Increased Frequency of T Cell Receptor Vα12.1 Expression on CD8+ T Cells: Evidence that Vα Participates in Shaping the Peripheral T Cell Repertoire

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Summary

The T cell receptor repertoire has a potential for vast diversity. However, this diversity is limited by the fact that the majority of thymocytes die as the repertoire is shaped by positive and negative selection events during development. Such thymic selection affecting TCR Vβ gene segment usage has been demonstrated in the mouse. However, similar data has not been forthcoming in man, and little is known about the role of the TCR α chain in antigen/major histocompatibility complex (MHC) recognition in any species. Here, we used a monoclonal antibody recognizing the TCR Vα12.1 gene product to assess the expression of this gene in the peripheral blood of man. In most individuals tested, the percentage of cells expressing Vα12.1 was significantly higher in CD8+ T cells than in CD4+ T cells. That the Vα gene product itself was responsible for this increased expression in CD8+ T cells was underscored by the lack of substantial skewing of Vβ usage in the Vα12.1-bearing T cells. Moreover, the skewed expression of Vα12.1 was already present at birth, indicating that it was likely to be due to a developmental process rather than the result of exposure to environmental antigens. Based on the established role for CD8 in binding to class I MHC molecules, we suggest that increased expression of Vα12.1 on CD8+ T cells points to a role for TCR's using Vα12.1 in class I MHC/Ag recognition. These results indicate that Vα gene usage in the peripheral blood of man is not random, and they support a role for Vα as a participant in the self-MHC recognition process that shapes the TCR repertoire.

T cells play a central role in immunity through specific recognition of foreign peptides bound to self-MHC molecules. This recognition of antigen and self-MHC is carried out by clonotypic αβ T cell receptors, present on the surface of T lymphocytes in association with the CD3 complex (1). TCR α and β chains are assembled by somatic recombination of discontinuous germline gene segments during T cell development. The generation of a diverse repertoire of human αβ TCRs is accomplished by the recombination of a single V gene segment (selected from a pool of approximately 100 Vαs and 80 Vβs) to an individual J gene segment (out of approximately 100 Jαs and 13 Jβs). Template-independent nucleotide (N-segment) insertions at the junctions, Dβ usage, and the imprecise joining of the germline gene segments further increase TCR diversity (2, 3). However, in a given individual the potentially large repertoire of TCR structures may not actually be used by mature peripheral T cells. For instance, thymocytes bearing TCRs strongly autoreactive to self-MHC molecules are clonally deleted (negative selection) to achieve self-tolerance during thymic maturation (4, 5). In addition, lymphocytes bearing transgenic TCRs restricted to a particular MHC allele fail to develop fully in mice lacking the appropriate MHC molecule. These and other data led to the suggestion that a process of positive selection may also shape the peripheral TCR repertoire in mice and relate to the self-MHC restricted recognition of antigens by peripheral T lymphocytes (6–8). With few exceptions, mature αβ bearing T lymphocytes in the periphery are either CD8+ and restricted to class I MHC molecules, or are CD4+ and restricted to class II MHC molecules (9).

Molecular modeling predicts that the V gene segment encoded CDR 1 and 2 may contact the MHC molecule, while the V-(Dβ)-J junctional sequence encoded CDR3 may be oriented to interact predominantly with the foreign antigen peptide (3, 10). However, most studies to date supporting the role of TCR segments in determining the fate of T cells during the process of thymic selection and in their subsequent function in cell-mediated immune responses are based largely on analyses of Vβ gene products (11, 12). In contrast, little correlation between the expression of particular TCR α chains and MHC recognition or thymic selection has been reported. The few studies available to date implicate the TCR

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α chain in foreign antigen specificity whereas little direct evidence has shown a role in MHC recognition.

Here, a Vα12.1-specific mAb enabled us to analyze the expression of this gene segment in peripheral blood T cell subsets by flow cytometry. Most individuals expressed the Vα12.1 gene segment with significantly higher frequency (up to threefold) on $CD8^+$ T cells in comparison with $CD4^+$ T cells. This phenomenon is likely to be mediated by a process acting on the Vα12.1 gene product itself, since the concomitant usage of Vβ genes was heterogenous and similar in both $CD4^+$ and $CD8^+$ subsets of T cells. Moreover, the biased expression of Vα12.1 on $CD8^+$ T cells was present at birth, before antigenic challenge, suggesting that it may be the result of thymic selection events and not of foreign antigen-driven peripheral expansion.

Materials and Methods

Antibodies. The mAb 6D6 was generated by injecting BALB/c mice at monthly intervals with TCR αβ complexes isolated from the human T leukemia cell line HPB-MLT. TCR complexes were partially purified by immunoprecipitation using anti-CD3 mAb adsorbed to Protein A bearing fixed *Staphylococcus aureus* bacteria (Pansorbin; Calbiochem Corp., San Diego, CA) using a protocol described previously (13). Immune spleen cells were fused with P3X63Ag8.653 myeloma cells in the presence of polyethylene glycol 1500 (British Drug House, Carlplace, NY). The fused cells were then selected in the presence of hypoxanthine-aminopterin-thymidine (Sigma Chemical Co., St. Louis, MO) at 10^5/well in 96-well plates. Supernatants from wells positive for growth were screened by immunoprecipitation on ^{125}I-labeled HPB-MLT cell lysates. One of the hybridomas that produced antibodies that specifically immunoprecipitated HPB-MLT TCR-complex was selected and subcloned for additional study. The hybridoma product, mAb 6D6, was identified as IgG1 by immunodiffusion analysis and is specific for Vα12.1 encoded products (see Text).

MAb 3A2 was also generated against the HPB-MLT TCR αβ-complex using the same protocol. It was shown to be specific for the β chain of HPB-MLT (Vβ5.3) by immunoprecipitation experiments (see Fig. 2 *B*, lane *3*). In addition to immunofluorescence staining of HPB-MLT cell surface, approximately 1% of all T cells in PBL stain with mAb 3A2 (data not shown). Anti-TCR β framework antibody, βF1, was also made in this laboratory (13). MAb obtained from other sources include Leu-4 (anti-CD3) (14), SPV-T3b (anti-CD3) (15), OKT4 (anti-CD4), OKT8 (anti-CD8) (16), C305 (anti-Vβ8) (17), αF1 (anti-TCR α chain framework) (T Cell Sciences, Inc., Cambridge, MA), P3 (control) (18) and 187.1 (Rat anti-mouse κ chain) (19).

Immunofluorescence and Flow Cytometry. Flow cytometric analyses were performed using directly conjugated antibodies. Two-color analyses on peripheral blood lymphocytes were carried out as described previously using a FACSCAN (Becton Dickinson and Co., Mountainview, CA) (20). Viable cells were analyzed by gating on the propidium iodide negative lymphocytes.

Radiolabeling and Immunoprecipitation. Cell surface radiolabeling of HPB-MLT cells was performed by using $Na^{125}I$ and lactoperoxidase as previously described (21). Biosynthetic labeling of TCR chains from HPB-MLT was carried out as follows. Cells were washed and incubated at 5×10^6 cells/ml in methionine- and cysteine-free RPMI-1640 containing 10% dialyzed FCS, 2 mM glutamine and 20 mM HEPES at 37°C in 5% CO_2 atmosphere. After 30 min, 0.5 mCi each of [^{35}S]-methionine and -cysteine were added and incubation continued for 4 h. Cells were pelleted and lysed in Tris-buffered saline (TBS, 50 mM Tris pH 7.5, 150 mM NaCl) containing 1% Triton X-100, 1 mM PMSF and 8 mM iodoacetamide. Cell lysates were pre-cleared twice using normal rabbit serum (NRS) and fixed *S. aureus* Cowan I (Pansorbin; Calbiochem-Boehring Corp., San Diego, CA). Specific immunoprecipitations were carried out using amounts of ascites determined to be optimal; 6D6 (0.1 μl), 3A2 (0.4 μl), Leu 4 (0.1 μl), βF1 (0.25 μl) or αF1 (0.1 μl). In each case 150 μl of mAb 187.1 (rat anti-mouse κ) culture supernatant was added as a second antibody. After 60 min incubation at 4°C, 100 μl of 10% Protein-A sepharose CL4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was added and incubated further for 30 min. Immune complexes were washed in 0.1% Triton X-100, resolved by SDS-PAGE and analyzed by autoradiography as described (22).

Derivation of $6D6^+$ T Cell Clones. PBMC were stimulated in vitro with 50 ng/ml of mAb 6D6 in RPMI-1640 containing 2 mM glutamine, 20 mM HEPES, and 10% pooled human serum. Secondary stimulation was carried out using autologous PBMC (4,000 rads) and 50 ng/ml of 6D6 antibody. After 3 wk in culture, the proportion of $6D6^+$ cells in the $CD3^+$ population rose from about 4% to 85%. Cells were cloned by plating at limiting dilutions in media containing IL-2 and a panel of $6D6^+$ and $6D6^-$ T cell clones was derived.

Southern Blot Hybridization. Genomic DNA was extracted from T cells by a standard technique (23). DNA was digested with *Bam* HI restriction enzyme, size fractionated on 0.7% agarose gel and transferred to Hybond-N nylon membranes (Amersham Corp.). DNA was cross-linked to the membranes by UV-irradiation and hybridized with ^{32}P-labeled probes. The Vα12.1 probe was an Eco RI-Acc I (nucleotides −100 to +322) fragment of the TCRα chain cDNA clone pGA5 (24). Vα DNA fragment was radiolabeled with [$^{\alpha-32}$P]-dGTP and -dCTP using random oligonucleotide primers and Klenow. Likewise, PCR products generated using the Vβ- and Cβ-specific primers were size fractionated on 2% agarose gels, transferred to nylon filters, and hybridized with mixed ^{32}P-labeled Cβ1-(oligo #3Cβ) and Cβ2-specific (oligo #4Cβ) probes (see below).

Statistical Analyses. To assess if the differences in expression of Vα12.1 in the $CD4^+$ and $CD8^+$ T cell subsets were significant, two-sided Wilcoxon signed-ranked sum tests were used (25).

Polymerase Chain Reaction. Total RNA was isolated from Ficoll-purified mononuclear cells according to Chomczynski and Sacchi (26). cDNA was synthesized at 42°C in 50 μl reactions using 0.5 μg oligo (dT) primer, 4 to 8 μg total RNA and 10 U of AMV reverse transcriptase (Promega Corp., Madison, WI). After 1 h, the reaction mixture was diluted to 100 μl, boiled and chilled, and centrifuged to remove insoluble material. PCR was performed in 25 μl reactions containing 1 μl of cDNA (1/100), 1 mm $MgCl_2$, 10 mM Tris pH 8.3, 1 mg/ml Gelatin, 5 pmole of each primer, 0.2 mM of each dNTP and 2 U of Taq-Polymerase (Thermus aquaticus DNA polymerase, Perkin-Elmer-Cetus Corp.). Briefly, cDNA and primers were pre-mixed with $MgCl_2$/Tris/Gelatin buffer and heated at 95°C for 7 min. Other components were added, the reaction mix was overlain with mineral oil, and 25 to 30 cycles of PCR were carried out in a thermocycler (Perkin-Elmer-Cetus Corp.) at the following settings: 0.7 min at 95°C for denaturation, 1 min at 56°C for annealing, and 1 min at 72°C for chain extension. To ensure complete synthesis, the last cycle at 72°C was extended to 10 min.

Cloning in M13 and Sequencing. Vα12.1-specific (5′ GGGG-TCGACTTGCCAGCCTGTTGAGGGCAG 3′) and Cα-specific (5′ GGGAAGCTTCTGGTACACGGCAGGGTCAGG 3′) primers

were used to generate PCR products using cDNA from 6D6+ T cell clones. Restriction sites in the PCR primers were used to generate sticky ends for cloning. Appropriately sized DNA products were then isolated from low melting point (LMP) agarose gels (BRL, Gaithersburg, MD) and ligated to M13 plasmids for cloning and sequencing by the dideoxy chain termination method using the modified T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH) (27). The sequencing products were resolved on polyacrylamide gels and autoradiography was carried out according to standard methods.

Oligonucleotide Probes and Primers. Vβ-specific PCR products were generated using primers based on published sequences with some modifications (28, 29). The oligonucleotide sequences for the Vβ-specific family members used in combination with the Cβ primers are listed below: Vβ1-GGGGTCGACGCACAACAGTTCCCTGACTTGCAC(+); Vβ2-GGGGTCGACTCATCAACCATGCAAGCCTGACCT(+); Vβ3-GGGGTCGACGTCTCTAGAGAGAAGAAGGAGCGC(+); Vβ4-GGGGTCGACACATATGAGAGTGGATTTGTCATT(+); Vβ5.1-GGGGTCGACATACTTCAGTGAGACACAGAGAAAC(+); Vβ5.2/3-GGGGTCGACTTCCCTAACTATAGCTCTGAGCTG(+); Vβ6.1/2/3-GGGGTCGACAGGCCTGAGGGATCCGTCTC(+); Vβ7-GGGGTCGACCCTGAATGCCCCAACAGCTCTC(+); Vβ8-GGGGTCGACATTTACTTTAACAACAACGTTCCG(+); Vβ9-GGGGTCGACCCTAAATCTCCAGACAAAGCTCAC(+); Vβ10-GGGGTCGACCTCCAAAAACTCATCCTGTACCTT(+); Vβ11-GGGGTCGACTCAACAGTCTCCAGAATAAGGACG(+); Vβ12-GGGGTCGACAAAGGAGAAGTCTCAGAT(+); Vβ13.1-GGGGTCGACCAAGGAGAAGTCCCCAATGGC(+); Vβ13.2-GGGGTCGACGGTGAGGGTACAACTGCCAAA(+); Vβ14-GGGGTCGACGTCTCTCGAAAAGAGAAGAGGAAT(+); Vβ15-GGGGTCGACAGTGTCTCTCGACAGGCACAGGCT(+); Vβ16-GGGGTCGACAAAGAGTCTAAACAGGATGAGTCC(+); Vβ17-GGGGTCGACCAGATAGTAAATGACTTTCAG(+); Vβ18-GGGGTCGACGATGAGTCAGGAATGCCAAAGGAA(+); Vβ19-GGGGTCGACCAATGCCCCAAGAACGCACCCTGC(+); Vβ20-GGGGTCGACAGCTCTGAGGTGCCCCAGAATCTC(+); #1Cβ-GGGAAGCTTCTGATGGCTCAAACACAG(−); #2Cβ-GGGAAGCTTACACCAGTGTGGCCTTTTGGGTG(−); #3Cβ-TCGGGTGGGAACACGTTTTTC(−); #4Cβ-TCGGGTGGGAACACCTTGTTC(−).

The 5′ sense strand primers Vβ11, Vβ14, Vβ15, Vβ18 and Vβ19 were used in combination with the 3′ anti-sense primer #2Cβ, while the rest of the Vβ primers were used in combination with the primer #1Cβ. This was to minimize PCR artifact (primer-dimer) formation due to possible 3′-end complementary bases shared between sense and anti-sense primers. Synthetic oligonucleotides were radiolabeled at the 5′ termini with [γ-^{32}P] using the enzyme bacteriophage T4 polynucleotide kinase. All of the PCR products were size fractionated, transferred and hybridized with internal region Cβ probes, #3Cβ and #4Cβ. Vβ-specific PCR products were visualized by autoradiography and radioactivity was counted directly by using a blot analyzer (Betascope 603; Betagen Corp.).

Results

MAb 6D6 is Specific for TCR Vα12.1. To facilitate direct examination of the TCR repertoire in man, a murine mAb directed against a Vα12.1 determinant was established (see Materials and Methods). MAb 6D6 bound specifically to the surface of HPB-MLT cells (the immunizing cell line) and to approximately 2% to 4% of peripheral T cells from normal

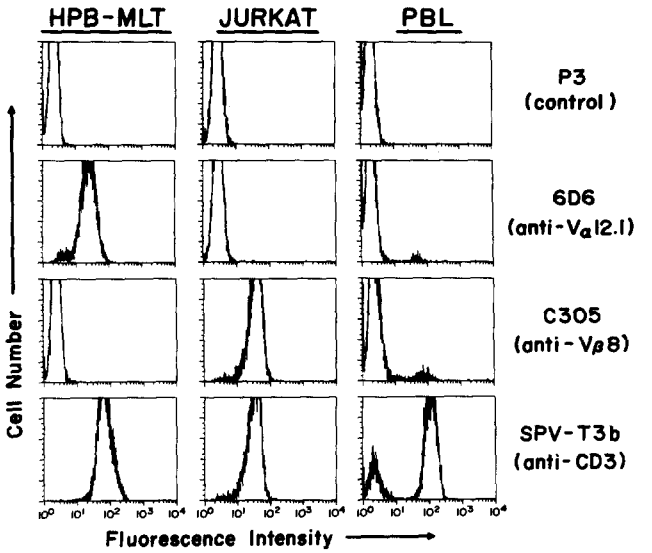

Figure 1. Cell surface reactivity of mAb 6D6. MAb 6D6 stained HPB-MLT cells and a small subpopulation (2–5%) of peripheral blood lymphocytes but not the Jurkat T leukemia cell line. Staining of Jurkat cells and PBL with the Vβ8-specific mAb C305 is shown for comparison (17). Isotype matched mAb P3 was used as a negative control and mAb T3b (anti-CD3) was used as positive control. MAb 6D6 was generated against the αβ TCR complex isolated from HPB-MLT as described in Materials and Methods. Cell number scale is linear, fluorescence intensity scale is log.

individuals (Fig. 1). For comparison, mAb C305, specific for Vβ8 encoded TCR products, also reacted with a few percent of peripheral blood T cells and the Jurkat cell line (which expresses Vβ8) but not with HPB-MLT cells (which express Vα12.1 and Vβ5.3) (Fig. 1).

To determine the nature of the molecule recognized by mAb 6D6, immunoprecipitations were performed on ^{125}I-labeled HPB-MLT cell lysates followed by SDS-PAGE and autoradiography. The mAb immunoprecipitated the TCR αβ heterodimer (85 kD) under nonreducing conditions (Fig. 2 *A*, lane *4*). This 85 kD heterodimer resolved into two species with Mr 46 kD (TCRα) and 39 kD (TCRβ) under reducing conditions (Fig. 2 *A*, lane *8*). For comparison, similar radiolabeled species were visualized after immunoprecipitations with TCR β-specific mAb βF1 and CD3ε-specific mAb Leu 4 (Fig. 2 *A*). In both anti-TCR and anti-CD3 immunoprecipitations, a variable degree of coimmunoprecipitation of the other components was noted.

To delineate the TCR chain specificity of mAb 6D6, immunoprecipitations were carried out on HPB-MLT cell lysates metabolically labeled with ^{35}[S]-methionine and -cysteine. Under these conditions a detectable fraction of the newly synthesized α and β TCR chains were still unpaired, such that mAb 6D6 immunoprecipitated both a free α chain (migrating as a sharp band at 43 kD), and the αβ-heterodimers as fully glycosylated (80–85 kD) and partially glycosylated (70–75 kD) structures (Fig. 2 *B*, lane *5*) based on sizes reported earlier (30). Immunoprecipitation with the anti-TCR α framework antibody, αF1, confirmed that the 43 kD species was the unpaired TCR α chain subunit (Fig. 2 *B*, lane *4*). The

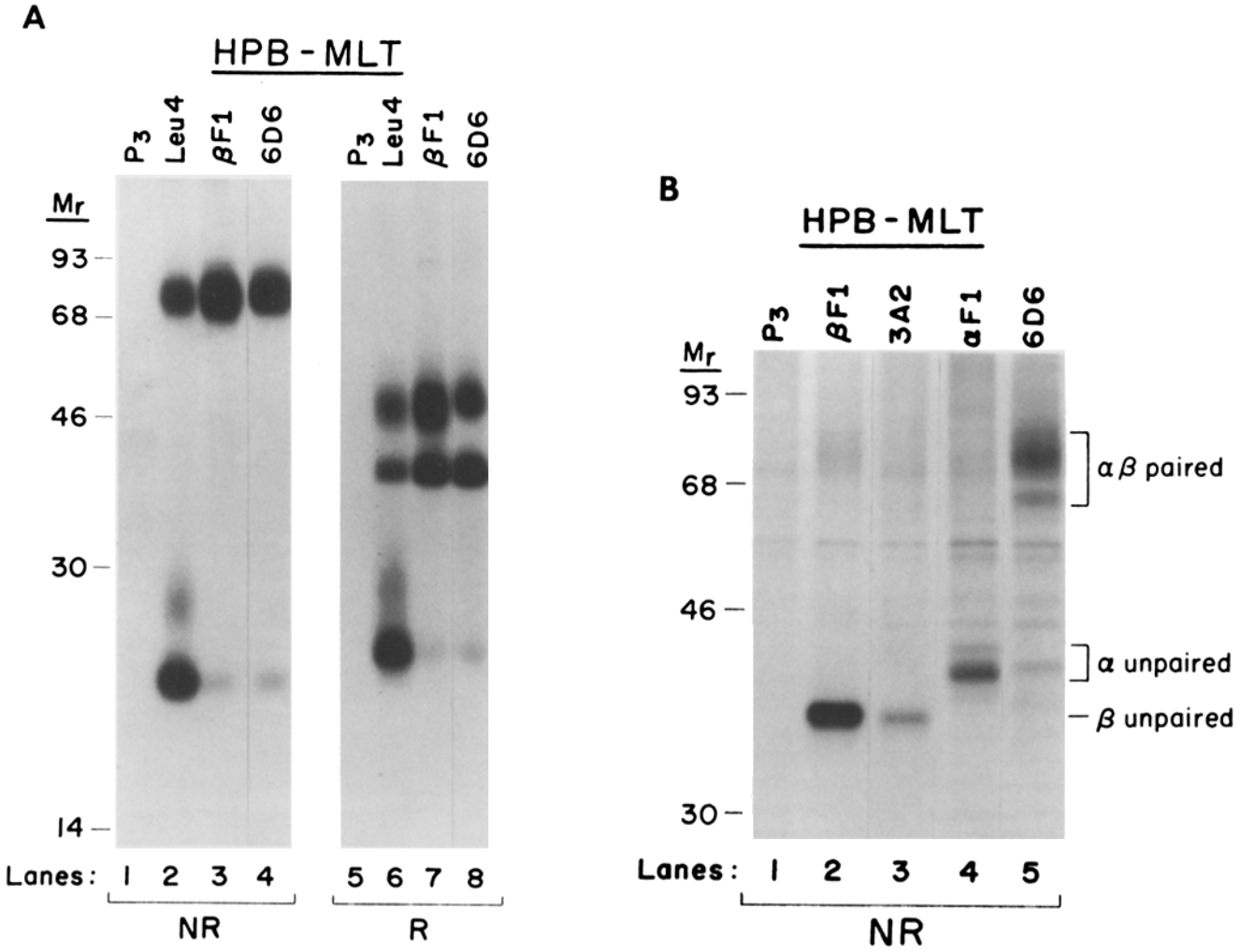

Figure 2. Immunochemical characterization of mAb 6D6 reactivity. (*A*) Immunoprecipitations of the αβ TCR complex from cell surface ^{125}I radiolabeled HPB-MLT cells were resolved by SDS-PAGE and analyzed by autoradiography. Antibodies were P3 (negative control), Leu4 (anti-CD3), βF1 (anti-β framework) and 6D6. Under nonreducing (NR) conditions (lanes *1–4*), the αβ TCR complex was resolved as a 85 kD species by direct immunoprecipitation with mAbs βF1 and 6D6 or by co-immunoprecipitation with anti-CD3 mAb Leu 4. Under reducing (R) conditions (lanes *5–8*), the TCR α and β subunits were visualized as 46 kD and 39 kD species, respectively. Note the CD3 subunits at 20–30 kD. (*B*) MAb 6D6 recognized a determinant present on the α chain of the HPB-MLT TCR complex. HPB-MLT cells were metabolically labeled with $^{35}(S)$-methionine and -cysteine for 4 h and solubilized in 1% Triton X-100. Immunoprecipitations were carried out with the indicated antibodies, and resolved by SDS-PAGE under nonreducing (NR) conditions and visualized by fluorography. MAbs βF1 (TCR β framework) and 3A2 (anti-Vβ5) immunoprecipitated the unpaired β chain (lanes *2* and *3*, respectively). In contrast, αF1 (anti-framework) and 6D6 antibodies (lanes *4* and *5*, respectively) immunoprecipitated the unpaired α chain from HPB-MLT cell lysates. While mAb αF1 immunoprecipitated all of the glycosylated α chain species identified in HPB-MLT (13), mAb 6D6 immunoprecipitated only one of these species. Note that mAb 6D6 also recognized the TCR αβ heterodimer as fully (80–85 kD) and partially (70–75 kD) glycosylated complexes (see text).

other radiolabelled species (41–44 kD) in the αF1 precipitation presumably corresponded to differentially glycosylated α chain species, as reported previously (13). By comparison, other mAbs that we generated including the anti-TCR β framework antibody, βF1 (lane *2*), and antibody against the Vβ product of HPB-MLT (3A2) (lane *3*) both immunoprecipitated the unpaired β-chain (38 kD) but not any of the free α chain species. Thus, mAb 6D6 reacted with a determinant expressed on the TCR-α chain.

To examine whether this determinant was encoded by Vα, Jα or a combination of both gene segments, 6D6$^+$ T cell clones derived from healthy individuals were analyzed. Southern blot analysis using the Vα12.1-specific (pGA5) probe showed this Vα gene to be rearranged in all the 6D6$^+$ T cell clones and the HPB-MLT leukemia cell line (Fig. 3 *A*). The fact that the rearranged fragments were of different sizes in each cell line indicated that the Vα12.1 gene segment which is part of a Vα family having only one member (24, 31), might be rearranging to several different Jα gene segments. This suggested that mAb 6D6 recognized a Vα rather than a Jα gene segment encoded determinant. Similarly, Northern blot analysis revealed expression of Vα12.1 transcripts in 6D6$^+$ T cell clones (data not shown). These conclusions were confirmed by determining the nucleic acid sequences

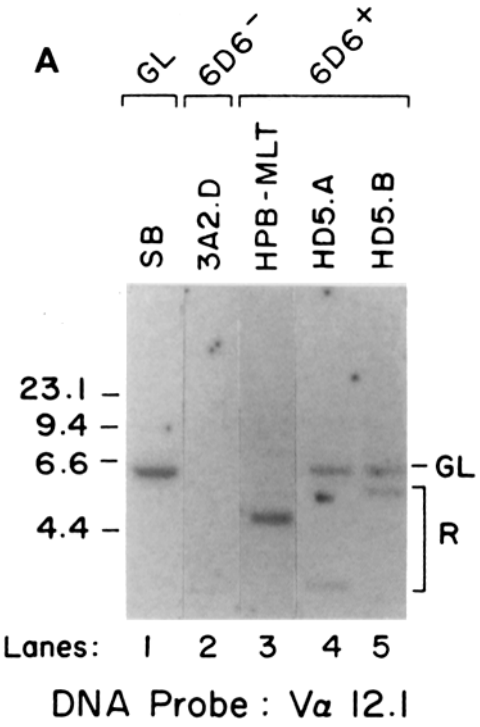

Figure 3. Vα12.1 gene segment encodes the determinant recognized by mAb 6D6. (*A*) 6D6⁺ T cells contained different Vα12.1 gene rearrangements. DNA from 6D6⁺ T cell clones HD5.A (lane *4*) and HD5.B (lane *5*), T cell leukemia cell line HPB-MLT (lane *3*), and the 6D6⁻ T cell clone 3A2.D were digested with *Bam* HI and analyzed by Southern hybridization using a Vα12.1-specific probe. Germline configuration (GL) for Vα12.1 gene was seen as a 6.3 Kb fragment in the B cell line, SB (lane *1*). While both Vα12.1 alleles in 3A2.D T cell clone were deleted, DNA from HD5.A, HD5.B and HPB-MLT showed distinct Vα12.1 rearrangements (R). (*B*) Partial nucleic acid sequences of HD5.A, HD5.B and HPB-MLT (24) TCR α chain transcripts showed in-frame Vα12.1 gene rearrangements with different Jα gene segments (JA30, Q, and A). cDNA sequences for HD5.A and HD5.B TCR α chain transcripts were generated by PCR using Vα12.1- and Cα-specific primers as described in Materials and Methods.

of the α chain junctional regions of 6D6⁺ T cell clones HD5.A and HD5.B, and compared with the reported HPB.MLT sequence (24). Each of the three 6D6⁺ T cell lines contained in-frame Vα12.1 rearrangements to distinct Jα gene segments (Fig. 3 *B*). These results demonstrated that mAb 6D6 specifically recognized the product encoded by the human Vα12.1 gene segment.

Increased Frequency of Vα12.1⁺ T Cells in the CD8⁺ Subset. Expression of the Vα12.1 gene segment in the total PBL varied from 1.4% to 4.5% of the CD3⁺ lymphocytes ($\bar{x}$ = 3.5%). When Vα12.1 gene expression was analyzed among the CD4⁺ or CD8⁺ T cells by two-color staining (an example is shown in Fig. 4 *A*), it was evident that most individuals had a non-random distribution of this gene segment among the two subsets (Fig. 4 *B*). Analysis of the two phenotypic subsets separately showed that approximately 80% of individuals had a 1.5– 3-fold higher frequency of Vα12.1 among CD8⁺ T cells ($\bar{x}$ = 4.2%; range 1.4% to 7.2%)

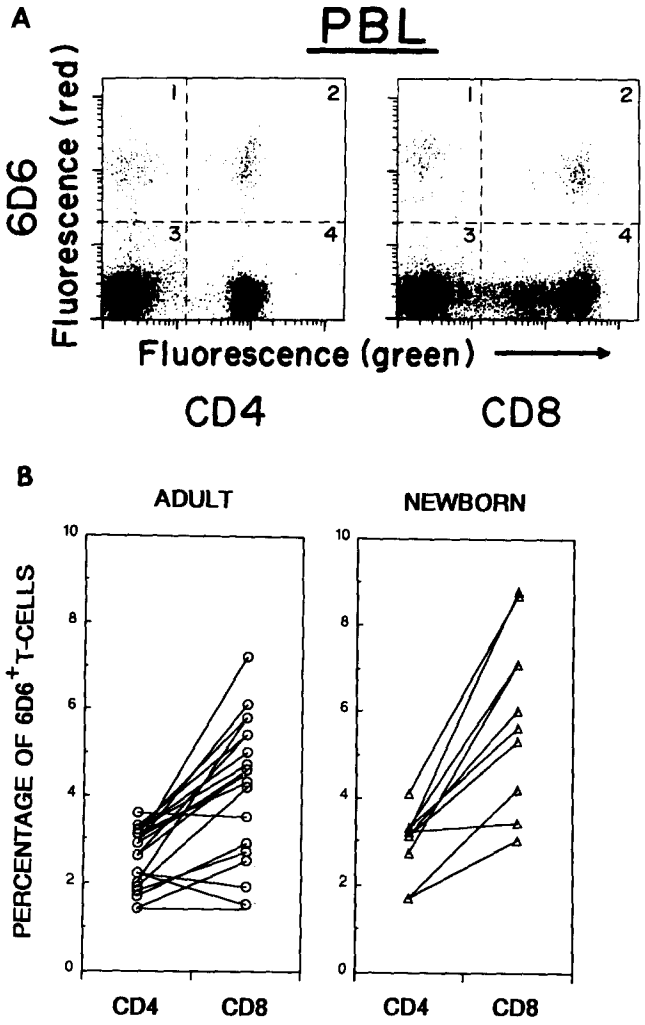

Figure 4. Two-color immunofluorescence analysis of 6D6 expression on CD4⁺ and CD8⁺ T cells. (*A*) PBMC from adult and newborn were stained with anti-CD4 (OKT4) or anti-CD8 (OKT8) (green fluorescence) and anti-Vα12.1 (6D6) (red fluorescence) mAbs as described in Materials and Methods. Lymphocytes were gated on the basis of forward and side scatter profiles (not shown) and analyzed for fluorescence intensity in log scale. Dot plots were divided into quadrants to represent unstained cells (lower left, quadrant 3), cells stained with FITC alone (lower right, quadrant 4), cells stained with PE alone (upper left, quadrant 1), and cells that double stained with FITC and PE (upper right, quadrant 2). (*B*) The Vα12.1 expression in CD4⁺ and CD8⁺ T cells from adult and newborn PBL was determined using data derived for individual blood samples stained as in *A*. The expression of Vα12.1 in the CD4⁺ and CD8⁺ subsets for each individual are connected by a line. The following formula was used to calculate the percentage value for 6D6 expression in CD4⁺ or CD8⁺ T cells: % 6D6⁺/CD4⁺ (or CD8⁺) cells = (6D6⁺ cells co-stained with CD4 (or CD8) in PBMC (2nd quadrant)/% T cells that were CD4⁺ (or CD8⁺)) × 100.

compared to CD4$^+$ T cells ($\bar{x}$ = 2.7%; range 1.4% to 3.6%) (p = 0.0044) (Fig. 4 *B*, Adult). Only 20% of individuals used Vα12.1 at similar frequency among CD4$^+$ and CD8$^+$ T cells. This result indicated that T cell receptor Vα gene usage in man is nonrandom.

This differential usage could have resulted either from developmental influences or from peripheral expansion as a consequence of foreign antigenic challenges. To distinguish these two possibilities, Vα12.1 expression was examined in newborn peripheral (umbilical cord) blood samples. Similar to the results seen in adult peripheral blood, significantly higher percentages of Vα12.1 expression were seen in the CD8$^+$ T cells of 10 newborns (p = 0.002) (Fig. 4 *B*, Newborn). These data in the peripheral blood of newborns suggested the skewing was not likely to be the result of peripheral antigenic challenges and pointed instead to a developmental mechanism, possibly thymic selection.

Analysis of Vβ Usage in Vα12.1$^+$ T Cells by Quantitative PCR. The preferential Vα12.1 expression on CD8$^+$ T cells was unexpected because essentially all known developmentally determined differences in TCR V gene usage are based on TCR Vβ expression and the presumed interaction of this gene segment with self-MHC or self-MHC antigen/superantigen complexes (32). Therefore, it became important to examine TCR Vβ usage in cells expressing the Vα12.1 gene product. For this analysis, we used the PCR with Vβ-specific oligonucleotides known to detect up to 90% of all known human Vβ genes. Such quantitative PCR techniques may not determine the precise percentage of T cells bearing the respective V genes (in-frame and out of frame sequences

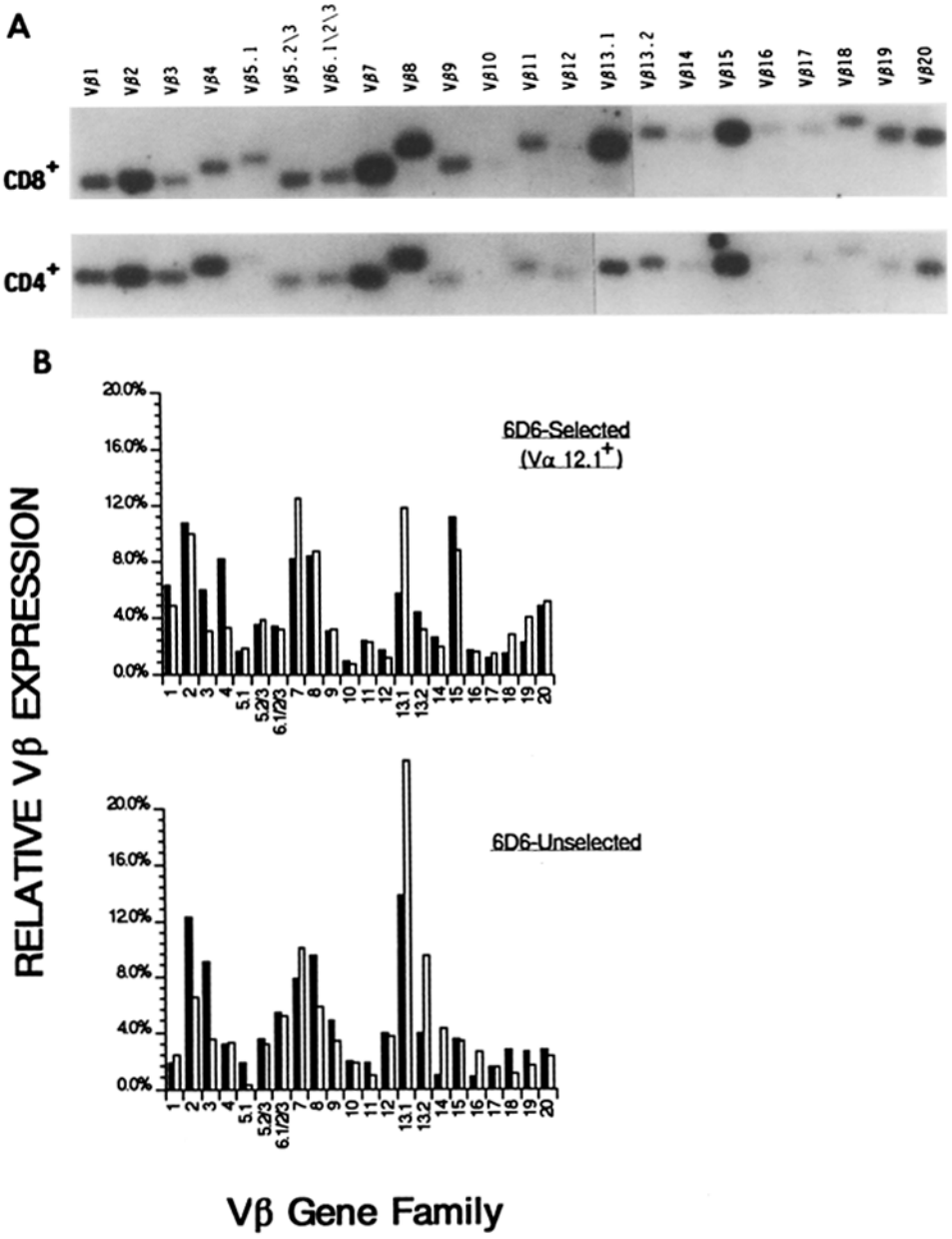

Figure 5. Vβ gene usage in Vα12.1$^+$ T cells analyzed by quantitative PCR-hybridization. cDNA from CD4$^+$ and CD8$^+$ T cells expressing the Vα12.1 (6D6$^+$) were used to generate PCR products using Vβ- and Cβ-specific primers, size fractionated, and hybridized with an internal constant region (Cβ) ^{32}P-labeled probe (see Materials and Methods). (*A*) An example of a southern blot analysis of the specific expression of Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2/3, Vβ6.1/2/3, Vβ7, Vβ8, Vβ9, Vβ10, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ14, Vβ15, Vβ16, Vβ17, Vβ18, Vβ19, and Vβ20 gene segments in the 6D6-selected (Vα12.1$^+$) CD4$^+$ and CD8$^+$ T cell subsets as represented in autoradiographic form. (*B*) Relative percentage of expression of each Vβ gene. The amount of Vβ-specific expression in CD4$^+$ and CD8$^+$ cells was quantitated by directly measuring the radioactivity hybridized to each Vβ-specific PCR products by a blot analyzer (Betascope 603; Betagen Corporation, Waltham, MA). The data are presented as percentages in a bar graph. The Y-axis represents percent Vβ expression in CD4$^+$ (*solid bars*) and CD8$^+$ (*open bars*) in Vα12.1$^+$ (6D6-selected; upper panel) and total (6D6-unselected; lower panel) T cells. The percent Vβ expression for each sample was calculated using the following formula: %Vβ = Hybridization to Vβ-specific PCR products in cpm/Sum of all Vβ-specific hybridizations in cpm) × 100.

are not distinguished for example), but such approaches are known to be useful in approximating relative V gene usage (29). Moreover, our own efforts to validate the procedure by mixing mRNA from T cell lines of known V gene usage revealed the expected proportions by PCR, at least for a sample of 3 Vβ genes analyzed (data not shown). In Vα12.1$^+$ (6D6 selected) T cells, quantitative hybridization of Vβ-specific PCR products revealed that the majority of the Vβ genes were shared equally between the CD4$^+$ and CD8$^+$ subsets (Fig. 5 *A* and 5 *B*, upper panel). No single Vβ gene dominated in its association with Vα12.1. Importantly, the overall similarity in Vβ usage on Vα12.1 bearing CD4$^+$ and CD8$^+$ T cells suggested that skewed Vβ usage was not responsible for the increased level of Vα12.1 expression on CD8$^+$ T cells. In addition, most of the Vβ genes that were studied in the 6D6 selected (Vα12.1$^+$) T cells were detected in similar amounts in CD4$^+$ and CD8$^+$ T cell subsets that had not been selected for Vα12.1 expression (Fig. 5 *B*, 6D6 unselected; lower panel). Thus Vβ2, Vβ7, Vβ8 and Vβ13 which were the most prominently used Vβ genes in the Vα12.1 selected T cells were also prominently used in CD4$^+$ and CD8$^+$ T cells not selected for Vα12.1 expression (Fig. 5 *B*, compare patterns in upper and lower panels). Moreover, the least frequently used Vβs (Vβ5, Vβ10, Vβ11, Vβ16, Vβ17, Vβ18 and Vβ19) were also similar.

Some differences in Vβ expression among the subgroups were noted. For example, Vβ7 and Vβ13.1 which accounted for 11% and 10.4% of the Vβ gene segments in the Vα12.1$^+$/CD8$^+$ subgroup were used at relatively lower levels in the Vα12.1$^+$/CD4$^+$ subset where they made up 7.5% and 5.3%, respectively (Fig. 5 *B*, upper panel). These usage levels together accounted for about a 9% difference between the two subsets. In fact, the sum of all differences in Vβ frequency between the Vα12.1$^+$/CD8$^+$ and Vα12.1$^+$/CD4$^+$ T cells was only 16%. This total difference in Vβ usage was substantially less than the 60% that would have been required if skewed Vβ usage was to account for the 2.5-fold increase in Vα12.1 expression in this individual's CD8$^+$ T cells.

While the above analysis examined Vβ expression on Vα12.1 selected CD4$^+$ versus CD8$^+$ T cells, we also compared Vβ expression in Vα12.1 selected and unselected T cell populations, we noted some evidence for preferential Vβ usage. For instance, in the CD4$^+$ subset, Vβ15 was present on approximately 11% of the Vα12.1$^+$ T cells compared with only approximately 3% of the T cell population not selected for Vα12.1 expression. In contrast, Vβ13.1 was less frequently used in Vα12.1$^+$ T cells (Fig. 5 *B*, compare upper and lower panels). Nevertheless, even in these few examples where Vβ differences were seen between Vα12.1 selected and unselected T cell populations, the differences were apparent to a nearly equal degree in both the CD4$^+$ and CD8$^+$ subsets, and therefore would seem not to be a likely explanation for the increased Vα12.1 expression on CD8$^+$ T cells.

It is important to note that all of the Vβs tested are capable of association with Vα12.1 and that no major Vβ product dominated in association with the Vα12.1 gene product. A similar analysis of Vβ usage in newborn umbilical cord blood also showed no dominant Vβ expression in association with Vα12.1 and no significant difference in Vβ usage by the CD4$^+$ and CD8$^+$ subsets (data not shown). Thus, one cannot readily account for the significantly higher expression of Vα12.1 on CD8$^+$ T cells based on Vβ skewing.

Discussion

Ever since early functional studies pointed to the requirement for self-restriction in T cell recognition of foreign antigens, immunologists have tried to understand this phenomenon in molecular terms (33). The binding of antigen peptides to MHC molecules has provided a molecular explanation for corecognition of Ag/MHC by the TCR (34–37), but the way in which different components of the TCR contribute to this Ag/MHC recognition has not been fully delineated. TCR gene sequences and hypothetical modeling based on immunoglobulin molecules suggest that the TCR V regions (especially those regions corresponding to CDR1 and CDR2) contact the MHC molecule, while the highly diverse V-(D)-J junctional region (corresponding to CDR3) contacts antigenic peptides bound to the MHC molecule (3, 10). If these assumptions are correct, it should be possible to correlate TCR germline gene segment usage with Ag/MHC recognition. In fact, several studies in mice have provided direct correlations between TCR Vβ gene segment usage and recognition of specific class II MHC/Ag complexes, mostly in association with the Mls antigens (38–41). However, the molecular basis of self-MHC recognition by the TCR is likely to be more complex than our current understanding which emphasizes only TCR Vβ gene products. The Vα encoded regions also are likely to be important in Ag/MHC recognition as predicted by the molecular model.

With regard to the role of the TCR α chain, a few studies have correlated Vα usage with foreign peptide or hapten recognition (42–45). Surprisingly, no molecular evidence has demonstrated a role for the Vα gene segment in the recognition of MHC or Ag/MHC complex. This is in part because few Vα-specific mAb have been available (46, 47). Moreover in man, the greater number of germline gene segments, the more polymorphic MHC, and the inability to control genetic variation account for the fact that data measuring the actual TCR α or β repertoire and its relation to MHC-linked recognition and selection are currently very limited.

This study analyzes the cell surface expression of the human TCR Vα12.1 gene product on CD4$^+$ versus CD8$^+$ T cells. Using a Vα specific mAb, expression of the Vα12.1 gene segment was found to be significantly higher on CD8$^+$ T cells in many of the individuals examined. Since CD8$^+$ T cells in the periphery are class I MHC-restricted, the predominant expression of Vα12.1 on CD8$^+$ T cells makes it likely that the product encoded by this gene segment is used preferentially on class I MHC-restricted T cells in man. Based on our current understanding of MHC molecule expression, it is likely that the class I MHC molecule is expressed on the cell surface only in association with foreign or self-peptides (36). Therefore, Vα12.1 may be interacting directly with MHC and/or with a peptide bound to it. While a precise

definition of what the Vα is interacting with in this case is not known, the data are consistent with the likelihood that Vα12.1 is playing a role on $CD8^+$ T cells in recognition of class I MHC/Ag complex in vivo.

The increased expression of Vα12.1 in $CD8^+$ T cells was apparent also in newborn samples, where environmental influences on the TCR repertoire via antigenic challenge would not yet have occurred. Thus it seems likely that the biased Vα12.1 usage results from developmental factors, rather than environmental ones. At present, we cannot distinguish thymic from extrathymic developmental influences, nor can we implicate a particular mechanism of thymic selection. In general (48), although not in every case (49), it has been suggested that negative selection of self-reactive thymocytes results in clonal deletion at the double positive ($CD4^+8^+$) stage of differentiation, resulting in deletion of the progenitors of both $CD4^+$ and $CD8^+$ mature T cells (50). In contrast, positive selection has been suggested to result in the unequal distribution of the TCR repertoire between the $CD4^+$ or $CD8^+$ T cell subsets (7, 10, 51). However, the actual mechanisms involved in thymocyte maturation are not well understood and selection may influence the T cell receptor repertoire at several intermediate stages during differentiation (52). We find it attractive to hypothesize that positive selection in the thymus plays a role in the preferential Vα12.1 expression in the $CD8^+$ T cells by expanding a subset of MHC class I-restricted T cell precursors bearing Vα12.1. Nevertheless, the data presented do not resolve the mechanism operating and further studies are needed to understand more fully the basis of biased Vα12 expression.

In order to confirm that the Vα12.1 gene product was directly responsible for this nonrandom usage, it was important to consider the contribution made by the other TCR elements. Data from two unrelated individuals expressing typically higher Vα12.1 levels in the $CD8^+$ T cell subset showed no substantial differences in Vβ usage between $CD4^+$ and $CD8^+$ T cells. Furthermore, Jα gene usage in Vα12.1 rearrangements was examined by nucleotide sequencing of 150 distinct inframe Vα12.1 transcripts. This showed extensive diversity and no relative difference in Jα usage on $V\alpha12.1^+/CD4^+$ and $V\alpha12.1^+/CD8^+$ cells (data not shown). These results indicated that expression of the Vα gene segment itself was the primary factor or element responsible for the relatively higher Vα12.1 expression on $CD8^+$ T cells, irrespective of concomitant Jα and Vβ usage. In mice, major Vβ mediated thymocyte deletions occur as a result of Mls and class II MHC corecognition. Thus, in the murine system for some receptors the Vβ segment dominates in selection with the α chain being more passive. However in man, no striking Vβ deletions have been reported, and we have found no major deletions among ten random donors. Thus, Mls-like/MHC class II based mechanisms of Vβ deletion may participate to a smaller extent in shaping the receptor repertoire of mature human T cells. Our data on the analysis of one Vα gene suggested that, at least in humans, some receptors may be selected during development with particular Vα products dominating in the selection process.

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