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CD4$^-$CD8$^-$ T cells bearing invariant Vα24JαQ TCR α-chain are decreased in patients with atopic diseases

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SUMMARY

Atopic disorders are caused by disregulated activation of T helper 2 (Th2) cells that produce IL-4 and IL-5. Because the presence of IL-4 potently augments the differentiation of naive T cells into Th2 cells, it is important to seek the cell population which provides IL-4 for naive T cells. Recently, a unique subpopulation of T cells, natural killer (NK) T cells, has been shown to produce a large amount of IL-4 upon activation, suggesting their regulatory role in initiation of Th2 cell differentiation. To determine whether NK T cells play a regulatory role in human Th2 cell-mediated atopic diseases, we analysed the frequency of invariant Vα24JαQ CD4$^-$CD8$^-$ double-negative (DN) T cells, human NK T cells, in patients with atopic asthma and atopic dermatitis. We also studied cytokine production from Vα24$^+$ Vβ11$^+$ DN T cells, which comprise most of Vα24JαQ DN T cells. We found that the invariant Vα24JαQ DN T cells were greatly diminished in patients with asthma and atopic dermatitis. On the other hand, there was no significant difference in Vα24$^+$ CD4$^+$ T cells possessing invariant Vα24JαQ TCR between healthy subjects and atopic patients. We also found that Vα24$^+$ Vβ11$^+$ DN T cells from healthy subjects predominantly produced interferon-gamma (IFN-γ) but not IL-4 upon activation. These results suggest that NK T cells may not be essential for human atopic disease and that the disappearance of NK T cells, most of which produce IFN-γ, may be involved in the pathogenesis of atopic diseases.

Keywords Vα24JαQ TCR α-chain natural killer T cells atopic diseases interferon-gamma

INTRODUCTION

A considerable amount of investigation has centred on the pathogenesis of atopic disorders, including asthma and atopic dermatitis (AD). The discovery of Th1 and Th2 subsets of CD4$^+$ T cells has helped to explain the immunological basis for the diversity of T cell responses; Th1 cells produce IL-2, interferon-gamma (IFN-γ), and lymphotoxin, while Th2 cells produce IL-4, IL-5, IL-10 and IL-13 [1–4]. Thus, cytokine production in atopic disorders is known to exhibit a bias toward the Th2 cytokines, IL-4 and IL-5 [5,6], and the requirement of these cytokines has been confirmed using murine models of allergic inflammation [7–9]. However, the mechanisms that lead to Th2 effector cell differentiation in atopic disorders are poorly understood.

In mice, one mechanism underlying the Th2 rather than Th1 bias may be promoted by the activation of natural killer (NK)1$^+$ T cells (reviewed in [10–12]). Murine NK1$^+$ T cells are a specialized subset of CD4$^-$CD8$^-$ double-negative (DN) T cell receptor (TCR) $\alpha\beta$ T cells that express the NK1 antigen, a member of the family of NKR-P1 natural killer cell receptors [10–12]. Moreover, a subpopulation of CD4$^+$ T cells also expresses the NK1 antigen on the cell surface in mice. These NK1$^+$ T cells have unusual features in comparison with the mainstream T cells. First, NK1$^+$ T cells possess an invariant TCR Vα14Jα281 that preferentially pairs with Vβ8, Vβ7, and Vβ2 [13,14]. This highly restricted TCR on NK1$^+$ T cells presumably recognizes a monomorphic MHC class I-like molecule CD1d, rather than polymorphic MHC molecules [15–18]. NK1$^+$ T cell development has recently been shown to be impaired in CD1-deficient mice [19–21]. Second, NK1$^+$ T cells can promptly produce a large amount of IL-4 by *in vivo* administration of anti-CD3 antibody and promote IgE production [22,23]. These observations suggest that the NK1$^+$ T cell is a unique T cell that is already programmed for the production of IL-4, and that NK1$^+$ T cells may play a regulatory role in Th2 cell differentiation.

Invariant Vα24JαQ DN T cells are thought to be a human counterpart of murine NK1$^+$ T cells [24–27]. The TCR Vα24JαQ chain has a high homology with murine Vα14Jα281 chain in both the amino acid and nucleotide sequences [24–27]. The Vβ chains pairing with the Vα24JαQ are Vβ11 and Vβ13, which also have a high homology with murine Vβ8 and Vβ7 [26,27]. It has recently been shown that human DN T cell clones

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bearing the invariant Vα24JαQ TCR also recognize CD1d molecule [28]. Moreover, Vα24JαQ DN T cells express NKR-P1A molecule on the surface [28,29]. Furthermore, Vα24JαQ DN T cell clones have been shown to produce both IL-4 and IFN-γ upon TCR stimulation [28,29]. However, the regulatory role of invariant Vα24JαQ DN T cells (human NK T cells) in the pathogenesis of atopic diseases has not yet been clarified.

In order to determine whether NK T cells play the regulatory role in the pathogenesis of human Th2 cell-mediated atopic diseases, we analysed the frequency of invariant Vα24JαQ DN T cells in patients with asthma and AD. We also studied cytokine production from $V\alpha24^+$ $V\beta11^+$ DN T cells, which comprise most of Vα24JαQ DN T cells. We found that the NK T cells are drastically decreased in patients with asthma and AD and that the majority of human NK T cells produce IFN-γ but not IL-4 upon activation. These results suggest that the lack of IFN-γ-producing NK T cells may be involved in the pathogenesis of atopic diseases.

PATIENTS AND METHODS

Patients

Six patients diagnosed with atopic asthma [30] and three patients diagnosed with AD [31] were studied (Table 1). All were atopic patients who had >300 U/ml of serum IgE determined with radioimmunosorbent test (RIST) and had positive results of radio-allergosorbent test (RAST) against at least one of 30 allergens, including *Dermatophagoides farinae*, house dust, cat dander, grass pollens and five moulds. Asthmatic patients were taking inhaled β_2-adrenergic agonists and/or oral theophyllines, some of them took inhaled beclomethasone, but none of them took oral corticosteroids. AD patients were treated with corticosteroid ointments, but no oral corticosteroids. All patients were in a stable condition of disease when analysed. Five healthy subjects with no history of atopic diseases and <100 U/ml of serum IgE were also examined as controls.

Flow cytometric analysis

Peripheral blood lymphocytes (PBL) were isolated from peripheral venous blood of nine atopic patients and five healthy subjects by Ficoll–Paque (Pharmacia Biotech Inc., Piscataway, NJ) density gradient centrifugation. For four-colour staining, cells were stained with FITC-, PE-, peridinin chlorophyll protein (PerCP)-, or allophycocyanin (APC)-conjugated MoAbs in PBS containing 1% fetal calf serum (FCS) for 30 min at 4°C. In some experiments, biotin-conjugated MoAbs were used and visualized by streptavidin-APC (Becton Dickinson, Mountain View, CA). The following MoAbs were used: CD4 (Leu-3a), CD8 (Leu-2a), TCRαβ (Becton Dickinson), TCR Vα24, and TCR Vβ11 (Cosmo Bio Co., Tokyo, Japan). Anti-human NKR-P1A MoAb (DX1) [32] was kindly provided by Dr L. Lanier (DNAX Research Institute, Palo Alto, CA). Stained cells were resuspended in PBS containing 1% FCS and analysed on FACScalibar (Becton Dickinson) using CELL Quest software.

Table 1. Characteristics of atopic patients studied

Patients	Age (years)	Sex	Diagnosis	Serum IgE (U/ml)	RAST	Blood eosinophils (/μl)
A-1	18	M	Asthma	680	HDM*	558
A-2	32	M	Asthma	700	HDM, C	364
A-3	41	M	Asthma	1500	HDM, G, JC	273
A-4	23	M	Asthma	2000	HDM, JC	224
A-5	59	F	Asthma	2400	HDM	233
A-6	39	M	Asthma	3500	HDM	648
A-7	25	F	AD	14 000	HDM, C	1460
A-8	24	F	AD	720	HDM, C, JC	1008
A-9	20	F	AD	1900	HDM, G, JC	376

M/F, Male/female; AD, atopic dermatitis.

*Positive results of radioallergosorbent test (RAST) against house dust mite (HDM), cat dander (C), grass pollens (G), and Japanese cedar pollen (JC).

Purification of $CD4^-CD8^-$ DN and $CD4^+$ T cells

$CD4^-CD8^-$ DN T cells were sorted from PBL of atopic patients and healthy subjects by FACStar (Becton Dickinson) using anti-CD4 and anti-CD8 MoAbs. $CD4^+$ T cells were also sorted from PBL by FACStar using anti-CD4 MoAb. The purity of the fractionated DN and $CD4^+$ T cells was $>99\%$.

Cloning and sequencing of cDNAs encoding TCR Vα24 genes

Total RNA was isolated from sorted DN or $CD4^+$ T cells using Isogen solution (Nippon Gene Co., Tokyo, Japan). The first strand complementary DNA (cDNA) was synthesized from total RNA using oligo-dT primer and avian myeloblastosis virus reverse transcriptase as described elsewhere [30]. cDNAs encoding TCR Vα24 were then amplified by polymerase chain reaction (PCR) using primers for Vα24 with an EcoRI restriction site (5′-CGAAT TCCTCAGCGATTCAGCCTCCTAC-3′) and Cα (5′-CGAATTC GGTGAATAGGCAGACAGACTT-3′). PCR products were purified by phenol extraction, precipitated with ethanol, and digested with excess amounts of EcoRI. The DNA fragments with expected sizes of the cDNAs were enriched by preparative low melting point agarose gel electrophoresis. The recovered DNA fragments were ligated to M13mp19 plasmids at EcoRI site. Phages were grown on TG-1 *Escherichia coli* cells. After hybridization with a Cα probe [33], recombinant phage DNAs were purified for DNA sequence determination. Sequencing reactions were performed by the dye primer method using an automated sequencer (Applied Biosystems, Foster City, CA).

Intracellular staining for IFN-γ and IL-4

To analyse the cytokine production from $V\alpha24^+$ $V\beta11^+$ DN T cells, sorted DN T cells were stimulated with phorbol myristate acetate (PMA; 25 ng/ml) and ionomycin (1 μM) for 4 h in RPMI 1640 medium supplemented with 10% FCS. Simultaneously, monensin (2 μM; Sigma, St Louis, MO) was added to prevent cytokine release. Cells were harvested, washed twice with PBS, and resuspended in PBS containing 1% FCS. Cells were incubated with biotin-conjugated anti-TCR Vα24 (mouse IgG1) and unconjugated anti-TCR Vβ11 (mouse IgG2a) for 30 min at 4°C and these antibodies were visualized with streptavidin–APC and anti-mouse IgG2a–PerCP (Becton Dickinson), respectively. After stained cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0·4% saponin, cells were incubated with anti-IL-4–PE and anti-IFN-γ–FITC (PharMingen, San Diego, CA) for 30 min at 4°C. Cells were washed, resuspended in PBS containing 1% FCS, and analysed on FACScalibar. To analyse cytokine production from $CD3^+$ T cells, unsorted PBL were stimulated with PMA and ionomycin in the presence of monensin as described above. After cells were

stained with anti-CD3–PerCP (Becton Dickinson), intracellular cytokines were analysed as described above.

Statistical analysis

Data are summarized as mean ± s.d. The statistical analysis of results was performed by the unpaired *t*-test. $P < 0.05$ was considered significant.

RESULTS

$V\alpha24^+$ DN T cells but not $V\alpha24^+$ $CD4^+$ T cells are decreased in patients with atopic diseases

To investigate the regulatory role of invariant Vα24JαQ DN T cells in the pathogenesis of Th2 cell-mediated atopic diseases, we evaluated the frequency of Vα24JαQ DN T cells in peripheral blood of patients with atopic asthma and AD (Table 1). We carried out a two-step frequency analysis of Vα24JαQ DN T cells as described in Patients and Methods; the frequency of $V\alpha24^+$ DN T cells was determined by three-colour FACS analysis and subsequently the frequency of Vα24JαQ rearrangement among $V\alpha24^+$ DN T cells was determined by sequencing. Results are summarized in Table 2 and representative FACS analyses of $V\alpha24^+$ DN T cells are shown in Fig. 1. Interestingly, while $V\alpha24^+$ $CD4^+$ T cells were present in peripheral blood of asthma and AD patients at a similar frequency (0·17 ± 0·06% of PBL, $n = 9$) to that of healthy subjects (0·16 ± 0·04% of PBL, $n = 5$) (R1 region of panels i and ii in Fig. 1a), $V\alpha24^+$ DN T cells were uniformly decreased in atopic patients (0·07 ± 0·04% of PBL, $n = 9$, $P < 0.001$) compared with those of healthy subjects (0·19 ± 0·05% of PBL, $n = 5$) (R2 region of panels iii and iv in Fig. 1a) (Table 2). In addition, there was no significant correlation between the decrease of $V\alpha24^+$ DN T cells and the treatments for atopic diseases (data not shown).

Since it has been shown that the majority of $V\alpha24^+$ DN T cells in normal subjects co-express TCR Vβ11 chain and a NK marker NKR-P1A [26–29], we then determined whether the decreased $V\alpha24^+$ DN T cells in atopic patients still co-expressed TCR Vβ11 chain and NKR-P1A. As controls, the expression of TCR Vβ11 chain and NKR-P1A was also analysed on $CD4^+$ T cells and $V\alpha24^+$ $CD4^+$ T cells. As shown in Fig. 1b, NKR-P1A molecule was present on approximately 15% of peripheral blood $CD4^+$ T cells in atopic patients as well as in healthy subjects, which was in agreement with the results of normal subjects reported originally by Lanier *et al*. [32]. Consistent with a similar frequency of $V\alpha24^+$ $CD4^+$ T cells between healthy subjects and atopic patients (Fig. 1a), the NKR-P1A$^+$ $V\beta11^+$ population among $V\alpha24^+$ $CD4^+$ T cells was found at a similar frequency between healthy subjects (11·0 ± 3·7%, $n = 5$) and atopic patients (10·3 ± 2·6%, $n = 9$) (Fig. 1b). However, the majority of $V\alpha24^+$ DN T cells in healthy subjects co-expressed TCR Vβ11 and NKR-P1A (55·7 ± 14·9%, $n = 5$), whereas the frequency of $V\alpha24^+$ DN T cells co-expressing NKR-P1A and TCR Vβ11 in atopic patients was significantly decreased (6·9 ± 1·4%, $n = 9$, $P < 0.001$) (Fig. 1b and Table 2).

Invariant Vα24JαQ DN T cells are diminished in patients with atopic diseases

Because Vα24JαQ DN T cells have been shown to co-express TCR Vβ11 and NKR-P1A [28,29], the low frequency of $V\beta11^+$ NKR-P1A$^+$ cells in $V\alpha24^+$ DN T cells in atopic patients suggested that the frequency of Vα24JαQ rearrangement among $V\alpha24^+$ DN T cells might be decreased. To investigate this possibility, we analysed nucleotide sequences of complementarity determining region 3 (CDR3) of TCR Vα24 genes in sorted DN T cells. In agreement with previous reports [24–27,33], the majority (86·7%) of $V\alpha24^+$ DN T cells exhibited invariant Vα24JαQ

Table 2. TCR Vα24 $CD4^-CD8^-$ double-negative (DN) T cell population in peripheral blood lymphocytes (PBL) from patients with atopic diseases

Materials	$V\alpha24^+$ DN T cells /PBL (%)	$V\beta11^+$ /$V\alpha24^+$ DN T cells (%)	NKR-P1A$^+$ /$V\alpha24^+$ DN T cells (%)	NKR-P1A$^+$ $V\beta11^+$ /$V\alpha24^+$ DN T cells (%)
Cont-1	0·24	47·2	45·2	45·8
Cont-2	0·13	44·9	51·2	43·7
Cont-3	0·17	51·0	61·7	49·4
Cont-4	0·19	60·8	62·3	59·5
Cont-5	0·21	83·3	81·4	80·1
Mean (s.d.)	0·19 (0·04)	57·4 (15·6)	60·4 (13·8)	55·7 (14·9)
A-1	0·06	9·9	15·3	7·5
A-2	0·14	12·3	11·2	8·6
A-3	0·07	8·2	14·4	7·6
A-4	0·08	7·3	11·2	6·2
A-5	0·06	11·4	12·6	8·8
A-6	0·11	7·7	9·2	6·5
A-7	0·03	5·2	21·2	4·2
A-8	0·03	8·1	10·0	6·1
A-9	0·06	9·3	9·9	6·3
Mean (s.d.)	0·07 (0·03)*	8·8 (2·1)*	12·8 (3·7)*	6·9 (1·4)*

The expression of TCR Vβ11 and NKR-P1A in peripheral blood Vα24 DN T cells from healthy subjects (cont-1 to -5) and patients with atopic diseases (A-1 to -9) was analysed by FACS using anti-CD4 peridinin chlorophyll protein (PerCP) plus anti-CD8 PerCP, anti-Vα24 biotin + streptavidin–allophycocyanin (APC), anti-Vβ11 FITC, and NKR-P1A + anti-mIgGl PE. PBL, Peripheral blood lymphocytes.

*Significantly different from the mean value of healthy subjects, $P < 0.001$.

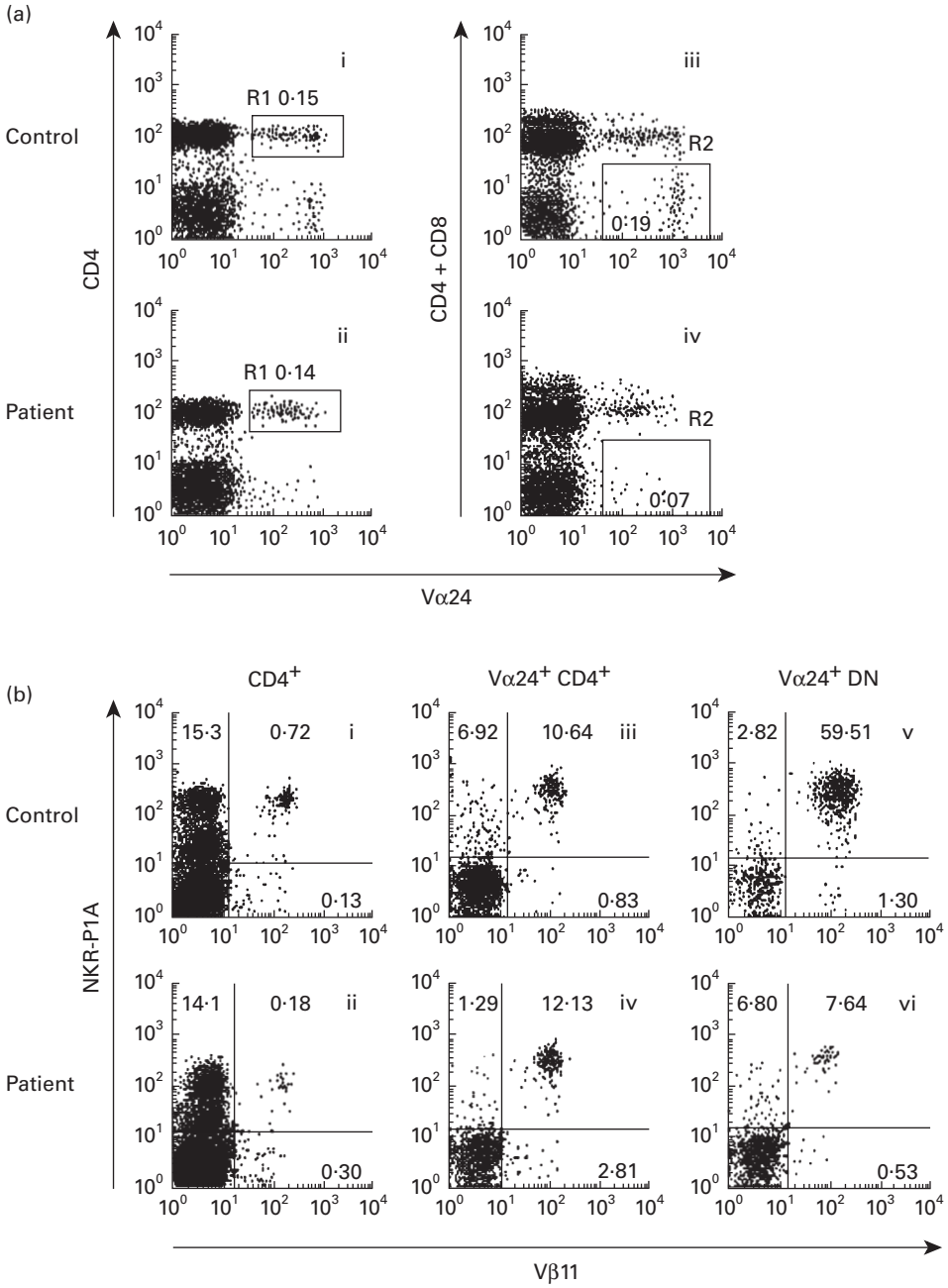

Fig. 1. (a) Vα24$^+$ double-negative (DN) T cells but not Vα24$^+$ CD4$^+$ T cells are decreased in patients with atopic diseases. TCR Vα24$^+$ CD4$^+$ T cells (left) and Vα24$^+$ CD4$^-$CD8$^-$ DN T cells (right) in peripheral blood lymphocytes (PBL) from healthy subjects (i, iii) and atopic patients (ii, iv) were analysed by FACS using anti-CD4 peridinin chlorophyll protein (PerCP) (plus anti-CD8 PerCP) and anti-TCR Vα24 biotin + streptavidin–allophycocyanin (APC). (b) Vα24$^+$ Vβ11$^+$ DN T cells are diminished in patients with atopic diseases. The expression of TCR Vβ11 chain and NKR-P1A was analysed on CD4$^+$ T cells, Vα24$^+$ CD4$^+$ T cells, and Vα24$^+$ DN T cells. i, ii: PBL were stained with anti-CD4 PerCP, anti-TCR Vβ11 FITC, and anti-NKR-P1A (mIgG1) + anti-mIgG1 PE. iii–vi: PBL were stained with anti-CD4 PerCP (plus anti-CD8 PerCP), anti-Vα24 biotin + streptavidin–APC, anti-Vβ11 FITC, and anti-NKR-P1A + anti-mIgG1 PE.

rearrangement in healthy subjects (Table 3). In contrast, the nucleotide sequences of CDR3 in Vα24$^+$ DN T cells were random in atopic patients and Vα24JαQ rearrangement was hardly detectable in the patients (Table 3). Thus, the actual number of Vα24JαQ DN T cells in peripheral blood was severely decreased in atopic patients ($0{\cdot}04 \pm 0{\cdot}06$/mm^3, $n = 9$, $P < 0{\cdot}001$) compared with that of healthy subjects ($2{\cdot}7 \pm 0{\cdot}5$/mm^3, $n = 5$) (Table 3). On the other hand, very few Vα24$^+$ CD4$^+$ T cells possessed invariant Vα24JαQ TCR in both healthy subjects ($2{\cdot}5 \pm 3{\cdot}5\%$, $n = 5$) and atopic patients ($3{\cdot}5 \pm 4{\cdot}5\%$, $n = 9$).

The majority of Vα24$^+$ Vβ11$^+$ DN T cells produce IFN-γ but not IL-4

Murine NK T cells have been shown to produce large amounts of

Table 3. Frequencies of invariant Vα24JαQ double-negative (DN) T cells in patients with atopic diseases

Source	Vα24JαQ/Vα24†	Vα24$^+$ DN T cells‡ (/mm^3)	Vα24JαQ DN T cells (/mm^3)
Cont-1	15/20	3·9	2·9
Cont-2	16/20	2·3	1·8
Cont-3	16/18	2·9	2·6
Cont-4	17/19	3·4	3·0
Cont-5	18/18	3·2	3·2
Mean (s.d.)	86·7% (9·6%)	3·1 (0·6)	2·7 (0·5)
A-1	2/17	1·0	0·11
A-2	1/16	2·9	0·18
A-3	0/16	1·2	0
A-4	0/16	1·6	0
A-5	0/16	1·4	0
A-6	0/16	2·5	0
A-7	3/16	0·4	0·05
A-8	0/16	0·5	0
A-9	0/16	0·8	0
Mean (s.d.)	4·1% (6·3%)*	1·4 (0·8)*	0·04 (0·06)*

*TCR Vα24 cDNA clones were randomly isolated from the polymerase chain reaction (PCR)-amplified libraries of DN T cells from healthy subjects (cont-1 to -5) and patients with atopic diseases (A-1 to -9), and the frequency of Vα24JαQ rearrangement among Vα24 cDNA clones was determined by sequencing of complementarity determining region 3 (CDR3) of TCR Vα24 genes.

†TCR Vα24 DN T cells in peripheral blood lymphocytes from healthy subjects and patients with atopic diseases were analysed by FACS.

*Significantly different from the mean value of healthy subjects, $P < 0{\cdot}001$.

IL-4 upon activation [22,23]. Human Vα24JαQ DN T cell clones have been shown to produce both IL-4 and IFN-γ upon TCR stimulation, and hence their cytokine profiles have been described as Th0 type [28,29]. Thus, in contrast to murine NK T cells, the production of IL-4 from human Vα24$^+$ Vβ11$^+$ T cells might be limited. Therefore, we decided to analyse cytokine production from Vα24$^+$ Vβ11$^+$ DN T cells, which are selectively decreased in atopic patients (Table 2). In these experiments, purified DN T cells from healthy subjects were stimulated with PMA and ionomycin for 4 h and subjected to four-colour FACS analysis with surface staining for Vα24$^+$ and Vβ11$^+$ and intracellular staining for IL-4 and IFN-γ. The majority (78·6%) of human Vα24$^+$ Vβ11$^+$ DN T cells produced IFN-γ but not IL-4 (Th1 type) and only a small portion (7·1%) of them produced both IL-4 and IFN-γ (Th0 type) ($n = 5$) (Fig. 2 and Table 4). Furthermore, the frequency of Th1 cells among Vα24$^+$ Vβ11$^+$ DN T cells was significantly higher than that among CD3$^+$ T cells (16·5 $\pm$ 2·7%, $n = 5$, $P < 0{\cdot}001$) (Fig. 2). These results indicate that, in contrast to murine NK T cells, human Vα24$^+$ Vβ11$^+$ DN T cells predominantly produce IFN-γ.

DISCUSSION

Cytokine production in atopic disorders is known to exhibit a bias toward the Th2 cytokines, IL-4 and IL-5. It has been shown that cytokines present during the initiation of an immune response determine the outcome of a particular Th subset [3,4]. Although IL-4 signal may not be essential for Th2 cell differentiation [34], the presence of IL-4 potently augments the differentiation of naive T cells into Th2 cells [35,36]. Most compelling evidence for this is obtained by studies showing that mice lacking either IL-4 [37,38] or Stat6 [39–41], a transcription factor activated by IL-4, fail to generate Th2 cells and the IgE responses. Despite the importance of IL-4, however, it is not clear how IL-4 synthesis is ever initiated. Several candidates for the initial source of IL-4 have been suggested. In addition to Th2 cells themselves, mast cells [42], $\gamma\delta^+$ T cells [43], and NK T cells [44] are all capable of producing IL-4. In this regard, it is striking that NK T cells can produce IL-4 without cell division, while naive $\alpha\beta$ T cells can not produce IL-4 until they proceed through at least three cell divisions even in the presence of IL-4 [45]. Nevertheless, it has become clear that murine NK T cells are not essential for *in vivo* Th2 responses, because mice lacking detectable NK T cells can still mount Th2 responses [19–21,46,47]. In addition, the Th2 allergic pulmonary responses induced by exogenous antigens have been shown to be independent of NK T cells in mice [47], although these responses are apparently IL-4-dependent [9].

In this study, we found that the invariant Vα24JαQ DN T cells, which are a human counterpart of murine NK T cells [24–27], were greatly diminished in patients with asthma and AD (Tables 2 and 3), suggesting that Th2 cytokines from NK T cells may not be essential for human Th2 cell-mediated atopic diseases. On the other hand, there was no significant difference in Vα24$^+$ CD4$^+$ T cells possessing invariant Vα24JαQ TCR between healthy subjects and atopic patients. Second, we also found that human NK T cells predominantly produced IFN-γ but not IL-4 upon activation (Fig. 2 and Table 4). Given that IFN-γ is known to inhibit Th2 responses in allergic airway inflammation [48], these results suggest that the disappearance of NK T cells, most of which produce IFN-γ, may be one of the mechanisms underling the pathogenesis of atopic diseases.

The number of NK T cells is regulated by the balance between their generation and disappearance. Thus, the diminished NK T cells in atopic patients should result from decreased generation and/or increased disappearance. Although there is as yet no information on the regulation of NK T cell development in humans, recent findings using gene-targeting mice revealed the mechanisms of NK T cell development in mice. Mice lacking rearrangement for Vα14Jα281 were found to lack NK T cells, suggesting that Vα14Jα281 rearrangement is essential for NK T cell development [49]. However, it is unlikely that atopic patients are defective in TCR Vα24JαQ rearrangement because all atopic patients exhibited Vα24JαQ rearrangement in CD4$^+$ T cells at a similar frequency to that of healthy subjects. Beside TCR Vα rearrangement, cytokine signalling from common cytokine receptor γ-chain (γc), which is a shared component of the receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 [50], is known to be essential for NK T cell development [51]. Because NK T cells can develop in mice lacking IL-2 or IL-7Rα [50] but not in mice lacking IL-2Rβ [52], which is a shared component of the receptors for IL-2 and IL-15, it is likely that IL-15 is important for NK T cell development. In fact, it has recently been reported that NK T cell development is impaired in IL-15R-deficient mice [53]. Therefore, it is possible that IL-15 itself or IL-15-related signalling molecules, such as Stat5, may be involved in the diminished NK T cells in atopic patients.

Recently, Eberl *et al*. [54] have shown that the activation of

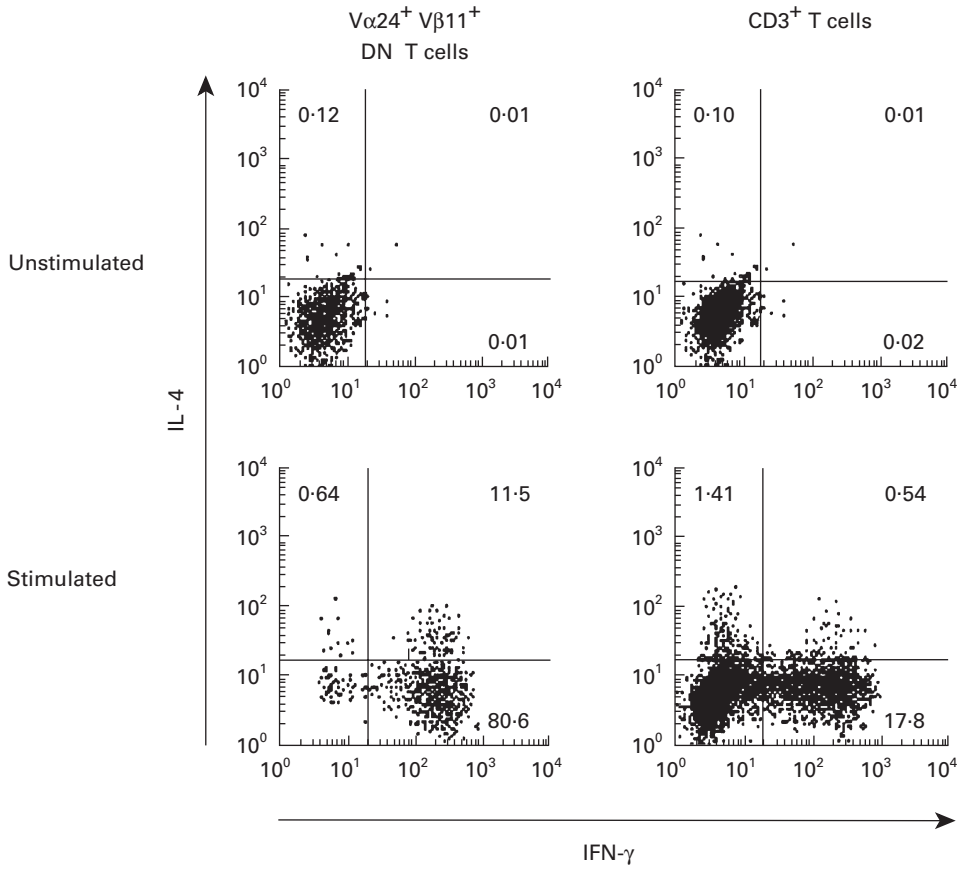

Fig. 2. IFN-γ production from Vα24$^+$ Vβ11$^+$ double-negative (DN) T cells upon activation. Left: sorted DN T cells from healthy subjects were unstimulated or stimulated with phorbol myristate acetate (PMA; 25 ng/ml) plus ionomycin (1 μM) for 4 h, and cytokine production was evaluated by intracellular staining for IL-4 and IFN-γ on Vα24$^+$ Vβ11$^+$ cells. Right: unsorted peripheral blood lymphocytes (PBL) were unstimulated or stimulated with PMA plus ionomycin and cytokine production was evaluated by intracellular staining for IL-4 and IFN-γ on the CD3$^+$ population.

murine NK T cells results in rapid disappearance of these cells. In contrast, unstimulated NK T cells exhibit a slow turnover rate [12]. Therefore, it is possible that NK T cells are continuously activated in patients with atopic diseases. Although it is difficult to analyse the turnover rate of NK T cells in human diseases, these analyses may give us a new insight into the role of NK T cells in human disease. In this regard, it remains unclear how NK T cells regulate the immune responses to exogenous antigens in an antigen-specific fashion. Because NK T cells express the limited range of TCR and because these TCR have been shown to recognize lipid ligands on CD1 molecules [16,17], the activation of NK T cells may be regulated by a distinct mechanism from that of mainstream $\alpha\beta$ T cells. Further studies including the mechanisms of NK T cell activation and the turnover of NK T cells in the site of allergic inflammation may reveal the role of NK T cells in atopic diseases.

We showed that the majority (79%) of human Vα24$^+$ Vβ11$^+$ DN T cells produced IFN-γ but not IL-4 (Th1 type) and only a small proportion (7%) of them produced both IL-4 and IFN-γ (Th0 type) (Fig. 2 and Table 4). In contrast, previous studies reported that most Vα24$^+$ Vβ11$^+$ DN T cell clones derived from peripheral blood T cells of healthy donors produced substantial amounts of both IL-4 and IFN-γ (Th0 type) upon stimulation [28,29]. Exley *et al*. [28] also described that, compared with CD4$^+$ T cells, there was a trend towards higher levels of IL-4 production by the Vα24$^+$ Vβ11$^+$ DN T cell clones, assessed by IL-4 production and IL-4/IFN-γ ratios. The difference of Th1 *versus* Th0 cytokine profiles may be due to the cells studied, i.e. uncultured peripheral blood Vα24$^+$ Vβ11$^+$ DN T cells in our

Table 4. Vα24$^+$ Vβ11$^+$ double negative (DN) T cells from healthy subjects predominantly produce IFN-γ

	Th0 (%)	Th1 (%)	Th2 (%)
Cont-1	8·51	75·20	1·39
Cont-2	5·80	73·41	1·10
Cont-3	11·45	80·60	0·64
Cont-4	6·30	81·20	0·54
Cont-5	3·52	82·59	0·82
Mean (s.d.)	7·11 (3·00)	78·60 (4·72)	0·90 (0·34)

Sorted DN T cells from healthy subjects were stimulated with phorbol myristate acetate (PMA; 25 ng/ml) plus ionomycin (1 μM) for 4 h, and cytokine production was evaluated by intracellular staining for IL-4 and IFN-γ on Vα24 Vβ11 cells. Th0, IL-4$^+$ IFN-γ$^+$; Th1, IL-4$^-$ IFN-γ$^+$ Th2, IL-4$^+$ IFN-γ$^-$.

study *versus* $V\alpha24^+$ $V\beta11^+$ DN T cell clones expanded by phytohaemagglutinin (PHA) and IL-2, which may have differentiated towards IL-4-producing cells (Th0 type). Prussin *et al.* [55] also found that using mixed $CD4^+$ and DN populations of human $V\alpha24^+$ $V\beta11^+$ T cells, cytokine profiles of those cells were either Th0 (IL-4^+ IFN-γ^+) or Th1 (IL-4^- IFN-γ^+) type.

At present there is no report on cytokine profiles of NK T cells in atopic patients. Because $V\alpha24J\alpha Q$ DN T cells were severely decreased in atopic patients compared with those of healthy subjects, and the majority of $V\alpha24^+$ $V\beta11^+$ DN T cells do not represent $V\alpha24J\alpha Q$ DN T cells in atopic patients (Table 3), it was extremely difficult to analyse cytokine profiles of $V\alpha24J\alpha Q$ DN T cells in atopic patients by our FACS analysis of $V\alpha24^+$ $V\beta11^+$ DN T cells. Therefore, this issue remains to be elucidated until cytokine profiles of $V\alpha24J\alpha Q$ DN T cells can be analysed by single-cell reverse transcriptase (RT)-PCR methods [56] in the future.

In conclusion, we have shown that the invariant $V\alpha24J\alpha Q$ DN T cells, human NK T cells, are greatly diminished in patients with atopic diseases and predominantly produce IFN-γ but not IL-4 upon activation. These results suggest that NK T cells may not be essential for human Th2 cell-mediated atopic diseases, and that the disappearance of NK T cells, most of which produce IFN-γ, may be involved in the pathogenesis of atopic diseases.

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