**Objective:** T lymphocytes have a central regulatory role in the pathogenesis of asthma. The objective of this study was to characterize immunologically the activation stage of asthma and the functional profile of lymphocytes from induced sputum, with particular emphasis on γδ T cells.

**Methods:** Induced sputum was collected from 10 patients with acute exacerbation of asthma, and from healthy controls. The expression of activation markers on freshly isolated induced sputum lymphocytes and T-cell subsets was analyzed by double immunofluorescent staining and flow cytometry. Fas ligand (FasL) was determined by reverse transcriptase-polymerase chain reaction analysis. The phenotype of γδ T-cell subpopulations was tested by A13 and BB3 monoclonal antibodies. In this context, the functional profile of γδ T cells was tested in a chromium releasing test.

**Results:** A significantly decreased proportion of αβ T cells and an increased proportion of γδ T cells, CD56+ cells and CD8+ γδ T cells were found in asthma patients compared with healthy controls. In asthmatic patients, there is a significantly increased proportion of T cells expressing CD69 and CD25 antigen. After stimulation of γδ T cells, an increased expression of intracellular tumour necrosis factor-α, interleukin (IL)-4 and IL10 cytokines were found at higher levels than controls. Interferon-γ was observed at similar levels in asthma patients and healthy controls. Freshly isolated T-cell receptor (TCR) γδ+ cells exhibited an increased percentage of FasL in our patient group. FasL mRNA was detected in TCR γδ+ cells before and after IL2 stimulation. TCR γδ+ cells were cytotoxic against the K562 cell line. This natural killer activity was mediated by the A13-positive subpopulation.

**Conclusion:** The presence of cytokines producing γδ cells in induced sputum of asthmatic patients is consistent with regulatory activities. These cells display also cytotoxic function.

**Keywords:** Asthma, γδ T cells, Inflammation, Fas ligand, Cytokines

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**Introduction**

Asthma is characterized by a chronic mucosal inflammation of the airways, which underlies the two main clinical features of the disease: bronchial constriction and bronchial hyper-responsiveness. The mechanisms regulating airway function are complex and still poorly understood. In diseases such as asthma, involvement of immune-dependent mechanisms has been suggested in causing changes in airway responsiveness to bronchoconstrictors. The role of T lymphocytes in the pathogenesis of asthma has been emphasized in recent years. T lymphocytes are increased in asthma and are activated in the bronchial mucosa of the asthmatics. Through secretion of cytokines, CD4+ T lymphocytes actively participate to the inflammatory process found in asthma. γδ T cells can regulate airway function in an αβ T cell-independent manner, identifying them as important cells in pulmonary homeostasis. γδ T cells can produce T helper (Th)2 cytokines, which suggests their possible participation in the development of Th2 responses and, thus, in the onset of pulmonary allergic reactions. In a model of pulmonary allergic inflammation, genetically γδ T-cell-deficient mice exhibited low specific immunoglobulin (Ig)E and interleukin (IL)-5 release, and diminished T-cell infiltration compared with wild-type mice. These response were restored by...
administration of IL-4. Thus, γδ T cells are essential for inducing IL-4 secretion and Th2-mediated airways inflammation. Therefore, the precise role of γδ T lymphocytes in the human lung and lower airways is not yet known. γδ T cells home in the epithelial surface, and induced sputum should be representative for γδ T cells in inflamed bronchi. Numerous effector functions have been attributed to γδ cells, and we are only now uncovering their potentially important contributions to immune responses.

The objective of this investigation was to determine whether there is a shift in the proportion of γδ T cells and their function in induced sputum of patients with asthma as compared with healthy controls.

**Patients and methods**

**Patients**

Induced sputum samples were collected from 10 successive patients with allergic asthma. All were inpatients, admitted by the emergencies for an acute exacerbation of dyspnea (mean age, 38.5 years; range, 22–80 years). Patient asthma was classified according to National Heart, Lung and Blood Institute criteria as persistent mild-to-moderate asthma. The sample was obtained the day after their admission. A precise history of the patient was subsequently obtained and, after recovery functional respiratory tests, skin tests and serum IgE dosages were realized. Patients were excluded due to the following: concomitant infectious pneumonia, tuberculosis, interstitial lung diseases, bronchiectasis, lung cancer, and associated acute pathologies: cardiac, renal, liver, or neurological diseases. Ten healthy subjects (mean age, 28.7 years; range, 22–36 years), who had normal pulmonary radiographs and showed no clinical signs of respiratory diseases, acted as controls. Informed consent was obtained from all the patients. The study was approved by the local Ethics Committee.

**Sputum**

Sputum induction and processing were realized following the method recently reported. Briefly, after the inhalation of salbutamol (2 × 200 μg), subjects were asked to inhale sterile, pyrogen-free, hypertonic saline in increasing concentrations for a duration of 10 min. Most patients were able to expectorate an adequate sample (5 ml and more) within the first 10 min. The cell pellet was resuspended in phosphate-buffered saline (PBS) in a volume equal to the sputum plus dithiothreitol solution volume. Total cell counting was carried out in a haemocytometer and the cell concentration was adjusted to 1.0 × 10^6 cells/ml. Cytospins were prepared by adding 75 μl of cell suspension into Shandon II cytocentrifuge cups (Shandon Southern Instruments, UK) and spun for 8 min at 500 rpm.

**Monoclonal antibodies**

Monoclonal antibodies (MoAbs) against human CD69, CD40 ligand and γδ T-cell receptor (TCR) were all from Coulter Electronics, Luton, UK. Fluorescein isothiocyanate (FITC)-labelled and unlabelled MoAbs against human αβ TCR (clone WT31), FITC-labelled MoAbs against human γδ TCR, and PE-labelled MoAbs against human CD4 and CD8 were all purchased from Becton Dickinson (Oxford, UK). MoAbs against human CD25 and FITC-labelled MoAbs against human CD56 were purchased from Immunotech (Marseille, France). Phycoeritrin (PE)-labelled or FITC-labelled goat anti-mouse immunoglobulin, the F(ab)_2 fragments, were purchased from Dako (Marseille, France). MoAbs anti-β3 (Vδ2) and anti-α13 (Vδ1) were used to determine anti-γδ TCR subpopulations. Optimal dilutions for use in immunofluorescence procedures were established in preliminary experiments.

**Double immunofluorescence staining**

To analyse expression of activation markers on induced sputum lymphocytes and T-cell subsets, 2 × 10^6 freshly isolated cells in 100 μl of staining buffer (PBS with 1% foetal calf serum (FCS) and 0.02% NaN₃) were incubated with 5 μl of MoAbs against CD25 or CD40L, or 30 μl of MoAbs against CD4 and CD8, or 30 μl of MoAbs against CD69, for 45 min on ice. Then the cells were washed twice with 1 ml of staining buffer at 160 × g for 5 min at 4°C, and subsequently incubated with 100 μl of PE-labelled goat anti-mouse immunoglobulin, diluted 1:100, for 30 min on ice, followed by washes as earlier. Unbound goat antibody sites were blocked by incubating the cells with 100 μl of PBS containing 10% normal human serum for 10 min on ice before the cells were incubated with 10 μl of FITC-labelled MoAbs against αβ or γδ TCR or FITC-labelled control mouse IgG1. The cells were washed as before and fixed in 200 μl of 1% paraformaldehyde in staining buffer. For co-receptor measurements, 2 × 10^5 cells were incubated for 45 min on ice with 10 μl of PE-labelled MoAbs against CD4 or CD8 or PE-labelled control mouse IgG1, and 10 μl of FITC-labelled antibodies against CD56, αβ TCR, or γδ TCR or FITC-labelled control mouse IgG1.

**Cell culture**

Cells were washed in RPMI medium and either stained immediately or cultured before further analysis. Cells were cultured in 24-well plates at a concentration of 1 × 10^5 cells/ml in RPMI medium.
with 10% FCS for 12 h at 37°C, 5% CO₂. Cells were cultured in the presence of phytohaemagglutinin (PHA) (Sigma Chemical Co., St Louis, MO, USA) at a concentration of 5 µg/ml or in the presence of phorbol myristate (PMA) (Sigma Chemical Co.) at a concentration of 50 ng/ml in combination with 1 µmol/ml of ionomycin (Sigma Chemical Co.) at 10 µg/ml. To prevent secretion of the cytokines, brefeldin A at 10 µg/ml (Sigma Chemical Co.) was added during the last 4 h of the incubation. Maximum production of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) was observed after stimulation with PMA in combination with ionomycin, whereas PHA was more effective in inducing production of IL-4 and IL-10.

Intracellular cytokines

Stimulated and unstimulated cells were stained in parallel by incubating 2 × 10⁵ cells in 100 µl of staining buffer for 45 min on ice with 10 µl of MoAbs against αβ TCR, diluted 1:10, followed by 100 µl of FITC-labelled goat anti-mouse immunoglobulin, diluted 1:100, for 30 min on ice. Before the second staining, the cells were fixed in 200 µl of 4% paraformaldehyde in PBS for 10 min on ice and then unbound goat antibody sites were blocked by incubating the cells with 100 µl of PBS containing 5% normal human serum and 5% normal mouse serum for 10 min on ice. To permeabilize the cells, 0.1% saponin was included in the staining buffer used for subsequent incubations and washing. The cells were incubated with 10 µl of PE-labelled antibodies against cytokines (IL-4, IL-10, TNF-α or IFN-γ) or PE-labelled control mouse IgG1, diluted 1:20, for 45 min on ice, washed and then fixed in 400 µl of 1% paraformaldehyde in staining buffer.

Flow cytometric analysis

Fluorescent labelled cells were analysed by a flow cytometry (FACScan; Becton Dickinson). Before each set of data analysis, the machine was standardized for data reproducibility using fluorescent labelled beads (Coulter). Ten thousand cells were collected. Plots showing the size and granularity were used to select the lymphocyte population for further analysis. Cells stained with isotype-matched antibody or a second antibody alone were used as controls, with positive cells set at 0.5%. For intracellular cytokine analysis, unstimulated cells were used as negative controls.

Statistical analysis

Results are expressed as mean ± standard error of the mean for each study group. Significance was evaluated using Student’s t-test for non-paired samples with unequal variance, with p < 0.05 being regarded as significant.

Results

Expression of αβ TCR, γδ TCR, CD56, CD4 and CD8 on lymphocytes and T-cell subsets in induced sputum cells from patients with asthma and from healthy controls

The expression of αβ TCR, γδ TCR, CD56, CD4 and CD8 on lymphocytes was analysed in induced sputum from patients with acute exacerbation of asthma and from healthy controls (Fig. 1). A significant decrease in the proportion of lymphocytes expressing αβ TCR and a significant increase in the proportion of lymphocytes expressing γδ TCR or CD56 were found.
in asthma patients (59.4 ± 3.4%, 16.2 ± 1.3%, and 14.2 ± 2.7%, for αβ TCR, γδ TCR and CD56, respectively) compared with healthy controls (72.5 ± 2.6%, \( p < 0.05 \); 7.4 ± 0.5%, \( p < 0.005 \); and 9.2 ± 0.4%, \( p < 0.005 \), for αβ TCR, γδ TCR and CD56, respectively), as shown in Fig. 1.

The proportion of CD4+ lymphocytes was raised in induced sputum of patients with asthma (52 ± 7.8%, \( p < 0.01 \)) compared with controls (42 ± 3.2%). There was no difference in the percentage of CD8+ lymphocytes between asthma patients (36.5 ± 8.2%, \( p < 0.01 \)) and the control group (34 ± 10.4) in induced sputum.

When the T-cell subsets were analysed separately, a significantly raised proportion of γδ T cells from asthma patients (10.5 ± 1.2%) co-expressed CD8 compared with γδ T cells from healthy controls (4.9 ± 3.4%, \( p < 0.01 \)) (Fig. 1).

We analyzed the TCR γδ+ cells subsets (A13+ cells and BB3+ cells). The significant increase in induced sputum of TCR γδ+ cells in patients with asthma, compared with normal controls, was exclusively due to the increase of the A13+ subset (asthma patients: A13+ cells, 26.5 ± 4.2%; BB3+ cells, 2.8 ± 0.4% versus control: A13+ cells, 4.4 ± 1.2%; BB3+ cells, 5.2 ± 1.2%).

Expression of activation markers on T cells in induced sputum from patients with asthma

A significantly increased percentage of CD69+ lymphocytes was observed in the induced sputum from asthmatic patients (15.3 ± 2.4%) compared with the controls (6.5 ± 1.7%, \( p < 0.01 \)).

An increase was observed for the proportion of γδ T cells expressing CD25 in patients with asthma (12.3 ± 2.6%, \( p < 0.01 \)) compared with healthy controls (2.5 ± 1.8%).

Expression of intracellular cytokines on γδ T lymphocytes in induced sputum from patients with asthma

The expression of intracellular IL-4, IL-10, IFN-γ and TNF-α in the γδ T-cell subset was analysed in induced sputum from patients with asthma and from healthy controls (Fig. 2).

Before stimulation, γδ T cells did not express any of the cytokines analysed. However, after appropriate mitogen stimulation of γδ T cells, there was expression of intracellular TNF-α, IFN-γ, IL-4 and IL-10 cytokines (Fig. 2). A significantly increased proportion of γδ T cells from asthma patients could be induced to express TNF-α after PHA stimulation compared with controls (\( p < 0.05 \)) and a similar proportion of γδ T cells expressing IFN-γ was observed for the asthmatic patients and the controls. The frequency of γδ T cells expressing IL-4 or IL-10 was significantly increased in asthma patients when compared with induced sputum from healthy controls.

Phenotype and expression of Fas ligand mRNA in TCR γδ+ cells

Freshly isolated TCR γδ+ cells isolated from induced sputum exhibited a high percentage of Fas ligand (FasL) in patients with acute exacerbation of asthma (14.7 ± 4.5%, \( p < 0.01 \)), when compared with healthy controls (3.6 ± 2.8%).

When TCR γδ+ cells from asthma patients and healthy controls were IL-2-activated, they expressed FasL mRNA (Fig. 3). This finding support the view that TCR γδ+ cells from asthma patients have the ability to lyse Fas-expressing targets by producing FasL, without IL-2 activation.

Functional profile of TCR γδ+ cells in induced sputum

Isolated induced sputum TCR γδ+ cells from eight patients with asthma compared with five healthy controls...
controls were tested for their cytotoxic activity against K562 cell line at a ratio of 10:1 (E:T) (Fig. 4). Fresh TCR γδ+ gave low but detectable cytotoxic activity in asthma patients (8 ± 2.5%), which was < 5% in healthy controls. When TCR γδ+ cells were cultured with IL-2 for 3 days, cytotoxic activity appeared in healthy controls (7.5 ± 4%) and increased significantly in patients with asthma (23 ± 2.4%, p < 0.001).

Cytotoxic activity of IL-2-activated TCR γδ+ cells was abolished when anti-FasL was added. Cytotoxic activity was lower than 5% both in asthmatic patients and in healthy controls. When IL-2-activated TCR γδ+ cells were incubated with anti-BB3 monoclonal antibody, natural killer (NK) activity decreased, compared with IL-2-activated TCR γδ+ cells alone in asthmatic patients, but did not reach statistical significance. Anti-BB3 monoclonal antibody did not reduce the lytic activity of IL-2-activated TCR γδ+ cells in healthy controls.

However, the addition of anti-A13 monoclonal antibody to activated TCR γδ+ cells induced the abolition of cytotoxic activity both in asthmatics and in healthy controls, which was lower than 5%. Lytic activity in healthy controls was identical to TCR γδ+ cells against K562 target cells. TCR γδ+ cells were purified from induced sputum from asthmatic patients and from healthy controls. The values represent the mean of triplicate assays at a 10:1 (E:T) ratio. Cytotoxic activity of asthmatics was significantly different from healthy controls (p < 0.001); N.S., not significantly different.

Discussion

We have observed a reduced number of αβ TCR and an increased number of γδ T lymphocytes in induced sputum from patients with acute asthma exacerbation. Studies revealed that the expression of αβ TCR by alveolar T cells was decreased as compared with autologous blood T cells, in a wide variety of pathological and physiological conditions of the lung. The expression of CD3 antigen by alveolar T cells is also likewise reduced in health and disease. We analysed the activation status, cytokine production and cytotoxic function of γδ T cells in induced sputum compared with those found in healthy controls.

The proportion of γδ T cells in BAL from patients with asthma was expanded as has been shown by Molfino et al. Asthma patients had a raised percentage of CD8+ T cells among the cells expressing the γδ TCR. This suggests a role for CD8+ T cells in asthma disease, with the γδ T cells being more prominent in asthma-induced sputum. It has been reported that, in allergic asthmatic subjects, T-cell expansion preferentially emerges in the lung CD8+ T-cell subset in BAL, with direct attention to the role of CD8 T lymphocytes in the pathogenesis of asthma. Furthermore, we reported a significant increase in the proportion of CD56+ cells in sputum from asthma patients compared with controls. CD56 is one of the markers of NK cells. The involvement of cytotoxicity in asthma pathways has been suggested, by the finding of induced sputum γδ T cells expressing increased percentages of CD56 and CD8 markers.

Studies that aimed at determining expansion of the γδ T-cell population in the human lung are scarce. Groh et al. did not detect γδ T cells in foetal and adult lung using MoAbs. Walker et al. found no difference in the number of γδ T cells between asthmatic and control subjects, which is in accordance with the findings of Fajac et al. in the normal bronchi mucosa and in alveolar parenchyma. Conversely, Spinozzi et al. reported an increased number of γδ T cells in asthmatic patients, which is in accordance with our finding. Agostini et al. recently reported that Vδ2+ T cells comprise approximately 70% of the γδ T cells in the normal lung. It has been reported that the number of γδ T lymphocytes in bronchi biopsies of asthmatics was not different from controls. These different results might be due to the diversity of the patients studied, since the airway inflammation may vary with the severity of the disease. An increased number of γδ T cells were found in the nasal mucosa of patients with perennial allergic rhinitis. γδ T cells express increased percentages of CD56 and CD8 markers. Studies that aimed at determining expansion of the γδ T-cell population were found nodifference in the number of γδ T cells expressing increased percentages of CD56 in the proximal bronchi. Yet in none of the bibliographic studies was the functional status of such cells investigated.
T-cell subset showed no increase in CD25 expression compared with controls. This observation indicates that the γδ T cells in sputum from asthma patients had already been activated. Whether the activation occurs as a result of the disease or whether the activated γδ T cells play a direct role in the pathogenesis needs to be elucidated.

It was of interest to investigate the expression of another activation molecule on the T cells that could interact with the various cells and epithelial proteins in the bronchial mucosa. Therefore, we analysed the expression of CD69. Our study found a raised percentage of γδ T cells expressing CD69 in asthma patients compared with controls. CD69, an early activation marker, is involved in lymphocyte proliferation, and functions as a signal-transmitting receptor in T and B lymphocytes, NK cells and platelets. The ligand for CD69 is still unknown.

Unbalanced expression of Th1 or Th2 cytokines has been associated with asthma and may play a role in human inflammatory diseases. Previous studies analysing cytokines in asthma have not been directed against individual cells, but have analysed cytokines in serum or in supernatants from bulk populations of peripheral blood, BAL, or induced sputum. The development of a method of detecting intracellular cytokines by flow cytometry meant that it was possible to study the contribution of individual cells in a heterogeneous cell population, to specific cytokine production. Asthma patients group had an increased sputum proportion of γδ T cells secreting TNF-α when compared with healthy controls. TNF-α is another cytokine involved in inflammation that plays a major role in induction of adhesion molecules and inflammatory cytokine production. It is mainly produced by activated monocytes and macrophages. Our results show, however, that in asthma patients a substantial proportion of γδ T cells is able to participate in TNF-α production. The observation of an increased proportion of γδ T cells that secrete TNF-α provides an interesting finding in asthma.

Our results reported increased IL-4 and IL-10 intracellular cytokines expression. This corroborates the recent inflammatory response in induced sputum in patients with acute exacerbation of asthma. Thus, Th2 cytokine-producing cells of the γδ TCR type are present in asthmatic sputum patients. These Th2 cytokine-producing cells may be upregulated as part of the general inflammatory response in the asthmatic bronchi. Th1 cytokines were at a normal level compared with controls.

Our findings reported a significant increase of γδ T cells associated with a non-specific lysis against the K562 cell line. One of the mechanisms of cell-mediated cytotoxicity established is the Fas/FasL system. Recent studies reported that NK cells in the inflammatory sites operate through the Fas/FasL interaction, and that FasL was expressed in the inflammatory sites. Interestingly mononuclear cells co-expressed both Fas and FasL, suggesting that Fas/FasL interactions may function in not only a paracrine way, but also an autocrine way. This may be relevant to the abnormal immune response in diseases.

The earliest contact between antigen and the innate immune system is thought to direct the subsequent antigen-specific T-cell response. It has been hypothesized that cells of the innate immune system, such as NK cells, NK1.1-positive T cells, and γδ T cells, may regulate the development of allergic airway disease. It has been demonstrated that depletion of NK cells before immunization inhibits pulmonary eosinophil and CD3-positive T-cell infiltration as well as increased levels of IL-4, IL-5, and IL-12 in bronchoalveolar lavage fluid in a murine model of allergic asthma.

We have reported that cells from induced sputum from patients with asthma exhibited an increased CD56+ cells within the γδ T-cell population, which in the majority is composed of A13+ cells. NK activity in sputum from patients with asthma was mediated by A13+ cells. The propensity of A13+ γδ T cells to accumulate in the airway of patients with asthma could be due to the better migratory capacities of these cells, as reported in other investigations. An increase of A13+ cells has been described in other inflammatory sites; it represents the major fraction of intraepithelial γδ T in the synovial fluid in rheumatoid arthritis. It was also reported in cerebrospinal fluid, and in bronchoalveolar lavage of patients with active Behet’s disease. The present results report that A13+ cells within the T γδ population acted as cells mediating NK activity, in sputum from patients with asthma.

The present study revealed that the expression levels of Fasl mRNA was high. Similar results were found in the liver, and the authors reported that this situation is compatible with the conclusion that the high level of autologous killing in TCRint cells is mediated by Fasl/Fas molecules, but not perforin. The Fasl-mediated cytotoxicity may be acquired at more mature stages of NK-cell maturation.

An interesting recent study from Zuany-Amorin et al. reported that the subset of γδ T cells directly involved in IL-4 production will represent an important step for understanding and modulating the development of Th2 responses, particularly in the context of allergic diseases such as bronchial asthma. A subset of γδ T cells, positive for A13 monoclonal antibody, in sputum from asthmatics acted as NK cells through the Fasl interaction. The knowledge of γδ T-cell function and specificity appears to be an important component of asthma. γδ T cells have proven biological activity in the lung. We therefore hypothesize that γδ T-cell activity elicited by infection early in life accelerates the bias in γδ T cells away for Th2.
References


