

The Role of Natural Killer T Cells in the Pathogenesis of Acute Exacerbation of Human Asthma

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Key Words

Asthma • Exacerbation • Infection • Natural killer T cells

Abstract

Background: Natural killer T (NKT) cells have been reported to play a crucial role in the pathogenesis of asthma in a mouse model of acute asthma. The present study aimed to investigate the role of NKT cells in the immune pathogenesis of acute exacerbation of human asthma. **Methods:** Blood and sputum were obtained at baseline and 8 h after a challenge in 20 asthmatics who underwent allergen bronchial provocation testing and during exacerbation and convalescence in 9 asthmatics who were admitted to hospital with an acute exacerbation after an upper respiratory tract infection. 6B11+ or V α 24+ NKT cells were measured with flow cytometry. Inflammatory cells, cytokines and chemokines were determined in sputum. **Results:** The number of blood NKT cells did not change after a positive allergen challenge compared to the baseline. However, blood CD4+V α 24+ NKT cells decreased during infection-associated asthma exacerbations compared to the convalescence measurements of the same patients ($p < 0.05$) or the baseline measurements of asthmatics who underwent allergen challenges ($p < 0.01$). The number of sputum NKT cells did not change after a positive al-

lergen challenge or during infection-associated asthma exacerbations. Eosinophils and various cytokines and chemokines increased in sputum during infection-associated asthma exacerbations. Blood CD4+V α 24+ NKT cells were inversely related to sputum eosinophils (Spearman's correlation coefficient = -0.62 ; $p < 0.01$). **Conclusion:** Blood NKT cells decreased during infection-associated asthma exacerbation and were inversely associated with eosinophilic airway inflammation, suggesting that blood NKT cells might be mobilized to the airways and lungs during asthma exacerbation in humans.

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Introduction

Asthma is characterized by chronic airway inflammation, airway hyperresponsiveness (AHR) and reversible airflow obstruction [1]. Asthma is well controlled with inhaled corticosteroids (ICS); however, acute exacerbations often occur and may be life-threatening. Asthma can be exacerbated by respiratory viruses, allergens, occupational agents, aspirin and air pollution [2]. Treatment of asthma attacks increases medical costs and can reduce the quality of life of patients and their families

[3–5]. There has been research to develop new medications to reduce the frequency and severity of acute asthma attacks [6]. However, an incomplete understanding of the immune pathogenesis of acute exacerbations has limited their development.

It has been reported that natural killer T (NKT) cells may play an important role in the pathogenesis of asthma [7]. NKT cells are unique CD1d-restricted T cells with surface markers of NK cells; they act as a bridge between the innate and acquired immune response. NKT cells secrete very large amounts of Th1 or Th2 cytokines immediately following activation and may affect dendritic cells, NK cells, B cells and conventional CD4+/CD8+ T cells [8]. The invariant NKT (iNKT) cells express very limited T cell receptors (TCRs), namely V α 14-J α 18 in mice and V α 24 in humans; they respond to α -galactosylceramide, a synthetic glycolipid from the marine sponge. Recently, iNKT cells have been reported to play an essential role in the development of ovalbumin-induced [9], α -galactosylceramide-induced [10] or ozone-induced [11] AHR in a mouse model of asthma. Non-invariant NKT cells may also play a role in the development of allergen-induced AHR [12].

It has been reported that NKT cells are increased in the airways of patients with asthma [13]; however, some investigations have not demonstrated an increase in NKT cells in the airways [14]. Recently, we measured V α 24+ NKT cells in the blood of stable asthma patients and showed an inverse association between blood NKT cells and atopic indexes, such as serum total immunoglobulin (Ig) E levels and the atopy score on skin prick testing [15]. In addition, NKT cells were analyzed in the induced sputum of patients with asthma and eosinophilic bronchitis, and the NKT cells were probably associated with the development of eosinophilic airway inflammation [16]. Therefore, it is probable that NKT cells play an important role in the immune pathogenesis of human asthma.

However, the role of NKT cells in the pathogenesis of acute asthma in humans has not been defined. The role of NKT cells in the pathogenesis of asthma has been demonstrated in an acute asthma model in mice. Therefore, it is reasonable to suppose that NKT cells may play a role in the pathogenesis of acute exacerbation of asthma in humans.

Asthma exacerbation occurs after allergen exposure and more commonly upper respiratory tract infection (URI). Thus, in the present study, NKT cells were analyzed in peripheral blood mononuclear cells (PBMCs) as well as in induced sputum before and after challenges with an allergen or during URI-associated asthma exacer-

beration and convalescence. The goal was to determine the role of NKT cells in the immune pathogenesis of acute asthma exacerbation.

Subjects and Methods

Subjects

Twenty asthmatics who underwent allergen bronchial provocation testing with *Dermatophagoides farinae* and 9 patients who were admitted to hospital with acute asthma exacerbation following URI were included in this study. Allergen challenge with *D. farinae* was performed in patients who had typical asthma symptoms, AHR or significant reversible airflow obstruction and sensitization to *D. farinae*. Reversible airflow obstruction was considered to be present if there was increased forced expiratory volume in 1 s (FEV₁) $\geq 12\%$ and ≥ 200 ml after bronchodilator testing. AHR was considered to be present if the provocative concentration of methacholine causing a 20% fall in FEV₁ from the saline value (PC₂₀) was less than 25 mg/ml on methacholine bronchial provocation testing. Subjects with acute asthma exacerbation were included if their asthma symptoms and lower lung function were newly worse following URI [17] and improved with asthma treatments.

Chronic asthma severity was classified according to the Global Initiative for Asthma guidelines of 2002 [17]. Briefly, the severity was classified as intermittent, mild persistent, moderate persistent or severe persistent asthma, based on the clinical features and lung function present before treatment was started. If the subject was already on treatment, the classification of severity was based on the clinical features present and the stage of the daily medication regimen that the subject was currently at. Additionally, asthma exacerbation severity in the emergency care setting was classified as mild, moderate, severe or respiratory arrest imminent according to the Global Initiative for Asthma guidelines of 2002. Tests for blood eosinophils and total serum IgE and skin prick testing were performed, if indicated. All subjects agreed to participate in the study and provided written consent. The ethical review board of Chonnam National University Hospital approved this study.

Skin Prick Tests and Atopy

A standardized skin prick test was performed using 12 common aeroallergens (*Dermatophagoides pteronyssinus*, *D. farinae*, cat, dog, *Alternaria tenuis*, Trichophyton, cockroach, alder, birch, orchard, mugwort and ragweed; Allergopharma, Reinbek, Germany). A subject was classified as atopic if any allergen caused a wheal 3 mm or larger in diameter compared to the negative controls. The degree of skin reaction was classified according to the ratio of the wheal size in response to allergen to that in response to histamine: 1+ = ≥ 25 and $< 50\%$; 2+ = ≥ 50 and $< 100\%$; 3+ = ≥ 100 and $< 200\%$; 4+ = $\geq 200\%$.

Allergen Bronchial Provocation Test

This test was performed with *D. farinae* extract (Arthropods of Medical Importance Resource Bank, Seoul, Korea). Undiluted stock solution of *D. farinae* was prepared, and the concentration of protein was 0.46 μ g/ml measured by the Lowry method [18]. After the baseline FEV₁ was measured (Spiro Analyzer ST-250,

Fukuda Sangyo, Tokyo, Japan), saline and the serial solutions of *D. farinae* were inhaled with a 2-min tidal breathing method using the DeVilbiss 646 nebulizer (DeVilbiss Co., Somerset, Pa., USA; output 0.13 ml/min). The FEV₁ was measured 5, 10 and 20 min and 1 h after inhalation and then at 1-hour intervals for 8 h. At first, a 10-fold diluted solution of *D. farinae* was inhaled, and then undiluted solution was inhaled if early asthmatic reactions did not occur.

An early asthmatic reaction was defined as having occurred when the FEV₁ fell by more than 20% during the first 2 h after the allergen challenge compared to saline. A late asthmatic reaction was defined as having occurred when the FEV₁ fell by more than 20% between 3 and 8 h [19]. Blood and induced sputum were obtained at baseline and 8 h after the challenges.

Asthma Exacerbation after URI

Patients presenting with an acute asthma attack received nebulized medications for quick relief, such as salbutamol and ipratropium bromide. Blood and induced sputum were obtained immediately. Other quick-relief medications, such as systemic corticosteroids and aminophylline, were also administered. After these treatments, medications used for control, such as ICS, a long-acting β_2 -agonist (LABA), a leukotriene receptor antagonist (LTRA) and sustained-release theophylline, were administered. After the asthma symptoms and FEV₁ improved, blood and induced sputum were obtained again during the convalescence stage.

Isolation of PBMCs

Heparinized blood was layered over an equal volume of Lymphoprep™ solution (Axis-Shield PoC AS, Oslo, Norway) and centrifuged at 2,000 rpm for 30 min. PBMCs were isolated from the interface and used to measure the NKT cells as previously described [15].

Sputum Induction and Processing

Sputum induction and processing were performed as previously described [16]. Sputum induction was performed by inhalation of hypertonic saline after premedication with 200 μ g of inhaled salbutamol. The aerosols were generated by an ultrasonic nebulizer (DeVilbiss-Sunrise Medical, Somerset, Pa., USA). For the patients with an acute exacerbation associated with a URI, sputum was obtained while they were inhaling a mixture of saline and salbutamol or ipratropium bromide. Selected sputum samples were weighed and treated with 4 times their volume of 0.1% dithiothreitol (Sigma, St. Louis, Mo., USA). This suspension was filtrated with a nylon filter (Spectra Mesh, Rancho Dominguez, Calif., USA). Cytospins (Sakura, Tokyo, Japan) were prepared on glass slides, and the preparations were stained with Diff-Quik (Sysmex Co., Kobe, Japan). The supernatants were kept at -70°C until the assays for cytokines and chemokines were performed. All cells were enumerated and counted, which allowed for the calculation of both the percentage of squamous cells among the total number of cells and the percentages of macrophages, neutrophils, eosinophils and lymphocytes among the nonsquamous cells. The remaining cells were used to measure the sputum NKT cells.

Flow Cytometric Analysis of NKT Cells

As previously described [15, 16], PBMCs or sputum cells were incubated at 4°C for 30 min with fluorochrome-conjugated monoclonal antibodies, including FITC-anti-CD4 (BD Biosci-

ences, San Jose, Calif., USA), phycoerythrin (PE)-6B11 (BD Biosciences) or PE-anti-TCRV α 24 (Beckman Coulter, Marseille, France), and peridinin-chlorophyll-protein-anti-CD3 (BD Biosciences). However, sputum cells were incubated with 2 mg/ml polyclonal human IgG (Sigma) for 15 min to block nonspecific binding of antibodies to Fc γ receptors prior to staining with the monoclonal antibodies. The 6B11 monoclonal antibody is directed against complementarity-determining region 3 of the V α 24-J α 18 TCR of iNKT cells [20]. PE-mouse IgG₁ (BD Biosciences) was used as isotype control antibody. Flow cytometry was carried out with the 4-color BD FACSCalibur (BD Biosciences). After gating the CD3+ T cells, the frequencies of CD3+6B11+, CD3+V α 24+, CD4+6B11+ and CD4+V α 24+ cells were determined in PBMCs and sputum, as shown in our previous studies [15, 16]. Additionally, absolute numbers of NKT cells were calculated. Blood NKT cell numbers are shown as the cell count per 4×10^4 CD3+ T cells, using a modified method described by Magnan et al. [21]. Sputum NKT cells are expressed as the absolute cell number per gram.

Determination of Cytokines and Chemokines in the Sputum Supernatant

Various cytokines and chemokines were measured with the Procarta cytokine assay kit (Affymetrix, Calif., USA). The sputum supernatants were diluted 5-fold with PBS, and the viscosity was reduced by body fluid buffer. The sample was incubated with antibody beads for 1 h and detection antibody for 30 min at room temperature, while being shaded from the light. After incubation with streptavidin-PE for 5 min, the following cytokines and chemokines were determined with the Luminex 200 (Mirai Bio, Calif., USA): interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, interferon (IFN)- γ , granulocyte macrophage colony-stimulating factor, tumor necrosis factor- α , granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1 β , monocyte chemotactic protein-1, eotaxin, basic fibroblast growth factor and vascular endothelial growth factor.

Statistical Analysis

Data are expressed as medians (interquartile range). Differences within groups were compared using the Wilcoxon test. Two different groups were compared by the Mann-Whitney U test or χ^2 test. Relationships between parameters were analyzed with Spearman's correlation coefficient (SPSS 18.0 for Windows, SPSS Inc., Chicago, Ill., USA). A p value less than 0.05 was considered significant.

Results

Characteristics of the Subjects

Patients Who Underwent the Allergen Bronchial Provocation Test

The characteristics of each of these patients are shown in table 1. Eight out of 20 patients (40%) who underwent the provocation test had early or late asthma reactions, and 12 (60%) had no asthma reactions. There were no differences in age, gender, the number of current smok-

Table 1. Characteristics of asthmatics who underwent the allergen bronchial provocation test

Patient No.	Age, years	Sex	FEV ₁ % predicted	Skin reactivity to <i>D. farinae</i>	Maximum fall in FEV ₁ after challenge, %	
					EAR ^a	LAR ^b
<i>Positive allergen challenge</i>						
1	49	male	72.7	4+	28.2	−2.3
2	19	male	80.7	4+	46.2	24.3
3	46	female	77.7	4+	37.3	23.0
4	50	female	59.2	3+	33.1	8.5
5	49	female	48.7	4.2 ^c	23.6	18.4
6	49	male	76.7	3+	23.2	6.4
7	29	male	68.9	4+	36.4	8.9
8	55	female	93.7	4+	20.5	6.8
Median (IQR)	49.0 (33.2–49.7)		74.7 (61.6–80.0)		30.7 (23.3–37.1)	8.7 (6.5–21.9)
<i>Negative allergen challenge</i>						
9	28	male	82.4	4+	3.1	0.5
10	20	male	80.7	4+	2.3	4.4
11	65	female	96.9	2+	16.5	−0.4
12	46	female	95.4	4+	8.3	19.1
13	69	male	104.8	2+	1.6	0.3
14	24	male	89.6	ND	6.7	7.5
15	28	male	79.9	0	4.8	2.5
16	33	male	92.6	2+	−0.7	−1.7
17	20	male	65.0	0	0	−32.3
18	29	male	115.0	3+	12.0	5.3
19	26	male	89.0	3+	14.7	18.9
20	39	female	90.5	4+	8.2	1.4
Median (IQR)	28.0 (24.5–44.2)		90.1 (81.1–96.6)		5.8 (1.8–11.1)	2.0 (−0.2–7.0)

EAR = Early asthmatic response; LAR = late asthmatic response; ND = not determined; IQR = interquartile range.

^a Defined as a $\geq 20\%$ fall in FEV₁ between 0 and 2 h after allergen challenge. ^b Defined as a $\geq 20\%$ fall in FEV₁ between 3 and 8 h after allergen challenge. ^c Denotes class determined by multiple-allergen simultaneous test.

ers (37.5 vs. 33.3%), the presence of atopy (100.0 vs. 91.7%), asthma duration [13.0 years (4.0–27.5) vs. 5.5 years (0.3–10.0)], methacholine PC₂₀ [2.3 mg/ml (0.3–12.9), n = 5, vs. 5.0 mg/ml (1.9–16.8), n = 9], serum total IgE [525.5 IU/ml (81.9–1,805) vs. 288.5 IU/ml (110.9–1,817.5)] or blood eosinophil counts [189.9/ μ l (138.0–505.8) vs. 340.4/ μ l (126.4–795.0)] between the positive and negative challenge groups ($p > 0.05$ for all). The baseline FEV₁ was significantly lower in the positive challenge group than in the negative challenge group ($p < 0.05$). The number of patients using ICS, LABAs, LTRAs and theophylline did not differ between the two groups. High doses of ICS were used by 3 patients (37.5%) with positive challenges, but none of the patients with negative challenges used high doses of ICS. The number of subjects with severe persistent asthma was higher in the positive challenge group than in the negative group (37.5 vs. 0%; $p < 0.05$).

Patients with Acute Exacerbation of Asthma following URI

The characteristics of these patients are summarized in table 2. Asthma exacerbations occurred following URI in all patients. They presented to the hospital 7.0 days (4.0–12.5) after URI. There was 1 current smoker (11.1%), and the asthma duration was 2.0 years (0.7–24.5). Total serum IgE and blood eosinophils on admission were 261.0 IU/ml (145.5–792.0) and 812.0/ μ l (367.8–1,238.5), respectively. Only 2 patients (22.2%) were taking medications to control their asthma, including ICS, before admission. All patients had severe asthma exacerbations, and their PaCO₂ levels on admission were 37.6 mm Hg (35.1–40.6). There was no pneumonia in any of the patients. Salbutamol and ipratropium were used in all patients; systemic steroids were administered in 8 patients (88.9%), and aminophylline was used in all patients. As the acute exacerbation improved, control medications,

Table 2. Characteristics of patients with asthma exacerbation following URI

Patient No.	Age, years	Sex	FEV ₁ on admission, % predicted	Exacerbation period, days ^a	Best FEV ₁ after treatment % predicted
1	51	female	59.8	0.5	101.5
2	18	male	22.6	7	110.1
3	33	male	50.5	15	100.7
4	48	male	29.8	5	73.7
5	21	female	35.4	4	82.3
6	56	female	59.5	7	90.4
7	56	female	46.6	4	102.4
8	47	female	10.4	15	101.6
9	40	male	45.5	10	100.0
Median (IQR)	47.0 (27.0–53.5)		45.5 (26.2–55.0)	7.0 (4.0–12.5)	100.7 (86.3–102.0)

IQR = Interquartile range.

^a Denotes the days elapsed from the onset of asthma exacerbation.

such as ICS, LABAs, LTRAs and theophylline, were used for treatment. Blood and sputum were obtained during convalescence, which occurred over a period of 10.0 days (7.0–13.0) after admission.

Change in the Numbers of Blood and Sputum NKT Cells

Change after Allergen Challenge

The numbers of blood CD3+6B11+ NKT cells [0.25% (0.07–0.34) of CD3+ T cells vs. 0.23% (0.07–0.30) of CD3+ T cells; 98 (25–134) cells/4 × 10⁴ CD3+ T cells vs. 92 (26–121) cells/4 × 10⁴ CD3+ T cells], CD3+Vα24+ NKT cells [0.30% (0.23–0.41) of CD3+ T cells vs. 0.35% (0.27–0.46) of CD3+ T cells; 120 (91–164) cells/4 × 10⁴ CD3+ T cells vs. 140 (109–185) cells/4 × 10⁴ CD3+ T cells] and CD4+Vα24+ NKT cells [0.20% (0.05–0.23) of CD3+ T cells vs. 0.18% (0.07–0.26) of CD3+ T cells; 80 (21–91) cells/4 × 10⁴ CD3+ T cells vs. 70 (29–102) cells/4 × 10⁴ CD3+ T cells] did not change after a challenge compared to the baseline in subjects with positive challenges ($p > 0.05$ for all). The numbers of blood CD3+6B11+ NKT cells [0.14% (0.09–0.19) of CD3+ T cells vs. 0.17% (0.12–0.24) of CD3+ T cells; 56 (37–75) cells/4 × 10⁴ CD3+ T cells vs. 66 (48–95) cells/4 × 10⁴ CD3+ T cells], CD3+Vα24+ NKT cells [0.31% (0.16–0.51) of CD3+ T cells vs. 0.36% (0.19–0.52) of CD3+ T cells; 124 (63–204) cells/4 × 10⁴ CD3+ T cells vs. 144 (76–207) cells/4 × 10⁴ CD3+ T cells] and CD4+Vα24+ NKT cells [0.17% (0.11–0.27) of CD3+ T cells vs. 0.21% (0.06–0.26) of CD3+ T cells; 68 (42–108) cells/4 × 10⁴ CD3+ T cells vs. 82 (25–103) cells/4 × 10⁴ CD3+ T cells] did not change after a challenge compared

to the baseline in subjects with negative challenges ($p > 0.05$ for all). However, CD4+6B11+ NKT cells increased slightly after a challenge compared to the baseline in subjects with a positive challenge [0.09% (0.04–0.12) of CD3+ T cells vs. 0.06% (0.04–0.09) of CD3+ T cells; 36 (15–48) cells/4 × 10⁴ CD3+ T cells vs. 24 (14–34) cells/4 × 10⁴ CD3+ T cells; $p = 0.04$] but not in those with a negative challenge [0.05% (0.04–0.08) of CD3+ T cells vs. 0.06% (0.04–0.09) of CD3+ T cells; 19 (15–31) cells/4 × 10⁴ CD3+ T cells vs. 23 (17–34) cells/4 × 10⁴ CD3+ T cells; $p > 0.05$].

The numbers of sputum CD3+6B11+ NKT cells [0.28% (0.00–0.66) of CD3+ T cells vs. 1.13% (0.41–1.33) of CD3+ T cells; 6 cells/g (0–17) vs. 4 cells/g (2–41)], CD3+Vα24+ NKT cells [0.00% (0.00–0.12) of CD3+ T cells vs. 0.25% (0.08–1.17) of CD3+ T cells; 0 (0–2) cells/g vs. 4 cells/g (0–9)], CD4+6B11+ NKT cells [0.22% (0.00–0.62) of CD3+ T cells vs. 0.61% (0.38–1.02) of CD3+ T cells; 5 cells/g (0–15) vs. 3 cells/g (1–32)] and CD4+Vα24+ NKT cells [0.00% (0.00–0.09) of CD3+ T cells vs. 0.25% (0.08–0.92) of CD3+ T cells; 0 cells/g (0–2) vs. 3 cells/g (0–8)] did not change after a challenge compared to the baseline in subjects with positive challenges ($n = 6$; $p > 0.05$ for all). The numbers of sputum CD3+6B11+ NKT cells [0.33% (0.01–1.86) of CD3+ T cells vs. 0.18% (0.00–1.57) of CD3+ T cells; 15 cells/g (1–83) vs. 1 cell/g (0–50)], CD3+Vα24+ NKT cells [0.01% (0.00–0.57) of CD3+ T cells vs. 0.06% (0.00–0.53) of CD3+ T cells; 1 cell/g (0–14) vs. 0 cells/g (0–10)], CD4+6B11+ NKT cells [0.31% (0.01–1.05) of CD3+ T cells vs. 0.13% (0.00–1.57) of CD3+ T cells; 13 cells/g (1–78) vs. 0 cells/g (0–41)] and CD4+Vα24+ NKT

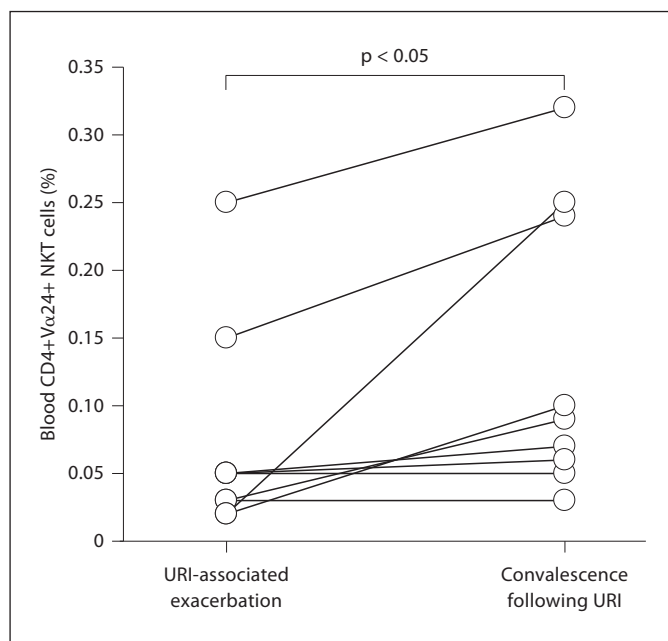


Fig. 1. Reduction in the frequency of blood CD4+Vα24+ NKT cells during URI-associated asthma exacerbation compared to the convalescence stage.

cells [0.00% (0.00–0.38) of CD3+ T cells vs. 0.06% (0.00–0.53) of CD3+ T cells; 0 cells/g (0–14) vs. 0 cells/g (0–10)] did not change after a challenge compared to the baseline in subjects with negative challenges (n = 8; p > 0.05 for all).

Change during URI-Associated Asthma Exacerbation

Blood CD3+Vα24+ NKT cells [0.07% (0.05–0.26) of CD3+ T cells vs. 0.16% (0.11–0.36) of CD3+ T cells; 26 (20–102) cells/ 4×10^4 CD3+ T cells vs. 64 (46–144) cells/ 4×10^4 CD3+ T cells; p = 0.07], CD4+6B11+ NKT cells [0.02% (0.02–0.07) of CD3+ T cells vs. 0.07% (0.03–0.13) of CD3+ T cells; 9 (7–26) cells/ 4×10^4 CD3+ T cells vs. 26 (10–50) cells/ 4×10^4 CD3+ T cells; p = 0.09] and CD4+Vα24+ NKT cells [0.05% (0.02–0.10) of CD3+ T cells vs. 0.09% (0.05–0.25) of CD3+ T cells; 20 (9–40) cells/ 4×10^4 CD3+ T cells vs. 36 (21–98) cells/ 4×10^4 CD3+ T cells; p = 0.02; fig. 1] decreased during exacerbation compared to the convalescence stage, while the numbers of blood CD3+6B11+ NKT cells [0.09% (0.04–0.18) of CD3+ T cells vs. 0.18% (0.08–0.27) of CD3+ T cells; 34 (14–72) cells/ 4×10^4 CD3+ T cells vs. 72 (32–108) cells/ 4×10^4 CD3+ T cells; p = 0.16] did not differ. Additionally, we analyzed whether the number of blood NKT cells was

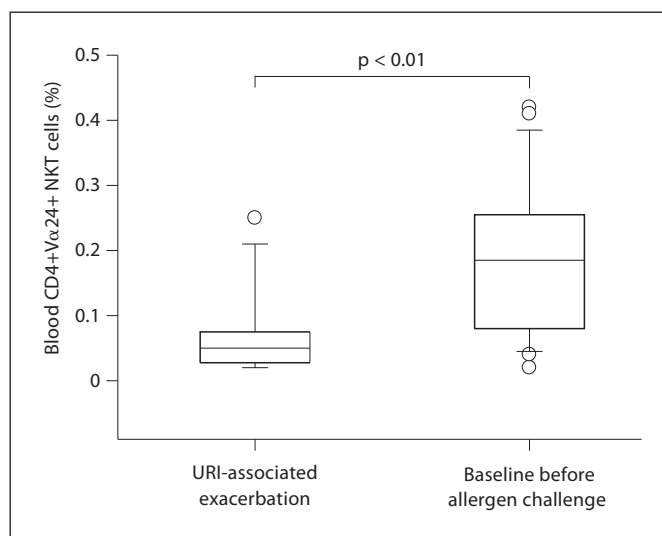


Fig. 2. Reduction in the frequency of blood CD4+Vα24+ NKT cells during URI-associated exacerbation compared to baseline measurements of patients who underwent allergen challenge. Medians and 10th, 25th, 75th and 90th percentiles are plotted as vertical boxes with error bars, and all data points that lie outside the 10th and 90th percentiles are plotted as open circles.

lower during URI-associated asthma exacerbation compared to chronic stable asthma. Blood CD3+6B11+ NKT cells (p = 0.05), CD3+Vα24+ NKT cells (p = 0.005), CD4+6B11+ NKT cells (p = 0.08) and CD4+Vα24+ NKT cells (p = 0.007; fig. 2) all decreased during URI-associated exacerbation compared to baseline measurements in patients who underwent allergen challenge.

The numbers of sputum CD3+6B11+ NKT cells [0.34% (0.19–0.65) of CD3+ T cells vs. 0.00% (0.00–2.08) of CD3+ T cells; 39 cells/g (37–64) vs. 0 cells/g (0–20)], CD3+Vα24+ NKT cells [0.10% (0.06–0.30) of CD3+ T cells vs. 0.05% (0.00–0.75) of CD3+ T cells; 18 cells/g (5–23) vs. 8 cells/g (0–36)], CD4+6B11+ NKT cells [0.30% (0.19–0.43) of CD3+ T cells vs. 0.00% (0.00–0.06) of CD3+ T cells; 31 cells/g (25–64) vs. 0 cells/g (0–20)] and CD4+Vα24+ NKT cells [0.10% (0.00–0.30) of CD3+ T cells vs. 0.10% (0.00–0.75) of CD3+ T cells; 18 cells/g (0–23) vs. 8 cells/g (0–36)] did not differ between the exacerbation and convalescence stages (n = 7; p > 0.05 for all).

Change in the Numbers of Inflammatory Cells in Sputum

Asthma patients who had an allergen provocation test had no change in the numbers of total cells, macrophages, lymphocytes, neutrophils and eosinophils after the chal-

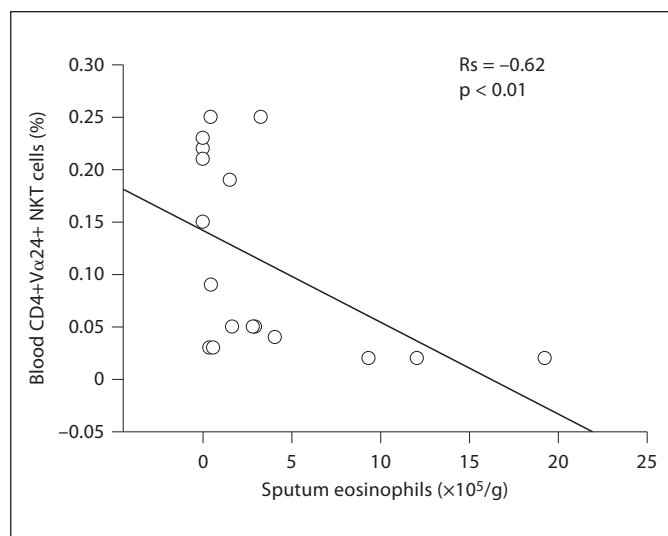


Fig. 3. Correlation between sputum eosinophils and blood CD4+V α 24+ NKT cells obtained 8 h after a positive allergen challenge and during asthma exacerbation associated with URI in patients with asthma. The number of NKT cells is expressed as the percentage of CD3+ T cells. Rs = Spearman's correlation coefficient.

lenge; this was true for patients with a positive allergen challenge as well as for those with a negative challenge. In patients with an asthma exacerbation associated with URI, the numbers of inflammatory cells did not change during exacerbation compared to the convalescence stage. However, when we compared the numbers of inflammatory cells to baseline measurements of patients who underwent allergen challenge, total cells ($p = 0.007$), lymphocytes ($p = 0.02$) and eosinophils ($p = 0.03$) increased during URI-associated asthma exacerbation (table 3).

Change in the Levels of Cytokines and Chemokines in Sputum Supernatant

After an allergen challenge, patients with a positive challenge showed no change in any of the cytokines or chemokines, while those with negative challenges had decreased levels of IL-4, IL-7, IL-10, IL-17, IFN- γ and G-CSF compared to the baseline. However, in patients with asthma exacerbations associated with URI, there were significant increases in IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-10 ($p = 0.07$), IL-12, IL-13, IL-17 ($p = 0.09$), IFN- γ , granulocyte macrophage colony-stimulating factor, tumor necrosis factor- α , G-CSF, MIP-1 β , monocyte chemoattractant protein-1 ($p = 0.06$), eotaxin and vascular endo-

thelial growth factor during exacerbation compared to the convalescence stage. Furthermore, most cytokines and chemokines increased during URI-associated exacerbation compared to baseline measurements of patients who underwent allergen challenge (table 4).

Relationship between Blood NKT Cells and Sputum Eosinophils

When the relationship between blood NKT cells and sputum eosinophils was analyzed in samples obtained after a positive allergen challenge and during a URI-associated asthma exacerbation, there was a negative correlation between blood CD3+V α 24+ NKT cells (Spearman's correlation coefficient = -0.51 ; $p = 0.04$) or CD4+V α 24+ NKT cells ($p = 0.008$; fig. 3) and sputum eosinophils.

Discussion

In the present study, blood NKT cells decreased during URI-associated acute exacerbation in patients with asthma, although they did not after a positive allergen challenge, suggesting that NKT cells may play an important role in the immune pathogenesis of acute asthma exacerbation in humans.

The reduction of blood NKT cells during an acute exacerbation may be associated with worsening airway inflammation. Blood NKT cells might be mobilized to the airways and lungs. The present study showed that the blood NKT cells decreased, while the sputum eosinophils increased during URI-associated asthma exacerbation. Furthermore, the blood NKT cells obtained after a positive allergen challenge or during a URI-associated acute exacerbation were inversely correlated with sputum eosinophils. Recently, we analyzed the number of sputum NKT cells in patients with chronic stable asthma and eosinophilic bronchitis and reported that NKT cells may be associated with eosinophilic airway inflammation [16]. Thus, in humans, the development or worsening of eosinophilic airway inflammation associated with asthma exacerbation might contribute to the mobilization of blood NKT cells into the airways and lungs. However, currently it is unclear whether the two are causally related or this is a chance association. This is mere speculation at this point.

Eosinophilic inflammation was found in sputum during URI-associated asthma exacerbation in the present study. Exposure to common cold viruses may induce eosinophilic airway inflammation in asthma [22, 23], which may occur more frequently in patients with atopic asth-

Table 3. Change in the number of inflammatory cells ($\times 10^5/\text{g}$) in induced sputum

	Allergen challenge				URI-associated asthma exacerbation (n = 8)	
	positive (n = 8)		negative (n = 11)		exacerbation	convalescence
	baseline	after challenge	baseline	after challenge		
Total cells	5.2 (3.0–17.0)	6.0 (2.9–16.5)	5.3 (2.3–11.3)	7.6 (4.2–12.3)	19.9 (12.6–46.7)**	15.6 (6.1–44.4)
Macrophages	2.0 (0.9–5.5)	2.6 (0.9–6.8)	2.0 (0.7–3.8)	3.8 (1.6–4.9)	5.3 (1.1–23.4)	3.8 (2.0–23.2)
Lymphocytes	0.4 (0.1–0.8)	0.3 (0.1–1.0)	0.3 (0.1–0.5)	0.2 (0.1–0.4)	0.5 (0.4–1.4)*	0.0 (0.0–0.6)
Neutrophils	2.3 (1.7–10.1)	2.8 (0.9–6.6)	3.0 (1.1–5.5)	2.9 (2.3–6.9)	8.1 (2.8–22.9)	4.0 (2.7–24.1)
Eosinophils	0.2 (0.0–1.8)	0.4 (0.0–3.4)	0.1 (0.0–0.6)	0.0 (0.0–1.5)	2.3 (0.4–7.8)*	0.0 (0.0–2.2)

Data are expressed as medians and interquartile range. * $p < 0.05$ and ** $p < 0.01$ compared to baseline measurements obtained from patients who underwent the allergen challenge (Mann-Whitney U test).

Table 4. Change in the production of cytokines and chemokines (pg/ml) in the supernatant of induced sputum

	Allergen challenge				URI-associated asthma exacerbation (n = 8)	
	positive (n = 8)		negative (n = 11)		exacerbation	convalescence
	baseline	after challenge	baseline	after challenge		
IL-1 β	48.5 (24.2–143.7)	37.5 (15.5–120.3)	31.0 (22.0–73.0)	27.0 (15.0–44.0)	113.5 (80.7–614.5) ^{a, b}	19.5 (13.2–37.2)
IL-2	6.0 (4.2–6.8)	5.5 (4.0–8.5)	5.0 (5.0–7.0)	5.0 (4.0–6.0)	8.5 (7.0–33.8) ^{a, c}	5.0 (4.2–6.0)
IL-4	6.0 (5.2–7.5)	6.2 (3.5–10.0)	6.5 (6.0–8.0)	5.0 (4.0–7.0) ^a	9.5 (6.2–30.5) ^{a, b}	6.0 (5.1–6.7)
IL-5	3.0 (3.0–5.0)	3.5 (2.0–5.5)	4.0 (3.0–5.0)	3.0 (3.0–5.0)	36.7 (8.0–93.2) ^{a, d}	4.0 (3.1–4.0)
IL-6	15.0 (8.2–31.7)	18.5 (13.0–37.5)	14.0 (9.0–28.0)	24.5 (7.0–34.0)	91.2 (58.6–151.5) ^{a, c}	11.0 (7.6–38.8)
IL-7	4.0 (3.2–5.0)	5.0 (3.0–14.5)	5.0 (3.0–7.0)	3.0 (3.0–4.0) ^a	7.0 (5.6–27.0) ^{a, c}	4.0 (3.2–4.7)
IL-8	207 (94–490)	219 (73–579)	164 (103–364)	164 (19–386)	634 (408–1,944) ^c	116 (87–671)
IL-10	10.0 (7.1–12.2)	11.0 (6.2–20.7)	9.0 (7.0–11.0)	6.0 (6.0–8.0) ^a	15.0 (10.0–29.7) ^b	7.0 (6.2–7.7)
IL-12	5.0 (4.0–5.0)	3.5 (2.2–12.5)	4.0 (3.0–7.0)	3.0 (3.0–4.0)	9.0 (5.2–31.5) ^{a, c}	3.5 (3.0–4.7)
IL-13	4.5 (4.0–5.0)	4.0 (3.2–13.0)	4.0 (4.0–5.0)	3.5 (3.0–4.0)	9.5 (5.2–26.5) ^{a, c}	4.5 (3.2–5.7)
IL-17	10.0 (7.5–14.0)	12.5 (8.5–15.1)	13.0 (12.0–16.0)	10.0 (8.0–14.0) ^a	16.2 (11.1–37.0)	10.0 (7.5–15.0)
IFN- γ	4.5 (4.0–6.0)	3.5 (3.0–8.2)	4.0 (4.0–6.0)	4.0 (3.0–5.0) ^a	8.0 (5.0–23.8) ^{a, c}	5.0 (3.2–5.0)
GM-CSF	3.0 (3.0–4.0)	3.0 (3.0–9.6)	3.0 (3.0–4.0)	2.0 (2.0–3.0)	5.7 (4.0–26.8) ^{a, c}	2.7 (2.0–3.7)
TNF- α	4.2 (3.2–5.8)	4.0 (4.0–13.2)	4.5 (4.0–5.0)	4.0 (3.0–5.0)	9.2 (5.2–25.7) ^{a, c}	4.5 (3.6–5.0)
G-CSF	5.0 (4.2–8.0)	7.5 (4.1–28.5)	7.5 (5.0–13.0)	5.0 (4.0–6.0) ^a	12.2 (7.2–30.0) ^{a, b}	5.5 (4.0–8.2)
MIP-1 β	41.0 (15.7–100.2)	34.0 (17.0–174.5)	19.0 (14.0–112.0)	37.0 (8.0–63.0)	1,985 (330–2,839) ^{a, d}	102 (41–521)
MCP-1	4.2 (4.2–7.5)	4.5 (3.2–41.0)	8.5 (4.0–14.0)	4.0 (4.0–7.0)	25.2 (8.2–88.5) ^c	10.5 (6.1–36.5)
Eotaxin	6.0 (6.0–6.8)	6.0 (5.2–16.0)	7.0 (5.0–8.0)	5.0 (5.0–6.0)	9.7 (7.0–22.5) ^{a, b}	7.5 (6.2–8.7)
bFGF	30.0 (22.2–46.5)	28.0 (16.5–56.0)	21.0 (15.0–33.0)	17.0 (14.0–20.0)	38.0 (21.5–62.7)	16.5 (13.2–20.7)
VEGF	11.0 (9.2–12.7)	14.5 (10.0–62.2)	20.0 (16.0–54.0)	19.0 (13.0–29.0)	40.0 (30.7–51.7) ^{a, b}	23.5 (13.6–56.6)

Data are expressed as medians and interquartile range. ^a $p < 0.05$ compared to baseline or convalescence (Wilcoxon test); ^b $p < 0.05$, ^c $p < 0.01$, ^d $p < 0.001$ compared to baseline measurements obtained from patients who underwent allergen challenge (Mann-Whitney U test). GM-CSF = Granulocyte macrophage colony-stimulating factor; TNF = tumor necrosis factor; MCP = monocyte chemotactic protein; bFGF = basic fibroblast growth factor; VEGF = vascular endothelial growth factor.

ma than in patients with asthma due to other causes [24]. Total serum IgE in the patients with URI-associated asthma exacerbations was higher, suggesting that they may have had atopic asthma. IL-5 [25], RANTES and eotaxin [26] may be involved in the pathogenesis of virus-induced eosinophilic airway inflammation. In the present study, IL-5 and eotaxin were increased in the sputum supernatant obtained during URI-associated asthma exacerbation. Furthermore, multiple cytokines including Th1 and Th2 cytokines were significantly increased in sputum during URI-associated asthma exacerbation.

NKT cells might be mobilized to the airways and lungs by a chemokine produced during an asthma exacerbation. In the present study, MIP-1 β was increased in sputum supernatant obtained during URI-associated asthma exacerbation. Based on the fact that MIP-1 β is a ligand of CCR5, which is expressed on blood NKT cells in humans [27], it can be speculated that blood NKT cells may be mobilized to the airways and lungs by MIP-1 β .

Viruses may activate the NKT-macrophage innate immune axis, which may play an important role in the airway inflammation of asthma and chronic obstructive lung disease [28]. Thus, it is possible that URI-associated asthma exacerbations may be associated with the activation of the NKT-macrophage immune axis.

However, in the present study, the number of both blood NKT cells and sputum eosinophils did not change after a positive allergen challenge. It is well known that an allergen challenge induces eosinophilic or neutrophilic airway inflammation in patients with asthma, which may be observed at 18, 24 or 42 h after a challenge [29–33]. However, airway eosinophilia may occur at an earlier time following the challenge. Sputum eosinophilia has been reported to occur at 4 [32] and 6 h [31] after an allergen challenge. However, it was reported that airway eosinophilia did not occur at 4 h after an allergen challenge [30]. It has been reported that earlier airway eosinophilia may be associated with late asthmatic responses [34, 35], which may be connected to the dose of inhaled allergen [36]. In the present study, all patients with a positive allergen challenge showed an early asthmatic reaction; however, only 2 among them had late asthmatic reactions. These findings may support the absence of sputum eosinophilia after the challenge. Additionally, no production of sputum cytokines and chemokines occurred after a positive allergen challenge. This observation might explain why blood NKT cells did not decrease after an allergen challenge. Rock et al. [37] did not find changes in the number of blood NKT cells until 7–8 h after allergen challenge. If sputum and blood are ob-

tained 24 h or more after an allergen challenge, airway eosinophilia and a reduced number of blood NKT cells might be observed.

Other mechanisms underlying the reduced number of blood NKT cells during asthma exacerbation may be considered. Recently, we showed that blood NKT cells were inversely associated with the degree of atopy in patients with chronic stable asthma [15]. Thus, blood NKT cells may behave like Th1-like NKT cells, in contrast to lung NKT cells, and might be decreased because Th1-like NKT cells are decreased in the peripheral blood during asthma exacerbation.

In the present study, sputum NKT cell counts did not change after a positive allergen challenge or during URI-associated asthma exacerbations. Rock et al. [37] and Thomas et al. [38] did not find any changes in the number of NKT cells in bronchoalveolar lavage fluid 24 h after an allergen challenge. However, Reynolds et al. [39] demonstrated an increase in the number of NKT cells in lung tissues 24 h after an allergen challenge. Examination of lung tissues might provide evidence for an increased number of NKT cells during URI-associated asthma exacerbation.

The asthma medications used in the present study, in particular ICS and systemic steroids, might have affected the results. However, it is unlikely that asthma medications alter the number of NKT cells, based on our prior studies [15, 16] and other studies [13]. For the asthma patients who underwent allergen challenge, the NKT cells and eosinophils were compared at baseline and after the challenges, regardless of the medications taken.

Recently, Brooks et al. [40] reported the possibility of methodological artifacts when using flow cytometry to detect NKT cells. Unless a stringent gating strategy is used when analyzing NKT cells, the observed number can be overestimated. However, in the present study the number of blood NKT cells was similar to that obtained by the stringent gating strategy in the study reported by Brooks et al. [40], although a simple gating strategy was used. We analyzed NKT cells by excluding nonspecific binding as much as possible, as previously described [15]. Additionally, it may be easier and better to determine blood NKT cells using whole blood, as used in the study by Magnan et al. [21].

It is possible that there are minor differences between V α 24+ NKT cells and 6B11+ NKT cells. Staining with V α 24 monoclonal antibody may include non-invariant and non-CD1d-restricted V α 24+ T cells, while 6B11 monoclonal antibody may recognize NKT cells expressing the invariant TCR α chain. Therefore, V α 24+ stain-

ing could lead to an overestimation of the NKT cell number. Both V α 24+ cells and 6B11+ cells were used to define NKT cells in the present study.

It may be reasonable to include a control disease group. Chronic obstructive pulmonary disease (COPD) patients with acute exacerbation might be included as a control group to confirm a specific role of NKT cells in the pathogenesis of exacerbation of asthma. For COPD to be included as an ideal control disease, NKT cells should not play a role in the pathogenesis of COPD. However, the possibility of a role of NKT cells in the pathogenesis of COPD cannot be excluded. Although Vijayanand et al. [14] revealed that NKT cells were not increased in the sputum from patients with stable COPD or COPD during exacerbation, Kim et al. [28] showed that V α 24+ NKT cells were increased in lung tissue from patients with COPD. Whether NKT cells may play a role in the pathogenesis of COPD exacerbation will be another important issue. Additionally, in the present study, we focused primarily on comparing the frequency of NKT cells before and after allergen challenge and also during exacerbation and convalescence within the same asthma patients.

Levels of sputum cytokines may be affected by any number of possible factors. Denaturation by dithiothreitol, degradation by proteases present in sputum and binding by sputum matrix proteins or plastic in processing may render the cytokines unavailable for immunodetection.

Additionally, there are the inherent problems of sampling errors in inducing sputum [41]. Simultaneous measurement of serum cytokines may be helpful, although serum cytokines seem to reflect the inflammatory status of the lungs and the airways to a lesser degree than sputum cytokines [42]. Serum cytokines may be easily measured compared to sputum cytokines. Unfortunately, serum samples were not available in the present study.

The present study is the first to show changes in the number of blood NKT cells during URI-associated asthma exacerbations; a few prior studies on NKT cells after allergen challenge have been performed [37–39]. Based on the findings of this study, it is most likely that NKT cells are involved in the immune pathogenesis of acute asthma exacerbation in humans. In terms of clinical implications, a new drug to control NKT cells will be effective for the treatment and prevention of asthma exacerbation in humans, which may prevent the development of fatal asthma.

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