A Distinct Wave of Human T Cell Receptor γ/δ Lymphocytes in the Early Fetal Thymus: Evidence for Controlled Gene Rearrangement and Cytokine Production

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Summary

The rearrangement and expression of human T cell receptor (TCR)-γ and -δ gene segments in clonal and polyclonal populations of early fetal and postnatal human TCR-γ/δ thymocytes were examined. The data suggest that the TCR-γ and -δ loci rearrange in an ordered and coordinated fashion. Initial rearrangements at the TCR-δ locus join Vδ2 to Dδ3, and initial rearrangements at the TCR-γ locus join downstream Vγ gene segments (Vγ1.8 and Vγ2) to upstream Jγ gene segments associated with Cγ1. These rearrangements are characterized by minimal junctional diversity. At later times there is a switch at the TCR-δ locus such that Vδ1 is joined to upstream Dδ gene segments, and a switch at the TCR-γ locus such that upstream Vγ gene segments are joined to downstream Jγ gene segments associated with Cγ2. These rearrangements are characterized by extensive junctional diversity. Programmed rearrangement explains in part the origin of discrete subpopulations of peripheral blood TCR-γ/δ lymphocytes that have been defined in previous studies. In addition, cytokine production by early fetal and postnatal TCR-γ/δ thymocyte clones was examined. Fetal thymocyte clones produced significant levels of IL-4 and IL-5 following stimulation, whereas postnatal thymocyte clones did not produce these cytokines. Thus, these cell populations may represent functionally distinct subsets as well.

Previous studies of clonal and polyclonal human TCR-γ/δ lymphocytes derived from peripheral blood have revealed the presence of two major subpopulations based upon variable (V) and constant (C) gene segment usage (1-11). The majority of peripheral blood TCR-γ/δ lymphocytes bear receptors using both Vδ2 and Vγ2 (Vγ9 in the nomenclature of Lefranc et al. [12]).1 The TCR-γ and -δ chains of these receptors are disulfide-linked, indicating that the TCR-γ chains are encoded by Cγ1 rather than Cγ2 (13, 14). A smaller population of peripheral blood TCR-γ/δ lymphocytes bear receptors using Vγ1. The majority of these lymphocytes use members of the Vγ1 gene family, rather than Vγ2, and the TCR-γ and -δ chains of these receptors are frequently non-disulfide linked, indicating that the TCR-γ chains are encoded by the Cγ2 gene segment. The explanation for the presence of relatively discrete populations of TCR-γ/δ lymphocytes is unclear.

Analysis of the ontogeny of murine TCR-γ/δ lymphocytes indicates that rearrangements at the TCR-γ and -δ loci occur in an orderly fashion (15-19). The first detectable wave of rearrangements in early fetal thymocytes involves the Vγ32 and Vγ1 gene segments. A distinct population of late fetal thymocytes displays rearrangements of the Vγ4 and Vγ1 gene segments. These early rearrangements are also characterized by low junctional diversity, since there is minimal incorporation of template independent N region nucleotides, and only the 3′ Dγ segment (Dγ2) is used. Murine dendritic epidermal γ/δ cells apparently originate from the early Vγ3-Vγ1 population of fetal thymocytes, and mucosal intraepithelial lymphocytes apparently originate from the subsequent Vγ4-Vγ1 population (20-22). Adult thymocytes display rearrangements

1 The nomenclature used for human Vγ and Jγ gene segments is that of Strauss et al. (66). The relationship between this scheme and that of Lefranc et al. (12, 67) is described in Ref. 63. The nomenclature for human Vδ gene segments is that of Hata et al. (8) and Takihara et al. (50), and for human Dδ and Jδ gene segments is that of Loh et al. (47).

2 The nomenclature for murine Vγ gene segments is that of Garman et al. (15), and the relationship between this scheme and those of others is described in Ref. 69.
involving distinct Vγ and Vδ gene segments, and display extensive junctional diversity (23, 24).

Based upon indirect evidence it has previously been suggested that rearrangements within the human TCR-γ locus may be ordered (25). It was proposed that the initial rearrangements at this locus join the downstream Vγ gene segments (i.e., Vγ2, Vγ3, Vγ4) to the upstream Jγ gene segments (i.e., those associated with Cγ1: Jγ1.1, Jγ1.2, Jγ1.3), whereas subsequent rearrangements join the upstream Vγ gene segments (i.e., members of the Vγ1 family) to the downstream Jγ gene segments (i.e., those associated with Cγ2: Jγ2.1, Jγ2.3). To date, this has not been confirmed by the direct analysis of gene rearrangements in human fetal thymocytes. Further, no information is available concerning the sequence of rearrangements at the human TCR-δ locus.

In this manuscript we have analyzed TCR-γ and TCR-δ gene rearrangement and expression in clonal and polyclonal early fetal and postnatal human TCR-γ/δ thymocytes. We show directly that early TCR-γ rearrangements join downstream V segments to upstream J segments, that these rearrangements occur coordinately with an early wave of Vγ2 rearrangements at the TCR-δ locus, and that these early TCR-γ and -δ gene rearrangements display limited junctional diversity.

Since our analysis showed the TCRs expressed by early fetal TCR-γ/δ thymocytes to be distinct from those of postnatal TCR-γ/δ thymocytes, we asked whether these cell populations represent functionally distinct subsets as well. We found that fetal TCR-γ/δ thymocyte clones were able to secrete significant levels of IL-4 and IL-5, whereas postnatal clones did not secrete these cytokines. Both sets of clones produce high levels of granulocyte/macrophage CSF (GM-CSF) and IFN-γ.

Materials and Methods

Establishment of TCR-γ/δ Thymocyte Clones. Postnatal thymuses were obtained from children undergoing corrective heart surgery and were kindly provided by Dr. D. Regal, Centre de Transfusion Sanguine, Lyon, France. Fetal thymic material was obtained from J. L. Touraine, Hopital Eduard Herriot, Lyon, France. Thymocyte suspensions were prepared by gently teasing thymus tissue into a single cell suspension. An entire lobe was used to ensure that both cortical and medullary cells were represented. The fetal thymocyte samples and the samples from child thymus OM were cultured in a mixture of 20 U/ml IL-2 and 100 U/ml IL-4 for 10 d. This procedure increased the number of TCR-γ/δ cells from <1% to 10% (OM), 5% (Solo), 6% (BB), and 7% (CC). The cells were then stained with anti-TCR-δ mAb TCR-δ1 (29) which recognizes an epitope on the TCR-δ chain, and were washed and incubated with goat anti-mouse-labeled human redbloodcells. The TCR-δ* cells were then stained with antiTCR-S1, which recognizes an epitope on the TCR-δ chain, and were washed and incubated with goat anti-mouse-labeled human redbloodcells. The TCR-δ*/δ cells were enriched as described previously (26). TCR-γ/δ cells of individual FH (4 mo old) were isolated from a fresh thymocyte sample using a combination of magnetic bead and FACS sorting as follows: thymocytes were incubated with anti-CD4 (RIV6; kindly provided by Dr. Kreeftenberg, RIV, Bilthoven, Netherlands) and anti-CD8 (WT-82; a kind gift from Dr. W. Tax, University of Nijmegen, Netherlands) mAbs for 30 min at 4°C, and were washed and then incubated with biotinylated goat anti-mouse antibody (Tago, Inc., Burlingame, CA) for 30 min. After washing, the cells were incubated for 5 min with FITC-labeled avidin (Becton Dickinson & Co., Mountain View, CA), and subsequently with biotinylated magnetic beads. CD4* CD8* cells were then removed on a MACS (27). The remaining FITC-labeled cells were then removed by sorting on a FACStar plus (Becton Dickinson) and <0.5% of the depleted cell population stained with PE-labeled anti-CD3 (PE-Leu 4, Becton Dickinson) as indicated by reanalysis of the sorted cells. The CD4* CD8* FH cells and the TCR-γ/δ enriched cultures of Solo, BB, CC, and OM were cloned by limiting dilution at 0.3 cell per well in a 96-well plate (Linbro, Flow Laboratories, McLean, VA) in the presence of a feeder cell mixture consisting of 5 x 10^5/ml irradiated (4,000 rad) PBL, 5 x 10^5/ml irradiated (5,000 rad) cells of the EBV-transformed B cell line JY, and 0.1 µg/ml purified PHA (Wellcome Diagnostics, Dartford, England). Yssel’s medium (28) was used for all cell cultures. The clones were expanded by weekly stimulations with the feeder cell mixture. 3 d after each stimulation, the cultures were split and 20 U/ml rIL-2 was added. The feeder cells deteriorated between day 2 and 4 at day 5 or 6 no feeder cells were visible or detectable with mAbs specific for B cells, monocytes, or TCR-α/β (data not shown). For molecular analysis of Vγ and Vδ usage, bulk cultures of the enriched BB and CC cells were maintained by weekly stimulations with feeder cells and PHA.

Monoclonal Antibodies and Immunofluorescence. The anti-TCR γ/δ mAb TCR-δ1 (29) was a kind gift of Dr. M. Brenner (Dana Farber Cancer Institute, Boston, MA); CTS1 (30) was purchased from T Cell Sciences Inc. (Boston, MA); and the mAbs BB3 (31) and Try A (32) and were gifts from Drs. L. Moretta (Genova, Italy) and D. Hercend (Institute Gustave Roussy, Paris, France), respectively. Characterization of the TCR-γ/δ T cell clones was carried out as described previously (28). Two hundred thousand cells were incubated with mAb for 30 min at 4°C, washed, and then incubated with FITC labelled goat anti-mouse F(ab)2fragments (Tago Inc., Burlingame, CA) for 30 min at 4°C. Cells were washed and analyzed on a FACScan.

DNA Fragments and Oligonucleotides. The 5′D probe is a 1.3-kb EcoRI-Bam HI fragment mapping 5′ of D1α that was isolated from the genomic clone BSKH (a gift of S. Ang, Harvard Medical School, Cambridge, MA). All other DNA probes have been described previously (8, 33–35). Oligonucleotides used for the PCR were: Vγ1, 5′GGGGTGCAAGAGTGTGGTAGCATTA; Vγ2, 5′GGGGTGACCTCCCTGAGTTGCAATGAG; Vδ3, 5′GGGCTGACACTGTATATCATAAATC; Vδ17 (Vδ5), 5′GGGTCGACTATCTAAACAGCATGTG; Vγ1, 5′TATACCTGATGTCATTACACCAGA; Vγ2, 5′GGGGTGACCTGAGTTGCAATGAG; Vδ1, 5′GGGGATCCTCCAGCTGCTGTTCCGTT; Vδ3, 5′GGGGACTACCCAATGAGATAGAGTCC; Vδ11.1, 5′GGGGATTCCAGTTAAGGATTTAGG; Vδ1.3, 5′GGGGATCCTGAGCTTACAACTGTTG.

Preparation and Blot Hybridization of Genomic DNA and RNA. Genomic DNA and RNA were prepared by established procedures (36, 37). Gel electrophoresis, blotting, hybridization with 32P-labeled probes, and washing were as described previously (8).

Polymerase Chain Reaction. Genomic DNA (0.5 µg) was heated to 94°C for 7 min and was amplified under mineral oil for 35 cycles in a 25 µl reaction containing 0.2 mM each deoxyribonucleotide triphosphate, 50 mM KCl, 1.25 mM MgCl2, 0.01% gelatin, 100 mM Tris-HCl (pH 8.3), 1 U Taq polymerase, and 20 pmol each oligonucleotide (38). Each cycle consisted of a 0.8 min denaturat-
tion step at 94°C, a 1.0-min annealing step at 51°C, and a 2.0-min extension step at 72°C. The extension step following the last cycle was for 9.9 min. One-fifth of each reaction was analyzed by agarose gel electrophoresis. The remainder was extracted with chloroform, phenol/chloroform (1:1), and chloroform, and was ethanol precipitated. Pelleted DNA was resuspended, digested using appropriate restriction enzymes, and purified through low gelling temperature agarose. Fragments were then cloned into appropriately digested vectors prepared from Bluescript KS+ (Stratagene, La Jolla, CA), and plasmid mini-preps were prepared. When DNA from a polyclonal cell source was amplified, secondary transformants and mini-preps were prepared in order to obtain pure clones. Nucleotide sequences were determined on both strands by the dideoxy chain termination method using double-stranded templates (39) and modified T7 polymerase (40) (Sequenase; U.S. Biomedical Corp., Cleveland, OH).

Stimulation of T Cell Clones and Assays for Cytokine Production. Cells were stimulated on day 7 after the last stimulation with feeder cells, and were collected, spun, and then washed two times with medium. The cells were stimulated at a concentration of 10³ cells/ml per well of a 24-well plate (Linbro). 24 h later the supernatant was removed, spun at 250 g, frozen, and kept at -20°C until testing. IL-4 (41), IL-5 (42), IFN-γ (43), and GM-CSF (42) were determined by ELISA as described. IL-2 levels were determined using the IL-2-dependent cell line CTLL-2 as described (44).

Results

Analysis of Cell Surface Expression of TCR-γ/δ on Thymocyte Clones. Thymocytes of three different fetal samples were obtained: Solo (8.5 wk of gestational age), BB (12 wk), and CC (15 wk). Postnatal thymus samples were obtained from two children: OM (6 mo old) and FH (4 mo old). The procedures to obtain the Solo, BB, CC, and OM clones were similar. Thymocytes were cultured in IL-2 and IL-4 for 10 d, and the CD4- CD8- cells were enriched by reversed rosetting and cloned by limiting dilution. The FH clones were obtained from freshly isolated purified CD3+ CD4- CD8- cells as indicated in Materials and Methods. To begin to investigate the Vδ and Vγ usage of these clones, the cells were incubated with mAbs that recognize epitopes encoded by different Vγ and Vδ gene segments. 9 of 15 postnatal thymocyte clones from sample FH reacted with the δTCS1 mAb (Table 1), indicating that these clones expressed Vδ1-Jδ1 determinants (10, 45). None reacted with mAb BB3, which detects a Vδ2 determinant (4, 9, 46). Most of the Vδ1+ clones were negative with the TiyA mAb, which detects a Vγ2 determinant (1), although two of the nine Vδ1+ clones did react with this antibody (Table 1). One of the FH clones (FH3) reacted with TiyA but with neither δTCS1 nor BB3, and the remaining FH clones were negative with all of these mAbs. Of the four OM clones, two (OM35 and OM64) reacted with the δTCS1 mAb, and one (OM35) reacted with the TiyA mAb. These observations confirm the findings of others that a large fraction of postnatal TCR-γ/δ thymocytes are Vδ1+ , that some of these cells coexpress Vδ1 and Vγ2, and that very few postnatal TCR-γ/δ thymocytes are Vγ2+ (5, 6, 10, 47).

In contrast to these results with postnatal thymocytes, all of the Solo, BB, and CC fetal thymus clones reacted with the BB3 mAb, and none reacted with δTCS1 (Table 1). Most of these were also TiyA+ , indicating coexpression of Vγ2 and Vδ2, although two of the three Solo clones (Solo 6 and 15) were TiyA- . In addition, at early stages in the expansion of the polyclonal BB and CC cultures a small fraction of the cells were BB3+ and TiyA- (data not shown), although all of the clones obtained were BB3+ TiyA+ . These data suggest that Vδ2 is the predominant Vδ gene segment used in early fetal thymocytes, and that although Vγ2 is also

Table 1. Reactivity of Fetal and Postnatal TCR-γ/δ Clones with mAbs Specific for Epitopes on the Constant and Variable Regions of the γ and δ Chains

| Donor | (Gestational) Age | Clones | TCR-δ1 | δTCS1 | BB3 | TiyA |
|-------|------------------|--------|--------|--------|------|------|
| Solo  | (8.5 wk)         | 6,15   | +      | -      | +    | -    |
|       |                  | 19     | +      | -      | -    | +    |
| BB    | (12 wk)          | 2,4,5,8,10 | +      | -      | +    | -    |
| CC    | (15 wk)          | 6,7,15,27,33,40 | +      | -      | +    | -    |
| FH    | 4 mo             | 1,6,9,20,26 | +      | -      | -    | -    |
|       |                  | 7,9,10,13,14,17,28 | +      | -      | -    | -    |
|       |                  | 16,31  | +      | -      | -    | -    |
|       |                  | 3      | +      | -      | -    | +    |
| OM    | 6 mo             | 47,54  | +      | -      | -    | -    |
|       |                  | 64     | +      | -      | -    | -    |
|       |                  | 35     | +      | -      | -    | +    |

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used with high frequency, V<sub>y2</sub> usage is not strictly linked to V<sub>y2</sub> usage within this cell population, despite coordinate usage in peripheral blood (4, 8-10).

TCR-γ and δ Rearrangements in Postnatal Thymocytes. To extend the above findings, we first analyzed the TCR-γ and TCR-δ gene rearrangements and transcripts in the OM postnatal thymocyte clones. By hybridization with a J<sub>y1.3</sub>/2.3 probe in KpnI and EcoRI digests of genomic DNA, and with V<sub>y2</sub> and V<sub>y3</sub> probes in KpnI digests, we identified two rearrangements in each T cell clone. Some rearrangements were further characterized by sequence analysis of PCR products amplified from genomic DNA using V and J specific oligonucleotide primers. We found the array of rearranged V<sub>γ</sub> gene segments to be heterogeneous, including multiple members of the V<sub>γ1</sub> family, as well as V<sub>γ2</sub> and V<sub>γ3</sub> (Table 2). Based on these results and on surface staining with the TiyA mAb (Table 1), OM35 carries an in-frame V<sub>y2</sub> rearrangement, and OM54 and OM64 carry in-frame V<sub>y1</sub>.4 and V<sub>y1</sub>.8 rearrangements, respectively. OM47 displays predominantly V<sub>y1</sub> transcripts, and therefore is likely to carry an in-frame V<sub>y1</sub>.3 rearrangement. Despite the observed heterogeneity in the rearranged V gene segments, it is striking that seven of the eight rearrangements in these cells involve J<sub>γ</sub> segments upstream of C<sub>y2</sub>, namely, J<sub>y2.1</sub> and J<sub>y2.3</sub>.

Assignment of the TCR-δ gene rearrangements in these cells was accomplished by hybridization of J<sub>a1</sub>, J<sub>a3</sub>, V<sub>a1</sub>, V<sub>a2</sub>, 5'D<sub>a</sub>, and V<sub>a17</sub> probes to XbaI digests of genomic DNA. The V<sub>a17</sub> probe was included because this V segment has been previously mapped to the genomic region between the V<sub>a1</sub> and V<sub>a2</sub> gene segments (35). We identified V<sub>a1</sub> rearrangements in OM35 and OM64, and a V<sub>a3</sub> rearrangement in OM54. Based upon nucleotide sequence anal-

Table 2. TCR-δ and -γ Gene Rearrangements in Fetal and Postnatal Thymocyte Clones

| Southern | Northern | J<sub>a1</sub> | J<sub>a3</sub> | V<sub>a1</sub> | V<sub>a17</sub> | V<sub>a2</sub> | 5'D<sub>a</sub> | Rearrangements |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Solo 6 | ND | 4.4, 3.4 | ND | ND | R, G | D, G | V<sub>a2</sub> - J<sub>a3</sub> | D-D-J<sub>a</sub> |
| Solo 15 | ND | 4.2 | 5.4, 3.4 | ND | ND | R, R | D, D | V<sub>a2</sub> - J<sub>a1</sub> | V<sub>a2</sub> - J<sub>a3</sub> |
| Solo 19 | V<sub>a2</sub> | 1.7 | 5.4, 3.4 | G, G | G, G | R, G | D, G | V<sub>a2</sub> - J<sub>a3</sub> | Germline |
| OM35 | V<sub>a1</sub> | 6.4 | 5.4 | R, R | D, D | D, D | D, D | V<sub>a1</sub> - J<sub>a1</sub> | V<sub>a1</sub> - J<sub>a3</sub> |
| OM47 | V<sub>a17</sub> | 2.9 | 5.4, 7.5 | G, G | G, G | D, G | D, G | V<sub>a17</sub> - J<sub>a3</sub> | D-D-J<sub>a1</sub> |
| OM54 | V<sub>a3</sub> | 3.9, 1.9 | 5.4 | G, G | G, G | D, G | D, G | V<sub>a3</sub> - J<sub>a1</sub> | - J<sub>a1</sub> |
| OM64 | V<sub>a1</sub> | 6.4, 3.2 | 5.4 | R, G | D, G | D, G | D, D | V<sub>a1</sub> - J<sub>a1</sub> | - J<sub>a1</sub> |

| Southern | KpnI digest | EcoRI digest |
|-----------------------------|---------------|---------------|
| J<sub>y1.3</sub>/2.3 | V<sub>y2</sub> | V<sub>y3</sub> | J<sub>y1.3</sub>/2.3 |
| Solo 6 | ND | 16, 14, 8.5 | R, D | D, D | 3.2, 1.5 | V<sub>y1.8</sub>-J<sub>y1.1</sub> | V<sub>y2</sub> - J<sub>y1.1</sub> |
| Solo 15 | ND | 16, 8.5 | D, D | D, D | 3.2, 1.5 | V<sub>y1.8</sub>-J<sub>y1.1</sub> | V<sub>y1.8</sub>-J<sub>y1.1</sub> |
| Solo 19 | V<sub>y2</sub> | 16, 9.0, 7.9 | R, G | D, G | 3.2, 3.1 | V<sub>y2</sub> - J<sub>y1.3</sub> | Germline |
| OM35 | V<sub>y2</sub> | 7.6, 7.2 | R, R | D, D | 2.3, 1.8 | V<sub>y2</sub> - J<sub>y1.3</sub> | V<sub>y2</sub> - J<sub>y1.3</sub> |
| OM47 | V<sub>y1</sub> | 16, 5.0, 1.8 | D, G | R, D | 5.0, 3.2 | V<sub>y1.3</sub> - J<sub>y1.3</sub> | V<sub>y3</sub> - J<sub>y2.1</sub> |
| OM54 | V<sub>y1</sub> | 7.6, 1.8 | R, D | D, D | 2.3, 0.9 | V<sub>y1.4</sub>-J<sub>y1.3</sub> | V<sub>y2</sub> - J<sub>y2.3</sub> |
| OM64 | V<sub>y1</sub> | 5.0, 1.8 | D, D | D, D | 4.0, 3.2 | V<sub>y1.8</sub>-J<sub>y1.3</sub> | V<sub>y1.8</sub>-J<sub>y2.1</sub> |

Assignment of TCR-δ gene rearrangements is according to reference 8, and assignment of TCR-γ gene rearrangements is according to references 66 and 67. Known or presumed in-frame rearrangements are in the first column, except for Solo 15, which displays two in-frame rearrangements. Assignments marked by an asterisk were analyzed and confirmed by PCR and nucleotide sequence analysis. The second J<sub>a1</sub> rearrangements in OM54 and OM64 are undefined. Sizes of the fragments detected by J<sub>a1</sub>, J<sub>a3</sub>, and J<sub>y1.3</sub>/2.3 probes are in kilobases. Fragments detected by other probes are either G, germline; R, rearranged; D, deleted. Germline fragments are: J<sub>a1</sub>, 1.7 kb XbaI; J<sub>a3</sub>, 5.4 kb XbaI; J<sub>y1.3</sub>, 9.0 kb KpnI and 1.5 kb EcoRI; J<sub>y2.3</sub>, 16 kb KpnI and 3.2 kb EcoRI.

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The sequence of a portion of the rearranged V segment is PCR amplification (Fig. 1A) demonstrated to be in-frame. The nucleotide sequence analysis of TCR-δ gene rearrangements in postnatal thymocyte clones. (A) Junctional sequences were determined from PCR products amplified using appropriate pairs of primers. D elements were assigned with the requirement for a minimum of three contiguous matches to the germline sequence. P nucleotides were assigned according to reference 19. Deduced amino acid sequences are in the single letter code; a shift to lowercase letters indicates a shift to an inappropriate reading frame. (B) The nucleotide sequence of the amplified portion of the OM47 V segment is compared with the analogous portions of three previously reported V sequences: L17 Vα (48), Vα5 (50), and Vα17.1 (49). The V-OM47 sequence was confirmed using multiple DNA templates. Differences between this sequence and the published sequences are noted.

The analysis of a truncated cDNA clone representing the 3.9-kb Jα1 rearrangement in OM54 revealed an out-of-frame rearrangement involving a previously undescribed V segment that maps between Vα17 (=Vα5) and Vα2 (data not shown). The 3.2-kb Jα1 rearrangement in OM64 was not characterized.

TCR-γ and -δ Gene Rearrangements in Early Fetal Thymocytes. We next analyzed the TCR-γ and -δ gene rearrangements in the 8.5-wk fetal thymus clones Solo 6, 15, and 19, and found them to be strikingly different from those observed in the postnatal thymocyte clones (Table 2). Southern blots revealed the rearrangement of both Vγ2 and Vγ1 gene segments. Notably, all five TCR-γ rearrangements were found to involve the Jγ1, rather than the Jγ2 cluster, whereas one chromosome was in the germline configuration. To identify the rearranged Vγ1 gene segments, each V-J rearrangement was amplified by PCR using an oligonucleotide that anneals to all Vγ1 sequences as a 5' primer, and a Jγ1 oligonucleotide as a 3' primer. Nucleotide sequence analysis indicated the rearrangement of Vγ1.8 in every instance and showed that Solo 6 and Solo 15 each carry a single in-frame Vγ1.8 rearrangement (Fig. 2). Similar PCR analysis revealed the Vδ2 rearrangement in Solo 19 to be in-frame, and that in Solo 6 to be out-of-frame (Fig. 2), in accordance with staining results (Table 1).

Together with the staining analysis of the Solo, BB, and CC clonal and polyclonal lines (Table 1), the above results indicate that whereas Vγ2 rearrangements are common in postnatal thymocytes, Vγ1 rearrangements occur as well. Further, these results suggested that the rearrangement of Vγ1 gene segments in early fetal thymocytes might not be random. To specifically address this issue, we analyzed DNA prepared from polyclonal BB and CC cultures by PCR using Vγ1 and Jγ1.1 primers. Six of six clones sequenced that were obtained following amplification of BB DNA used Vγ1.8 (Fig. 3). Two of four clones obtained following amplification of CC DNA used Vγ1.8, whereas one clone each used Vγ1.3 and Vγ1.4. All clones were independent based upon their V-J junction nucleotide sequences. Further, 8 of 10 were out-of-frame, in accordance with staining data indicating the BB and CC cultures to be composed primarily of Thy1+ TCR-γ/δ lymphocytes and TCR-α/β lymphocytes. These results provide strong evidence that Vγ1 rearrangements in early fetal thymocytes involve Vγ1.8 almost exclusively, at least
sequences with differences noted. They are compared with the germline V\(_{\gamma}\) polyclonal fetalthymocyte functionalsequences were determined from PCR products amplified using V\(_{\gamma}\) primers. They are compared with the relevant germline gene segment maps 3' of C\(_{\gamma}\) and would not delete the region hybridizing to the 5'D\(_{\gamma}\) probe upon rearrangement, the predicted size of a V\(_{\gamma}3\)-J\(_{\gamma}2\) rearranged fragment is only 3.3 kb.

The V-D-J junctional regions of the V\(_{\gamma}2\) rearrangements in these cell lines were amplified by PCR and their nucleotide sequences were determined (Fig. 2). Notably, all four rearrangements were found to be functional. Solo 15 therefore displays two in-frame rearrangements, and possibly two \(\gamma/\delta\) TCRs on its cell surface. This apparent violation of allelic exclusion could have arisen if the two rearrangements occurred nearly simultaneously. However, it is intriguing that other reports have suggested that allelic exclusion at the TCR \(\alpha\)-locus may not be complete (51–53). Nevertheless, these data, along with the results of surface staining (Table 1), argue that V\(_{\gamma}2\) rearrangements predominate in early fetal thymocytes, even though, as demonstrated in this and in other studies, they are rare in postnatal thymocytes (5, 6, 10, 47).

**Minimal Diversity of Early Fetal Thymocyte TCR-\(\gamma\) and \(\delta\) V(D)J Junctions.** A striking feature of the TCR-\(\gamma\) and \(\delta\) junctional sequences presented in Fig. 2 is the limited diversity they display relative to the junctions of rearrangements in postnatal thymocytes (Fig. 1A) (47) and peripheral blood lymphocytes (7, 8, 34, 47). Two of the five TCR \(\gamma\) V-J junctions analyzed display no incorporation of template independent N-region nucleotides, and a total of only six N-region nucleotides are seen in the five junctions analyzed. The TCR-\(\gamma\) V-D-J junctions are even more striking. They reveal only two N-region nucleotides among the four sequences, with three of the junctions displaying no N-nucleotide incorporation. Further, the only D\(_{\gamma}\) segment used is D\(_{\gamma}3\). Hence, these V-D-J junctional sequences bear marked similarities to those described for TCR-\(\delta\) gene rearrangements in very early murine fetal thymocytes: the use of a single V gene segment, the use of only the 3' D gene segment, and minimal N nucleotide incorporation (16–19).

To confirm and extend these findings, we analyzed the junctions of V\(_{\gamma}2\) rearrangements in the polyclonal BB and CC fetal thymocyte cultures. Southern blot analysis of Xbal

![Figure 2](image2.png)

![Figure 3](image3.png)
digested BB and CC genomic DNA using a radiolabeled Vα2 probe revealed that in both populations Vα2 rearrangements to Jα3 predominated over those to Jα1 (data not shown). We then used PCR to amplify both types of junctions in each sample. The analysis of 13 junctions of 12-wk fetal thymocytes and 17 junctions of 15-wk fetal thymocytes is presented in Fig. 4. All 30 junctions display portions of the Dα3 gene segment. By contrast, only three display possible contributions from Dα1, and two from Dα2. However, since in these instances the maximal contribution is only three nucleotides, apparent Dα1 and Dα2 contributions could have arisen by chance. We conclude that Dα1 and Dα2 rearrangements are rare events in early fetal thymocytes.

These junctions also display minimal incorporation of N-region nucleotides. Roughly one-third of the 15-wk junctions, and one-half of the 15-wk junctions, display no N nucleotides. The average number of N-nucleotides per junction increases from 0.5 at 9 wk to 2.4 at 12 wk and 2.7 at 15 wk. When only N-nucleotide positive junctions are considered, these numbers increase to 2.0 at 9 wk, 3.7 at 12 wk, and 5.4 at 15 wk. By contrast, the sequences of five different junctions from the OM postnatal thymocyte γ/δ T cell clones (using Vα1, Vα3, and Vα5) reveal in most instances the usage of both Dα3 and Dα2, and display much more extensive N-nucleotide incorporation (15.8 per V-D-D-J junction) (Figs. 1 and 4).

To determine whether the differences noted between early fetal and postnatal thymocyte TCR-δ junctions are related to the age of the sample rather than the particular V gene segment rearranged, we amplified by PCR a single rare example of Vα1 to Jα3 rearrangement in each of the polyclonal fetal thymus samples (Fig. 4). Both junctions display the use of only the Dα3 gene segment, and display no N-nucleotide incorporation. These data therefore argue that TCR-δ junctional diversity increases in an age-related fashion throughout early thymic development, and is only indirectly associated with V segment usage.

**Cytokine Production by Fetal and Postnatal TCR-γ/δ Thymocyte Clones.** In order to reveal possible functional differences between the Vα2+ fetal and Vα2− postnatal thymocyte clones, we analyzed the capacity of the clones to produce cytokines upon stimulation. Therefore the clones were stimulated with 30 μg/ml ConA, a powerful polyclonal stimulant for human T cell clones (54), and the supernatants were assayed for IL-2, IL-4, IL-5, IFN-γ, and GM-CSF. Table 3 shows the cytokine production profiles of seven fetal TCR-γ/δ clones from samples Solo, BB and CC, and seven postnatal TCR-γ/δ clones from donors OM and FH. All fetal clones produced significant levels of IL-4 after activation with ConA. The fetal clones also produced IL-5 and high levels of GM-CSF and IFN-γ. Three additional fetal clones also produced IL-4 and IL-5 after activation, but one (CC40) consistently failed to produce IL-4 or IL-5, and secreted low levels of GM-CSF but significant amounts of IFN-γ (data not shown). In contrast to the fetal clones, the clones derived from postnatal thymic material did not produce IL-4 or IL-5 above background (Table 3). In general, the clones produced less GM-CSF than the fetal clones, although the levels of IL-2 and IFN-γ were comparable (Table 3). The same pattern of cytokine production was observed with five other postnatal TCR-γ/δ thymocyte clones (data not shown).

To determine the capacity of these clones to produce cytokines after activation via the TCR/CD3 complex or CD2, they were stimulated with a CD3 mAb in the presence or absence of the phorbol ester PMA or by a mitogenic combination of anti-CD2 mAbs. As a control, the clones were stimulated with PMA alone, a treatment that normally cannot activate mature T cells on its own (54). The cytokine production pattern of three representative fetal and three postnatal TCR-γ/δ clones is presented in Table 4. The combination of anti-CD2 mAbs was able to induce cytokine production in all clones. As expected, the anti-CD3 mAb SPVT3b only induced cytokine production in combination with PMA. Interestingly, PMA alone activated the cells to produce cytokines. Whereas 1 ng/ml PMA was sufficient to trigger secretion of significant levels of cytokines (data not shown), 10 ng/ml was found to be optimal. The patterns of cytokine production after activation via CD2 or TCR/CD3 were the same as after activation via ConA, inasmuch as the fetal thymo-
Each value is the mean ± SD of duplicate determinations. One of three representative experiments is shown. Cytokine production varied quantitatively, but not qualitatively, between experiments.

**Table 3. Cytokine Production by Fetal and Postnatal Thymocyte TCR-γ/δ Clones Stimulated with 30 μg/ml ConA**

| Clone | ConA | IL-2  | IL-4  | IL-5  | IFN-γ | GM-GSF |
|-------|------|-------|-------|-------|-------|--------|
| Solo 6 | -    | <0.5 | <50   | ND    | <0.3  | <0.05  |
|       | +    | 4.0  | 2,397 ± 289 | ND    | 125 ± 3 | 22 ± 3  |
| Solo 15 | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 10.0 | 200 ± 27 | 796 ± 102 | 25 ± 4 | 16 ± 1.3 |
| Solo 19 | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 6.7  | 496 ± 104 | 575 ± 43 | 89 ± 3 | 26 ± 9  |
| BB2   | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 6.5  | 1,658 ± 108 | 270 ± 39 | 220 ± 57 | >20     |
| BB5   | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 5.0  | 502 ± 100 | 542 ± 88 | 110 ± 16 | 23 ± 7  |
| CC6   | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 9.6  | 1,096 ± 31 | 1,540 ± 500 | 81 ± 8 | 19 ± 3  |
| CC7   | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 8.0  | 286 ± 9 | 96 ± 20 | 121 ± 25 | 2 ± 0.2 |
| OM35  | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 4.3  | 502 ± 100 | 542 ± 88 | 110 ± 16 | 23 ± 7  |
| OM47  | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 3.9  | 1,096 ± 31 | 1,540 ± 500 | 81 ± 8 | 19 ± 3  |
| OM64  | -    | <0.5 | <50   | <50   | <0.3  | 0.5 ± 0.02 |
|       | +    | 5.0  | 1,096 ± 31 | 1,540 ± 500 | 81 ± 8 | 19 ± 3  |
| FH3   | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 9.3  | 502 ± 100 | 542 ± 88 | 110 ± 16 | 23 ± 7  |
| FH13  | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 4.6  | 502 ± 100 | 542 ± 88 | 110 ± 16 | 23 ± 7  |
| FH26  | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 9.6  | 1,096 ± 31 | 1,540 ± 500 | 81 ± 8 | 19 ± 3  |
| FH31  | -    | <0.5 | <50   | <50   | <0.3  | <0.05  |
|       | +    | 7.3  | 286 ± 9 | 96 ± 20 | 121 ± 25 | 2 ± 0.2 |

Each value is the mean ± SD of duplicate determinations. One of three representative experiments is shown. Cytokine production varied quantitatively, but not qualitatively, between experiments.

**Discussion**

By analyzing the rearrangement and expression of the TCR-γ and -δ genes in early human fetal thymocytes we have obtained strong evidence that rearrangement at these loci occurs in an orderly and coordinated fashion, summarized schematically in Figure 5. Our data indicate that the initial rearrangement events at the TCR-δ locus involve the joining of the Vδ2 gene segment to the Dδ3 gene segment. The initial rearrangement events at the TCR-γ locus involve the joining of downstream Vγ gene segments, primarily Vγ1.8 and Vγ2, to Jγ gene segments of the Jγ1 cluster. At later times there is a switch in the rearrangement pattern at the TCR-δ locus such that the Vδ1 gene segment (as well as some other Vδ gene segments), is joined primarily to the upstream Dδ3 gene segments, Dδ1 and Dδ2. Similarly, there is a switch in the rearrangement pattern at the TCR-γ locus such that upstream Vγ gene segments in the Vγ1 family are joined to downstream Jγ gene segments of the Jγ2 cluster. Superimposed on this highly ordered rearrangement pattern is a gradual increase in diversification at the junctions of the rearranged gene segments through the incorporation of template-independent N region nucleotides. Our results are almost certainly due to control at the level of gene rearrangement rather than to selection based upon surface TCR expression, since the out-of-frame rearrangements that were ana-
lyzed displayed properties that were highly similar to the in-frame rearrangements.

Previous studies have shown that the predominant population of TCR-γ/δ lymphocytes in human peripheral blood displays cell surface TCRs that pair TCR δ chains using V\(_{δ2}\) with TCR γ chains using V\(_{γ2-C_γ1}\) (4, 8–10). We have previously demonstrated in transfection experiments that there is no physical barrier that prevents V\(_{δ2}\)+ TCR δ chains from pairing with V\(_{γ1-C_γ2}\) TCR γ chains (46). Consistent with this result, and the recent analysis of a large panel of postnatal thymocyte clones (10), we find that two of three early thymocyte clones display cell surface TCRs composed of V\(_{δ2}\)+ TCR δ chains paired with V\(_{γ1,8-C_γ1}\) TCR γ chains. We therefore suggest that the predominant V\(_{δ2}\)/V\(_{γ2-C_γ1}\) peripheral blood population arises as the result of two distinct processes. Coordination between rearrangement events at the TCR-γ and -δ loci as described here dictates that TCR-γ/δ lymphocytes that use V\(_{δ2}\) will also express TCR γ

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**Table 4. Cytokine Production by Fetal and Postnatal Thymocyte TCR-γ/δ Clones after Activation via CD3 or CD2**

| Clone   | Stimulus | IL-2 U/ml | IL-4 pg/ml | IL-5 pg/ml | IFN-γ ng/ml | GM-CSF ng/ml |
|---------|----------|-----------|------------|------------|-------------|--------------|
| BB2     |          |          |            |            |             |              |
|         | –        | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | αCD2     | 2.0       | 400 ± 3    | 389 ± 39   | 27 ± 4.4    | 3.4 ± 0.3    |
|         | αCD3     | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | PMA      | 0.9       | <50        | 550 ± 160  | 5 ± 0.06    | 2.1 ± 0.2    |
|         | αCD3 + PMA | 5.5     | 538 ± 16   | 1,072 ± 180| 93 ± 0.4    | 61 ± 14      |
| BB8     |          |          |            |            |             |              |
|         | –        | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | αCD2     | 7.6       | 1,814 ± 22 | 7,020 ± 2,670 | 39 ± 0.2   | 23 ± 6.9    |
|         | αCD3     | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | PMA      | 0.9       | <50        | 622 ± 218  | 6 ± 0.1     | 2.1 ± 0.1    |
|         | αCD3 + PMA | 4.2     | 203 ± 10   | 3,034 ± 24 | 38 ± 8      | 5.4 ± 1.7    |
| BB10    |          |          |            |            |             |              |
|         | –        | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | αCD2     | 28.2      | 1,270 ± 50 | 5,104 ± 1,048 | 38 ± 3     | 27 ± 0.5    |
|         | αCD3     | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | PMA      | 1.5       | <50        | 4,452 ± 460| 14 ± 6      | 1.8 ± 0.1    |
|         | αCD3 + PMA | 4.0     | 678 ± 118  | >10,000    | 38 ± 9      | 12.2 ± 0.3   |
| FH3     |          |          |            |            |             |              |
|         | –        | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | αCD2     | 4.5       | <50        | <50        | 93 ± 3.8    | 93 ± 0.6     |
|         | αCD3     | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | PMA      | <0.5      | <50        | 26 ± 0.6   | 2.4 ± 0.2   |
|         | αCD3 + PMA | <0.5     | <50        | 55 ± 3     | 1.2 ± 0.03  |
| FH13    |          |          |            |            |             |              |
|         | –        | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | αCD2     | 11.0      | <50        | 176 ± 55   | 30 ± 0.2    | 2.4 ± 0.4    |
|         | αCD3     | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | PMA      | <0.5      | <50        | <50        | <0.3        | 1.8 ± 0.02   |
|         | αCD3 + PMA | <0.5     | <50        | 40 ± 1.6   | 0.7 ± 0.1   |
| FH26    |          |          |            |            |             |              |
|         | –        | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | αCD2     | 1.0       | <50        | <50        | 11 ± 0.5    | 1.2 ± 0.1    |
|         | αCD3     | <0.5      | <50        | <50        | <0.3        | <0.05        |
|         | PMA      | <0.5      | <50        | <50        | 52 ± 0.1    | 2.3 ± 0.1    |
|         | αCD3 + PMA | 1.0     | <50        | <50        | 32 ± 11     | 1.2 ± 0.05   |

7 d after the previous stimulation, cells were washed twice and stimulated at a concentration of 10\(^6\) cells/ml with a combination of 1 μg/ml purified SPV-T3b (70) and 1 ng/ml PMA, 10 ng/ml PMA, or a 1:2,500 dilution of ascites of the anti-CD2 mAbs X.11-1 and D66 (71). Each value is the mean ± SD of duplicate determinations. One of three representative experiments is shown.
phocytes with a distinct anatomical localization cannot be

tion of the V62/Vy2-Cy lymphocytes in peripheral blood

teous, which apparently predominates in early fetal thymic

dominated by multiple D6 segments, cumulated from peri-

tal blood TCR-γ/β lymphocytes. These observations argu-

the V61+ and V62+ subsets arise via rearrangements occur-

ting at distinct phases of thymic ontogeny. Peri-

tal blood and postnatal thymocyte V61 junctions typi-

cally display contributions from multiple D6 segments,

chains using downstream Vγ segments (Vγ1, Vγ2, etc.)

tial, however. Among these TCR-γ chains, only those

Figure 5. Controlled and coordinate TCR-γ and TCR-δ
gene rearrangement. TCR-γ locus organization is ac-

ted references 66 and 72. Open boxes represent non-

tic D6 locus organization is according to references 8, 35,

Our data demonstrate remarkable similarities between the

earliest rearrangement events at the human and murine TCR-γ

and δ loci. In both systems the repertoire of rearrangeable

V gene segments is initially highly restricted, and the usage

of homologous D6 gene segments (D63 in the human, D62

in the mouse) and J6 gene segments (J63 in the human, J62

in the mouse) predominates (16, 18, 19). Further, junc-

tional diversification by the incorporation of N-region nucleo-

tides is minimal or absent in both systems at early times, pre-

sumably as the result of low levels of terminal transferase ac-

tivity (56). However, the analysis of the V-(D)-J junctions of early

murine fetal thymocytes that use specific pairs of Vγ and Vδ

gene segments has also shown them to be highly homoge-

neous, and to encode the TCRs expressed on adult lympho-

cytes in certain epithelial locations (19–22). In marked con-

trast, the Vγ2-D63-J63 junctions analyzed here are quite

heterogeneous. The junctions of the Solo clones were all dis-

tinct, and did not match any of the junctions obtained from

the polyclonal BB and CC cultures. None of the CC junc-

tions occurred more than twice among the sequences ex-

amined, and although one BB junction was detected at high

frequency, it seems most plausible to attribute this result to

oligoclonality as the result of expansion of the BB culture

in vitro. These results would argue that early human TCR-

γ/δ lymphocytes might not be subjected to the same types

of intrathymic selection that limit the repertoire of certain

intraepithelial lymphocytes in the mouse (19–22).

Striking differences were found in the capacities of fetal

and postnatal TCR-γ/δ thymocytes to produce cytokines upon

activation, suggesting that these two sets of TCR-γ/δ thymo-

cyte clones represent functionally distinct subpopulations. In

contrast to postnatal TCR-γ/δ thymocyte clones, the fetal

cloned TCR-γ/δ thymocyte clones produced significant levels of IL-4 and IL-5 after acti-

vated with either the lectin ConA, anti-CD3 mAb plus PMA,

or a mitogenic pair of anti-CD2 mAbs. Both sets of clones

were able to secrete GM-CSF and IFN-γ. The failure of post-

natal TCR-γ/δ thymocyte clones to produce IL-4 is probably
due to a lack of IL-4 gene transcription, because with a highly

sensitive PCR technique no IL-4 mRNA could be detected

after activation of the clones with ConA (De Waal Malefyt, R.,

and H. Spits, unpublished data). Since the postnatal and fetal

clones were cultured under identical conditions, the inability

of the postnatal clones to produce IL-4 and IL-5 is not ac-

quired in vitro. Moreover, freshly isolated day 13–15 murine

CD3+ fetal thymocytes that contain only TCR-γ/δ cells pro-

duce IL-4 upon stimulation with anti-CD3 mAb (57).

We believe, therefore, that the fetal TCR-γ/δ cells have the

ability to produce IL-4 and IL-5 in vivo. The cloned TCR-

γ/δ lines produced cytokines upon activation via the

CD3/TCR-γ/δ complex or via CD2, but whether or not

TCR-γ/δ cells are actually stimulated in vivo to produce IL-4

and IL-5 is unknown. In situ hybridization should be carried
out to investigate this point.

TCR-γ/δ thymocytes might play a role in early TCR-α/β

T cell development, and if so, cytokines are presumably in-

volved. Our data could therefore be an indication that IL-4,

IL-5, or both, are important regulators of early thymic de-

velopment. In man, the main target cell of IL-5 is the eosino-
In addition, some effects of IL-5 on human B cells have been documented (58, 59), but so far no effects of IL-5 on human T cells have been reported. It is therefore unclear what the biological significance is of IL-5 for thymic development. On the other hand, T cells can respond to IL-4. While activated mature human T cells proliferate in response to IL-4 (60), this factor can inhibit activation of resting human T cells under certain conditions (61). In the mouse, CD4+CD8- day 15 fetal thymocytes respond to IL-4 in the presence of PMA, although they do not acquire CD4 or CD8 under these conditions (62). In addition, mature CD4+CD8- and CD4-CD8+ thymocytes proliferate in response to IL-4 both in mouse and in man (60, 63). Fetal human thymocytes cultured for 14 d in IL-4 contain an elevated percentage of proliferating TCR-γ/δ T cells (data not shown), indicating that unstimulated TCR-γ/δ thymocytes can respond to IL-4. Not unexpectedly, the TCR-γ/δ fetal clones respond well to IL-4 (data not shown). Further, IL-4 can induce CD8 on mature CD4+ T cells (64), which raises the possibility that IL-4 plays a role in the regulation of CD8 expression in the thymus. Lastly, it has been reported that IL-4 has the ability to induce class II MHC antigens on murine thymic macrophages, resulting in enhanced antigen presenting abilities in these cells (65). Thus, IL-4 has the potential to have pleiotropic effects on cells in the thymus. Further research will be necessary to elucidate the possible regulatory role of IL-4 in early thymic development.

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