# Reduced CD4+ subset and Th1 bias of the human iNKT cells in Type 1 diabetes mellitus

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**Abstract: Invariant NKT (iNKT) cells are considered to be important in some autoimmune diseases including Type 1 diabetes mellitus (T1DM). So far, the published data are contradictory in regard to the role of iNKT cells in T1DM. We aimed to study iNKT cell frequency and the function of different iNKT cell subgroups in T1DM. We compared the results of four subject groups: healthy (H), long-term T2DM (ltT2DM; more than 1 year), newly diagnosed T1DM (ndT1DM; less than 3 months), and ltT1DM (more than 1 year) individuals. We measured the iNKT cell frequencies by costaining for the invariant TCR α-chain with 6B11-FITC and Vα24-PE. After sorting the Vα24+6B11+ cells, the generated iNKT clones were characterized. We tested CD4, CD8, and CD161 expression and IL-4 and IFN-γ production on TCR stimulation. The CD4+ population among the iNKT cells was decreased significantly in ltT1DM versus ndT1DM, ltT2DM, or H individuals. The T1DM iNKT cell cytokine profile markedly shifted to the Th1 direction. There was no difference in the frequency of iNKT cells in PBMC among the different patient groups. The decrease in the CD4+ population among the iNKT cells and their Th1 shift indicates dysfunction of these potentially important regulatory cells in T1DM. *J. Leukoc. Biol.* 81: 654–662; 2006.**

***Key Words:*** *autoimmunity · Vα24+6B11+ cells · cytokines*

## INTRODUCTION

NKT cells represent a specialized subset of thymus-derived T cells, which express the TCR and NK cell markers. A majority of the NKT cells expresses an invariant TCR, consisting of the Vα24–JαQ α-chain (Vα14–Jα18 in mice) without nucleotide insertions. Thus, these cells are referred to as invariant NKT (iNKT) cells. They are also known as Type I or classical NKT cells [1]. The invariant Vα24–JαQ α-chain preferentially pairs with the Vβ11 β-chain in humans [2].

The invariant germ-line TCR, expressed by these iNKT cells, is unique, as it interacts with the nonclassical MHC molecule CD1d. The subset of NKT cells with diverse or semi-invariant α-chains is Type II or nonclassical NKT cells, which are also CD1d-dependent. Mice lacking CD1d or the Jα18 of the TCR α-chain have no iNKT cells. One of the candidates for natural ligand presented by CD1d to the NKT cells is thought to be a GPI [3]. Others reported that a lysosomal glycosphingolipid (isoglobotrichexosyl-ceramide) may be the endogenous ligand of the iNKT cells [4]. After TCR stimulation, iNKT cells can rapidly produce large quantities of Th1 cytokines (IFN-γ) as well as Th2 cytokines (IL-4) [5]. Secreted cytokines can affect the differentiation of naive T cells and can also influence the inflammatory or anti-inflammatory response, thereby contributing to the innate and adaptive immune responses [6]. Specifically, iNKT cells have an important role, not only in innate or early immune response but also in tumor surveillance and in adaptive immune regulation [5]. The iNKT cells are defective in NOD mice [7] and may also be defective in some human autoimmune diseases, such as Type 1 diabetes mellitus (T1DM), sclerosis multiplex, systemic sclerosis, systemic lupus erythematosus, and rheumatoid arthritis [8]. Recently, there has been great interest in the possible applications of iNKT cells as a therapeutic modality for treating autoimmune diseases and malignancies [9, 10].

The origin and development of iNKT cells are currently the subject of intensive research. Current data indicate that the thymus is involved in early iNKT cell development [11]. In mice, the peripheral distribution of iNKT cells varies between organs. For example, in the liver, 30–40% of T cells are iNKT cells and in bone marrow, only 20–30%. The data about human iNKT cell frequencies in different tissues are essentially limited to blood; however, some results indicate that iNKT cells are also present in the liver [12–15].

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Received November 1, 2006; revised November 9, 2006; accepted November 10, 2006.

doi: 10.1189/jlb.1106654

 

TABLE 1. Characteristics of the Patient Cohorts

| | Healthy | ndT1DM | ltT1DM | ltT2DM |
|---|---|---|---|---|
| Age (years) | 29.7 ± 4.2 | 28.7 ± 1.6 | 24.2 ± 1.4 | 58.7 ± 2.9[a] |
| Gender | 2 F/4 M | 3 F/9 M | 4 F/2 M | 4 F/3 M |
| Body mass index (kg/m$^2$) | 23.8 ± 0.5 | 22.8 ± 1.0 | 23.9 ± 1.3 | 36.4 ± 2.2[b] |
| IAA value (<39 nU/ml) | 0.84 ± 7.04 | 170 ± 79.7 | 1043 ± 547.9 | 64 ± 52.4[c] |
| GAD index (<0.1) | 0.02 ± 0.02 | 0.58 ± 0.10 | 0.11 ± 0.08 | 0.04 ± 0.01 |
| IA2 index (<0.1) | 0.01 ± 0.00 | 1.45 ± 0.74 | 3.15 ± 1.91 | 0.00 ± 0.02 |
| Duration of disease (months) | | <3 | >12 | >12 |

As expected, the body mass index (BMI) and age of the ltT2DM patients were higher than the other groups. Mean ± SEM; [a]age: H-ltT2DM, $P = 0.002$; ndT1DM–ltT2DM, $P = 0.026$; ltT1DM–ltT2DM, $P = 0.027$; [b]BMI: H-ltT2DM, $P < 0.0001$; ndT1DM–ltT2DM, $P < 0.0001$; ltT1DM–ltT2DM, $P = 0.0003$. [c]Two ltT2DM patients had elevated IAA levels as a result of insulin treatment.

In humans, the iNKT cells are segregated into three subsets: CD4+, CD4–CD8– [double-negative (DN)], and CD8+ [2]. In mice, there are only two subsets: CD4+ and DN. Experimental human data suggest that upon stimulation, the CD4+ subset produces relatively more IL-4 than the DN subset [16]. This may indicate a more prominent, immune-regulatory function for the CD4+ than for the DN or CD8+ subgroups.

The CD161 (NKR-P1) is a NK locus encoded on a C-type lectin, and its expression is dependent on the maturation level of the iNKT cell [17]. The CD161 may function as a costimulatory molecule for TCR-mediated recognition of CD1d by human iNKT cells [18].

The data about iNKT cell frequency in human T1DM are contradictory. The frequency of iNKT cells has been reported to decrease [19, 20], increase [21], or remain unaltered [16] in the peripheral blood of T1DM patients as compared with control subjects. In these studies, different combinations of TCR-specific antibodies [Vα24, Vβ11, CD3, 6B11 (antibody against the invariant part of the TCR α-chain)], different surface markers (CD161, CD56), and α-galactosyl-ceramide (α-GalCer)-loaded tetramer (CD1d tetramer) were used to identify iNKT cells. The application of these diverse methods may be the source of the inconsistent findings, as the different methods may identify different populations [1, 8, 22, 23].

We aimed to study the frequency and the function of different iNKT cell subgroups in patients with T1DM. We concluded that the Vα24/6B11 costaining, described earlier by Exley, Wilson, and co-workers [24, 25], is a reliable method to detect iNKT cells. We used this costaining to single cell-sort the Vα24+6B11+ cells. We characterized the clones by CD4, CD8, and CD161 expression and measured the IL-4 and IFN-γ production upon TCR stimulation.

## MATERIALS AND METHODS

### Patients

The iNKT cells were isolated from 31 individuals [six healthy (H), 12 with newly diagnosed T1DM (ndT1DM; less than 3 months), six with long-term T1DM (ltT1DM; more than 1 year), and seven with ltT2DM]. All of the subjects were characterized by serum autoantibodies [insulinoma-associated protein-tyrosine phosphatase antibody (IA2-Ab), glutamic acid decarboxylase (GAD65)-Ab, and insulin autoantibody (IAA)]. The IA2- and GAD65-Ab were measured by radioimmunoassay using $^{35}$S-labeled GAD65 [26] and IA2 antigens [27]. The IAA [28] was measured by competitive radioimmunoassay using $^{125}$I-labeled insulin. The patient cohorts are shown in **Table 1**.

### Single cell sorting from PBMC

Isolation of PBMC from fresh blood was performed by gradient centrifugation using Ficoll (Amersham Pharmacia Biotech Europe GmbH, Uppsala, Sweden), and the cells were frozen in heat-inactivated, human AB serum (Omega, Tarzana CA, USA) with 10% DMSO (Sigma-Aldrich, St. Louis, MO, USA) and stored at −80°C until assayed.

The frozen PBMC were thawed, washed twice with PBS, and stained with 6B11-FITC (1:20 dilution, BD Biosciences PharMingen, San Diego, CA, USA) and Vα24-PE (1:50 dilution, Beckman Coulter, Fullerton, CA, USA). Both antibody preparations were dialyzed in sterile PBS using Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL, USA). The staining media consisted of PBS and 0.5% heat-inactivated, human AB serum (Omega). Ten million cells, in 0.5 ml staining media, were incubated on ice for 30 min with labeled antibodies. The Vα24- and 6B11-positive cells were sorted (FACSVantage cell sorter, Becton Dickinson, San Jose, CA, USA) into 96-well, round-bottom, polystyrene plates (Corning, Corning, NY, USA). The plates were prepared with irradiated (4000 rad), allogenic feeder cells (150,000/wells) and media [200 μl/wells (RPMI-1640, supplemented with 1% 1 M HEPES, 1% sodium pyruvate, 1% glutamate, 1% penicillin/streptomycin, all from BioWhittaker Cambrex, Walkersville, MD, USA), 1% MEM (amino acid solution from Gibco, Invitrogen, Grand Island, NY, USA), and 5% heat-inactivated, human AB serum (Omega)] with 20 U/ml recombinant (r)IL-2 (Tecin™ Teceleukin Bulk Ro 23-6019, National Cancer Institute-Frederick, Frederick, MD, USA) and 3 μg/mL PHA-P (Remel Inc., Lenexa, KS, USA). Every 2nd day, 100 μl media was replaced containing 40 U/ml rIL-2. No NKT cell-specific stimulation was used. The surviving clones became visible after 9–11 days of culturing.

### Second staining of the NKT cell clones

The surviving clones were cultured and divided into new wells over 8–10 additional days to get 0.5–2 × 10$^6$ cells from each clone. During this culturing phase, we did not use NKT cell-specific stimulation. The media were supplemented with 40 U/ml rIL-2. The clones were restained with Vα24-FITC, Vβ11-PE (Beckman Coulter), CD4-FITC, CD8-PECy5, 6B11-PE, and CD161-PE (all from BD Biosciences PharMingen) and were measured by Beckman Coulter XL FACS (Beckman Coulter). FITC-labeled antibody preparations were used in 1:20; PE-labeled antibodies were used in 1:50 dilutions. FACS results were analyzed with WinMDI 2.8. The percentage of cells with identical staining was evaluated to verify the purity of each clone.

### RNA extraction

After thawing the clones, the RNA extraction was carried out using RNAEasy Microkit® (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol (including DNase digestion). The matrix-bounded RNA was resuspended in diethylpyrocarbonate-treated water and quantified at 260 nm. The quality of the total RNA was confirmed by 1% agarose gel analysis.

## RT-PCR and sequencing

The cDNA was produced from the total RNA with Superscript II RT (Invitrogen, Carlsbad, CA, USA) in 25 μl reactions with random hexamers following the manufacturer's protocol. The resulting cDNA was stored at −20ºC. Primers were designed around the V-J-C rearrangement region of the α-chain of the TCR [29, 30]. Forward primer (Vα24 region-specific): 5′-GATATACAGCAACTCTGGATGCA-3′; reverse primer (C region-specific): 5′-GGCAGACAGACTTGTCACTGGAT-3′. PCR was performed on the cDNA using Platinum® *Taq* DNA polymerase (Invitrogen) in 50 μl reactions, according to the manufacturer's protocol. Final primer concentration of 10 μM was used to amplify the DNA. The annealing temperature was 52ºC. The cDNA quality was confirmed in all cases by using GAPDH as a housekeeping gene. Amplified DNA was visualized on 2% agarose gels containing 1 μg/ml ethidium bromide, run in 0.5× Tris-boric acid-EDTA buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). DNA templates (230 bp) were recovered from PCR products with a QIAquick™ PCR purification kit (Qiagen GmbH), and quality was confirmed by 2% agarose gel analysis before sequencing. Templates were sequenced with a ABI3100 sequencer from both sides with forward and reverse primers.

## Measurement of cytokine production by ELISA

Round-bottom polystyrene plates (Corning) were coated with 1 μg/ml anti-CD3 (α-CD3; Exalpha Biologicals Inc., Watertown, MA, USA) in 50 μl/well PBS at 37°C for 2 h. We cultured the iNKT cells (50,000/well) from all of the clones that yielded enough cells to run the stimulation assay with and without α-CD3 in duplicate for a total of 24 h. After 4 and 24 h, the supernatants were taken out for cytokine ELISA. The antibodies and standards for IL-4 and IFN-γ were purchased from BD Biosciences PharMingen and used according to the manufacturer's protocol. Avidin-peroxidase conjugate and 3,4,5-trimethoxybenzoic acid substrate (all from BD Biosciences PharMingen) were used to develop the assay.

## Statistical analysis

Statistical analysis was performed by StatView for Windows 5.0.1. (SAS Institute Inc., Cary, NC, USA). Group comparisons for data with normal distribution were performed by one-way ANOVA and Scheffe post hoc tests; in case of non-Gaussian distribution, the Kruskal-Wallis test was used, followed by the Mann-Whitney U test with Bonferroni correction of the α as post hoc analysis. Results are expressed as mean ± SEM; $P < 0.05$ was considered statistically significant.

# RESULTS

## PBMC single cell sorting

After staining with 6B11-FITC and Vα24-PE, the double-positive cells were single cell-sorted into 96-well plates. Because of the low frequency of iNKT cells, we used at least 10 million cells for each frequency analysis and sorting (**Fig. 1A**). To check the reproducibility, we stained the same samples at two different times and FACS analyzed them using two different instruments. The correlation between the frequencies of the iNKT cells was $r = 0.97$ (Fig. 1B).

Following ~20 days of culturing, each of the generated clones contained 0.5–2 million cells. The survival rate of the sorted cells was between 5% and 25%.

## Confirmation of the invariant TCR of the clones by second staining and by RT-PCR and sequencing

All clones were restained with Vα24-FITC, and 80% (285/354) were positive. All of the randomly selected, Vα24-positive clones were also 6B11-positive (67/67; **Fig. 2A**). The Vα24-negative clones were excluded from the following characterization, or they were used as negative controls.

The RNA was isolated from 52 Vα24-positive and from five Vα24-negative clones. The produced cDNA quality was confirmed in all cases (GAPDH was used as a housekeeping gene). Using the Vα24 region-specific forward primer and the C region-specific reverse primer, 230 bp DNA templates were recovered from all Vα24+ clones (52/52), but there was no relevant PCR product from any of the Vα24 negative clones. The sequence of the templates in all of the clones (52/52) showed the canonical TCR sequence: TGTGTGGTGAGCGA-CAGAGGCTCAACC, Cys-Val-Val-Ser-Asp-Arg-Gly-Ser-Thr.

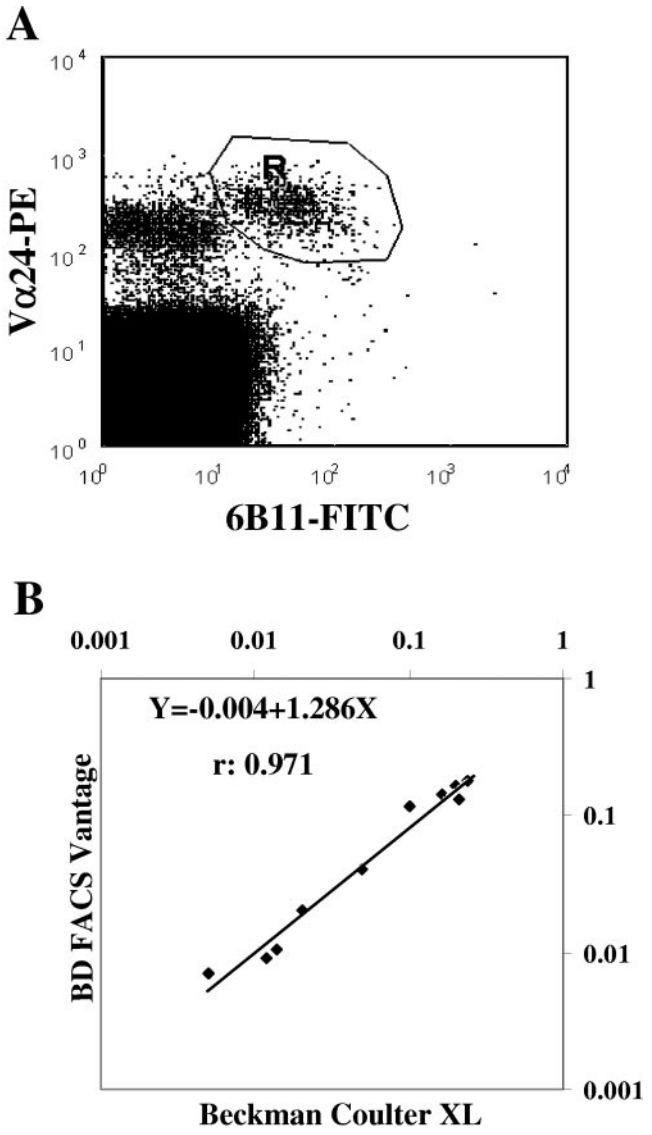


**Fig. 1.** FACS analysis of iNKT cells from peripheral blood and the reproducibility of the method. Double-staining with Vα24 and 6B11 antibodies identifies iNKT cells. (A) A representative example where the Vα24+/6B11+ cell frequency was 0.18 (R region/lymphocytes). The cells were single-sorted from the R region. (B) Comparison of the results of the Vα24+/6B11+ cell frequencies (R region/lymphocytes) of the same samples stained on two different occasions and read on two different instruments (correlation coefficient, $r$=0.971).

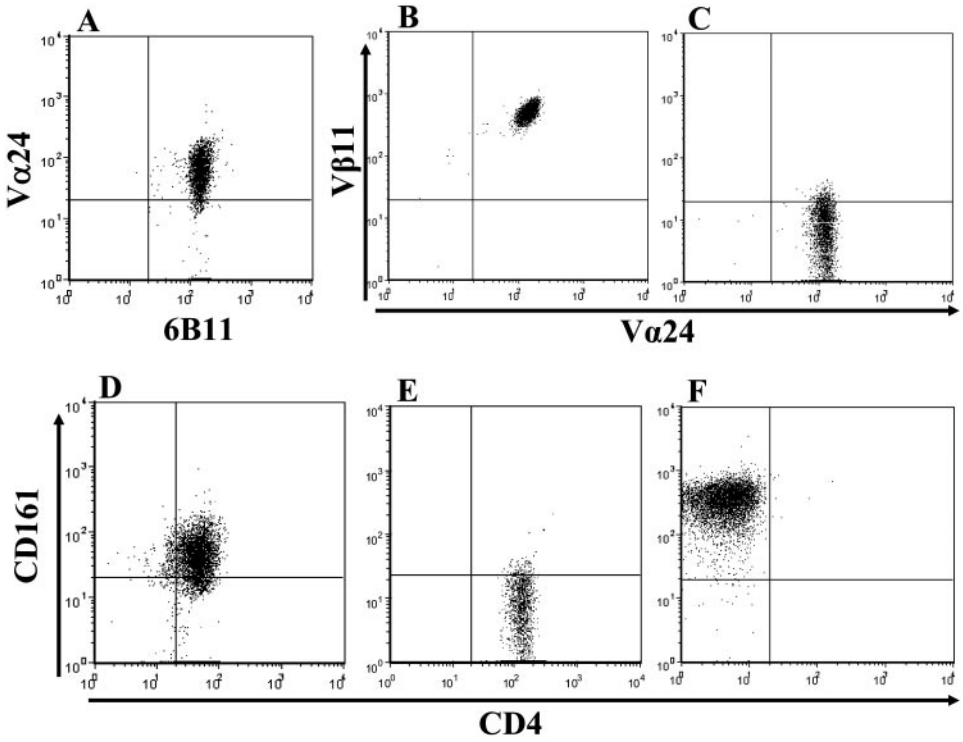


**Fig. 2.** Characterization of the cultured iNKT cell clones. (A–F) Representative examples of various single cell clones isolated by the Vα24 and 6B11 double-staining. All Vα24+ iNKT cell clones were positive for 6B11 antibody (antibody for the germ-line CDR3 region of the TCR α-chain; A). The Vα24 chain most often paired with Vβ11 (B); few of them were Vβ11-negative (C). The clones were also classified by CD4 and CD161 expression; examples for double-positive (D) and single-positive clones (E and F) are shown.

## The frequency of the iNKT cells

As we confirmed the Vα24 and 6B11 costaining technique to be reproducible and as it identifies iNKT cells with a high positive predictive value, we used it to measure the iNKT cell frequency in PBMC. There was a wide range in the percentage of Vα24+ and 6B11+ cells (0.002–0.4%) among the lymphocytes; however, there was no difference among the subject groups (**Fig. 3**).

## Characterization of the iNKT cell clones

All of the Vα24-positive clones were characterized by the expression of Vβ11, CD4, CD8, and CD161. Figure 2, B–F, shows representative examples of different clones. **Table 2** shows the detailed characterization of the clones. As expected, the majority of the iNKT cells had the most common (Vβ11) β-chain, and there was no significant difference among the different sets of patients. The expression of CD161 was highly variable, without significant difference among the different sets of patients. On the iNKT cells, the expression of CD4 was frequent among H subjects, ltT2DM, and ndT1DM patients and was found reduced significantly (one-way ANOVA, $P=0.0032$; Scheffe post hoc test, H–ltT1DM, $P=0.0181$; ndT1DM–ltT1DM, $P=0.0376$; ltT1DM–ltT2DM, $P=0.0082$) in ltT1DM patients (**Fig. 4A**). As the frequency of the clones positive for these surface characteristics was calculated from a limited number of clones for each patient, we also confirmed these findings with direct staining from PBMC. We used only three samples with high iNKT cell frequency from each patient group, as it is technically difficult to calculate the ratios of the iNKT cell subpopulations in PBMC with low iNKT cell frequency. After staining with the same stains that we used for sorting and frequency measurement (Vα24, 6B11) and with CD4-PECy5, we gated on the Vα24+6B11+ cells and measured the CD4+ ratio. The results were similar (one-way ANOVA, $P=0.0007$; Scheffe post hoc test, H–ltT1DM, $P=0.0017$; ndT1DM–ltT1DM, $P=0.0023$; ltT2DM–ltT1DM, $P=0.0091$; Fig. 4, B and C).

The number of CD8+ iNKT clones was low; we found four of 60 among the H iNKT clones, three of 102 among ndT1DM, eight of 69 among ltT1DM, and two of 54 among the ltT2DM iNKT cell clones.

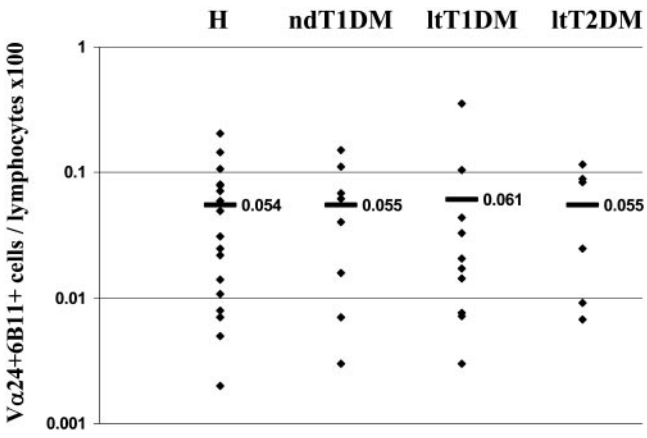


**Fig. 3.** The Vα24+6B11+ cell frequencies in different patient groups. The Vα24+6B11+ cell frequency (Vα24+6B11+ cells/lymphocytes×100) was in a wide range, as shown on the logarithmic scale (*y*-axis). The mean values were between 0.054% and 0.061%. There was no difference among the patient sets.

## Cytokine production of the clones

After TCR stimulation of the iNKT cell (50,000/well) clones, IL-4 and IFN-γ production was measured by ELISA from the

TABLE 2. Summary of the Characteristics of the iNKT Clones

| H | | | | ndT1DM | | | | ltT1DM | | | | ltT2DM | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *n* | Vβ11 | CD161 | CD4 | *n* | Vβ11 | CD161 | CD4 | *n* | Vβ11 | CD161 | CD4 | *n* | Vβ11 | CD161 | CD4 |
| *6* | 67 | 100 | 100 | *12* | 92 | 33 | 67 | *9* | 78 | 0 | 33 | *11* | 100 | 18 | 82 |
| *19* | 79 | 0 | 100 | *5* | 60 | 40 | 20 | *10* | 100 | 10 | 60 | *10* | 70 | 0 | 100 |
| *10* | 100 | 0 | 80 | *4* | 50 | 50 | 75 | *10* | 100 | 10 | 40 | *5* | 0 | 0 | 100 |
| *6* | 83 | 0 | 83 | *5* | 20 | 20 | 80 | *10* | 100 | 10 | 10 | *3* | 100 | 0 | 100 |
| *11* | 100 | 0 | 82 | *3* | 100 | 0 | 100 | *21* | 86 | 5 | 90 | *3* | 67 | 0 | 100 |
| *8* | 100 | 0 | 75 | *21* | 100 | 76 | 67 | *9* | 56 | 11 | 33 | *7* | 71 | 0 | 71 |
| *60* | | | | *3* | 100 | 0 | 100 | *69* | | | | *15* | 87 | 0 | 87 |
| | | | | *14* | 100 | 43 | 64 | | | | | *54* | | | |
| | | | | *9* | 100 | 33 | 78 | | | | | | | | |
| | | | | *13* | 100 | 15 | 92 | | | | | | | | |
| | | | | *7* | 86 | 100 | 71 | | | | | | | | |
| | | | | *6* | 100 | 0 | 100 | | | | | | | | |
| AV: | 88% | 17% | 87% | *102* | 84% | 34% | 76% | | 87% | 8% | 45% | | 71% | 3% | 91% |

Each row represents a different patient. The numbers in italics represent how many clones were isolated from a patient (*n*). The percentages of clones positive for a given marker are shown. In all four subject groups, the Vβ11 β-chain was the most frequent (71–88%). The CD161 expression was highly variable, and there was no significant difference between the patient cohorts. The CD4+ ratio of the clones was high among the H (87%), ltT2DM (91%), and the ndT1DM (76%) individuals but low among the ltT1DM patients (45%). The data analysis of the CD4+ ratio is shown (see Fig. 4A). AV, Average.

supernatant. There was no correlation among the stimulation index values measured by $H^3$-methylthymidine incorporation and the cytokine production (data not shown). Only clones from the T1DM groups (the ndT1DM and ltT1DM) produced more IFN-γ than the mean + 3 SD of the IFN-γ values of the H iNKT cell clones, suggesting that the T1DM clones are higher IFN-γ producers (**Fig. 5**).

We did not have enough data from the infrequent CD8+ iNKT cell clones for statistical analysis; therefore, we evaluated only the CD4+ and DN clones. There was no significant difference in IL-4 production among the different patient groups (data not shown).

The iNKT cells of ndT1DM patients produced more IFN-γ than those of the H and ltT2DM subjects (H–ndT1DM, $P=0.0054$; ndT1DM–ltT2DM, $P=0.0006$; **Fig. 6A**). Among the ndT1DM iNKT clones, the CD4+ ones produced more IFN-γ than those of the ltT1DM and ltT2DM groups ($P=0.0024$ and $P=0.0126$, respectively), and the CD4–CD8– (DN) clones of the ndT1DM group produced more than those of the H group ($P=0.036$). In the ltT1DM group, significantly higher IFN-γ production of the CD4+ clones was found compared with the DN cells ($P=0.0399$; Fig. 6B).

## DISCUSSION

iNKT cells may have an important role in controlling immune response, specifically, autoimmunity and tumor surveillance. Studying iNKT cells in humans has been difficult as a result of the low frequency of these cells in the peripheral blood [16, 31]. Limited data are available regarding iNKT cells in other human tissues [12–14]. Moreover, contradictory results have been published about the role of the iNKT cells in T1DM. Some studies suggest that iNKT cells have a protective role in T1DM [19, 20], and others did not find any connection between the development of the disease and the frequency or function of the iNKT cells [16]. The use of different techniques of identification may be a factor for these inconsistent findings. The α-GalCer-loaded CD1d tetramer was designed to be a specific and sensitive method to identify iNKT cells [32]. Lee et al. [16] reported high specificity of this tetramer in identifying iNKT cells in conjunction with Vα24 staining. However, others have suggested that not only the iNKT cells but also other Vα24-negative T cells with diverse Vα and Vβ TCR chains could recognize the α-GalCer-loaded CD1d tetramer [17, 22, 23].

We confirmed by two independent techniques that the Vα24/6B11 costaining is a reliable method to detect iNKT cells. Eighty percent of the clones obtained this way were subsequently confirmed to be iNKT cells. The remaining 20% represented unspecific binding and also other technical challenges (insufficiency of the sorting and possible contamination with other cells during the culturing period, among others). There was no correlation between the Vα24+6B11+ cell frequency in PBMC and the effectiveness of the sorting (data not shown). The reproducibility of this method was $r = 0.97$. We tested the specificity of this method with two independent techniques (second staining and RT-PCR/sequencing of the invariant part of the TCR). These independent confirmations support the notion that the method using this double-staining (Vα24 and 6B11) identifies iNKT cells reliably; thus, it is well-suited for studying these cells from human peripheral blood.

We did not find differences in the Vα24+6B11+ cell frequency among the H, ndT1DM, ltT1DM, and ltT2DM subjects. By including not only the H but also the ltT2DM patient

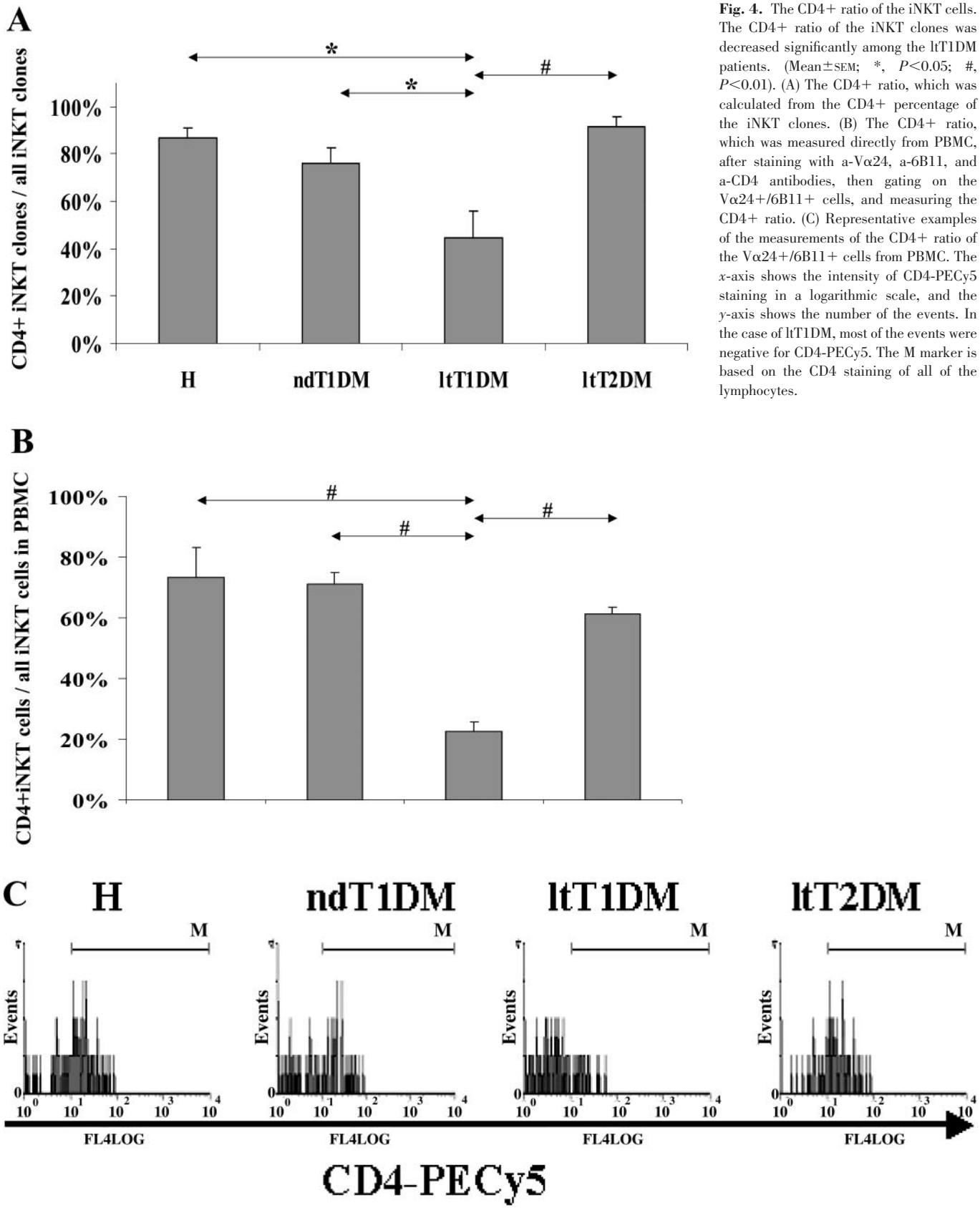


**Fig. 4.** The CD4+ ratio of the iNKT cells. The CD4+ ratio of the iNKT clones was decreased significantly among the ltT1DM patients. (Mean±SEM; *, $P<0.05$; #, $P<0.01$). (A) The CD4+ ratio, which was calculated from the CD4+ percentage of the iNKT clones. (B) The CD4+ ratio, which was measured directly from PBMC, after staining with a-Vα24, a-6B11, and a-CD4 antibodies, then gating on the Vα24+/6B11+ cells, and measuring the CD4+ ratio. (C) Representative examples of the measurements of the CD4+ ratio of the Vα24+/6B11+ cells from PBMC. The *x*-axis shows the intensity of CD4-PECy5 staining in a logarithmic scale, and the *y*-axis shows the number of the events. In the case of ltT1DM, most of the events were negative for CD4-PECy5. The M marker is based on the CD4 staining of all of the lymphocytes.

group as controls, we could rule out the effect of glucose homeostasis on these results.

There is also contradictory data about ratios of the iNKT cell subpopulations. Previous studies have reported the ratios in wide ranges: DN, 17–71%; CD4+, 25–90%; CD8+, 1–55% [33, 34]. They used different methods to detect iNKT cells and/or used different techniques to expand them [17, 35, 36].

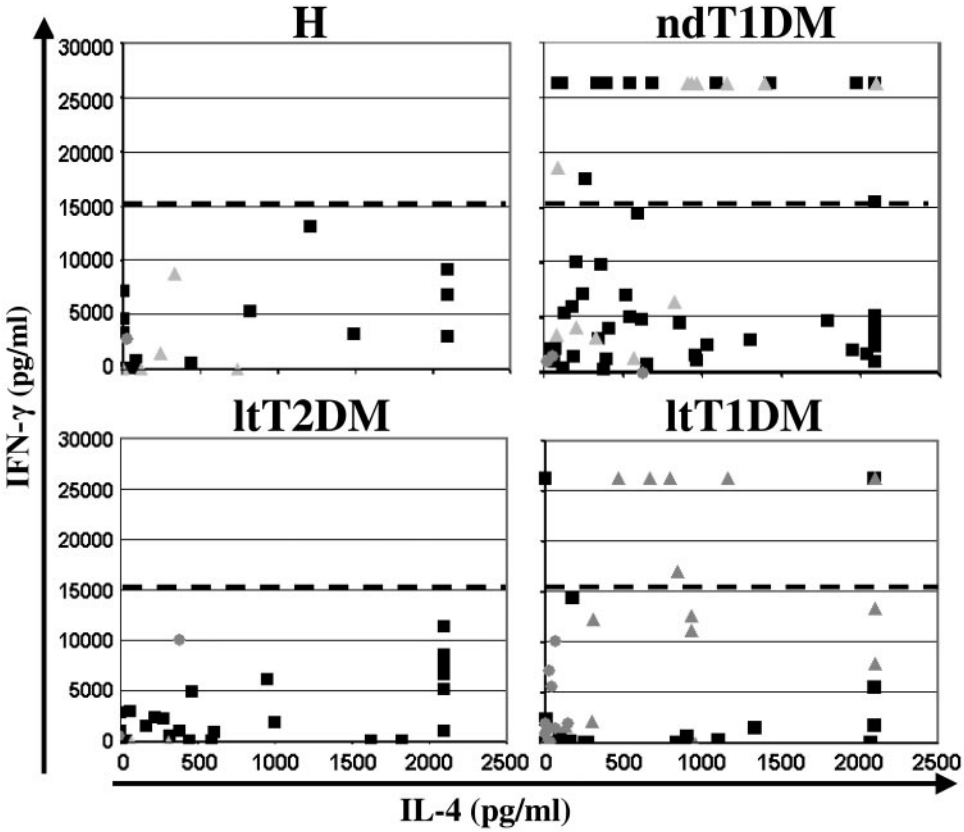


**Fig. 5.** The cytokine profiles of the stimulated iNKT cell clones. The figure shows the raw data of all of the stimulated iNKT cell clones. The IL-4 (*x*-axis) and the IFN-γ (*y*-axis) production of the TCR-activated iNKT cell clones (50,000/well) were measured. ■, CD4+; ▲, CD4−CD8− (DN); ●, CD8+ iNKT cell clones. The dashed lines show the average and three times the standard deviation ($x+3$ SD) IFN-γ production of the iNKT clones from healthy subjects. Only clones from the T1DM groups produced IFN-γ values above the mean + 3 SD.

Similar to most of the reports about the CD8+ cell frequency, in our experiments, the CD8+ subpopulation frequency was low. There was no significant difference among the patient sets. However, the CD4+ ratio of the iNKT clones was decreased significantly in ltT1DM patients. This result was confirmed using direct staining of the nonmanipulated PBMC. There was also a tendency of a lower CD4+ ratio in the ndT1DM patients compared with the H or ltT2DM subjects. The reason for this finding is unclear. One might hypothesize that the current technology is not sensitive enough to detect the decreased CD4+ ratio early in the course of the disease, or there is a slow, continuous decrease of the CD4+ cells in T1DM. It is not likely to be connected to glucose homeostasis or insulin treatment, as it was not observed in the ltT2DM group.

There are limitations to direct staining and cloning approaches. For example, when the CD4+ ratio was calculated from the clones, the relatively low numbers of the clones could influence this ratio. Similarly, the direct measurement can be technologically difficult because of the low frequency of iNKT cells. However, there was no correlation between the original Vα24+6B11+ cell frequency and the number of yielded clones. As expanding or cloning the cells can result in a biased population, we confirmed the decrease of the CD4+ iNKT cell population directly from PBMC.

In T1DM, especially in ndT1DM, the iNKT clones produced more IFN-γ than in H or ltT2DM patients. This was also the case when we looked at the subpopulations (CD4+ and DN) separately.

It has been reported in healthy individuals and in T1DM patients that CD4+ iNKT cells produce relatively more IL-4 than the DN cells. The dominant IL-4 production suggests a possible regulatory effect of the CD4+ subpopulation [16, 35]. In our dataset, there was no significant difference in IL-4 production between the CD4+ and DN iNKT clones in any of the subject groups.

Conversely, among the T1DM iNKT cells, the DN clones produced more IFN-γ than the CD4+ clones. Our clones were prestimulated during the cloning, which can influence the cytokine production; however, the CD4+ and DN clones were stimulated the same way. Our results are in agreement with previous publications that the DN iNKT cells produce more Th1 cytokines.

There has been little published regarding CD8+ iNKT cell function. We studied 14 clones and found that they are low producers of IL-4 and IFN-γ cytokines (data not shown). Although we analyzed a large number of iNKT cell clones (285), the number of CD8+ clones in each of the subject groups was too low to be included in the functional statistical analysis.

The significantly decreased CD4+ population, which favors the Th1 shift itself, and the increased IFN-γ production of the total iNKT cell population, which is independent from the ratios of the subpopulations, cause a strong Th1 bias. These findings underline the importance of the Th1/Th2 theory and support the notion of Th1 bias in T1DM.

In conclusion, we identified a reliable and effective method to study iNKT cells in humans using 6B11/Vα24 costaining, which we confirmed with two independent methods (second staining and PCR/sequencing). Using these techniques, we compared the frequency of Vα24+6B11+ cells in PBMC and found no difference among ndT1DM,

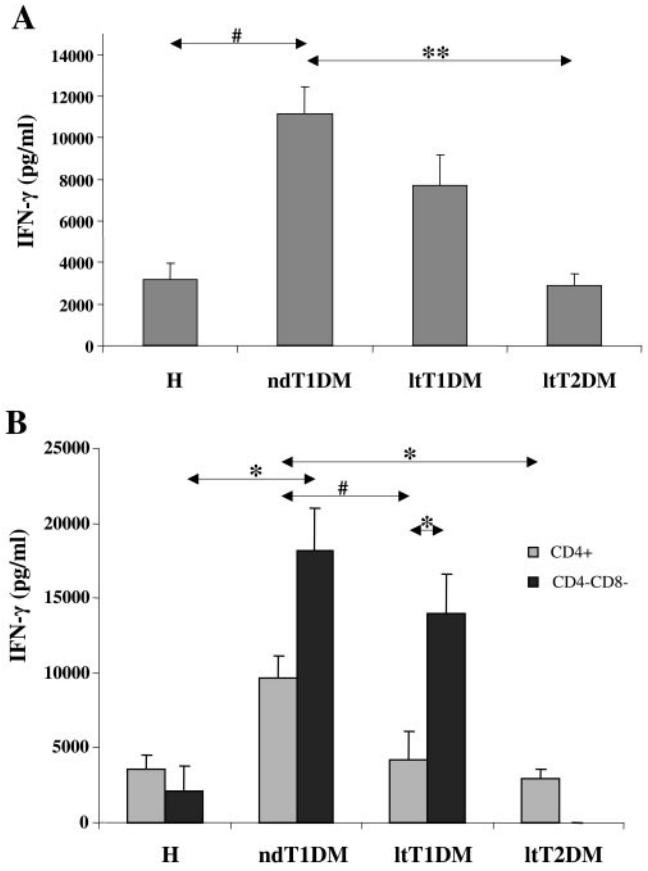


**Fig. 6.** Analysis of IFN-γ production of the iNKT clones in the different patient groups. (A) The data of all of the iNKT cell clones (CD4+, CD8+, and DN). The iNKT cell clones of ndT1DM patients produced more IFN-γ than those of the H and ltT2DM subjects. (Mean±SEM; #, $P<0.01$; **, $P<0.001$). (B) The analysis of IFN-γ production of the CD4+ and DN iNKT cell clones in the four subject groups. Among the ndT1DM clones, the CD4+ ones produced more IFN-γ than those of the ltT1DM and ltT2DM groups, and the DN clones of the ndT1DM group produced more than those of the H group. In the ltT1DM group, a significant difference was found between the IFN-γ production of the CD4+ and DN iNKT cell clones. (Mean±SEM; *, $P<0.05$; #, $P<0.01$).

ltT1DM, ltT2DM, and H subjects. We characterized a large number (285) of iNKT clones, which produced significantly more Th1 cytokines in the ndT1DM group, signifying a Th1 bias in T1DM. We determined the ratios of the iNKT cell subpopulations and observed a decrease of the CD4+ ratio in ltT1DM patients.

## ACKNOWLEDGMENTS

This work was supported in part by an Immune Tolerance Network grant (NO1-AI-15416). We thank the National Cancer Institute–Frederick, Cancer Research and Development Center, for providing us the rIL-2 (Teceleukin).

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