

Peripheral Blood CD161⁺ T Cells from Asthmatic Patients are Activated During Asthma Attack and Predominantly Produce IFN- γ

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Abstract

In humans, T cells expressing the CD161 molecule NKR-P1A constitute around 20% of the circulating CD3⁺ cells and are potentially immunoregulatory in several diseases. Their role in asthma is not well known, but they could participate in asthma attacks. To determinate whether activation of CD161⁺ T cells and their cytokine production correlate with clinical status of asthma, we analysed blood samples from asthma attack patients (AAP) and stable asthma patients (SAP) in comparison with healthy non-atopic controls (HC). There was a significant higher baseline expression of CD69 on T cells from AAP and the difference was more notorious on CD161⁺ T cells; upregulation of CD69 was observed on both CD161[−] and CD161⁺ T cells driven by *Dermatophagoides pteronyssinus* crude extract, whereas polyclonal stimulation with phorbol 12-myristate 13-acetate plus ionomycin predominantly induced IFN- γ but no IL-4, IL-5 and IL-13 by CD161⁺ T cells in all groups; upon polyclonal stimulation, there were more CD161⁺ T cells producing IFN- γ and less CD161[−] T cells producing this cytokine, contrasting with the opposite results observed in SAP and HC groups. Our results indicate that, during asthma attack, CD161⁺ T cells are activated and are able to produce predominantly IFN- γ but no Th2 cytokines. We hypothesize that during an asthma attack, IFN- γ produced by CD161⁺ T cells could help to reestablish the Th1/Th2 equilibrium. These observations may contribute to the understanding of the immune mechanisms involved in asthma attacks.

Introduction

Asthma is one of the most common and important chronic, non-infectious disorders [1]. Paediatric asthma is a leading cause of health-care request, school absences, as well as activity restriction for children and adolescents. Exacerbations of the disease, known as acute asthma attacks, represent one of the most common reasons for which patients seek emergency care from general practitioners and hospital emergency departments [2, 3]. The predominant Th2-biased immune response to allergens is the hallmark of asthma, which is downregulated by IFN- γ produced by Th1 cells [4] as well as by other cell subsets such as CD8⁺ T cells, $\gamma\delta$ T cells, NK and NKT cells [5]. Demonstration of an increase of serum IFN- γ in asthma attacks with its further decrease as the patients improve clinically after therapy has been reported [6].

Some of the T cells able to produce IFN- γ constitutively express the molecule CD161 (NKR-P1A, an NK-cell receptor equivalent to the antigen NK1.1 in mice), a homodimeric C-type lectin-like receptor [7]. In human blood, CD3⁺ CD161⁺ circulating cells account for approximately 20% of the total CD3⁺ population and include CD4[−] CD8[−] (double negative, DN) as well as CD4⁺ and CD8⁺ T cells [8, 9]; simultaneous expression of other NK cell markers on CD161⁺ cells, such as CD16 and CD56, are relatively low [8–10]. In general, T CD4⁺ CD161⁺ cells are central (CD45RA[−] C-D45RO⁺ CD62L⁺ CCR7⁺) and/or effector (CD45RA[−] C-D45RO⁺ CD62L[−] CCR7[−]) memory cells, while T CD8⁺ CD161⁺ cells are mainly effector memory cells [11]. An interesting subset of T CD161⁺ cells are the invariant NKT (iNKT, V α 24–J α 18–V β 11 TCR) cells, which recognize glycolipid antigens presented by CD1d

[12], which represent a small minority (0.1–1%) of the circulating T CD161⁺ population and have been intensively studied because of their possible role in the pathogenesis of asthma [13–17]. Also, CD8⁺ CD56⁺ cells, another interesting group of cells closely related to CD161⁺ T cells, have been implicated in the pathogenesis of severe asthma [18]. However, the participation of the significantly broader group of other CD161⁺ T cells in asthma pathogenesis has not been assessed.

In this study, we investigated cellular activation and cytokine production in peripheral circulating CD161⁺ T from patients with an asthma attack or with stable asthma, in comparison with healthy subjects. As peripheral blood lymphocytes can be recruited to the bronchial tree during asthma attack, determination of frequency, activation and cytokine patterns of CD161⁺ cells might be very informative to understand the pathogenic mechanisms in individuals during an ongoing asthma attack.

Materials and methods

Subjects. Forty-seven subjects aged 7–19 years, selected in the Department of Paediatric Allergy and Clinical Immunology of the Hospital Infantil de México 'Federico Gomez', in Mexico City, were enrolled in this study. The Ethical Committee of the hospital approved this study and parents of all participating children signed an informed written consent. Asthma diagnosis was established by patient history, clinical manifestations, <60% of predicted forced expiratory volume (FEV₁) and peak expiratory flow (PEF) variability >30% of predicted value. Atopic condition was defined as positive skin prick-test (>10 mm) to the dust mite *Dermatophagoides pteronyssinus* total crude preparation (Dp; International Pharmaceutical Immunology, Madrid, Spain) and positive to specific IgE against *Der p* 1 and *Der p* 2 (Indoor Biotechnologies, Charlottesville, VA, USA) using Schuurman method [19]. Patients were considered free from recent upper respiratory tract infections and did not use corticosteroids (inhaled, systemic or topical) during the month previous to bleeding. Participants were classified into the following groups. *Group I: asthma attack patients (AAP)*, defined by a progressive increase in asthma symptoms and a decrease in PEF levels resulting in a hospital visit and was classified as severe, moderate or mild in accordance with GINA criteria [20]. Twelve children (six males and six females), aged 7–14 years (mean 12 ± 3), two with intermittent (step 1), six mild persistent (step 2) and four moderate persistent (step 3) asthma were included in this group. Blood samples were taken during the first 24 h immediately after the asthma attack and before the commencement of treatment. *Group II: stable asthma patients (SAP)*: this group included patients with a diagnosis of asthma without symptoms at the moment of bleeding: 14 patients (11 males and three females), aged

8–17 years (mean 12 ± 2) were included, five patients had previous diagnosis of intermittent asthma (step 1), six mild persistent (step 2) and three moderate persistent (step 3). *Group III: healthy controls (HC)*. Twenty-one healthy subjects were included as controls: 12 males and nine females, aged 7–19 years (mean 14 ± 4). Subjects in this group had no history of atopic diseases, normal lung function, Dp-negative skin-prick test and negative to specific IgE against *Der p* 1 and *Der p* 2. Exclusion criteria for all groups included a recent history of infection of the upper respiratory tract or any other clinical illness.

In vitro cell stimulation to assess CD69 expression and IFN-γ production. Heparinized peripheral blood samples were collected and 1 ml whole blood per well was stimulated for 6 h, at 37 °C and 5% CO₂, with 25 ng phorbol 12-myristate acetate (PMA; Sigma Chemical, St Louis, MO, USA) plus 1 µg ionomycin (Sigma), or 10 µg Dp crude extract (Indoor Biotechnologies), or 20 µl PBS as negative control in tissue culture plates (Nunc, Roskilde, Denmark). After stimulation, triple fluorescence FACS analysis was performed using saturating amounts of anti-CD161-PE, anti-CD69-FITC and anti-CD3-PerCP (Becton-Dickinson, San Jose, CA, USA) for CD161⁺ T and T cell identification. Specificity of staining was assessed using FITC or PE conjugated isotype controls. Erythrocytes were lysed with 500 µl of FACS lysing solution (Becton-Dickinson) for 10 min. After washing with 1 ml PBS containing 2% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 0.1% sodium azide (Sigma), cells were resuspended in 300 µl PBS containing 1% paraformaldehyde and analysed in a FACSort (Becton-Dickinson). At least 20,000 events were collected for each tube-condition and analysed using CELLQuest software version 3.1 (Becton-Dickinson).

Lipopolysaccharide (LPS) mitogen-activity inhibition assays [21] were performed with polymixin B sulfate (Gibco/BRL, Grand Island, NY, USA) at 10 µg/ml in whole blood cells activated with Dp crude extract. LPS of *Escherichia coli* 044:B5 twice purified by phenol extraction (donated by Dr. D. Golenbock, University of Massachusetts, MA, USA) was used as positive control.

Intracellular cytokine detection was performed as recommended by the antibody provider. Briefly, 1 ml aliquots of whole blood were cultured for 6 h, at 37 °C in 5% CO₂ atmosphere in the presence of 10 µg brefeldin A (Sigma) and 25 ng PMA plus 1 µg ionomycin, or 10 µg Dp crude extract, or 20 µl of PBS as control. FACS analysis was performed in 0.1 ml of the sample incubated for 15 min with anti-CD161-PE for IFN-γ and IL-4 detection or anti-CD161-FITC for IL-5 and IL-13 identification, or anti-CD3-PerCP for NKT and T cells, respectively. Erythrocytes were lysed for 15 min with 500 µl of FACS lysing solution. After washing, cells were treated with 500 µl of 1X FACS permeabilizing solution (Becton-Dickinson) for 15 min and washed again with

PBS–FBS. Cells were incubated for 30 min with any of the following antibodies: anti-IFN- γ -FITC, anti-IL4-FITC, anti-IL-5-PE or IL-13-PE (Becton-Dickinson). Cells were washed and resuspended in 300 μ l of PBS containing 1% paraformaldehyde and analysed on a FACSsort flow cytometer. Acquisition and analysis were performed using CELLQuest 3.1. For each tube, 20,000 events were collected and stored for analysis.

Statistical analysis. Percentages of CD69-positive and IFN- γ -positive cells were compared between groups by Mann–Whitney rank sum test. Analysis was performed using the Sigma Stat software (3.1 Sigma Stat Software Inc., Richmond, CA, USA). Quantitative differences between samples with P values of ≤ 0.05 were considered significant.

Results

There is no difference in CD161⁺ T cells frequency among patients with asthma attack, stable asthma or healthy control individuals

We sought for the frequency of CD161⁺ T cells in peripheral blood by flow cytometry. Cells with simultaneous expression of both antigens CD3⁺ and CD161⁺ were considered as CD161⁺ T cells. The selection and percentages of whole-blood CD161⁺ T cells are shown in Fig. 1. CD161 was present in 15–18% of peripheral blood CD3⁺ T cells and there was no statistical difference

among groups: ranges of CD161⁺ T cells were 4.0–40.5% for AAP (median = 16.5), 9.3–29.1% in SAP (median = 16.3) and 6.1–27.8% in HC (median = 14.9). Therefore, although there was a tendency to observe higher percentages of these cells in patients with asthma attacks, equivalent proportions of CD161⁺ T cells were found in the studied groups.

CD161⁺ T cells are activated *in vivo* in patients with asthma attack

In order to determine the *in vivo* baseline activation level during asthma episodes, three-colour flow cytometry analysis was used to identify CD69, an early activation marker, on CD161⁺ T cells as well as on CD161[−] T cells (Fig. 2, upper panel). Percentages of CD69 spontaneously expressed on CD161⁺ T cells (background), shown at the right side of the lower panel, were significantly higher in AAP (median 16.7, range 7.8–36.0%) compared with SAP (median 6.7, range 2.3–14.0%, $P = 0.001$) or HC (median 4.8, range 2.5–13.2%, $P = 0.001$). No differences were found between SAP and HC ($P = 0.08$). Similarly, unstimulated T cells (CD3⁺ CD161[−] cells, lower panel, left, Fig. 2) from AAP expressed more CD69 (median 8.1, range 1.4–27%) than cells from SAP (median 2.7, range 0.3–7.3%, $P = 0.05$), or HC (median 2.1, range 0.09–8.4%, $P = 0.007$) (Fig. 2, lower panel left). These data suggest that CD161⁺ T cells and CD161[−] T cells were activated during asthma attack.

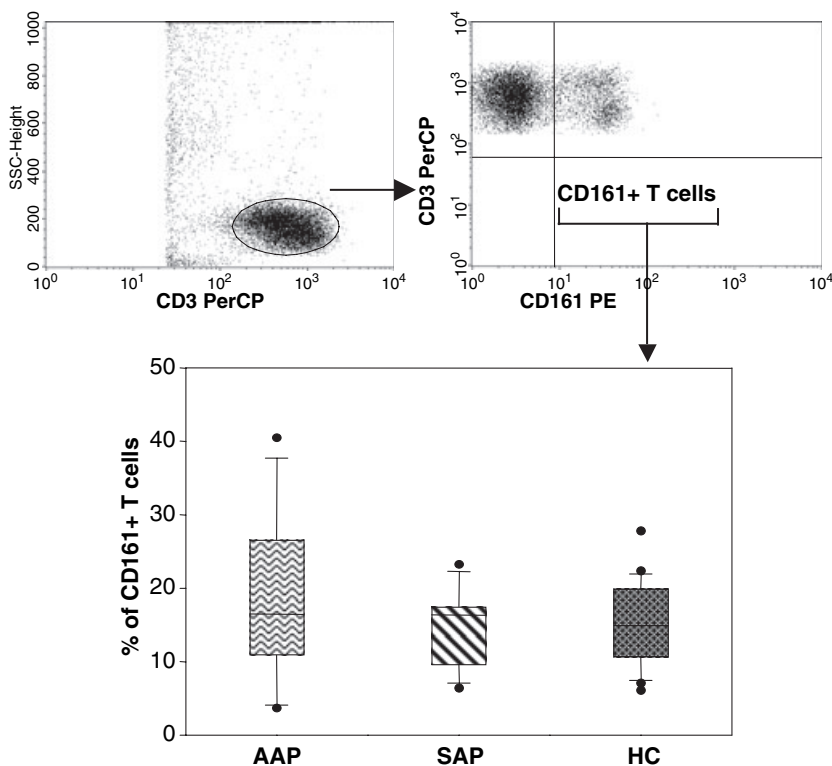


Figure 1 Percentage of CD161⁺ T cells in blood samples. Fresh blood samples from asthma attack patients, stable asthma patients and healthy non-atopic controls were analysed by two-colour immunofluorescence flow cytometry (anti-CD161-PE and anti-CD3-PerCP) (upper panel). T lymphocytes were gated on CD3⁺ cells and percentages of double positives (CD3⁺ CD161⁺) cells were plotted in 95th percentile box graphics for the groups (lower panel). Lines inside the boxes represent medians. Statistical analysis was performed using non-parametric Mann–Whitney rank sum test in all graphics. No significant differences were observed between groups.

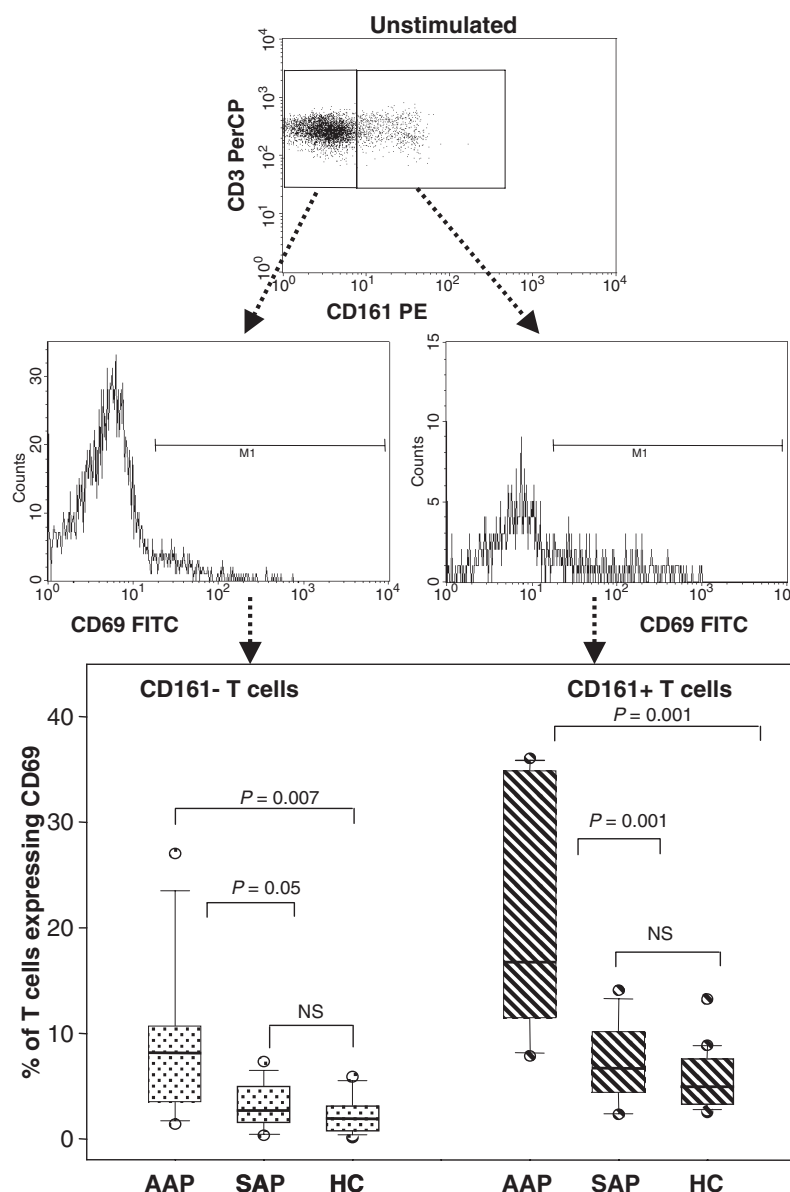


Figure 2 CD69 spontaneous expression on blood CD161-positive and -negative T cells. Blood samples were incubated with PBS during 6 h and analysed by fluorescence-activated cell sorting by three-colour immunofluorescence. CD161⁺ and CD161⁻ T cells were defined as shown in upper graphic and analysed for CD69 expression as shown in the middle panel. Percentages of CD69⁺ cells for each subset were plotted in 5th and 95th percentile box graphics of the lower panel. Horizontal bars represent medians.

CD69 expression on CD161⁺ T cells of asthma patients induced by specific stimulation with *D. pteronyssinus*

The previous results showed more T cells *in vivo* activated (both CD161⁺ and CD161⁻ cells) in patients during asthma attack; therefore, we tested whether these cells could specifically increase *in vitro* CD69 expression responding to activation with crude Dp antigen. Percentage of CD161⁺ T cells expressing CD69 molecule on AAP increased from a median of 16.5 in the resting cells up to a median of 28 upon Dp stimulation ($P = 0.24$), while in SAP increment was from 6.7 to 10.4 ($P = 0.001$) and in HC from 4.8 to 10.5% ($P = 0.001$) (Fig. 3. lower panel). A similar increased response in CD69 expression was observed on T cells (CD3⁺ CD161⁻ cells) comparing the same groups: in AAP, median value

went from 8.1 to 12.2 ($P = 0.07$), in SAP from 2.7 to 4.4 ($P = 0.06$) and in HC from 2.1 to 4.6 ($P = 0.004$). The high frequency of cells responding to Dp even in healthy controls is consistent with published information [22] suggesting a 'mitogen-like' effect because of the presence of contaminants in the crude extract, such as LPS. To test this possibility, cell-activation inhibition assays with polymyxin B were performed. Results showed that polymyxin B abrogated CD69 expression in cells stimulated with LPS, but not with Dp (data not shown). Finally, no differences were found in percentages of CD161⁺ T cells expressing CD69 comparing the three groups in polyclonally stimulated cells: median = 95.2% for AAP, 82.3% for SAP and 88.5% for HC, indicating that all the cells had the same capacity to respond to *in vitro* stimulation. Considering the previous results, it

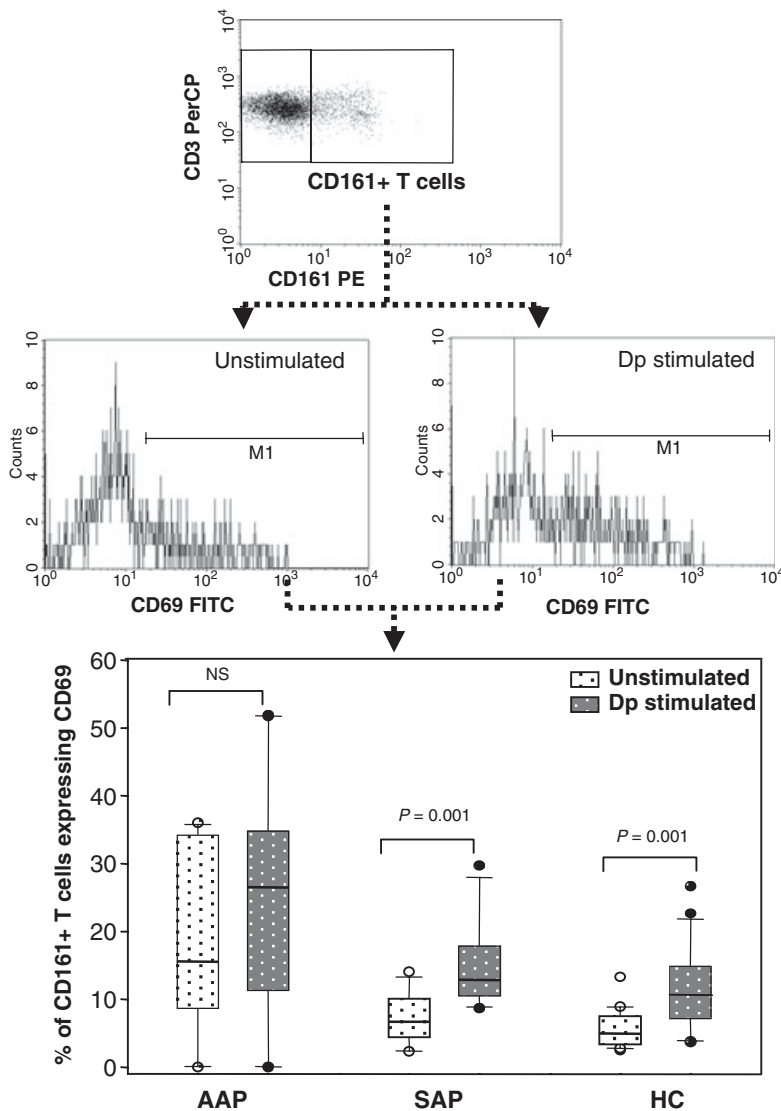


Figure 3 Comparison of spontaneous versus allergen-specific induced CD69 expression on CD161⁺ T blood cells. Whole-blood samples were incubated for 6 h with PBS or Dp crude extract and analysed by flow cytometry as indicated in Figs 1 and 2. CD161⁺ T cells were defined as indicated in the upper panel graphic. M1 in the middle panel was set on the basis of matched negative isotype controls and percentages of CD69⁺ cells were used to construct the lower box chart.

may be concluded that CD161⁺ T cells are *in vivo* activated during asthma attack and these cells can still be further specifically *in vitro* stimulated to express more CD69.

There are more CD161⁺ T cells and less T cells (CD3⁺ CD161⁻) able to produce IFN- γ *in vitro* in patients with asthma attack

We compared the number of CD161⁺ T cells producing IFN- γ , IL-4, IL-5 and IL-13 versus T CD161⁻ cells under a polyclonal stimulation with PMA by multicolour FACS analysis, combining surface phenotyping and intracellular cytokine detection. As the production of any of the Th2 cytokines (IL-4, IL-5 and IL-13) was negligible, either spontaneously or under polyclonal stimulation in all the groups (data not shown), we focused just on the IFN- γ production. Spontaneous production of IFN- γ was not detected

in any of the studied subjects of any group, but after polyclonal stimulation with PMA during 6 h, IFN- γ production was observed in both CD161⁺ and CD161⁻ T cells in all samples (Fig. 4). IFN- γ -positive CD3⁺ cells were gated to determine the proportions of CD161-negative and -positive cells producing this cytokine (Fig. 4, upper panel). In all the groups, there were more CD3⁺ CD161⁻ T cells able to produce IFN- γ upon polyclonal stimulation. Patients suffering asthma attack had the lowest percentages of IFN- γ producing cells, while the control group (healthy donors) showed the highest percentages (Fig. 4, lower panel). It is noteworthy that when the analysis was carried out focusing on the CD161⁺ T cells, the results among these groups were opposite to what we observed on the T cells; the AAP group had significantly more cells producing IFN- γ than the HC group ($P = 0.0006$, Fig. 4, lower panel right). Thus, CD161⁺ T cells are able to produce IFN- γ significantly with more frequency during the

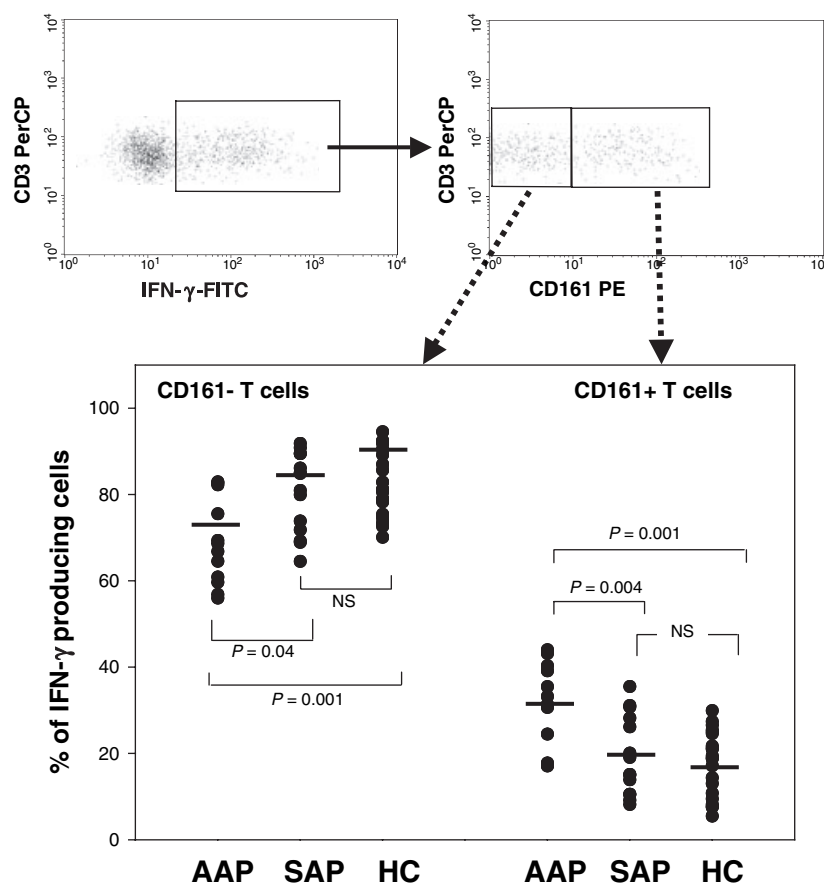


Figure 4 Comparison of IFN- γ producing cell percentages between CD161-negative and -positive T cells. Blood samples were stimulated with phorbol 12-myristate 13-acetate plus ionomycin for 6 h in the presence of BFA, surface labelled with anti-CD3 and anti-CD161 antibodies and intracellularly for IFN- γ . T cells producing IFN- γ were selected as shown in the upper left graphic and analysed to distinguish between CD161-negative and -positive T cells as depicted in the upper right graphic. Taking the IFN- γ -positive cells as 100%, the lower box chart was drawn and comparison between T cell subsets in the groups is shown.

asthma attack, whereas T cells predominate as a source of IFN- γ in SAP or HC.

Discussion

In this study, we addressed the frequency and activation of blood CD161⁺ T cells in patients with stable and exacerbated asthma. We found that CD161⁺ T cells constitute an important fraction of T blood lymphocytes with similar levels in asthmatic patients and healthy subjects. Moreover, in patients under asthma attack, circulatory CD161⁺ T cells are activated and produce IFN- γ in greater proportion than patients with stable asthma or healthy controls.

CD161⁺ T cells constitute a heterogeneous cell subset that represents a significant proportion of the total T-cell population [11]. In the three groups studied here, CD161⁺ cells accounted for approximately 17% of the total CD3⁺ cell population; these data are consistent with information published by other authors [10, 11, 23]. However, percentages of these cells in humans can vary in a broad range depending on the patient selected as well as the organ or tissue where cells are sought for [23, 24]. Although equivalent percentages of CD161⁺ T cells were found in all groups, cells of patients with asthma

attack showed higher basal activation measured by the expression of CD69, suggesting that *in vivo* activation might be associated with the stage of disease. Correlation between severity of disease and a high proportion of baseline activated CD161⁺ cells has also been reported in patients with intestinal cancer [24]. When the CD161⁺ T cells from AAP were stimulated with Dp crude antigen, no significant increase of CD69 was observed. On the contrary, in cells from SAP and HC groups there was an evident upregulation of this molecule. This difference suggests that the cell activation capacity of the former group was almost fulfilled and no further induction was possible. Dp crude extract could contain other components, such as LPS, which could induce unspecific cell activation; this possibility was ruled out as polymyxin B did not abrogate Dp-driven activation.

The most interesting finding in the present study was the inverse pattern of IFN- γ production by CD161⁺ and CD161⁻ T cells between AAP and SAP groups, i.e. polyclonal activation of cells from AAP induced more CD161⁺ cells to produce IFN- γ than those from SAP and HC groups, whereas the opposite effect was observed in CD161⁻ T cells. In our experiments, we detected intracellular IFN- γ production by CD161⁺ T cells, but percentages of IL-4, IL-5 and IL-13 producing cells in

asthmatic patients were negligible (data not shown). Our results tend to agree with other studies that have shown that circulating CD161⁺ T cells [12] or iNKT cells [14, 25] produce higher amounts of IFN- γ , compared with Th2 cytokines production [24–26]. Increased activation of CD161⁺ cells with a Th1 cytokine-profile polarization in AAP may suggest that these cells are part of the mechanisms to compensate the Th2-biased response associated with asthma during the attack [7, 27, 28].

The role of CD161⁺ T cells in asthma has not been studied, contrasting with iNKT cells (which are part of the CD161⁺ T cell subset) that had been studied in both mice and humans (revised in [16]). CD161 has been shown to be a regulatory molecule of lymphocyte transmigration [29], and CD161⁺ T cells are mainly memory cells [11]; therefore, they might have a potential participation in the control of asthma condition, and especially in asthma attacks, through their migration to the airways and the local production of IFN- γ . The importance of 161⁺ T-cell migration to the lungs in asthma pathogenesis can be inferred from the finding that about 60% of the T cells present in bronchoalveolar-lavage fluid from patients with moderate-to-severe asthma are CD4⁺ iNKT cells [13, 14], which are mainly CD161⁺. iNKT cells in the lung produce more Th2 cytokines [13], while our results show more capacity to produce IFN- γ by blood CD161⁺ T during asthma attack, signifying that the migration of the latter cells to the lung could potentially provide a regulatory physiological mechanism to compensate the deleterious effects of the Th2 response. More studies are necessary to understand the balance between these two branches of the immune response.

In conclusion, findings here reported are of considerable significance, as there has been little information concerning the participation of CD161⁺ T cells in asthma. CD161⁺ T cells comprise functionally distinct cell populations, including a cell subset that is activated during asthmatic attack and produced important amounts of IFN- γ . This information supports a possible immunoregulatory role of CD161⁺ T cells in the reestablishment of the altered homeostasis in asthma attack.

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