



Research article

Inverse association of peripheral blood CD4⁺ invariant natural killer T cells with atopy in human asthma

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ABSTRACT

Invariant natural killer T (iNKT) cells have been reported to play a role in the pathogenesis of murine asthma. However, the role for iNKT cells in the pathogenesis of human asthma is not defined. In this study we aimed to determine how blood iNKT cells are associated with atopy in asthmatic individuals. Peripheral blood mononuclear cells were isolated from 45 asthmatic subjects. iNKT cells were stained with 6B11 mAb, anti-TCR α 24 mAb, or α -galactosylceramide (GalCer)-loaded CD1d-tetramer and analyzed with flow-cytometric assays. Increased serum total IgE or one or more positive skin reactions to common allergens were used as atopic indexes. Asthmatic subjects with IgE > 500 IU/ml showed lower frequency of CD4⁺ 6B11⁺ iNKT cells ($p < 0.01$) or CD4⁺ V α 24⁺ iNKT cells ($p < 0.01$) compared with subjects with IgE $\leq$ 500 IU/ml. Asthmatic subjects with atopy on skin tests had lower frequency of CD4⁺ α -GalCer-loaded CD1d-tetramer⁺ iNKT cells compared with those without atopy ($p < 0.05$). The frequency of CD4⁺ V α 24⁺ iNKT cells was negatively correlated with total IgE in asthmatic subjects ($r = -0.33$, $p < 0.05$). In summary, blood CD4⁺ iNKT cells were inversely associated with atopic indexes in asthmatic individuals. We hypothesize that blood CD4⁺ iNKT cells might behave like T_H1-like iNKT cells in human asthma.

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1. Introduction

It has been reported that natural killer T (NKT) cells may play an important role in the pathogenesis of asthma [1]. NKT cells are unique CD1d-restricted T cells with surface markers of NK cells and act as a bridge between innate and adaptive immune responses. NKT cells secrete huge amounts of T_H1 or T_H2 cytokines immediately after activation [2]. In particular, invariant NKT (iNKT) cells express very limited TCR, V α 14-J α 18 in mice, and V α 24 in human beings, and respond to α -galactosylceramide (α -GalCer) synthetic glycolipids from a marine sponge [3]. Recently, iNKT cells have been reported to play an essential role in the development of ovalbumin-induced [4,5], α -GalCer-induced [6], or ozone-induced [7] airway hyperresponsiveness (AHR) in a murine model of asthma.

However, a role for iNKT cells in the pathogenesis of atopy remains controversial. Murine iNKT cells were reported to be essential [8] or nonessential [9] for IgE production. α -GalCer-activated murine iNKT cells were reported to inhibit IgE production by producing a large amount of interferon (IFN)- γ [10]. In addition, in human beings, the

relationship of iNKT cells to atopy has not been clearly defined. It has been variously reported that the number of peripheral blood iNKT cells is decreased in patients with atopic dermatitis [11,12], is not different according to atopic status [13,14], or is increased in atopic subjects [15].

Human iNKT cells can be identified with the different markers. In the TCR α chain in human iNKT cells, the V α 24 segment is joined with J α 18 in a germ-line configuration, resulting in an invariant complementarity-determining region (CDR) 3 loop encoded by the mature TCR α chain. Therefore, human iNKT cells have been identified using mAbs against the V α 24 chain [16] or the CDR 3 region [17]. In addition, the development of CD1d-tetramers loaded with α -GalCer has enhanced the specificities of iNKT cell evaluation [18].

In the present study, we analyzed the frequency of peripheral blood iNKT cells according to atopic indexes in stable asthmatic individuals. It has been reported that more than 90% of iNKT cells in bronchoalveolar lavage (BAL) fluid of asthmatic subjects were CD4⁺ cells, indicating that CD4⁺ iNKT cells may play a major role in the pathogenesis of human asthma [19]. However, it is not clear how peripheral blood CD4⁺ iNKT cells contribute to the asthma pathogenesis. Thus, we focused on blood CD4⁺ iNKT subset and its role in the pathogenesis of atopy in human asthma. We clearly showed that blood CD4⁺ iNKT cells were inversely associated with atopic indexes in human asthma.

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2. Subjects and methods

2.1. Subjects

A total of 45 asthmatic individuals and 10 normal control individuals were included. Asthma was defined by the presence of reversible airflow obstruction, i.e., increases in forced expiratory volume in 1 second (FEV_1) $\geq 12\%$ and ≥ 200 ml after bronchodilator test [20] or AHR to methacholine was demonstrated. Bronchodilator tests were performed in asthmatic subjects with initial FEV_1 predicted values of $<60\%$. Methacholine bronchial provocation tests were performed in asthmatic subjects with FEV_1 predicted values of $>60\%$. Asthmatic subjects had no signs or symptoms of acute exacerbations. Asthma severity was classified according to the Global Initiative for Asthma guideline 2002 [21]. Briefly, severity was classified into intermittent, mild persistent, moderate persistent, and severe persistent types based on the clinical features and lung functions present before treatment was begun. When the subject was already on treatment, the classification of severity was based on the clinical features present and the step of the daily medication regimen that the subject was currently taking. Normal controls had no respiratory symptoms, history of allergic diseases, or AHR. All were residents or medical students, and all were non-smokers. Bronchodilator tests or methacholine bronchial provocation tests, skin tests, and blood sampling were performed. Some asthmatic subjects had received asthma medications, which were withdrawn for 5–7 days before the tests. All subjects agreed to participate in the study and gave written consent, and the ethics review board of Chonnam National University Hospital approved the study.

2.2. Spirometry and methacholine bronchial provocation tests

Spirometry was carried out with a spirometer (Spiro Analyzer ST-250, Fukuda Sangyo, Tokyo, Japan). Methacholine (Sigma, St. Louis, MO) aerosols from 0.075 to 25 mg/ml were administered during tidal breathing for 2 minutes. The percentage FEV_1 fall was calculated from the postsaline FEV_1 . The test was continued until FEV_1 fell by $>20\%$ or to last dose if not. The airway response was expressed as the concentration of methacholine that provoked a 20% fall in FEV_1 (PC_{20}). A subject was considered as having AHR if PC_{20} was <25 mg/ml.

2.3. Skin prick tests and atopy

A standardized skin prick test was performed using 12 common aeroallergens (*Dermatophagoides pteronyssinus*, *D. farinae*, *Alternaria tenuis*, *Trichophyton*, cat, dog, cockroach, alder, birch, orchard, mugwort, and ragweed) (Allergopharma, Reinbeck, Germany). A subject was classified as atopic if any allergen caused a wheal ≥ 3 mm in diameter than in negative controls. Atopy score was defined as the number of “+” for skin reactions to the 12 allergens. Reactions to allergen producing a wheal similar in size to that produced by histamine were to be graded 3+, those twice that size 4+, those half that size 2+, and those between the negative control and 2+ in size were to be graded 1+.

2.4. Serum total IgE

Blood samples for serum preparation were drawn for measurement of total IgE concentration. After blood sampling, clotting occurred for 2 hours at 37°C . Thereafter, blood samples were centrifuged for 10 minutes at 1350 g. Subsequently, the serum samples were stored at -20°C until analysis. IgE concentration was measured by nephelometry (Dade Behring, Marburg, Germany).

2.5. Isolation of peripheral blood mononuclear cells

Heparinized blood was layered over equal volume of Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) solution, which was

centrifuged at 2000 rpm for 30 minutes. Peripheral blood mononuclear cells (PBMCs) were isolated from the interface and used to measure NKT cells.

2.6. Flow-cytometric analysis of NKT cells

PBMCs were incubated at 4°C for 30 minutes with fluorochrome-conjugated monoclonal antibodies, including FITC-anti-CD4 (BD Biosciences, San Jose, CA), PE-6B11 (BD Biosciences), or PE-anti-TCR α 24 (Beckman Coulter, Marseille, France), PerCP-anti-CD3 (BD Biosciences), and APC-anti-CD56 (BD Biosciences). 6B11 monoclonal antibody is directed against the CDR 3 of the $V\alpha 24$ -J $\alpha 18$ T cell receptor of iNKT cells [17]. PE-mouse IgG $_1$ (BD Biosciences) and APC-mouse IgG $_1$ (BD Biosciences) were used for isotype control antibodies. In addition, α -GalCer-loaded PE-CD1d–tetramer was used to detect iNKT cells in 25 asthmatic subjects and 10 controls. α -GalCer (Alexis Biochemicals, Lausen, Switzerland) was conjugated with PE-CD1d–tetramer (Proimmune, Oxford, UK) according to the manufacturer's instructions. Unloaded PE-CD1d–tetramer was used for negative control. Flow cytometry was done with the four-color BD FACS Calibur (BD Biosciences). After gating $CD3^+$ T cells, the frequency of $CD3^+ CD56^+$ NKT cells was expressed as percentage of $CD3^+$ T cells and was considered to be equivalent to human NKT cells [22]. iNKT cells were defined as $CD3^+ 6B11^+$ cells, $CD3^+ V\alpha 24^+$ cells or $CD3^+ \alpha$ -GalCer-loaded CD1d–tetramer $^+$ cells, the frequency of which was expressed as percentage of $CD3^+$ T cells. Representative analytic results of iNKT cells is shown in Fig. 1. The frequency of $CD4^+$ iNKT cells, such as $CD4^+ 6B11^+$ cells, $CD4^+ V\alpha 24^+$ cells, or $CD4^+ \alpha$ -GalCer-loaded CD1d–tetramer $^+$ cells, was determined. The ratio of $CD4^+$ iNKT cells to $CD3^+$ iNKT was also calculated.

2.7. Statistical analysis

Data were expressed as mean $\pm$ SEM. To examine differences between the two groups, we used Student's *t* test for continuous variables. Categorical variables were analyzed with Pearson χ^2 -test to determine the association between different variables. Correlations between parameters were analyzed with Pearson's correlation coefficient (SPSS 17.0 for Windows; SPSS, Inc., Chicago, IL). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Characteristics of subjects

Of all asthmatic subjects, 41 were diagnosed with methacholine bronchial provocation tests and the rest of the subjects with bronchodilator tests. In the 41 asthmatic subjects, PC_{20} ranged from 0.1 to 22.6 mg/ml. Skin prick tests were performed in 44 asthmatic subjects, half of whom were atopic. Serum total IgE levels were determined in 42 asthmatic subjects and ranged from 17 to 4,020 IU/ml. Fifteen asthmatic subjects had received various asthma medications, such as inhaled corticosteroids, at least for 3 months before the study. Asthmatic subjects were older ($p < 0.001$), included fewer male subjects ($p < 0.01$), and had lower lung function values ($p < 0.05$) than controls. The proportions of atopic subjects did not differ between the two groups (Table 1). Asthma severity was classified into intermittent or mild persistent in 15 (33%) patients, moderate persistent in 17 (38%) patients and severe persistent in 13 (29%) patients. There were no differences in age, gender, presence or degree of atopy, or level of serum total IgE among asthmatic subjects with different clinical severities, although the patients with moderate or severe asthma had lower lung function and PC_{20} values and used more asthma medications (Table 2).

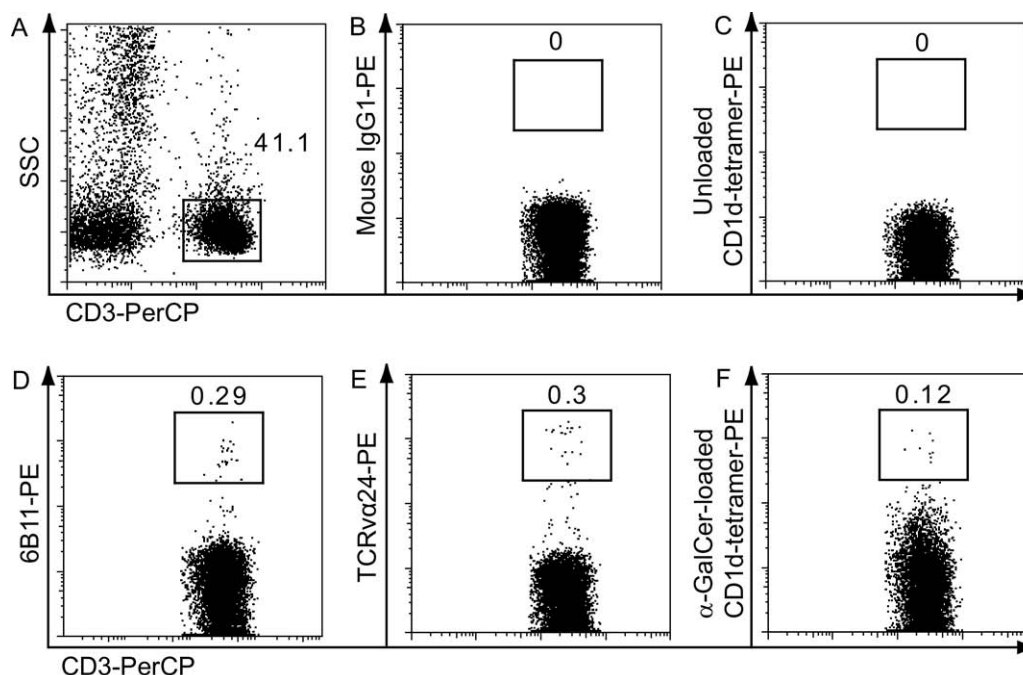


Fig. 1. A representative example of flow cytometric analysis of blood CD3⁺ 6B11⁺ invariant natural killer T (iNKT) cells (B and D), CD3⁺ TCRvα24⁺ iNKT cells (B and E) and CD3⁺ α-galactosylceramide (GalCer)-loaded CD1d – tetramer⁺ iNKT cells (C and F), which was analyzed after gating CD3⁺ T cells (A). Mouse IgG1-PE was isotype for 6B11-PE and TCRvα24-PE and unloaded PE-CD1d – tetramer was negative control for α-GalCer-loaded PE-CD1d – tetramer. The number of iNKT cells was shown as the percentage of CD3⁺ T cells.

3.2. Frequency of blood iNKT cells according to different markers in normal controls

We determined the frequency of blood iNKT cells using anti-TCRvα24 and 6B11 mAbs and α-GalCer-loaded CD1d tetramer in normal controls. We found that anti-TCRvα24 mAb detected a higher percentage of iNKT cells ($0.42\% \pm 0.06\%$) when compared with 6B11 mAb ($0.24\% \pm 0.08\%$, $p < 0.05$) or α-GalCer-loaded CD1d tetramer ($0.22\% \pm 0.07\%$, $p < 0.01$).

3.3. Lower number of blood iNKT cells in asthmatic subjects with higher IgE levels

We compared the frequency of blood NKT cells between asthmatic subjects and controls. There were no differences in the

frequency of CD3⁺ CD56⁺ NKT cells ($1.98\% \pm 0.37\%$ vs. $1.29\% \pm 0.37\%$, $p > 0.05$), CD3⁺ 6B11⁺ iNKT cells ($0.24\% \pm 0.04\%$ vs. $0.24\% \pm 0.08\%$, $p > 0.05$), CD3⁺ Vα24⁺ iNKT cells ($0.48\% \pm 0.04\%$ vs. $0.42\% \pm 0.06\%$, $p > 0.05$), CD3⁺ α-GalCer-loaded CD1d – tetramer⁺ iNKT cells ($0.22\% \pm 0.05\%$ vs. $0.22\% \pm 0.07\%$, $p > 0.05$), CD4⁺ 6B11⁺ iNKT cells ($0.07\% \pm 0.01\%$ vs. $0.05\% \pm 0.01\%$, $p > 0.05$), CD4⁺ Vα24⁺ iNKT cells ($0.24\% \pm 0.02\%$ vs. $0.19\% \pm 0.03\%$, $p > 0.05$), and CD4⁺ α-GalCer-loaded CD1d – tetramer⁺ iNKT cells ($0.09\% \pm 0.02\%$ vs. $0.05\% \pm 0.01\%$, $p > 0.05$) between asthmatic subjects and controls.

We divided asthmatic subjects into low (≤ 500 IU/ml) and high IgE (> 500 IU/ml) groups according to serum total IgE levels. All

Table 1
Characteristics of subjects

	Asthmatics	Controls
Age (y)	45.6 $\pm$ 2.4 ^a	26.8 $\pm$ 0.7
Female gender (n, %)	26 (57.8) ^b	0 (0)
Smoking habit (n, %)		
Current	9 (20)	0 (0)
Ex	7 (15.6)	0 (0)
Never	29 (64.4)	10 (100)
Asthma duration (yr)	6.7 $\pm$ 1.0	—
Atopy (n, %)	22 (50)	2 (20)
Serum total IgE (IU/ml)	515.9 $\pm$ 127.1	—
FEV ₁ (% predicted)	89.6 $\pm$ 2.8 ^c	101.2 $\pm$ 3.3
PC ₂₀ (mg/ml)	6.6 $\pm$ 1.1	—
Medications (n, %)		
Inhaled corticosteroids	11 (24)	—
Long-acting β ₂ -agonists	9 (20)	—
Leukotriene receptor antagonists	6 (13)	—
Theophylline	11 (24)	—
Oral corticosteroids	1 (2)	—

FEV₁, forced expiratory volume in 1 second; PC₂₀, concentration of methacholine that provoked a 20% fall in FEV₁.

^a $p < 0.001$, ^b $p < 0.01$, and ^c $p < 0.05$ vs. controls.

Table 2
Demographic data of asthmatics according to clinical severity

	Intermittent or mild persistent (n = 15)	Moderate persistent (n = 17)	Severe persistent (n = 13)
Age (y)	41.1 $\pm$ 3.6	47.1 $\pm$ 4.6	49.0 $\pm$ 4.5
Female (n, %)	9 (60.0)	9 (52.9)	8 (61.5)
Atopy (n, %) ^a	8 (53.3)	9 (56.3)	5 (38.5)
Atopy score ^a	4.9 $\pm$ 1.5	7.3 $\pm$ 2.0	4.5 $\pm$ 2.1
Serum total IgE (IU/ml) ^a	593.3 $\pm$ 222.8	445.5 $\pm$ 128.9	507.2 $\pm$ 322.9
FEV ₁ (L)	3.1 $\pm$ 0.1	2.6 $\pm$ 0.2	2.4 $\pm$ 0.1 ^c
(% predicted value)	99.2 $\pm$ 2.8	84.8 $\pm$ 4.9 ^c	84.7 $\pm$ 5.7 ^c
FVC (L)	3.9 $\pm$ 0.2	3.4 $\pm$ 0.2	3.3 $\pm$ 0.1
(% predicted value)	103.0 $\pm$ 3.3	90.3 $\pm$ 3.7 ^c	91.6 $\pm$ 4.6
PC ₂₀ (mg/ml) ^a	11.5 $\pm$ 1.7	4.3 $\pm$ 1.6 ^d	3.0 $\pm$ 1.3 ^d
One or more medications (n, %) ^b	0 (0)	9 (52.9) ^d	6 (46.2) ^d

FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; PC₂₀, concentration of methacholine that provoked a 20% fall in FEV₁.

^aData are obtained from 15 patients with intermittent or mild persistent asthma, 15–16 patients with moderate persistent asthma, and 11–13 patients with severe persistent asthma.

^bNumber of subjects who used any of the following asthma medications: inhaled corticosteroids, long-acting β₂-agonists, leukotriene receptor antagonists, theophylline, or oral corticosteroids.

^c $p < 0.05$ and ^d $p < 0.01$ vs. intermittent or mild persistent.

though high-IgE asthmatic subjects were younger and included more male subjects than low-IgE asthmatic subjects, there were no differences in smoking habits, duration of asthma, lung function, degree of AHR, medication use, or clinical severity between the two groups. The frequency of CD4⁺ 6B11⁺ iNKT cells ($p < 0.01$) or CD4⁺ Vα24⁺ iNKT cells ($p < 0.01$) was significantly lower in high-IgE asthmatic subjects than in low-IgE asthmatic subjects, although there was no significant difference in the frequency of CD3⁺ CD56⁺ NKT cells, CD3⁺ 6B11⁺ iNKT cells, CD3⁺ Vα24⁺ iNKT cells, CD3⁺ α-GalCer-loaded CD1d– tetramer⁺ iNKT cells or CD4⁺ α-GalCer-loaded CD1d– tetramer⁺ iNKT cells between the two groups. The ratio of CD4⁺ 6B11⁺ iNKT cells to CD3⁺ 6B11⁺ iNKT cells was significantly lower in high-IgE asthmatic subjects than in low-IgE asthmatic subjects ($p < 0.01$). However, for Vα24⁺ iNKT cells or α-GalCer-loaded CD1d– tetramer⁺ iNKT cells, the ratio of CD4⁺ iNKT cells to CD3⁺ iNKT cells did not differ between the two groups (Table 3).

Log-transformed IgE levels were negatively correlated with the frequency of CD4⁺ Vα24⁺ iNKT cells ($p < 0.05$, Fig. 2), the ratio of CD4⁺ 6B11⁺ iNKT cells to CD3⁺ 6B11⁺ iNKT cells ($r = -0.49$, $p = 0.001$, $n = 40$), and the ratio of CD4⁺ Vα24⁺ iNKT cells to CD3⁺ Vα24⁺ iNKT cells ($r = -0.27$, $p = 0.08$, $n = 41$).

3.4. Lower number of blood iNKT cells in atopic asthmatic subjects

We classified asthmatic subjects into nonatopic and atopic groups on the basis of the results of skin tests. Although atopic asthmatic subjects were younger and included more male subjects than nonatopic asthmatic subjects, there were no differences in smoking habits, duration of asthma, lung function, the degree of AHR, medication use, and clinical severity between the two groups. The frequency of CD3⁺ α-GalCer-loaded CD1d– tetramer⁺ iNKT cells ($p < 0.05$) or CD4⁺ α-GalCer-loaded CD1d– tetramer⁺ iNKT cells ($p < 0.05$) was significantly lower in atopic asthmatic subjects than in nonatopic asthmatic subjects, although there was no significant difference in the frequency of CD3⁺ CD56⁺ NKT cells, CD3⁺ 6B11⁺ iNKT cells, CD3⁺ Vα24⁺ iNKT cells, CD4⁺ 6B11⁺ iNKT cells, or CD4⁺ Vα24⁺ iNKT cells between the two groups. The ratio of CD4⁺ 6B11⁺ iNKT cells to CD3⁺ 6B11⁺ iNKT cells ($p < 0.01$) or CD4⁺ Vα24⁺ iNKT cells to CD3⁺ Vα24⁺ iNKT cells ($p = 0.07$) was lower in atopic asthmatic subjects than in nonatopic asthmatic subjects. However, for α-GalCer-loaded CD1d– tetramer⁺ iNKT cells, the ratio of CD4⁺ iNKT cells to CD3⁺ iNKT cells did not differ between the two groups (Table 4).

Table 3
Frequency of blood natural killer T cells in low-IgE and high-IgE asthmatic subjects

	Serum total IgE (IU/ml)	
	≤500 (N = 30)	>500 (N = 12)
CD3 ⁺ CD56 ⁺ NKT cells (%) ^a	2.22 ± 0.51	1.48 ± 0.42
CD3 ⁺ iNKT cells (%) ^a		
CD3 ⁺ 6B11 ⁺ iNKT	0.28 ± 0.05	0.16 ± 0.04
CD3 ⁺ Vα24 ⁺ iNKT	0.52 ± 0.06	0.37 ± 0.06
CD3 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	0.23 ± 0.07	0.25 ± 0.13
CD4 ⁺ iNKT cells (%) ^a		
CD4 ⁺ 6B11 ⁺ iNKT	0.09 ± 0.01	0.03 ± 0.01 ^c
CD4 ⁺ Vα24 ⁺ iNKT	0.28 ± 0.02	0.17 ± 0.02 ^c
CD4 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	0.08 ± 0.02	0.13 ± 0.07
Ratio of CD4 ⁺ iNKT cells to CD3 ⁺ iNKT cells (%)		
CD4 ⁺ /CD3 ⁺ 6B11 ⁺ iNKT	45.5 ± 4.2	22.7 ± 6.7 ^c
CD4 ⁺ /CD3 ⁺ Vα24 ⁺ iNKT	61.0 ± 4.2	53.9 ± 7.3
CD4 ⁺ /CD3 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	45.5 ± 6.1	61.8 ± 11.1

α-GalCer, α-galactosylceramide; iNKT, invariant natural killer T cells.

^aData are expressed as percentage of CD3⁺ T cells.

^bData are obtained from 15–17 asthmatic subjects with low IgE and six asthmatic subjects with high IgE values.

^c $p < 0.01$ vs. low-IgE asthmatic subjects.

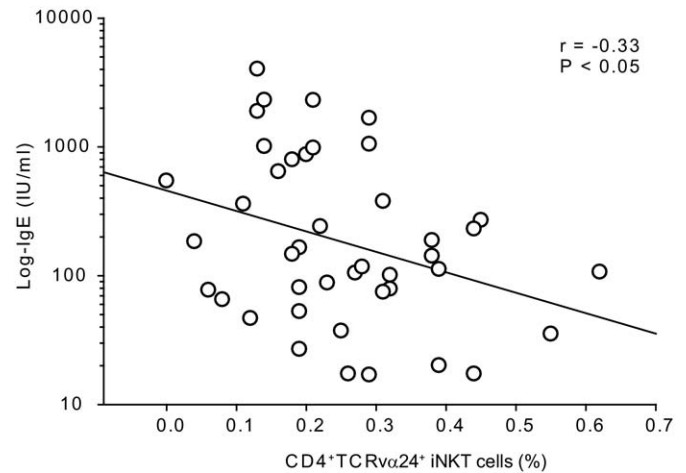


Fig. 2. Negative correlations between the frequency of CD4⁺ Vα24⁺ invariant natural killer T (iNKT) cells and serum total IgE levels in asthmatics. The frequency of iNKT cells was expressed as % of CD3⁺ T cells, and IgE levels was log transformed.

Atopy scores were negatively correlated with the frequency of CD4⁺ 6B11⁺ iNKT cells ($r = -0.28$, $p = 0.06$, $n = 44$), CD4⁺ Vα24⁺ iNKT cells ($r = -0.25$, $p = 0.09$, $n = 44$), ratio of CD4⁺ 6B11⁺ iNKT cells to CD3⁺ 6B11⁺ iNKT cells ($r = -0.46$, $p = 0.002$, $n = 42$), and ratio of CD4⁺ Vα24⁺ iNKT cells to CD3⁺ Vα24⁺ iNKT cells ($r = -0.35$, $p = 0.01$, $n = 43$).

3.5. No effect of asthma medications on association between blood iNKT cells and atopy

We aimed to determine whether asthma medications would affect the frequency of blood iNKT cells. There were no differences observed in the frequency of CD3⁺ 6B11⁺ iNKT cells ($0.19\% \pm 0.03\%$ vs. $0.27\% \pm 0.06\%$, $p > 0.05$), CD3⁺ Vα24⁺ iNKT cells ($0.44\% \pm 0.04\%$ vs. $0.50\% \pm 0.06\%$, $p > 0.05$), CD3⁺ α-GalCer-loaded CD1d– tetramer⁺ iNKT cells ($0.24\% \pm 0.09\%$ vs. $0.21\% \pm 0.07\%$, $p > 0.05$), CD4⁺ 6B11⁺ iNKT cells ($0.06\% \pm 0.01\%$ vs. $0.07\% \pm 0.01\%$, $p > 0.05$), CD4⁺ Vα24⁺ iNKT cells ($0.24\% \pm 0.03\%$ vs. $0.25\% \pm 0.02\%$, $p > 0.05$), and CD4⁺ α-GalCer-loaded CD1d– tetramer⁺ iNKT cells ($0.14\% \pm 0.05\%$ vs. $0.05\% \pm 0.01\%$, $p > 0.05$) between asthmatic subjects with medications and those without medications. To exclude a possible confounding effect of the medications on the association between iNKT cells and atopy, we limited the analyses to asthmatic

Table 4
Frequency of blood natural killer T cells in nonatopic and atopic asthmatic subjects

	Nonatopy (n = 22)	Atopy (n = 22)
CD3 ⁺ CD56 ⁺ NKT cells (%) ^a	1.79 ± 0.56	2.13 ± 0.50
CD3 ⁺ iNKT cells (%) ^a		
CD3 ⁺ 6B11 ⁺ iNKT	0.24 ± 0.06	0.25 ± 0.05
CD3 ⁺ Vα24 ⁺ iNKT	0.48 ± 0.06	0.49 ± 0.07
CD3 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	0.33 ± 0.08	0.07 ± 0.01 ^c
CD4 ⁺ iNKT cells (%) ^a		
CD4 ⁺ 6B11 ⁺ iNKT	0.08 ± 0.01	0.06 ± 0.01
CD4 ⁺ Vα24 ⁺ iNKT	0.27 ± 0.02	0.22 ± 0.02
CD4 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	0.13 ± 0.04	0.02 ± 0.01 ^c
Ratio of CD4 ⁺ iNKT cells to CD3 ⁺ iNKT cells (%)		
CD4 ⁺ /CD3 ⁺ 6B11 ⁺ iNKT	46.8 ± 5.2	27.8 ± 4.5 ^d
CD4 ⁺ /CD3 ⁺ Vα24 ⁺ iNKT	64.0 ± 4.8	51.3 ± 4.8
CD4 ⁺ /CD3 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	49.2 ± 6.3	43.5 ± 10.3

α-GalCer, α-galactosylceramide; iNKT, invariant natural killer T cells.

^aData are expressed as percentage of CD3⁺ T cells.

^bData were obtained from 14 to 15 nonatopic asthmatic subjects and nine to 10 atopic asthmatic subjects.

^c $p < 0.05$ and ^d $p < 0.01$ vs. nonatopic asthmatic subjects.

subjects without medications. The frequency of CD3⁺ 6B11⁺ iNKT cells ($0.12\% \pm 0.03\%$ vs. $0.34\% \pm 0.08\%$, $p < 0.05$), CD3⁺ Vα24⁺ iNKT cells ($0.29\% \pm 0.06\%$ vs. $0.57\% \pm 0.08\%$, $p = 0.07$), CD4⁺ 6B11⁺ iNKT cells ($0.03\% \pm 0.01\%$ vs. $0.09\% \pm 0.01\%$, $p < 0.05$), or CD4⁺ Vα24⁺ iNKT cells ($0.15\% \pm 0.02\%$ vs. $0.28\% \pm 0.03\%$, $p < 0.05$) was lower in asthmatic subjects with high IgE compared with those with low IgE. Log-transformed IgE levels were correlated with the frequency of CD4⁺ Vα24⁺ iNKT cells ($r = -0.37$, $p < 0.05$). The frequency of CD4⁺ α-GalCer-loaded CD1d– tetramer⁺ iNKT cells was lower in atopic asthmatic subjects compared with nonatopic asthmatic subjects ($0.02\% \pm 0.01\%$ vs. $0.08\% \pm 0.02\%$, $p = 0.06$).

3.6. Frequency of blood iNKT cells according to asthma severity

We further analyzed the frequency of blood iNKT cells according to the clinical severity in asthmatic subjects. However, there were no difference in the frequency of blood iNKT cells among patients with intermittent or mild persistent, moderate persistent, and severe persistent asthma (Table 5).

4. Discussion

The present study showed that the number of peripheral blood iNKT cells was reduced in atopic asthmatic subjects compared with nonatopic asthmatic subjects and was negatively correlated with serum total IgE levels in asthmatic subjects, indicating that blood iNKT cells may be inversely associated with atopy in human asthma.

Human iNKT cells segregate into CD4⁺, CD4[−]CD8[−] (double negative [DN]), and CD8⁺ cell subsets [23], which differ in their functional properties. CD4⁺ iNKT subsets produce both T_H1 and T_H2 cytokines, whereas DN iNKT subsets produce T_H1 cytokines [24]. Thus, CD4⁺ iNKT cells may promote T_H2 responses in some conditions and T_H1 in others. Interestingly, in the present study, the number of CD4⁺ iNKT cells or the fraction of CD4⁺ iNKT cells to CD3⁺ iNKT cells was inversely related to atopic index, which indicates that blood CD4⁺ iNKT cell subsets are likely to promote T_H1 responses in human asthma. iNKT cells are also known to play crucial roles in anti-infectious responses. It has been reported that human CD4⁺ iNKT cells have antibacterial activity partly via IFN-γ production [25]. In addition, Liu et al. [26] reported that dendritic cells stimulated by CD4⁺ iNKT cell subsets preferentially induced T_H1 responses, whereas dendritic cells reacted with DN iNKT cell subsets induced a shift toward T_H2. Thus, it is possible that blood CD4⁺ iNKT cells behave like T_H1-like iNKT cells in human asthma.

Blood CD4⁺ iNKT cell subsets might differ functionally from lung CD4⁺ iNKT cells. It has been reported that CD4⁺ iNKT cells in the lungs of asthmatic subjects produced both IL-4 and IL-13 but little IFN-γ on intracellular cytokine staining after activation with phorbol myristyl acetate and ionomycin or by measurement of

cytokines in supernatants after activation with α-GalCer [19], which indicates that lung CD4⁺ iNKT cells may be a T_H2-like iNKT cells.

Thus, CD4⁺ iNKT cells might function differently according to cellular location. iNKT cells could be categorized into subsets on the basis of chemokine-receptor expression or tissue of origin [27]. However, we could not exclude the possibility that T_H2-like CD4⁺ iNKT cells are recruited into the lungs because of airway inflammations and that, as a consequence, T_H1-like CD4⁺ iNKT cells are left in the blood of asthmatic individuals. However, our finding that the number of blood CD4⁺ iNKT cells was not lower in asthmatic subjects compared with normal controls may not support that possibility. Further studies are needed to determine the cytokine expression profiles of blood CD4⁺ iNKT cells. The present study showed that the frequency of blood CD4⁺ iNKT cells did not differ according to clinical severity in asthmatic subjects, although it has been suggested that the level of lung CD4⁺ iNKT cells may be related to asthma severity [19,28]. BAL was not performed in the present study, because we focused on blood iNKT cells but not lung iNKT cells to determine the role for blood iNKT cells in the pathogenesis of atopy in asthma. However, it is needed to measure simultaneously iNKT cells in both blood and BAL fluid to elucidate the mechanism of how they differ from each other in asthma.

Although Ikegami, et al. [14] reported that the number of blood iNKT cells did not differ according to the atopic status in asthmatic subjects, they did not examine blood CD4⁺ iNKT subsets. To the best of our knowledge, our study may be the first report to show clearly that blood CD4⁺ iNKT cells are inversely associated with atopic index in asthmatic individuals. However, in contrast to our findings, Magnan et al. [15] reported that blood CD4⁺ iNKT cells were positively related to atopy and total IgE levels in healthy volunteers. We can speculate that human blood CD4⁺ iNKT cells might behave like as T_H2-like iNKT cells before the development of asthma and allergic diseases but as T_H1-like iNKT cells after establishment of the diseases.

In the present study, atopic asthmatic subjects were younger and included more male subjects compared with nonatopic asthmatic subjects. It has been reported that human blood iNKT cells decrease with age [29] and are not affected by gender [30]. Our finding that young atopic asthmatic subjects had lower numbers of CD4⁺ iNKT cells may suggest that our results are unlikely to be affected by age. For similar reasons, it is less likely that differences in age and gender between asthmatic subjects and controls may affect the statistical results.

We included some asthmatic subjects with medications, such as inhaled corticosteroids, in the present study. However, the medications did not affect the number of blood iNKT cells and our results. If blood CD4⁺ iNKT cells are clarified to play a role in the pathogenesis of atopy in human asthma, any new therapeutic agents to enhance the function of blood CD4⁺ iNKT cells will be effective for difficult-to-treat asthma resistant to current asthma medications. It has been reported that α-GalCer-activated NKT cells may induce expansion of CD4⁺ CD25⁺ T-regulatory cells, which in turn mediate the therapeutic effects of α-GalCer in experimental autoimmune myasthenia gravis [31].

It is possible that there are minor differences among Vα24⁺ iNKT cells, 6B11⁺ iNKT cells, and α-GalCer-loaded CD1d– tetramer⁺ iNKT cells. Staining with Vα24 monoclonal antibodies may include non-invariant and non-CD1d– restricted Vα24⁺ T cells. Therefore, Vα24⁺ staining could lead to an overestimation of iNKT cells number. In the present study, the frequency of Vα24⁺ iNKT cells was higher than that of 6B11⁺ iNKT cells or α-GalCer-loaded CD1d– tetramer⁺ iNKT cells. In addition, α-GalCer-loaded CD1d– tetramer may bind to a small, diverse population of noninvariant T cells not recognized by 6B11 or Vα24 antibodies, but 6B11 or Vα24

Table 5

Frequency of peripheral blood iNKT cells according to asthma severity

	Intermittent or mild persistent (n = 15)	Moderate persistent (n = 17)	Severe persistent (n = 13)
CD3 ⁺ iNKT cells (%) ^a			
CD3 ⁺ 6B11 ⁺ iNKT	0.35 ± 0.09	0.22 ± 0.06	0.15 ± 0.03
CD3 ⁺ Vα24 ⁺ iNKT	0.60 ± 0.10	0.42 ± 0.06	0.42 ± 0.07
CD3 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	0.24 ± 0.08	0.25 ± 0.12	0.17 ± 0.07
CD4 ⁺ iNKT cells (%) ^a			
CD4 ⁺ 6B11 ⁺ iNKT	0.08 ± 0.01	0.05 ± 0.01	0.08 ± 0.02
CD4 ⁺ Vα24 ⁺ iNKT	0.27 ± 0.03	0.21 ± 0.02	0.25 ± 0.04
CD4 ⁺ α-GalCer-CD1d ⁺ iNKT ^b	0.10 ± 0.02	0.08 ± 0.05	0.09 ± 0.05

α-GalCer, α-galactosylceramide; iNKT, invariant natural killer T cells.

^aData are expressed as percentage of CD3⁺ T cells.

^bEach group included seven to nine asthmatic subjects. There were no statistically significant differences between any two groups.

antibodies may bind to invariant T cells that recognize a lipid antigen different from α -GalCer [32,33].

In conclusion, we hypothesize that peripheral blood CD4⁺ iNKT cells may behave similarly to T_H1-like iNKT cells in individuals with chronic asthma, which might inhibit IgE production in human asthma. The development of a new immunomodulator to activate blood CD4⁺ iNKT cells will be useful to control asthma and allergic diseases.

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