Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12082

# Immune response to *Streptococcus pneumoniae* in asthma patients: comparison between stable situation and exacerbation

C. Otero,* R. D. Paz,† N. Galassi,* L. Bezrodnik,† M. R. Finiasz*‡ and S. Fink*‡

*Immune Response to Human Infections Laboratory, IMEX-CONICET-Academia Nacional de Medicina, and †Immunology Unit, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina*

Accepted for publication 23 January 2013
Correspondence: S. Fink, Immune Response to Human Infections Laboratory, IMEX-CONICET-Academia Nacional de Medicina, Pacheco de Melo 3081, C1425 AUM Buenos Aires, Argentina.
E-mail: sfink@hematologia.anm.edu.ar; finksusana@gmail.com

‡These two authors contributed equally to this paper.

## Summary

**In Argentina, more than 3 million people suffer from asthma, with numbers rising. When asthma patients acquire viral infections which, in turn, trigger the asthmatic response, they may develop subsequent bacterial infections, mainly by *Streptococcus (S.) pneumoniae*. This encapsulated Gram$^+$ bacterium has been considered historically a T cell-independent antigen. Nevertheless, several papers describe the role of T cells in the immune response to *S. pneumoniae*. We evaluated the response to *S. pneumoniae* and compared it to the response to *Mycobacterium (M.) tuberculosis*, a different type of bacterium that requires a T helper type 1 (Th1) response, in cells from atopic asthmatic children, to compare parameters for the same individual under exacerbation and in a stable situation whenever possible. We studied asthma patients and a control group of age-matched children, evaluating cell populations, activation markers and cytokine production by flow cytometry, and cytokine concentration in serum and cell culture supernatants by enzyme-linked immunosorbent assay (ELISA). No differences were observed in γδ T cells for the same patient in either situation, and a tendency to lower percentages of CD4$^+$CD25$^{hi}$ T cells was observed under stability.**

**A significantly lower production of tumour necrosis factor (TNF)-α and a significantly higher production of interleukin (IL)-5 was observed in asthma patients compared to healthy individuals, but no differences could be observed for IL-4, IL-13 or IL-10. A greater early activation response against *M. tuberculosis*, compared to *S. pneumoniae*, was observed in the asthmatic patients' cells. This may contribute to explaining why these patients frequently acquire infections caused by the latter bacterium and not the former.**

**Keywords:** activation, asthma, atopy, infections, T cells

## Introduction

In Argentina more than 3 million people suffer from asthma, and the number is rising. This is also true for many other countries [1]. Asthma is a complex and heterogeneous disease characterized by chronic airway inflammation and airway hyperreactivity. This inflammatory pathology is characterized by oedema and mucosal hyperaemia with infiltration by mast cells, an overabundance of eosinophils and activated T helper lymphocytes [2,3]. These inflammatory cells release mediators that trigger bronchoconstriction, mucus secretion and airway remodelling. The inflammatory mediators that drive this process include cytokines, chemokines, growth factors, lipid mediators, immunoglobulins and histamine. In asthma, inflammation is generally directed by T helper type 2 (Th2) cytokines, which can act by positive feedback mechanisms to promote the production of more inflammatory mediators, including other cytokines and chemokines [4,5].

The chronic airway inflammation present in asthma patients may contribute to impaired immunity and predispose to infections that are more severe and long-lasting than in healthy individuals [6–8]. These infections, often caused by a virus, then trigger the asthmatic response, which involves inflammation and which may elicit subsequent bacterial infections, most frequently by *Streptococcus (S.) pneumoniae* or pneumococcus [9]. This may be due to the fact that, in asthma patients, *S. pneumoniae* colonization

is higher than in healthy controls. This, associated with inflammatory stimuli that alter endothelium permeability, would favour the development of lung infection, generating pneumonia. Talbot *et al*. have described that asthma patients have double the risk of invasive pneumococcal disease than healthy individuals. In asthma airway remodelling, on one hand, and increased mucin production as well as altered secreted mucus, on the other hand, result in impaired clearance of pathogenic bacteria of the airway, generating the presence of debris that may harbour infectious agents and contribute to the increased risk of pneumococcus invasive infection [6].

*S. pneumoniae* is an encapsulated Gram$^+$ bacterium saprophyte of the high airways [10]. Host defence against *S. pneumoniae* involves mainly acute phase responses and antibodies against surface proteins, as well as against polysaccharides. This pathogen has always been considered a T cell-independent antigen, but a number of papers describe the role of T cells in the immune response to *S. pneumoniae* [11–14]. The immunoglobulin (Ig)G-specific antibody response to this bacterium depends upon CD4 T cells, B7/CD28 co-stimulation and the interaction of CD40 with its ligand, while the IgM response is T-independent [4]. It has been observed that T lymphocytes are involved in the early stages of the host immune response to pneumococcal infection [15]. Other studies have described that the T help required for induction of an antigen- specific humoral response *in vivo* depends upon the type of pneumococcal antigen [5]. Snapper *et al*. [14] have studied many aspects of the immune response to *S. pneumoniae*. They have demonstrated recently in a murine model that Toll-like receptor (TLR)-2 expression by B and CD4 T cells is critical for induction of the T cell-dependent humoral immune response to intact pneumococci [13]. Evidence is also accumulating that the Th1 response is important for elimination of this pathogen, as interleukin (IL)-12-deficient patients develop serious pneumococcal infections, and interferon (IFN)-γ has also been involved in the defence against this pathogen [12]. Recently, the involvement of Th1 and Th17 cells in the response to *S. pneumoniae* has been reported [16].

Asthmatic patients are not more susceptible than healthy individuals to other bacterial respiratory infections such as tuberculosis. Therefore, we undertook to analyse if an ongoing Th2 response, such as that present in atopic asthmatic children, could modulate the response to *S. pneumoniae* and compare it to the response to *Mycobacterium (M.) tuberculosis*, a different type of bacterium that requires a Th1 response. Considering that asthma patients undergo periods of exacerbation of their symptoms, triggered by several stimuli, we were interested in determining whether or not, in that situation, they had the same or a different response to the evaluated bacteria in our experimental system.

## Materials and methods

### Subjects

We recruited patients aged 7–18 years who met the asthma diagnostic criteria. They were selected by high IgE levels, a history of bronchial hyperreactivity, more than two bronchospasm crises per year and positive for cutaneous tests to different allergens. We studied a total of 41 mild asthma patients who had received bacilli Calmette–Guérin (BCG) vaccination early in life and were on maintenance treatment of inhaled fluticasone $\leq 250$ μg/day. Samples were obtained during either the remission period or stable condition ($n = 39$) and, in some cases, at exacerbation ($n = 19$). In the latter situation samples were taken from the patients before the onset of oral corticosteroid treatment, provided to relieve the symptoms of exacerbation. We compared the whole group of patients in stable condition to a control group. Then, for 17 patients, we compared the same parameter in the stable condition and undergoing exacerbation of their symptoms, prior to rescue medication.

The control group consisted of age-matched healthy children (aged 7–20 years), non-asthmatic, non-atopic, undergoing routine blood tests prior to surgery (healthy individuals) ($n = 17$), who had also received BCG vaccination early in life. In Argentina the conjugate pneumococcal vaccine was incorporated into the National Vaccination Calendar during the second semester of 2011 for children up to 2 years of age. Hence, none of the individuals included into this study had received any pneumococcal vaccine. Informed consent was obtained for sample extraction as well as inclusion into the study, which was approved by the Ethics Committee of the Gutiérrez Children Hospital and the Ethics Committee of the Institutes of the Academia Nacional de Medicina.

### Antigens

American Type Culture Collection (ATCC) 49619 *S. pneumoniae* (Spn), cultured, washed and heat-inactivated, was kindly provided by the central Bacteriology Laboratory of the National Institute of Infectious Diseases ANLIS Dr Malbrán, and H37-RV *M. tuberculosis* (Mtb), cultured, washed and heat-inactivated was kindly provided by the Mycobacteria Service of the same institution. The correct antigen concentration used was determined at the beginning of the study by a dose–response curve.

### Cell purification

Heparinized blood was collected and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation. Cells were collected from the interphase and resuspended in RPMI-1640 tissue culture medium (Gibco Laboratory, Grand Island, NY,

USA) containing gentamycin (85 μg/ml) and 15% heat-inactivated fetal calf serum (Gibco) (complete medium).

### Cell culture

PBMC were cultured at $1 \times 10^6$ cells/ml in 1 ml of complete medium in Falcon 2003 tubes, at 37°C in a humidified 5% $CO_2$ atmosphere, in the presence or absence of 0·1 UDO/ml of either Spn ($2 \times 10^6$ bacteria/ml) or Mtb ($3 \times 10^6$ bacteria/ml) for different time-periods. Due to the small sample size, the same culture was used both for activation marker evaluation by flow cytometry and collection of supernatants, and the 48-h time-point ($t = 48$) was chosen as a compromise to obtain peak levels of cytokines and measurable levels of both activation markers.

### Cell populations and subpopulations

Eosinophils, basophils and B cells expressing bright IgE were evaluated by flow cytometry on the day of sample collection ($t = 0$) in whole blood by the use of anti-CD45 phycoerythrin-cyanin 5 (PE-Cy5) (BD Pharmingen, San Diego, CA, USA), anti-CD19 fluorescein isothiocyanate (FITC) (Cytognos, Salamanca, Spain) and biotinylated anti-IgE (Vector, Ontario, Canada), followed by streptavidin-PE (Vector). Basophils were defined as $CD19^-CD45^+IgE^+$ cells.

Populations expressing CD3, CD4, CD8, CD25, CD69 and TCR γδ were also evaluated in PBMC by flow cytometry using anti-CD3 PE-Cy5 (BD Pharmingen), anti-CD4 PE (BD Pharmingen), anti-CD8 FITC (Caltag Laboratories Burlingame, CA, USA), anti-CD25 FITC (BD Pharmingen), anti-CD69 PE (BD Pharmingen) and anti-TCR γδ FITC (BD Biosciences, Franklin Lakes, NJ, USA).

### Cytokine evaluation

Intracellular cytokines were evaluated by flow cytometry in whole blood cells from patients and healthy individuals. Cells were stimulated with phorbol myristate acetate (PMA) (50 ng/ml)/ionomycin (1 μM) in the presence of GolgiPlug (BD Biosciences) for 5 h and then stained with membrane anti-CD3 PE-Cy5 and anti-CD8 FITC. After fixation and permeabilization using the Fix & Perm kit (Caltag Laboratories), cells were stained with intracellular PE-conjugated antibodies for several cytokines: tumour necrosis factor (TNF)-α (Caltag Laboratories), IL-4 (BD FastImmune), IL-5 (BD Pharmingen), IL-13 (BD FastImmune), IFN-γ (Caltag Laboratories) and IL-10 (BD Pharmingen).

Cytokine production by PBMC was evaluated by commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Jose, CA, USA). The IL-5, IFN-γ and IL-10 concentrations were measured in supernatants of PBMC cultured for 48 h, with or without stimulation with Spn and Mtb. In order to evaluate spontaneous production, cytokine concentrations were measured in serum; we also evaluated the presence of IgE with the UBI Magiwell ELISA kit (United Biotech Inc., Mountain View, CA, USA).

### Statistical analysis

GraphPad Prism version 5·0 was used for statistical analysis. Statistical significance between parameters, measured in healthy individuals and asthma patients at the time of the sample extraction ($t = 0$), was determined by the Mann–Whitney *U*-test. To compare the response to the same stimulus (Spn or Mtb) in healthy individuals and asthma patients, we used the Mann–Whitney *U*-test. The same treatment of the data was performed to compare asthma patients in stable *versus* exacerbation periods, but statistical significance was determined by the Wilcoxon signed-rank test, given that we studied the same asthma patients in both conditions. To compare the different culture conditions within a specific group ($t = 48$), statistical significance was determined by a repeated-measures one-way analysis of variance (anova) test, followed by Tukey's *post-hoc* multiple comparison test.

## Results

### T cells

Taking into account that recent reports have described the participation of T cells in acquired immunity to *S. pneumoniae* [10,13,16], showing that clearance of *S. pneumoniae* depends upon $CD4^+$ T cells in colonization studies, and considering that this may also be true for mucosal disease [10], we decided to study some T cell parameters.

We evaluated T cell subpopulations comparing healthy individuals to asthma patients. In particular, we studied γδ T cells, as these have been implicated as important players in the response to *M. tuberculosis* [17] and, to a lesser degree, to *S. pneumoniae* [18]. As shown in Table 1a, γδ T cell percentages are significantly lower in asthma patients than in healthy individuals. Comparison of cells from the same patient in both stable and exacerbation conditions are shown in Table 1b; no differences were observed in γδ T cells for the same patient in both conditions.

It has been suggested that $CD4^+CD25^{hi}$ T cells may be involved in regulating the development of allergies and asthma [19,20]; therefore, we decided to evaluate $CD4^+CD25^{hi}$ T cells in our patients. As shown in Table 1a, no difference in the percentages of $CD4^+CD25^{hi}$ T cells was observed in asthma patients compared to healthy individuals. When patients were compared in both exacerbation and stable conditions, a tendency towards lower percentages of $CD4^+CD25^{hi}$ T cells could be observed in the latter (Table 1b).

### Activation markers

Expression of the CD69 and CD25 activation markers was also evaluated in these *ex-vivo*-obtained T cells. As shown in

**Table 1.** T cell subsets in healthy and asthmatic individuals.

(a)

| $t = 0$ | Healthy individuals | Asthma patients |
|---|---|---|
| % T cells | | |
| $CD3^+$ | 68·90 ± 4·35 | 63·69 ± 3·35 |
| $CD3^+ \gamma\delta$ | 7·85 ± 1·40 | 4·00 ± 0·44* |
| $CD3^+ CD69^+ \gamma\delta$ | 9·86 ± 3·00 | 7·05 ± 1·68 |
| $CD3^+ CD69^+$ | 2·95 ± 0·60 | 2·61 ± 0·28 |
| $CD3^+ CD25^+$ | 7·38 ± 2·00 | 8·43 ± 0·80 |
| $CD4^+ CD25^+$ | 6·31 ± 1·72 | 7·24 ± 0·87 |
| $CD8^+ CD25^+$ | 1·01 ± 0·36 | 0·63 ± 0·08 |
| $CD4^+CD25^{hi}$ | 1·14 ± 0·22 | 1·28 ± 0·28 |

(b)

| | Asthma patients | |
|---|---|---|
| $t = 0$ | Stability | Exacerbation |
| % T cells | | |
| $CD3^+ \gamma\delta$ | 2·96 ± 0·41 | 3·11 ± 0·46 |
| $CD3^+CD69^+ \gamma\delta$ | 5·20 ± 0·95 | 6·61 ± 1·19 |
| $CD3^+CD69^+$ | 2·51 ± 0·36 | 2·18 ± 0·45 |
| $CD3^+CD25^+$ | 8·40 ± 0·96 | 7·60 ± 1·24 |
| $CD4^+ CD25^+$ | 7·50 ± 1·11 | 7·65 ± 4·50 |
| $CD8^+ CD25^+$ | 0·64 ± 0·11 | 0·88 ± 0·19 |
| $CD4^+CD25^{hi}$ | 1·27 ± 0·41 | 1·64 ± 0·40 |

(a) Percentage of T cell subsets analysed *ex vivo* ($t = 0$), from healthy individuals *versus* asthma patients. Significance: $^*P < 0{\cdot}05$ by Mann–Whitney *U*-test. (b) Percentage of T cell subsets analysed *ex vivo* ($t = 0$), from asthma patients during the stable condition *versus* exacerbation. No significant differences were found by Wilcoxon's signed-rank test.

Table 1a, no significant differences could be observed in the expression of these markers when healthy individuals were compared to asthma patients. Similarly, no significant differences were observed for the same patient in the stable or exacerbation conditions (Table 1b).

### Peripheral blood B cells and other leucocyte populations

We then evaluated basophils, eosinophils and B cells expressing IgE ($IgE^+$ B cells), all of which are involved in asthma. The data are shown in Table 2. No difference was observed in basophil percentages when asthma patients were compared to healthy individuals. Significantly higher percentages of eosinophils were present in asthma patients.

$IgE^+$ B cells were also increased significantly in asthma patients. A significantly higher level of IgE was observed in the sera of asthma patients compared to that of healthy individuals, both in the stable and exacerbation conditions (Table 3). No significant differences were observed for the two patient conditions.

### T cell activation

In order to evaluate the T cell response to the bacteria, we cultured PBMC for 48 h in the presence or absence of Spn or Mtb and then evaluated CD69 and CD25 activation markers.

As shown in Fig. 1a,b, a significant difference can be observed in cells from patients as well as from healthy individuals in the expression of CD69 in response to both bacterial antigens when compared to non-stimulated culture. Expression of CD69 was significantly higher in cells from asthma patients stimulated with Mtb with respect to Spn. A significant difference was observed in response to Mtb when compared to the response to Spn in asthma patients, which cannot be seen in cells from healthy individuals. This appears to be due to a lower response to pneumococci in the cells from asthma patients. When the same patient was evaluated in both the stable and exacerbation conditions, significant differences were observed in response to the bacterial antigens compared to non-stimulated cells (data not shown), but no differences were found between the two patient conditions (Fig. 2a,b).

Expression of CD69 by stimulated γδ T cells was significantly different when compared to control for each bacterial stimulus, but no differences were observed in healthy individuals by stimulation with Spn and Mtb (Fig. 1c). In asthma patients, expression of CD69 was significantly higher when stimulated with Mtb with respect to control cells or those stimulated with Spn (Fig. 1d). CD69 expression was not increased significantly with Spn when compared to control.

As shown in Fig. 3, CD25 expression was increased significantly both in healthy individuals and asthma patients when stimulated with Mtb, but not with Spn, for CD3 as well as CD4 lymphocytes. Some authors have described a role for $CD8^+$ T cells in the antibody response to pneumococcal polysaccharides [21]. In our study, the CD8 subpopulation showed an increase in CD25 expression only with Mtb in cells from asthma patients, not in cells from healthy individuals. When the expression was compared in both the stable and exacerbation conditions for the same patient, no significant differences could be detected in response to the same bacterial antigen. Only the percentage of $CD3^+CD25^+$ T cells from asthma patients in the stable condition is significantly higher than in the exacerbation period (Fig. 4). This may be due to the migration of activated cells to the inflammation site during asthmatic exacerbation.

**Table 2.** Peripheral blood B cells and other leucocyte populations in healthy and asthmatic individuals.

| $t = 0$ | Healthy individuals | Asthma patients |
|---|---|---|
| % Basophils | 0·91 ± 0·17 | 0·76 ± 0·11 |
| % Eosinophils | 2·47 ± 0·17 | 6·39 ± 0·61*** |
| % $IgE^+$ B cells | 4·55 ± 0·92 | 6·55 ± 0·71* |

Percentage of basophils, eosinophils and immunoglobulin (Ig)$E^+$ B cells, analysed *ex vivo* ($t = 0$), from both healthy individuals and asthma patients. Significance: $^*P < 0{\cdot}05$; $^{***}P < 0{\cdot}0001$ by Mann–Whitney *U*-test.

**Table 3.** Serum soluble immune factors in healthy and asthmatic individuals.

| | | Asthma patients | |
|---|---|---|---|
| Blood serum ($t = 0$) | Healthy individuals | Stability | Exacerbation |
| IFN-γ (pg/ml) | > 500 ($n = 9$) | 118·30 ± 51·94 ($n = 7$) | 85·31 ± 41·05 ($n = 6$) |
| IL-5 (pg/ml) | < 3·9 ($n = 9$) | 80·19 ± 41·41 ($n = 7$) | 66·31 ± 29·09 ($n = 9$) |
| IL-10 (pg/ml) | 12·70 ± 3·56 ($n = 10$) | 22·46 ± 7·00 ($n = 11$) | 23·10 ± 7·16 ($n = 11$) |
| IgE (IU/ml) | 60·15 ± 11·96 ($n = 11$) | 1262·00 ± 304·80 ($n = 13$)** | 1545·00 ± 319·90 ($n = 11$)*** |

Concentration of interferon (IFN)-γ, interleukin (IL)-5, IL-10 and immunoglobulin (Ig)E in serum samples from healthy individuals and asthma patients during both stable condition and exacerbation. Significance: $^{**}P < 0{\cdot}001$; $^{***}P < 0{\cdot}0001$ by Mann–Whitney *U*-test.

## Cytokine production

We first evaluated the cytokine production capacity of T cells by non-specific stimulation and flow cytometry in whole blood cells from asthmatic patients in the stable condition and in healthy individuals. Data are presented in Table 4. Significantly lower production of TNF-α was observed in asthma patients compared to healthy

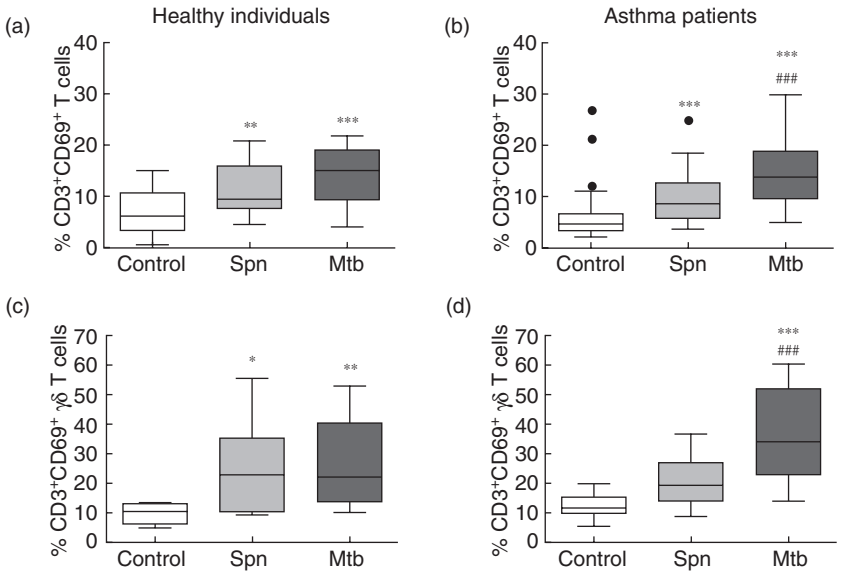


**Fig. 1.** CD69 expression induced by bacterial antigens in T cells. Percentage of $CD3^+CD69^+$ T cells and $CD3^+CD69^+$ γδ T cells from healthy individuals (a,c) and asthma patients (b,d), respectively, after stimulation in culture with *Streptococcus pneumoniae* (Spn), *Mycobacterium tuberculosis* (Mtb) or without stimulus (control). Significance: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}001$; $^{***}P < 0{\cdot}0001$ (*versus* control); $^{\#\#\#}P < 0{\cdot}0001$ (*versus* Spn); by one-way analysis of variance (ANOVA) with Tukey's *post-hoc* multiple comparison test.

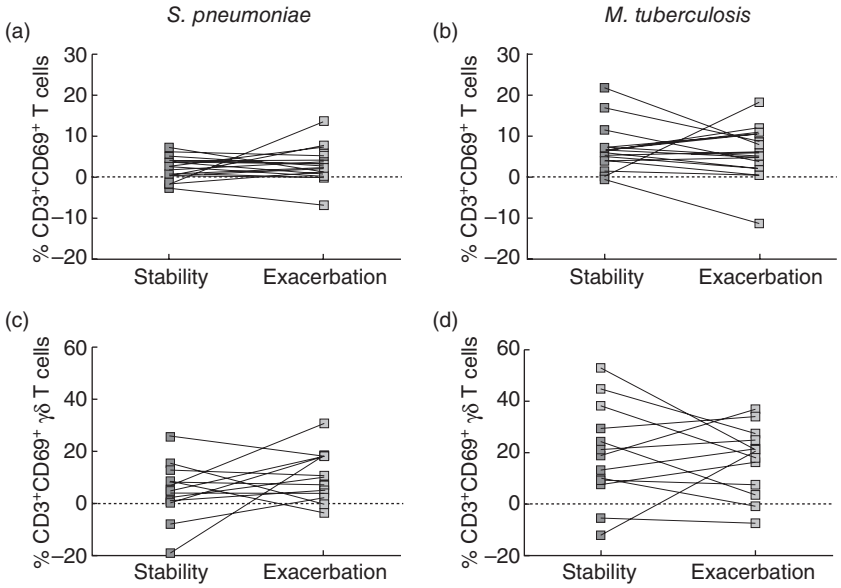


**Fig. 2.** CD69 expression induced by bacterial antigens in T cells from asthma patients in the stable condition and in exacerbation. Percentage of $CD3^+CD69^+$ T cells and $CD3^+CD69^+$ γδ T cells, from the same asthma patients in both conditions, after stimulation in culture with *Streptococcus pneumoniae* (a,c) or *Mycobacterium tuberculosis* (b,d). No significant differences were found by Wilcoxon's signed-rank test.

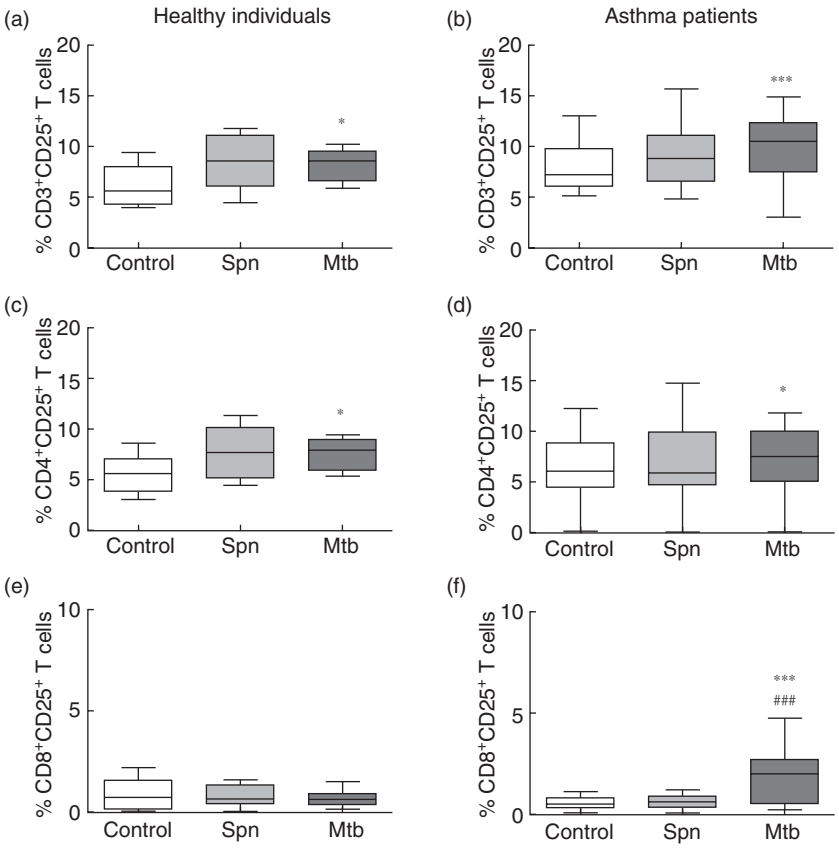


**Fig. 3.** CD25 expression induced by bacterial antigens in T cells. Percentage of $CD3^+CD25^+$ T cells, $CD4^+CD25^+$ T cells and $CD8^+CD25^+$ T cells from healthy individuals (a,c,e) and asthma patients (b,d,f), respectively, after stimulation in culture with *Streptococcus pneumoniae* (Spn), *Mycobacterium tuberculosis* (Mtb) or without stimulus (control). Significance: $^*P < 0{\cdot}05$ and $^{***}P < 0{\cdot}0001$ (*versus* control); $^{\#\#\#}P < 0{\cdot}0001$ (*versus* Spn); by one-way analysis of variance (ANOVA), with Tukey's *post-hoc* multiple comparison test.

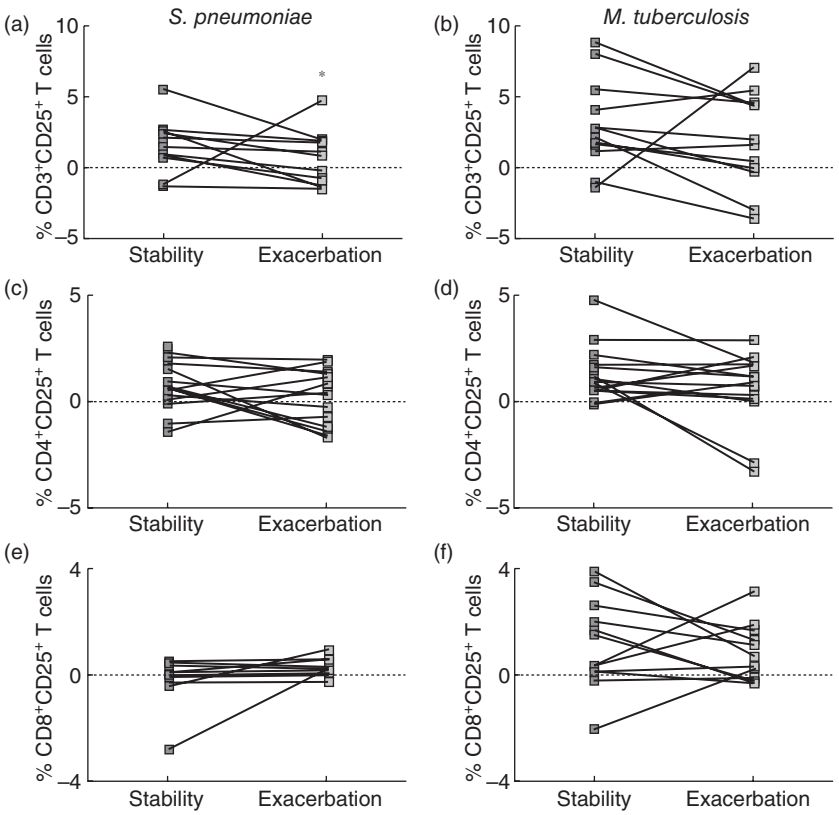


**Fig. 4.** CD25 expression induced by bacterial antigens in T cells from asthma patients in stable condition and in exacerbation. The percentage of $CD3^+CD25^+$ T cells, $CD4^+CD25^+$ T cells and $CD8^+CD25^+$ T cells, from the same asthma patients in both conditions, after stimulation in culture with *Streptococcus pneumoniae* (a,c,e) or *Mycobacterium tuberculosis* (b,d,f). Significance: $^*P < 0{\cdot}05$; by Wilcoxon's signed-rank test.

**Table 4.** Cytokine production capacity of T cells by non-specific stimulation.

| Whole blood cultures | Healthy individuals | Asthma patients |
|---|---|---|
| % T cells | | |
| $CD3^+$ TNF-$\alpha^+$ | 28·52 ± 5·70 ($n = 13$) | 6·78 ± 2·73 ($n = 10$)** |
| $CD3^+$ IFN-$\gamma^+$ | 15·99 ± 3·09 ($n = 13$) | 8·59 ± 3·71 ($n = 10$) |
| $CD3^+$ IL-$4^+$ | 0·41 ± 0·11 ($n = 10$) | 0·39 ± 0·18 ($n = 10$) |
| $CD3^+$ IL-$5^+$ | 0·31 ± 0·04 ($n = 12$) | 1·78 ± 0·73 ($n = 10$)* |
| $CD3^+$ IL-$13^+$ | 0·62 ± 0·22 ($n = 13$) | 0·60 ± 0·27 ($n = 5$) |
| $CD3^+$ IL-$10^+$ | 0·19 ± 0·05 ($n = 12$) | 0·17 ± 0·05 ($n = 8$) |

Percentage of $CD3^+$ T cells expressing tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-4, IL-5, IL-13 or IL-10 after stimulation of whole blood cells with phorbol myristate acetate (PMA)/ionomycin for 5 h, from both healthy individuals and asthma patients. Significance: $^{**}P < 0{\cdot}001$; $^{*}P < 0{\cdot}05$ by Mann–Whitney *U*-test.

individuals. A marked tendency to lower production of IFN-γ, a Th1 cytokine, although non-significant, was also observed in the cells from asthma patients.

In contrast, a significantly higher production of IL-5, a characteristic Th2 cytokine, was observed in these patients, but no differences could be observed for IL-4 and IL-13, hallmarks of asthma and other allergic conditions, or for IL-10, a regulatory cytokine.

In order to gain insight into the cytokine environment to which the peripheral blood cells were exposed *in vivo*, the concentration of some cytokines was also determined in blood serum. The results presented in Table 3 demonstrate that serum IFN-γ was lower and IL-5 higher in asthma patients compared to healthy individuals, while no significant differences were evident for IL-10.

As a second measure of T cell activation, using ELISA we also evaluated cytokine production in response to bacterial antigens by culturing PBMC in the presence or absence of Spn or Mtb. Cytokines representative of three T cell subpopulations (Th1, Th2 and regulatory) were evaluated in patients in the stable condition and at exacerbation of their symptoms (Table 5). The concentration of IFN-γ was above detection limit in the cells from asthma patients in exacerbation, even with no stimulus. For healthy individuals and patients in the stable condition, that type of concentration was reached by stimulation with either one of the bacteria. Nevertheless, a tendency to lower levels of the Th2 cytokine IL-5 were observed under exacerbation with respect to the stable condition, but differences were non-significant.

Finally, IL-10 was evaluated as a cytokine produced by regulatory cells. IL-10 production was stimulated greatly by both Spn and Mtb when compared to non-stimulated cultured cells. Its level was not significantly different with both bacterial stimuli and in both patient conditions.

## Discussion

Asthma patients suffer acute exacerbation of their symptoms triggered by several stimuli, associated with an increase of airway inflammation, increased levels of inflam-

**Table 5.** Cytokine production of peripheral blood mononuclear cells (PBMC) stimulated with bacterial antigens.

| | | Asthma patients | |
|---|---|---|---|
| Supernatants | Healthy individuals | Stability | Exacerbation |
| IFN-γ (pg/ml) | | | |
| | $n = 3$ | $n = 7$ | $n = 5$ |
| Control w/o bacteria | 96·10 ± 60·10 | 79·20 ± 51·6 | > 500 |
| *Streptococcus pneumoniae* | > 500 | > 500 | > 500 |
| *Mycobacterium tuberculosis* | > 500 | > 500 | > 500 |
| IL-5 (pg/ml) | | | |
| | $n = 3$ | $n = 7$ | $n = 5$ |
| Control w/o bacteria | < 3·9 | < 3·9 | 2·22 ± 2·22 |
| *S. pneumoniae* | < 3·9 | 6·50 ± 4·30 | 4·20 ± 2·60 |
| *M. tuberculosis* | < 3·9 | 25·90 ± 17·00 | 14·30 ± 9·40 |
| IL-10 (pg/ml) | | | |
| | $n = 8$ | $n = 7$ | $n = 5$ |
| Control w/o bacteria | 16·67 ± 6·42 | 20·58 ± 4·10 | 17·34 ± 6·46 |
| *S. pneumoniae* | 181·20 ± 55·31 | 169·80 ± 49·21 | 174·60 ± 27·54 |
| *M. tuberculosis* | 102·20 ± 24·13 | 140·80 ± 41·57 | 187·20 ± 39·64 |

Concentration of interferon (IFN)-γ, interleukin (IL)-5 and IL-10 in supernatants obtained after 48 h stimulation in culture with *S. pneumoniae*, *M. tuberculosis* or without stimulus (control). Data correspond to healthy individuals and asthma patients during both the stable condition and exacerbation. No significant differences were found by Mann–Whitney *U*-test.

matory factors and higher numbers of infiltrating cells. This represents a severe burden on the health-care system, accounting for approximately 50% of the costs linked to asthma, but it also affects the patient's quality of life [22]. Nevertheless, most of the time asthma patients are in the stable condition, with absence of asthma symptoms and normal peak expiratory flow (PEF) and forced expiratory volume in 1 s ($FEV_1$) [23]. Viral infections are one of the most common causes of exacerbations of asthma [24] and, they may open the way for bacterial infections, and *S. pneumoniae* is frequently involved in respiratory infections associated with wheezing [25].

Recent evidence suggests that allergic asthma is a multifaceted condition controlled actively by effector as well as regulatory T cells ($T_{regs}$). Effector cells increase airway inflammation, while $T_{regs}$ are anti-inflammatory. Cytokines are involved in the development and activation of all T cell subpopulations [20]. They are also involved directly or indirectly in most approaches to asthma treatment.

The classical view considers the immune response to *S. pneumoniae* to be based on anti-capsular antibodies, but several reports have described the involvement of T cells [10,16,20]. Mureithi *et al.* have demonstrated the presence of T cell memory in response to this pathogen in human PBMC [26]. Recently, Olliver *et al.* have characterized the immune response to this pathogen in humans [16], while several reports have described T cell involvement in the immune response to *S. pneumoniae* in animal models [27,28].

Kirby *et al.* have reported evidence for the involvement of lung-specific γδ T cell subsets in local responses to *S. pneumoniae* infection [18]. This T cell subset is considered important in the immune response to *M. tuberculosis* [29]. The lower percentage of γδ T cells that we observed in asthma patients would affect the immune response to Spn as well as to Mtb. It may therefore be detrimental in both cases, but this would not explain a differential susceptibility to infection with these agents.

Some authors have described a role for $CD8^+$ T cells in the immune response to pneumococcus [21,30]. In our experiments, the CD8 subpopulation showed an increase in CD25 expression only with Mtb in cells from asthma patients, and not in cells from healthy individuals. This activation may also be a contributory factor in the defence against Mtb, and not to Spn, although the overall immune response will depend upon the balance of all the T cell subpopulations.

An important role has been ascribed to $T_{reg}$ cells in the development of atopic asthma. Several authors have suggested that $T_{reg}$ cell deficiency could be the cause of allergies and atopic asthma. Some authors have reported lower levels of $T_{reg}$ cells in asthma patients [31]. Pumputiene *et al.* have recently studied $CD4^+CD25^{hi}$ regulatory cells in atopic asthmatic children, both during remission and exacerbation [32]. They observed no significant correlation of $CD4^+CD25^{hi}$ T cell percentages. They also report no reduction in peripheral blood $T_{reg}$ cells compared to controls. This is in accordance with our data, with no statistical differences in the proportion of this T cell subpopulation in either condition of the patients and in healthy individuals. We also agree with this group, in that if one could study cells from the local tissue, differences both in numbers and function might be observed.

T cells are well characterized with respect to their function in protective humoral immune responses; however, their roles during early stages of infection and invasive pneumococcal disease are less well defined. Using a mouse model of pneumococcal sepsis, LeMessurier *et al.* found that $CD4^+$ T cells were recruited to the lung as early as 12 h after intranasal infection. Recruitment was accompanied by up-regulation of CD69 and B7-H1, reflecting T cell activation. They suggest an important and adverse role of $CD4^+$ T cells in the pathogenesis of invasive pneumococcal disease [33].

The immune response to an antigen can be evaluated by the expression of activation markers on lymphocytes as well as cytokine production after stimulation of PBMC with the antigen. CD69 is an early activation marker of lymphocytes. In a recent report, Martin *et al.* proposed that CD69 expression can modulate T lymphocyte differentiation towards the Th17 lineage in allergic asthma and other inflammatory diseases. CD69 promotes activation of the Jak3-signal transducer and activator of transcription 5 (STAT-5) signalling pathway, which inhibits Th17 cell differentiation, thus providing a mechanistic link between CD69 and the regulation of Th17 responses [34]. Th17 cells have been described in turn as relevant in the immune response to *S. pneumoniae* [10]. Other authors have reported that CD69 plays a crucial role in the pathogenesis of allergen-induced eosinophilic airway inflammation and hyperresponsiveness [35]. Taking all this information into consideration, the expression of CD69 was evaluated. A greater early activation response against Mtb, compared to Spn, was observed in the cells from asthma patients. This difference in CD69 expression was not observed in the cells from healthy individuals. A deficient early activation in response to Spn in the cells from asthmatic individuals, which would also impair the subsequent T helper effector response, may help to explain the different frequencies of infection with these two pathogens reported in asthma patients when compared to healthy individuals.

The immune response to bacterial antigens also involves the production of cytokines, which may be biased in asthma patients by their underlying condition. TNF-α is a proinflammatory cytokine that is considered to be responsible for several aspects of the airway pathology in asthma. In our experimental system we evaluated the cytokine production capacity of PBMC by non-specific stimulation and flow cytometry. A significantly lower percentage of TNF-α and a marked tendency towards a lower percentage of IFN-γ-

expressing cells was observed in asthma patients compared to healthy individuals. These results are partially in accordance with those of Machura *et al.*, which describe a decrease in IFN-γ production, evaluated by intracellular cytokine staining in T cells from asthma patients compared to healthy subjects, while TNF-α was similar in both groups [36]. Several clinical trials have evaluated biological therapies that target inflammatory mediators such as TNF-α. This cytokine, considered initially as a product of macrophages, can be expressed by a broad range of cell types [37], so it is possible that the elevated levels reported by others are derived from cells other than those we evaluated, or are generated at local sites.

In our data, levels of IFN-γ in serum were lower in asthmatics with respect to the control group. However, in non-stimulated supernatants from cultured PBMC, high production was detected in the cells from asthma patients under exacerbation. This may be due to an *in-vivo* partial stimulation related to the exacerbation state, which results during the culture in the production of this cytokine. A significantly higher percentage of cells expressing IL-5, a typical Th2 cytokine, was observed in these patients, but no differences could be observed for IL-4 and IL-13, hallmarks of asthma and other allergic conditions. This is in agreement with the results of Kuo *et al.*, who reported significantly higher IL-5 and lower IFN-γ production from activated PBMC of asthmatic patients than of normal controls [38].

The specific response to the bacterial antigens was also evaluated by the concentration of cytokines in supernatants of cultured cells. Higher levels of IFN-γ, our marker of Th1 cytokine production, were observed under exacerbation in the non-stimulated samples. IFN-γ production has also been reported in response to Spn [39]. Lama *et al.* have reported that serum levels of IFN-γ were significantly lower in both steroid-naive and steroid-treated groups of asthmatic children compared to healthy control subjects, but they do not make reference to exacerbation [40].

The cytokine evaluated for Th2 cells was IL-5. A tendency to lower values was observed in the exacerbation condition in response to the bacterial antigens when compared to the stable condition, although the response to Mtb was always higher than to Spn. Robroeks *et al.* have proposed IL-5 levels in exhaled breath as a significant predictor of asthma exacerbations [41]. It is possible, then, that the IL-5 *in-vitro* production by antigen-stimulated PBMC is reduced in asthma patients under exacerbation because these cells have exhausted their capacity *in vivo*, explaining the tendency observed in our data. This may also be due to the fact that in the presence of Spn, an immune response is elicited that may counteract or interact with the ongoing Th2 response present in asthma through other mediators.

Asthma has been related to defects in the regulation of the immune response, and IL-10 is a regulatory cytokine involved in the physiopathology of this disease. We evaluated the production of this cytokine in our experimental model. IL-10 production was greatly stimulated by both Spn and Mtb when compared to non-stimulated cultured cells, with similar levels to both bacterial stimuli and in both patient conditions.

A recent study on the immune response elicited by heat-killed and live pneumococci in human cells reports that the former triggered a Th1-biased response while the latter triggered a Th17 response [16]. Therefore, our experimental data provide useful information but cannot be extrapolated directly to the pathophysiological *in-vivo* situation. Important differences have been reported for cells from the periphery and cells from the local environment, in our case the lung. Several studies in HIV infection have reported increased numbers of $T_{reg}$ cells in local tissue, while the percentage of these cells in the peripheral blood remained similar to that of healthy individuals [42]. This may be also the case in our experiments, but we had no access to broncheoalveolar lavage or patient sputum. Cho *et al.* have also published that findings in blood did not reflect those in the airways [43]. It is possible that more differences could be observed with those sample types. Further studies on these materials might provide a clearer insight into the immune response to *S. pneumoniae* in asthma patients.

## Acknowledgements

We are grateful to all patients and their parents or guardians who consented to participate in this study. We are also grateful to Drs A. Teper and M. Kohan for referral of patients and M. M. de E. de Bracco for critical reading of the manuscript. This work was supported by grants from CONICET (PIP6067) and Fundación A.J. Roemmers.

## Disclosure

The authors have no financial conflicts of interest.

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