

Characteristics of T-cell receptor V α 24J α Q T cells, a human counterpart of murine NK1⁺ T cells, from normal subjects

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Background: In mice, natural killer (NK) T cells are specialized subsets of T cells that express an invariant T cell receptor (TCR) α chain and NK markers. In particular, murine NK1⁺ T cells rapidly produce IL-4 and function as regulatory T cells.

Objective: We investigated the distribution of invariant TCR V α 24J α Q T cells in CD4⁺CD8⁻ double-negative (DN) and CD4⁺ T cell populations of healthy individuals. We also studied the NK phenotypes and IL-4 production of V α 24J α Q T cells.

Methods: The frequency of V α 24⁺ DN or CD4⁺ T cells was determined by three-color FACS analysis, and subsequently the frequency of V α 24J α Q rearrangement among V α 24⁺ DN or CD4⁺ T cells was determined by sequencing.

Results: While the majority of DN V α 24⁺ T cells (68% to 88%) possessed TCR V α 24J α Q, few of CD4⁺ V α 24⁺ T cells (0.4% to 4%) did, indicating that V α 24J α Q T cells are a major population of DN T cells, but not of CD4⁺ T cells, in healthy subjects. The DN V α 24J α Q T cells expressed a natural killer surface receptor NKR-P1A and CD56, but not CD16, on the cell surface. Moreover, DN V α 24J α Q T cells promptly expressed IL-4 mRNA by stimulation with anti-V α 24 monoclonal antibody *in vitro*.

Conclusion: From these phenotypic and functional similarities of human DN V α 24J α Q T cells with murine NK1⁺ V α 14J α 281 T cells, we conclude that DN V α 24J α Q T cells are a counterpart of murine NK1⁺ T cells, suggesting that they may play a regulatory role in autoimmune responses *in vivo*. (J Allergy Clin Immunol 1999;103:S445-51.)

Key words: V α 24J α Q T cells, double-negative TCR $\alpha\beta$ T cells, CD4⁺ T cells, NKR-P1A, IL-4

CD4⁺CD8⁻ double-negative T-cell receptor (TCR) $\alpha\beta$ T cells are present in normal mice,¹⁻⁴ and some of them express the natural killer cell 1 (NK1) antigen, a member

Abbreviations used

cDNA: Complementary DNA
CDR3: Complementarity determining region 3
NK1: Natural killer cell 1
NKR-P1: Natural killer cell receptor-P1
TCR: T-cell receptor

of the family of natural killer cell receptors (NKR-P1).⁵ 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 and may play an important role in the regulation of some immune responses. First, NK1⁺ T cells possess an invariant TCR V α 14J α 281 that preferentially pairs with V β 8.2, V β 7, and V β 2.⁶ Second, CD4⁺ NK1⁺ T cells can promptly produce IL-4 by stimulation with anti-CD3 mAb.⁷ Finally, NK1⁺ T cells are decreased in autoimmune-prone mice in correlation with the disease activity and have been suggested to regulate autoimmune symptoms in lupus erythematosus⁸⁻¹⁰ and diabetes.¹¹

Double-negative invariant V α 24J α Q T cells are present in normal subjects.^{12,13} The TCR V α 24J α Q chain has a high homology with murine V α 14J α 281 chain in both the amino acid and nucleotide sequences. The V β chains pairing with the V α 24J α Q are V β 11 and V β 13, which also have a high homology with murine V β 8 and V β 7.^{14,15} Furthermore, selective reduction of V α 24J α Q T cells was observed in patients with systemic sclerosis,¹⁶ suggesting that V α 24J α Q T cells may play a critical role in controlling the development of autoimmune disease. However, the functional role and phenotypic characteristics of invariant V α 24J α Q T cells have not yet been clarified.

Therefore we investigated the distribution of invariant TCR V α 24J α Q T cells in double-negative and CD4⁺ T-cell populations of healthy individuals. We also studied the NK phenotypes and IL-4 production of V α 24J α Q T cells. Our results indicate that most double-negative V α 24⁺ T cells bear TCR V α 24J α Q, but few CD4⁺ V α 24⁺ T cells do, and that V α 24J α Q T cells express NKR-P1A on the cell surface and have the ability of IL-4 production.

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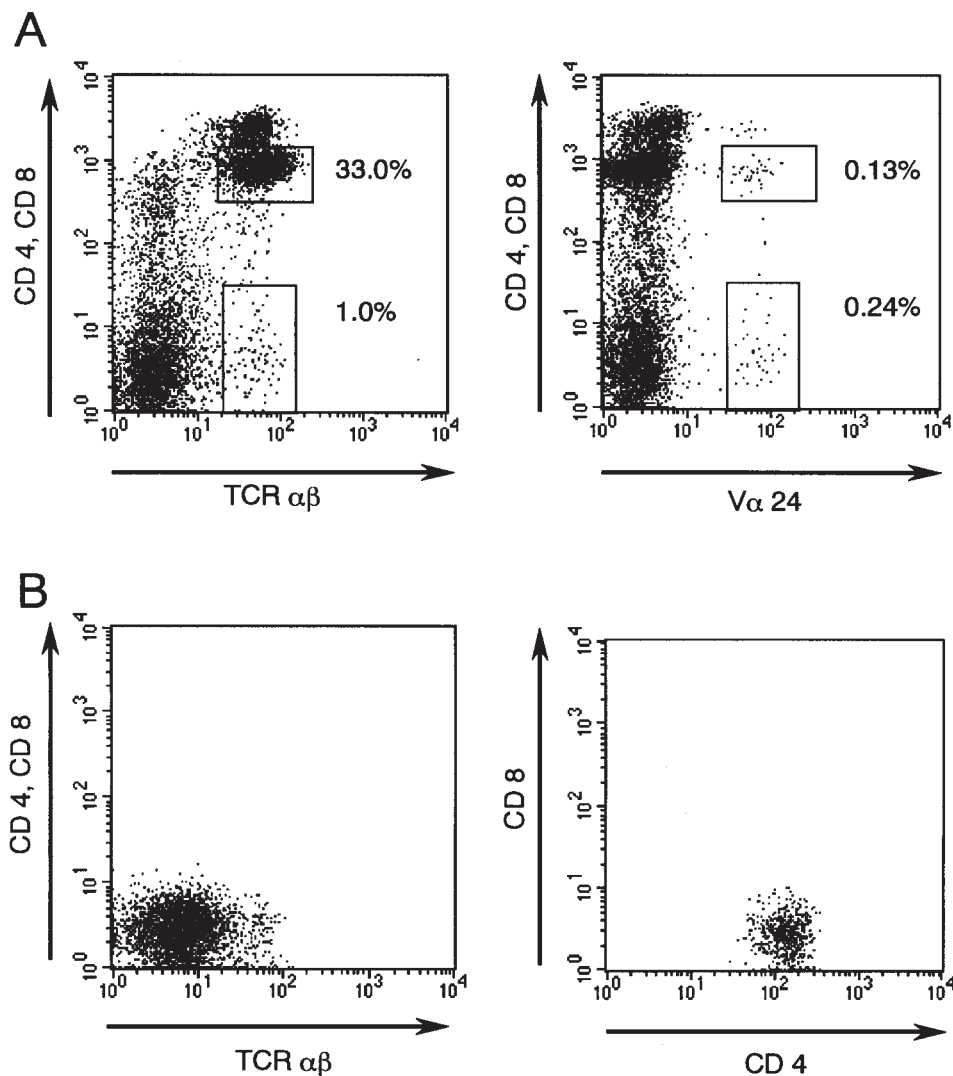


FIG 1. Fluorescence-activated cell sorter (FACS) profiles and purification of CD4-CD8⁻ double-negative and CD4⁺ TCR $\alpha\beta$ T cells and double-negative and CD4⁺ V α 24⁺ T cells in the peripheral blood of a healthy subject. **A**, Double-negative and CD4⁺ TCR $\alpha\beta$ T cells and double-negative and CD4⁺ V α 24⁺ T cells in PBL from a healthy subject were analyzed by FACS with phycoerythrin-conjugated anti-CD4 plus anti-CD8 mAb and FITC-conjugated anti-TCR $\alpha\beta$ mAb or anti-V α 24 mAb. **B**, FACS profiles of double-negative TCR $\alpha\beta$ T cells and CD4⁺ T cells isolated from the PBL of a healthy subject by anti-CD4 and anti-CD8 mAb depletion or by positive selection with anti-CD4 mAb. The purities of the fractionated double-negative and CD4⁺ T cells were more than 99%, respectively.

METHODS

Flow cytometry

PBL were isolated from 20 mL of heparinized peripheral venous blood of 4 healthy subjects by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation.

Cells (1×10^6) were stained with fluorescence- or biotin-conjugated antibodies in PBS containing 1% FCS for 30 minutes at 4°C. The following FITC-, phycoerythrin-, or biotin-conjugated mAbs were used: CD4 (Leu-3a), CD8 (Leu-2a), TCR $\alpha\beta$, CD16 (Leu-11c), CD56 (Leu-19; Becton Dickinson, Mountain View, Calif) and TCR V α 24 (Cosmo Bio Co, Tokyo, Japan). Anti-NKR-P1A mAb DX1 was provided by Dr L. Lanier (DNAX Research Institute, Palo Alto, Calif).¹⁷ Cells stained with biotinylated mAb were then incubated with streptavidin-phycoerythrin or -tricolor (Caltag, San Francisco, Calif). Stained cells were resuspended in PBS containing

1% fetal calf serum and analyzed by FACScan (Becton Dickinson) with the Cell Quest program (Becton Dickinson).

Purification of CD4-CD8⁻ double-negative and CD4⁺ T Cells

CD4-CD8⁻ double-negative TCR $\alpha\beta$ T cells were sorted from the PBL of healthy subjects by FACStar (Becton Dickinson) with anti-CD4 plus anti-CD8 mAb. CD4⁺ T cells were also sorted by anti-CD4 mAb. The yields of double-negative and CD4⁺ T cells were approximately 1×10^5 and 1×10^6 cells, respectively.

Cloning and sequencing of cDNAs encoding TCR V α genes

Total RNA (0.1 to 10 μ g) was prepared from sorted double-negative or CD4⁺ T cells by the method of acid guanidinium thio-

TABLE I. Frequency of invariant V α 24J α Q T cells in the peripheral blood double-negative (DN) and CD4⁺ T cells of healthy subjects

Source*	V α 24 ⁺ /PBL* (%)	V α 24 ⁺ /CD4 ⁺ or DN* (%)	V α 24J α Q/V α 24 ⁺ (%)
DN-1	0.24	24.0	236/349 (67.6)
DN-2	0.12	19.7	384/481 (79.8)
DN-3	0.17	18.3	500/570 (87.7)
DN-4	0.19	17.9	210/242 (86.8)
CD4-1	0.13	0.39	2/360 (0.5)
CD4-2	0.20	0.47	13/304 (4.2)
CD4-3	0.11	0.19	9/413 (2.2)
CD4-4	0.15	0.74	1/285 (0.4)

*CD4⁺CD8⁻ double-negative and CD4⁺ TCR V α 24⁺ T cells in the peripheral blood of 4 healthy subjects were counted by flow cytometry and expressed as the percentage against PBL, CD4⁺ T cells or double-negative T cells. Double-negative 1 to 4 represents the double-negative T cells from subjects 1 to 4; CD4-1 to 4 represents CD4⁺ T cells from subjects 1 to 4.

†TCR V α 24 cDNA libraries generated by PCR from double-negative and CD4⁺ T cells were blotted on 2 separate filters and hybridized with either V α 24-specific oligonucleotide probe or J α Q probe. The ratio of invariant V α 24J α Q/V α 24 cells was calculated from the number of positive plaques.

cyanate/phenol/chloroform extraction¹⁸ with Isogen solution (Nippon Gene Co, Tokyo, Japan). First strand complementary DNA (cDNA) was then synthesized from 0.1 to 1 μ g of total RNA in 20 μ L of reaction buffer containing oligo-dT primer with avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated at 25°C for 10 minutes and then at 42°C for 60 minutes.

TCR V α 24 cDNAs from double-negative and CD4⁺ T cells were amplified by PCR with primers for V α 24 with an EcoRI restriction site (5'-CGAATTCCTCAGCGATTACGCTCTAC-3') and C α (5'-CGAATTCGGTGAATAGGCAGACAGACTT-3'). Denaturing was performed at 95°C for 1.5 minutes; annealing was performed at 60°C for 1 minute, and extension was performed at 72°C for 1 minute, for 30 cycles on a DNA thermal cycler (Perkin-Elmer Corp, Norwalk, Conn). PCR products were purified by phenol extraction, precipitated with ethanol, and digested with excess amounts of EcoRI. DNA fragments with the expected sizes of the cDNAs were enriched by preparative low melting-point agarose gel electrophoresis. The recovered DNA fragments were ligated to M13mp19 plasmids obtained by EcoRI digestion. Phages were grown on TG-1 *Escherichia coli* cells. After hybridization with a C β probe was performed, a single phage was allowed to grow, and recombinant phage DNA was purified for DNA sequence determination. Sequencing reactions were performed by the dye primer method with an automated sequencer (Applied Biosystems).

Plaque hybridization

TCR V α 24 cDNA libraries were generated by PCR with RNA from the double-negative and CD4⁺ T cells with primers for the V α 24 and C α . Recombinant plaques were transferred from agar plates to a nitrocellulose membrane (Schleicher & Schnell, Dassel, Germany) and were hybridized with either a V α 24 probe (5'-CTCAGCGATTACGCTCTAC-3') or a 53-base pair (bp) J α Q probe. The latter probe was synthesized by PCR with 5'-J α Q (5'-CAACCCTGGGGAGGCTATAC-3') and 3'-J α Q (5'-AGGCCA-GACAGTCAACTGAG-3') primers and purified by electroelution.

Functional analysis of T cells

Double-negative T cells were cultured for 1.5, 6, 12, 24, 48, or 72 hours at 37°C in RPMI 1640 medium containing 10% human serum in 24-well flat-bottomed plates coated with anti-CD3, anti-V α 24, or anti-V α 12 mAb (10 mg/mL). Then, total RNA was prepared from stimulated T cells, and first strand cDNAs were synthesized. PCR was performed with 5'IL-4 (CTTCCCCCTCT-

GTTCTTCCT) and 3'IL-4 (TTCCTGTCGAGCCGTTTCAG) primers or 5'C α and 3'C α primers at 95°C for 1.5 minutes for denaturation, 55°C for 1 minute for annealing, and 72°C for 1 minute for extension, for 30 cycles on a DNA cycler. The PCR products were hybridized with a digoxigenin-labeled IL-4 probe of 317 bp in length or a C α probe of 155 bp in length.

Data analysis

Data are summarized as mean $\pm$ SD. The statistical analysis of the results was performed by the unpaired *t*-test. *P* < .05 was considered significant.

RESULTS

Relative number of double-negative and CD4⁺ V α 24⁺ T cells in the peripheral blood of healthy subjects

CD4⁺CD8⁻ double-negative and CD4⁺ TCR V α 24⁺ T cells in the peripheral blood of healthy subjects were counted by flow cytometry. As shown in Fig 1, A, and Table I, the percentage of double-negative and CD4⁺ V α 24⁺ T cells was similar in the peripheral blood of healthy subjects (double-negative V α 24⁺/PBL 0.18% $\pm$ 0.05% vs CD4⁺ V α 24⁺/PBL 0.15% $\pm$ 0.04%, mean $\pm$ SD; *n* = 4). However, double-negative V α 24⁺ T cells accounted for a large proportion of the double-negative T cells, whereas CD4⁺ V α 24⁺ T cells were very few among CD4⁺ T cells (V α 24⁺/double-negative 20.0% $\pm$ 2.3% vs V α 24⁺/CD4⁺ 0.5% $\pm$ 0.2%; *P* < .0005; Table I).

V α 24J α Q T cells are a major population of double-negative T cells, but not of CD4⁺ T cells

To determine the clonality of TCR V α 24 genes in peripheral blood double-negative and CD4⁺ T cells, we analyzed the nucleotide sequences of complementary determining region 3 (CDR3) of TCR V α 24 genes in the peripheral blood double-negative and CD4⁺ T cells of healthy subjects. Double-negative T cells were isolated from PBL of 4 healthy subjects by flow cytometry with

TABLE II. Junctional sequences of TCR V α 24 genes obtained from T cells of healthy subjects

Source	V α 94	N	J α 104	Frequency
DN-1				
GTGGTGAGC			GACAGAGGCTCAACCCTG J α Q	15/15
DN-2				
GTGGTGAGC			GACAGAGGCTCAACCCTG J α Q	14/16
GTGGTGAGC	CGGGAAG		CTGGCAACAACCGTAGC IGRJ α 14	2/16
DN-3				
GTGGTGAGC			GACAGAGGCTCAACCCTG J α Q	14/14
DN-4				
GTGGTGAGC			GACAGAGGCTCAACCCTG J α Q	11/11
CD4-1				
GTGGTG	TTATCTA		ACGACTACAAGCTC J α N	2/14
GTGGTGAGC		GGAGGAAGCCAAGGAAATCTC	IGRJ α 06	2/14
GTGGTGAGC	GCGTTATCTT	CTGGTGGCTACAATAAGCTG	IGRJ α 08	2/14
GTGGTGAGC		GATAACAATAACAATCAGATG	IGRJ α 10	1/14
GTGGTGA	CCTCGGTGG	ACACCAATCGAGGCAAATCA	IGRJ α 01G	1/14
GTGGTGAGC	CGTTATGGAGGAAA	CCAAGGAAATCTC	IGRJ α 06	1/14
GTGGTGAGC	CCGTTTTTC	AGATGGCCAGAAGCTG	J α L	1/14
GTGGTGAGC	GCGGGG	AGCAGTGCTTCCACGATA	J α A	1/14
GTGGTGAGC	GGT	GCATCAGGAGGAAGCTACATACCT	JHAP51G	1/14
GTGGTGAGC	CGTAGTGGT	GGAGCCAATAGTAAGCTG	IGRJ α 07	1/14
GTGGTGAG	TTTCATACTG	GATGGCCAGAAGCTG	J α L	1/14
CD4-2				
GTGGTG	GTTATA	CCCTCAGGAACCTACAAA	J α AP511	14/16
GTGGTGAGC		GACAGAGGCTCAACCCTG	J α Q	2/16
CD4-3				
GTGGTG	TTCGATT	CAGGATACAGCCACCCTC	J α AD17	12/12
CD4-4				
GTGGTGAGC	TTCT	TAGGGAACAACAGA	IGRJ α 11	7/16
GTGGTGAGC	GCGAGTTTAC	ATGGCCAGAAG	J α L	9/16

DN, Double-negative.

TCR V α 24 cDNA clones were randomly isolated from PCR-amplified libraries of DN and CD4⁺ T cells and sequenced. The nucleotide sequences of the 3' end of TCR V α , the N region, and the 5' end of the J α region are aligned. The frequency of identical sequences defined is shown on the extreme right.

anti-CD4 and anti-CD8 mAb. CD4⁺ T cells were isolated by positive selection with anti-CD4 mAb. The purities of fractionated double-negative and CD4⁺ T cells were more than 99%, respectively (Fig 1, B).

cDNAs encoding V α 24 genes from double-negative and CD4⁺ T cells were cloned and sequenced. The invariant J α Q gene was detected in double-negative T cells of all 4 subjects and was the major population of double-negative T cells (Table II). In 3 of 4 subjects, only the invariant J α Q gene was detected in V α 24 cDNA clones from double-negative T cells. In 1 subject, double-negative-2 in addition to J α Q gene, another J α gene IGRJ α 14, was detected at a low frequency (2 of 16 subjects) in double-negative T cells. In contrast, the invariant

J α Q gene was mostly undetected in V α 24 cDNA clones from CD4⁺ T cells of the same individuals, and only 1 subject CD4-2 expressed the J α Q gene at a low frequency (2 of 16 subjects; Table II).

To further determine whether the invariant V α 24J α Q T cells are few among the CD4⁺ T cells of healthy subjects in comparison with those of double-negative T cells, TCR V α 24 cDNA libraries generated by PCR from double-negative and CD4⁺ T cells were hybridized with either a V α 24-specific oligonucleotide probe or a J α Q probe. The V α 24J α Q gene was hardly detected in CD4⁺ T cells of all 4 subjects ($1.8\% \pm 1.8\%$; $n = 4$), although double-negative T cells mostly used V α 24J α Q TCR at a high frequency ($80.5\% \pm 9.3\%$; $n = 4$; $P < .0005$; Table I).

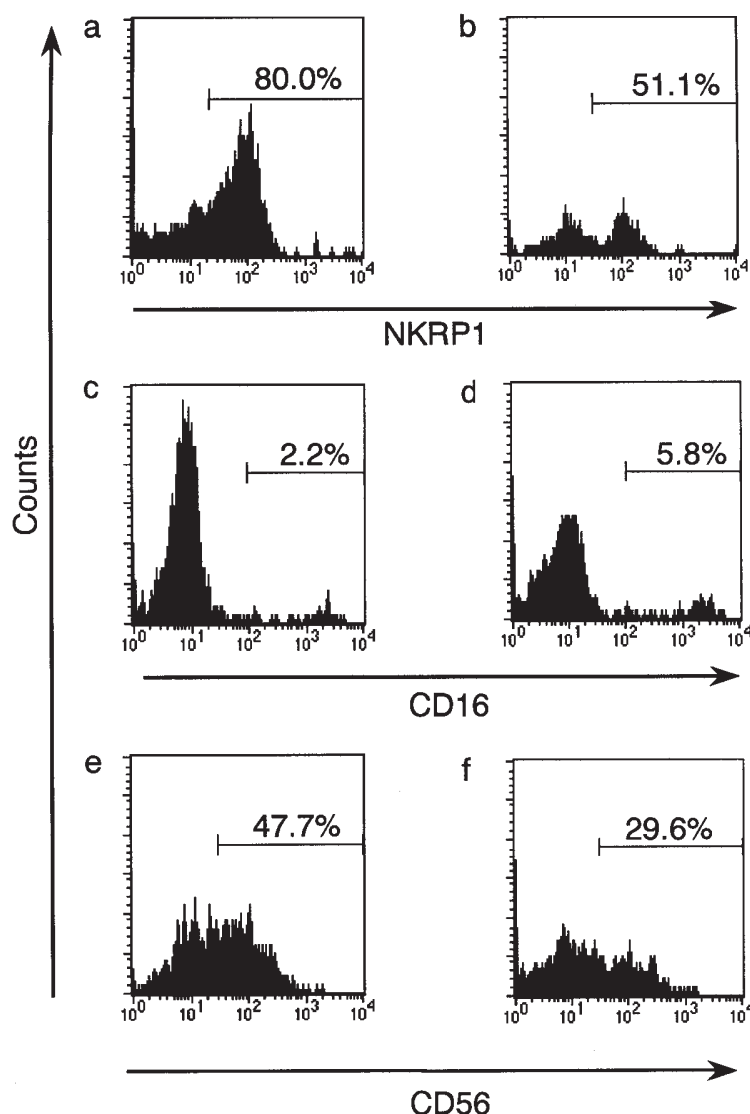


FIG 2. Cell surface phenotypes of double-negative Vα24⁺ T cells and CD4⁺ Vα24⁺ T cells. T cells were stained with anti-CD4, anti-CD8, and anti-Vα24 mAb; and double-negative and CD4⁺ Vα24⁺ T cells were then analyzed for cell surface antigens NKRP-1A (a and b), CD16 (c and d), and CD56 (e and f).

Double-negative Vα24⁺ T cells from healthy subjects express NKRP-1 on the cell surface

To determine whether double-negative and CD4⁺ Vα24⁺ T cells from healthy subjects express NK markers on the surface, we analyzed the expression of CD16, CD56, CD57, and NKRP-1A on double-negative and CD4⁺ Vα24⁺ T cells from healthy subjects by a 3-color flow cytometry (Fig 2). NKRP-1A was expressed by 80% of double-negative Vα24⁺ T cells and 51.5% of CD4⁺ Vα24⁺ T cells, but only by 9.2% of CD4⁺ Vα24⁺ T cells. CD56 antigen was expressed by 47.7% of double-negative Vα24⁺ T cells, but CD16 and CD57 antigens were not expressed.

Double-negative Vα24JαQ T cells express IL-4 mRNA by stimulation with anti-Vα24 mAb

Because it has been shown that murine CD4⁺ NK1.1⁺ T cells can promptly produce IL-4 by stimulation with anti-CD3 mAb,⁷ we examined whether Vα24JαQ T cells have the ability to produce IL-4. We stimulated double-negative T cells from healthy subjects with plate-coated anti-Vα24, anti-CD3, or anti-Vα12 mAb for 1.5 to 72 hours in vitro and measured the expression of IL-4 mRNA in those cells. By stimulation with anti-CD3 or anti-Vα12 mAb, the expression of IL-4 mRNA reached a maximum at 48 hours after the stimulation (Fig 3). In contrast, by stimulation with anti-Vα24 mAb, the

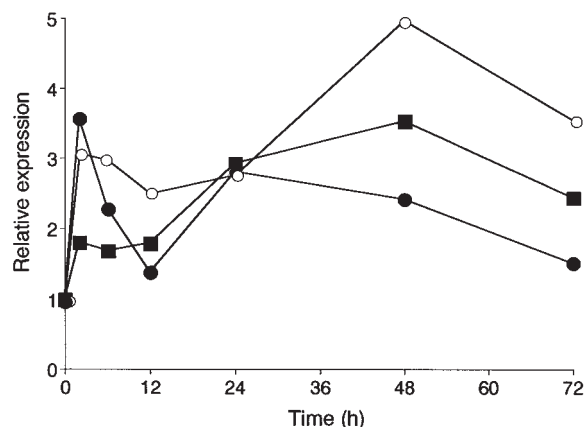


FIG 3. Analysis of IL-4 mRNA expression in double-negative TCR $\alpha\beta$ T cells. Double-negative T cells were cultured for 1.5 to 72 hours in plates coated with anti-CD3 (○), anti-V α 24 (●), or anti-V α 12 (■) mAb. Total RNA was prepared with and IL-4 mRNA was amplified by PCR with IL-4-specific primers. The PCR products were hybridized with an IL-4-specific probe, and the amount of IL-4 mRNA was normalized to that of unstimulated T cells.

expression of IL-4 mRNA became maximal at 1.5 hours (Fig 3).

DISCUSSION

In this study, we show that human double-negative V α 24J α Q T cells have phenotypic and functional similarities with murine V α 14J α 281 NK T cells. We found that V α 24J α Q T cells were a major population of double-negative T cells, but not of CD4⁺ T cells, in the peripheral blood of healthy subjects. We also found that V α 24J α Q T cells had an NK surface receptor NKR-P1A and CD56, but not CD16, and that they also promptly produced IL-4 by stimulation with anti-V α 24 mAb *in vitro*.

In murine NK T cells, the number of CD4⁺ V α 14J α 281 T cells is almost the same as that of double-negative V α 14J α 281 T cells,¹⁹ and both NK T-cell populations play an important role in controlling the T_{H1}/T_{H2} balance and in IgE production.^{7,20} In contrast, we found that an invariant V α 24J α Q gene was expressed in a very small proportion of CD4⁺ T cells, suggesting that the developmental pathway of CD4⁺ and double-negative NK T cells in humans may be different from that in mice or that there may be CD4⁺ NK T-cell populations other than V α 24J α Q T cells in humans. Our findings are consistent with previous observations that V α 24J α Q T cells were present predominantly among double-negative TCR $\alpha\beta$ T cells¹²⁻¹⁶ and that they were only a small population of CD4⁺ T cells.^{12,14}

We found that almost all double-negative V α 24J α Q T cells expressed NKR-P1A antigen at high levels comparable with the expression of NK1 (NKR-P1C) in the murine homologue V α 14J α 281 T-cell population. However, we found that double-negative V α 24J α Q T cells did

not express CD16 or CD57 NK cell antigens and that only one half of double-negative V α 24J α Q T cells expressed CD56 antigen. Exley et al²¹ recently established IL-2-dependent double-negative V α 24J α Q T-cell clones and demonstrated that NKR-P1A (but not CD16, CD56, or CD57) was also expressed in the T-cell clones. These findings suggest that there may be some relationship between double-negative V α 24J α Q T cells and NK cells in terms of transcriptional activation of NK locus antigens but that double-negative V α 24J α Q T cells may share a limited functional overlap with NK cells.

Approximately one half of CD4⁺ V α 24⁺ T cells also expressed NKR-P1A antigen, and the frequency of expression was much higher than that in conventional CD4⁺ V α 24⁻ T cells, although V α 24J α Q T cells were sparsely found in CD4⁺ T cells. These findings also suggest that there may be CD4⁺ NK T cells other than V α 24J α Q T cells.

We also studied IL-4 production in double-negative V α 24J α Q T cells and found that these cells expressed IL-4 mRNA at an early phase by stimulation with anti-V α 24 mAb, which is consistent with the observation by Yoshimoto et al.⁷ of the prompt expression of IL-4 mRNA in murine NK1⁺ T cells with *in vivo* challenge by anti-CD3. This indicates that human double-negative V α 24J α Q T cells also have the ability of IL-4 production at an early phase by TCR stimulation.

Double-negative TCR $\alpha\beta$ T cells have been shown to be increased in patients with autoimmune diseases such as systemic lupus erythematosus and systemic sclerosis.^{22,23} Furthermore, those double-negative T cells can produce IL-2 and help anti-DNA antibody synthesis *in vitro* in patients with systemic lupus erythematosus.²² Therefore those cells are thought to be similar to murine B220⁺ double-negative TCR $\alpha\beta$ T cells that accumulate in autoimmune *lpr* or *gld* mice and recognize a self antigen.²⁴⁻²⁹ On the other hand, double-negative V α 24J α Q T cells have been shown to be decreased in patients with systemic sclerosis¹⁶ and are thought to play a regulatory role in controlling the development of autoimmune disease.

In summary, we have shown that human V α 24J α Q T cells are a major population of double-negative T cells, but not of CD4⁺ T cells, in healthy subjects, express an NK surface receptor NKR-P1A and CD56, and promptly produce IL-4 by TCR stimulation. These results suggest that double-negative V α 24J α Q T cells are a counterpart of murine NK1⁺ T cells and may play a regulatory role in autoimmune responses *in vivo*.

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