

Effect of allergen challenge on the percentage of natural killer T cells in patients with atopic asthma

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Background: Invariant natural killer T (iNKT) cells produce cytokines that can influence the immune response to infection or allergen. Controversy surrounds their role in exacerbations of human atopic asthma.

Objectives: To determine the effect of allergen challenge on iNKT cells' mobilization to the airways and blood and to establish the relationship between airway iNKT cells and bronchial sensitivity to methacholine and allergen in patients with atopic asthma.

Methods: We performed flow cytometry analysis for the iNKT cell receptor $V\alpha 24$ and $V\beta 11$ on bronchoalveolar lavage (BAL) cells at baseline and 24 hours after segmental antigen challenge (SAC) ($n = 8$) and on peripheral blood mononuclear cells (PBMCs) at baseline and 6 to 7 hours after inhaled allergen ($n = 10$). Challenges were performed using standardized protein allergens to which the participants were sensitive.

Results: The number of BAL eosinophils increased 24 hours after SAC. The low mean (SEM) baseline percentage of iNKT cells in the population of BAL $CD4^+$ T cells remained unchanged 24 hours after SAC (0.035% [0.01%] vs 0.049% [0.02%]; $n = 8$; $P = .50$). Likewise, the mean (SEM) percentage of iNKT cells in PBMCs was unchanged after inhaled allergen provocation (0.068% [0.033%] vs 0.057% [0.026%]; $n = 10$; $P = .10$). No correlation was found between iNKT cells in BAL and the sensitivity to inhaled methacholine or allergen.

Conclusions: The percentages of both BAL and peripheral blood iNKT cells did not increase during allergen provoked asthmatic responses. Determination of iNKT cells in airway biopsy specimens would allow conclusively ruling against mobilization of iNKT cells in allergen-induced asthma exacerbation in humans.

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INTRODUCTION

Invariant natural killer T (iNKT) cells belong to a distinct small subset of lymphocytes that express surface markers characteristic of both natural killer cells and T lymphocytes. Human iNKT cells possess a highly restricted T-cell receptor consisting of α chain ($V\alpha 24$ - $J\alpha 18$) and invariant β chain ($V\beta 11$) that recognizes conserved glycolipid antigens presented by the major histocompatibility complex class I-like protein CD1d expressed on dendritic cells, monocytes, and subtypes of B cells. The antigens recognized by iNKT cells include endogenous, bacterial, and plant pollen glycolipids and α -galactosylceramide. Activated iNKT cells produce potent immunomodulatory mediators, including interferon γ , interleukin (IL) 4, IL-13, IL-5, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor α , and

thereby play important regulatory roles in both innate and adaptive immune responses.¹

Recently, a prominent pathogenic role of iNKT cells has been suggested in human asthma. The origin of this controversial hypothesis comes from experiments that revealed an important role of iNKT cells in allergen-, ozone-, and viral-induced airway hyperreactivity in mice.^{2–7} However, in human asthmatic patients the pathogenic role of iNKT cells has been less well documented. Sen and colleagues⁸ reported increased numbers of $V\alpha 24^+$ iNKT cells in the bronchial mucosa from asthmatic patients compared with controls. Interestingly, they also provided evidence that CCR9 expression was up-regulated in asthmatic iNKT cells as opposed to normal cells. Abkari and coworkers⁹ found that unlike healthy volunteers and patients with sarcoidosis, up to 60% of lymphocytes in bronchoalveolar lavage (BAL) and in the lamina propria of patients with moderate-to-severe asthma were iNKT cells producing T_H2 cytokines. Most recently, Kim and collaborators⁷ described an increased number of IL-13–positive macrophages in BAL from severe asthmatic patients of unknown phenotype compared with healthy subjects imposing the presence of an activated natural killer T cell–macrophage immune axis in asthma. However, neither Vijayanand and coworkers¹⁰ nor Mutalithas and colleagues¹¹ were able to find similarly high numbers of iNKT cells in

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patients with asthma or chronic obstructive pulmonary disease compared with healthy subjects.^{10,11} Therefore, at present the pathogenic role of iNKT cells in asthma in humans remains uncertain.

We tested the hypothesis that allergen provocation would cause mobilization of iNKT cells in the lung and peripheral blood of human allergic asthmatic patients *in vivo*. Both segmental and inhaled challenges using specific allergen are established models of exacerbation in allergic asthmatic patients and provide important tools for the study of the pathophysiology of allergic asthma *in vivo*.^{12,13}

METHODS

Study Participants

Ten patients with mild allergic asthma according to National Asthma Education and Prevention Program guidelines¹⁴ were recruited from the middle Tennessee community (Table 1). All volunteers had positive skin test results with standardized aeroallergens, methacholine challenge, and inhaled allergen challenge during screening before enrollment into the study. Patients who used oral antihistamines and/or nasal or inhaled corticosteroids were asked to discontinue use of the medications at least 48 hours before airway challenges. Pregnant

women were excluded by urine human chorionic gonadotropin testing. No volunteer had a history of a viral respiratory infection in the 6 weeks preceding the study. Volunteers consented to our protocol verbally and in writing. The protocol was approved by the Vanderbilt University Committee for the Protection of Human Subjects.

Study Protocol

Eight of the 10 volunteers underwent bronchoscopy with baseline BAL followed by a segmental antigen challenge (SAC) in a subsegment of the opposite lung. The allergen-challenged subsegment was lavaged 24 hours later. At least 3 weeks after the bronchoscopy studies patients underwent methacholine challenge followed by an inhaled allergen provocation the next day. All 10 patients underwent inhaled allergen challenge. Heparinized blood for isolation of peripheral blood mononuclear cells (PBMCs) was collected before and between 6 and 7 hours after inhalation of allergen.

Allergens

Standardized aeroallergens used in the study included *Dermatophagoides pteronyssinus*, short ragweed, Kentucky bluegrass, Bermuda grass, and cat hair (Greer Laboratories, Lenoir, North Carolina).

Allergy Skin Testing

Skin prick testing was performed with diluent and histamine controls in parallel with a battery of standardized aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat hair, Bermuda grass, Kentucky bluegrass, fescue meadow grass, orchard grass, redtop grass, ryegrass, sweet vernal grass, timothy grass, short ragweed [Greer Laboratories]).¹⁵ The allergen that showed the strongest wheal reaction was selected and subsequently tested intradermally. Intradermal testing was performed by injecting of 0.02 mL of serially diluted antigen extracts from 10⁻⁵ to 10⁻³ (wt/vol) dilutions. The diluent and histamine, 0.01 mg/mL, were used as negative and positive controls, respectively.

Segmental Allergen Challenge

Volunteers were fasted overnight. A questionnaire that targeted asthma symptoms and medications was administered. Spirometry, including forced vital capacity (FVC), forced expiratory volume in 1 second (FEV₁), and FEV₁/FVC ratio, was measured in triplicate and was more than 70% of predicted. None of the patients received inhaled bronchodilators before bronchoscopy. Midazolam, 1 to 2 mg, and fentanyl, 50 µg, were administered intravenously if patients wished conscious sedation. Topical lidocaine (≤7 mg/kg) was used for airway anesthesia. First, the lingual subsegment was lavaged with two 50-mL aliquots of sterile saline and immediately suctioned back. The bronchoscope was then wedged in a subsegment of the right middle lobe, and 2 incremental doses of the allergen to which the volunteers had positive skin test results were delivered, each dose in a 5-mL aliquot of normal saline. The first dose of the allergen was 10-fold higher than the concentration that gave a 2+ skin reaction on intradermal

Table 1. Patient Demographics, Baseline Characteristics, and History of Atopy, Asthma, and Asthma Medication Use

Characteristic	No. of patients ^a
Sex, F/M	5/5
Age for F/M, mean (range), y	31.8 (25–43)/27.2 (23–29)
Race	
White	9
African American	1
Weight, mean, kg	72.8
Mean height, cm	169.7
Family history of atopy	10
History of allergic rhinitis	10
Frequency of daily asthma symptoms	
≤2 days per week	6
>2 days per week but not daily	4
Nighttime awakenings	
≤2 days per week	10
>2 days per week but not daily	0
Baseline spirometry, % predicted	
FEV ₁ , mean (range), % predicted	90 (75–101)
FEV ₁ /FVC, mean (range)	0.69 (0.56–0.76)
Methacholine PC ₂₀ , mean (range), mg/mL	2.49 (0.14–10)
Medications	
Histamine ₁ -antihistamine	8
Nasal corticosteroids	1
Low-dose inhaled corticosteroid	2
Albuterol ≤2 days per week	10

Abbreviations: FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; PC₂₀, provocation concentration causing a decrease of more than 20% in the baseline FEV₁.

^aData are number of patients unless otherwise indicated.

testing (5- to 8-mm wheal, 20- to 30-mm erythema). The subsegment was watched for 3 minutes. None of the volunteers developed visible local inflammatory reaction after the first dose of allergen. Thus, in each volunteer the second dose of the same allergen was instilled at a concentration 100-fold higher than the 2+ skin reaction, and the bronchoscope was then removed.¹⁶ Postbronchoscopy monitoring included spirometry. Four patients received albuterol metered-dose inhaler treatment because of a mild decrease in FEV₁ (not exceeding 10% of the baseline) and/or mild asthma symptoms. The following day another bronchoscopy was performed and the allergen challenge subsegment was identified and lavaged.

Methacholine Challenge

The methacholine (Provocholine; Methapharm Inc, Brantford, Ontario, Canada) challenge was undertaken according to the American Thoracic Society guidelines.¹⁷ Doubly increasing aerosolized concentrations were given by inhalation of 5 slow inspiratory capacity breaths starting at a concentration of 0.075 mg/mL to a maximum of 20.0 mg/mL or until a decrease of more than 20% in the baseline FEV₁ occurred (methacholine PC₂₀). Methacholine solutions were aerosolized through a DeVilbiss 646 nebulizer connected via a dosimeter set to open for the first 0.6 second of inspiration to a wall air source at 20 psi. Measurements of pulmonary function FEV₁ were made using a Renaissance II spirometer (Puritan-Bennett, Carlsbad, California). After demonstrating a more than 20% decrease in FEV₁, all patients were treated with albuterol metered-dose inhaler and monitored until spirometry results returned to baseline.

Inhaled Allergen Challenge

Allergen inhalation challenge was performed 24 hours after methacholine challenge as recently described.¹⁸ In brief, the initial dose of allergen was calculated based on the value of methacholine PC₂₀ and the skin prick test end point. Allergen aerosols were generated using a Wright nebulizer connected to a 2-way Hans Rudolph valve mouthpiece (Roxon, Montreal, Canada) attached to a wall oxygen source at 50 psi. Before the challenge the asthma questionnaire was administered and baseline pulmonary function was measured in triplicate.

All patients manifested baseline FEV₁ and FEV₁/FVC within 10% of the previous day's measurements; thus, provocations were performed in all volunteers. Doubling increases in allergen concentration were inhaled for 2 minutes during tidal breathing using a nose clip and separated by intervals of 12 minutes. The range of allergen concentrations sufficient to provoke at least a 20% decrease in FEV₁ (allergen PC₂₀) was 1:8 to 1:512 of the stock solution. Triplicate measurement of pulmonary function was performed at baseline, then every 15 minutes for the first hour after allergen inhalation, and then hourly for 8 hours after inhalation challenge. The greatest decrease in FEV₁ between 0 and 3 hours after allergen was taken as the early airway response. Like-

wise, the greatest FEV₁ decrease at 3 to 8 hours was the late airway response. The end point was a 20% or more decrease in FEV₁. None of the participants was treated with albuterol during the 8-hour period of the challenge.

Isolation of PBMCs

Blood samples were collected, labeled for identification, held at room temperature, and processed within 2 hours. PBMCs were isolated by Ficoll-Hypaque (Sigma-Aldrich, St Louis, Missouri) density gradient centrifugation¹⁹ and resuspended at a concentration of 5×10^6 cells/mL in freezing medium containing 90% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, California) and 10% dimethyl sulfoxide. The cells were then transferred to cryogenic vials, in turn placed in freezing containers (Nalge Nunc International, Rochester, New York), and stored at -80°C . Frozen specimens were transferred to a liquid nitrogen freezer and stored in the vapor phase. At the time of analysis, cryopreserved cells were thawed in a 37°C water bath, incubated with 20 $\mu\text{g/mL}$ of DNase (Roche, Basel, Switzerland), and washed twice. Viability was determined by trypan blue exclusion. Only samples with viability of 80% or more were used for analysis.

Analysis of iNKT Cells by Flow Cytometry

iNKT cells were quantified using thawed PBMC or fresh BAL samples by multicolor flow cytometry. Samples were washed and cells resuspended in minimal volume of FACS buffer (phosphate-buffered saline +1% bovine serum albumin). Surface staining for phenotypic markers was performed with directly conjugated antibodies for 30 minutes at room temperature. If excess red blood cells were present, samples were treated with BD Lyse solution per manufacturer's protocol before staining. After a final wash, cells were resuspended in 0.5% paraformaldehyde. Multiparametric flow cytometry was performed using a LSRII cytometer (BD Immunocytometry Systems, Franklin Lakes, New Jersey), equipped with 3 lasers (488-nm solid state, 405-nm solid state, and 633 HeNe). Instrument compensation was performed with antibody capture beads (BD Pharmingen, Franklin Lakes, New Jersey) stained singly with individual antibodies used in the test samples. In all cases a total of 500,000 cells were acquired. Data analysis was performed using Flow Jo version 8.7 (Tree Star, Ashland, Oregon). All flow cytometric dot plots were displayed with 5-decade logarithmic axes (except forward and side scatter, linear) with a data transformation that allows for improved visibility of events that fall at the lower end of the log scale. The following antihuman antibodies were used: V α 24 fluorescein isothiocyanate (Coulter clone C15), V β -11 phycoerythrin (PE) (Coulter clone C21), CD4 Cy5.5-peridinin chlorophyll protein (BD Bioscience clone SK3), CD8 Cy7-PE (BD Bioscience clone RPA-T8), and CD3 allophycocyanin (BD Bioscience clone HIT3a). All antibodies were pretitered to optimal concentrations. Figure 1 shows representative flow cytometric analysis of iNKT cells in BAL fluid and blood.

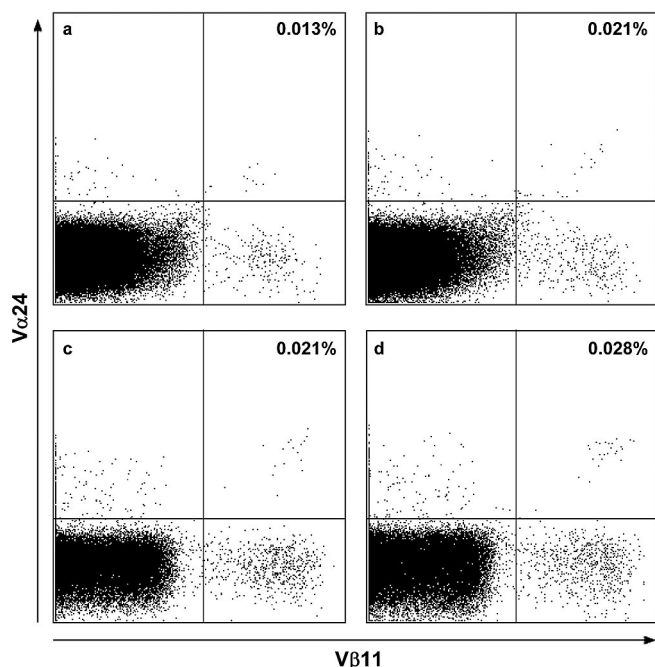


Figure 1. Representative flow cytometric dot plots comparing population of Va24- and Vβ11-positive cells among bronchoalveolar lavage cells at baseline and 24 hours after segmental antigen challenge (A and B) and among peripheral blood mononuclear cells at baseline and 6 hours after inhaled allergen challenge (C and D).

Statistical Analysis

Comparisons between baseline variables and those after allergen challenge were made using a nonparametric paired Wilcoxon signed rank test and Pearson correlation coefficient using the software package SPSS (SPSS Inc, Chicago, Illinois). Significance was accepted when $P < .05$. Means (SEMs) were calculated.

RESULTS

Recruitment of Inflammatory Cells Into BAL After SAC

At 24 hours after SAC, a significant increase in the percentage of eosinophils (0.7 [0.36] vs 15.7 [8.3]; $n = 8$; $P = .01$) occurred. The percentage of neutrophils was also increased and nearly reached statistical significance (4.3 [1.2] vs 6.6 [17.1]; $P = .06$).

Effect of SAC on iNKT cells in BAL

The frequency of iNKT cells in BAL (percentage of CD4⁺ lymphocytes) was low at baseline and did not increase significantly 24 hours after SAC (0.035% [0.01%] vs 0.049% [0.02%]; $P = .50$) (Fig 2).

Effect of Inhaled Allergen Challenge on iNKT Cells in Peripheral Blood

All patients had positive methacholine challenge results (Table 1 for the range of PC₂₀) and positive inhaled allergen challenge results. Four asthmatic patients developed late air-

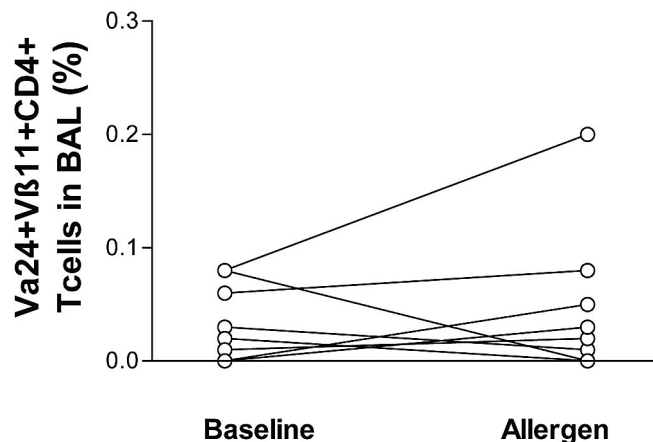


Figure 2. Percentage of invariant natural killer T cells in the population of bronchoalveolar lavage (BAL) lymphocytes at baseline and after segmental antigen challenge for each study participant.

way response. In all study participants ($n = 10$) the baseline percentage of iNKT in peripheral blood (presented as the percentage of CD4⁺ cells) was low and did not increase at 6 to 7 hours after inhalation of specific allergen (0.068% [0.033%] vs 0.057% [0.026%]; $P > .10$) (Fig 3). Additional analysis of peripheral blood iNKT at baseline and in 2-hour intervals after allergen challenge in 3 of these patients did not show any changes at any time point (data not shown).

Correlation Among BAL iNKT Cells, Methacholine PC₂₀, and the Provoking Dose of Allergen

No correlation was found between baseline frequencies of iNKT cells in BAL and methacholine PC₂₀ (Pearson correlation coefficient, $P > .05$). Likewise, no association between baseline iNKT cells in BAL and allergen PC₂₀ was present.

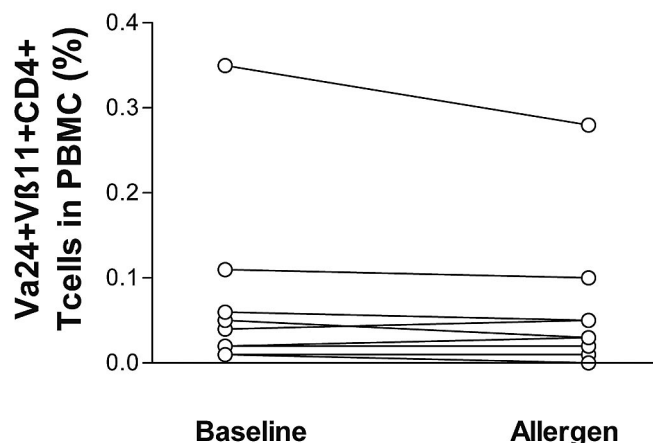


Figure 3. Percentage of invariant natural killer T cells in the population of peripheral blood mononuclear cells (PBMC) at baseline and after inhaled allergen provocation for each study participant.

DISCUSSION

Our study showed no change in the percentage of iNKT cells in BAL at 24 hours after allergen challenge compared with control BAL in well-characterized human allergic asthmatic patients *in vivo*. The patients had a small percentage of iNKT cells at baseline that did not significantly increase 24 hours after allergic stimulation. The significant increase in eosinophils recovered at 24 hours demonstrates the successful induction of allergic inflammation by the instilled allergen. Likewise, the small subpopulation of peripheral blood iNKT cells detected at baseline was not affected by an inhaled allergen that produced significant bronchoconstriction in all studied patients.

Recently, some investigators have proposed a prominent pathogenic role for iNKT cells in asthma.⁹ iNKT cells, with their capacity to rapidly produce large amounts of proallergic and proasthmatic mediators, could be important in asthma pathogenesis.¹ A vital role of iNKT cells has been documented in animals.²⁻⁷ However, little is known about the function of these cells in human asthmatic patients. Therefore, determination of whether iNKT cells are mobilized into asthmatic airways by specific allergen is an important step in unraveling the role of these cells in asthma.

Thomas and coworkers²⁰ recently showed no influx of iNKT cells into the airways of 5 patients with mild asthma after SAC. In a monkey model of dust mite asthma, no increase in the iNKT cells in BAL fluid during either early or late asthmatic responses was found.²¹ Our study strengthens these data and provides new information about the lack of effect of allergen exposure on circulating iNKT cells as well as those in the BAL fluid of asthmatic patients. A potential limitation of our and other investigators' studies is lack of data showing effects of allergen exposure on iNKT cells in the airway tissue, because biological fluids may not exactly mirror the traffic of inflammatory cells in bronchial mucosa in asthma.²² Hence, it cannot be concluded with certainty that allergen exposure does not provoke influx of iNKT cells into asthmatic airways. Another potential weakness of our study is the lack of serial BAL sampling after provocation because the allergen-induced airway inflammation is a dynamic process that involves a time-dependent chain of cellular and biochemical responses. However, although not assuring, the lack of an increase in the number of peripheral blood iNKT cells after exposure to allergen supports the supposition that iNKT cells are not mobilized by protein allergens in allergic asthmatic patients.

We found a low percentage of iNKT cells in our patients. Because the study did not include healthy subjects, we are unable to conclude if the frequency of iNKT cells is elevated in asthmatic airways even if the cells represent only a small fraction of the BAL CD4⁺ cell population. One study showed a slightly increased percentage of iNKT cells among T cells in BAL fluid of children with atopic asthma compared with healthy controls and children with bronchiectasis, although the iNKT cells consisted of only 0.4% of the BAL T cells.²³

Nevertheless, our data concur with several investigations that demonstrated a similarly low percentage of iNKT cells in the BAL fluid of asthmatic patients.^{10,11,24-26} Only 1 study⁹ found an abundantly increased frequency of iNKT cells in the BAL fluid of asthmatic patients compared with healthy patients and patients with sarcoidosis. In addition to an analytical error causing overestimation of the real percentage of iNKT cells suggested by some authors,¹⁰ another possible explanation of the discrepancy could rely on a different selection of patients. Our study included only mild allergic, predominantly white asthmatic patients. Two volunteers who used low doses of inhaled corticosteroids discontinued the treatment for 5 days before each study. Patients with moderate-to-severe asthma and nonatopic asthmatic patients were not allowed to participate in our study. On the other hand, the low frequency of iNKT cells in peripheral blood in our patients concurs with other publications.^{9,20,27,28}

We found no association between the number of iNKT cells in BAL fluid and either the provocative dose of methacholine or the specific allergen that caused a 20% decrease in FEV₁ in our patients. This new observation may suggest that unlike in the mouse model of airway hyperreactivity,^{2-4,6} in human allergic asthmatic patients neither nonspecific airway reactivity nor hypersensitivity to specific allergens critically depends on iNKT cells. The discrepancy is not unexpected because significant phenotypical and functional distinctions between the mouse and human iNKT cells have been described.²⁹ On the other hand, our study does not exclude an important pathophysiologic role of iNKT cells in nonatopic asthma.³⁰ As already mentioned, on activation through binding of glycolipid antigens to the V α 24V β 11 in a CD1d-restricted fashion, iNKT cells can rapidly produce large quantities of cytokines.¹ Distinct functional lineages of human iNKT cells have been described in peripheral blood. Whereas double negative iNKT cells (CD4⁻CD8⁻) strictly produce T_H1 cytokines, CD4⁺ iNKT cells predominantly generate T_H2 cytokines, particularly IL-4 and IL-13.^{26,31,32} It has been mentioned (although solid data not provided) that iNKT cells recovered from asthmatic airways are particularly T_H2 skewed.³³ If this is true it will be important to establish if the predominantly T_H2 cytokines producing iNKT cells are specifically attracted to the asthmatic lungs or if local mechanism cause up-regulation of T_H2 cytokines genes in the existing cells. Currently, however, neither mechanisms regulating homing nor activation of iNKT cells, especially in humans, has been precisely characterized. Although protein allergens are not capable of activating iNKT cells, one could speculate that certain ligands could be of importance in provoking or promoting allergic inflammation in human asthmatic patients through stimulation of iNKT cells. These hypothetical ligands could include some pollen, bacterial, and even endogenous glycolipids.^{34,35} Furthermore, this supposition could be tested in carefully designed studies in humans.

Allergists should be familiar with the novel hypothesis about possible involvement of iNKT cells in asthmatic inflammation. iNKT cells have the potential to profoundly

affect human asthma and atopy, but the initial observations of markedly increased numbers of these cells in human asthma have not been confirmed. Although iNKT cells have the potential to modify atopic responses, we could find no evidence in our study that they do so. Given the lack of adequate animal models of asthma, human studies are particularly valuable in exploring mechanisms of allergic and asthmatic airway inflammation,

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