Analysis of T Cell Antigen Receptor (TCR) Expression by Human Peripheral Blood CD4-8- α/β T Cells Demonstrates Preferential Use of Several Vβ Genes and an Invariant TCR α Chain

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Summary

CD4-CD8- (double negative [DN]) α/β T cells are a largely uncharacterized subpopulation of unknown function. To investigate whether these cells are selected to recognize particular antigens or antigen-presenting molecules, DN α/β T cells were purified from the peripheral blood of five normal donors and their T cell receptor (TCR) α and β chains were examined. Random cloning of TCR α chains by single-sided polymerase chain reaction (PCR) amplification identified an invariant rearrangement between Vα24 and JαQ, with no N region diversity, which was expressed preferentially by DN α/β T cells from all donors. Random cloning also identified a precise VαH.2-Jα(IGRJa14) rearrangement, with two variable amino acids encoded in the V-J junction, which was enriched in the DN α/β T cell preparations from some, but not all, donors. Analysis of TCR β chains by quantitative PCR amplification demonstrated that the expression of four Vβ gene families, Vβ2, 8, 11, and 13, was markedly increased in these DN α/β T cell preparations. The expression of particular TCRs by DN α/β T cells from multiple donors indicates that these cells, or at least a subpopulation of cells with this phenotype, recognize a limited spectrum of antigens and suggests that they may use nonpolymorphic antigen-presenting molecules.

Human peripheral blood contains small numbers of T cells that express the TCR α/β, lack CD4, and express low or undetectable levels of CD8 (1-4). These double-negative (DN)1 α/β T cells comprise <1% of the T cells in normal peripheral blood from most donors, but their numbers may be higher in some tissues, in particular epidermis (5). Increases in DN α/β T cells have been observed in patients with SLE (2) and systemic sclerosis (6), in rare immunodeficient patients with a graft-vs.-host-like disease (7), and in occasional normal donors (3). These cells are also massively increased in lpr and gld mice, which share features of human autoimmune disease (8, 9). However, the normal function of DN α/β cells and the role they may play in autoimmune diseases are uncertain, nor is it clear if they represent a functionally or developmentally discrete subpopulation of mature T cells.

A small population of mature DN α/β T cells is also found in murine thymus (10-16) and peripheral lymphoid tissues (15, 17-19). Investigations of TCR Vβ expression by these cells have demonstrated preferential usage of Vβ8 in some strains of mice (10-12, 14). A second population of murine DN α/β T cells that preferentially expresses Vβ2 has been demonstrated in bone marrow (20, 21). Studies of DN α/β T cells in strains of mice expressing endogenous superantigens (19) and in mice with an autoreactive transgenic TCR (22, 23) suggest that these cells are selected by a mechanism different from that operating on CD4+8- and CD4-8+ (single-positive) T cells. These observations suggest that murine DN α/β T cells may represent a developmentally distinct subpopulation of T lymphocytes, perhaps mediating a specialized function.

Murine and human T cells with the DN α/β TCR phenotype have been identified that can carry out several effector functions (4, 24-32), but the precise antigens recognized by these cells have not been defined in most cases. The absence of CD4 and CD8 expression suggests that these cells, or a subpopulation of cells with the DN α/β TCR phenotype, may use antigen-presenting molecules other than the classical MHC class I or II proteins. Consistent with this hy-
Cytometry, and used for preparation of total cellular RNA.

One approach to address the question of whether specific antigens or antigen-presenting molecules are recognized by human \( \alpha/\beta \) T cells is to identify the TCRs used by these cells. If the function of \( \alpha/\beta \) T cells involves recognition of a limited number or specific type of antigen, or if antigen presentation to these cells is carried out by proteins other than the classical MHC class I and II molecules, then the TCRs expressed by these cells may use particular V region genes or have some common structural features in their V-J joints. In this study, TCR expression by freshly isolated human \( \alpha/\beta \) T cells was assessed using a combination of random cloning, sequencing, and quantitative PCR amplification methods. This analysis identified at least two TCR \( \alpha \) chains with distinctive structural features that were expressed preferentially by purified \( \alpha/\beta \) T cells from multiple donors. V\( \beta \) usage by \( \alpha/\beta \) T cells was also biased towards a small number of \( \beta \) gene families in each donor. These results demonstrated distinctive features of TCRs expressed by \( \alpha/\beta \) T cells and provide further evidence that DN \( \alpha/\beta \) T cells may carry out a specialized immunological function.

**Materials and Methods**

**Isolation of DN \( \alpha/\beta \) T Cells.** Leukocyte concentrates of human peripheral blood were obtained as a byproduct of platelepheresis of randomly selected healthy blood donors. PBMC were purified by Ficoll-Hypaque gradient centrifugation (lymphocyte separation medium; Pharmacia Fine Chemicals, Piscataway, NJ). A total of \( 4\times 10^8 \) to \( 10^9 \) PBMC were suspended at \( 5 \times 10^7 \) cells/ml in PBS containing 2% human serum (HS) and a 1:1,000 dilution of ascites fluid containing mAbs OKT4 (anti-CD4) (33) and OKT8 (anti-CD8\( \alpha \)) (33), and incubated on ice for 2 h. Cells were then pelletted by centrifugation, resuspended in 25 ml of 20% rabbit serum (Accurate Chem. & Sci. Corp., Westbury, NY), diluted in RPMI 1640 (Gibco Laboratories, Grand Island, NY) as a source of complement activity, and incubated 1 h at room temperature followed by an additional 15 min at 37°C. Viable cells were collected after complement-mediated lysis by Ficoll-Hypaque centrifugation and cultured at 2.5 \( \times 10^6 \) cells/ml in RPMI 1640 + 10% FCS in a single 150-cm\(^2\) tissue culture flask. After incubation for 90 min at 37°C, nonadherent cells were collected, washed, and resuspended at 5 \( \times 10^7 \) cells/ml in PBS containing 2% HS with 1:500 dilutions of ascites fluids containing mAbs OKT4, OKT8, and (in four of five preparations) anti-TCR \( \delta \) (specific for the C region of the TCR \( \delta \) chain) (34). After incubation on ice for 1 h, the cells were washed twice and resuspended at 2 \( \times 10^7 \) cells/ml in PBS + 2% HS containing 6 \( \times 10^7 \) ml goat anti-mouse Ig-coupled magnetic beads (Dynabeads M450; Dynal, Inc., Great Neck, NY). The mixture of cells and magnetic beads was incubated with gentle agitation on an orbital shaker for 1 h at 4°C, after which the beads and bound cells were removed by magnetic separation. Unbound cells representing purified CD4 8" nonadherent (and TCR \( \delta \) in four of five cases) PBMC were collected, analyzed by flow cytometry, and used for preparation of total cellular RNA.

**Flow Cytometry Analysis.** Aliquots of purified CD4 8" nonadherent PBMC and unfractionated PBMC from each donor were stained with mAbs for two-color flow cytometry analysis as previously described (35). CD4 and CD8 expression was assessed using a mixture of FITC-conjugated mAbs Leu-3a (anti-CD4; reference 36) and B9.2 (anti-CD8\( \alpha \); reference 37), each diluted to 10 \( \mu \)g/ml. These particular anti-CD4 and -CD8 mAbs were chosen because they bind to epitopes that are not blocked by the OKT4 and OKT8 mAbs used in the immunoselection procedure of CD4 8" cells (S. Porcelli, unpublished data). Expressions of CD3, TCR \( \alpha/\beta \), and TCR \( \gamma/\delta \) were assessed using biotin-conjugated mAbs OTK3 (anti-CD3\( \varepsilon \); reference 33), BMA-031 (anti-TCR \( \alpha/\beta \); reference 38) and anti-TCR \( \delta \), each diluted to 2 \( \mu \)g/ml. Binding of biotinylated mAbs was detected by staining with a 1:500 dilution of PE-conjugated streptavidin (Tago, Inc., Burlingame, CA). Background staining was determined using FITC and biotin conjugates of IgG1 myeloma protein P3 (39). Immunofluorescence was analyzed using a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA). 10,000 cells of each sample were analyzed using forward and 90° light scatter gates to select for lymphocytes.

**Preparation of Whole Cellular RNA and cDNA.** For each of the five different donor preparations analyzed, between 1 and \( 5 \times 10^7 \) purified CD4 8" nonadherent PBMC were used for cDNA preparation. For each donor, an identical number of unfractionated PBMC was used. Whole cellular RNA was extracted in 3 M LiCl/6 M urea and first-strand cDNA synthesis was carried out using oligo(dT) priming and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), as described (40).

**Single-sided PCR Amplification and Cloning.** TCR \( \alpha \) transcripts were randomly cloned using a modification of the rapid amplification of cDNA ends (RACE) (41) or anchored PCR methods (42), as described previously (40). Aliquots of cDNA from the DN \( \alpha/\beta \) T cell preparations were homopolymer tail with terminal deoxynucleotidyltransferase (TdT) and deoxyctosine triphosphate (dCTP), as described (40). Second-strand synthesis was carried out using the Klenow fragment of DNA polymerase I and an oligo(dG) priming (GdATGTCGACGGGCGGGGG, Sall-diG) (40). TCR \( \alpha \) chains were then amplified by PCR using an antisense C\( \alpha \) primers located at the 3' end of the C region (GAGGAGCAAGCA-GGTGTTCTT) and the nonspecific Sall-diG10 primer (40). PCR products were then reamplified using internal antisense C\( \alpha \) primers in conjunction with the Sall-diG10 primer. The internal antisense C\( \alpha \) primers are either GAGGACAGACACTTGTGAC-TGAT or GAAGTTTTAGGTCTGTAAGGTTCA, located just 3' to PV1 (C\( \alpha \)-pre-Pvu II) or HindIII sites (C\( \alpha \)-pre-HindIII in C\( \alpha \), respectively. The PCR products were then digested with Sall and PvuII or HindIII, and cloned into pBlueScript (Stratagene, La Jolla, CA). Positive colonies were identified by hybridization with an internal C\( \alpha \) probe (CGCGAGGGTCAGGGTCTT) and multiple isolates were then picked and sequenced.

**PCR Amplification and Cloning of Specific \( V_\alpha \) Transcripts.** Anti-sense primers specific for C\( \alpha \) (TGGTTACTATTTCGAGGTA, C\( \alpha \), or C\( \alpha \) 2) (4AGAGATTCCATGCTGGTTCA) were included in conjunction with the 3' C\( \alpha \) primer to amplify by PCR the respective V\( \alpha \). The PCR reactions contained cDNA, 20 pmol of each primer, 0.1 mg/ml BSA, 0.2 mM dNTPs, 1.5 mM MgCl\( \text{2} \), 1 U Taq polymerase (Promega Biotec, Madison, WI), and buffer supplied by the manufacturer in a final 50-\mu l volume. Each cycle was 94°C for 20 s, 55°C for 30 s, and 72°C for 90 s for 30 cycles in some cases, 1 \mu l of the PCR product was reamplified using an internal C\( \alpha \) primer located 3' to the HindIII site in C\( \alpha \) (see above). The ends of the PCR products were then blunted with T4 DNA polymerase, digested with HindIII, and cloned into pBlueScript.
Quantitative PCR Amplification of Vα Transcripts. PCR amplifications with a pair of Vα primers were carried out initially on cDNA from PBLs and DN α/β T cells to estimate the relative amount of total TCR α cDNA in each sample. The 5' sense Vα primer was CCAGAACCCTGACCTGAGT and the Cα-pre-HindIII oligonucleotide was the 3' antisense primer. PCR reactions were carried out in 20 μl containing 100 ng of each primer, using the buffer and conditions described above. The PCR reactions were carried out for 24, 30, and 36 cycles, and the products were hybridized with an internal oligonucleotide probe (Cα-pre-PvuII). The relative amount of cDNA in each sample was estimated at a cycle number before the reaction reached a plateau.

Approximately equal amounts of cDNA from each sample (based upon the above Vα amplifications) were then amplified by PCR with the Vα17, 23, or 24 primer in conjunction with the antisense, Cα-pre-HindIII primer. The PCR reactions contained 15 pmol of each primer in 30 μl and were carried out for 26 and 32 cycles. The products were analyzed by Southern blot with an internal oligonucleotide probe (Cα-pre-PvuII) and quantitated on a Phosphorimag (Molecular Dynamics, Inc., Sunnyvale, CA). The lower cycle number was used to quantitate; the higher cycle number was used only to confirm that the reactions had not reached a plateau at the lower number of cycles. In every experiment, the Cα amplification was repeated with each cDNA to precisely normalize for the amount of cDNA. The results were expressed as the ratio of Vα/Cα in the DN population divided by the ratio of Vα/Cα in the matched PBLs.

To estimate the frequency of each Vα in the PBLs, the efficiencies of the Cα versus the Vα amplification primer pairs were compared. Plasmids containing full-length Vα17, 23, or 24 TCR α cDNAs (V and C region) were amplified by PCR using the pair of Cα primers or the Cα and appropriate Vα primers. The amount of product amplified by each primer pair, using an identical amount of template, was then compared. The efficiency of each Vα-Cα pair was calculated as the ratio of Vα product/Cα product. The frequency of each Vα in the Vα-Cα product was then estimated by taking the ratio of Vα/Cα products amplified from PBL cDNA and dividing by the efficiency of the respective primer pair.

Analysis of TCRs Amplified by PCR on High-Resolution Polyacrylamide Gels. TCR α chains were first amplified with the Cα-pre-HindIII primer and a specific Vα primer, as described above. The PCR products (0.5 μl) were then reamplified in 20 μl containing the same Vα primer, an internal antisense Cα primer located at the 5' end of the C region (CCCTCCAGCTGCTCTTCT, 100 ng), buffer, nucleotides, and enzyme as above, and 0.1 μl of [32P]dCTP (3,000 Ci/mmol, 10 mCi/ml). Alternatively, in some experiments the internal Cα primer was end labeled with 32P, using polynucleotide kinase. Each cycle was 94°C for 20 s, 48°C for 30 s, and 72°C for 30 s, for 20–25 cycles. The products were denatured by heating at 80°C in 50% formamide and ran on a 6% polyacrylamide DNA sequencing gel. Analysis of Vβ transcripts was carried out similarly using external (GCTTCTCTCTCTGCTGAGT) and internal (GGCTCAAAACACACGGCACCT) antisense Cβ primers. The spacing of the TCR bands was determined by running DNA sequencing ladders next to the TCR samples.

Quantitative PCR Amplification of Vβ Transcripts. A series of Vβ-specific primers and PCR amplification were used to estimate the expression of each Vβ gene family (40, 44, 45). The DN α/β T cell cDNA were amplified in a series of 30-μl reactions containing cDNA, 10 pmol of a Cβ antisense primer (GCTTCTCTCTCTGCTGAGT), 10 pmol of a Vβ primer (see below), 0.1 mg/ml BSA, 0.5 U Taq polymerase (Promega Biotec), 0.2 mM dNTPs, and buffer supplied by the manufacturer. Each cycle was 94°C for 20 s, 55°C for 30 s, and 72°C for 60 s. Aliquots of 5 μl were withdrawn after 24, 30, and 36 cycles and hybridized with a 32P-labeled internal antisense Cβ probe (GGCTCAAAACACACGGCACCT). Quantitation was performed on a Phosphorimag (Molecular Dynamics, Inc.) after 24 or 30 cycles. The 36-cycle aliquots were useful only to confirm that the reactions had not reached a plateau at the lower cycle numbers. Each Vβ was expressed as a percent of the total product amplified by all of the Vβ primers.

One- or twofold degenerate primers were synthesized to recognize all members of each Vβ gene family (Vβ1-20), except Vβ6, which required two primers to recognize all members. The TCR nomenclature is based on reference 46, unless otherwise indicated. The α primers were Vβ1, GAGAGCAAAAAGAATCCATCTGGAC; Vβ2, GAGAGCATAGAGCAAGGGTTC; Vβ3, GAGAGCATAGAGCAAGGGTTC; Vβ4, CCACATATGAGAGTGGATTTTAC; Vβ5, GATTTGACCTGCGCAGTTTC; Vβ6, CAGTGGATGCTTCTCAGA; Vβ7, GTCAGGAGATGAGCAAGGGTTC; Vβ8, GATGATGATGGAGAGTGGATTTT; Vβ9, TTATATGAAGGACAGGTAATACGGT; Vβ10, TACGAGGAGATGAGCAAGGGTTC; Vβ11, GATGATGATGGAGAGTGGATTTT; Vβ12, TGGCAGGAGATGAGCAAGGGTTC; Vβ13, CCGT(GA)ATGAGCAAGGAGGAGGC; Vβ14, CTTGCGAACAGATGAGCAAGGGTTC; Vβ15, GACGAGGAGATGAGCAAGGGTTC; Vβ16, TGGCAGGAGATGAGCAAGGGTTC; Vβ17, GACGAGGAGATGAGCAAGGGTTC; Vβ18, GACGAGGAGATGAGCAAGGGTTC; Vβ19, GACGAGGAGATGAGCAAGGGTTC; Vβ20, GACGAGGAGATGAGCAAGGGTTC.

Results

Enrichment of DN α/β T Cells from Human Peripheral Blood. Preparations of PBLs enriched for CD4-8- TCR α/β+ T cells (DN α/β T cells) from five random donors were prepared by extensive depletion of CD4+ and CD8+ T cells, and plastic-adherent cells from PBMC. In four of the five preparations (donors 1–4), T cells expressing TCR γ/δ were also depleted. An example of two-color flow cytometry analysis of one representative preparation is shown in Fig. 1, along with an identical analysis of the unfraccionated PBMC for the same donor. Contrary to the earlier description of reduced TCR levels on murine peripheral lymph node DN α/β T cells (19), human peripheral blood DN α/β T cells expressed the same level of TCR as did their CD4+ or CD8+ counterparts (Fig. 1).

The enrichment of DN α/β T cells in the CD4- and CD8-depleted preparations (DN samples) relative to the corresponding unfraccionated PBMC (PBL samples) from the same donor is shown in Table 1 for each of the five preparations. DN α/β T cells comprised <1.5% of the total lymphocytes and <2.0% of the TCR α/β+ cells in all five donor PBL samples. In the five DN samples, DN α/β T cells accounted for >94% of the total TCR α/β+ cells, representing an enrichment of between 50- and 136-fold relative to PBL. In preparations from which TCR γ/δ+ cells were specifically depleted (donors 1–4), DN α/β T cells represented >90% of the total TCR γ/δ+ cells (donor 5), whereas in the single preparation lacking depletion of TCR γ/δ+ cells (donor 5), these represented 21.8% of the CD3+ cells.
Figure 1. FACS® analysis of PBLs and purified DN α/β T cells from donor 1. Unfractionated PBLs and purified DN α/β T cells from donor 1 were analyzed for expression of CD3, TCR α/β, TCR γ/δ, CD4, and CD8. On the horizontal axis in all samples is a mixture of FITC-conjugated CD4 and CD8 antibodies (CD4 + CD8). The horizontal and vertical cursors were placed to yield <1% positive cells with control antibodies. The horizontal and vertical scales are log10 fluorescence intensity.

In all of the DN samples, the majority of the lymphocytes were CD3−, and probably represented non-T lymphocytes, including B and NK cells. Since these CD3− lymphocytes express low or undetectable levels of rearranged TCR α or β chain transcripts, their presence would be predicted to have no effect on the analysis of these transcripts (47, 48). Similarly, the presence of TCR γ/δ+ cells in the DN preparation from donor 5 should not significantly affect the TCR α/β analysis, since TCR α transcripts are absent from the vast majority of γ/δ T cells and TCR β gene rearrangements are not expressed at high levels (49).

Single-sided PCR Amplification, Cloning, and Sequencing of TCR α Chains from DN α/β T Cells. The initial aim was to sample the TCR α chains expressed by DN α/β T cells and determine if particular Vα genes were expressed at an increased frequency relative to single-positive PBLs. A concern in analyzing TCR α chains from minor subpopulations, such as DN α/β T cells, was that they could use previously undescribed Vα genes. A single-sided PCR amplification method (40–42) was used initially, therefore, to randomly clone and sequence all potential TCR α chains from two preparations of DN α/β T cells. Using this approach, it would be expected that Vα genes enriched in the DN α/β T cell population would be found in multiple independent clones.

A total of 48 TCR α chains were sequenced, 29 from donor 1 and 19 from donor 2. Two new Vαs were identified, but they were only single isolates and their expression by DN α/β T cells was not, therefore, characterized further (data not shown). Three previously described Vαs were, however, used by multiple TCRs (Table 2). TCRs using Vα24 (43) were isolated 14 times from the first donor (48% of isolates from this donor) and once from the second donor. In the majority of these isolates from donor 1 (12/14) and in the single isolate from donor 2, Vα24 was recombined with JoQ. Moreover, no V-J junctional diversity was evident in any of these 13 Vα24-JoQ recombination.

The isolation of the identical Vα24-JoQ recombination from donors 1 and 2 (Table 2) and subsequently from three additional donors (Table 3, see below) suggested that they may represent precise rearrangements of the germline Vα24 and JoQ genes, with minimal or no N region additions or deletions. The germline sequence of Vα24 is not available, but the multiple Vα24 cDNA isolates in Tables 2 and 3 (see below) indicate that the 3' end of the Vα24 gene is at the AGC (encoding serine), as shown in Table 2. The structure of the JoQ gene can be deduced from a germline JoQ transcript we have isolated and sequenced (Fig. 2). Similar germline transcripts encoding other JoQs have been described previously and presumably initiate from a promoter upstream of the unrearranged Joα gene (43, 50). A comparison between

| Table 1. Frequency of CD4−8− TCR α/β+ Lymphocytes in Cell Preparations Used for TCR Analysis |
|---------------------------------------------------------------|
| CD4−8− TCR α/β+ in PBL samples | CD4−8− TCR α/β+ in DN samples |
| Donor | Percent of total lymphocytes | Percent of CD3+ lymphocytes | Percent of TCR α/β+ lymphocytes | Percent of total lymphocytes | Percent of CD3+ lymphocytes | Percent of TCR α/β+ lymphocytes |
|-------|-----------------------------|-----------------------------|-------------------------------|-----------------------------|-----------------------------|-------------------------------|
| 1     | 1.0                         | 1.3                         | 1.4                           | 7.0                         | 97.2                        | 97.2                          |
| 2     | 1.4                         | 1.9                         | 1.9                           | 7.6                         | 90.5                        | 97.4                          |
| 3     | 0.5                         | 0.6                         | 0.7                           | 4.1                         | 93.2                        | 95.3                          |
| 4     | 1.3                         | 1.7                         | 1.7                           | 3.0                         | 94.9                        | 98.7                          |
| 5     | 1.4                         | 1.9                         | 1.9                           | 5.4                         | 21.8                        | 94.7                          |
| Donor | V      | N                          | J                               | Times isolated | Rearrangement |
|-------|--------|----------------------------|---------------------------------|----------------|---------------|
|       | ilecysvalvalser | aspargglyserthreuglyargleutyrphegyl  | | JaQ | 12 | + |
| 1 Va24 | ATCTGTGTGGTGAGC | pheleumetielylysaspala | GACAGAGGCTCAACCGTGAGGCTATACTTTGGA- | | |
|       | ilecysvalvalser | threutyrphegyl | GGAACAAAGGCTATACTTTGGAG- | | |
| 1 Va24 | ATCTGTGTGGTGAGC | ilecysvalvalser | JqA3 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | GATAGCAACTATCGTTAATCCTGGGC- | | IGRLJa06 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va24 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |
| 1 Va7.2 | CTCTGTGCT | ilecysvalvalser | JqA14 | 1 | + |
|       | ilecysvalvalser | ilecysvalvalser | ATGAGCAACTATCGTTAATCCTGGGC- | | |

TCR α transcripts were cloned randomly by single-sided PCR amplification, and sequences containing Vαs isolated more than once are shown. The 3' end of each Va, the N region, and the 5' end of each J region are shown along with the predicted amino acid sequence (three-letter code). The Jα nomenclature is from reference 46 unless otherwise indicated. The IGR through the Va7.2, 23, and 24 sequences have been described recently (43). The complete sequences of Jα9A3 and Jα8A1 will be reported elsewhere (S. P. Balk, manuscript in preparation).

* Number of times each TCR was isolated.

† Whether rearrangement is in frame (+) or out of frame (−).
| Donor | V       | N            | J                                      | Times isolated | Rearrangement |
|-------|---------|--------------|----------------------------------------|----------------|---------------|
| 1 DN  | Va24    | TACATCTGGTGTTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 8/8            | +             |
|       |         | TACATCTGTTGGTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 6/11           | +             |
|       |         | GCCCTGGGG     | GAGAGGAGCTACATACCTACATTTTGA - JaT     | 1/11           | +             |
|       |         | GCCCTGGGG     | CCGAGATGACACTCACTCTTAAG - IGR.Ja8     | 1/11           | +             |
|       |         | GCCCTGGGG     | GACACTTTAACTTTGGA - JαN                | 1/11           | +             |
|       |         | GCCCTGGGG     | GAGAAATTACCTTTGGGA - JαP                | 1/11           | +             |
| 2 DN  | Va24    | TACATCTGTTGGTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 7/14           | +             |
|       |         | TACATCTGTTGGTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 3/8            | +             |
|       |         | AAGGGG        | TGGAGGAAGCAGAAATCTCATTTTGA - IGR.Ja6   | 1/8            | +             |
|       |         | GAGCA         | AGCAGTCTCAGATCATATCTTTGGA - JαA        | 1/8            | +             |
|       |         | GCCGCAGAGGTGCTTGCAGGACT | ACCTTTTGGC - JαW                      | 1/8            | +             |
|       |         | GCCCTGGGG     | TAAAGCTGTATTTTTGA - JαP17              | 1/8            | +             |
|       |         | GCCCTGGGG     | AACACCGAACAAGCTCATTTTGA - JaI          | 1/8            | +             |
| 3 DN  | Va24    | TACATCTGTTGGTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 10/13          | +             |
|       |         | TACATCTGTTGGTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 9/13           | +             |
|       |         | GGGTTTTT      | CTCGTTGCTACAAATAGCCTATTTGA - IGR.Ja8   | 1/3            | +             |
|       |         | CTCTCC         | ATAGCCGGAGCAGCAACCTTTGGA - JaI          | 1/3            | +             |
|       |         | CCCCCTTTTTT   | CTGTTGCTTAAATAGCCTATTTGA - IGR.Ja8    | 1/3            | +             |
| 3 PBL | Va24    | TACATCTGTTGGTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 0/7            | +             |
|       |         | TACATCTGTTGGTGAGC | GACAGAGGCTCAACCCTGGGAGGCTATACTTTGGA - JaQ | 0/7            | +             |
|       |         | GCCCTA         | GGGCTACAGAAAGTTACCTTTTGA - JαO        | 1/7            | +             |
|       |         | CCCCCTTTTTT   | GATAGCCTTTGGAAGTTGCTTGCATTGCGGG - IGR.Ja02 | 1/7            | +             |
TCRs using Va24 were amplified from cDNA by PCR with Va24 and Ca primers. Multiple isolates from each cDNA were picked and sequenced. The 3' end of Va24, the N region, and 5' end of the J region are shown. JA210 has been reported (43, 72) and the complete sequences of Ja0P251, 9P30, 8De, 8P171, and 6D10 will be reported elsewhere (S. P. Balk, manuscript in preparation). In several isolates, Va24 was spliced directly to Ca with no Ja; the 5' end of Ca is shown in these cases (underlined).

* Number of times each transcript was isolated.

1 Whether the rearrangement is in frame (+) or out of frame (−).

---

| Transcript 1 | Transcript 2 | Frequency | Frame |
|--------------|--------------|-----------|-------|
| TACATCTGTTGAGC T | TACATCTGTTGAGC T | JA210 1/7 | - |
| TACATCTGTTGAGC TAGGGATG | TACATCTGTTGAGC TAGGGATG | JaP 1/7 | - |
| TACATCTGTTGAGC ACGGCA | TACATCTGTTGAGC ACGGCA | JaA 1/7 | - |
| 4 DN Va24 -TACATCTGTTGAGC | 4 DN Va24 -TACATCTGTTGAGC | JaQ 10/15 | + |
| TACATCTGTTGAGC TACGG | TACATCTGTTGAGC TACGG | JaF 1/15 | - |
| TACATCTGTTGAGC CCAATCACAC | TACATCTGTTGAGC CCAATCACAC | JaN 1/15 | - |
| TACATCTGTTGAGC ACCTTTAACCAGAC | TACATCTGTTGAGC ACCTTTAACCAGAC | - |
| TACATCTGTTGAGC CCCAATCAAC | TACATCTGTTGAGC CCCAATCAAC | - |
| 4 PBL Va24 -TACATCTGTTGAGC | 4 PBL Va24 -TACATCTGTTGAGC | JaQ 1/8 | + |
| TACATCTGTTGAGC ACC | TACATCTGTTGAGC ACC | JaQ 1/8 | + |
| TACATCTGTTGAGC GAA | TACATCTGTTGAGC GAA | JaT 1/8 | + |
| TACATCTGTTGAGC GAA | TACATCTGTTGAGC GAA | JaT 1/8 | + |
| 5 DN Va24 -TACATCTGTTGAGC | 5 DN Va24 -TACATCTGTTGAGC | JaQ 5/5 | + |
| TACATCTGTTGAGC GGGGCGGAGGAGGAAACAAACTCACCTTTGGG | TACATCTGTTGAGC GGGGCGGAGGAGGAAACAAACTCACCTTTGGG | 1/9 | + |
| TACATCTGTTGAGC GGGGCGGAGGAGGAAACAAACTCACCTTTGGG | TACATCTGTTGAGC GGGGCGGAGGAGGAAACAAACTCACCTTTGGG | 1/9 | + |
| TACATCTGTTGAGC CCTTAATCGA | TACATCTGTTGAGC CCTTAATCGA | 1/9 | - |
| TACATCTGTTGAGC ATATCCAGAACCC | TACATCTGTTGAGC ATATCCAGAACCC | 1/9 | - |
| TACATCTGTTGAGC AACTGGGGCAAACAACGCTCTTCTTTGGG | TACATCTGTTGAGC AACTGGGGCAAACAACGCTCTTCTTTGGG | 5/5 | + |
| TACATCTGTTGAGC ACAAGCTGGTCTTTGGCGC | TACATCTGTTGAGC ACAAGCTGGTCTTTGGCGC | 1/9 | + |
| TACATCTGTTGAGC ACTATCAGTTAATCTGGGGC | TACATCTGTTGAGC ACTATCAGTTAATCTGGGGC | 6D10 1/8 | + |
| TACATCTGTTGAGC ACTATCAGTTAATCTGGGGC | TACATCTGTTGAGC ACTATCAGTTAATCTGGGGC | 6D10 1/8 | + |
| TACATCTGTTGAGC ACTATCAGTTAATCTGGGGC | TACATCTGTTGAGC ACTATCAGTTAATCTGGGGC | 6D10 1/8 | + |
were between 2.2 and 6.5% (data not shown). These frequencies were consistent with the results from random cloning from these five donors, while the Vot23 and Vot24 transcripts varied from 3.0 to 10% of the total TCR $\alpha$ cDNA in PBLs versus the Cot primers were determined (see Materials and Methods). This analysis indicated that the Vot7.2 transcript was joined to IGRJa14 (43) (4/5 from donor 1 and 4/4 from donor 2). Furthermore, these V-J joints were similar in that they occurred to the same nucleotide in IGRJa14 and appeared to contain six N region nucleotides. Alternatively, the Vot7.2 gene shown in Table 2 may encode several additional 3' bases (G or GT being most likely; see below). In either case, the TCR $\alpha$ chains encoded by these transcripts had the 3' alanine in Vot7.2 and two variable amino acids in the V-J junction joined precisely to the 5' aspartate in IGRJa14. Interestingly, the only Vot7.2 isolate that was joined to a different J was out of frame (Table 2, donor 1), further indicating that this rearrangement to IGRJa14 predominates among functional Vot7.2 rearrangements.

The third Vot identified more than once was Vot23 (43), which occurred in three isolates from each donor (Table 2). A previously undescribed Jot, Jot8A1, was used by one of these Vot23 TCRs from each donor, but each of these Vot23-Jot8A1 TCRs had a distinct N region and the rearrangement was out of frame in donor 1. There were otherwise no noteworthy similarities between the TCRs using Vot23.

Analysis of Vot7.2, 23, and 24 Expression by Quantitative PCR Amplification. To confirm the results from random cloning, the expression of Vot7.2, 23, and 24 by donors 1 and 2 and by three additional donors was assessed by quantitative PCR amplification. The frequency of these Vots in unfractionated PBLs from each donor was first assessed. Primers specific for Vot7.2, 23, or 24, in conjunction with a Ca primers, were used to amplify PBL cDNA from each of the donors. Two specific Ca primers were used to estimate the total amount of TCR $\alpha$ cDNA, and the efficiencies of the Vot primers versus the Ca primers were determined (see Materials and Methods). This analysis indicated that the Vot7.2 transcript varied from 3.0 to 10% of the total TCR $\alpha$ cDNA in PBLs from these five donors, while the Vot23 and Vot24 transcripts were between 2.2 and 6.5% (data not shown). These frequencies were consistent with the results from random cloning of TCR $\alpha$ chains using these Vots from peripheral blood (43). The expression of these Vots in the DN population versus the PBLs from the same donor was then compared to determine if any were enriched in the DN population. cDNA from the DN and corresponding unfractionated PBL samples were used as templates for quantitative PCR amplification using primers specific for Vot7.2, 23, or 24. The amplifications were normalized in each experiment by amplifying parallel samples with two Ca primers. The amount of PCR product was quantitated and the ratio of DN versus PBL transcripts was calculated.

Fig. 3 shows that the expression of Vot7.2 and Vot24 by DN $\alpha$/B T cells was increased relative to PBLs in the majority of donors, although most of the increases were less than fourfold. In contrast, Vot23 was not increased in any of the DN $\alpha$/B T cell preparations, indicating a discrepancy between the random cloning method (Table 2) and this quantitative PCR method in assessing the expression of Vot23 by donors 1 and 2. The reason for this discrepancy is not clear, but it emphasizes the need for a second method to confirm results derived from random cloning. The frequencies of the Vot7.2 and Vot24 transcripts in the DN $\alpha$/B T cell populations from donors 1 and 2 estimated by this quantitative PCR method were, however, consistent with the random cloning results (Table 2). Both methods indicated that TCRs using Vot7.2 were frequent transcripts in donors 1 and 2 and that TCRs using Vot24 were particularly abundant in donor 1.

Sequence Analysis of TCR $\alpha$ Transcripts from PBLs and DN $\alpha$/B T Cells Using Vot7.2 and Vot24. Although the expression of the Vot7.2 and Vot24 transcripts was not markedly increased in most of the DN $\alpha$/B T cell preparations relative to the PBLs, the sequencing data in Table 2 also indicated that DN $\alpha$/B T cells using Vot7.2 and Vot24 may select particular Jot genes and junctional sequences. To further assess the expression of Vot7.2 and Vot24 and determine if they undergo precise rearrangements with specific Jot genes in DN $\alpha$/B T cells, multiple additional TCRs using these Vots were

Figure 2. Nucleotide sequence of a germline JotQ-Ca transcript. A cDNA containing the Jot gene spliced precisely to Ca was isolated by single-sided PCR amplification. The sequence of the 5' upstream region, complete Jot, and the 3' end of Ca are shown. The heptamer and nonamer recombination sequences are underlined and the 5' end of Ca is boldfaced.

Figure 3. Comparison of Vot7.2, 23, and 24 expression by DN $\alpha$/B T cells versus PBLs. The expression of each Vot relative to Ca in the DN and PBL samples was estimated by quantitative PCR amplification. The ratio of Vot expression in the DN versus the matched PBL samples was determined. The results from two experiments are shown.
Table 4. Sequences of TCRs Using Vα7.2 from DN α/β T Cells and PBLs

| N region        | Donor | Other Vα7.2 joints                                      |
|-----------------|-------|--------------------------------------------------------|
|                 |       | N region Donor                                         |
|                 |       | J region Donor                                         |

| Precise Vα7.2-IGRJa14 joints | | Other Vα7.2 joints |
|-------------------------------|-------------------------------|
| GTGAAG (vallys) DN1           | GTGAGAGATGAGTA                | CAATAACAATGACATGCCTTTGGA-  |
| GTGAGA (valarg) DN1           | GTGGCTTTAGGG                  | GGGGTACCAAGAAATCTCCTTTGGA-  |
| GTGGGG (valgly) DN1           | GTCCCTC                      | AATTCCGGGTATGCACTCAACTCGGC-  |
| GTACTG (valleu) DN1           | GTGAGAGTT                    | ATACTCACGGAGGAGGAAAAAATCTCCTTTGGA- |
| GTGGTG (valval) DN2           | GTGAAG                      | GGCTGG (glytrp) DN2          |
| GGCTGG (glytrp) DN2           | GTGACCTTGACAG                | GTCATG (valmet) DN4          |
| GGCATG (glymet) DN3           | GTGAGATCAGGG                 | GTGAGAGATCGGG                |
| GTGAAG (vallys) DN4           |                             |                             |
| GTGAGA (valarg) DN4           |                             |                             |
| GTCATG (valmet) DN4           |                             |                             |
| GTGATG (valmet) DN4           |                             |                             |
| GTGATG (valmet) PBL1          |                             |                             |
| ATCATG (ilemet) PBL1          |                             |                             |
| GTGAGA (valarg) PBL2          |                             |                             |
| GCCGGT (alaal) PBL2           |                             |                             |
| CTACTC (leuleu) PBL3          |                             |                             |
| GTAAGA (valarg) PBL4          |                             |                             |
| ACAATG (thrmet) PBL4          |                             |                             |
| GTGAGA (valarg) PBL4          |                             |                             |

TCRs were amplified from cDNA by PCR using Vα7.2 and Cε primers and multiple isolates were sequenced. The majority contained the precise Vα7.2-IGRJa14 joint and only the six-base (two-amino acid) V-J junctional sequences are shown for these. All of the other isolates also contained functional rearrangements and the N region and 5' end of the J region are shown for these Vα7.2 TCRs. The Vα7.2 sequence in all of these extended to the TGTGCT (cysala) indicated as the 3' end of this Vα gene in Table 2. The sequences shown here do not include those shown in Table 2 from donors 1 and 2. The complete Jα6Pa2 sequence will be reported elsewhere (S. P. Balk, manuscript in preparation).
cloned and sequenced. TCR α transcripts were amplified by PCR from unfractionated PBLs or DN α/β T cells using an antisense Ca primer and Vα7.2- or Vα24-specific sense primers. The amplified TCRs were then cloned into pBlue-script and multiple isolates were sequenced.

Table 3 shows the N region sequence and J region usage of Vα24 TCRs from five donors. In all five DN populations, the invariant rearrangement to JαQ comprised the majority of functional TCRs. In donors 1 and 5, every isolate from the DN preparation had this rearrangement. The invariant Vα24-JαQ rearrangement was also found in the PBL samples from all but one donor, but the frequency of this rearrangement in the PBLs was in all cases less than in the matched DN preparations. Moreover, some of the invariant Vα24-JαQ transcripts in the unfractionated PBLs were likely derived from DN α/β T cells. These results demonstrated that the invariant Vα24-JαQ rearrangement was enriched in the DN α/β T cell populations of all donors and indicated that some degree of selection for this TCR may also occur in single-positive T cells from some donors.

TCRs using Vα7.2 were examined similarly to determine if the precise rearrangement to IGRJa14, with two variable amino acids in the V-J junction (see Table 2), was enriched in the DN α/β T cell population. Consistent with the results in Table 2, this precise rearrangement to IGRJa14 was found in the majority (11/12) of Vα7.2 TCRs isolated from the DN α/β T cell preparations (Table 4). However, this Vα7.2-IGRJa14 rearrangement was also found in many of the Vα7.2 isolates from unfractionated PBLs (8/14), which contained predominantly single-positive T cells. These results showed that the Vα7.2 gene had a marked preference for this particular rearrangement to IGRJa14 in the DN α/β T cell populations and suggested that a similar bias was present in the single-positive T cells from some donors (see also Fig. 4 B).

Table 4 shows the junctional nucleotides and amino acids from each of the precise Vα7.2-IGRJa14 joints isolated. The two junctional amino acids were not highly conserved, although the first one was neutral in all cases and the second was neutral or basic. This larger sampling of Vα7.2 sequences also indicated that the germline Vα7.2 gene probably extended past the 3′ alanine shown in Table 2 and includes, at least, the GTG (valine), which is shown in Tables 2 and 4 as being part of the N region. Furthermore, the finding of GTGAGA (valine) at the 3′ end of several Vα7.2 isolates (including three that did not have the precise joint to IGRJa14) suggested that this GTGAGA was the actual 3′ end of the Vα7.2 gene.

Figure 4. Analysis of Vα24 and Vα7.2 transcripts by PCR amplification and gel electrophoresis. cDNA from DN or PBL samples were amplified with a Vα24 primer (A) or Vα7.2 primer (B) and an external Ca primer. They were reapplied using an internal Ca primer and labeled by incorporation of α-[βP]dCTP. The labeled PCR products were then analyzed on a 6% denaturing polyacrylamide gel. (Lanes +) The template was a plasmid containing the precise Vα24-JαQ joint (A) or the Vα7.2-IGRJa14 joint (B). (Lane −) No added cDNA or plasmid.
these PCR products were then reamplified with the Va24 primer and an internal Ca primer, in the presence of α-[³²P] dCTP, and analyzed on a DNA sequencing gel.

The DN α/β T cell cDNA from each of the donors yielded predominantly a single band (Fig. 4 A). The size of this band was identical to the band generated by amplification from a plasmid containing the invariant Va24-JαQ rearrangement. In contrast, the matched PBL samples showed a more heterogeneous banding pattern, consistent with the presence of N region diversity and usage of multiple J regions (Fig. 4 A). These results were in good agreement with the sequencing data (Table 3) and supported the conclusion that the invariant Va24-JαQ rearrangement was enriched in the DN α/β T cell population.

A similar analysis was carried out for TCRs using the Va7.2 gene (Fig. 4 B). In these experiments all of the DN and PBL samples generated a prominent doublet that was identical to the doublet generated by amplification from a plasmid containing the precise Va7.2-IGRJa14 rearrangement. The bands appear as doublets, rather than single bands, due to incomplete addition of a non-template-encoded adenosine at the 3' end by the Taq polymerase (51). This incomplete addition of an adenosine at the 3' has been seen in some PCR amplifications, but not all (see Fig. 4 A), and the variables involved are not clear.

Large doublets were also seen at intervals of three bases in most samples, indicating that they were derived from functional V-J rearrangements (Fig. 4 B). The sequencing data in Table 4 similarly showed that most Va7.2 rearrangements to Jαs other than IGRJa14 were functional. In all cases except one (DNs from donor 3), these additional bands were significantly less intense than the Va7.2-IGRJa14 rearrangement. The bands appear as doublets, rather than single bands, due to incomplete addition of a non-template-encoded adenosine at the 3' end by the Taq polymerase (51). This incomplete addition of an adenosine at the 3' has been seen in some PCR amplifications, but not all, (see Fig. 4 A), and the variables involved are not clear.

Large doublets were also seen at intervals of three bases in most samples, indicating that they were derived from functional V-J rearrangements (Fig. 4 B). The sequencing data in Table 4 similarly showed that most Va7.2 rearrangements to Jαs other than IGRJa14 were functional. In all cases except one (DNs from donor 3), these additional bands were significantly less intense than the Va7.2-IGRJa14 rearrangement, particularly in the DN preparations from donors 1, 2, 4, and 5. These results demonstrated that the Va7.2 gene had a very strong bias towards one particular type of rearrangement in both the DN and single-positive T cell populations, with an even more pronounced bias in the DN α/β T cell population from some, but not all, donors.

Quantitative PCR Analysis of Vβ Usage by DN α/β T Cells. Further evidence that certain TCRs are used preferentially by DN α/β T cells was provided by an analysis of Vβ expression. Quantitative PCR amplification was performed on unfractionated PBLs and DN α/β T cells from donor 1, using a panel of primers specific for Vβ1-20 and a Cβ primer specific for Cβ1 and Cβ2. Fig. 5 shows that the pattern of Vβ usage by the PBLs versus the DN α/β T cells was significantly different. In particular, there was clearly enrichment for Vβ2 and Vβ11 in the DN α/β T cell population versus the PBLs from this donor.

A similar analysis from a second donor is shown in Fig. 5, and the results from analyses of all five donors are summarized in Table 5. These data showed a marked preference for the use of one or several Vβ genes in every donor. Moreover, the dominant Vβ gene families expressed by every donor were Vβ2, 8, 11, or 13. These results supported the conclusion that DN α/β T cells used certain TCRs preferentially. The

Table 5. Quantitative PCR Analysis of Vβ Expression by DN α/β T Cells from Peripheral Blood of Normal Donors

| TCR  Vβ | Donor 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---------|--------|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|
| %       |        |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |
| 1 DN    | 15     | 18 | 4 | 5 | 12 | 1 | 9 | 6 | 1 | 1  | 36 | 3 | 1  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| 2 DN    | 0      | 16 | 3 | 5 | 0  | 0 | 0 | 0 | 0 | 0  | 1  | 72 | 1  | 0  | 0  | 0  | 0  | 1  | 0  | 0  |
| 3 DN    | 1      | 8  | 7 | 2 | 1  | 5 | 7 | 37| 3 | 1  | 15 | 2 | 8  | 1  | 0  | 0  | 0  | 0  | 0  | 0  |
| 4 DN    | 0      | 7  | 0 | 0 | 0  | 0 | 0 | 1 | 73| 4  | 1 | 2  | 2  | 1  | 0  | 0  | 7  | 0  | 0  | 0  |
| 5 DN    | 0      | 26 | 0 | 0 | 3  | 0 | 2 | 6 | 1 | 1  | 33 | 3 | 16 | 2  | 2  | 1  | 3  | 0  | 0  | 0  |
finding of similar Vβ gene usage in multiple unrelated donors further suggested that polymorphisms in the classical MHC class I and II molecules did not influence the selection of Vβ genes by these cells. Additional studies will be needed to determine if TCR β chains using these predominant Vβ genes pair with invariant TCR α chains or TCR α chains with other distinct structural features.

Assessment of Vβ Heterogeneity by High-Resolution PAGE. A gel electrophoresis method was used to determine whether the TCR-β chains using predominant Vβs from each donor were monoclonal or multiclonal. The TCRs were amplified by PCR with Vβ2, 8, 11, or 13 primers and a Cβ primer. The PCR products were then reamplified with the same Vβ primer and an internal, 32p-labeled Cβ primer, and analyzed by gel electrophoresis. PBL cDNA was analyzed similarly with each Vβ primer.

The PBL cDNA yielded multiple bands with each Vβ primer (Fig. 6), indicative of differences in length of the N region in a polyclonal population. The major PBL bands were all separated by three bases, consistent with most TCR transcripts being derived from functional rearrangements. A similar pattern was seen for the DN TCR β chains using Vβ11, indicating that multiple DN α/β T cell clones using Vβ11 were present. In contrast, several predominant bands were detected among the DN TCR β chains using Vβ2, 8, and 13, suggesting an oligoclonal expansion of T cells using these Vβs. Taken together, the results of these analyses indicated that DN α/β T cells preferentially expressed particular Vβs, with Vβ2, 8, 11, and 13 being over represented, and suggested that diversity at the Vβ-Jβ joint in DN α/β T cells using some Vβ genes was also limited compared with PBLs. Sequencing of multiple DN TCR β chains will be required, however, to determine precisely the complexity of TCR β chains expressed by DN α/β T cells.

Discussion

Human peripheral blood contains a small population of DN α/β T cells, but whether these cells are functionally or developmentally distinct from their CD4- or CD8-positive counterparts has not been clear. The data presented here demonstrate significant differences between the TCR α and β chains used by DN and single-positive α/β T cells in the peripheral blood of normal human donors. The expression of one particular TCR α chain, a rearrangement between Vα24 and JαQ with no diversity at the V-J joint, was increased in the DN α/β T cells from all five donors examined. The preferential use of certain Vβ genes was also found in all five donors. These results indicate that DN α/β T cells may arise through a different pathway of T cell development or be selected for recognition of different ligands than single-positive α/β T cells.

The invariant Vα24-JαQ TCR found increased in each DN α/β T cell preparation was most likely generated by the removal of two bases from the germline JαQ gene, with no N region additions. These invariant Vα24-JαQ TCRs could be derived from a single expanded clone in each donor, but their occurrence in all donors suggests that this rearrangement occurs in multiple independent clones in each donor. Examples of invariant TCR chains with no N region additions have been described for certain γ/δ T cells. Murine dendritic epidermal T cells (DECs) express invariant TCR γ and δ chains that are generated by independent recombination events in multiple clones (52, 53). In humans, a significant proportion of the joints between Vγ9 and JγP are generated by the excision of a single JγP-encoded base, with no N region additions (54). Whether this invariant Vγ9-JγP rearrangement occurs in one or multiple independent clones has not been determined.

The relatively high frequency of these invariant Vα24-JαQ TCRs in multiple donors indicates that some mechanism exists that favors their occurrence. Although this mechanism may be simply positive selection of randomly generated clones, a number of factors could increase the frequency of this rearrangement directly. For example, the germline Vα24 and JαQ genes may be particularly accessible to each other due to close physical proximity or because they become activated for recombination at the same time during development. The lack of template-independent insertions in the invariant Vα24-JαQ TCR could be due to development of DN α/β T cells in

![Figure 6](image-url)
the thymus before TdT is active, as is the case for the invariant TCR \( \gamma \) and \( \delta \) chains of murine DECs (55, 56). If the high frequency of the invariant \( \text{V}a24-\text{JaQ} \) joint is indeed due to these or other mechanisms operating to favor invariant \( \text{V}a-\text{Ja} \) recombinations in DN \( \alpha/\beta \) T cells, then TCRs with other invariant germline \( \text{V}a-\text{Ja} \) joints may be present and predominate in the DN \( \alpha/\beta \) T cells from other donors. Random TCR \( \alpha \) cloning from additional DN \( \alpha/\beta \) T cell preparations and further information on genomic \( \text{V}a \) and \( \text{Ja} \) sequences will be necessary to address this question.

An alternative possibility is that invariant \( \text{V}a24-\text{JaQ} \) joints could be generated at random in the thymus and then undergo positive selection followed by ligand-dependent clonal expansion in the periphery. The ligands recognized by DECs or by TCRs using the invariant \( \text{V}a24-\text{JaQ} \) or \( \text{V}g9-\gamma \text{P} \) rearrangements have not been identified. The expression of the \( \text{V}a24-\text{JaQ} \) TCR in multiple donors suggests that the ligand, or at least the epitope, recognized by this TCR is nonpolymorphic. Similar arguments have been made for the ligands recognized by DECs (52, 53, 57) and the \( \text{V}g9-\gamma \text{P} \) recombination (54). If the ligand recognized by the \( \text{V}a24-\text{JaQ} \) TCR is an MHC class I or II molecule or a nonpolymorphic MHC class I–like molecule, then the lack of CD4 or CD8 expression may be necessary to prevent autoreactivity. Analysis of T cell clones using this invariant \( \text{V}a24-\text{JaQ} \) TCR will be necessary to identify the ligand recognized by this TCR and determine if it arises in multiple independent clones in each donor.

A second interesting group of TCR \( \alpha \) chains identified in these studies were recombinations between \( \text{V}a7.2 \) and IGRJa14. These \( \text{V}a7.2-\text{IGRJa14} \) joints were enriched in some, but not all, DN \( \alpha/\beta \) T cell preparations. In the majority of DN \( \alpha/\beta \) T cells and unfractionated PBLs using \( \text{V}a7.2 \), the joint was to the same nucleotide in IGRJa14 with two variable amino acids in the V-J junction. The conserved structure of this \( \text{V}a7.2-\text{IGRJa14} \) joint in multiple clones from five donors suggests that this TCR \( \alpha \) chain may be selected by a nonpolymorphic ligand. Nonpolymorphic MHC class I–like (58) or class II–like (59, 60) molecules are candidate ligands, as these \( \text{V}a7.2-\text{IGRJa14} \) TCRs are expressed by single-positive as well as DN T cells. Alternatively, the \( \text{V}a7.2 \) gene may have a structural limitation such that only these particular recombinations with IGRJa14 allow it to fold correctly and undergo positive selection by conventional MHC molecules. A similar recombination between murine \( \text{V}a14 \) and \( \text{Ja281} \) with a one-base N region has been demonstrated in multiple strains of mice, and these TCRs appear to be positively selected by a non-MHC-linked monomorphic ligand (61).

The analysis of TCR \( \beta \) chains provided further evidence that DN \( \alpha/\beta \) T cells use particular TCRs preferentially. The majority of DN \( \alpha/\beta \) T cells from each donor used only one or two \( \text{V}B \) gene families, with the predominant \( \text{V}B \) genes being \( \text{V}B2 \), 8, 11, and 13 (Table 5). Further analysis of these TCR \( \beta \) chains on DNA sequencing gels indicated that they were less heterogeneous than the corresponding TCRs from PBLs. This suggests that their expansion was probably not due to \( \text{V}B \)-specific polyclonal stimulation by a superantigen (62), although the possibility that chronic stimulation by superantigens might result in this pattern of restricted TCR \( \beta \) expression cannot be ruled out. The preferential expression of these \( \text{V}B \)s by DN \( \alpha/\beta \) T cells from multiple unrelated donors suggests, therefore, that these cells may recognize a limited number of conventional antigens and may use nonpolymorphic antigen-presenting molecules. Alternatively, if the TCR \( \beta \) chains using these predominant \( \text{V}B \)s are paired with invariant TCR \( \alpha \) chains or other TCR \( \alpha \) chains with particular structural features, then the ability to pair with these TCR \( \alpha \) chains could be the major factor responsible for selecting these \( \text{V}B \)s.

Normal mice also have a small population of mature DN \( \alpha/\beta \) T cells (10–19) that preferentially use the \( \text{V}B8 \) gene family (10–12, 14, 16), but the function of these cells and their relationship to single-positive \( \alpha/\beta \) T cells have not been resolved (16, 19, 22, 23, 63, 64). Studies of the CD8\( \alpha \) gene in DN \( \alpha/\beta \) cells have shown that this gene is demethylated at some sites, providing evidence that it was expressed at one point by these cells (16, 65). However, the demethylation pattern appears to be less complete in DN \( \alpha/\beta \) thymocytes than in CD4 single-positive cells, suggesting that DN \( \alpha/\beta \) T cells have never fully expressed the CD8\( \alpha \) gene (65). A recent report demonstrates that CD4+CD8+ thymocytes, which have been proposed as the immediate precursors of CD4+CD8+ thymocytes, express low levels of functional \( \alpha/\beta \) TCRs and that ligation of their TCRs blocks their expression of CD4 and CD8, but does not lead to cell death (66). Taken together, these observations suggest that CD4+CD8+ thymocytes expressing TCRs with a high affinity for some intrathymic antigens could be the precursors of mature DN \( \alpha/\beta \) T cells (67).

The antigens recognized by murine DN \( \alpha/\beta \) T cells have not been identified, but candidate ligands for these cells in humans are the MHC-like CD1a, b, and c proteins, which are expressed by immature thymocytes (68–70). DN \( \alpha/\beta \) T cell clones that specifically lyse target cells expressing CD1a (4, 32) or CD1c (4), with no apparent requirement for an exogenous antigen, have been isolated from human peripheral blood. A possible function for such CD1-specific DN \( \alpha/\beta \) T cells could be the elimination of malignant or autoimmune immature T cells that escape from the thymus into the periphery. DN \( \alpha/\beta \) T cells that display CD1b-restricted recognition of a processed mycobacterial antigen have also been described (4), suggesting a role for these cells in immunity to microbial pathogens. Our finding that TCRs expressed by DN \( \alpha/\beta \) T cells have restricted \( \text{V}B \) gene usage and distinctive structural features in their V-J junctions, in conjunction with these observations on CD1 recognition, provide further evidence for the hypothesis that a significant proportion of T cells lacking CD4 and CD8 recognize antigen-presenting molecules other than the classical MHC class I and II proteins.

DN \( \alpha/\beta \) T cells are increased in some patients with SLE (2) and systemic sclerosis (6), and restricted \( \text{V}B \) gene usage has been reported in the latter disease (6). However, the relationship between these DN \( \alpha/\beta \) T cells in autoimmune diseases and in normal peripheral blood is unclear, nor is it known if DN \( \alpha/\beta \) T cells play a direct causative role in these diseases. Mice with the lpr defect, caused by a mutant \( \text{Fas} \) gene (71), similarly have a SLE-like disease and greatly increased
numbers of DN \( \alpha/\beta \) T cells (8). This suggests that expansion of DN \( \alpha/\beta \) T cells in some cases may be secondary to an underlying immunological defect causing autoimmunity, rather than a primary cause of the disease. Nevertheless, further studies on the relationship between expansion of DN \( \alpha/\beta \) T cells and autoimmune disease are warranted and could lead to important insights into the function of these cells. The data in this report on the structure of the TCRs expressed by DN \( \alpha/\beta \) T cells in normal peripheral blood provide a useful baseline against which the structure of these TCRs in various diseases can be compared.

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