

Participation of CD161⁺ and invariant natural killer T cells in pediatric asthma exacerbations

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ABSTRACT

Asthma has been defined as a disease of chronic airway inflammation in which many cells and cellular products participate with variable degrees of airflow obstruction and hyperresponsiveness that lead to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing. Prominent among these cellular elements are two cell types referred to as the invariant natural killer T (iNKT) cells and a subpopulation of T cells expressing the molecule CD161, which are both thought to play a role in the pathogenesis of asthma. Although the presence of iNKT and other CD161⁺ cells in murine models has been associated with asthma, relatively few studies have been performed in the adult patient with asthma that have been often conflicting and even fewer studies are available in children. The present study was performed to investigate the peripheral blood frequencies of iNKT and CD161⁺ T cells in children with asthma. A total of 35 children, 19 stable asthmatic patients, 6 who had experienced an asthmatic attack within 24 hours and had not received any treatment, and 10 healthy controls, aged 6–12 years, were enrolled in the study. iNKT and CD161⁺ T-cell frequencies in blood were measured together with quantitative levels of IL-4 and interferon (IFN) γ using a cytofluorimetric approach. The results show that iNKT cells are increased in pediatric asthmatic patients undergoing exacerbations of asthma. These cells also produced less IFN- γ and more IL-4 than children with stable asthma and in healthy control children. These results suggest that iNKT cells might participate in the development of the asthmatic exacerbations. The increased production of IL-4 in conjunction with the decrease of IFN- γ may be mechanistically responsible, at least partially, for the heightening of the immunologic response leading to the asthmatic attack in children. Knowledge of these interactive mechanisms involving the iNKT cell and our understanding of its role in the exacerbation of asthma hold great promise in the development of better diagnostic predictive markers of disease progression as well as new forms of therapeutic interventions.

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Asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness leading to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing.¹ Although asthma is generally well controlled when properly managed with the administration of inhaled cor-

ticosteroids,^{2,3} exacerbations do occur as an effect of a number of triggers, including respiratory viral infection, allergens, occupational agents, drugs, and air pollution.^{4,5} The frequency of exacerbations is highly variable depending on factors such as the severity and degree of control of the underlying disease process.^{6,7} Although mortality due to asthma is relatively low, poorly managed patients, often young individuals, tragically and frequently succumb to preventable complications associated with the disease.⁸

Recently, a major investigative effort has been directed to the study of the underlying mechanisms of the inflammatory response that contribute to the tissue injury associated with asthma, an understanding of which is critical to the management of the disease. Although many cell types have been identified, a major pathogenic finding seems to be the result of intensive T helper (Th) 2-biased immune response and its cytokines.⁹ The participation of other immune cells and their cytokines has also been implicated¹⁰ including a subpopulation of T cells expressing the molecule CD161,

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a homodimeric C-type lectin-like receptor,¹¹ which produces interferon (IFN) γ constitutively, which are thought to play a role in the pathogenesis of asthma and allergic rhinitis.^{12,13} In blood, double-positive CD3⁺/CD161⁺ circulating T cells account for ~20% of the total CD3⁺ population; this includes CD4⁺ and CD8⁺ as well as CD4⁻/CD8⁻ double-negative cells.¹⁴ In general, CD4⁺/CD161⁺ T cells are central or effector memory cells, and CD8⁺/CD161⁺ T cells are mainly effector memory cells.¹⁵

Prominent among these cells is the T-cell subpopulation called invariant natural killer T (iNKT) cells, characterized by the coexpression of both NK and T-cell markers.¹⁶⁻¹⁸ A main feature of the iNKT cells is their limited expression of T-cell receptors (V α 24J α 18 and V β 11 in humans), which recognizes glycolipid antigens and nonglycosidic molecules presented by CD1 d.^{19,20} In circulation, iNKT cells represent ~5% of all T cells, and can display CD4⁺ or CD8⁺ markers or neither.²¹ Subsets of iNKT cells may perform relevant immunoregulatory functions.²² On activation, iNKT produces large amounts of IL-4 or IFN- γ more rapidly than conventional T cells.²³ In turn, these cells exert effector functions over other cells such as dendritic, B and T cells.^{19,24} Thus, the main functionality of iNKT cells is to act as a bridge between the innate and acquired immune responses.²⁵ Although iNKT cells have been extensively studied in mice and have been shown to be involved in asthma and allergy, their role in humans with asthma is less clear and studies have yielded conflicting results.²⁶⁻³² The role of iNKT and other CD161⁺ cells in the pathogenesis of exacerbation of asthma in children is even less understood. The present study was performed to investigate the frequencies of iNKT and CD161⁺ T cells and their cytokine production in peripheral blood cells from children suffering an asthmatic attack in comparison with subjects with stable asthma and healthy children.

METHODS

Study Participants

Children enrolled in the study were from the Department of Pediatric Allergy and Clinical Immunology, Hospital Infantil de Mexico "Federico Gomez" in Mexico City. The children's parents or guardians were informed about the study and the corresponding written consents were signed. The study was approved by the Institutional Review Boards of the Hospital Infantil de México "Federico Gómez" and the Instituto de Diagnóstico y Referencia Epidemiológicos. Inclusion criteria for asthmatic patients included compatible medical history, clinical manifestations of asthma, <60% of predicted forced expiratory volume in 1 second, and peak expiratory flow variability >30% of predicted value. The atopic condition was defined as a positive skin test with dust-mite antigen *Dermatophagoides*

pteronysinus. Exclusion criteria included any upper respiratory tract infection and/or history of therapy with corticosteroids 1 month before sample collection.

Subjects were classified into three groups: (a) group 1, asthma attack patients (AAPs), cases experiencing acute asthmatic exacerbation; blood samples were strictly collected within the first 24 hours after the attack of asthma and before beginning of any treatment; additionally, all AAPs were admitted to the allergy medical unit and showed evidence of airflow obstruction on spirometry (forced expiratory volume in 1 second <70%); (b) group 2, stable asthma patients (SAPs); individuals who had been previously diagnosed with asthma and who had not experienced any symptoms for at least 1 month before sampling; and (c) group 3, a nonatopic, healthy cohort (HC) defined as subjects who did not have any history of asthma, atopic diseases, and who with presented normal lung function.

Flow Cytometric Analysis

Blood collection and cytometric analyses were performed as reported elsewhere.¹² Briefly, whole blood was incubated with brefeldin A (Sigma-Aldrich, Saint Louis, MO). Red blood cells were lysed and cells were washed, fixed, and resuspended in fixation/permeabilization solution (Becton Dickinson, San Jose, CA). A four-color staining was performed using the following antibodies (Becton Dickinson): PerCP anti-CD3, FITC or PerCP anti-CD4, FITC or PerCP anti-CD8, APC anti-CD161, FITC or APC anti-TCRV α 24, PE anti-IFN- γ , and PE anti-IL-4. For color generation, fluorochrome-conjugated antibody or the corresponding isotype control was added. Cells were washed twice with PBS with 1% bovine albumin solution, resuspended in fixation buffer (PBS with 1% paraformaldehyde), and analyzed in a FACSCalibur flow cytometer (Becton Dickinson) using the CellQuest Pro software (Becton Dickinson).

Statistical Analysis

A descriptive analysis was performed to establish the distribution of all variables including box plots. The Kolmogorov-Smirnov test was performed to determine whether or not the data followed a normal distribution. The Mann Whitney *U* test was used to analyze non-parametric data, and ANOVA was performed on all data after a normal distribution.

RESULTS

Patients' Characteristics

The AAP group consisted of 6 patients, 4 male and 2 female patients (average age, 10 years old; range, 8-12 years). The SAP group consisted of 19 patients, 14 male and 5 female patients (average age, 9.3 years

old; ranged 7–12 years). The nonatopic, HC consisted of 10 individuals, 6 male and 4 female patients (average age, 9.9 years old; range, 7–12 years).

The Frequency of Total T and CD161⁺ Cells are Unaltered during an Asthma Attack

The frequencies of CD161⁺ and T cells (CD3⁺, CD4⁺, and CD8⁺) were measured in all three groups. T lymphocytes were gated according to cell size using a side versus CD3⁺ dot plots and the gated subpopulation was used to generate the corresponding dot plots (Fig. 1 A). The aforementioned analysis did not show significant differences in the frequencies of T cells and subpopulations, among all three groups. CD3⁺ cells were slightly higher among individuals in the SAP group (42.9 [72.8–13.7]), in comparison to the AAP and HC groups (34.0 [59.8–16.7] and 35.2 [70.4–10.1], respectively). On the other hand, CD4⁺ and CD8⁺ cell percentages were more evenly distributed; CD4⁺ cell percentage for the AAP group was 38.7 (47.5–33.3), and for the SAP and the control groups CD4⁺ cell percentages were 40.1 (58.9–28.4) and 41.6 (56.5–11.1), respectively. The CD8⁺ cell percentages were higher among individuals in the SAP group, 30.5 (44.3–16.0), followed by the AAP, 28.5 (45.7–12.9), and the HC, 26.4 (43.9–16.4), groups. The percentages of total CD161⁺ cells were 7.2 (10.7–4.1), 6.6 (20.2–1.1), and 6.8 (12.5–2.4) for AAP, SAP, and HC groups, respectively, without any significant difference between groups.

IL-4-Producing CD161⁺/CD8⁺ T Cells Are Increased in Asthmatic Patients

Although the total number of CD161⁺ cells was shown to be initially unaltered among asthmatic children, a separate set of studies was performed to determine if the CD161⁺ cell subpopulation remained unchanged during the occurrence of the asthmatic crisis. As shown in Fig. 1 B, the CD161⁺ cell subpopulations did not show any statistically significant differences. CD161⁺/CD4⁺ double-positive cell percentages were 29.5 (34.7–23.9), 29.0 (43.0–15.3), and 24.0 (35.4–5.2) for the AAP, SAP, and HC groups, respectively. Likewise, the CD161⁺/CD8⁺ cells did not show any significant differences. The highest percentage of CD161⁺/CD8⁺ cells was seen among individuals in the control group (47.3 [58.7–34.3]). Both asthmatic groups, AAP and SAP, exhibited lower percentages of CD161⁺/CD8⁺ cells (35.1 [49.7–20.9] and 42.1 [62.7–17.6], respectively). The cytokine profiles for each subset were measured next. Interestingly, the CD161⁺/CD8⁺ cells that produce IL-4 were significantly increased among asthmatic stable individuals in contrast to the healthy controls (Fig. 2). The percentages for AAP and SAP were 0.7 (2.4–0.0) and 2.2 (9.2–0.0), respectively, higher than in the control group (0.4 [2.3–0.0]). On the other hand, no differences were

observed in the frequencies of CD161⁺/CD8⁺ cells producing IFN- γ between all groups. The percentages for AAP, SAP, and HC were 1.6 (5.5–0.2), 3.3 (11.4–0.0), and 0.7 (2.1–0.0), respectively.

Interestingly, no differences were observed among CD161⁺/CD4⁺ cells producing either IL-4 or IFN- γ . However, the SAP group consistently displayed the highest percentages of either IL-4- or IFN- γ -producing cells (4.1 [19.8–0.4] and 4.0 [13.6–0.5]), followed by the AAP group (2.3 [5.1–0.2] and 2.5 [4.2–0.2]). The frequencies of CD161⁺/CD4⁺ cells producing either cytokine were lower (1.2 [3.2–0.0] and 1.8 [6.2–0.1] for the AAP and SAP groups, respectively).

iNKT Cells Are Increased during an Asthma Exacerbation Attack

The frequencies of iNKT cells among all groups were assessed next. Shown in Fig. 3 is the distribution of cells simultaneously expressing the invariant TCRV α 24 and either CD4 or CD8 markers. Total iNKT cells were significantly higher among AAP children than in either the SAP and HC groups. The percentage of cells for AAP was 2.4 (range, 3.6–1.7), followed by the control group (1.5 [2.4–0.9]) and finally the SAP group (0.7 [1.0–0.4]).

To analyze the distribution of iNKT in more detail, we assessed the frequencies of both iNKT expressing either CD4 or CD8 markers (Fig. 3 A). However, no statistical differences were observed among all of the different subpopulations between all groups. The percentages of all subpopulations were more evenly distributed among all subjects. iNKT CD4⁺ cells were slightly higher among the AAP (0.7 [1.3–0.1]) and HC (0.7 [1.6–0.1]) groups, followed by the SAP groups (0.4 [1.3–0.1]). Likewise, iNKT CD8⁺ cells were slightly higher among children from the AAP and HC groups (0.6 [0.9–0.2] and 0.7 [1.5–0.1], respectively).

iNKT Cells Tend to Produce Less IFN- γ

A separate set of studies were performed to analyze the cytokine synthesis profile for the iNKT CD4⁺ or CD8⁺ subpopulation cells in whole blood (Fig. 3 B). No significant differences were observed between all subpopulations and the production of IL-4 or IFN- γ . Overall, the HC group showed higher frequencies of both iNKT CD4⁺ and CD8⁺ producing either IL-4 or IFN- γ , respectively. In contrast, AAP and SAP groups exhibited slightly lower frequencies of all iNKT cells producing cytokines. The iNKT CD4⁺ cells producing IL-4 frequencies in the AAP were 1.7 (6.3–0.1) or IFN- γ , 4.8 (9.8–1.9), respectively, and for the SAP group 1.9 (5.7–0.1) and 4.9 (7.5–2.7). Similarly, iNKT CD8⁺ cells producing either cytokine were somewhat higher in the

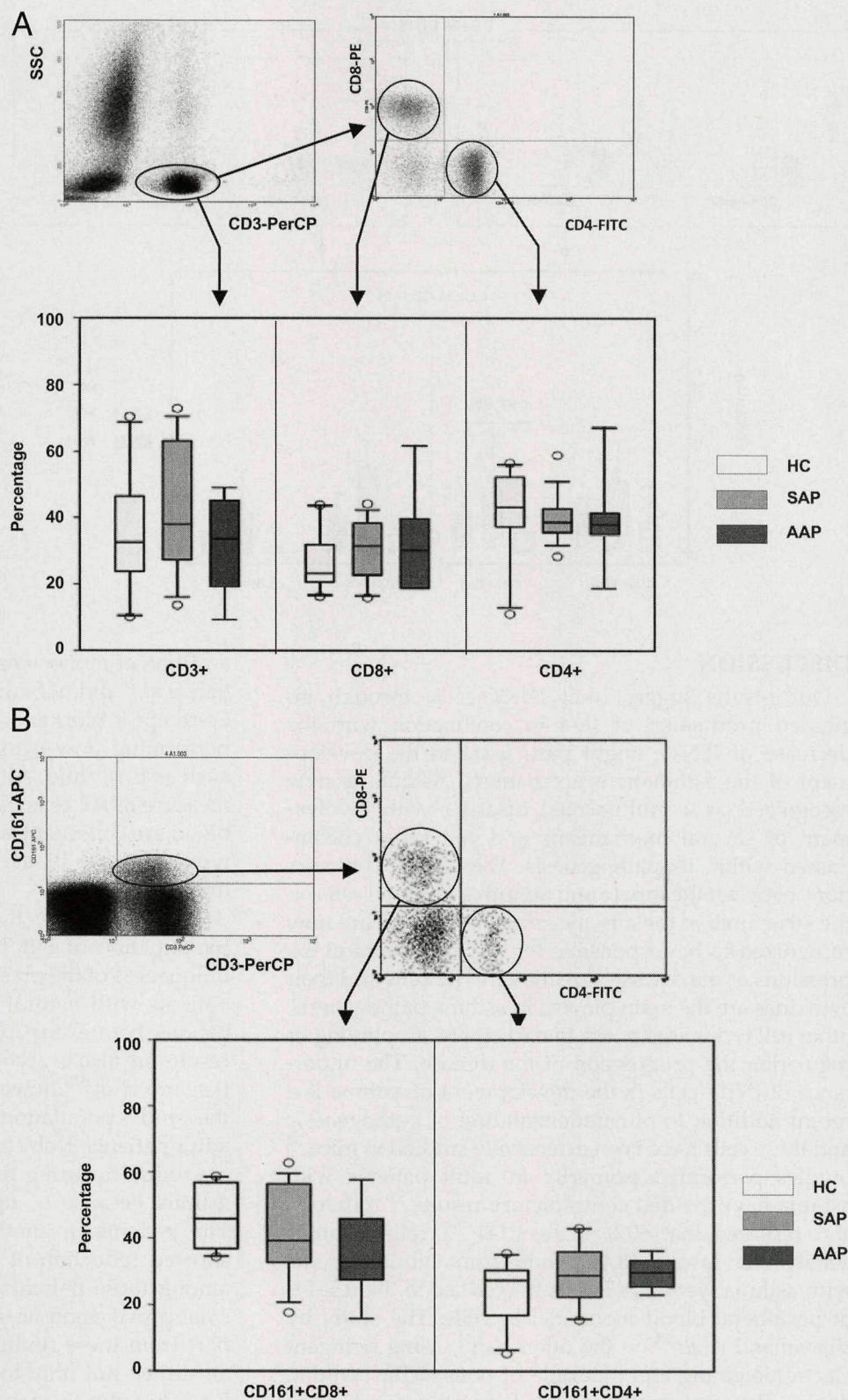


Figure 1. (A) Percentage of circulating T cells. Whole blood from asthma attack patients (AAPs), stable asthma patients (SAPs), and healthy cohort (HC) individuals was subjected multicolor immunofluorescence analysis using a flow cytometric approach. T lymphocytes were gated using side scatter versus CD3 dot plots as shown in the upper left panel. The percentages of both CD4⁺ and CD8⁺ T cells were assessed by dot plots (upper right panel). Overall percentage for all groups was plotted using a 95th percentile box graphic (lower panel). (B) CD161⁺ subpopulations. CD161⁺ cells were gated from CD3 versus CD161⁺ dot plots. Total percentages of CD161⁺/CD4⁺ and CD161⁺/CD8⁺ cells were calculated from the corresponding dot plot analysis (upper right panel). Overall percentages for both subpopulations were plotted as box graphics as shown in panel A.

HC group when compared with the AAP group. However, these differences were not statistically significant. IFN- γ -producing cells were slightly higher among children in the SAP and HC groups (1.2 [2.6–0.0] and 1.4 [4.5–0.2], respectively). The AAP group exhibited

lower frequencies (0.5 [1.1–0.1]). On the other hand, the percentages of IL-4-producing cells were more homogeneous between the three groups (1.3 [4.2–0.3], 1.5 [3.0–0.3], and 1.2 [2.8–0.1] for the AAP, SAP and HC groups, respectively).

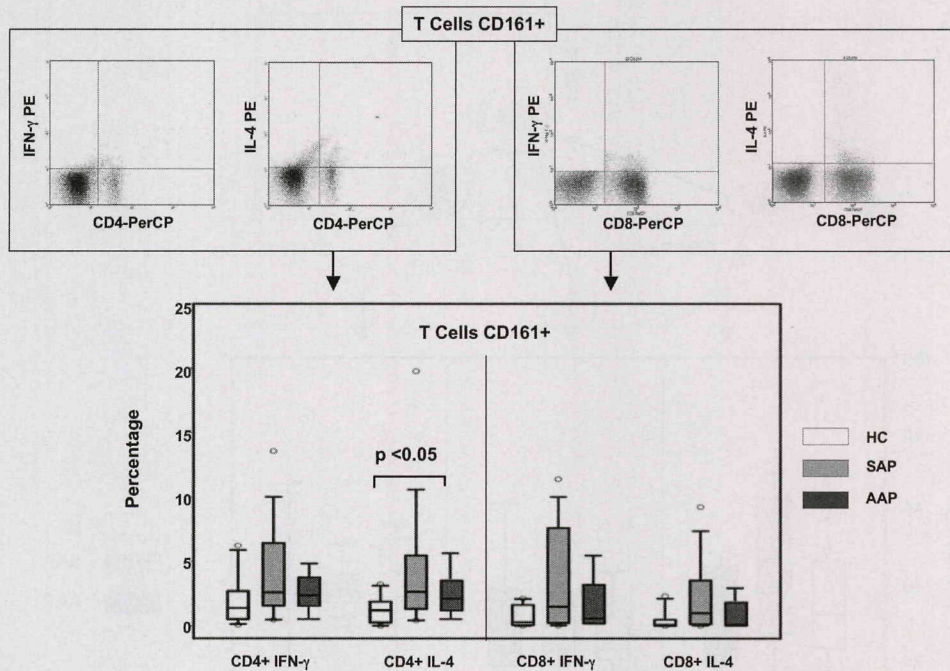


Figure 2. CD161⁺ cells cytokine production. Expression of IL-4 and interferon (IFN) γ was assessed by flow cytometry using an intracellular staining method. Dot plots for each CD161⁺ subset and both cytokines were used to calculate the percentage of cells producing either IL for the asthmatic and control groups. Box graphics were used to depict the overall percentages of cell among all groups. The *p* values from statistically different groups are shown for each independent graphic.

DISCUSSION

Our results suggest that iNKT cells, through increased production of IL-4 in conjunction with the decrease of IFN- γ , might participate in the development of the asthmatic exacerbations. Asthma is now recognized as a multifaceted disorder with involvement of several mechanisms and cell types encompassed within its pathogenesis. The complex interactions between the innate and adaptive immune cells on the structures of the airways are relevant and are now recognized to be responsible for the major clinical expressions of the disease.³³ Although Th2 cells and their cytokines are the main players in asthma pathogenesis, other cell types are known to participate, amplifying or regulating the progression of the disease. The importance of iNKT cells in the development of asthma is a recent addition to our understanding of pathogenesis and these cells have been extensively studied in mice.³⁴ Studies performed primarily in adult patients with asthma have yielded contradictory results.³⁵ Akbari *et al.*²⁶ reported that 60% of the CD4⁺ T cells in bronchoalveolar lavage (BAL) fluid from adult patients with asthma were iNKT cells in contrast to the 0.5–1% of peripheral blood mononuclear cells. The study by Vijayanand *et al.*,²⁸ on the other hand, using stringent electronic gating and blockade of nonspecific staining during flow cytometry, showed that the numbers of NKT cells obtained from samples of BAL from asthmatic patients were not significantly different from samples from healthy volunteers. In their study, Fc γ -receptors, commonly responsible for nonspecific interaction with monoclonal antibodies used in flow cytometry, were blocked by polyclonal human IgG before the

addition of monoclonal antibodies.³⁶ The study by Akbari *et al.*²⁶ did not take into consideration the need for Fc-receptor blockade, now known to be critical when performing flow cytometry in a “cluttered” medium such as BAL fluid. Although the present study did not measure iNKT cells in BAL, the increased quantities in blood are consistent with those of Akbari *et al.*²⁶ and it represents one of the first studies performed in children with asthma.

Our results are, therefore, in agreement with the participation of iNKT cells in asthmatic attacks. The uniqueness of the present study is its focus on pediatric patients with natural occurrence of asthmatic exacerbations before any pharmacologic intervention. Our results are also in accordance with findings reported by Ikegami *et al.*³⁷; however, the major difference is that the study population consisted mostly of asthmatic adult patients. Koh *et al.*³⁰ have shown that iNKT cells are reduced during the acute exacerbation episode of asthma because of upper respiratory tract infection. The patients in another study by Koh *et al.*³¹ only showed reduction of iNKT cells on infection but not among those patients who underwent allergen bronchial provocation testing. Our results significantly depart from these findings with differences that can be attributed not only to the age of the individuals analyzed but also the nature of the asthmatic exacerbation. Because the participants in the present study were children experiencing naturally occurring exacerbation episodes, this might offer an explanation for the differences observed between the two studies. Alternatively, the small number of patients included might have also contributed to the differences observed between the

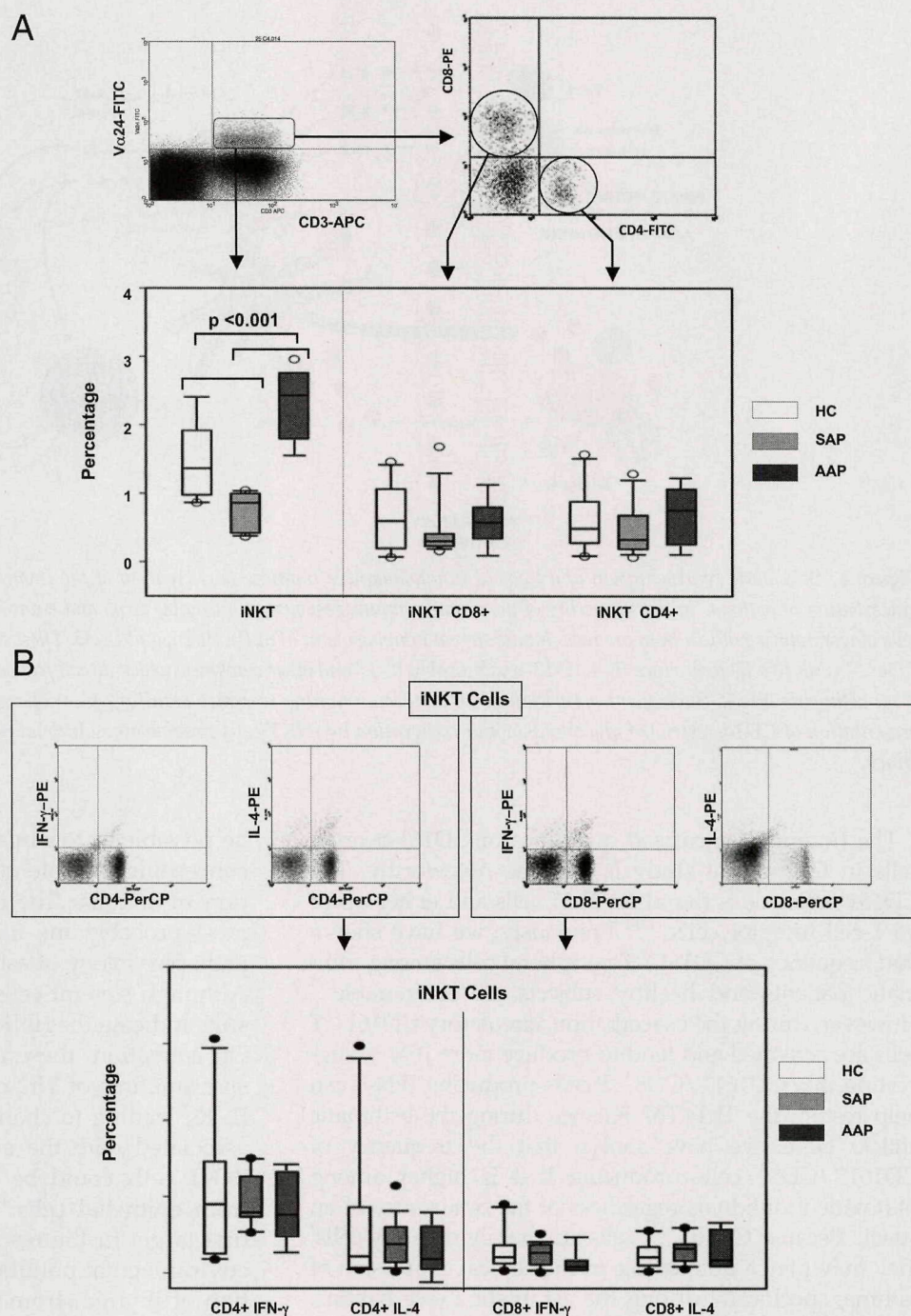


Figure 3. Invariant natural killer T (iNKT) cells. (A) Total iNKT as well as CD4⁺ and CD8⁺ iNKT cells were measured by flow cytometry. Dot plots were used to assess each individual subpopulation in all experimental and control groups. (B) Cytokine production by iNKT cell subsets was approached by intracellular staining as in Fig. 2. The p values from statistically different groups are shown in each independent graphic.

two reports; this small number of AAP subjects resulted from the rigorous inclusion criteria used for selection of the patients.

The rapid secretion of cytokines observed after activation of iNKT cells may be their contribution to the development of asthma exacerbations, probably through the promotion of T-cell polarization and recruitment of immune and inflammatory cells.³⁸ Our data show a lower frequency of iNKT cells (1.5% in HC) from those reported in normal individuals from other Latin American origin (3–3.5%).³⁹ Interestingly, iNKT values obtained from the

SAP were lower than the HC group; however, they were not statistically different. On the other hand, the frequency of iNKT cells in the AAP group was considerably higher than in the SAP and HC groups. Surprisingly, the analysis of the iNKT subpopulations did not show significant statistical differences between the three groups. More recently, Bienneman *et al.*⁴⁰ suggested that the number of iNKT cells could be constant through the life of the subject. Therefore, the high variability in the values found could be credited to unique factors pertinent to each subject.

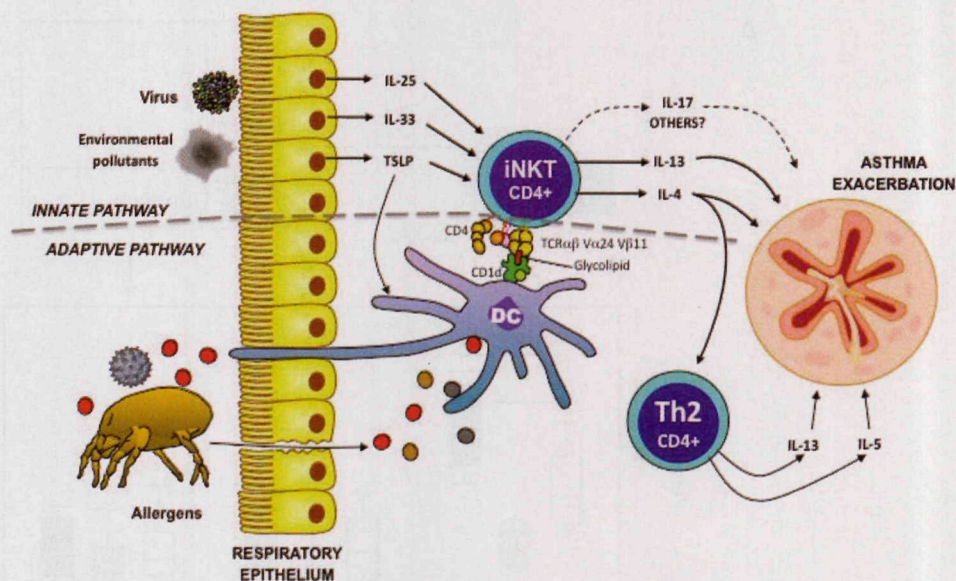


Figure 4. Schematic representation of a twofold innate/adaptive immune participation of the invariant natural killer T (iNKT) cells in the exacerbation of asthma. Initial triggering of the innate immune response pathway by virus and environmental pollutants can activate epithelial cells of respiratory epithelium to produce thymic stromal lymphopoietin (TSLP), IL-25, and IL-33. These cytokines, in turn, can stimulate invariant CD4⁺ T (iNKT) cells to produce IL-4, IL-13, and probably IL-17 and other cytokines, which directly drive airway changes resulting in exacerbation of an asthmatic attack. Subsequent activation of an adaptive immune response resulting from allergen processing in dendritic cells (DCs), presentation of CD1d-restricted glycolipids and recognition by iNKT cells provide an additional pathway for exacerbation of an asthmatic attack.

The finding of increased quantities of CD161-bearing cells in the present study is likewise noteworthy. The CD161 molecule is found on Th17 cells and either $\alpha\beta$ or $\gamma\delta$ T-cell receptor cells.^{41,42} Previously, we have shown that frequency of CD161⁺ T peripheral cells among asthmatic patients and healthy subjects are comparable.¹² However, during the exacerbation, circulatory CD161⁺ T cells are activated and tend to produce more IFN- γ , suggesting that CD161⁺/CD8⁺ T cells producing IFN- γ can help restore the Th1/Th2 balance during the asthmatic attack. Here, we have shown that the frequency of CD161⁺/CD8⁺ cells producing IL-4 is higher among asthmatic individuals regardless of the occurrence of an attack. Because CD161⁺ T cells are mainly memory cells, they may play a unique role in the clinical expression of asthma, specifically during the asthmatic exacerbations. Moreover, their presence in the BAL fluid from patients with moderate-to-severe asthma also supports their participation in asthma. Thus, the characteristic cytokine profile of this cell type most probably has important implications in the pathogenesis of asthma both locally and systemically during the occurrence of exacerbations.

The Question Important for the Health Care Provider Entrusted to the Care of the Patient with Asthma is, What is the Clinical Significance of These Findings of Increased iNKT Cells?

Based on the findings of the present study and from available knowledge derived from the literature it may

be possible to formulate a central unifying hypothesis concerning the role of the iNKT cells in the exacerbation of asthma. The early innate immune response is most probably the initial mechanism involved in the pathophysiology of asthma exacerbation attacks (Fig. 4). Although several cells could be participating, our results indicate that iNKT cells could play a critical role. On activation, these cells immediately produce massive amounts of Th2 cytokines, most notably IL-4 and IL-13, leading to changes in the bronchial physiology associated with the exacerbation.⁴³ This activation of iNKT cells could be the result of cytokines derived from epithelial cells.⁴⁴ The airway epithelium is the first target in the responses to allergens, viruses, and environmental pollutants^{45–48} resulting in the production of thymic stromal lymphopoietin, IL-25, and IL-33.⁴⁹ Therefore, interaction between the epithelial cells and iNKT cells as well as other innate immune cells (nuocytes, natural helper cells, and others),^{10,50,51} triggers cytokine and chemokine releasing and the subsequent molecular cascade leading to exacerbation. In advanced phases of the process, adaptive immune mechanisms can participate through the allergen glycolipids recognition by iNKT cells⁵² and thymic stromal lymphopoietin-mediated stimulation of dendritic cells.⁵³ Differentiation of naïve T CD4⁺ cells into Th2 cells and expansion of CD4⁺ Th2 memory cells leads to the synthesis and release of Th2 cytokines responsible of perpetuation of the bronchial dysfunction.

In summary, our results support the participation of CD161⁺ and iNKT cells in asthmatic attacks. However, it is of the utmost importance to elucidate the cellular mechanisms by which both cell types participate. Knowledge of these interactive mechanisms involving the iNKT cell and our understanding of its role in the exacerbation of asthma hold great promise in the development of better diagnostic predictive markers of disease progression as well as new forms of therapeutic interventions. Future studies will undoubtedly be enhanced by the availability of a never-ending list of novel immunologic and molecular tools that will most likely help to unravel the pivotal mechanisms responsible for the triggering of the asthmatic attack.

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