In vitro and in vivo modulation of human T lymphocytes from allergic asthmatic subjects
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Segmental allergen challenge in allergic asthma results in early activation of local T lymphocytes

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submitted for publication
Abstract

We have analysed the effects of segmental allergen challenge (SAC) of the airways on T-lymphocyte activation in allergic asthma patients and non-allergic healthy control (C) subjects at 4h after SAC. Lymphocyte subpopulations in blood and bronchoalveolar lavage (BAL) fluid were measured by flow cytometry. Intracellular IFN-γ, IL-4 and IL-5 proteins were analysed with an immunocytochemical assay. SAC caused specific effects in allergic asthma patients. The percentage of CD4⁺CD27⁺CD103⁺ cells increased in BAL fluid (p<0.02), and there was a tendency for a decrease of the percentage of CD8⁺CD27⁺CD103⁺ cells (p = 0.08). The changes in the BAL fluid of the allergic asthma patients were accompanied by reciprocal changes in the blood. SAC resulted in an increase of IFN-γ⁺CD4⁺ lymphocytes in BAL fluid of allergic asthma subjects, whereas in C subjects IFN-γ⁺ cells tended to decrease. These effects were detected in cells that were not stimulated in vitro. We conclude that SAC results in T-cell activation in the airway compartment within 4h after allergen challenge.

Introduction

Patients with allergic asthma may experience two phases of bronchoconstriction after inhalation of allergens [1], the early asthmatic reaction and after 4 to 12h the late phase asthmatic reaction (LAR). The LAR is accompanied with an influx of inflammatory cells into the lungs, among which eosinophils are most characteristic for asthma [2]. The precise mechanisms causing the LAR are not known. However, T lymphocytes may play an important immunoregulatory role. Gratiou et al. [3] have shown a fall in the proportion of CD3⁺ lymphocytes in the bronchoalveolar lavage (BAL) fluid of patients with allergic asthma within 10 min after allergen exposure. Furthermore, they showed increased CD3⁺ lymphocyte counts in the mucosa at 6h after allergen exposure [4] and an increased HLA-DR expression by T lymphocytes in the BAL fluid suggestive for activation of the cells [5]. At 12 to 48h after allergen challenge, T lymphocytes were clearly increased and they had acquired an activated state as concluded from increased percentages of CD25 positive T cells and FAS-L positive cells [6-11], and from increased percentages of T cells expressing cytokine mRNA and protein [10,12-14]. Also soluble products from T cells are present in increased amounts in BAL fluid at those later time points after allergen challenge [11,12,15].
If T lymphocytes play a role in the onset of the LAR one would expect to see changes in their numbers and activation state before and at the early onset of the LAR. We tested this hypothesis in the model of segmental allergen challenge (SAC) in patients with allergic asthma. Analysis of T cells from the site of allergen challenge was performed before and at 4h after SAC. We have focused on T-cell activation by measuring the expression of CD27, on the activation of mucosal T cells by analysing CD103+ T cells, and on the expression of intracellular cytokine proteins in bronchoalveolar lavage (BAL) fluid derived T cells.

CD27 is a membrane surface protein on T cells which is upregulated on naive T cells after first contact with antigen via antigen presenting cells [16]. At the same time the cells are induced to express high levels of the ligand CD70. Subsequently, the CD27/CD70 pair will support clonal expansion and cytokine production by specific T lymphocytes [17]. After repeated antigenic stimulation the CD27 molecule is shed [18]. Thus CD27+ T cells and CD27- T cells are considered to be naive T cells and memory-type T cells activated by antigen, respectively. In a house dust mite-reactive donor the allergen-reactive T cells in the blood were exclusively found within the CD27+ population [19].

CD103 (integrin αEβ7) is a membrane surface molecule that is predominantly expressed on mucosal leukocytes [20]. It specifically binds to E-cadherin expressed on the basolateral surface of epithelial cells [21]. Furthermore, it has been described as an activation antigen which can in vitro be upregulated by mitogens, phorbolesters, antigens and IL-2 [22,23]. It has been shown indeed that the percentage CD103+ T cells is higher in BAL fluid than in peripheral blood [24].

T-cell cytokines and in particular the Th2 cytokines IL-4 and IL-5 mediate several reactions in allergic asthma, e.g. supporting recruitment of eosinophils into the airways [25,26]. Th2 cytokine mRNA and the protein levels have been shown to be increased in lung-biopsy tissues from symptomatic allergic asthma patients and from patients with severe asthma [27]. Controlled allergen exposure resulted in an increase of these cytokines in the lungs of allergic subjects at 12 to 48h after allergen exposure. However, Th2 cytokines were not predominant in stable patients with mild asthma [28], and there is no information on their possible role in the early onset of the LAR in the lungs. We have developed an immunocytochemical procedure for the staining of intracellular cytokine proteins in BAL fluid T cells [29]. With this method the T-cell subset can be identified and there is no need for in vitro stimulation of the cells. The omission of in vitro stimulation may avoid masking of differences between allergic and C subjects [30].

Here we report the results of our analysis of those parameters of T-cell activation in BAL fluid and blood in our model of SAC [31,32] in allergic patients with mild to moderate
asthma and in non-allergic healthy subjects. We have observed changes in numbers of CD27+CD103+ T-cell subpopulations, and activation of especially IFN-γ production in the BAL fluid of allergic asthma, which was different from the effects in controls. This provides evidence for the involvement of T lymphocytes in the onset of allergen-induced inflammatory reactions in the lungs within 4h after allergen contact.

Material and Methods

Subjects
Sixteen volunteers (8 non-smoking allergic asthmatics and 8 non-smoking non-allergic healthy controls) were selected for the study. The groups were similar with respect to age and sex (Table 1). Asthma was defined according to the American Thoracic Society (ATS) criteria and included a history of recurrent episodes of wheezing, chest tightness and dyspnoea and a normal FEV1 between asthmatic attacks [33]. Asthma severity ranged from Grade I (episodic) to Grade III (moderate persistent), according to the staging as proposed in the Global Initiative for Asthma [34]. All asthma patients showed bronchial hyperreactivity. None had experienced an exacerbation of asthma during at least 2 months before the SAC. None of the subjects used anti-inflammatory medication, in particular they did not use glucocorticosteroids for at least 6 weeks prior to the study. Inhaled bronchodilators were withheld at least 8 h before all investigations. All asthma subjects had a positive RAST and a positive skin prick test for house dust mite allergens. The control subjects had no past or present history of wheezing, chest tightness, recurrent episodes of reversible airway obstruction, or allergy. The control subjects were RAST negative for a standard panel of allergens tested. They were skin prick test negative for house dust mite allergens. None of the subjects had experienced recent airway infection. 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.

Lung function tests
The FEV1 and inspiratory vital capacity were measured with a dry rolling-seal spirometer (Sensor Medics BV, Bilthoven, The Netherlands) according to standardized guidelines [35]. Bronchial reactivity to histamine was determined by a two-minute tidal-breathing method [36]. The histamine threshold (PC20histamine) was defined as the interpolated concentration of histamine that caused a fall in FEV1 of 20% of the baseline value.
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Age (Yr)</th>
<th>Sex</th>
<th>FEV$_1$ (%pred)</th>
<th>PC$_{20}$hist (mg/ml)</th>
<th>Skin threshold (BU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>1</td>
<td>29</td>
<td>F</td>
<td>103</td>
<td>3.2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>F</td>
<td>100</td>
<td>0*</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>F</td>
<td>111</td>
<td>4.5</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>M</td>
<td>92</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>F</td>
<td>95</td>
<td>1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>F</td>
<td>104</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>F</td>
<td>67</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>M</td>
<td>108</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>C group</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>39</td>
<td>F</td>
<td>114</td>
<td>&gt;32</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>F</td>
<td>121</td>
<td>&gt;32</td>
<td>&gt;300</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>M</td>
<td>100</td>
<td>&gt;32</td>
<td>&gt;300</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>F</td>
<td>106</td>
<td>23.1</td>
<td>&gt;50</td>
</tr>
<tr>
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<td>20</td>
<td>F</td>
<td>103</td>
<td>&gt;32</td>
<td>&gt;50</td>
</tr>
<tr>
<td>7</td>
<td>38</td>
<td>M</td>
<td>110</td>
<td>&gt;32</td>
<td>&gt;50</td>
</tr>
<tr>
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<td>29</td>
<td>M</td>
<td>110</td>
<td>21.5</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

* subject showed a fall in FEV$_1$ of 20% when breathing PBS only.

**Study design**

The study design was as described before [31,32]. At an initial visit, all subjects completed a respiratory and allergy questionnaire. A blood sample was taken to determine allergen-specific and total IgE. Baseline spirometry and a histamine provocation test were performed. On the day that the subjects returned to the clinic for allergen challenge, a second baseline spirometry was done and intracutaneous dose response series with house dust mite were performed to determine the allergen dose for the allergen provocation procedure.

**Determination of allergen dose**

Skin test titrations were performed to determine the concentration of the allergen producing a 10 mm wheal response at 15 min after intracutaneous injection [37]. The allergic asthma group underwent an allergen challenge with 1 ml of this allergen concentration brought to a final volume of 5 ml with saline. The control (C) group was challenged with either 300 or 50 BU house dust mite allergen in 5 ml saline. For the amounts of allergen applied see Table 1. The highest level of LPS delivered was 0.25 pg/ml (in 3 C subjects). Samples containing less than 2.1 pg/ml are within United States Pharmacopeia standards for endotoxin concentrations and are considered safe and non-pyrogenic for intravenous injection [38].
**Fiberoptic bronchoscopy and Segmental Allergen Challenge**

A baseline BAL was performed of the lingula and a postchallenge BAL of the right middle lobe segment at 4h after SAC. Bronchoscopy was performed as described previously [39]. Briefly, 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 or middle lobe. Seven successive 20 ml aliquots of sterile, prewarmed NaCl (154 mM) were instilled and each aspirated immediately with low suction. After the baseline lavage, allergen was administered into a segment of the right middle lobe. Four h after challenge, bronchoscopy was repeated and the allergen challenged segment of the right middle lobe was lavaged. Transcutaneous oxygen saturation was monitored throughout the bronchoscopy. Subjects were observed for at least 3h after the last bronchoscopy.

**Bronchoalveolar lavage fluid**

The aliquots four to seven were combined and used for this study. The pooled BAL fluid was immediately centrifuged at 500 g and 4°C. The cell-free supernatant was stored in aliquots at -80°C. BAL fluid cells were resuspended in phosphate buffered saline (140 mM NaCl (Merck, Munich, Germany) and 10 mM sodium phosphate (Merck), pH 7.4) containing 0.5% (w/v) bovine serum albumin (BoseraI DEM, Organon Teknika b.v., Boxtel, the Netherlands) (PBA). The number of erythrocytes was counted with Daecie-suspension (1.2% (w/v) trisodiumcitrate.2H$_2$O (Merck) + 0.4% (v/v) formaldehyde 37% (w/v; Merck)) in a Fuchs-Rosenthal counting chamber. All pools contained less than 1 x 10$^5$ erythrocytes per ml, indicating no significant bleeding during bronchoscopy. Total cell number was determined by counting manually in a Bürker counting chamber. Cells were cytocentrifuged at 500 rpm for 2 min in a Shandon Cytocentrifuge (model cytospin 2) and stained with Romanovsky (Diff-Quick) and Jenner-Giemsa. For differential cell counts, a total of at least 1,000 cells were enumerated. Epithelial cells, macrophages, lymphocytes, neutrophils and eosinophils were identified.

**Peripheral blood (mononuclear) cells**

Blood was collected from the subjects via venepuncture before and at 4h after allergen challenge. The collected blood was anticoagulated with 0.105 M (3.2%) buffered sodium citrate (Becton Dickinson (BD), San Jose, CA). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of the blood over Lymphoprep (Nycomed Pharma, Oslo,
T-cell activation after allergen challenge

Norway). Total and differential leukocyte counts were performed in EDTA-blood by using an automated method (H$_3$-RTX, Bayer-Technicon, Tarrytown, NY).

**Flow cyt fluorimetry**
Inert mouse IgG1, inert mouse IgG2a, goat-anti-mouse-IgG1, anti-LeucoGate$^\text{Tm}$, anti-CD2, anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD27, anti-CD28, anti-CD45RA, anti-CD45RO, anti-NK mix$^\text{Tm}$, anti-Leu8 and anti-HLA II were from BD. Anti-CD103 (2G5) was from Beckman-Coulter (Mijdrecht, The Netherlands). All antibodies were FITC, phycoerythrin (PE) or PE-Cy5 labeled.

Cells (from blood and BAL fluid) were kept in tubes on melting ice at all times. Cells were collected, washed with PBA and incubated for 30 minutes with different combinations of monoclonal antibodies in PBA. After washing, analysis of cell suspensions was performed on a FACSscan (BD) [40]. Lymphocytes were gated on the basis of forward and side light scatter. The occurrence of dead cells in the gate was checked by analyzing samples after propidium iodide incubation. In all experiments, parallel incubations were performed with irrelevant antibodies matched for the isotypes of the detecting antibodies. Minimal 50,000 events where counted per sample until at least 5,000 cells were detected in the lymphocyte gate.

**Intracellular labeling and staining**
BAL fluid cells and PBMC were treated as described before [29]. Cells were scored with a light microscope. The origin of the cells was not known to the person scoring the slides. At least 1,000 cells were scored. Absolute numbers of cytokine-positive cells were calculated by multiplying the percentage cytokine-positive cells by the absolute number of lymphocytes present as deduced from the cytospin preparations. The method was validated by using T-cell clones which were stable producers (as detected by ELISA) of one or more of the cytokines studied.

**Statistical analysis**
The non-parametric Mann Whitney U (MWU) test was used to evaluate differences between the asthmatic and the control group. The non-parametric Wilcoxon matched pair signed rank (WR) test was used to evaluate differences due to SAC within individual subjects. Probability values less than 0.05 were considered significant.
Chapter 9

Results

The subject characteristics, FEV₁, PC₂₀, histamine and skin allergen threshold are given in Table 1. The percentage of BAL fluid recovered was similar in both groups. At baseline, the recovery in the allergic asthma group was 76.3% (71.3% - 96.3%) (median and range) and in the C group 78.8% (62.5% - 82.5%). At 4h, the recovery in the allergic asthma group was 71.9% (56.3% - 88.8%) and in the C group 81.9% (60.0% - 93.8%).

Total and differential cell counts in BAL fluid and blood

At baseline, there was no difference in total cell counts in BAL fluid between the patients with allergic asthma and non-allergic healthy subjects (Table 2). The allergic asthma patients had higher numbers of eosinophils (mean ± SD: 1.36 ± 1.2 x 10³/ml vs. 0.33 ± 0.3 x 10³/ml; p < 0.01, MWU) and neutrophils (2.53 ± 2.7 x 10³/ml vs. 0.84 ± 0.6 x 10³/ml; p < 0.05, MWU) than C subjects. No differences were observed for the numbers of macrophages, lymphocytes, monocytes and epithelial cells.

Table 2. Cells (x10³/ml) in BAL fluid pool 2 before and at 4h postchallenge

<table>
<thead>
<tr>
<th>Time</th>
<th>Allergic asthma (n=8)</th>
<th>C group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (95-186)</td>
<td>123 (82-306)</td>
</tr>
<tr>
<td></td>
<td>121 (86-172)</td>
<td>90 (47-212)</td>
</tr>
<tr>
<td></td>
<td>7.6 (3.6-27.6)</td>
<td>6.1 (2.1-46.8)</td>
</tr>
<tr>
<td></td>
<td>0.89³ (0.14-3.5)</td>
<td>0.88 (0.09-12.2)</td>
</tr>
<tr>
<td></td>
<td>1.3³ (0.52-7.9)</td>
<td>17.0⁴ (3.4-53.2)</td>
</tr>
<tr>
<td></td>
<td>0.27 (0-0.75)</td>
<td>0.50 (0-2.4)</td>
</tr>
<tr>
<td></td>
<td>0.07 (0-0.83)</td>
<td>0.20 (0-1.8)</td>
</tr>
<tr>
<td></td>
<td>0.0 (0-0.0)</td>
<td>0.26 (0-0.77)</td>
</tr>
<tr>
<td></td>
<td>0.0 (0-0.0)</td>
<td>0.22 (0-0.47)</td>
</tr>
</tbody>
</table>

0, before challenge; 4h, at 4h postchallenge. Median and range are shown. ³p = 0.02 (compared to baseline); ⁴p = 0.02 (compared to control); ³p = 0.02 (compared to control); ⁴p = 0.05 (compared to baseline); ⁵p = 0.01 (compared to baseline).
SAC had no effect on total cell numbers in BAL fluid, neither in the allergic asthma nor in the C subjects. Both in allergic asthma and C subjects there was an increase in the numbers of neutrophils (p<0.05 and p<0.01, respectively, WR). In C subjects this was accompanied by a significant decrease in the numbers of macrophages (p<0.02, WR). No effects of SAC were observed for the numbers of lymphocytes, eosinophils, monocytes and epithelial cells.

At baseline, blood cells from allergic asthma patients did not differ from the cells from C subjects for all parameters tested: total number of leukocytes/L, total number of eosinophils/L, % neutrophils, % eosinophils, % basophils, % lymphocytes, % monocytes.

**Lymphocyte subpopulations**

Cell surface markers were analysed in all subjects. At baseline, the percentages of both CD4+CD27+ and CD8+CD27+ cells were much lower in BAL fluid than in blood (p<0.05), both in allergic asthma and controls. The CD4+CD103+ and CD8+CD103+ cells were higher in BAL fluid than in blood (p<0.02) (Table 3). No significant differences between allergic asthma and controls were found in the numbers of CD3+ cells/ml (median 5.36 x 10^3 cells/ml of BAL fluid in the allergic asthma group versus 5.02 x 10^3 cells/ml of BAL fluid in the C group). No differences were found between BAL fluid cells of the allergic asthma and C group for the percentages CD3+, CD4+, CD8+, CD4+CD27+, CD8+CD27+, CD4+CD103+ and CD8+CD103+ cells.

In allergic asthma patients, SAC resulted in an increase of the percentage of BAL fluid CD4+CD27+CD103+ cells (% of CD4+CD103+; p<0.02; WR test; Figure 1a). This was significantly different from the changes in the C group (p<0.02; Figure1a). On the other hand, there was a tendency for a decrease of the percentage of CD8+CD27+CD103+ cells (% of CD8+CD103+) after SAC in allergic asthma and not in controls (p=0.08; WR test; Figure 1b). When the CD103+ subpopulations were studied or when these four markers were studied separately no significant effects of SAC were detected. The numbers of CD3+ cells in both groups were not significantly changed after SAC. SAC had no effect on the percentages CD4+ and CD8+ cells in BAL fluid (in % of CD3).

In the blood at baseline, no significant differences were found between the groups for lymphocyte subpopulations. After SAC, the percentage CD4+CD27+CD103+ cells tended to decrease in the allergic asthma patients (Figure 2a, p=0.07). The changes in the patients were significantly different from those in controls (p<0.02; MWU; Figure 2b).
Table 3. FACS analysis of blood and BAL fluid cells (% of positive cells; median and range)

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>BAL fluid cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Allergic asthma (n = 8)</td>
<td>Control (n = 8)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>4h</td>
</tr>
<tr>
<td>CD4CD27</td>
<td>44.6*</td>
<td>47.3</td>
</tr>
<tr>
<td>(%CD4)</td>
<td>(2.3-68.0)</td>
<td>(24.1-70.7)</td>
</tr>
<tr>
<td>CD8CD27</td>
<td>61.3*</td>
<td>56.2</td>
</tr>
<tr>
<td>(%CD8)</td>
<td>(7.2-67.6)</td>
<td>(37.5-86.9)</td>
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<tr>
<td>CD4CD103</td>
<td>18.5*</td>
<td>16.9</td>
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<tr>
<td>(%CD4)</td>
<td>(7.6-45.7)</td>
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<tr>
<td>CD8CD103</td>
<td>68.9</td>
<td>72.4</td>
</tr>
<tr>
<td>(%CD8)</td>
<td>(0.3-86.2)</td>
<td>(59.3-86.9)</td>
</tr>
</tbody>
</table>

0, before challenge; 4h, at 4h post-challenge. * p = 0.01 blood compared to BAL fluid; † p = 0.04 blood compared to BAL fluid; § p = 0.02 blood compared to BAL fluid.
T-cell activation after allergen challenge

Figure 1. A. Percentage of CD4⁺CD103⁺ and BAL fluid cells expressing CD27 before and 4h after SAC. Circles, asthmatic subjects; Triangles, healthy control subjects. B. The same analysis for the CD8⁺CD103⁺ cells.

Figure 2. A. Percentage of CD4⁺CD103⁺ peripheral blood cells expressing CD27 before and 4h after SAC 4. Circles, asthmatic subjects; Triangles, healthy control subjects. B. The changes in the percentage CD4⁺CD27⁺CD103⁺ (% of CD4⁺CD103⁺) in asthmatic subjects were significantly different from those in C subjects (p<0.02).
Intracellular cytokines

Cells from BAL fluid without in vitro stimulation

At baseline, the total number of IFN-γ+ lymphocytes per ml BAL fluid tended to be lower in allergic asthma than in C subjects (median (range): <1/ml (<1-92.5) vs. 66/ml (<1-2,174); p = 0.09; MWU). When analysed for CD4+ and CD8+ cells separately this difference was detected in the CD8+ lymphocyte subpopulation. IL-4+ cells were detected in 5 of 8 asthma patients and in 3 of 7 controls. IL-5+ cells were detected in 3 of 8 asthma patients and in 2 of 7 controls. No differences were found for numbers of IL-4+ and IL-5+ cells between patients controls (IL-4: 24.4/ml (<1-800) vs. <1/ml (0 - 490); IL-5: <1/ml (<1-85.7) vs. <1/ml (<1-66.4)).

At 4h after challenge there was an increase of IFN-γ+ T cells in the asthma patients only (from 39.4 ± 29.2/ml to 410.4 ± 804.9/ml (mean ± SD); p = 0.03) (Figure 3a). The increase of IFN-γ+ cells in asthma was significantly different from the changes in controls (p = 0.01). The increase of IFN-γ+ cells was found mainly in the CD4+ cells (Figure 3b) indicating that at this early time-point CD4+ and not CD8+ T cells responded to the allergen challenge by cytokine production. Numbers of IL-4+ and IL-5+ cells were not significantly changed by SAC at 4h. When the cytokine-positive cells were expressed in percentage of lymphocytes essentially the same results were found.

Cells from BAL fluid after in vitro stimulation

Before SAC, the total numbers of IFN-γ+ cells tended to be lower in the patients than in controls (median (range) 1,170/ml (585 - 5,111) vs. 559/ml (<1 - 10,128); p = 0.08; MWU), with contributions both by CD4+ and CD8+ cells. The total numbers of IL-4+ cells were significantly higher in asthma than in C subjects (357/ml (117-2,276) vs. 98.6/ml (<1-1,454); p = 0.02; MWU), whereas for IL-5+ cells a trend for higher numbers in asthma was found (206/ml (<1-2,975) vs. 27.4/ml (<1-1,152); p = 0.06; MWU). IL-4+ CD4+ lymphocytes were now detected in 7 of 8 asthma patients and 5 of 7 C subjects, and IL-5+ CD4+ lymphocytes also in 7 of 8 asthma patients and 5 of 7 C subjects. IL-4+ CD8+ lymphocytes were detected in 4 of 8 patients and in 3 of 7 C subjects, whereas IL-5+ CD8+ lymphocytes were detected in 5 of 8 patients and 1 of 7 C subjects. SAC did not significantly affect the numbers of IFN-γ+, IL-4+ and IL-5+ cells. There was no difference between the changes observed in the patients and those in controls.
T-cell activation after allergen challenge

Figure 3. A. IFN-γ⁺ lymphocytes (10⁴/L) in BAL fluid before and 4h after SAC. Cells were not stimulated in vitro. Circles, 8 asthmatic subjects; Triangles, 7 healthy control subjects. B. as A but now IFN-γ⁺CD4⁺ lymphocytes.

Cells from blood without in vitro stimulation
At baseline, there were no differences in cytokine-positive cells between patients and controls. SAC resulted in a significant increase of the number of IL-4⁺ cells both in allergic asthma patients and in C subjects. This increase in IL-4⁺ cells resided in the CD4⁺ cells mainly (Figure 4) indicating that also in the blood at this early 4h time-point the CD4⁺ and not CD8⁺ T cells are responding to the allergen challenge by cytokine production.

Cells from blood after in vitro stimulation
At baseline, no significant differences were found in the number of cytokine-positive cells between asthma patients and controls. SAC resulted in a tendency for increased numbers
of IFN-γ⁺ cells (CD8⁺ plus CD4⁺) in the patients (from 59.6/ml (25.5-199.6) to 90.8/ml (27.2-209.9); p < 0.08). There were no statistically significant effects on numbers of IL-4⁺ and IL-5⁺ cells, neither in the patients nor in controls.

Discussion

Segmental allergen challenge had marked effects on T-cell subpopulations and intracellular cytokine protein production in BAL fluid and blood T cells from allergic asthmatic patients within 4 h after allergen challenge. The percentage of CD4⁺CD27⁺CD103⁺ cells increased in BAL fluid (p < 0.02). Furthermore, SAC resulted in an increase of IFN-γ⁺CD4⁺ cells in the BAL fluid of asthma patients, whereas in C subjects IFN-γ⁺ cells tended to decrease.

The changes in the CD27⁺ population indicate antigen-specific events in the BAL fluid T-cell population. It is important to note that these changes were restricted to the mucosal CD103⁺ T cells. Both CD4⁺ and CD8⁺ lymphocytes were implicated in the allergen-induced reactions in the airways at 4h after SAC. The increase of the percentage CD4⁺CD27⁺CD103⁺ T cells in BAL fluid points to an increase of the percentage naive T cells in this subpopulation. This may have been the result of a migration of antigen-experienced T cells into the mucosal tissue and/or a migration of CD27⁺ T cells from the blood into the epithelial lining fluid. The decrease of the percentage CD4⁺CD27⁺CD103⁺ T cells in the blood is in line with this explanation. It would be very interesting to know the precise events occurring in the tissue as we did not observe a significant increase of the numbers of the CD4⁺CD27⁺CD103⁺ cells in the BAL fluid. This was related to a decrease of total CD3⁺ T cells in the BAL fluid in 4 patients which contributed to a net decrease of the CD27⁺ subpopulation in these patients.

The studies from Gratzioiu et al. [3] provided evidence for an early involvement of T lymphocytes in the reactions after allergen challenge by showing a decrease of CD3⁺ cells in BAL fluid within 10 min after SAC, an increase of T cells in the mucosal tissue and a decrease of the T cells expressing adhesion molecules in BAL fluid at 6h after SAC [4,5]. Our results provide further support for a role of T cells in the onset of the late phase asthmatic reaction and pinpoint such a role to antigen-related events and to the mucosal CD103⁺ T cells.

Further evidence for the activation of T cells within 4h after SAC derives from the increase in cytokine-positive cells in BAL fluid and blood. When we omitted in vitro stimulation of the cells, SAC resulted in an increase of IFN-γ⁺ T cells in BAL fluid from
asthma patients, which were CD4+ cells in particular. Furthermore, there was an increase in IL-4+ cells in the blood both in asthma and control. When we stimulated the cells in vitro the outcome was different: no significant changes were observed in BAL fluid cells and there was an increase in IFN-γ+ cells in the blood. Differences in cytokine staining in relation to the stimulus applied have been described earlier [30,41,42]. We assume that results of the procedure without in vitro stimulation closely reflect the actual in vivo situation. The results after in vitro stimulation may provide information on the total cytokine production capacity of the cell populations.

Our results of an increase in IFN-γ+ cells in BAL fluid after SAC in asthma patients are in line with the increase in BAL fluid IFN-γ levels after SAC [43] and with the observation that T-cell clones derived from BAL fluid T cells obtained after allergen inhalation showed high IFN-γ production [44]. The increase of IFN-γ+ cells may be related to activation of mast cells in the allergic asthmatic patients. We have reported earlier that there is release of tryptase after SAC indicating the activation of mast cells under the conditions applied [30]. In vitro studies performed by our group showed that mast cells products enhanced IFN-γ production by T cells [45]. Furthermore, differences in characteristics of alveolar macrophages may cause the differences between asthma patients and healthy subjects. In asthma patients a considerable part of alveolar macrophages belongs to the type that may activate T cells, whereas in healthy subjects most of the alveolar macrophages belong to the type that suppresses T-cell functions [46,47]. Thus, activation of macrophages by the SAC may result in activation of T cells resulting in increased cytokine production in asthma patients. Alternative explanations may invoke differences between asthma and control subjects with respect to the redistribution of T cells in various compartments.

For IL-4 and IL-5 the 4h time-point after SAC might have been too early to detect any increase in their expression. Teran et al. [48] found a less than 3-fold increase of IL-5 at 4h after allergen challenge but a 20-fold increase of IL-5 at 24h after challenge.

Besides the effects of SAC, there are a number of observations in our study at baseline that showed important differences between BAL fluid and blood T lymphocytes. Both in asthma and controls the CD27+ cells were much lower in BAL fluid than in blood. This confirms earlier results observed for BAL fluid T lymphocytes in sarcoidosis patients [40], and demonstrates the activated state of T cells in the epithelial lining fluid. In agreement with Erle et al. [24] CD103+ cells were higher in BAL fluid than in blood.

Our baseline cytokine results, for cells that had not been stimulated in vitro, showed a trend for lower percentages of IFN-γ+ T cells in BAL fluid from asthma patients as
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compared to healthy subjects. CD8⁺ T cells of the asthmatic patients studied showed no IFN-γ staining at all, which was different from control subjects. No differences were detected in percentages IL-4⁺ or IL-5⁺ cells. An earlier report about immunohistochemical staining of bronchial biopsies failed to show cytokine staining in T cells [14], which was explained by the limited capacity of T cells to store cytokines. Our method of immunocytochemical double staining of BAL fluid cells seems to be sensitive enough to detect T-cell cytokine production even in unstimulated cells and shows that a decreased IFN-γ production rather than increased production of IL-4 and IL-5 is found in asthmatic subjects at baseline.

After in vitro stimulation of BAL fluid cells there was still a tendency for lower percentages of IFN-γ⁺ cells in asthmatic subjects; the numbers of IL-4⁺ cells were clearly elevated and there was a tendency for higher numbers of IL-5⁺ cells than in control subjects. However, after in vitro stimulation, numbers of IFN-γ⁺ cells exceeded numbers of Th2-cytokine producing cells both in asthmatic and control subjects. Krug et al. [49] also found a predominant production of IFN-γ by BAL fluid cells stimulated in vitro.

We conclude that flow cytometrical study of combinations of cell surface markers and immunocytochemical study of both unstimulated and in vitro stimulated cells provide us with new insights in T-cell inflammatory reactions after SAC. The flow cytometrical study revealed that in particular mucosal CD4⁺ and CD8⁺ T lymphocytes were activated and that this activation seems to be antigen-related. Though it is believed that CD8⁺ T cells preferentially interact with peptides derived from intracellular antigens, in several model systems CD8⁺ T cells have been shown to interact with antigenic fragments from soluble antigens as well [50]. Any role of allergen-specific CD8⁺ cells is still controversial. A recent study in a rat model, however, showed that antigen-primed CD8⁺ cells downregulated the late airway response [51]. Most striking was the early rise in IFN-γ production by CD4⁺ BAL fluid cells in asthmatics after SAC, pointing at an initial Th1 reaction. Although a role for Th2-like cells in the pathophysiology of allergic asthma has been convincingly demonstrated, IFN-γ also is implicated in allergic reactions. Whether the role of IFN-γ is proinflammatory (e.g. via stimulating TNF-α production by alveolar macrophages) and thus exacerbating allergic inflammation, or downregulatory (dampening Th2 reactions) still needs further investigation. It is interesting to note that recently published data in a mouse model showed that after airway challenge Th1 cells were efficiently recruited to the lungs and that Th2 cells needed the help of Th1 cells for successful recruitment [52]. It might well be that in humans the same mechanisms play a role and that after airway challenge an initial Th1 reaction mediates the subsequent Th2 cell recruitment and Th2 reactions.
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