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

By Steven Porcelli,* Courtland E. Yockey,‡ Michael B. Brenner,* and Steven P. Balk‡

*From the *Lymphocyte Biology Section, Department of Rheumatology/Immunology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115; and the ‡Hematology-Oncology Division, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215*

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 Vα7.2-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-

[1] *Abbreviations used in this paper:* DEC, dendritic epidermal T cell; DN, double negative; HS, human serum.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/07/0001/16 $2.00
Volume 178 July 1993 1–16

pothesis, the nonpolymorphic MHC-like CD1 molecules have been identified as the ligands for several human DN α/β T cell clones (4, 32).

One approach to address the question of whether specific antigens or antigen-presenting molecules are recognized by human DN α/β T cells is to identify the TCRs used by these cells. If the function of DN α/β 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 DN α/β T cells was assessed using a combination of random cloning, sequencing, and quantitative PCR amplification methods. This analysis identified at least two TCR α chains with distinctive structural features that were expressed preferentially by purified DN α/β T cells from multiple donors. Vβ usage by DN α/β T cells was also biased towards a small number of Vβ gene families in each donor. These results demonstrate distinctive features of TCRs expressed by DN compared with single-positive α/β T cells and provide further evidence that DN α/β T cells may carry out a specialized immunological function.

Materials and Methods

Isolation of DN α/β T Cells. Leukocyte concentrates of human peripheral blood were obtained as a byproduct of plateletpheresis 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 4–14 × 10^8 PBMC were suspended at 5 × 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-CD8α) (33), and incubated on ice for 2 h. Cells were then pelleted 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 × 10^8 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 × 10^7 cells/ml in PBS + 2% HS with 1:500 dilutions of ascites fluids containing mAbs OKT4, OKT8, and (in four of five preparations) anti-TCR δ1 (specific for the C region of the TCR δ chain) (34). After incubation on ice for 1 h, the cells were washed twice and resuspended at 2 × 10^7 cells/ml in PBS + 2% HS containing 6 × 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 δ^- in four of five cases) PBMC were collected, analyzed for purity 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-CD8α; reference 37), each diluted to 10 μg/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 α/β, and TCR γ/δ were assessed using biotin-conjugated mAbs OKT3 (anti-CD3ϵ; reference 33), BMA-031 (anti-TCR α/β; reference 38) and anti-TCR δ1, each diluted to 2 μg/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 × 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 α 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 α/β T cell preparations were homopolymer tailed with terminal deoxynucleotidyltransferase (TdT) and deoxycytosine triphosphate (dCTP), as described (40). Second-strand synthesis was carried out using the Klenow fragment of DNA polymerase I and an oligo-(dG) primer (GATAGTCGACGGGGGGGGGG, SalI-dG_{10}) (40). TCR α chains were then amplified by PCR using an antisense Cα primer located at the 3′ end of the C region (GAGGGAGCACAGGCTGTCTT) and the nonspecific SalI-dG10 primer (40). The PCR products were than reamplified using internal antisense Cα primers in conjunction with the SalI-dG_{10} primer. The internal antisense Cα primers were either GGCAGACAGACTTGTCACTGGAT or GAAAGTTTAGGTTCGTATCTGTTTCA, located just 3′ to PvuII (Cα-pre-Pvu II) or HindIII sites (Cα-pre-HindIII) in Cα, respectively. The PCR products were then digested with SalI and PvuII or HindIII, and cloned into pBluescript (Stratagene, La Jolla, CA). Positive colonies were identified by hybridization with an internal Cα probe (CGGCAGGGTCAGGGTTCT) and multiple isolates were then picked and sequenced.

PCR Amplification and Cloning of Specific Vα Transcripts. Antisense primers specific for Vα7.2 (TCCTTCGTCGGTCTAAAGGGTACA), Vα23 (AGTGGAAGACTTAATGCCTCGCT), and Vα24 (GATATACAGCAACTCTGGATGCA) (43) were used in conjunction with the 3′ Cα primer to amplify by PCR the respective Vαs. The PCR reactions contained cDNA, 20 pmol of each primer, 0.1 mg/ml BSA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U Taq polymerase (Promega Biotec, Madison, WI), and buffer supplied by the manufacturer in a final 50-μl 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 μl of the PCR product was reamplified using an internal Cα primer located 3′ to the HindIII site in Cα (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 Cα 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 Cα primer was CCAGAACCCTGACCCTGCAGT 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 Cα amplifications) were then amplified by PCR with the Vα7.2, 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 Phosphorimager (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 Vα7.2, 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 PBL samples 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 (CGGCAGGGTCAGGGTTCT, 100 ng), buffer, nucleotides, and enzyme as above, and 0.1 μl of [^{32}P]dCTP (3,000 Ci/mmol, 10 mCi/ml). Alternatively, in some experiments the internal Cα primer was end labeled with ^{32}P, 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 to 80°C in 50% formamide and run on a 6% polyacrylamide DNA sequencing gel. Analysis of Vβ transcripts was carried out similarly using external (GCCTTTTCCCTGTGGGAGAT) and internal (GGCTCAAACACAGCGACCT) 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 (GCCTTTTCCCTGTGGGAGAT), 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 ^{32}P-labeled internal antisense Cβ probe (GGCTCAAACACAGCGACCT). Quantitation was performed on a Phosphorimager (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 Vβ primers were Vβ1, GAGAGCAAAAGGAAACATTCTTGAAC; Vβ2, GGCCACATACGAGCAAGGCGTC; Vβ3, GGAGATATTCCTGA(GA)GGGTACA; Vβ4, CCACATATGAGAGTGGATTTGTCA; Vβ5, GATTCTCAGCTCGCCAGTTC(TC)CTAACT; Vβ6, CAGTGATCGCTTCT(TC)TGCAGA, GTGCGGCAGATGACTCAGGGCT; Vβ7, GCTTCTCACCTGAATGCCCCAACA; Vβ8, GATAGATGATTCAGGGATGCCCGA; Vβ9, TTATAAATGAAACAGTTCCAAATCGCT; Vβ10, TTCAGAAAGCAGAAATAATCAATGAG; Vβ11, GAGAAGGGAGATCTTTCCTCTGA; Vβ12, TACTGACAAAGGAGAAGTCTCAGA; Vβ13, CCCT(AG)ATGGCTACAATGTCTCCAGAT; Vβ14, CTGATAAGGGAGATGTTCCTGAAG; Vβ15, GATGTGAAAGATATAAACAAAGGAGAGA; Vβ16, GATAATCTTTATCGACGTGTTATGGGA; Vβ17, GAAAGGAGATATAGCTGAAGGGTAC; Vβ18, GTCAGGAATGCCAAAGGAACGATTT; Vβ19, GAAACGGAGATGCACAAGAAGCGA; Vβ20, GCCTCCAGCTGCTCTTCTACTCCA.

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$^+$, CD8α$^+$, 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 unfractionated 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 unfractionated 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 T cells (CD3$^+$), whereas in the single preparation lacking depletion of TCR γ/δ$^+$ cells (donor 5), these represented 21.8% of the CD3$^+$ cells.

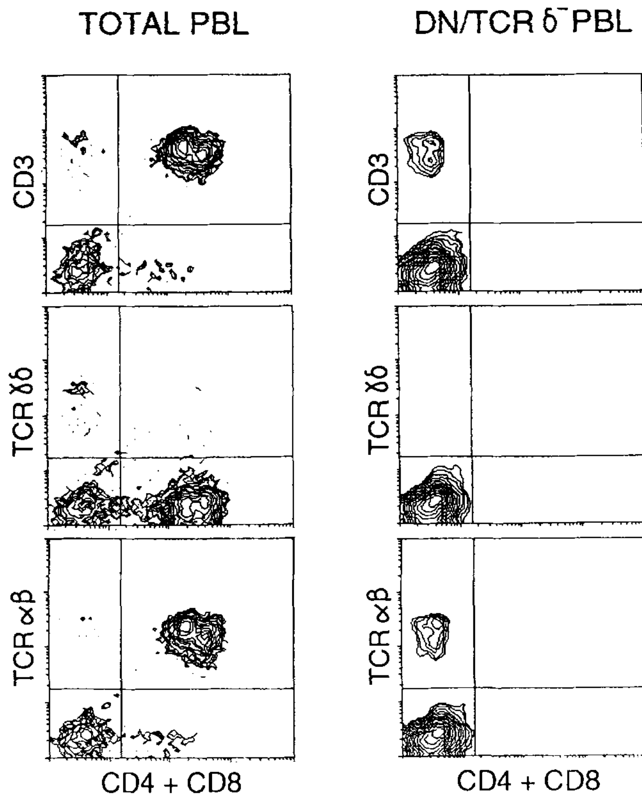

Figure 1. FACS® analysis of PBLs and purified DN α/β T cells from donor 1. Unfractionated PBLs and purified DN α/β T cells from donor 1 were analyzed for expression of CD3, TCR α/β, TCR γ/δ, CD4, and CD8. On the horizontal axis in all samples is a mixture of FITC-conjugated CD4 and CD8 antibodies (CD4 + CD8). The horizontal and vertical cursors were placed to yield <1% positive cells with control antibodies. The horizontal and vertical scales are $\log_{10}$ 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 JαQ. Moreover, no V-J junctional diversity was evident in any of these 13 Vα24-JαQ recombinations.

The isolation of the identical Vα24-JαQ 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 JαQ 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 JαQ gene can be deduced from a germline JαQ transcript we have isolated and sequenced (Fig. 2). Similar germline transcripts encoding other Jαs have been described previously and presumably initiate from a promoter upstream of the unrearranged Jα 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

Table 2. *Sequences of Vα-Jα Joints Containing Vαs Isolated More than Once from Two DN α/β T Cell cDNA Libraries*

Donor		V	N	J		Times isolated*	Rearrangement‡
1	Vα24	ilecysvalvalser -ATCTGTGTGGTGAGC		aspargglyserthrleuglyargleutyrphegly GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	12	+
1		-ATCTGTGTGGTGAGC	pheleumetilelysaspala TTTCTTATGATCAAAGATGCA	glyasnlysleuthrphegly GGCAACAAGCTAACTTTTGGA-	Jα9A3	1	+
1		-ATCTGTGTGGTGAGC	thrmetasn ACCATGAATT	tyrglyglyserglnglyasnleuilephegly ATGGAGGAAGCCAAGGAAATCTCATCTTTGGA-	IGRJa06	1	+
1	Vα7.2	leucysala -CTCTGTGCT	glytrp GGCTGG	aspserasntyrglnleuiletrpgly GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	1	+
1		-CTCTGTGCT	valleu GTACTG	GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	1	+
1		-CTCTGTGCT	valmet GTGATG	GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	1	+
1		-CTCTGTGCT	valarg GTGAGA	GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	1	+
1		-CTC	CCCTCCGGA	GGGCAAACAACGTCTTCTTTGGG-	IGRJa05	1	−
1	Vα23	leucysala -CTCTGTGCT	val GTTT	trpmetaspserseryrlysleuilephegly GGATGGATAGCAGCTATAAATTGATCTTCGGG-	JαB	1	+
1		-CTCTGTGCT	valargphe GTGAGGTTC	asnthrasplysleuilephegly AACACCGACAAGCTCATCTTTGGG-	JαI	1	+
1		-CTCTGTGCT	GTCCCGGAATA	CCGGCACTGCCAGTAAACTCACCTTTGGG-	Jα8A1	1	−
2	Vα24	ilecysvalvalser -ATCTGTGTGGTGAGC		aspargglyserthrleuglyargleutyrphegly GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	1	+
2	Vα7.2	leucysala -CTCTGTGCT	glyleu GGCTTG	aspserasntyrglnleuiletrpgly GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	1	+
2		-CTCTGTGCT	valarg GTGAGA	GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	2	+
2		-CTCTGTGCT	valthr GTGACT	GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	1	+
2		-CTCTGTGCT	sermet TCTATG	GATAGCAACTATCAGTTAATCTGGGGC-	IGRJa14	1	+
2	Vα23	leucysala -CTCTGTGCT	leuaspasn CTAGACAAT	alaasnserglytyralaleuasnphegly GCAAATTCCGGGTATGCACTCAACTTCGGC-	IGRJa12	1	+
2		-CTCTGTGCT	vallysasnthrarg GTGAAAAATACCC	thralaserlysleuthrphegly GCACTGCCAGTAAACTCACCTTTGGG-	Jα8A1	1	+
2		-CTCTGTGCT	valgluproalagly GTGGAACCTGCAGGG	glnleuthrphegly CAACTGACCTTTGGA-	JαC	1	+

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 Vα, 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 IGRJa1-14 sequences and the Vα7.2, 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 (−).

Table 3. *Nucleotide Sequences of TCRs Using $V\alpha24$ from DN α/β T Cells and PBLs*

Donor	V	N	J		Times isolated*	Rearrangement‡
1 DN Vα24	-TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	8/8	+
1 PBL Vα24	-TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	6/11	+
	TACATCTGTGTG	TCCCTGGGG	GGAGGAAGCTACATACCTACATTTGGA-	JαT	1/11	+
	TACATCTGTGTGGTGAGC	GCTCTAAA	CGGGTATGCACTCAACTTCGGC-	IGRJa12	1/11	+
	TACATCTGTGTGGTG	GCGAAGGTGAACACAGGC	TTTCAGAAACTTGTATTTGGA-	Jα9P251	1/11	+
	TACATCTGCGTGGTGAG	GCCGACTTTTAAC	GACTACAAGCTCAGCTTTGGA-	JαN	1/11	+
	TACATCTGTGTGGT	TAATCTAACTTTGGAAAT	GAGAAATTAACCTTTGGG-	Jα9P30	1/11	–
2 DN Vα24	-TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	7/14	+
	TACATCTGTGTGGTGAG	GGCCCCCCGTG	ACAATGACATGCGCTTTGGA-	IGRJa10	1/14	+
	TACATCTGTGTGGTGAGC	GGATTC	AGAGATGACAAGATCATCTTTGGA-	JαP	1/14	+
	TACATCTGTGTGGTGAG	GCCGAATGCT	GGTGGTACTAGCTATGGAAAGNTGAGATTTGGA-	Jα8De	1/14	+
	TACATCTGTGTG		ATATCCAGAACCC-	Cα	4/14	–
2 PBL Vα24	-TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	3/8	+
	TACATCTGTGTGGTGAG	AAGGGG	TGGAGGAAGCCAAGGAAATCTCATCTTTGGA-	IGRJa06	1/8	+
	TACATCTGTGTGGTGAG	GGCA	AGCAGTGCTTCCAAGATAATCTTTGGA-	JαA	1/8	+
	TACATCTGTGTGGTGAGC	GCCAGGAGGTGCTGACGGACT	ACCTTTGGC-	JαW	1/8	–
	TACATCTGTGTGGTGAGC	CCCTCAGGTGGCTACAA	TAAGCTGATTTTTGGA-	Jα8P171	1/8	–
	TACATCTGTGTGGTGAGC	GGGGACCAAT	AACACCGACAAGCTCATCTTTGGA-	JαI	1/8	–
3 DN Vα24	-TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	10/13	+
	TACATCTGTGTGGTGAG	GGGGTTTT	CTGGTGGCTACAATGAGCTGATTTTTGGA-	IGRJa08	1/13	+
	TACATCTGTGT	CTTCC	ATAACACCGACAAGCTCATCTTTGGG-	JαI	1/13	+
	TACATCTGTGTGGTGA	CCCCTTTTT	CTGGTGGCTACAATAAGCTGATTTTTGGA-	IGRJa08	1/13	+
3 PBL Vα24	-TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA-	JαQ	0/7	
	TACATCTGTGTGGTGAGC	GGCCTA	GGAGGAAGCTACATACCTACATTTGGA-	JαT	1/7	+
	TACATCTGTGTGGT	CCCTTCCTCTGTAA	GGGCTACCAGAAAGTTACCTTTGGA-	JαO	1/7	+
	TACATCTGTGTGGTGAGC	TC	GATAGGCTTTGGGAATGTGCTGCATTGCGGG-	IGRJa02	1/7	+
	TACATCTGTGTG	TCCCGG	GCAGGAACTGCTCTGATCTTTGGG-	JαS	1/7	+

Source	V	3′ end of V	N region	5′ end of J	J	No.*	Frame‡
		TACATCTGTGTGGTGAGC	T	CTCACGGGAGGAGGAAACAAACTCACCTTTGGG–	JA210	1/7	–
		TACATCTGTGTGGTGAGC	TAGGGATG	AACAGAGATGACAAGATCATCTTTGGA–	Jα P	1/7	–
		TACATCTGTGTGGTGAG	AGGCC	GTACAGCAGTGCTTCCAAGATAATCTTTGGA–	Jα A	1/7	–
4 DN	Vα24 –	TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA–	Jα Q	10/15	+
		TACATCTGTGTGGTGAGC	TACGG	AATTCAGGAAACACACCTCTTGTCTTTGGA–	Jα F	1/15	–
		TACATCTGTGTGGTGAGC	CCCAATCAAC	GACTACAAGCTCAGCTTTGGA–	Jα N	1/15	–
		TACATCTGTGTGGTGA	ACCTTTAACACCGAC	AAGCTCATGTTTGGG–	Jα6D10	1/15	–
		TACATCTGTGTGGTGAG	TTCGACG	CAATAACAATGACATGCGCTTTGGA–	IGRJa10	1/15	–
		TACATCTGTGTGGTGAGC	CAGACGCA	TCGGGAGGAAGCTACATACCTACATTTGGA–	Jα T	1/15	–
4 PBL	Vα24 –	TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA–	Jα Q	1/8	+
		TACATCTGTGTGGT	ACC	GGGGAGGCTATACTTTGGA–	Jα Q	1/8	+
		TACATCTGTGTGGTGAGC	GA	CACGGGAGGAGGAAACAAACTCACCTTTGGG–	JA210	1/8	+
		TACATCTGTG	ATACTCAC	GGAGGAGGAAACAAACTCACCTTTGGG–	JA210	1/8	+
		TACATCTGTGTGGTGAGC	GAA	GGAAGCTACATACCTACATTTGGA–	Jα T	1/8	+
		TACATCTGTGTGGTGA	CCGTCA	AACTGGGGCAAACAACGCTCTTCTTTGGA–	IGRJa05	1/8	+
		TACATCTGTGTGGTGA	CCTTAATCGA	TACAGCAGTGCTTCCAAGATAATCTTTGGA–	Jα A	1/8	–
		TACATCTGTGTG		ATATCCAGAACCC– (underlined)	Cα	1/8	–
5 DN	Vα24 –	TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA–	Jα Q	5/5	+
5 PBL	Vα24 –	TACATCTGTGTGGTGAGC		GACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGA–	Jα Q	3/9	+
		TACATCTGTGTGGTGAGC	GCGTGGG	ACAAGCTGGTCTTTGGCGC–	IGRJa09	1/9	+
		TACATCTGTGTGGTGAGC	CGTTATT	ATAACACCGACAAGCTCATCTTTGGG–	Jα I	1/9	+
		TACATCTGTGTGGTGAGC	GACG	ACTATCAGTTAATCTGGGGC–	IGRJa14	1/9	+
		TACATCTGTGTGGTGAG	AGGCCGA	ACAGCAGTGCTTCCAAGATAATCTTTGGA–	Jα A	1/9	–
		TACATCTGTGTG	CCTTCGTTCTAAC	GACTACAAGCTCAGCTTTGGA–	Jα N	1/9	–
		T	TTGAATT	ATGGAGGAAGCCAAGGAAATCTCATCTTTGGA–	IGRJa06	1/9	–

TCRs using Vα24 were amplified from cDNA by PCR with Vα24 and Cα primers. Multiple isolates from each cDNA were picked and sequenced. The 3′ end of Vα24, the N region, and 5′ end of the J region are shown. JA210 has been reported (43, 72) and the complete sequences of Jα9P251, 9P30, 8De, 8P171, and 6D10 will be reported elsewhere (S. P. Balk, manuscript in preparation). In several isolates, Vα24 was spliced directly to Cα with no Jα; the 5′ end of Cα is shown in these cases (underlined).

* Number of times each transcript was isolated.

‡ Whether the rearrangement is in frame (+) or out of frame (–).

```
TTTTCAACCCTGTACTCCAAAGCTGAGGGGAGAGGGGATGGAAACATTAAGGGCTGGGTTCATG

                         aspargglyserthrleuglyargleutyrpheglyargglyth
TAAAGGGACCAGCATTGTGCCGACAGAGGCTCAACCCTGGGGAGGCTATACTTTGGAAGAGGAAC

rglnleuthrvaltrpproaspile
TCAGTTGACTGTCTGGCCTGATATCC-Cα
```

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

the Vα24-JαQ rearrangement in Table 2 and the germline JαQ gene in Fig. 2 shows that this Vα24-JαQ joint contains the entire JαQ gene segment except for the two cytosines immediately after the heptamer recombination signal. The generation of this invariant Vα24-JαQ joint required, therefore, the deletion of two bases but no N region additions.

A second Vα isolated multiple times from both donors was Vα7.2 (43) (Table 2). In the majority of these TCRs, Vα7.2 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 Vα7.2 gene shown in Table 2 may encode several additional 3′ bases (G or GT being most likely; see below). In either case, the TCR α chains encoded by these transcripts had the 3′ alanine in Vα7.2 and two variable amino acids in the V-J junction joined precisely to the 5′ aspartate in IGRJa14. Interestingly, the only Vα7.2 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 Vα7.2 rearrangements.

The third Vα identified more than once was Vα23 (43), which occurred in three isolates from each donor (Table 2). A previously undescribed Jα, Jα8A1, was used by one of these Vα23 TCRs from each donor, but each of these Vα23-Jα8A1 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 Vα23.

Analysis of Vα7.2, 23, and 24 Expression by Quantitative PCR Amplification. To confirm the results from random cloning, the expression of Vα7.2, 23, and 24 by donors 1 and 2 and by three additional donors was assessed by quantitative PCR amplification. The frequency of these Vαs in unfractionated PBLs from each donor was first assessed. Primers specific for Vα7.2, 23, or 24, in conjunction with a Cα primer, were used to amplify PBL cDNA from each of the donors. Two specific Cα primers were used to estimate the total amount of TCR α cDNA, and the efficiencies of the Vα primers versus the Cα primers were determined (see Materials and Methods). This analysis indicated that the Vα7.2 transcript varied from 3.0 to 10% of the total TCR α cDNA in PBLs from these five donors, while the Vα23 and Vα24 transcripts were between 2.2 and 6.5% (data not shown). These frequencies were consistent with the results from random cloning of TCR α chains using these Vαs from peripheral blood (43).

The expression of these Vαs 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 Vα7.2, 23, or 24. The amplifications were normalized in each experiment by amplifying parallel samples with two Cα 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 Vα7.2 and Vα24 by DN α/β T cells was increased relative to PBLs in the majority of donors, although most of the increases were less than fourfold. In contrast, Vα23 was not increased in any of the DN α/β T cell preparations, indicating a discrepancy between the random cloning method (Table 2) and this quantitative PCR method in assessing the expression of Vα23 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 Vα7.2 and Vα24 transcripts in the DN α/β 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 Vα7.2 were frequent transcripts in donors 1 and 2 and that TCRs using Vα24 were particularly abundant in donor 1.

Sequence Analysis of TCR α Transcripts from PBLs and DN α/β T Cells Using Vα7.2 and Vα24. Although the expression of the Vα7.2 and Vα24 transcripts was not markedly increased in most of the DN α/β T cell preparations relative to the PBLs, the sequencing data in Table 2 also indicated that DN α/β T cells using Vα7.2 and Vα24 may select particular Jα genes and junctional sequences. To further assess the expression of Vα7.2 and Vα24 and determine if they undergo precise rearrangements with specific Jα genes in DN α/β T cells, multiple additional TCRs using these Vαs were

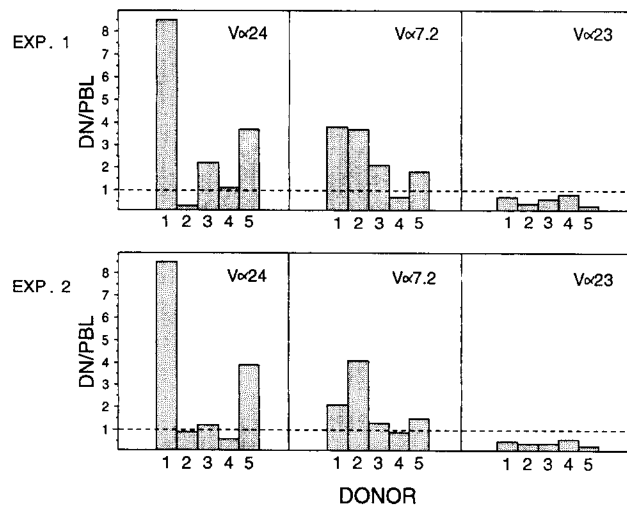

Figure 3. Comparison of Vα7.2, 23, and 24 expression by DN α/β T cells versus PBLs. The expression of each Vα relative to Cα in the DN and PBL samples was estimated by quantitative PCR amplification. The ratio of Vα expression in the DN versus the matched PBL samples was then determined. The results from two experiments are shown.

Table 4. *Sequences of TCRs Using Vα7.2 from DN α/β T Cells and PBLs*

Precise Vα7.2-IGRJa14 joints			Other Vα7.2 joints			
N region		Donor	N region	J region		Donor
GTGAAG	(val lys)	DN1	GTGAGAGATGAGTA	CAATAACAATGACATGCGCTTTGGA-	IGRJa10	DN1
GTGAGA	(val arg)	DN1	GTGGCCTTAGGG	GGGGTTACCAGAAAGTTACCTTTGGA-	JαO	PBL1
GTGGGG	(val gly)	DN1	GTCCTC	AATTCCGGGTATGCACTCAACTTCGGC-	IGRJa12	PBL2
GTACTG	(val leu)	DN1	GTGAGAGTT	ATACTCACGGGAGGAGGAAACAAACTCACCTTTGGG-	JA210	PBL3
GTGGTG	(val val)	DN2	GCT	AATTCAGGAAACACACCTCTTGTCTTTGGA-	JαF	PBL3
GGCTGG	(gly trp)	DN2	GTGACCTTGGACACG	GGCAGGAGAGCACTTACTTTT-	Jα6Pa2	PBL4
GGCATG	(gly met)	DN3	GTGAGAGATCGGG	ACTATCAGTTAATCTGGGGC-	IGRJa14	PBL4
GTGAAG	(val lys)	DN4				
GTGAGA	(val arg)	DN4				
GTCATG	(val met)	DN4				
GTGATG	(val met)	DN4				
GTGATG	(val met)	PBL1				
ATCATG	(ile met)	PBL1				
GTGAGA	(val arg)	PBL2				
GCGGTG	(ala val)	PBL2				
CTACTC	(leu leu)	PBL3				
GTAAGA	(val arg)	PBL4				
ACAATG	(thr met)	PBL4				
GTGAGA	(val arg)	PBL4				

TCRs were amplified from cDNA by PCR using Vα7.2 and Cα 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 Cα primer and Vα7.2- or Vα24-specific sense primers. The amplified TCRs were then cloned into pBluescript 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 (valarg) 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.

Enrichment of the Vα24-JαQ Rearrangement in DN α/β Demonstrated by Direct Analysis of PCR Products on Denaturing Polyacrylamide Gels. To further support the results in Table 3, that the predominant Vα24 rearrangement in the DN cells was to JαQ with no diversity at the V-J joint, PCR products generated with the Vα24 primer were analyzed on high-resolution denaturing polyacrylamide gels (DNA sequencing gels). The PBL or DN cDNA were amplified by PCR initially with the Vα24 primer and a Cα primer. Aliquots of

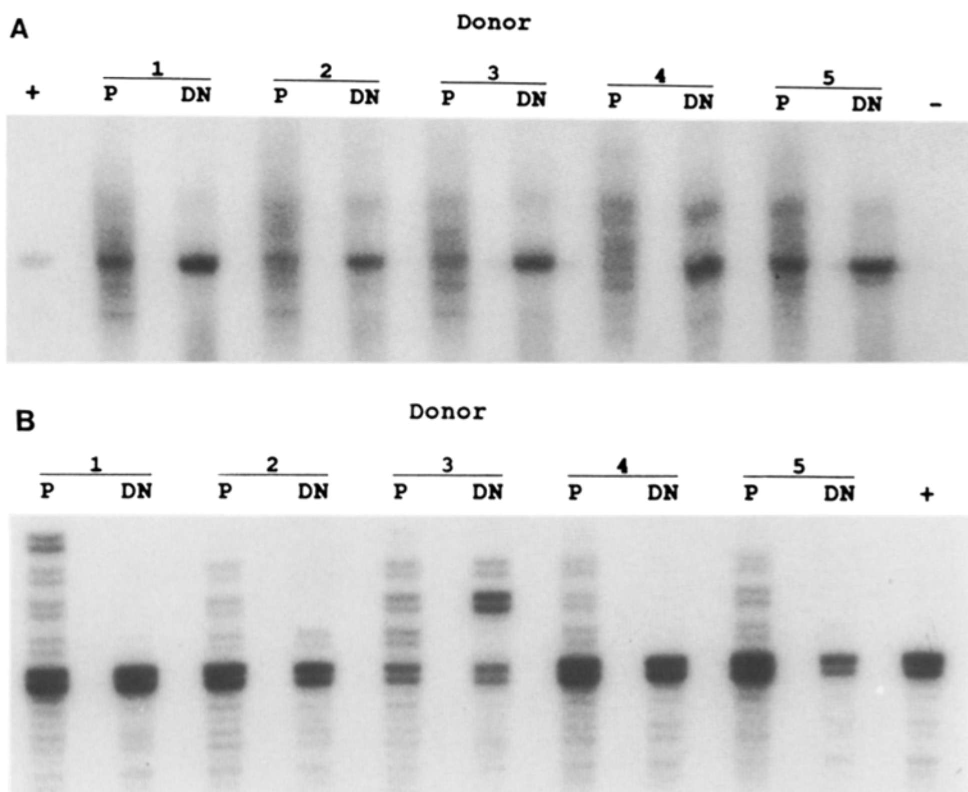

Figure 4. Analysis of Vα24 and Vα7.2 transcripts by PCR amplification and gel electrophoresis. cDNA from DN or PBL samples were amplified with a Vα24 primer (*A*) or Vα7.2 primer (*B*) and an external Cα primer. They were reamplified using an internal Cα primer and labeled by incorporation of α-[^{32}P]dCTP. 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 Vα24 primer and an internal Cα primer, in the presence of α-[^{32}P] 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 Vα24-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 Vα24-JαQ rearrangement was enriched in the DN α/β T cell population.

A similar analysis was carried out for TCRs using the Vα7.2 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 Vα7.2-IGRJa14 rearrangement. The bands appear as doublets, rather than single bands, due to incomplete addition of a nontemplate-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 Vα7.2 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 Vα7.2-IGRJa14 rearrangement, particularly in the DN preparations from donors 1, 2, 4, and 5. These results demonstrated that the Vα7.2 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

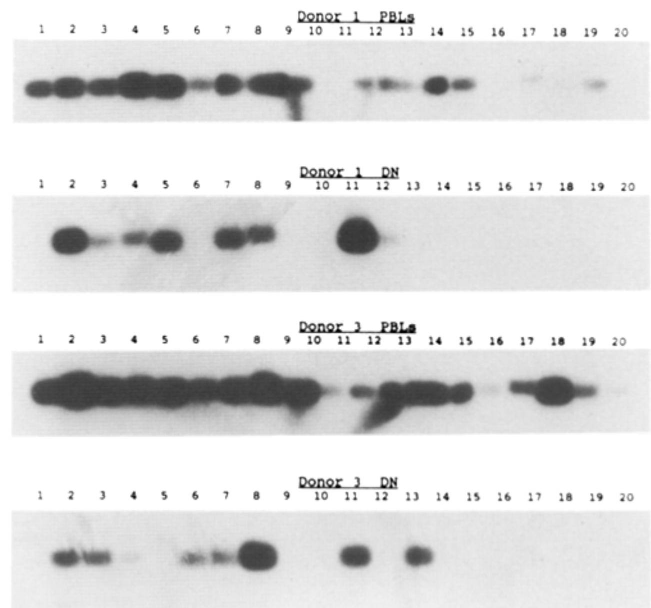

Figure 5. Quantitative PCR amplification analysis of Vβ expression by DN α/β T cells and PBLs. PBL and DN α/β T cell cDNA from donors 1 and 3 were amplified by PCR for 30 cycles with a series of primers specific for Vβ1–20 and the products were hybridized with an internal oligonucleotide probe, as described in Materials and Methods. The sources of the cDNA and the Vβ primers are indicated.

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	1	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	0	1	72	1	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	1	73	4	1	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, ^{32}P-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

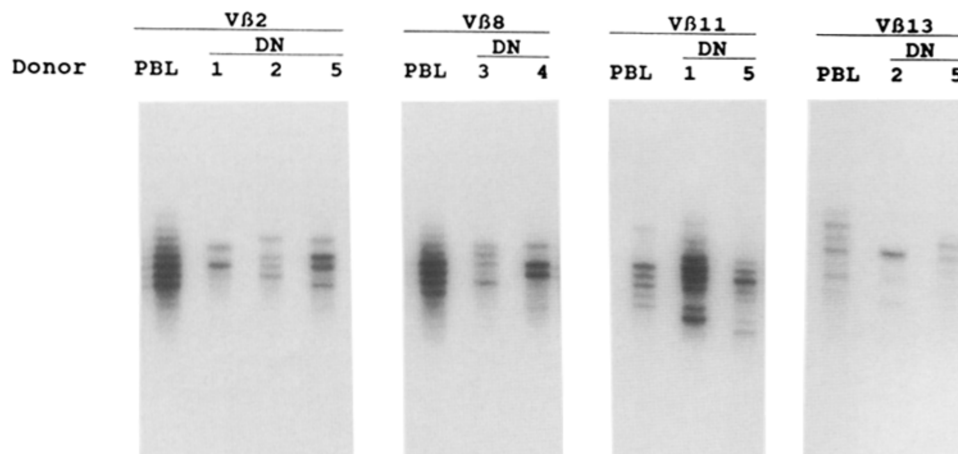

Figure 6. Analysis of Vβ transcripts by PCR amplification and gel electrophoresis. cDNA was amplified with Vβ primers and a Cβ primer and then reamplified using an internal, ^{32}P-labeled Cβ primer. The labeled PCR products were analyzed on a 6% denaturing polyacrylamide gel. The Vβ primers used and the sources of the cDNA are indicated.

the thymus before TdT is active, as is the case for the invariant TCR γ and δ chains of murine DECs (55, 56). If the high frequency of the invariant Vα24-JαQ joint is indeed due to these or other mechanisms operating to favor invariant Vα-Jα recombinations in DN α/β T cells, then TCRs with other invariant germline Vα-Jα joints may be present and predominate in the DN α/β T cells from other donors. Random TCR α cloning from additional DN α/β T cell preparations and further information on genomic Vα and Jα sequences will be necessary to address this question.

An alternative possibility is that invariant Vα24-JαQ 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 Vα24-JαQ or Vγ9-JγP rearrangements have not been identified. The expression of the Vα24-JαQ 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 Vγ9-JγP recombination (54). If the ligand recognized by the Vα24-JαQ 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 Vα24-JαQ 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 α chains identified in these studies were recombinations between Vα7.2 and IGRJa14. These Vα7.2-IGRJa14 joints were enriched in some, but not all, DN α/β T cell preparations. In the majority of DN α/β T cells and unfractionated PBLs using Vα7.2, the joint was to the same nucleotide in IGRJa14 with two variable amino acids in the V-J junction. The conserved structure of this Vα7.2-IGRJa14 joint in multiple clones from five donors suggests that this TCR α 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 Vα7.2-IGRJa14 TCRs are expressed by single-positive as well as DN T cells. Alternatively, the Vα7.2 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 Vα14 and Jα281 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 β chains provided further evidence that DN α/β T cells use particular TCRs preferentially. The majority of DN α/β T cells from each donor used only one or two Vβ gene families, with the predominant Vβs being Vβ2, 8, 11, and 13 (Table 5). Further analysis of these TCR β 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 Vβ-specific polyclonal stimulation by a superantigen (62), although the possibility that chronic stimulation by superantigens might result in this pattern of restricted TCR β expression cannot be ruled out. The preferential expression of these Vβs by DN α/β 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 β chains using these predominant Vβs are paired with invariant TCR α chains or other TCR α chains with particular structural features, then the ability to pair with these TCR α chains could be the major factor responsible for selecting these Vβs.

Normal mice also have a small population of mature DN α/β T cells (10–19) that preferentially use the Vβ8 gene family (10–12, 14, 16), but the function of these cells and their relationship to single-positive α/β T cells have not been resolved (16, 19, 22, 23, 63, 64). Studies of the CD8α gene in DN α/β 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 α/β thymocytes than in CD4 single-positive cells, suggesting that DN α/β T cells never fully expressed the CD8α gene (65). A recent report demonstrates that $CD4^-CD8^{lo}$ thymocytes, which have been proposed as the immediate precursors of $CD4^+CD8^+$ thymocytes, express low levels of functional α/β 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 $CD4^-CD8^{lo}$ thymocytes expressing TCRs with a high affinity for some intrathymic antigens could be the precursors of mature DN α/β T cells (67).

The antigens recognized by murine DN α/β 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 α/β 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 α/β T cells could be the elimination of malignant or autoimmune immature T cells that escape from the thymus into the periphery. DN α/β 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 α/β T cells have restricted V 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 α/β T cells are increased in some patients with SLE (2) and systemic sclerosis (6), and restricted Vβ gene usage has been reported in the latter disease (6). However, the relationship between these DN α/β T cells in autoimmune diseases and in normal peripheral blood is unclear, nor is it known if DN α/β T cells play a direct causative role in these diseases. Mice with the *lpr* defect, caused by a mutant *Fas* gene (71), similarly have a SLE-like disease and greatly increased

numbers of DN α/β T cells (8). This suggests that expansion of DN α/β 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 α/β 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 α/β T cells in normal peripheral blood provide a useful baseline against which the structure of these TCRs in various diseases can be compared.

We thank Dr. R. G. Kurrle (Boehringwerke, Marburg, Germany) for providing the BMA-031 antibody.

This work was supported by grants from the Council for Tobacco Research (to S. P. Balk) and the National Institutes of Health (AI-28973 to M. B. Brenner). S. Porcelli is the recipient of an NIH Clinical Investigator Award, M. B. Brenner is a scholar of the Leukemia Society of America, and S. P. Balk is the recipient of a Junior Faculty Research Award from the American Cancer Society.

Address correspondence to Steven Balk, Division of Hematology-Oncology, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.

Received for publication 8 February 1993 and in revised form 17 March 1993.

References

1. Londei, M., A. Verhoef, P. De Berardinis, M. Kissonerghis, B. Grubeck-Loebenstein, and M. Feldman. 1989. Definition of a population of CD4$^-$8$^-$ T cells that express the $\alpha\beta$ T-cell receptor and respond to interleukins 2, 3, and 4. *Proc. Natl. Acad. Sci. USA.* 86:8502.
2. Shivakumar, S., G.C. Tsokos, and S.K. Datta. 1989. T cell receptor α/β expressing double-negative (CD4$^-$/CD8$^-$) T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. *J. Immunol.* 143:103.
3. Kusunoki, Y., Y. Hirai, and M. Akiyama. 1992. Evidence for *in vivo* clonal proliferation of a unique population of blood CD4$^-$/CD8$^-$ T cells bearing T-cell receptor α and β chains in two normal men. *Blood.* 79:2965.
4. Porcelli, S., C. Morita, and M.B. Brenner. 1992. CD1b restricts the response of human CD4$^-$8$^-$ T cells to a microbial antigen. *Nature (Lond.).* 360:593.
5. Groh, V., M. Fabbi, F. Hochstenbach, R.T. Masiarz, and J.L. Strominger. 1989. Double-negative (CD4$^-$CD8$^-$) lymphocytes bearing T-cell receptor α and β chains in normal human skin. *Proc. Natl. Acad. Sci. USA.* 86:5059.
6. Sakamoto, A., T. Sumida, T. Maeda, M. Itoh, T. Asai, H. Takahashi, S. Yoshida, T. Koike, H. Tomioka, and S. Yoshida. 1992. T cell receptor Vβ repertoire of double-negative α/β T cells in patients with systemic sclerosis. *Arthritis Rheum.* 35:944.
7. Wirt, D.P., E.G. Brooks, S. Vaidya, G.R. Klimpel, T.A. Waldman, and R.M. Goldblum. 1989. Novel T-lymphocyte population in combined immunodeficiency with features of graft-versus-host disease. *N. Engl. J. Med.* 321:370.
8. Davidson, W.F., F.J. Dumont, H.G. Bedigian, B.J. Fowlkes, and H.C. Morse. 1986. Phenotypic, functional, and molecular genetic comparisons of the abnormal lymphoid cells of C3H-*lpr/lpr* and C3H-*gld/gld* mice. *J. Immunol.* 136:4075.
9. Yui, K., S. Wadsworth, A. Yellen, Y. Hashimoto, Y. Kokai, and M.I. Greene. 1988. Molecular and functional properties of novel T cell subsets in C3H-*gld/gld* and nude mice. Implications for thymic and extrathymic maturation. *Immunol. Rev.* 104:121.
10. Fowlkes, B.J., A.M. Kruisbeek, H. Ton-That, M.A. Weston, J.E. Coligan, R.H. Schwartz, and D.M. Pardoll. 1987. A novel population of T-cell receptor $\alpha\beta$-bearing thymocytes which predominantly expresses a single Vβ gene family. *Nature (Lond.).* 329:251.
11. Budd, R.C., G.C. Miescher, R.C. Howe, R.K. Lees, C. Bron, and H.R. MacDonald. 1987. Developmentally regulated expression of T cell receptor β chain variable domains in immature thymocytes. *J. Exp. Med.* 166:577.
12. Ceredig, R., F. Lynch, and P. Newman. 1987. Phenotypic properties, interleukin 2 production, and developmental origin of a "mature" subpopulation of Lyt-2$^-$ L3T4$^-$ mouse thymocytes. *Proc. Natl. Acad. Sci. USA.* 84:8578.
13. Crispe, I.N., M.W. Moore, L.A. Husmann, L. Smith, M.J. Bevan, and R.P. Shimonkevitz. 1987. Differentiation potential of subsets of CD4$^-$8$^-$ thymocytes. *Nature (Lond.).* 329:336.
14. Wilson, A., T. Ewing, T. Owens, R. Scollay, and K. Shortman. 1988. T cell antigen receptor expression by subsets of Ly-2$^-$ L3T4$^-$ (CD8$^-$CD4$^-$) thymocytes. *J. Immunol.* 140:1470.
15. Guidos, C.J., Weissman, I.L., and B. Adkins. 1989. Developmental potential of CD4$^-$8$^-$ thymocytes. Peripheral progeny include mature CD4$^-$8$^-$ T cells bearing $\alpha\beta$ T cell receptor. *J. Immunol.* 142:3773.
16. Takahama, Y., A. Kosugi, and A. Singer. 1991. Phenotype, ontogeny, and repertoire of CD4$^-$CD8$^-$ T cell receptor $\alpha\beta^+$ thymocytes. *J. Immunol.* 146:1134.
17. De Talance, A., D. Regnier, S. Spinella, J. Morisset, and M. Seman. 1986. Origin of autoreactive T helper cells. I. Characterization of Thy-1$^+$, Lyt2$^-$, L3T4$^-$ precursors in the spleen of normal mice. *J. Immunol.* 137:1101.
18. Morisset, J., E. Trannoy, A. De Talance, S. Spinella, P. Debre, P. Godet, and M. Seman. 1988. Genetics and strain distribution of concanavalin A-reactive Ly-2$^-$, L3T4$^-$ peripheral precursors of autoreactive T cells. *Eur. J. Immunol.* 18:387.
19. Huang, L., and I.N. Crispe. 1992. Distinctive selection mechanisms govern the T cell receptor repertoire of peripheral CD4$^-$CD8$^-$ α/β T cells. *J. Exp. Med.* 176:699.

20. Sykes, M. 1990. Unusual T cell populations in adult murine bone marrow. Prevalence of $CD3^{+}CD4^{-}CD8^{-}$ and $\alpha\beta TCR^{+}NK1.1^{+}$ cells. *J. Immunol.* 145:3209.
21. Kubota, H., H. Okazaki, M. Onuma, S. Kano, M. Hattori, and N. Minato. 1992. CD3+4−8− $\alpha\beta$ T cell population with biased T cell receptor V gene usage: presence in bone marrow and possible involvement of IL-3 for their extrathymic development. *J. Immunol.* 149:1143.
22. Scott, B., H. Bluthmann, H.S. Teh, and H. von Boehmer. 1989. The generation of mature T-cells requires interaction of the α/β T-cell receptor with major histocompatibility antigens. *Nature (Lond.).* 338:591.
23. von Boehmer, H., J. Kirberg, and B. Rocha. 1991. An unusual lineage of α/β T cells that contains autoreactive cells. *J. Exp. Med.* 174:1001.
24. Seman, M., S. Boudaly, T. Roger, J. Morisset, and G. Pham. 1990. Autoreactive T cells in normal mice: unrestricted recognition of self peptides on dendritic cell I-A molecules by $CD4^{-}CD8^{-}$ T cell receptor α/β^{+} T cell clones expressing $V\beta8.1$ gene segments. *Eur. J. Immunol.* 20:1265.
25. Strober, S., S. Dejbachsh-Jones, P. van Vlasselaer, G. Duwe, S. Salimi, and J.P. Allison. 1989. Cloned natural suppressor cell lines express the $CD3^{+}CD4^{-}CD8^{-}$ surface phenotype and the α, β heterodimer of the T cell antigen receptor. *J. Immunol.* 143:1118.
26. Yankelvich, B., C. Knobloch, M. Nowicki, and G. Dennert. 1989. A novel cell type responsible for marrow graft rejection in mice. T cells with NK phenotype cause acute rejection of marrow grafts. *J. Immunol.* 142:3423.
27. Yui, K., Y. Hashimoto, S. Wadsworth, and M.I. Greene. 1987. Characterization of $Lyt\text{-}2^{-}$, $L3T4^{-}$ class I-specific cytolytic clones in C3H-*gld/gld* mice. Implications for functions of accessory molecules and programmed development. *J. Exp. Med.* 166:1026.
28. Mieno, M., R. Suto, Y. Obata, H. Udono, T. Takahashi, H. Shiku, and E. Nakayama. 1991. $CD4^{-}CD8^{-}$ T cell receptor $\alpha\beta$ T cells: generation of an in vitro major histocompatibility complex class I-specific cytotoxic T lymphocyte response and allogeneic tumor rejection. *J. Exp. Med.* 174:193.
29. Quaratino, S., G. Murison, R.E. Knyba, A. Verhoef, and M. Londei. 1991. Human $CD4^{-}CD8^{-}$ $\alpha\beta^{+}$ T cells express a functional T cell receptor and can be activated by superantigens. *J. Immunol.* 147:3319.
30. Brooks, E.G., D.P. Wirt, R.M. Goldblum, S. Vaidya, M.T. Asuncion, J.C. Patterson, C.F. Ware, and G.R. Klimpel. 1990. Double negative ($CD4^{-}CD8^{-}$) T cells with an α/β T cell receptor: non-MHC-restricted cytolytic activity and lymphokine production. *J. Immunol.* 144:4507.
31. Rivas, A., J. Koide, R. Laus, and E.G. Engleman. 1990. Alloantigen-specific cytotoxic clones bearing the α,β T cell antigen receptor but not CD4 or CD8 molecules. *J. Immunol.* 145:470.
32. Porcelli, S., M.B. Brenner, J.L. Greenstein, S.P. Balk, C. Terhorst, and P.A. Bleicher. 1989. Recognition of cluster of differentiation 1 antigens by human $CD4^{-}CD8^{-}$ cytolytic T lymphocytes. *Nature (Lond.).* 341:447.
33. Reinherz, E., P.C. Kung, G. Goldstein, R.H. Levey, and S.F. Schlossman. 1980. Discrete stages of human intrathymic differentiation. Analysis of normal thymocytes and leukemic lymphoblasts of T lineage. *Proc. Natl. Acad. Sci. USA.* 77:1588.
34. Band, H., F. Hochstenbach, J. McClean, S. Hata, M.S. Krangel, and M.B. Brenner. 1987. Immunochemical proof that a novel rearranging gene encodes the T cell receptor delta subunit. *Science (Wash. DC).* 238:682.
35. Groh, V., S. Porcelli, M. Fabbi, L.L. Lanier, L.J. Picker, T. Anderson, R.A. Warnke, A.K. Bhan, J.L. Strominger, and M.B. Brenner. 1989. Human lymphocytes bearing T cell receptor $\gamma\delta$ are phenotypically diverse and evenly distributed throughout the lymphoid system. *J. Exp. Med.* 169:1277.
36. Ledbetter, J.A., R.L. Evans, M. Lipinski, C. Cunningham-Rundles, R.A. Good, and L.A. Herzenberg. 1981. Evolutionary conservation of surface molecules that distinguish T lymphocytes helper/inducer and T cytotoxic/suppressor subpopulations in mouse and man. *J. Exp. Med.* 153:310.
37. Malissen, B., N. Rebai, A. Liabeuf, and C. Mawas. 1982. Human cytotoxic T cell structures associated with expression of cytolysis. I. Analysis at the clonal cell level of the cytolysis inhibiting effect of 7 monoclonal antibodies. *Eur. J. Immunol.* 12:747.
38. Schlitt, H.J., R.G. Kurrle, and K. Wonigeit. 1989. T cell activation by monoclonal antibodies directed to different epitopes on the human T cell receptor/CD3 complex: evidence for two different modes of activation. *Eur. J. Immunol.* 19:1649.
39. Koehler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.).* 256:495.
40. Balk, S.P., E.C. Ebert, R.L. Blumenthal, F.V. McDermott, K.W. Wucherpfenning, S.B. Landau, and R.S. Blumberg. 1991. Oligoclonal expansion and CD1 recognition by human intestinal intraepithelial lymphocytes. *Science (Wash. DC).* 253:1411.
41. Frohman, M.A., M.K. Dush, and G.R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer (polymerase chain reaction/5′ and 3′ cDNA ends/cDNA cloning/low-abundance mRNAs/*int*-2 gene). *Proc. Natl. Acad. Sci. USA.* 85:8998.
42. Loh, E.Y., J.F. Elliott, S. Cwirla, L.L. Lanier, and M.M. Davis. 1989. Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. *Science (Wash. DC).* 243:217.
43. Roman-Roman, S., L. Ferrandini, J. Azocar, C. Genevee, T. Hercend, and F. Triebel. 1991. Studies on the human T cell receptor α/β variable region genes. I. Identification of 7 additional V_{α} subfamilies and $14\text{-}J_{\alpha}$ gene segments. *Eur. J. Immunol.* 21:927.
44. Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA.* 86:8941.
45. Oksenberg, J.R., S. Stuart, A.B. Begovich, R.B. Bell, H.A. Erlich, L. Steinman, and C.C.A. Bernard. 1990. Limited heterogeneity of rearranged T-cell receptor $V\alpha$ transcripts in brains of multiple sclerosis patients. *Nature (Lond.).* 345:344.
46. Toyonaga, B., and T.W. Mak. 1987. Genes of the T-cell antigen receptor in normal and malignant T cells. *Annu. Rev. Immunol.* 5:585.
47. Lanier, L.L., S. Cwirla, N. Federspiel, and J.H. Phillips. 1986. Human natural killer cells isolated from peripheral blood do not rearrange T cell antigen receptor β chain genes. *J. Exp. Med.* 163:209.
48. Calman, A.F., and B.M. Peterlin. 1986. Expression of T cell receptor genes in human B cells. *J. Exp. Med.* 164:1940.
49. Brenner, M.B., J.L. Strominger, and M.S. Krangel. 1988. The gamma delta T cell receptor. *Adv. Immunol.* 43:133.
50. Yoshikai, Y., N. Kimura, B. Toyonaga, and T.W. Mak. 1986. Sequence and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. *J. Exp. Med.* 164:90.

51. Clark, J.M. 1988. Novel nontemplated nucleoside addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerases. *Nucleic Acids Res.* 16:9677.
52. Asarnow, D.M., W.A. Kuziel, M. Bonyhadi, R.E. Tigelaar, P.W. Tucker, and J.P. Allison. 1988. Limited diversity of $\gamma\delta$ antigen receptor genes of Thy-1$^+$ dendritic epidermal cells. *Cell.* 55:837.
53. Asarnow, D.M., T. Goodman, L. LeFrancois, and J.P. Allison. 1989. Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. *Nature (Lond.).* 341:60.
54. Delfau, M.-H., A.J. Hance, D. LeCossier, E. Vilmer, and B. Grandchamp. 1992. Restricted diversity of Vγ9-JP rearrangements in unstimulated human γ/δ T lymphocytes. *Eur. J. Immunol.* 22:2437.
55. Havran, W.L., and J.P. Allison. 1988. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature (Lond.).* 335:443.
56. Bogue, M., S. Gilfillan, C. Benoist, and D. Mathis. 1992. Regulation of N-region diversity in antigen receptors through thymocyte differentiation and thymus ontogeny. *Proc. Natl. Acad. Sci. USA.* 89:11011.
57. Havran, W.L., Y.-H. Chien, and J.P. Allison. 1991. Recognition of self antigens by skin-derived T cells with invariant $\gamma\delta$ antigen receptors. *Science (Wash. DC).* 252:1430.
58. Strominger, J.L. 1989. The $\gamma\delta$ T cell receptor and class Ib MHC-related proteins: enigmatic molecules of immune recognition. *Cell.* 57:895.
59. Tonnelle, C., R. DeMars, and E.O. Long. 1985. DO beta: a new beta chain gene in HLA-D with a distinct regulation of expression. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2839.
60. Servenius, B., L. Rask, and P.A. Peterson. 1987. Class II genes of the human major histocompatibility complex: the DOβ gene is a divergent member of the class II β gene family. *J. Biol. Chem.* 262:8759.
61. Koseki, H., A. Hidefumi, T. Inaba, N. Miyashita, K. Moriwaki, K. Fischer Lindahl, Y. Mizutani, K. Imai, and M. Taniguchi. 1991. Dominant expression of a distinctive V14$^+$ T-cell receptor α chain in mice. *Proc. Natl. Acad. Sci. USA.* 88:7518.
62. Herman, A., J.W. Kappler, P. Marrack, and A.M. Pullen. 1991. Superantigens: mechanisms of T-cell stimulation and role in immune responses. *Annu. Rev. Immunol.* 9:745.
63. Egerton, M., and R. Scollay. 1990. Intrathymic selection of murine TCR$\alpha\beta^+$ CD4$^-$CD8$^-$ thymocytes. *Int. Immunol.* 2:157.
64. Singer, P.A., R.S. Balderas, R.J. McEvilly, M. Bobardt, and A.N. Theofilopoulos. 1989. Tolerance-related Vβ clonal deletions in normal and CD4$^-$8$^-$, TCR-α/β^+ and abnormal *lpr* and *gld* cell populations. *J. Exp. Med.* 170:1869.
65. Wu, L., M. Pearse, M. Egerton, H. Petrie, and R. Scollay. 1990. CD4$^-$CD8$^-$ thymocytes that express the T cell receptor may have previously expressed CD8. *Int. Immunol.* 2:52.
66. Takahama, Y., and A. Singer. 1992. Post-transcriptional regulation of early T cell development by T cell receptor signals. *Science (Wash. DC).* 258:1456.
67. Takahama, Y., E.W. Shores, A. Singer. 1992. Negative selection of precursor thymocytes before their differentiation into CD4+CD8+ cells. *Science (Wash. DC).* 258:653.
68. Martin, L.H., F. Calabi, F.-A. Lefebvre, C.A.G. Bilsland, and C. Milstein. 1987. Structure and expression of the human thymocyte antigens CD1a, CD1b, and CD1c. *Proc. Natl. Acad. Sci. USA.* 84:9189.
69. Balk, S.P., P.A. Bleicher, and C. Terhorst. 1989. Isolation and characterization of a cDNA and gene coding for a fourth CD1 molecule. *Proc. Natl. Acad. Sci. USA.* 86:252.
70. Calabi, F., and A. Bradbury. 1991. Review: the CD1 system. *Tissue Antigens.* 37:1.
71. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (Lond.).* 356:314.
72. Klein, M.H., P. Concannon, M. Everett, L.D.H. Kim, T. Hunkapiller, and L. Hood. 1987. Diversity and structure of human T-cell receptor α-chain variable region genes. *Proc. Natl. Acad. Sci. USA.* 84:6884.