Brief Definitive Report

Interleukin 7–induced Expression of Specific T Cell Receptor γ Variable Region Genes in Murine Fetal Liver Cultures

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Summary

We previously reported that culture of murine fetal liver (FL) cells with interleukin 7 (IL-7) results in expression of high levels of T cell receptor (TCR) γ transcripts by a population of cells expressing Thy-1 and Pgp-1, suggesting that IL-7 promotes the growth and/or differentiation of pre-T cells. We demonstrate herein that culture of FL cells for 7 d with IL-7 caused the rearrangement and expression of TCR γ variable (V) region genes Vγ4 and Vγ6, but not Vγ5 or Vγ7. Since this effect was not blocked by hydroxyurea, it appeared to represent induction of expression of these genes by IL-7 rather than expansion of a preexisting positive population. We also show that IL-7 induced RAG-1 and RAG-2 mRNA expression by FL cells. These data provide evidence that specific TCR γ/δ V region genes can be rearranged and expressed by T lineage cells before their migration to the thymus, in response to IL-7.

Lymphocytes expressing the TCR γ/δ are a unique subset of T lymphocytes, as defined by their developmentally regulated and tissue-specific expression of specific variable (V) regions of the TCR γ and δ genes, and their unique specificities and junctional region diversity (1–6). Fetal liver (FL) cells differ from the bone marrow in the capacity to give rise to specific types of γ/δ T cells, i.e., those utilizing Vγ5 genes (Tonegawa nomenclature) (7), or thymocytes with canonical Vγ4-Jγ1 transcripts (8), perhaps due to the existence of different subsets of γ/δ pre-T cells in the fetus vs. the adult. It is clearly of interest to understand the signals involved in the development of γ/δ T cells, in particular, the signals that regulate rearrangement and expression of the TCR γ/δ V region genes.

IL-7 is a cytokine synthesized by bone marrow stromal cells that has growth-promoting effects on pre-B cells and immature thymocytes (9). We previously reported that culture of murine FL cells with IL-7 results in expression of high levels of TCR γ transcripts by Thy-1$^+$ Pgp-1$^+$ cells, suggesting that IL-7 promotes the growth and/or differentiation of pre-T cells (10). We were, therefore, interested in determining whether FL cells cultured with IL-7 expressed a restricted pattern of expression TCR γ V regions. Our findings herein support the hypothesis that TCR Vγ4 and Vγ6 region genes can be rearranged and expressed by prethymic T lineage cells in response to IL-7.

Materials and Methods

Animals and Cultures. 8–10-wk-old CBA and C57BL/6 mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and bred as described (10). FL cultures were established as described previously (10). Human rIL-7 was a gift of Sterling Research Group (Malvern, PA).

Northern Blot Analysis. RNA was prepared and Northern blot analysis performed as described (10). Probes included TCR Cγ1.2-specific cDNA, provided by Dr. T. W. Mak (University of Toronto, Toronto, Canada); RAG-1– and RAG-2–specific cDNA probes were a gift of Dr. David Schatz (Howard Hughes Medical Institute, Yale University, New Haven, CT).

Reverse Transcription and PCR. cDNA synthesis was performed using the Superscript Preamplification System from GIBCO BRL (Gaithersburg, MD). For each reaction, 2 μg of RNA were reverse transcribed using a random hexamer according to the protocol supplied by GIBCO BRL, in the presence of 40 U of RNase inhibitor (Clontech, Palo Alto, CA). The cDNA was amplified using 100 pmol each of antisense Cγ1 oligonucleotide (5′-AAATGTCTGCATCAAGTCT-3′), sense Vγ4 (5′-CTTGCAACCCCTACCCATAT-3′), Vγ5 (5′-CCGCTTGGAAATTGATGAGA-3′), Vγ6 (5′-AGAGGAAAGGAAATACGGC-3′), or Vγ7 (5′-AACTTCGTCAGTTCCACAAC-3′), as described by Kyes et al. (5). Samples were also reverse transcribed and amplified using primers specific for actin (Clontech), to verify that the RNA was intact and could be reverse transcribed and amplified. We also performed reactions without RNA, to verify that samples were not contaminated with DNA that could be amplified. Samples were amplified for 30 cycles in

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a DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) using the following conditions: 94°C for 1 min, 55°C for 2 min, 72°C for 3 min.

Southern Blot Analysis of PCR Products. PCR products were analyzed on 1% agarose gels. Gels were alkaline denatured and blotted onto nylon membrane (Boehringer Mannheim Corp., Indianapolis, IN) overnight with 10× SSC, or were transferred using a vacuum blot apparatus (Hoefer Scientific Instruments, San Francisco, CA). DNA was UV cross-linked onto the membranes and blots were hybridized, washed, and incubated with anti–digoxigenin antibody conjugated to alkaline phosphatase, as described in the Genius Non-Radioactive Labeling Kit (Boehringer Mannheim Corp.). For labeling, cDNA inserts were isolated in low melting point agarose and random primed with digoxigenin-dUTP. For detection, Lumi-Phos (Boehringer Mannheim Corp.) was pipetted onto membranes in plastic sheet protectors, and then the blots were exposed to x-ray film.

Thymidine Incorporation Assay. FL cells (10^6/ml) were added to flat-bottomed microtiter wells, six wells per sample. Medium, IL-7, and hydroxyurea (HU) were added and the plate was incubated for 8 d at 37°C, 10% CO_2. Methyl-[^{3}H]thymidine (NEN-DuPont, Boston, MA) (2 μCi/well) was then added, and incorporation of ^{3}H was determined after a 4-h incubation, by harvesting the cells onto glass fiber filters and liquid scintillation counting.

Results

Expression of TCR γ V Region Genes in Response to IL-7. Specific TCR γ V regions are expressed in a developmentally regulated pattern (2, 4, 11) and different V regions predominate in different tissues (3–5, 12, 13). Thus, we sought to determine which TCR γ V region genes were expressed in the FL in response to IL-7. Since we used a cDNA probe encoding the Cγ1 region for our previous analyses of TCR γ RNA expression (10), we focused on the V regions that recombine with Cγ1 and are abundant in the fetal thymus, i.e., Vγ4, Vγ5, Vγ6, and Vγ7 (14).

Transcripts encoding each of these γ V regions were detected from fetal thymus RNA (day 18 of gestation) (Fig. 1). The same analyses were then performed using RNA from day 14 FL cells after 7 d of culture in medium alone or with IL-7 (Fig. 2). Expression of TCR γ transcripts in response to IL-7 were first verified by Northern blot (Fig. 2 *A*). The same RNA was then reverse transcribed and γ V genes amplified. We identified transcripts specific for Vγ4 and Vγ6 of the expected size, using RNA from IL-7-stimulated cultures (Fig. 2 *B*). However, TCR γ V region transcripts could not be detected after culture in medium alone (Fig. 2 *B*). We have, in some experiments, observed a smeared band spanning the expected size for a Vγ5 PCR product (Fig. 2 *B*). Fig. 3 *A* shows similar results from another set of day 14

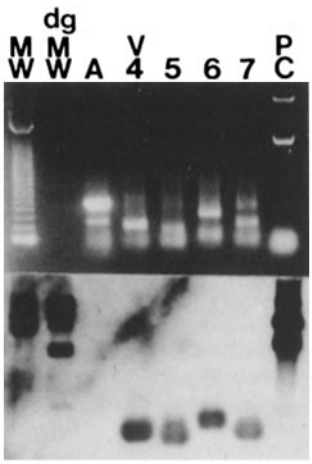
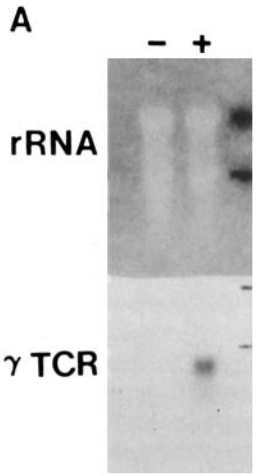


Figure 1. Vγ4–7 expression in day 18 fetal thymus. Reverse-transcriptase-PCR analysis of TCR Vγ4, -5, -6, and -7 expression using RNA from day 18 fetal thymus. (*Top*) Ethidium bromide–stained agarose gel; (*bottom*) Southern blot analysis. *MW*, 123-bp molecular weight markers; *DIG MW*, digoxigenin-labeled molecular weight markers; *A*, amplification with actin-specific primers; *V4*, *V5*, *V6*, *V7*, amplification with Vγ specific primers; PC, positive control for hybridization (Cγ1.2 cDNA).



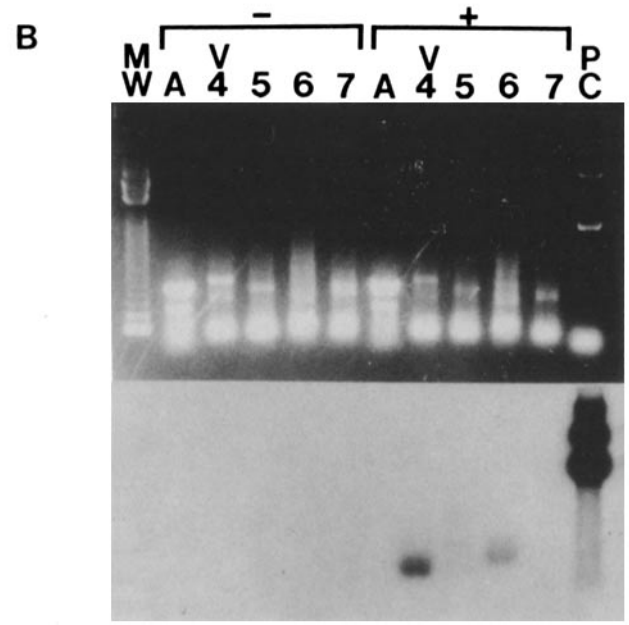


Figure 2. Vγ4–7 expression in fetal liver cells in response to IL-7. (*A*) 10 μg of total cellular RNA from FL cells cultured in medium alone (–) or with 250 U/ml IL-7 (+) for 7 d was analyzed by Northern blot for expression of TCR γ mRNA. (*Top*) Ribosomal RNA bands; (*bottom*) autoradiogram of Northern blot. (*B*) RNA was reverse transcribed and cDNA amplified using primers specific for Vγ4, Vγ5, Vγ6, Vγ7, and Cγ1, or actin. (*Top*) Ethidium bromide–stained agarose gel; (*bottom*) Southern blot analysis. *B*, blank; see Fig. 1 for other labels.

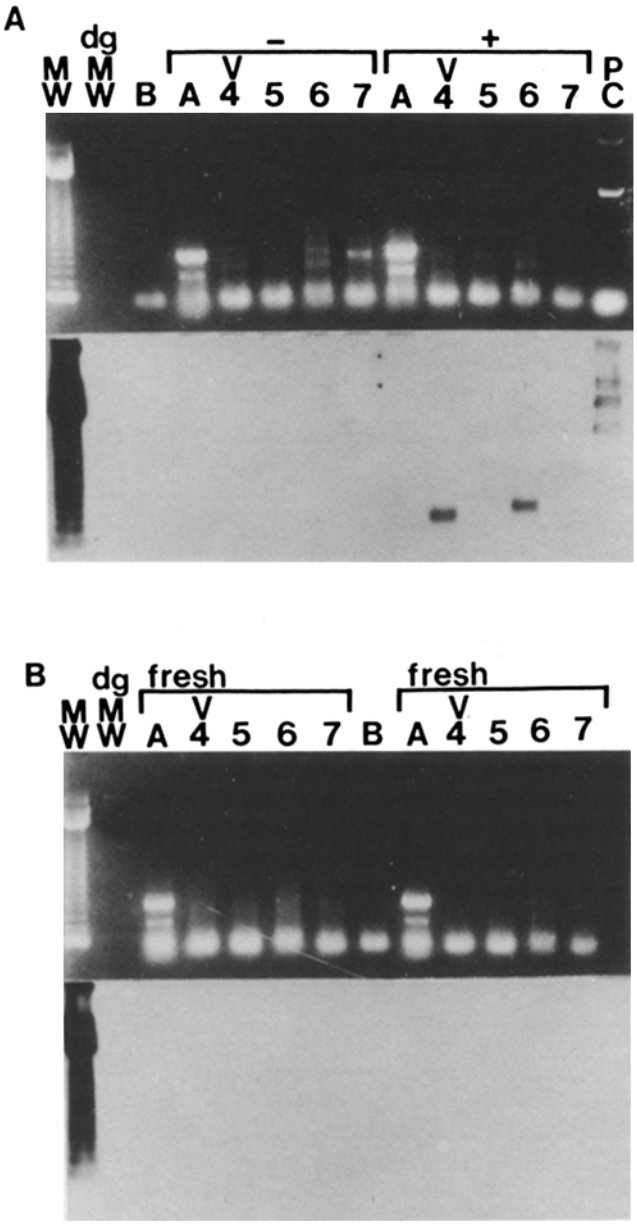


Figure 3. Vγ4–7 expression in cultured or fresh day 14 FL. Total cellular RNA from (*A*) FL cells cultured with or without 250 U/ml IL-7, or (*B*) FL of day 14 C57Bl/6 fetuses, reverse transcribed and cDNA amplified using primers specific for Vγ4, Vγ5, Vγ6, Vγ7, and Cγ1, or actin. (*Top*) Ethidium bromide–stained agarose gel; (*bottom*) Southern blot analysis. *B*, blank; see Fig. 1 for other labels.

FL cultures in which we identified transcripts specific for Vγ4 and Vγ6 in response to culture with IL-7 only. We also isolated RNA from two separate preparations of fresh (gestational day 14) FL cells and could not detect any TCR transcripts (Fig. 3 *B*). We have not detected Vγ7 PCR products in fresh or cultured FL cells (Figs. 2 and 3).

Since one possible explanation for our findings is that IL-7 induced the expansion of a small population of FL cells already expressing Vγ4 and/or Vγ6, not detectable by reverse transcriptase–PCR analysis of fresh FL, we determined whether inhibition of proliferation would affect expression of these TCR genes. We cultured FL cells with 0.01–0.08 mM of the DNA synthesis inhibitor, HU, to determine the optimal concentration to inhibit growth in response to IL-7 (Fig. 4); from these experiments, we chose a concentration of 0.02 mM HU. As illustrated by a representative experiment (Fig. 5 *A*), culture with 0.02 mM HU completely blocked the IL-7-induced growth of FL cells, since cultures were initiated with 10×10^6 cells per dish and only 7.8×10^6 cells remained after culture with IL-7 and HU. Culture with HU had no detectable effect on expression of TCR Vγ4 and Vγ6 transcripts in response to IL-7 (Fig. 5 *B*). We detected no PCR products when the reverse transcription reaction was omitted (Fig. 5 *B*), confirming that the Vγ4 and Vγ6 products represented amplification from RNA transcripts converted to cDNA.

RAG-1 and RAG-2 mRNA Expression in Response to IL-7. We were also interested in determining whether IL-7 affected expression of genes related to the regulation of TCR gene expression or other components of the TCR complex. The V(D)J recombinase activating genes RAG-1 and RAG-2 are expressed only in the least mature cells of the B and T cell lineage, i.e., those cells in which V(D)J recombination is initiated and TCR or Ig genes are transcribed (15, 16). RAG-1 and RAG-2 transcripts were undetectable in fresh day 14 FL cells, FL cells cultured in medium alone, or mature B cells (Fig. 6, *top*). In contrast, the levels of RAG-1 and RAG-2 transcripts were very high in day 14 FL cells after culture for 7 d with IL-7. We did not detect expression of CD3ε transcripts in fresh or cultured FL cells, or B cells (Fig. 6, *bottom*). RAG-1, RAG-2, and CD3ε transcripts all were expressed in the adult thymus (Fig. 6).

Discussion

Lymphocytes expressing the TCR γ/δ are a small population of T lymphocytes predominantly expressed during fetal

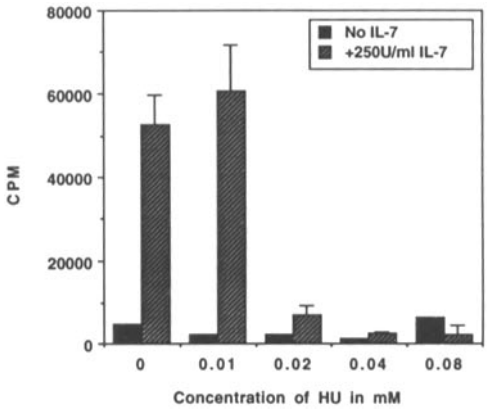


Figure 4. Effect of HU on IL-7–induced increase in thymidine incorporation by cultured FL cells. FL cells from mice at day 14 of gestation were cultured with or without IL-7 (250 U/ml) and with or without the indicated concentration of HU for 8 d. Incorporation of [^{3}H]thymidine in the last 4 h of culture was measured.

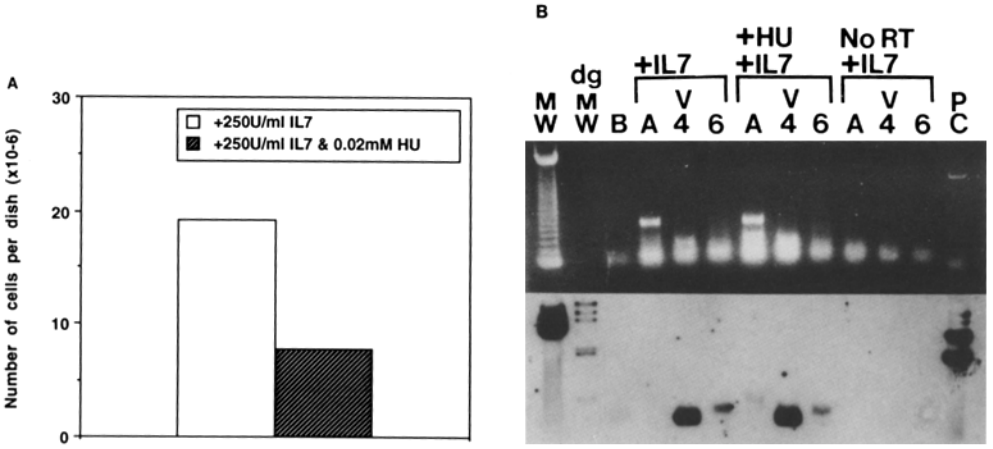


Figure 5. Effect of HU on IL-7–induced expression of TCR Vγ4 and Vγ6 transcripts. (*A*) Effects of HU on the number of cells generated per dish. (*B*) Effects of 0.02 mM HU on TCR Vγ4 and Vγ6 transcripts. FL cells (10×10^6/dish) from mice at day 14 of gestation were cultured for 7 d in medium with 250 U/ml IL-7 alone, or IL-7 and 0.02 mM HU. Total cellular RNA was isolated, reverse transcribed (except the samples *No RT + IL7*), and cDNA amplified using primers specific for Cγ1 and Vγ4 or Vγ6, or actin. (*Top*) Ethidium bromide stained agarose gel; (*bottom*) Southern blot analysis. *B*, blank; see Fig. 1 for other labels.

thymic development (1, 2) and in certain epithelia (5, 17). Evidence exists to suggest that γ/δ T cells can recognize antigen in the absence of class II MHC restriction, and may participate in the first line of host defense (18). The finding that FL, but not bone marrow, can give rise to γ/δ T cells utilizing the TCR Vγ5 gene in fetal thymic organ cultures suggests that there are subclasses of pre-T cells that exist in the FL and not in the bone marrow (19). The restricted expression of TCR γ V regions could result in unique functional properties of V region subsets. Thus, it is of obvious interest to examine the mechanisms regulating development of TCR γ/δ cells. Our previous finding that culture of FL cells with IL-7 resulted in TCR γ expression (10) led us to address whether these effects were restricted to expression of specific TCR γ V regions. It is apparent from our results that IL-7 stimulates selective expression of TCR Vγ4 and Vγ6, but not Vγ7, and probably not Vγ5 transcripts.

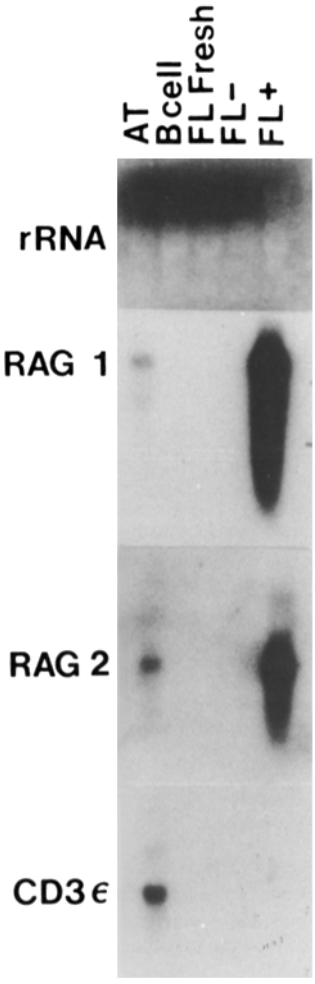


Figure 6. RAG1 and RAG2, but not CD3ϵ, expression in the FL in response to IL-7. 10 μg of total cellular RNA from adult thymocytes (*AT*), a B cell clone (*B cell*), day 14 FL (*FL Fresh*), FL cells cultured in medium alone (*FL−*), or with 250 U/ml IL-7 (*FL+*) for 7 d were analyzed by Northern blot for expression of TCR-γ transcripts using cDNA probes specific for RAG-1, RAG-2, or CD3ϵ. (*Top*) Ribosomal RNA bands; (*bottom*) autoradiograms of the Northern blot analysis.

Our data demonstrating that HU, which inhibits growth of FL cells, does not block IL-7-induced expression of Vγ4 or Vγ6 suggests that expansion of cells expressing these genes is not the primary basis for the effects of IL-7 on TCR gene expression. It also seems unlikely that IL-7 is merely a viability factor for pre-T cells already expressing V region genes, since we did not detect Vγ transcripts in fresh FL where viability was always greater than cultured FL cells. Carding et al. (4) also did not detect either Vγ4 or Vγ6 transcripts in the fetal gut before day 17 of murine development. We have observed that HU causes a slight reduction in the IL-7-induced increase in the percentage of Thy-1^+ Pgp-1^+ cells (Appasamy, P., and Kost, J., unpublished data). Thus, we hesitate to completely rule out the possibility that proliferation is not important in expanding some small subpopulation of FL pre-T cells expressing TCR genes. However, our data suggest that proliferation would play a minimal role in the effects of IL-7 on TCR gene expression.

A more conceivable explanation for our results is that IL-7 stimulates the rearrangement and transcription of Vγ genes. Consistent with this notion is our finding that IL-7 stimulated very high levels of transcripts encoding the recombinase activating genes, RAG-1 and RAG-2, in cultures of day 14 FL. This explanation is also supported by the recent findings of Muegge et al. (20), who showed that IL-7 stimulated rearrangement of TCR β genes in fetal thymocytes and maintained RAG-1 and RAG-2 gene expression. We have cloned and sequenced a number of the PCR products from these

experiments and have identified some in-frame Vγ4-Jγ1 and Vγ6-Jγ1 rearranged transcripts expressed in response to IL-7 (Appasamy, P., Y. Weng, and T. Kenniston, unpublished data). IL-7 did not induce expression of CD3ε mRNA, suggesting that the mechanisms of regulation of at least this member of the CD3 complex are distinct from the mechanisms of regulation of TCR γ genes.

Our findings that Vγ4 and Vγ6 transcripts were expressed in response to IL-7 provides additional evidence for subsets of FL pre-T cells destined to express Vγ4 or Vγ6 genes, since the Vγ6 gene would probably be deleted during rearrangement of Vγ4 to Jγ1. Alternatively, pre-T cells expressing Vγ6 transcripts could be the precursors of cells expressing Vγ4, given the developmental pattern of rearrangement and expression of Vγ6 before Vγ4 in the thymus (1). It is possible that other growth or differentiation factors are necessary for development of pre-T cells expressing other V region genes, i.e., Vγ5 and Vγ7. Vγ4-expressing T cells predominate in the thymus, blood, spleen, and lymph nodes after birth, while the Vγ6 subset is primarily found in the mucosal surfaces of the uterus, vagina, and tongue (6). It remains to be seen whether the FL cells expressing Vγ4 or Vγ6 in response to IL-7 can acquire the properties of functional T cells and whether they would preferentially home to these different anatomical sites.

A growing body of literature exists demonstrating that IL-7 preferentially supports the development of fetal thymocytes bearing TCR γ/δ (21, 22). For example, Leclercq et al. (22) showed that IL-7 stimulates growth of $V\gamma5^+$ thymocytes. Plum et al. (23) recently showed that IL-7 increases the frequency of γ/δ T cells, including Vγ5-, Vγ4-, and Vδ4-expressing cells, in fetal thymic organ cultures. We propose that IL-7 induces rearrangement and transcription of specific TCR genes as part of the early stages in pre-T cell differentiation within the fetal thymus, and extrathymically for pre-T cells in the FL, since IL-7 is also produced in the FL (24). We can now use these findings to begin to address the mechanisms by which IL-7 drives TCR γ gene rearrangement and expression by FL cells, which may be related to commitment of stem cells to the T lineage.

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