Inflammatory response in induced sputum mononuclear cells from patients with acute exacerbation of asthma

A. Hamzaoui1,6CA, M. Ben Brahim2, A. Zhioua3, Kh. Ayed4 and K. Hamzaoui3

1A. Mami Hospital, Department of Pediatric Respiratory Diseases, Pavillon B, Ariana;
2Department of Physiology, Medicine University of Tunis, Pavillon 6; 3Immuno-Histology Laboratory, Medicine University of Tunis, Pavillon 6. Research Unit of the ‘Ministère de l’Enseignement Supérieur et de la Recherche Scientifique’, Code: 99/UR/08–40;
4Department of Immunopathology, Ch. Nicole Hospital.

EXAMINATION of sputum provides a direct method to investigate airway inflammation non-invasively in particular Th1 (IL–2, IFN–γ) and Th2 (IL–4, IL–10) cytokine production.

IL–2, IL–4, IL–10 and IFN–γ cytokine were studied in induced sputum mononuclear cells of asthmatic patients.

Sputum induction was performed on 10 patients and 10 normal controls. Basal and mitogen-stimulated cytokine production was determined in induced sputum T-cell culture. Supernatants were collected and assayed not only with specific ELISA but also with polymerase chain reaction (PCR) techniques.

Data showed a significantly higher production of IL–10 by both the ELISA and the RT-PCR techniques in asthmatic patients compared with sputum mononuclear cells from healthy controls. IL–4 production was detected at a low level using the ELISA method in asthmatic patients. The RT-PCR analysis detected a significantly IL–4-mRNA expression in all asthmatic patients, compared with controls. Results of IL–10 and IL–4 mRNA expression were reproducible. We did not find any alteration in the expression of the type 1 derived cytokines (IL–2 and IFN–γ) in asthmatic patients or in healthy controls.

Our study showed a tendency of induced sputum mononuclear cells to express a Th2-like cytokine pattern in acute exacerbation of asthmatic patients, where IL–10 and IL–4 are synthesized in larger amounts. The combination of sputum induction as a non-invasive tool to explore the lung and the identification of disease-associated cytokine expression and of specific cytokine mRNA should help elucidate mechanisms of the immunologically mediated inflammatory responses in asthma.

Key words: Asthma, Th1 and Th2, Inflammation

Introduction

Chronic mucosal inflammation plays an important role in the pathogenesis of asthma. Several airway cells such as mast cells, macrophages, eosinophils, endothelial or epithelial cells, and lymphocytes have been postulated to participate in this inflammatory response. Cytokines and mediators produced by these airway inflammatory cells could provide a local mechanism to induce, amplify or modulate the ongoing inflammation.

T lymphocytes play a vital role in the regulation and coordination of immune responses. Activation of T-cells leading to secretion of cytokines may be involved in the pathogenesis of asthma. Activated T-cells can be recognized by the expression of particular surface markers, such as the interleukin–2 (IL–2) receptor (CD25), the human leukocyte antigen HLA–DR and the very late activation antigen VLA–1.1,2 T-cells may orchestrate inflammatory responses to inhaled allergen and other stimuli in asthma by production of several cytokines.

T-cell derived cytokines IL–4, IL–5 and interferon-γ (IFN–γ) are intimately involved in the regulation of IgE production which is fundamental to the pathogenesis of allergic asthma.3–5 Furthermore, several studies have suggested a role for T-cell derived cytokines in the initiation of eosinophil inflammation.6,7 Interleukin–5 (IL5), granulocyte-macrophage colony stimulating factor (GM-CSF) and IL–3 have pronounced effects on eosinophil tissue localisation, prolongation of survival, maturation and activation. The description of a subset of murine T helper cell clones (termed TH2) that produces IL–5, IL–3 and GM-CSF together with IL–4 or IL–2 raised the possibility that activation of a Th2-like subset of CD4+ T-lymphocytes might contribute to eosinophil infiltration.8,9 Indeed, in atopic individuals, allergen specific T-cell clones produce a Th2 like pattern of cytokines, whereas other antigen specific T-cell clones from the same
Patients have a Th1-like pattern of cytokine production including IL–2 and IFN–γ but no IL–4 or IL–5.\textsuperscript{10}

Induced sputum provides a direct method to investigate airway inflammation non-invasively. When sputum cannot be produced spontaneously, it usually can be successfully induced by inhalation of an aerosol of hypertonic saline. Sputum cell counts can be reproducible, reliable and valid. New methods of examination using cytopsins of dithiothreitol-treated sputum improve reliability and reduce the time of examination.\textsuperscript{11} The cells can also be stained immunohistochemically for activation markers and the cell supernatant can be used to investigate fluid phase constituents. The use of sputum allows us to study inflammatory mononuclear cells and programmed cell death in asthma.\textsuperscript{11,12} Its application to the study of Th1 and Th2 in asthma disease should improve understanding of the pathogenesis.

The present paper describes the role of induced sputum T-lymphocytes in the inflammatory response of different forms of asthma patients, focusing mainly on the analysis of the Th1 (IL–2 and IFN–γ) and Th2 (IL–4 and IL–10) lymphocyte, using the ELISA method and mRNA analyses. The role of Th1 and Th2 cells in mediating the selective infiltration of inflammatory cells will be discussed.

Patients and methods

Patients

Induced sputum samples were collected from 10 successive patients with asthma. All were inpatients, admitted for an acute wheezing exacerbation of dyspnoea, usually as an emergency. The sample was obtained the day after admission. A precise history of the patient was subsequently obtained, and after recovery functional respiratory tests and eventually skin tests were carried out. Patients with the following diagnoses were excluded: concomitant infectious pneumonia, tuberculosis, interstitial lung diseases, bronchiectasis, lung cancer, and associated acute pathologies: cardiac, renal, liver, or neurological diseases. The induced sputum from 10 healthy subjects (mean age 28.7 yrs; range 22–36), who had normal pulmonary radiographs and showed no clinical signs of respiratory disease, acted as controls. Informed consent was obtained from all patients. The study was approved by the local Ethics Committee.

Serum IgE levels were elevated in most patients (range 110–940 U/ml). In healthy controls the serum IgE levels were < 100 U/ml.

Sputum induction

After the inhalation of salbutamol (2 × 200 mg), subjects were asked to inhale sterile, pyrogen-free, hypertonic saline in increasing concentrations for a duration of 10 min. The hypertonic saline was nebulized via an ultrasonic nebulizer. Subjects were encouraged to cough throughout the procedure. Most patients were able to expectorate an adequate sample (7 ml and more) within the first 10 min.

Sputum processing

In order to reduce salivary contamination, plugs were selected and transferred into an Eppendorf tube. A freshly prepared 10% solution of dithiothreitol (1 ml) (DTT) was added. The tube was vortex mixed and the sputum was incubated for 5 min at room temperature, filtered through 52 μm nylon gauze to remove debris and mucus, and subsequently centrifuged at 450 × g for 10 min. The cell pellet was resuspended in phosphate-buffered saline (PBS) in a volume equal to the sputum plus DTT 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 ml cell suspension into Shandon II cytocentrifuge cups (Shandon Southern Instruments) and spun for 8 min at 500 rpm. Two slides were stained with Wright-Giemsa for an overall differential cell count of leukocytes, bronchial epithelial cells and squamous cells. Slides were coded and counted blind by two investigators. None of the cytopsins contained > 5% squamous epithelial cells. For cell differentiation, 400 nucleated cells per slide were counted and expressed as a percentage of intact round nucleated cells, excluding the squamous epithelial cells.

T-cell culture

Lymphocytes were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and washed twice with PBS at 4°C, as we have recently reported.\textsuperscript{5} The cell pellet expressed more than 80% CD3-positive cells as determined by anti-CD3 monoclonal antibody (Becton Dickinson).

T lymphocytes were diluted in RPMI 1640, and 1 ml aliquots were deposited in 2 ml wells of a 24-well plate (Falcon). Basal and mitogen stimulated [phytohaemagglutinin (PHA); Sigma, St Louis, M final concentration of 5 μg/ml]; and lipopolysaccharide (LPS, from Salmonella enteritidis; Sigma, final concentration 25 μg/ml)] conditions were evaluated. The plates were incubated at 37°C in a CO2 atmosphere. After culture periods of 24 h and 48 h the contents of the wells were harvested, spun at 2000 × g for 2 min and the supernatants were collected and stored frozen at −80°C until use. Sputum induced-cells were used for mRNA analysis.
Cytokine assays

Culture supernatants were collected after 24 h to measure the IL-4 and IFN-γ contents and after 48 h to evaluate IL-10.

Supernatant cytokine concentrations were determined by ELISA (Immunotech, Bio-Care, Tunis). The positivity thresholds were 10 pg/ml for IL-2, 0.08 U/ml for IFN-γ, 1.5 pg/ml for IL-4 and 3 pg/ml for IL-10 (Immunotech, Bio-Care, Tunis).

RNA extraction

After incubation for an appropriate time, cells were isolated by brief centrifugation and then stored in 1 ml Trizol (Gibco, at -80°C until further processing. RNA extraction was performed using phenol chloroform extraction and ethanol precipitation following the manufacturer’s instructions. The RNA content of the solution was quantified using the optical density (OD) at 260 nm measured on a SECOMAN spectrophotometer and the RNA aliquots were stored at -80°C until analysed. The ratio 260/280 nm was always more than 1:8.

Reverse transcription and PCR

cDNA was synthesized from oligo-dT-primed RNA by reverse transcription (RT) with M-MLV superscript reverse transcriptase (Gibco). The total RNA mixture was incubated with 200 UM-MLV reverse transcriptase, 20 U RNasin, 0.5 mM dNTPs, 25 mg/ml primer dT, 10 mM dithiothreitol, 50 mM Tris-HCl pH 8.3, 75 mM KCl and 3 mM MgCl₂, in a final volume of 20 ml for 50 min at 42°C. The final cDNA product was stored at -20°C for subsequent cDNA amplification by PCR.

Reaction mixtures for PCR of β-actin and cytokines contained 2 ml sample, 0.5 mM dNTP, 0.5 U Taq polymerase, 1 mg of each primer, 50 mM Tris-HCl, 3 mM MgCl₂, 75 mM KCl 0.01% gelatin in a final volume of 100 ml. PCR was performed on a thermal cycler (Personal cycler Biometra, Germany). Reaction times for β-actin were 94°C 1 min, 65°C 1 min, and 72°C 1 min for 28 cycles, followed by 10 min extension at 72°C. Reaction times for cytokine PCR were as follows: for IL-4 and IL-10, 94°C 1 min, 65°C 1 min and 72°C 1 min; for IL-2 and IFN-γ, 94°C 1 min, 55°C 1 min and 72°C 1 min for 38–40 cycles followed by 10 min extension at 72°C for all cytokines.

Primer sequences for the internal control, β-actin, were 5'-TAC ATG GCT GGG GTG TTG AA-3' for the downstream primer, and 5'-AAC AGA GGC ATC CTC ACC CT-3' for the upstream primer. Primer sequences for cytokines were as follow: for IL-4, 5'-CTT CCC CCT CTG TTC TTC CT-3' for the 59 primer, and 5'-TTC CTG TCG AGC CTG TTT AG-3' for the 39 primer; for IL-10, 5'-ATG CCC CAA GCT GAG AAC CAA GAC CCA-3' for the 59 primer, and 5'-TCT CAA GGG GCT GGG TCA GCT ATC CCA-3' for the 39 primer; for IL-2, 5'-AAT CTC TTG CTT GCA TTG CAC TA-3', for the 59 primer, and 5'-TTG CTT CAG ATT AAG TCC CTG GTG-3' for the 5 primer; for IFN-γ, 5'-AGT TAT ATC TTG GTT TTT CA-3' for the 59 primer, and 5'-ACC GAA TAA TTA GTC AGC TT-3' for the 39 primer. The primer sequences were chosen in two different exons. These primer sequences were confirmed to specifically amplify the corresponding cytokine cDNA by Southern blot analysis using a fluorescein-labelled internal probe. cDNA products were visualized by gel electrophoresis in 20% agarose after ethidium bromide staining. A cDNA positive control, a negative control and a DNA ladder were run with all PCR reactions. Amplifications resulted in 200–400 bp product, as determined by electrophoresis on 2% TEA agarose gel containing bromide ethidium.

Dot-blot analysis

PCR products were denatured and vacuum dot blotted onto Hybond-N membrane (Amersham, France). Specific probes were 32P-end labelled with fluorescein-11-dUTP using the ECL 32P-oligolabelling reagents (RPN 2130; Amersham). The sequences of internal probes were as follows: for β-actin, 5'-CCA ACT GGG AGC ACA TGG AGA AAA – 3' for IL-2, 5'-GGC CAC AGA ACA TGG AGA AAA - 3' for IL-4, 5'-CTC GGT GCT CAG AGT CTT CTG CTC T-3' for IL-10, 5'-CAG GTG AAG AAT GCC TTT AAT AAG CTCCAA CAG AAA GGC ATC TAC AAA GCC ATG AGT GACTTT GAC ATC-3' for IFN-γ, 5'-ATT TGG CTC TGC ATT TTT CT GT -3'. Following hybridization to the dot blots and incubation with anti-fluorescein-horseradish peroxidase (HRP) conjugate, detection of the bound peroxidase was performed using hydrogen peroxide and luminol. The amount of each spot was determined by densitometry analysis. All of the cytokine PCR products were analysed comparatively to the amount of β-actin detected in the same mRNA sample. All samples were checked in the same test run for each cytokine analysed. Separate cycle course experiments confirmed linearity of amplification for β-actin and cytokine cDNA over 20–35 cycles and 30–45 cycles, respectively. For each PCR, linearity of amplification relative to cDNA dilutions was over 1/5–1/20 for IL-4 and IFN-γ, 1/5–1/40 for IL-2, 1/5–1/80 for IL-10 and β-actin.

Statistical analysis

The distributions of cytokine concentrations are reported as their median values, first and third
quartiles. Comparisons between two sample populations were made with the non-parametric Mann-Whitney U-test, with the level of significance set at 0.05. Tests were performed with the statistical software STATISTICA. Correlations were determined by linear regression and Spearman’s rank correlation.

Results

Cytokine production

**Th1 cytokines**

In the absence of exogenous stimuli, the spontaneous production of IL–2 was at normal values in asthmatic patients and healthy controls (Table 1). After 24 h in the presence of LPS + PHA, IL–2 levels were higher in supernatants from both populations, with asthma patients’ IL–2 concentrations being significantly higher than those of healthy controls (Table 1).

As for IL–2, basal IFN–γ production was comparable for both populations. LPS + PHA stimulation increased these concentrations, without differences between patients and healthy controls (Table 1).

**Th2 cytokines**

Basal IL–10 production was significantly higher in asthmatic patients than healthy controls. Significantly higher amounts of IL–10 were also detected in samples from asthmatic patients compared with controls under culture conditions (Table 1).

Spontaneous IL–4 production differed between asthmatic patients and healthy controls (Table 1), but significance was not notable. Mitogen-activated sputum T-cells from both populations generated enhanced IL–4 concentrations, and low statistical difference between groups was observed (Table 1). All asthmatic patients stimulated sputum T-cells produced high amounts of IL–4.

Expression of cytokine mRNA in sputum mononuclear cells from asthmatic patients and healthy controls

Because the spontaneous secretion of IL–4 cytokine in sputum mononuclear cells is very low by ELISA, we studied the expression of cytokine genes in sputum cells with a semi-quantitative PCR technique. Cytokine-specific cDNA were normalized to the intensity of the β-actin product as a standard marker.

Data revealed spontaneous IL–4 mRNA expression in all asthmatic patients, whereas normal donors in most cases did not show specific signals (p < 0.0001) (Fig. 3). Representative examples of autoradiographs showing signals from sputum cells of asthmatics and normal donors after PCR amplification and specific hybridization are shown in Fig. 1.

The technique of RTPCR amplification used in this study remained semi-quantitative; IL–10 mRNA expression appeared to be significantly enhanced in asthmatic patients compared with healthy controls (p < 0.001) (Fig. 3).

Table 1.

| Cytokine | Healthy controls | Asthmatic patients |
|----------|------------------|--------------------|
|          | Basal            | LPS+PHA            | Basal            | LPS+PHA            |
| IL–2     | 14.79 (10.58–22.75) | 16.92 (12.42–68.32) | 11.62 (10.39–36.93) | 43.6* (12.43–125.30) |
| IFN–γ    | 0.163 (0.092–0.746) | 17.824 (8.53–42.29) | 0.172 (0.128–4.96) | 22.183 (3.56–98.32) |
| IL–10    | 22.7 (2.36–75.46) | 1407 (870–1913) | 64.3* (37.41–131.6) | 3058.6** (214.7–6592) |
| IL–4     | 1.912 (1.66–2.94) | 34.25 (2.63–98.4) | 2.21 (1.97–7.94) | 67.35* (32.6–110.9) |

Results are given as medians and ranges, and expressed in pg/10⁶ sputum T-cells. Results for IFN–γ are expressed in U/10⁶ sputum T-cells. *Statistical significance comparing asthmatic patients versus healthy controls: p < 0.01 is considered significant.
Data shown in Fig. 2 indicate that the expression of mRNA for IFN-γ and IL-2 in asthmatic patients did not differ at all from healthy controls ($p = 0.42$ and $p = 0.36$, respectively for IFN-γ and IL-2).

Using RT-PCR techniques, our results show a clear enhancement of spontaneous IL-10 mRNA expression in asthmatic patients over healthy controls. In addition, mRNA expression of the Th1 type derived cytokines (IL-2 and IFN-γ) in the same experimental conditions did not differ between sputum induction cells of healthy controls and asthmatic patients.

**Correlations**

There is no correlation between the amount of IL-4 mRNA expression, IL-10 mRNA expression, IFN-γ mRNA expression and serum IgE levels. An inverse correlation is observed between IL-2 mRNA expression and serum IgE levels ($r = -0.72$, $p = 0.007$).

**Discussion**

Thelper lymphocytes type 2 (Th2) are considered to play an essential role in orchestrating the inflammatory response associated with asthma. Th2 lymphocytes express cytokine-associated cell differentiation, eosinophil differentiation, mucus secretion, airway smooth muscle hyperreactivity, and mast cell hyperplasia. The aim of the present study was to determine the cytokine pattern during acute exacerbation of asthma, using the non-invasive method of induced sputum combined with the polymerase chain reaction. Our study focused on the cytokine production in culture supernatant sputum-T cells, of IL-2, IFN-γ, IL-10 and IL-4.

Total cell count was elevated in patients with asthma compared with healthy controls, as we have recently reported. Sputum cells of patients with asthma expressed high percentages of eosinophils and lymphocytes. The lymphocytes were in the majority CD3-positive (T lymphocytes). The leukocyte count and phenotype of the lymphocytes showed
that sputum traduced an inflammatory state in patients with asthma. Our results obtained from analysing induced sputum in asthma patients reflect the inflammatory status of the bronchi.\textsuperscript{12} Th2 T-lymphocytes, characterized by the ability to produce cytokines such as IL–4 and IL–10, have been largely implicated in the pathogenesis of allergic inflammation. In support of these results, studies on sputum cells from asthmatic individuals show high levels of IL–4 and IL–10 in culture supernatants of mononuclear cells. These results were corroborated with the expression of mRNA expression of Th2-associated cytokines IL–4 and IL–10. Interleukin–10 secretion by monocytes and lymphocytes was responsible for the heightened immunoglobulin production.\textsuperscript{13} IL–10 production was also found to be enhanced in disorders characterized by prominent B lymphocyte hyperactivity, which results in increased production of immunoglobulin.\textsuperscript{14} IL–10 was originally characterized as a factor generated by Th2 cells that inhibits cytokine synthesis by Th1 cells.\textsuperscript{15} However, several other cell types have been further identified as a source of IL–10, including CD4\textsuperscript{+} and CD8\textsuperscript{+} T-lymphocytes, natural killer (NK) cells, monocytes/macrophages, B cells, mast cells, eosinophils, and bronchial epithelial cells. The concept that IL–10 acts as an anti-inflammatory molecule emerged from its capacity to down-regulate the synthesis of a broad spectrum of proinflammatory cytokines produced by monocytes/macrophages and neutrophils.\textsuperscript{16} The immunosuppressive properties of IL–10 are mainly related to inhibition of antigen-presenting cell (APC) functions. Indeed, IL–10 downregulates the expression of class II major histocompatibility complex (MHC) and costimulatory molecules (e.g. intercellular adhesion molecule–1 (ICAM–1) on the membrane of monocytes/macrophages and inhibits their synthesis of inflammatory cytokines (IL–1, IL–6, IL–8 and tumour necrosis factor-\textalpha) (TNF-\textalpha). The latter effect was also documented on alveolar macrophages.\textsuperscript{17} The inhibition of the local synthesis of TNF-\textalpha secretion and the down-regulation of pulmonary vascular ICAM-1 expression were shown to be involved in the protective effects of IL–10 against immunoglobulin G (IgG) immune complex-induced lung injury.

The increased expression of IL–4 mRNA in asthmatic-induced sputum cells that we observed is consistent with the findings obtained by Brown \textit{et al.}\textsuperscript{18} in peripheral blood cells. Cytokine requirement for primary generation of the Th2 response in the lung was dependent on IL–4.\textsuperscript{20} The ELISA method that we used displayed only 2.21 pg/ml in culture supernatant sputum mononuclear cells; one could suggest that sputum mononuclear cells needed more days stimulation and the sensitivity of the method remained insufficient for the detection of cytokine production. The lack of spontaneous IL–4 secretion reported by other authors\textsuperscript{19} in peripheral circulation indicated that this method is not representative of an inflammatory process localised in the lung. Cells obtained by induced sputum were more representative of the \textit{in situ} inflammation in asthmatic patients, and use of the mRNA-PCR method was important. Our study ruled out the possibility of a lack of correlation between gene transcription and active synthesis of the relevant protein, since the increased spontaneous mRNA expression for IL–4 as detected by PCR is compatible with the secretion of this cytokine.

IFN–\textgamma protein in sputum culture supernatant cells from asthmatic patients was comparable to healthy controls, after mitogenic stimulation. This result was consistent with other data.\textsuperscript{21} Our results demonstrated a tendency of T cells from a group of asthmatic patients to express a Th2 pattern with a high level of expression of IL–4 and IL–10 cytokines. However, the classified Th2 cytokines reported in this paper can also be produced by Th1 cells such as IL–10.\textsuperscript{22} The study of IL–4 and IL–10 production in T cells from induced sputum could be the description of localised inflammatory process. A similar study should be corroborated with local B cell-IgE production. Such investigation should give information about the local T and B cell interaction.

Given that IgE production is considered to involve the production of Th2 cytokines, we were surprised at the lack of correlation of IL–4 mRNA, IL–10 mRNA and serum IgE levels. Other groups have shown that high and low IgE responders are under the control of IL–4 expression, and that IL–4 production correlates with serum IgE.\textsuperscript{23,24} The lack of correlation between serum IgE level and IL–10 mRNA expression could be explained by the fact that IL–10 induces B cell proliferation and production of isotypes IgM, IgG and IgA, with no effect on IgE synthesis.\textsuperscript{25} In these conditions, the IL–10/IL–2 and IL–10/IFN–\textgamma ratios were examined (data not given). Enhanced ratios attributable to high IL–10 production were found for all asthmatic patients, and might trigger the B lymphocyte hyperactivity. Low ratios reflecting elevated IL–10 and IL–2 or IFN–\textgamma levels could be taken as marker of inflammation and tissue injury. Such interpretation could help us to understand the modulation of Th1 and Th2 cytokines.

Matzinger\textsuperscript{26} proposed the hypothesis that antigenic stimuli presented to a mucosal barrier interface direct the immune response towards the Th2 phenotype and that the normal response is effectively to inhibit these processes through a variety of pathways, including tolerance, anergy and apoptosis. Th2 cytokine profiles were significantly more resistant to activation-induced apoptosis and bcl–2 expression was reported to be higher in Th2 cells than in those producing Th1 cytokines.\textsuperscript{24} Our recent results concerning apoptosis...
in acute exacerbation of asthma showed an increased bcl–2 expression in induced sputum mononuclear cells and the percentage of apoptosis was decreased.\textsuperscript{1,2} Resistance to activation-induced apoptosis may explain the expansion of cells producing Th2 cytokines.\textsuperscript{27}

Our results provided indirect evidence \textit{in vivo} for increased mRNA expression for IL–4 and IL–10. The Th1 and Th2 cytokine profile may be significant in the analysis of clinical allergic responses in asthmatic patients. An improved understanding of the cellular and molecular mechanisms resulting in Th2 responses has suggested potential therapeutic strategies to inhibit Th2 responses associated with asthma. Further studies will need to determine whether alternative approaches to inhibit Th2 cells are practical in human subjects. The Th2-induced sputum cell appears to be a useful, non-invasive tool to explore the inflammation of asthma.

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