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Frequency of Vα24⁺CD161⁺ natural killer T cells and invariant TCRAV24-AJ18 transcripts in atopic and non-atopic individuals

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Abstract

Th2 cells play a central role in type I allergies. However, the source of interleukin-4 which may lead to a Th1/Th2 imbalance is unknown. $V\alpha24^+CD161^+$ Natural killer T (NKT) cells secrete high amounts of interleukin-4 and/or interferon-γ and are assumed to participate in the initiation of Th1/Th2 immune responses. Their contribution to the development of Th2-dependent type I allergies is controversial.
Our objective in this paper was to determine whether $V\alpha24^+CD161^+$ NKT cells differ in atopic and non-atopic adults.
Venous blood was obtained from thirteen atopic and sixteen healthy adult probands. $V\alpha24^+CD161^+$ NKT cells were determined in $CD4^+$, $CD8^{bright/dim}$ and $CD4^-CD8^-$ lymphocytes by flow cytometry. At the molecular level, the amounts of T cell receptor (TCR) AV24-AJ18 transcripts were quantified with respect to TCRAV24 chain transcripts alone or to all TCR alpha chain transcripts. To detect potential inserted nucleotides in the N-region, a novel real-time PCR-based technology was applied.
Both $CD4^+$ and $CD4^-CD8^-$ NKT cells were present at higher frequencies than $CD8^+$ NKT cells in all probands. $CD8^{dim}$ NKT cell levels were lower in healthy individuals, although not statistically significantly different to the patients. Amounts of AV24-AJ18 transcripts in relation to total TCR alpha-chains and to TCRAV24 alone were equal in both proband groups. N-region diversity was detected in four clones from four different individuals, but altered the amino acid sequence in only one clone of an atopic donor.
Analysis of $V\alpha24^+CD161^+$ NKT cell frequencies at both the cellular and molecular levels failed to reveal significant differences in peripheral blood of atopic and non-atopic probands. If NKT cells contribute to development of type I allergies they must do so at earlier times or in other locations.

Abbreviations: CDR3 = complementarity determining region; DN = double negative; FRET = fluorescence resonance energy transfer; aGalCer = alphaGalactosylceramide;

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IFN-γ = interferon-γ; IL-4 = interleukin-4; NKT = natural killer T cell; TCR = T cell receptor; TCRAV/TCRAJ = variable and joining genes of the TCR α-chain; TCRAC = constant gene of T cell receptor constant α-chain.

Introduction

The concept of a disturbed balance between Th1 and Th2 $CD4^+$ effector cells plays a central role in the current understanding of allergic diseases. Several environmental factors that are positively or negatively associated with allergic diseases have been identified (Strachan, 1989; Braun-Fahrländer et al., 1999). The circumstances that initiate a Th2-biased imbalance in $CD4^+$ lymphocyte populations are less well understood. IL-4 is a key cytokine in the establishment of a Th2-prevalence, although other IL-4-independent mechanisms exist (Hwang et al., 2002; Ritz et al., 2002). In particular, the cellular source of IL-4 that promotes the initial growth and differentiation of Th2 cells remains unknown. Potential cellular candidates that are able to synthesize high amounts of IL-4 include mast cells (Bradding et al., 1994), dendritic cells (Chang et al., 2000) and the more recently described natural killer T (NKT) cells (Bendelac et al., 1995). Once a Th2-preponderance is established, Th2-lymphocytes perpetuate the Th2 imbalance themselves. NKT cells are highly conserved between mouse and humans (Davodeau et al., 1997) and are characterized by the simultaneous expression of a T cell receptor (TCR) and the NK cell markers CD161 (NKR-P1A) and/or CD56 (Prussin & Foster, 1997). Other NK markers such as CD16, CD94 or KIR proteins are not, or only seldomly, expressed (Davodeau et al., 1997; Exley et al., 1997; Prussin & Foster, 1997) but can be induced by cytokine stimulation (Assarsson et al., 2000). Most human NKT cells derived from peripheral blood are $CD4^+$ or $CD4^-CD8^-$ (DN), whereas $CD8^+$ NKT cells are rare (Dellabona et al., 1994; Davodeau et al., 1997). Furthermore, NKT cells express an activated memory phenotype ($CD25^{hi}$, $CD45RO^{hi}$, $CD45RB^{low}$, $CD62L^{low}$) (Davodeau et al., 1997; Prussin & Foster, 1997). The most characteristic feature of NKT cells is their extremely restricted TCR alpha chain repertoire, with constant expression of the TCRAV24 chain that rearranges almost exclusively with TCRAJ18 and corresponds to a AV14-AJ281 recombination in the mouse (Makino et al., 1993). Furthermore, the N-region of the TCR alpha chain is devoid of inserted nucleotides (Dellabona et al., 1994; Han et al., 1999), with very rare reported exceptions from this general rule (Dellabona et al., 1994; Exley et al., 1997). Further restrictions of the TCR repertoire include the frequent pairing of TCRAV24-AJ18 with polyclonal TCRBV11 chains that have multiple, diverse CDR3 regions and use different TCRBJ gene segments. While beta chains other than BV11 have not been demonstrated in NKT cells in humans, three different beta chains are used in the murine system (BV7, BV2 and the murine BV11 homologue BV8.2). In contrast to conventional T cells, NKT cells recognize glycosylated lipid antigens, such as aGalCer and glycosylphosphatidylinositols (Joyce et al., 1998). These are presented by CD1d molecules (Sieling et al., 1995; Porcelli & Modlin, 1999) which are highly conserved in mammalian evolution, showing complete interspecies cross-reactivity between mouse and humans (Brossay et al., 1998). However, aGalCer which was originally purified from a marine sponge is not the natural ligand for mammalian NKT cells. An additional characteristic element of NKT cells is their ability to secrete high levels of IFN-γ and IL-4, as well as other cytokines, within an extraordinarily short time. However, the precise cytokine profile of NKT cells depends on various parameters. These include the engagement of different surface molecules with diverse signaling pathways on the NKT cells themselves as well as the presence of different NKT cell subpopulations, and the expression of different costimulatory factors (Gombert et al., 1996; Leite-de-Moraes et al., 1998, 2001) and ligands (Spada et al., 1998; Kitamura et al., 2000) in their microenvironment. Thus, TCR cross-linking generally leads to a predominant IL-4 synthesis (Yoshimoto & Paul, 1994; Leite-de-Moraes et al., 1995) whereas cross-linking of NKR-P1A leads to prevalent IFN-γ production (Arase et al., 1996). A predominant IFN-γ synthesis was found in $CD8^+$ NKT cells (Emoto et al., 2000), while $CD4^+$ and DN NKT cells produced IL-4 (Davodeau et al., 1997; Prussin & Foster, 1997). Th0 cytokine patterns have also been described in several instances (Doherty et al., 1999).

The ability of NKT cells to rapidly produce large amounts of Th1/Th2 cytokines upon primary stimulation has led to the hypothesis that they may regulate secondary adaptive immune responses. This concept is strengthened by studies of Th1- and Th2-associated diseases in different mouse models. Thus, adoptive transfer of NKT cells or transgenic expression of a TCRAV14-AJ281 protects NOD mice from disease in an IL-4 dependent manner (Baxter et al., 1997; Lehuen et al., 1998; Poulton et al., 2001), which leads to a Th2 shift (Laloux et al., 2001). Adoptive transfer of aGalCer-activated mouse splenic NKT cells attenuated dextran sulfate induced colitis (Saubermann et al., 2000) and hepatic NKT cells activated via aGalCer injection inhibited hepatitis virus B replication with an increase of interferons (Kakimi et al., 2000).

Several observations in the human system also support the concept that NKT cells are important immune regulatory cells. A selective reduction of NKT cells was observed in autoimmune diseases such as IDDM (Wilson et al., 1998), systemic sclerosis (Illes et al., 2000; Kojo et al., 2001), SLE (Sumida et al., 1998; Kojo et al., 2001) and rheumatoid arthritis (Maeda et al., 1999; Kojo et al., 2001), whereas an increase of NKT cells was observed in myasthenia gravis (Reinhardt & Melms, 1999) and T cell-reactive leprosy (Mempel et al., 2000). The majority of these are Th1-mediated immune responses. A potential role for NKT cells in Th2-mediated immune responses was postulated by studies in NKT transgenic mice, which synthesized high amounts of IL-4 and produced elevated basal IgE levels in the serum (Bendelac et al., 1996). Activation of NKT cells with aGalCer in an ovalbumin-primed mouse led to elevated serum levels of ovalbumin-specific IgE and to the generation of antigen-specific Th2 cells (Singh et al., 1999). In contrast, NKT deficient, $\beta_2m^{-/-}$ mice were unable to synthesize IL-4. A single administration of exogenous IL-4 restored their capacity to produce IL-4 since it led to a differentiation of Th precursor cells into Th2 cells (Yoshimoto et al., 1995). These data are contrasted by the finding that NKT cell deficient, $CD1^{-/-}$ mice were still able to mount Th2 responses and $\beta_2m^{-/-}$ mice were shown to generate ovalbumin-specific IgE responses (Zhang et al., 1996). Furthermore, aGalCer-activated murine NKT cells suppressed antigen-specific IgE production and Th2 lymphocyte differentiation (Cui et al., 1997). Thus, the circumstances leading to the contribution of NKT cells to either Th1- or Th2-associated diseases are still unclear.

The aim of this study was to determine whether NKT cells participate in the pathogenesis of human allergic diseases. For this purpose, the frequencies of $V\alpha24^+CD161^+$ NKT cells were investigated within peripheral blood lymphocyte (PBL) subpopulations of adults with type I allergies in comparison to non-atopic individuals. Furthermore, the frequency of TCRAV24-AJ18 transcripts was quantified and their N-regions were analyzed for possible differences between the two groups.

Methods

Proband selection

Venous blood samples were obtained from 29 selected adult volunteers following informed consent. 26 samples were stored in guanidine isothiocyanate (GT) buffer at − 80 °C for molecular analyses. Furthermore, peripheral whole blood was available in 22 probands for flow cytometric analyses (Table 1). Serum IgE levels were determined using a commercial kit (UniCAP 100, Pharmacia; IgE-Elecsys, Fa. Roche, Basel, Switzerland). The results of a radio allergy sorbent test (RAST) for forty inhalation and food allergens (CAP System IgE FEIA, Pharmacia) were available at the time of venipuncture for 22 of 29 participants. In the remaining seven patients, specific IgE was assessed with MAST CLA® Allergen specific IgE Assay (Hitachi Chemical Diagnostics, California, USA). The participants were classified into two groups according to the following criteria: a proband was classified "atopic" when clinical symptoms of type I allergy were present, together with elevated IgE serum levels greater than 150 kU/ml and specific IgE greater than RAST class 3 against at least one allergen. A proband was classified as "non-atopic" if no clinical symptoms were present, IgE serum levels were below 100 kU/ml and no specific IgE was detectable (RAST class 0). Only probands fulfilling all three criteria of the respective groups were included in the study.

Flow cytometry

Four-color flow cytometry was performed on freshly drawn whole blood samples, anticoagulated with EDTA, as described elsewhere (Gruber et al., 1993). The following primary antibodies were used: CD4-APC mouse IgG_1 (BD Pharmingen, Heidelberg, Germany), CD8-PC5 mouse IgG_1 (Immunotech, Marseille, France), $V\alpha24$-FITC mouse IgG_1 (Immunotech) and CD161 mouse IgG_1 (Pharmingen).

Table 1. Analyses and number of probands.

	Probands (n = 29)
Molecular and cellular samples	17
Molecular samples only	7
Cellular samples only	5

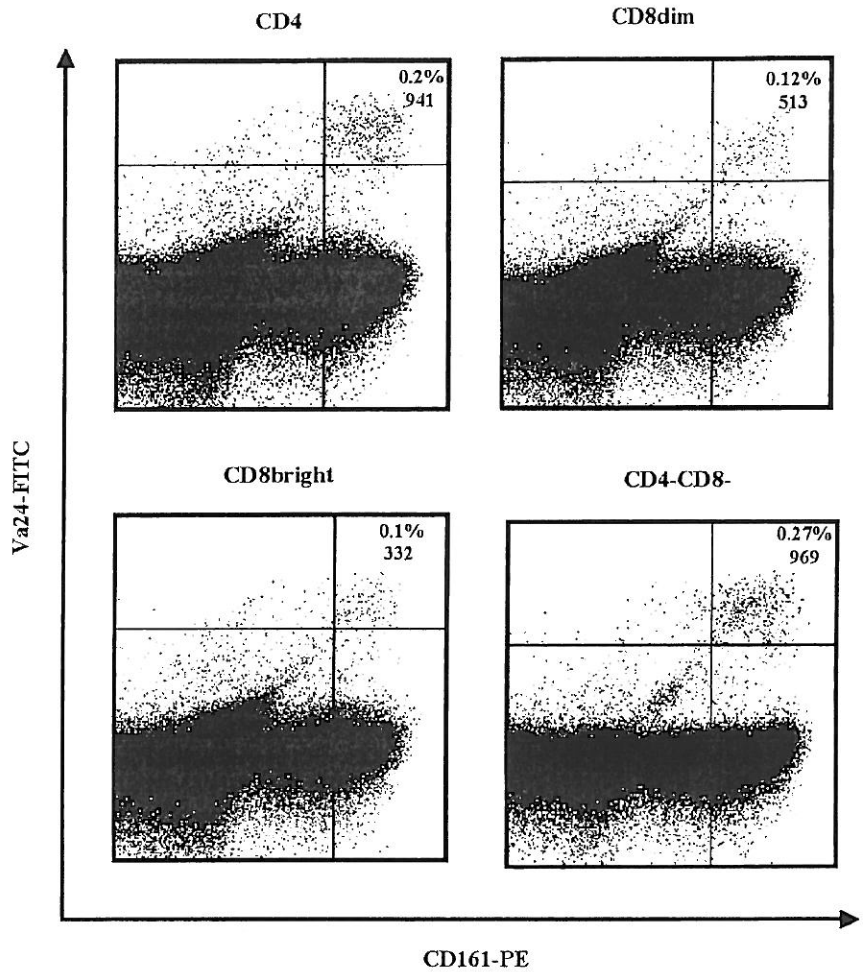

Fig. 1. Lymphocyte subsets were localized in a CD4-CD8 plot. NKT cells were identified within each lymphocyte subset by their coexpression of Vα24 and CD161. In the example presented here, 5×10^5 events were acquired from a non-atopic donor.

Mouse IgG_1-FITC (Immunotech) was used as isotype control. Samples were immediately processed for flow cytometry after venipuncture and calculations were performed with Cell Quest analysis software (FACS Calibur, Becton-Dickinson, Heidelberg, Germany). From each sample a mean of 4.5×10^5 lymphocytes (range 6×10^4 to 1.43×10^6) was acquired. To determine the percentage of $V\alpha24^+CD161^+$ T cells, lymphocytes were first gated according to their forward/side-scatter characteristics. A second gate was set on $CD4^+$, $CD8^{bright}$, $CD8^{dim}$ or DN cells and the percentage of $V\alpha24^+CD161^+$ cells was calculated within each lymphocyte subpopulation, after defining a cutoff value according to the isotype controls (Fig. 1).

Semiquantitative analysis of TCRAV24-AJ18 transcripts

Total RNA was extracted from whole blood with a modification of the phenol extraction method, according to Chomczynski et al. cDNA was synthesized using random hexamer primers and reverse transcriptase (Superscript II, GIBCO, Karlsruhe, Germany). A reference cDNA, consisting of pooled cDNAs from multiple donors, was prepared for further comparative analysis. T cell receptor constant alpha chain (TCRAC) transcripts were amplified from samples and the titrated reference cDNA using a TCRAC sense primer (5′ CTT GCC TCT GCC GTG AAT GT 3′) and a TCRAC antisense primer (5′ CTG TGC TAG ACA TGA GGT CT 3′). After denaturation at 94 °C for 1 minute, 36 and 40 cycles were run at 94 °C for 40 s, at 58 °C for 40 s and at 72 °C for 60 s, followed by one cycle at 72 °C for 7 minutes in a thermal cycler (UNO II, Fa. Biometra, Göttingen, Germany). PCR products were collected at both 36 and 40 cycles to avoid PCR endpoint analysis.

PCR products were subjected to 3% agarose ethidium bromide gel electrophoresis and further analyzed by densitometry in a fluoroimager (Vistra FluorImager SI, Molecular Dynamics, Sunnyvale, CA; software ScanControl, ImageQuaNT). The cDNA content of each probe was determined semiquantitatively by comparing the relative fluorescence intensity (rfu) of the titrated reference cDNA with the PCR products of each proband cDNA sample. In order to use equal amounts of cDNA from each donor, the cDNAs were further standardized by diluting the probes with reference to the probe with the lowest cDNA content. This standardization was verified by repeating the TCRAC PCR five times after dilution of the cDNAs. Finally, the median value of all repeated PCRs was determined for each proband. Subsequently, a TCRAV24-AJ18 PCR was performed from the standardized patient cDNA using the following primers (Porcelli et al., 1996): AV24 (5′ GAT ATA CAG CAA CTC TGG ATG CA 3′) and AJ18 (5′ GAG TTC CTC TTC CAA AGT ATA GCC 3′). After denaturation at 94 °C for 1 minute, 36 and 40 cycles were run at 94 °C for 40 s, at 61 °C for 40 s and at 72 °C for 60 s, followed by one cycle at 72 °C for 7 minutes. PCR products were collected at 36 and 40 cycles. The amount of AV24-AJ18 PCR products from each individual was calculated on a semiquantitative basis by densitometric comparison with a titrated standard DNA derived from a plasmid containing AV24-AJ18. The AV24-AJ18 PCR was repeated five times to confirm the obtained results. Results are expressed as ratios according to the following algorithm:

$$\frac{\text{median AV24} - \text{AJ18 transcripts}_{\text{proband}}}{\text{median TCRAC transcripts}_{\text{proband}}}$$

Cloning of cDNAs encoding TCRAV24

The cDNAs encoding TCRAV24-TCRAC were amplified using newly designed primers with *Xho*I and *Spe*I restriction sites: AV24 (5′ ATC C▲TC GAG GGA AAG AAC TGC ACT 5′); TCRAC (5′ GGT GAA TAG GCA GAC AGA ▼ CTA GTC ACT GGA 3′). The TCRAV24-TCRAC PCR products were digested with *Spe*I and *Xho*I (Roche, Basel, Switzerland) and purified from 3% agarose with DEAE dextran cellulose (Schleicher & Schuell, Dassel, Germany) as described elsewhere (Dretzen et al., 1981). After ligation of TCRAV24-TCRAC into pBS II SK + plasmid (Boehringer, Ingelheim, Germany) the plasmids were transfected into *E. coli* (DH5a) (Roche, Basel, Switzerland). TCRAV24-TCRAC-positive clones were analyzed for the frequency of JA18 rearrangements by PCR (AV24: 5′ GAA CTG CAC TCT TCA ATG C 3′ and AJ18: 5′ TCC AAA GTA TAG CCT CCC CAG 3′). Between 94 and 188 clones were screened per proband. The identity of TCRAV24-AJ18 TCRs was further confirmed by direct sequencing of 40 of 304 TCRAV24-AJ18 positive clones (MWG, Ebersberg, Germany).

N-region analysis using fluorescence resonance energy transfer (FRET) and melting curve analysis

In addition to the TCRAV24-AJ18 primers, two probes labeled with different dyes were designed (Tib MolBiol, Berlin, Germany). An LC-Red 640-labeled detection probe complementary to the sense strand was constructed covering the 3′ end of AV24 and the 5′ end of AJ18. LC-Red does not fluoresce by itself. A FITC-labeled anchor probe was designed 5′ upstream in direct proximity to the detection probe where an N-region would not be expected (Fig. 2A). A PCR was performed in a thermal cycler (Light Cycler, Roche Diagnostics, Mannheim, Germany). During PCR laser excitation, fluorescence of the FITC molecule (wavelength 530 nm) is transferred to the LC-Red molecule, which then starts to emit a signal at 640 nm wavelength (Fig. 2B). Single nucleotide inserts lead to a lower affinity of the detection probe thereby reducing the melting temperature. With greater mismatches the detection probe is released and its fluorescence decreases as a result of ceasing FRET. A melting curve analysis was performed at the end of each PCR run. The first negative derivative of the fluorescence ($-dF2/dT$) was plotted against the temperature. If no N-region was present, the detection probe matched the target DNA completely and the melting temperature analysis revealed a melting temperature of ~71 °C. Introduction of one single nucleotide reduced the melting temperature to ~62 °C (Fig. 2C).

Statistical analysis

Mann-Whitney *U* test was applied to test differences between two groups. A probability of $p \leq 0.05$ was regarded as statistically significant. Spearman *rho* test was performed to test the correlation between cell subsets. A correlation was considered positive if the correlation coefficient r was greater than 0.3. Statistical analysis was performed with SPSS statis-

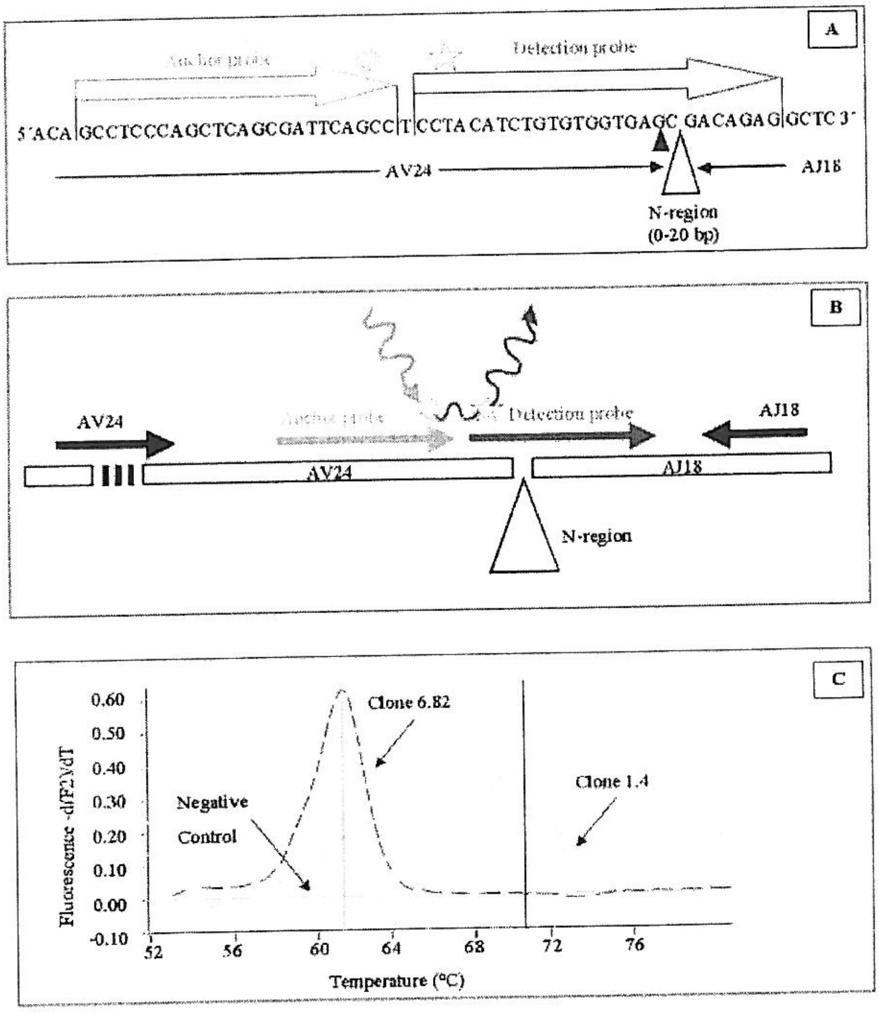

Fig. 2. (A) Schematic presentation of the positioning of the anchor probe (FITC-labelled) and the detection probe (LC-Red-labelled). (B) Principle of FRET analysis showing the conventional primers AV24 and AJ18 and the additional hybridisation probes (anchor and detection probe). Fluorescence of the FITC molecule is transferred to the LC-red molecule only if anchor and detection probe are in close proximity. (C) Melting curve analysis of two TCRAV24-AJ18 clones. Clone 1.4 has the classical AV24-AJ18 sequence without inserted nucleotides at the N-region. Clone 6.82 has a single nucleotide exchange (C to G), at the position indicated in Fig. 1A by a black arrowhead, that leads to a reduced melting temperature of the probe.

tical program (Version 10, SPSS Inc. Chicago, Illinois, USA).

Results

Characterization of the probands

According to the proband selection criteria 29 individuals were selected for the study. Thirteen probands fulfilled all three criteria for "atopic" and sixteen probands fulfilled all three criteria for "healthy" individuals and were included in the study (Table 2). All atopic donors had specific IgE (>RAST class 3) against at least one of 40 allergens, including house dust mite, furred pets, grass and tree pollen, egg, milk, nuts, fruits and vegetables. Six patients had more than one clinical diagnosis. One asthmatic patient took inhaled β_2-adrenergic agonists and/or theophylline. None of the patients used oral corticosteroids. No medications were used by the healthy subjects. IgE levels differed significantly between atopic and non-atopic probands ($p <$ 0.0001; Mann-Whitney-U).

Table 2. Proband characteristics. * $p < 0.0001$, Mann-Whitney-*U*

	Atopic (n = 13)	Non-atopic (n = 18)
Age (median, range)	33 yrs (21–41)	27 yrs (22–47)
Sex (m/f)	6/7	7/9
IgE (kU/ml)	621 (151–1 923)	21* (5–86)
Clinical diagnosis[a]	13	0
Atopic dermatitis	8	0
Allergic rhinitis	7	0
Allergic asthma	4	0
RAST class > 3[b]	13	0

[a] Six patients had more than one clinical diagnosis.
[b] All atopic patients had RAST class 4 for 3 up to 12 allergens.

Table 3. Percentages of Vα24⁺CD161⁺ cells within T-lymphocytes subsets (left columns) and calculated absolute numbers of NKT cells per 10^5 total lymphocytes (right columns). The values given are medians with the ranges in parentheses. * $p < 0.2$ (Mann-Whitney-*U*)

	Atopic Vα24⁺ CD161⁺ n = 8/13		Non-atopic Vα24⁺ CD161⁺ n = 14/18	
	%	per 10^5 lymphocytes	%	per 10^5 lymphocytes
CD4	0.09 (0.03–0.5)	42 (8–194)	0.1 (0–0.23)	52 (0–200)
CD8^{bright}	0.02 (0–0.55)	8 (0–116)	0.03 (0–0.13)	10 (0–69)
CD8^{dim}	0.31* (0–8.28)	9 0–236	0.04* (0–1.41)	21 (0–106)
DN	0.12 (0–2.26)	37 (0–685)	0.09 (0–1.27)	58 (0–398)

Table 4. Ratios of TCRAV24 transcripts to all TCR α-chain transcripts. The values given are medians with the ranges in parentheses.

atopic n = 13	non-atopic n = 11
21.26 (2.18–71.44)	18.07 (8.13–48.64)

Phenotypic characterization of Vα24⁺CD161⁺ NKT cells in peripheral blood of atopic and non-atopic adults

The frequency of Vα24⁺CD161⁺ NKT cells was evaluated by flow cytometry within CD4⁺, CD8^{bright}, CD8^{dim} and DN lymphocyte subsets in peripheral blood from 22 probands (Table 3). Samples from eight of thirteen atopic and fourteen of sixteen non-atopic adults were available for phenotype analysis. The percentage of total Vα24⁺ cells within all probands was 0.35% (median), with a range of 0.03–1.45% and did not differ between the two groups, but correlated strongly with CD4⁺Vα24⁺CD161⁺ cells ($r = 0.55$; $p < 0.008$) and CD8^{dim}Vα24⁺CD161⁺ cells ($r = 0.648$; $p < 0.03$; Spearman *rho*). CD4⁺ and DN NKT cells, defined by expression of Vα24 and CD161, were consistently verifiable in 20 of 22 probands with higher frequencies compared to CD8^{dim} and CD8^{bright} Vα24⁺CD161⁺ NKT cells. One non-atopic proband had no Vα24⁺CD161⁺ cells in any lymphocyte compartment. Levels of CD4⁺Vα24⁺CD161⁺ cells were equal in atopic and non-atopic individuals. No Vα24⁺CD161⁺ NKT cells were detectable within the CD8^{bright} and CD8^{dim} lymphocytes in six of 22 individuals, without disparity between the groups (three atopic, three non-atopic individuals). The remaining sixteen probands had only very few CD8^{bright} NKT cells. In contrast, CD8^{dim} NKT cells, if present, were more frequent than CD8^{bright} NKT cells and tended to be more numerous in allergic *vs.* non-atopic individuals (statistically not significant). Within the DN lymphocyte compartment, NKT cells were evenly distributed between both groups. Within the group of atopic individuals, no correlation between the numbers of any NKT cell subtype and IgE levels was present.

Frequency of TCRAV24-AJ18 rearrangement in TCRAV24⁺ clones of atopic and non-atopic individuals

Since Vα24⁺CD161⁺ cells are not necessarily identical to TCRAV24-AJ18 bearing cells, we next analyzed samples of 13 atopic and 11 non-atopic probands to determine whether the frequency of TCRAV24-AJ18 transcripts among all TCR alpha chains differed between the two groups. For this purpose, the TCRAV24-AJ18 transcripts were assessed semi-quantitatively in relation to all TCRAC transcripts of the same individual. The TCRAV24-AJ18/TCRAC ratios ranged from 2.18–71.44 (median 20.69) and were evenly distributed between both groups (Table 4). Since the frequency of AV24-AJ18 transcripts among the total TCR alpha chains might be too low to detect quantitative differences between allergic and non-atopic individuals, TCRAV24 transcripts were cloned into *E. coli* and an analysis was made restricted to the quantitation of AV24-AJ18 rearrangements among total TCRAV24 transcripts (Fig. 3). To verify the specificity of transcripts, 40 clones were additionally sequenced: 39 clones had the canonical AV24-AJ18 rearrangement and one clone of a non-atopic donor

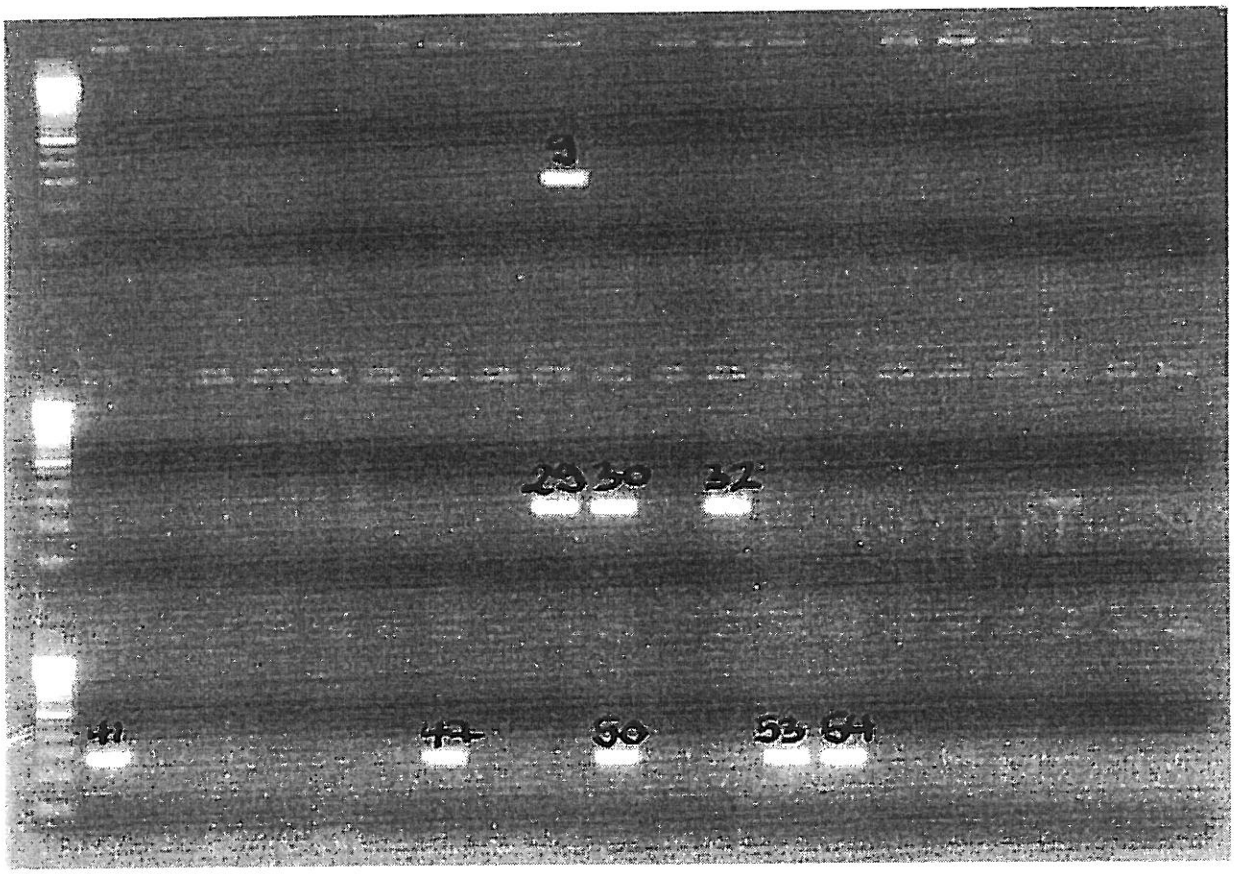

Fig. 3. Quantitation of AV24-AJ18 rearrangements among total TCRAV24 transcripts. In the example shown here, 94 TCRAV24-TCRAC-positive clones that were obtained from a non-atopic donor were analyzed for the frequency of JA18 rearrangements by PCR and yielded nine TCRAV24-AJ18 clones.

yielded only a very faint PCR product that was revealed to be AJ29. The percentage of AV24-AJ18 among total AV24-transcripts in all individuals ranged from 0–55%. No significant differences were found between atopic and non-atopic individuals (Table 5) nor did the percentage of AV24-AJ18 positive clones correlate with the serum IgE levels within the atopic group (data not shown). When it was analyzed how NKT cells, as defined by the frequency of AV24-AJ18 transcripts, related to NKT cells defined by the expression of surface molecules, a correlation was found between the percentage of clones and DN Vα24$^+$CD161$^+$ cells ($r = 0.588$; $p < 0.05$).

N-region analysis of AV24-AJ18 transcripts in atopic and non-atopic individuals by a modified real-time PCR technology

To test the possibility that NKT cells differed within the CDR3 region between the two proband groups, we analyzed the N-region between AV24 and AJ18 at the single nucleotide level with the aid of fluorescence resonance energy transfer (FRET), combined with melting curve analysis. For this purpose, an anchor and a detection probe labeled with different dyes and covering the AV24-AJ18 junction were constructed, in addition to the AV24 and AJ18 primers as described in materials and methods. Based on the literature, we hypothesized that the N-region of AV24-AJ18 TCR transcripts would not contain inserted nucleotides. 33% of all AV24-AJ18 positive PCR clones were analyzed from each donor. A total of 72 from 221 TCRAV24-AJ18$^+$ clones were analyzed, including 46 clones derived from atopic donors and 26 clones derived from non-atopic donors. To confirm reproducibility, FRET was performed five times with one PCR clone and demonstrated always to yield the same results (data not shown). According to FRET analysis, 56 of 72 clones had a melting temperature (Tm) consistent with an absence of N-region diversity (Fig. 2C).

Table 5. Percentage of TCRAV24-AJ18$^+$ clones among all TCRAV24$^+$ clones. The values given are medians with the ranges in parentheses.

atopic n = 13	non-atopic n = 11
5.26 (0–55.32)	9.04 (0–40)

Sequencing of 21 of these 56 clones confirmed the canonical AV24-AJ18 sequence containing no inserted nucleotides at the V-J junction (Table 6). In the remaining 16 of 72 clones, a reduced Tm was found, indicating a mismatch within the N-region covered by the anchor and/or detection probe. Since a reduced Tm does not differentiate between an inserted nucleotide in the N-region or a polymorphism within the AV24 or the AJ18 gene segment, all 16 clones with reduced Tm were sequenced. Of these 16 clones, 11 had single nucleotide exchanges within the AV24 gene and one clone within the AJ18 gene. In the remaining four clones (two each from allergic donors and non-atopic donors) three to four nucleotides were replaced, yielding an N-region diversity (Table 6, bottom). In one clone the mutations led to an altered amino acid sequence; whereas an amino acid sequence identical to the classical AV24-AJ18 sequence in the other three clones was retained.

Discussion

This study compared the frequency of NKT cells in adult atopic and non-atopic individuals at both the cellular and molecular levels. At the cellular level, we determined the frequency of NKT cells as defined by the simultaneous surface expression of Vα24 with CD161 in CD4$^+$, CD8^{bright}, CD8^{dim} and DN lymphocyte subpopulations. No clear differences in the numbers of Vα24$^+$CD161$^+$ NKT cells were found between the two groups, although CD8^{dim} NKT cells tended to lower numbers in the non-atopic group. Considerably larger proband populations have to be screened to confirm these results on a statistically significant level. Consistent with previous results (Dellabona et al., 1994; Davodeau et al., 1997) CD8^{bright} NKT cells, corresponding to heterodimeric CD8$^+$ cells, were barely detectable. In contrast, CD8^{dim} Vα24$^+$CD161$^+$ cells, most likely corresponding to CD8$\alpha\alpha$ NKT cells (Prussin & Foster, 1997), were considerably more frequent.

Other studies investigating the frequency of NKT cells in atopic adults give conflicting results since a selective decrease of DN Vα24$^+$Vβ11$^+$CD161$^+$ cells was found in patients with asthma or atopic dermatitis (Oishi et al., 2000; Takahashi et al., 2003) or a significant increase of CD4$^+$Vα24$^+$Vβ11$^+$ cells was found in atopic individuals (Magnan et al.,

Table 6. Sequences of 72 TCR AV24-AJ18 clones with potential N-region and deduced amino acid sequences. AV24AJ18+ clones were analyzed with hybridisation probes covering the N-region. The presence of an N-region was suspected in clones where the hybridisation probe had a reduced melting temperature. These clones were sequenced (shown here) to differentiate between single nucleotide polymorphisms in the adjacent gene regions and true N-region inserts.

	AV24	N-region	AJ18
Classical AV24-AJ18 sequence (56 clones)	ile cys val val ser ATC TGT GTG GTG AGC		asp arg gly ser thr leu GAC AGA GGC TCA ACC CTG
	ACC TGT GTG GTG AGC		GAC AGA GGC TCA ACC CTG
	ACC TGT GTG GTG AGC		GAC AGA GGC TCA ACC CTG
Single nucleotide exchange within AV24 or AJ18 (12 clones)	ATC TGT GTG GTG AGT		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG ACC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG AAC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG AAC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG AAC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG GGC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GCG AGC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG ACC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG ACC		GAC AGA GGC TCA ACC CTG
	ATC TGT GTG GTG AGC		GAT AGA GGC TCA ACC CTG
AV24-AJ18 with N-region(4 clones)	ATC TGT GTG GTG	tyr ile TAC ATC	AGA GGC TCA ACC CTG
	ATC TGT GTG	val ser GTC TCC	GAC AGA GGC TCA ACC CTG
	ATC TGT GTG	val ser GTC TCC	GAC AGA GGC TCA ACC CTG
	ATC TGT	val val ser GTA GTA TCC	GAC AGA GGC TCA ACC CTG

2000). These differences may be explained, at least in part, by different and sometimes insufficient criteria for patient selection (Finkelman et al., 2000; Magnan et al., 2000; Oishi et al., 2000). We set very strict standards through the use of three parameters that clearly demark the two groups. In contrast to others (Takahashi et al., 2003), our patients with atopic dermatitis, who represented two third of the probands, did not receive immunomodulating ointments. Furthermore, a high inter-individual variability in the frequency of $V\alpha24^{+}V\beta11^{+}$ cells has been found in healthy subjects (Davodeau et al., 1997; Prussin & Foster, 1997). Thus, the investigation of small groups of 14 blood-donors (Oishi et al., 2000) may lead to erroneous estimates of the association of NKT cells with atopic diseases. This holds especially true when parametric statistical tests are applied (Oishi et al., 2000) since a normal distribution can not be expected in small groups. For this reason we utilized non-parametric statistical analyses. Another crucial factor is the number of cells analyzed by flow cytometry since the frequency of NKT cells in any subset is extremely low. Some analyses did not provide this information (Oishi et al., 2000) rendering interpretation of the results difficult. Our studies relied on measurement of larger sample sizes of more than 1×10^5 cells in most probands. Interestingly, we saw a small predominance of $CD8^{dim}$ NKT cells in the atopic individuals.

Because of these difficulties we also performed molecular TCR studies. Two strategies were pursued in parallel. First, we determined the frequency of TCRAV24-AJ18 transcripts among all TCR alpha chains. Since the frequency of TCRAV24-AJ18 among total TCR alpha chains may be too low to detect significant differences between the two groups, we further sought to determine the frequency of AV24-AJ18 compared with TCRAV24 transcripts alone. A priori, this second procedure could have been hampered by an overly strong selectivity, thereby justifying the need for the first approach as well. Nonetheless, both procedures failed to reveal any significant differences between the atopic and non-atopic groups.

Four of 72 TCRAV24-AJ18^{+} clones had N-region inserts. Interestingly, in three clones the amino acid sequence remained the same as the classical invariant AV24-AJ18 NKT cells. If these transcripts are derived from conventional AV24-AJ18^{+} T cells, the finding that conservation of the amino acid sequence has been retained despite multiple nucleotide variations would suggest that some functional selection for antigen specificity has occurred. Alternatively, these cells may be derived from a small subset of NKT cells that contain N-nucleotides even though NKT cells are considered to develop before lymphocytes have the capacity to express terminal deoxynucleotidyl transferase, which is required for N-region diversification. Although these clones clearly represent exceptions, their occurrence is surprising but others have made similar observations (Dellabona et al., 1994; Exley et al., 1997). In 11 clones, single nucleotide exchanges altered the amino acid sequence within the CDR3 region. If these changes were due to erroneous amplification by the *Taq* polymerase this would represent an unexpected high rate of mistakes, since the provider declares an average error rate of 42×10^{-6}. Four of the eleven clones that were obtained from different donors had identical mutations within the CDR3 region, which makes erroneous amplification unlikely.

The failure to find significant differences among NKT cells in the atopic and non-atopic individuals, at either the cellular or the molecular level, may be due to several factors. First, we analyzed adult donors and allergic diseases frequently begin during childhood. Thus, it is possible that after the initiation of a Th2-prone imbalance at the onset of disease, NKT cells may return to normal levels and a Th2 bias is further sustained by IL-4-producing Th2 cells themselves. That NKT cells may indeed play a role early in life is indicated by the finding that AV24-AJ18 transcripts are present in high frequencies in human cord blood where a Th2 bias is present (Prescott et al., 1999). Other studies have shown (Alm et al., 1999; Leynaert et al., 2001) that early exposure to high levels of house-dust endotoxin may be associated with the development of type 1 T cells (Gereda et al., 2000). Although the precise antigen ligands of NKT cells remain to be defined, several studies demonstrated that NKT cells are involved in the host response against microbial infections. Thus, NKT cells exert protective effects against *Plasmodium* (Pied et al., 2000), protozoans such as *Leishmania* (Ishikawa et al., 2000)or *Toxoplasma gondii* (Denkers et al., 1996) and against bacteria such as *Listeria* (Flesch et al., 1997) and Bacillus Calmette Guerin – the latter shifting the cytokine synthesis of NKT cells from a high IL-4/IFN-γ ratio to a very low ratio (Emoto et al., 1999). In a more direct approach it was shown that NKT cells recognizing GPI-anchored proteins from *Plasmodium falciparum* and *Trypanosoma brucei*, presented by CD1d, contributed to the IgG response against these microbes (Schofield et al., 1999). Therefore, it can be speculated that the recognition of microbial products by NKT cells in early life could influence their cytokine production, thereby favoring or suppressing the development of Th2 responses. Epidemiological investigations have demonstrated that the exposure to microbes in early life protects from atopy (von Mutius et al., 2000). Whether this correlates with NKT cells and their cytokine profile remains to be determined.

The failure to find differences between the two groups at the molecular level may be partly explained by the analysis of whole blood samples. In future studies it is possible that differences between atopic and non-atopic donors might be detected using isolated subpopulations, encompassing for example $CD4^+$ or DN cells. One report found a decrease in the frequency of the AV24-AJ18 rearrangements in the DN population of atopic individuals as compared to healthy controls (Oishi et al., 2000). However, in our analysis the calculation of the absolute numbers of $V\alpha24^+CD161^+$ cells revealed that the majority of NKT cells belonged to the $CD4^+$ subset, followed by DN NKT cells. Furthermore, AV24-AJ18 transcripts correlated strongly with $CD4^+$ and DN NKT cells, indicating that the molecular cloning strategy primarily detected NKT cells belonging to these subsets. Since both the cellular and molecular assessments failed to reveal differences with respect to these parameters in the two groups, a substantial difference at the subpopulation level is not readily apparent. While our studies show that the frequency of AV24-AJ18 transcripts in freshly isolated unstimulated peripheral blood does not differ between atopic and healthy adults, it is possible that NKT cells in peripheral blood do not exert a regulatory influence on cells that function in diseased skin or in the lung *in situ*. Analysis of lymphocytes present at these locations might reveal alterations in NKT cell numbers in the adult atopic individual. Studies in animal models have shown that the phenotype and function of NKT cells can vary among different organs (Hammond et al., 1999; Emoto et al., 2000; Matsuura et al., 2000) and also within a given tissue (Apostolou et al., 2000). Similarly, studies of healthy liver specimens of human donors indicate that such heterogeneity of NKT cells also exists in man (Doherty et al., 1999). In this respect peripheral blood may not be the optimal compartment to detect differences in NKT cells of atopic and non-atopic donors.

Although NKT cell numbers did not differ in atopic and non-atopic individuals in this study, functional studies are necessary to examine the role of NKT cells in the development of atopic disease. Depending on the microenvironment (Trop & Ilan, 2003) or their individual maturity (Loza et al., 2002) NKT may express IL-4 and IFN-γ in different ratios. Therefore, we can not exclude the possibility that NKT cells still contribute to the generation of Th1/Th2 imbalance in atopic individuals by the expression of different sets of cytokines. The isolation of these cells from PBL of atopic and non-atopic probands is crucial to further investigate the cytokine profile of NKT cells. In this study, the amount of blood available from the donors was far too small for such an approach.

On the other hand, it will be essential to perform longitudinal studies in children to clarify whether NKT cells contribute to the development of allergic disease in early life. Furthermore, longitudinal studies may reveal whether NKT cells contribute to initiation or protection from allergic disease, dependent upon their cytokine profile. Such studies will also enable one to elucidate whether NKT cells containing N-nucleotides represent persisting clones.

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