytes and eosinophils to the site of inflammation and activating the cells locally.

Other chemokines able to attract lymphocytes, monocytes, and/or eosinophils are eotaxin, MCP-1, MCP-3, MCP-4, MDC/TARC, I-309, IL-2, IL-8, RANTES, MIP1-α and MIP1-β [13,25]. It is not clear whether all of these chemokines contribute in a major way to in vivo chemotaxis. For the T lymphocytes, IL-16 and MIP1-α were shown to represent the major chemoattractant activity in BAL fluid at 6 h after allergen challenge of asthmatic subjects [13].

Because we wanted to further characterize the properties of the T cells present in the BAL fluid, we studied other T-cell cytokines like IFN-γ, IL-4 and IL-5. The characteristics of the release of these cytokines were markedly different from those of IL-16. In contrast to IL-16 we did not find increases of IFN-γ, IL-4 and IL-5 in the BAL fluid at 4 h after SAC, implying that CD8⁺ or CD4⁺ T lymphocytes are not releasing large amounts of these cytokines at this time-point. Calhoun and coworkers [26] found a significant increase of IFN-γ at 5 min but not at 48 h after SAC. IL-5 was found to be slightly but significantly increased at 4 h after allergen challenge as compared to saline-challenged sites [27]. Baseline levels were not measured in this study. At 24 h or 48h IL-5 was clearly increased [27,28].

In our unstimulated cultures of BAL fluid cells we found that IFN-γ was present in some supernatants of unstimulated cells from both AA and C subjects. This is in agreement with Walker and coworkers [29] who studied purified BAL fluid lymphocytes instead of total BAL cell populations. In contrast, the latter group found levels of IL-4 to be increased in allergic asthmatic subjects, whereas we did not observe increased levels of IL-4 nor of IL-5. These differences may be explained by differences in asthma severity or by a possible suppressive influence of alveolar macrophages present in our cultures. In unstimulated total BAL cells of our asthmatic population we found a predominance of IFN-γ over the Th2 cytokines IL-4 and IL-5.

Our group reported before about production of IFN-γ and IL-4 by CD4⁺ T-cell clones obtained from BAL fluid [22,23]. In the present study we more extensively investigated CD8⁺ T-cell clones from BAL fluid. IFN-γ, IL-4 and IL-5 were hardly secreted by unstimulated clonal T cells, in contrast to IL-16. Stimulated cells produced substantial amounts of the Th2 cytokines IL-4 and IL-5. To our knowledge this is the first report about cytokine release by CD8⁺ T cell clones from BAL fluid of subjects with allergic asthma. CD8⁺ T cells have long been considered as potent producers of IFN-γ. More recently it has become clear that CD8⁺ T cells like CD4⁺ T cells can develop into Th2 type cells,
producing IL-4 and IL-5 [30,31]. This has also been shown for CD8$^+$ T cells in the airway tissue [32,33]. It is unknown whether CD8$^+$ T cells significantly contribute to the allergic inflammatory reaction in the airways of asthma patients. Our *in vitro* cytokine production studies show relatively high IFN-γ production, low IL-4 production, but equal IL-5 production in comparison with CD4$^+$ T cells from the same patients. The presence of increased CD8$^+$ T cells in BAL fluid has been linked to the absence of late phase bronchus obstructive reactions after inhalation of allergen challenge [34,35]. Thus, despite CD8$^+$ T cells being potential producers of IL-16 by which they facilitate the infiltration of inflammatory CD4$^+$ cells, they may play mainly a protective role under the conditions of experimental allergen challenge. This may be related to their high IFN-γ production capacity upon activation and the dampening of Th2 reactions by IFN-γ. Another possibility which was raised recently, is that IL-16 might prevent antigen-specific T-cell activation [9].

In conclusion, we showed early *in vitro* release of bioactive IL-16 by airway-derived CD8$^+$ T cells of allergic asthmatic subjects. *In vivo* allergen challenge of the airways induced rapid (5 min and 4 h) increases in IL-16 in asthmatic patients. Our results point to an important regulatory role of CD8$^+$ T cells in the early events after allergen challenge.

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