Putative Prethymic T Cell Precursors within the Early Human Embryonic Liver: A Molecular and Functional Analysis

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Summary

Hematopoietic cells present in the liver in early human fetal life were characterized by phenotypic analysis using a broad panel of monoclonal antibodies. Expression of very late antigen 4 and leukocyte function-associated antigen 3 cell adhesion receptors and 4F2 cell activation molecules was found in all fetal liver hematopoietic cells before acquisition of T cell–, B cell–, or myeloid-specific surface markers, and before the time of intrathymic colonization. Molecular studies showed that expression of the interleukin 2 receptor β (IL-2Rβ) also occurred in the embryonic liver at this early ontogenic stage. In contrast, no expression of IL-2Rα or IL-2 transcripts was found in fetal liver cells, whereas transcription of the IL-4 gene was detected in a small fetal liver cell subset. Putative T cell precursors were identified among the hematopoietic fetal liver cells by the expression of genes encoding the γ, δ, ϵ, and ζ invariant chains of the CD3–T cell receptor (TCR) complex. However, no transcription of the polymorphic α and β TCR genes was detected. Functional in vitro assays further demonstrated that fetal liver hematopoietic cells from those early embryos were capable of proliferating in response to T cell growth factors, including IL-4 and IL-2. However, whereas IL-4-induced proliferation paralleled the appearance in vitro of $CD45^{+}CD7^{-}CD4^{dull}$ cells expressing the CD14 myeloid antigen, as well as of $CD34^{+}$ primitive hematopoietic progenitors, differentiation into $CD45^{+}CD7^{+}CD8^{+}CD3^{-}$ immature T cells was observed when using IL-2. Moreover, coculture with thymic epithelial cell monolayers provided additional evidence that early fetal liver hematopoietic cells may include very primitive T cell precursors, which were able to differentiate in vitro into TCR α/β^{+} mature T cells. Therefore, our results indicate that, after triggering of the T cell–specific maturation program in primitive fetal liver hematopoietic progenitors, specific signals provided intrathymically by epithelial cells may fulfill the requirements to drive terminal differentiation of prethymically committed T cell precursors.

During human fetal development, hematopoietic stem cells home to the fetal liver at 5–6 wk of gestation. The liver then becomes, until week 24, the major site of hematopoiesis in humans, supporting the proliferation of progenitor cells for all hematolymphoid series (including the erythroid, myelomonocytic, and lymphoid lineages) (1, 2). Differentiation of fetal liver progenitors along a particular hematopoietic lineage is further dependent upon epigenetic influences provided by specific cellular microenvironments.

The development of immunocompetent T lymphocytes occurs primarily in the embryonic thymus (3, 4). At 8.5–10 wk of gestation, hematopoietic precursors migrating from the fetal liver colonize the thymic rudiment (5), where they undergo a complex series of differentiation events leading to the expression of the TCR-CD3 complex (6, 7). Efficient performance of this complex program appears to be dependent upon cellular interactions between developing thymocytes and thymic stromal components (i.e., epithelial cells, macrophages, fibroblasts, and dendritic cells) (8). In addition, the coordinated production of multiple growth factors by both hematopoietic and stromal thymic cells throughout ontogeny may be critically involved in the T cell differentiation process (6, 9).

Less is known, however, about the developmental programs

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that may operate at very early stages in fetal T cell ontogeny to drive hematopoietic stem cell differentiation towards the T cell lineage. Considerable insight into this process has been gained by the identification of fetal hematopoietic $CD7^+$ cells as the earliest prethymic T cell precursors (5, 10, 11). Phenotypic studies have shown that cells expressing the CD7 T cell marker were present in the fetal liver before hematopoietic cell colonization of the epithelial thymic rudiment (10, 11), and concentrated in the upper thorax mesenchyme just before and during the time of T cell precursor migration to the embryonic thymus (11). Functional studies further demonstrated that $CD7^+$ cells from fetal liver and thorax were enriched for T cell precursors capable of giving rise to mature T cells (11), indicating that T cell commitment events may occur very early in human ontogeny. Nonetheless, the observation that both prethymic and intrathymic $CD7^+$ cells can also be driven to undergo myeloerythroid differentiation by particular combinations of cytokines (11, 12) suggested that certain subsets of $CD7^+$ cells are not irreversibly committed to the T cell lineage (7).

In the present report, phenotypic and molecular studies were performed to determine whether prethymic differentiation events of human T cells might be identified in primitive hematopoietic precursors from the early embryonic liver, before T cell marker expression. In addition, functional studies were done to analyze the differentiation requirements of the putative T cell precursors present in the liver in early human fetal life. Our results showed that T cell–specific maturation events, including the expression of genes encoding the γ, δ, ϵ, and ζ invariant components of the CD3-TCR complex, were initiated early in ontogeny in fetal liver hematopoietic precursors that lacked CD7 molecules but expressed very late antigen 4 (VLA-4)[1] and leukocyte function-associated antigen 3 (LFA-3) cell adhesion receptors. Initial differentiation of these primitive fetal liver hematopoietic progenitors along the T cell lineage was demonstrated by their ability to proliferate in response to rIL-2 and to give rise in vitro to $CD7^+$ $CD8^+CD3^-$ immature T cells. In addition, fully differentiated CD3-TCR α/β^+ mature T cells were obtained after coculture with thymic epithelial cell monolayers plus IL-2. Therefore, our results provide evidence that specific signals delivered by the thymic epithelium are required to drive terminal differentiation of prethymically committed fetal liver T cell precursors.

Materials and Methods

Cell Samples. Human fetal tissues were obtained after legal termination of pregnancy. Gestational age determined by crown/rump length, foot length, and menstrual records (13) ranged from 7 to 22 wk. Infant thymus samples were obtained from 1 mo-old to 3 yr-old patients undergoing corrective cardiac surgery. Cell suspensions were obtained from thymus, spleen, and liver samples, and mononuclear cells were separated by centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), through which mature red blood cells and hepatocytes sedimented. The cells at the interface were then washed three times in RPMI 1640 (Gibco, Paisley, Scotland) containing 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FCS (Gibco) (complete medium).

Intrathymic T cell precursors, referred to as pro-T cells (14), were isolated from neonatal thymi by consecutive treatments with anti-CD1a (Na1.34), -CD4 (HP2/6), -CD8 (B9.4), -CD3 (SPV-T3b), and -CD2 (Leu-5b) mAbs plus rabbit complement (Sera Labs Ltd., Sussex, UK), as described previously (14). After Ficoll-Hypaque centrifugation, recovered viable cells were treated again, in one step, with a cocktail of the indicated mAbs and incubated 30 min at 4°C with magnetic beads coated with affinity-purified sheep anti–mouse IgG (Dynabeads M-450; Dynal, Oslo, Norway) following manufacturer's recommendations. This procedure allowed the isolation of a highly purified preparation of pro-T cells (>99% $CD45^+$, 50–95% $CD7^+$, $CD1a^-2^-3^-4^-8^-$) (14, 15).

Activated peripheral T cells were obtained after culture of PBMC in complete medium containing 1% PHA (Difco Laboratories, Detroit, MI) for 4 d.

Quantitative Flow Cytometry. Flow cytometry studies were performed by direct or indirect immunofluorescence as described (14). Cells were incubated with saturating amounts of the corresponding mAb (Table 1) at 4°C for 30 min, followed by two washes. FITC-conjugated $F(ab')_2$ goat anti–mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) was used as a second-step reagent. For dual parameter analyses, cells stained following the described procedure, or directly labeled with available FITC-coupled mAbs: FITC-GAP8.3 (Table 1) and FITC-OKT3, -OKT4, -OKT8 (Ortho Diagnostic, Raritan, NJ), were further incubated either with biotin-conjugated mAbs (3A1, MAR-108) (Table 1) followed by PE-coupled streptavidin (Southern Biotechnology Associates, Inc.) or with available PE-coupled mAbs (T11-RD1; Coulter Immunology, Hialeah, FL; PE-Leu-4, PE-Leu-2a, PE-Leu-3a; Becton Dickinson & Co., Mountain View, CA). Irrelevant isotype-matched mAbs (Becton Dickinson & Co.) were used as negative controls.

Quantitation of the surface staining of 10^4 viable cells was performed using an EPICS Profile flow cytometer (Coulter Electronics, Hialeah, FL). Cells were considered positive for the tested antigen when fluorescence intensity was above the upper limit of the negative control. The data were computed for graphic display using a program developed in our laboratory (46).

Fetal Liver Cultures. Freshly isolated fetal liver mononuclear cells (2×10^6) were cultured in 24-well macroplates (Costar, Cambridge, MA) at 10^6 cells/ml in complete medium alone, or in complete medium containing optimal mitogenic doses of either rIL-2 (10 nM) (Hoffman-La Roche, Basel, Switzerland) or rIL-4 (1 nM) (Sandoz, Basel, Switzerland); or were cocultured with monolayers of the ET-VII thymic epithelial cell line (2×10^4 cells/well) in complete medium with or without 10 nM rIL-2.

Preparation of Thymic Epithelial Cell Monolayers. Monolayers of thymic epithelial cells were prepared from neonatal or fetal thymi as previously described (47). The cultures were initiated in six-well plates (Costar) in D-valine-supplemented MEM (Gibco) containing 2 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FCS (D-valine medium). D-valine was used to hinder the growth of fibroblasts without affecting that of epithelial cells (49). After 5–7 d, cells were incubated at 37°C for 2 d in D-valine medium containing 5 μg/ml mycophenolic acid (Sigma Chemical Co., St. Louis, MO) to further eliminate hematopoietic cells. The cell monolayers were subsequently removed from the wells every 7 d by treatment with 1.5 ml/well Puck's-modified solution containing trypsin and EDTA (Gibco) and, after washing,

[1] *Abbreviations used in this paper:* TEC, thymic epithelial cells; VLA, very late antigen.

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cells were propagated in D-valine medium. Functional and phenotypic studies of epithelial cells growing under these culture conditions were performed after 2–3 mo. For phenotypic analyses, epithelial cells, either untreated or treated with digitonin (0.005%) as previously described (49), were subjected to indirect immunofluorescence staining by using the following mAbs: AE-3, anti–high molecular weight basic keratins; TE-3A, anti–cortical thymic epithelium; and TE-4, anti-subcapsular cortical and medullary epithelium (8), kindly provided by Dr. B. F. Haynes (Duke University Medical Center, Durham, NC); RFD4, anti– medullary thymic epithelium (50), the kind gift of Dr. G. Janossy (Royal Free Hospital School of Medicine, London, UK); TE-7, anti–mesoderm-derived thymic epithelium (8) (Sera Labs); TS 2/9, anti-LFA3 (41), kindly donated by Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain); and W6/32, anti-MHC class I (A, B, C) (43).

cDNA Preparation. Total cellular RNA was isolated from fetal and adult tissues by the acid phenol procedure (51). The integrity of mRNA was verified by optical density, ethidium bromide staining, and Northern blot hybridization after electrophoresis on formaldehyde-agarose gels, as described elsewhere (14). Reverse transcription of total RNA (5 μg) into single-strand cDNA was performed using AMV reverse transcriptase and oligo(dT)$_{15}$ primers (Boehringer Mannheim GmbH, Mannheim, Germany), according to the manufacturer's recommendations.

PCR Analysis. PCR amplifications (52) were performed with 5% of each cDNA sample, in a 100-μl reaction volume containing 2.5 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) and 100 pmol of each of the two gene-specific primers. DNA was amplified by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, in a thermal cycler (Perkin Elmer Cetus). PCR amplifications of genes encoding the γ, δ, ϵ, and ζ chains of the CD3-TCR complex and IL-2Rα and IL-2Rβ were performed by using the following oligonucleotide primers: 5′ CD3-γ, 5′-CAGGAATTCGGGCTGCTCCACGCTTTTGC-3′; 3′ CD3-γ, 5′-CAGAAGCTTTTCCCCAATAGGTGGCGC-3′; 5′ CD3-δ, 5′-TTCCGGTACCTGTGAGTCAGC-3′; 3′ CD3-δ, 5′-GGTACA-GTTGGTAATGGCTGC-3′; 5′ CD3-ϵ, 5′-GAAATGGGTGGTAT-TACACAGACACC-3′; 3′ CD3-ϵ, 5′-GGCCTCTGTCAACATTT-ACCCAGTCC-3′; 5′ TCR-ζ, 5′-CAGAAGCTTCTGCCTCAGC-CTCTGCCT-3′; 3′ TCR-ζ, 5′-TATGAATTCGCCTTTGAGTGG-TGAAAT-3′; 5′ IL-2Rα, 5′-TTCACTGCGGCCGCTGGTCCCA-AGGGTCAG-3′; 3′ IL-2Rα, 5′-GCTTGCCTGAGGCTTCTCT-TCACCTGGAAA-3′; 5′ IL-2Rβ, 5′-CCAGCTGAGCTCAGAGC-ATGG-3′; 3′ IL-2Rβ, 5′-CCAAGTGAGTTGGGTCCTGACC-3′.

To avoid unspliced mRNA or DNA amplification, each primer of every pair was located in different exons. IL-2 and IL-4 transcription studies were performed by the MAPPing™ technique (Clontech Laboratories, Inc., Palo Alto, CA), using appropriate oligonucleotide primers (Clontech Laboratories, Inc.). Previously reported degenerate primers (53), developed on the basis of the conserved sequences at the 5′ end of human Vα and Vβ gene segments, in combination with either Cα or Cβ primers, enabled the amplification of all known human Vα and Vβ chain TCR genes. As described (53), these sets of primers also proved successful in the amplification and cloning of VDJ regions for which no prior sequence data are known, indicating their general applicability in TCR gene expression studies.

Equivalent amounts of cDNA among different samples could be estimated by comparable capacity to act as substrate for amplification directed by β-actin gene-specific primers (Clontech Laboratories, Inc.) at 15, 20, and 30 cycles of amplification. Titration of cycle number allowed us to perform densitometric analyses of the β-actin amplifications among different cDNA samples under nonsaturating conditions. Thus, since all samples analyzed for a given pair of primers were processed simultaneously, comparisons could be made for the expression of a particular gene in different cell samples. The β-actin control shown in Fig. 2 applies for studies shown in both Figs. 2 and 4, which were performed on exactly the same cDNA samples. The specificity of the amplified products was demonstrated by Southern blot hybridization. PCR products (10 μl) were run on 1.5% agarose gels, transferred to nylon membranes, and hybridized as described elsewhere (14) using the corresponding gene-specific probes. The CD3-ϵ (54) and CD3-δ (55) probes were the kind gift of Dr. C. Terhorst (Dana Farber Cancer Institute, Boston, MA), and the CD3-γ (56) and TCR-ζ (57) probes were kindly provided by Drs. M. J. Crumpton (Imperial Cancer Research Foundation, London, UK) and R. D. Klausner (National Institutes of Health, Bethesda, MD), respectively. The IL-2 (58) and IL-2Rβ (59) probes were generously provided by Dr. T. Taniguchi (Osaka University, Osaka, Japan), and the IL-4 (60) and IL-2Rα (61) probes were the generous gift of Drs. K. Arai (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) and T. Honjo (Kyoto University, Kyoto, Japan), respectively. Specific Cα (62) and Cβ (63) probes were kindly provided by Dr. T. Mak (The Ontario Cancer Institute, Toronto, Ontario, Canada).

In Situ Hybridization. Freshly isolated hematopoietic fetal liver cells (10^5 cells/slide) were subjected to in situ hybridization by using cDNA probes labeled with digoxigenin-11-dUTP (Boehringer Mannheim), as previously described (46). Antidigoxigenin antibodies conjugated to alkaline phosphatase (Boehringer Mannheim) were used for the immunological detection according to the manufacturer's recommendations. The number of positive cells was determined over background labeling detected after hybridization of fetal liver cells with an irrelevant mouse Ig probe (J11). The CD3-ϵ (54), CD3-δ (55), CD3-γ (56), TCR-ζ (57), IL-2Rα (61), IL-2Rβ (59), IL-2 (58), and IL-4 (60) cDNA probes used in this study have previously proved successful in the in situ hybridizations of intrathymic T cell precursors and mature T cells, whereas background labeling was detected with mouse B cells (46, and our unpublished results).

IL-2 Binding Assays. Aliquots of freshly isolated fetal liver cells (2×10^7) were extensively washed and treated for 3 min at 4°C with 0.15 M NaCl, 0.1 M glycine (pH 4) and washed again three times. Cells (10^6) were then incubated with serial dilutions (1 pM to 25 nM final concentration) of ^{125}I-labeled IL-2 (Amersham Corp., Amersham, UK) for 1 h at 4°C, as previously described (15). Specifically bound and free ^{125}I-labeled IL-2 was determined by solid scintillation counting. The calculated values for the dissociation constant and the number of binding sites per cell were derived by Scatchard computer analysis of equilibrium binding data, according to Munson and Rodbard (64), assuming a uniform distribution of sites. The data were corrected for nonspecific binding of IL-2, determined in the presence of a 250-fold molar excess of unlabeled rIL-2, and for the loss of ligand binding activity incurred during the IL-2 binding procedure.

Results

Phenotypic Characterization of Primitive Hematopoietic Cells in Human Fetal Liver: Early Ontogenic Expression of VLA-4 and LFA-3 Cell Adhesion Molecules. To address the ontogeny of hematopoietic cells in the human fetal liver, cytofluorometric analyses were performed in freshly isolated mononuclear cells from different 7–22-wk-old fetal liver samples, using the panel of mAbs listed in Table 1. These studies revealed that

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Table 1. *mAbs Used for the Phenotypic Analysis of Hematopoietic Cells in Human Fetal Samples*

Differentiation cluster (CD)[*]	mAb	Antigen recognized	Reference
CD1a	Na1.34,T6[‡]	T6/HTA1,gp49	16,17
CD2	OKT11,[§]Leu5b,[‖]T11[‡]	T11/LFA2,gp50	18,19
CD3	SPV/T3b,OKT3,[§]Leu4[‖]	T3,gp26,20,16	20-22
CD4	HP2/6,OKT4,[§]Leu3a[‖]	T4,gp59	23-25
CD5	OKT1,[§]Leu1[‖]	T1,gp67	20,26,27
CD7	3A1[¶]	gp40	28
CD8	B9.4,OKT8,[§]Leu2a[‖]	T8,gp32	17,24,29
CD11b	Mo1[‡]	C3biR,gp155/95	30
CD13	My7[‡]	gp150	31
CD14	Mo2[‡]	gp55	30
CD16	Leu11c[‖]	FcRIII,gp50-65	32
CD19	B4[‡]	gp95	33
CD20	B1[‡]	p37/32	34
CD25	MAR-108	IL-2Rα/Tac,gp55	25
CD28	9.3[**]	T44,gp44	35
CD29	Ts2/16	VLA-β,gp130	36
CD34	HPCA1[‖]	gp105-120	37
CD38	OKT10[§]	T10,p45	17
CD45	GAP8.3[¶]	T200/LCA,gp180-220	38
CDw49d	HP2/1	VLA-4,gp150	39
CD57	Leu7[‖]	HNK-1,gp110	40
CD58	Ts2/9	LFA3,gp40-65	41
CD71	OKT9[§]	T9/TfR,gp180	17
–	4F2[¶]	gp120	42
–	W6/32[¶]	HLA A,B,C;gp40	43
–	anti-HLA-DR[‖]	HLA-DR;gp29/34	44

* From references 44a and 45.
‡ Coulter Clone.
§ Ortho Diagnostic (Raritan, NJ).
‖ Becton Dickinson & Co.
¶ American Type Culture Collection (Rockville, MD).
** New England Nuclear (Boston, MA).

none of the fetal livers analyzed from 7 to 8 wk of gestation (the earliest time examined) contained cells expressing T cell markers on the cell surface, as assessed by staining with mAbs specific for CD1a, CD2, CD3, CD4, CD8 (Table 2), CD28, or IL-2Rα (CD25) (data not shown). We observed reactivity of these samples neither with mAbs that recognize the early T lineage molecule CD7 (Table 2) nor with antibodies specific for myeloid (CD13, CD14), NK cell (CD11b, CD16, CD57), or B cell (CD19, CD20) differentiation antigens (data not shown). Similarly, CD34 molecules (normally expressed on primitive hematopoietic progenitors) were rarely found in the fetal liver at this early gestational age, although CD34$^+$ cells were observed in older liver and spleen samples (Table 2). Expression of the pan-leukocyte molecule CD45 was detected on only a small, but measurable, fraction (0–8%) of fetal liver cells before 9 wk of gestation. The proportion of CD45$^+$ cells in each particular sample was slightly higher than the number of cells reactive with an anti-HLA heavy chain–specific mAb (0–4%), while expression of class II MHC molecules could not be detected (Table 2). With increasing gestational age, the percentage of cells in the fetal liver expressing the CD45 marker and class I MHC molecules gradually rose to 41 and 22%, respectively, at week 22 of gestation (the latest gestational age examined). Surface expression of the CD45 leukocyte–associated marker paralleled the acquisition of the CD7 T cell molecule, which was detected on 0.5–6% of fetal liver cells at weeks 11–13, and reached 13% at week 22. Very few CD7$^+$ cells, however, expressed CD3 molecules on the membrane (Table 2). Mature T cells progressively increased in fetal spleen and thymus following the sequence of appear-

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Table 2. *Surface Expression of Lymphoid Antigens on Hematopoietic Cells from Human Fetal Tissues*

Antigen	Fetal tissue				
	Liver			Thymus	Spleen
	7–8 wk*	11–13 wk	22 wk	22 wk	22 wk
CD45	4.5 ± 3.5 (6)	10.2 ± 4.6 (4)	33.0 ± 8.5 (3)	95.0 ± 2.0 (3)	47.0 (1)
CD34	<0.1 (3)	0.5 ± 0.3 (2)	6.0 (1)	ND	11.0 ± 5.0 (2)
CD7	<0.1 (6)	3.2 ± 2.7 (3)	13.0 (1)	87.5 (1)	ND
CD1a	<0.1 (6)	<0.1 (3)	3.0 ± 2.0 (3)	82.0 ± 5.0 (3)	1.5 ± 0.3 (3)
CD2	<0.1 (6)	1.5 ± 0.4 (3)	8.5 ± 2.5 (3)	96.0 ± 2.0 (3)	15.5 ± 4.5 (3)
CD3	<0.1 (6)	1.3 ± 0.2 (3)	5.1 ± 1.1 (4)	71.5 ± 4.5 (3)	12.5 ± 5.0 (3)
CD4	<0.1 (6)	<0.1 (3)	7.5 ± 4.0 (4)	70.5 ± 8.0 (3)	8.5 ± 2.0 (3)
CD8	<0.1 (6)	<0.1 (3)	6.5 ± 2.0 (4)	82.5 ± 4.5 (3)	7.5 ± 2.0 (3)
MHC class I	2.3 ± 1.3 (5)	11.5 ± 4.5 (3)	15.0 ± 7.0 (3)	17.5 ± 2.5 (2)	19.5 ± 2.5 (2)
MHC class II	0.5 ± 0.3 (4)	4.4 ± 2.8 (3)	8.0 ± 3.0 (2)	1.0 ± 0.5 (2)	9.5 ± 1.5 (2)

Values are expressed as the mean ± SEM of the percent of nucleated positive cells determined by flow cytometry. Number of fetal samples tested is in parentheses.
* Gestational age (wk) was determined by crown/rump length, foot length, and menstrual records.

ance of CD7⁺ T cell precursors in fetal liver. As shown in Table 2, a phenotypic distribution similar to that of the neonatal thymus was observed in fetal thymi at week 22, and both mature B and T cells were detected in the spleen at this gestational age.

In spite of the very low proportion of liver cells that express conventional leukocyte markers in early fetal life, 4F2 molecules, commonly expressed on rapidly dividing and activated cells, were detected on most (>95%) liver cells from as early as 7–8-wk-old embryos. Expression of transferrin receptors (Tfr, CD71) was also detected on a significant fraction (75–90%) of fetal liver cells (Fig. 1). Up to 50% of the fetal liver cells were in the S or $G_2 + M$ phases of the cell cycle (data not shown), which correlated with their activation/proliferation surface phenotype. Surprisingly, a majority of hematopoietic fetal liver cells (>99%) at this gestational age also showed high levels of expression of different cell adhesion receptors. As shown in Fig. 1, LFA-3 (CD58) and the β1 subunit of the integrins (VLA-β, CD29) were detected in virtually all fetal liver cells. The expression of CD29 directly paralleled that of the VLA-4 (CDw49d) member of the β1 integrin subfamily (Fig. 1), whereas the expression of other β1 integrins, such as VLA-1, was undetectable (data not shown).

Prethymic Transcription of Genes Encoding the γ, δ, ϵ, and ζ Invariant Chains of the CD3-TCR Complex in the Early Human Embryonic Liver. To analyze whether T cell–specific maturation events can occur in the human fetal liver before the acquisition of conventional T lineage markers, we studied the expression of genes coding for the CD3-TCR complex molecules in hematopoietic cells present in the liver from as early as at 8 wk of gestation, when no hematopoietic cell precursors have yet colonized the thymic rudiment. Transcription of genes encoding the γ, δ, ϵ, and ζ invariant components of the CD3-TCR complex was assessed by the PCR technique (52) (Fig. 2). Aliquots of single-strand cDNAs synthesized from similar amounts of total RNA obtained from freshly isolated cells were amplified by using specific oligo-

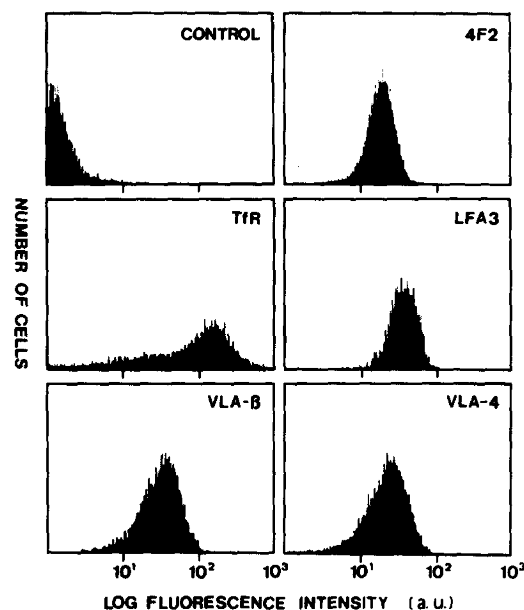

Figure 1. Expression of activation antigens and adhesion molecules on early human fetal liver hematopoietic cells. Freshly isolated 8-wk fetal liver cells were stained with the indicated mAbs followed by FITC-conjugated goat anti–mouse $F(ab')_2$ IgG. The number of positive cells (98% 4F2⁺, 82% Tfr⁺, >99% LFA3⁺, 98% VLA-β⁺, 97% VLA-4⁺) was determined over background staining observed with irrelevant mAbs used as negative controls. Results are representative of five independent experiments.

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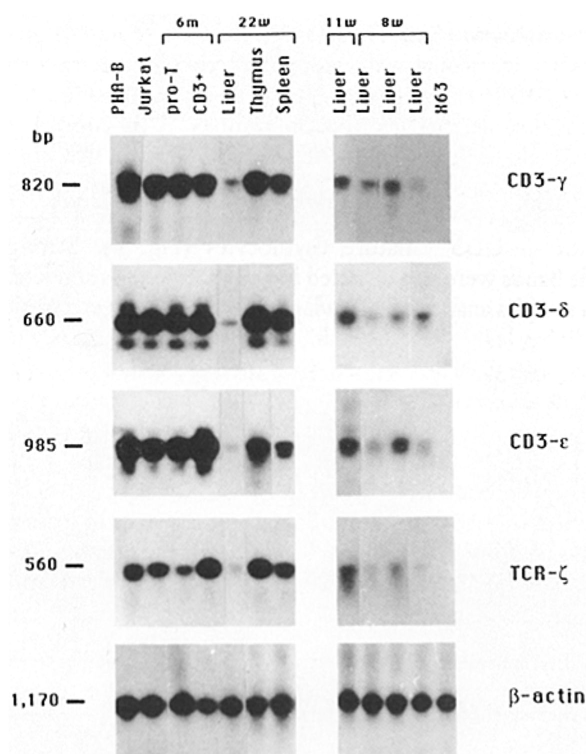

Figure 2. Expression of genes encoding the γ, δ, ε, and ζ invariant chains of the CD3-TCR complex in human fetal liver hematopoietic cells. Amplification of identical amounts of cDNA obtained from the indicated RNA samples was performed using CD3-γ, CD3-δ, CD3-ε, and TCR-ζ chain-specific oligonucleotides. PCR-amplified products (10 μl) were subjected to Southern blot hybridizations using gene-specific probes. Films were exposed for 1-6 h and 1-4 d (*left* and *right*), respectively. The capacity of cDNA generated from RNA to be amplified by PCR was in every case tested first by use of β-actin-specific primers and subsequent hybridization with a β-actin probe. β-actin films were exposed for 30 min (*left* and *right*). Use of the 1-kb ladder marker indicated that products detected were of the expected size.

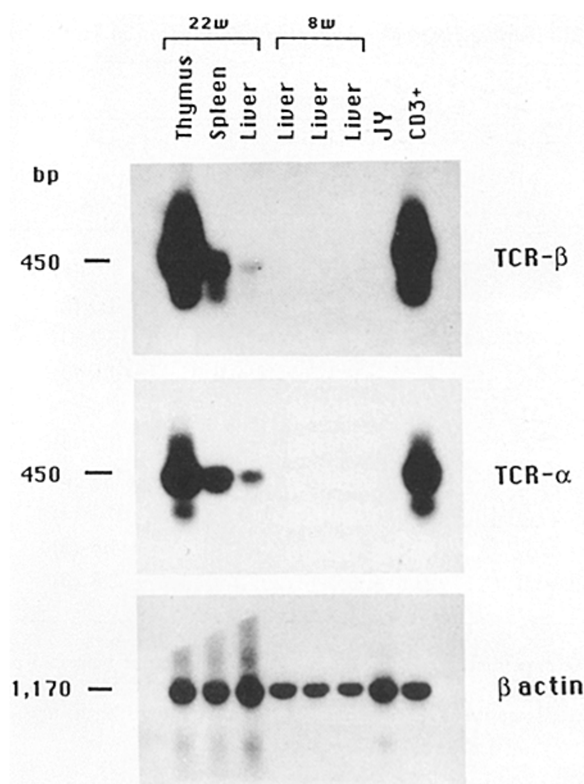

Figure 3. Analysis of the expression of TCR α and TCR β RNA transcripts in human fetal hematopoietic tissues. Equivalent amounts of cDNA obtained from the indicated sources were PCR amplified using pan Vα or pan Vβ primers, in combination with Cα or Cβ gene-specific primers, respectively. Southern blot hybridization of PCR amplifications was then performed using Cα or Cβ probes. Exposure to film was for 1 and 2 h, respectively. β-actin gene PCR amplifications of the expected size verified the integrity of cDNA samples.

nucleotide primers (see Materials and Methods). Checking of equivalence of cDNAs was assessed by use of specific primers for the β-actin gene (Fig. 2), as described in Materials and Methods. All primer combinations failed to amplify a product when irrelevant cDNA (from mouse hybridoma X63) was used, or when cDNA was omitted from the reaction. The specificity of amplification was determined by further electrophoresis, blotting, and hybridization of amplified products with specific probes.

As shown in Fig. 2, CD3-γ-, CD3-δ-, CD3-ε-, and TCR-ζ-specific bands were detected in the following samples: (*a*) Jurkat T cells and PHA-activated peripheral T cell blasts (PHA-B), included in the study as positive controls; (*b*) mature $CD3^+$ thymocytes and $CD7^+CD3^-$ intrathymic T cell precursors (referred to as pro-T cells; reference 14), obtained from a 6-mo-old thymus; and (*c*) fetal thymus and spleen from a 22-wk-old embryo. In addition, expression of genes encoding all γ, δ, ε, and ζ invariant chains of the CD3-TCR complex was clearly evident in the fetal liver at all tested gestational times (8–22 wk), although less intense PCR signals requiring longer film exposures were observed in the earliest (8–11-wk) fetal liver samples (Fig. 2). Comparable results were obtained with independent RNA preparations from the same fetal sample, or from different samples at equivalent ontogenic stages. Parallel analyses of CD3-TCR gene expression carried out by in situ hybridization independently confirmed the data derived by PCR (see below). Our results, therefore, provide molecular evidence that at early ontogenic stages, when T lymphoid markers are undetectable on the cell surface, the transcription of genes encoding the γ, δ, and ε chains of the T cell-specific CD3 complex and the TCR-associated ζ chain has already been initiated in the human fetal liver in putative prethymic T cell precursors.

In contrast to the early ontogenic expression observed for the genes encoding the invariant components of the CD3-TCR complex, no rearrangement of genes coding for the polymorphic α and β chains of the TCR could be detected by Southern blot hybridization in the 8-wk-old fetal liver samples studied. Neither did we find expression of functional TCR α and TCR β RNA transcripts in those samples by Northern blotting (data not shown). To confirm these negative results, functional V-(D)-J-C rearrangements were sought by the use of a highly sensitive PCR technique proved to detect transiently expressed rearranged genes in very small

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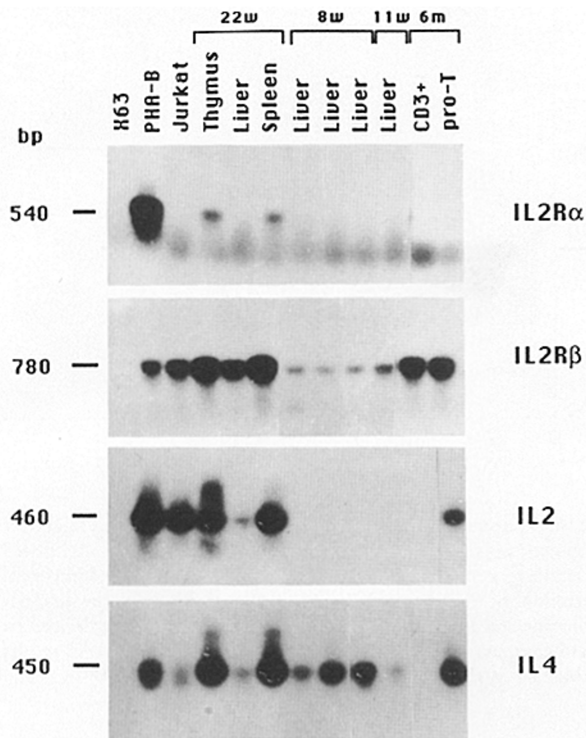

Figure 4. Cytokine gene expression in human fetal hematopoietic tissues. cDNA obtained from the indicated samples was PCR amplified using IL-2, IL-4, IL-2Rα, and IL-2Rβ gene-specific primers. Amplified products were then subjected to Southern blot hybridization using gene-specific probes. Films were exposed for 1–3 d. cDNA sample integrity and checking of equivalence of cDNAs was assessed by β-actin gene amplifications. This control is shown in Fig. 2 in a different order.

percentages of cells in embryonic tissues (65). As described in Materials and Methods, amplifications were performed by using pan Vα or pan Vβ primers specific for all human Vα or Vβ gene families, in concert with either Cα or Cβ gene–specific primers (53), and followed by blotting and hybridization either with Cα or Cβ gene–specific probes. The results confirmed the absolute lack of expression of both rearranged TCR α and TCR β genes in the fetal liver by 8 wk of gestation (Fig. 3). As a negative control, TCR transcripts were also undetectable in the lymphoblastoid B cell line JY. In contrast, both α and β TCR gene transcripts were clearly evident in the liver, and strongly expressed in the thymus and spleen from a 22-wk-old embryo, as well as in CD3$^+$ neonatal thymocytes (Fig. 3). Identical results were obtained independently of the amount of PCR products loaded, and upon longer film exposures (10 d; data not shown).

Developmental Regulation of Cytokine/Cytokine Receptor Gene Expression. Studies performed in the mouse embryo have shown that the genes coding for certain cytokines and their receptors, particularly IL-2 and IL-4, are activated in vivo in the fetal thymus during early T cell development. Similar ontogenic studies have not been performed in humans as yet. Thus, cDNA samples identical to those used for Fig. 2, were amplified by PCR with appropriate oligonucleotide primers, and the amplified products were further analyzed by Southern blot hybridization (Fig. 4). As described in Materials and Methods, equivalence in the amount of cDNA could be estimated by their comparable capacity to act as substrate for amplification directed by β-actin primers. This control is shown in Fig. 2. As expected, these studies showed that high levels of IL-2 transcripts were expressed in activated peripheral T cells, as well as in neonatal intrathymic pro-T cells, but not in CD3$^+$ mature thymocytes (Fig. 4). Strong specific bands were also detected in the fetal thymus and fetal spleen samples analyzed at 22 wk of gestation, whereas a weak hybridizing band was detected in the liver at this gestational age (Fig. 4). By contrast, no IL-2 transcripts could be detected in the liver from 8-wk- and 11-wk-old fetuses (Fig. 4), even after 60 cycles of amplification and upon long (10 d) exposure time (data not shown). This suggests that IL-2 gene expression detected in liver and spleen at 22 wk most probably reflected recirculating intrathymic precursors seeding those peripheral organs. In addition, IL-4-gene expression was detected in all 8-, 11-, and 22-wk-old fetal liver samples exam-

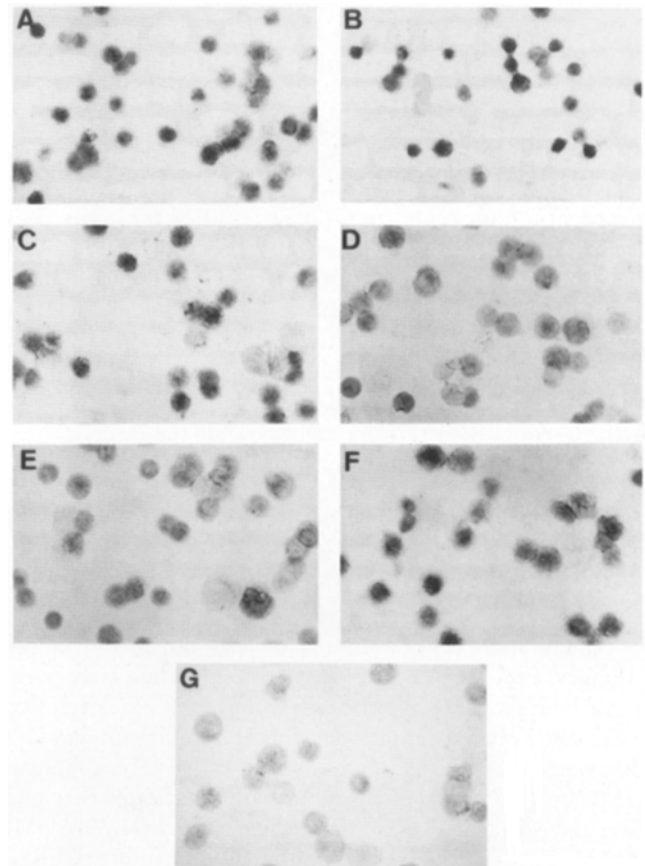

Figure 5. Expression of CD3 and IL-2Rβ transcripts in human fetal liver hematopoietic cells. Cytocentrifuge preparations of freshly isolated 8-wk-old fetal liver cells (10^5 cells/slide) were hybridized in situ with cDNA digoxigenin-11-dUTP-labeled probes specific for the genes encoding the ϵ (*A*), δ (*B*), γ (*C*), and ζ (*D*) invariant chains of the CD3-TCR complex, and for the IL-2Rα (*E*) and IL-2Rβ (*F*) genes. An irrelevant mouse Ig probe (J11) was included as negative control (*G*). Representative fields are shown (× 440).

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ined; as well as in fetal thymus, fetal spleen, and neonatal pro-T cells, but not in mature thymocytes (Fig. 4).

Parallel studies also showed that expression of the gene coding for the light chain of the IL-2R (IL-2Rα; p55, Tac) was not detectable in the fetal liver at any gestational age, neither in the early intrathymic pro-T cell subset, nor in mature thymocytes (Fig. 4), independently of the amount of PCR product loaded, and even upon long exposure times. Weak specific bands were observed, however, in fetal thymus and spleen, while high levels of IL-2Rα transcripts were produced in activated peripheral T cells. Expression of the gene encoding the heavy IL-2R chain (IL-2Rβ; p75) was observed in all fetal and adult tissues analyzed, including the youngest (8 wk) fetal liver samples (Fig. 4), and a similar pattern of expression was detected for the IL-4R gene (data not shown).

In situ hybridization studies were next performed to obtain quantitative information on the expression of the analyzed genes, and to determine whether the observed pattern of cytokine production and cytokine receptor expression could be selectively attributed to a single subset of hematopoietic fetal liver cells, particularly to putative T cell precursors. Expression of genes encoding the ϵ, δ, and γ CD3 subunits was detected in 40 ± 7, 35 ± 5, and 15 ± 5%, respectively, of hematopoietic cells within the 8-wk-old fetal liver, whereas ζ gene transcription was restricted to 6–8% of the cells (Fig. 5). As previously shown by PCR, no IL-2Rα expression could be found in the embryonic liver by in situ hybridization (<1% of positive cells). In contrast, IL-2Rβ transcripts were detected in a high proportion (70 ± 7%) of fetal liver cells (Fig. 5). Expression of IL-4 and IL-2 genes was also found to be limited to 15–20 and <1%, respectively, of the cells (data not shown). These quantitative results indicate that at least a minor fraction of putative fetal liver T cell precursors, shown to transcribe the CD3-ϵ and/or CD3-δ genes, may also express IL-2Rβ, but not IL-2Rα or IL-2 transcripts. However, similar crosscorrelation analyses could not be performed for the IL-4 gene.

Surface Expression of IL-2Rβ Molecules on Hematopoietic Cells within the Early Human Embryonic Liver. The functional relevance of IL-2Rβ gene expression detected in the early embryonic liver was assessed at the protein level by IL-2 binding assays performed in freshly isolated fetal liver cells. Because of the low numbers of mononuclear cells obtained from the youngest available fetal liver samples, IL-2 binding assays were carried out on hematopoietic cells from 13-wk-old fetal livers. As shown in Fig. 6, Scatchard analysis of equilibrium binding data obtained by using serial dilutions of ^{125}I-labeled human rIL-2 (1 pM to 25 nM final concentration) indicated that fetal liver cells displayed 1,605 intermediate-affinity (K_d, 936 pM) IL-2 binding sites per cell (assuming a uniform distribution of sites in all cells) (Fig. 6). However, since IL-2Rβ transcripts were expressed at most in 70% of fetal liver cells, a higher number of IL-2Rβ molecules per cell may be expressed by a subset of fetal liver cells. According to the observed lack of IL-2Rα transcripts, low and high affinity IL-2Rs were not detected (Fig. 6). Therefore, IL-2Rβ is the chief IL-2 binding component expressed on the surface of hematopoietic fetal liver cells.

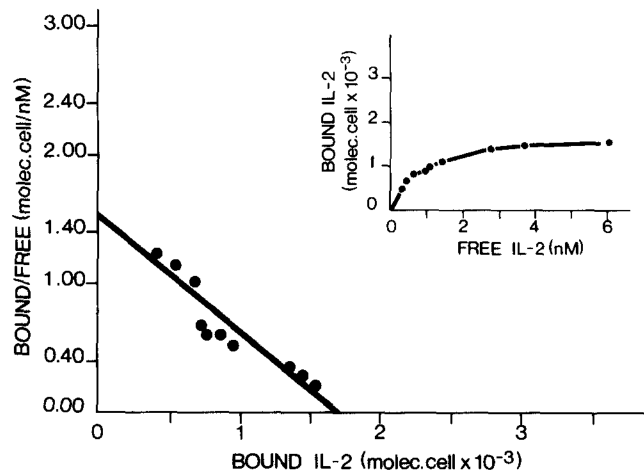

Figure 6. Expression of IL-2Rβ molecules on early human fetal liver hematopoietic cells. IL-2 binding assays were performed in freshly isolated 13-wk fetal liver cells incubated with serial dilutions (1 pM to 25nM final concentration) of ^{125}I-labeled IL-2. The calculated values for the dissociation constant and for the number of IL-2 binding sites were derived by Scatchard computer analysis of equilibrium binding data. Similar results were obtained in three independent experiments.

Hematopoietic Cells Present in the Early Human Fetal Liver Contain Precursors of $CD7^+CD8^+CD3^-$ Immature T Cells. Fetal liver cells were next functionally assayed for their ability to proliferate to defined T cell growth factors. Thus, freshly isolated mononuclear cells from 8-wk fetal liver samples were cultured in vitro with either IL-2 or IL-4; i.e., two lymphokines known to play a key role as growth factors for intrathymic

Table 3. *In Vitro Induction of T Cell Marker Expression on Human Cultured Fetal Liver Cells*

	Culture Conditions				
Antigen	–	IL-2	IL-4	ET-VII	IL-2 + ET-VII
CD45	98	97	70	98	94
CD34	ND	8	38	45	ND
CD7	<0.1	94	5	63	53
CD1a	<0.1	<0.1	<0.1	<0.1	<0.1
CD2	<0.1	12	<0.1	<0.1	45
CD3	<0.1	<0.1	<0.1	<0.1	31
CD4	27^{dull}	<0.1	25^{dull}	<0.1	30^{bright} + 9^{dull}
CD8	<0.1	63	<0.1	<0.1	12
CD14	ND	5	30	ND	9
IL-2Rα	<0.1	3	<0.1	ND	47

Shown are percentages of positive cells analyzed by flow cytometry. Freshly isolated 8-wk fetal liver cells (10^6/ml) were cultured (15 d) in medium alone or in medium containing either rIL-2 (10 nM) or rIL-4 (1 nM), or were cocultured with monolayers of the ET-VII thymic epithelial cells (2 × 10^4 cells/well) in medium either with (20 d) or without (15 d) 10 nM rIL-2. Results are representative of three independent experiments.

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T cell precursors in both mice and humans (6). Although functional responses varied between different samples, a dose-dependent cellular proliferation was repeatedly observed in cultures set up with either IL-2 or IL-4. To determine the phenotype of hematopoietic cells within the embryonic liver able to proliferate in response to these cytokines, the expression of distinct differentiation molecules was next analyzed by flow cytometry after culture in the presence of optimal mitogenic doses of rIL-2 (10 nM) or rIL-4 (1 nM). A gradual increase in the expression of MHC class I and CD45 molecules was observed in both culture conditions, as well as in cultures devoid of exogenous lymphokines. CD45$^+$ leukocytic cells arising in these cultures reached maximal levels (≤98%) at days 8–15 (in five different experiments) (Table 3). The acquisition of lineage-specific differentiation antigens and the quantitative outcome of the progeny differed, however, between IL-2- and IL-4-driven cultures. Upon culture with IL-2, acquisition of CD45 molecules paralleled the expression of the CD7 T cell marker and, thus, both molecules were coexpressed on most (94%) cells by day 15 (Table 3, and Fig. 7). In contrast, no CD7 expression was detected in the absence of exogenous lymphokines, and rare (<5%) CD7$^+$ cells were observed after supplementation with IL-4 (Table 3). Interestingly, the lack of CD7 molecules in both culture conditions correlated with the acquisition of low levels of CD4 molecules (CD4^{dull}) on a significant proportion (≤30%) of CD45$^+$CD7$^-$ cells. These cells also expressed variable levels of CD14 molecules (Table 3), classically adscribed to the myeloid lineage. Rare CD4^{dull} and CD14$^+$ cells were also observed at initial periods of culture in the presence of IL-2. However, the increase in CD7 molecules in IL-2 driven cultures paralleled a gradual decrease in CD14$^+$ cells (Table 3). Special mention also has to be made of the differential expression of CD34 molecules, normally expressed on primitive hematopoietic stem cells, in cultures supplemented with IL-4 (38%) or IL-2 (8%) (Table 3).

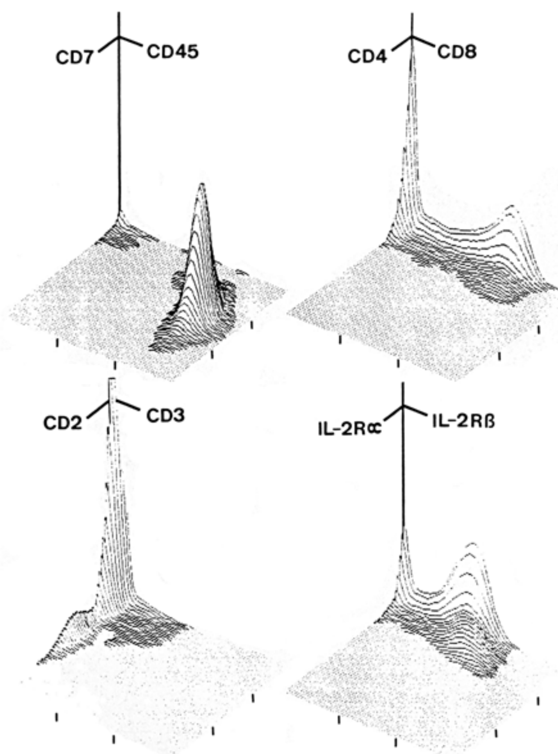

Figure 7. Hematopoietic cells from 8-wk human fetal liver contain precursors of CD7$^+$CD8$^+$CD3$^-$ immature T cells. Freshly isolated 8-wk fetal liver cells displaying the phenotype shown in Table 2 were cultured with rIL-2 (10 nM) for 15 d. Two-color immunofluorescence analyses were then performed using the following combinations of mAbs: FITC-GAP8.3 (anti-CD45) and biotin-coupled 3A1 (anti-CD7) plus streptavidin; FITC-OKT8 (anti-CD8) and PE-Leu-3a (anti-CD4); FITC-OKT3 (anti-CD3) and T11-RD1 (anti-CD2); and MiK-β1 (anti-IL-2Rβ) plus FITC-goat anti-mouse F(ab')$_2$ IgG and biotinilated MAR-108 (anti-IL-2Rα) plus PE-streptavidin. Identical results were obtained in three independent experiments.

Our functional data demonstrated that hematopoietic cells found in the fetal liver as early as 8 wk of gestation can give rise to cells of the T cell lineage. This was further supported by the observation that acquisition of CD7 molecules was followed by the expression of low levels of CD2 and high levels of CD8 T cell antigens on 12 and 63%, respectively, of IL-2-cultured fetal liver cells, whereas CD2$^+$ and CD8$^+$ cells were not detected in cultures devoid of IL-2 or in the presence of IL-4 (Table 3). It must be stressed, however, that the CD8$^+$ T cell subset comprised large-sized blastic cells that did not include mature T cells, since no expression of the CD3-TCR complex was detectable (Table 3, and Fig. 7). CD7$^+$CD8$^+$CD3$^-$ cells lacked the IL-2Rα (Table 3), while most cells in the culture (79%) were reactive with a recently available anti-IL-2Rβ mAb (66) (Fig. 7).

CD7$^-$ Hematopoietic Cells from 8-wk Fetal Liver Give Rise to TCR α/β^+ Mature T Cells in the Presence of Thymic Epithelium. The finding that CD7$^+$CD8$^+$CD3$^-$ immature T cells, but not mature T lymphocytes, were derived from 8-wk-old fetal liver hematopoietic precursors in IL-2-driven cultures led us to analyze whether additional costimulatory signals delivered in vivo within the thymic microenvironment were required to efficiently drive terminal differentiation of primitive fetal liver T cell precursors into TCR-CD3$^+$ mature T cells in vitro. To this end, we prepared monolayers of thymic adherent stromal cells derived from neonatal and fetal thymi. Hematopoietic cells were depleted from these preparations by treatment with mycophenolic acid (see Materials and Methods). After 3 mo of culture, stromal cell preparations were shown to be homogeneous for the expression of cytokeratins recognized by the AE-3 mAb (>95% AE3$^+$), as well as for the lack of hematopoietic antigens (<0.1% T cell–, B cell–, or myeloid-specific markers) or fibroblast-specific markers (<5% TE-7$^+$), with all these criteria supporting their thymic epithelial origin. The capacity of these thymic epithelial cells (TEC) to support T cell differentiation was assessed in vitro by coculturing freshly isolated 8-wk fetal liver mononuclear cells with monolayers of established TEC. All experiments reported in this study were performed with a TEC preparation (ET-VII) that was homogeneous for the expression of MHC class I and LFA-3 molecules and expressed both cortical and medullary thymic epithelium markers: 100% AE-3$^+$, 90% TE-4$^+$, 20% RFD4$^+$, 30%

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TE-3$^+$, <1% TE-7$^+$. As assessed by PCR, ET-VII cells were also shown to transcribe the IL-1β, IL-3, IL-6, and IL-7 genes, while transcription of IL-2 and IL-4 was not detected (data not shown).

Fetal liver cells cultured in the presence of ET-VII epithelial cells without exogenous cytokine supplementation showed limited proliferative responses. This hampered performing extensive phenotypic analyses, although expression of CD45 and MHC class I molecules could be detected in a major proportion of cultured cells (Table 3, and data not shown). CD34 molecules were also found on up to 45% of cultured fetal liver cells, while no expression of T cell markers such as CD2, CD3, CD4, or CD8 was detected during the culture period. By contrast, CD7 expression increased throughout culture, and was detected on 63% of cells at day 15 (Table 3). Since high proliferation of fetal liver cells was observed in response to rIL-2, phenotypic studies were next performed in cultures set up in the presence of ET-VII epithelial cells plus 10 nM rIL-2. Fetal liver cells growing under these culture conditions showed proliferation kinetics similar to those observed in the presence of IL-2 alone. Cellular proliferation also paralleled a sequential acquisition of CD45 and CD7 molecules, which were first detected on 49 and 14% of cells, respectively, at day 7, and reached 94 and 53% of cells, respectively, at day 20 (Table 3, and Fig. 8). Expression of CD45 and CD7 was followed by the acquisition of CD2 and IL-2Rα molecules by a variable proportion of fetal liver cells (17 and 15%, respectively, at day 13), that increased throughout culture (45 and 47% of cells, respectively, at day 20) (Table 3). Thus, after 20 d of culture, most CD45$^+$CD7$^+$ cells (85%) also expressed CD2 molecules in high levels, and some of them (11%) did so in low levels. As shown in Fig. 8, expression of IL-2Rα was detected in the fetal liver subset shown to coexpress high CD2 levels.

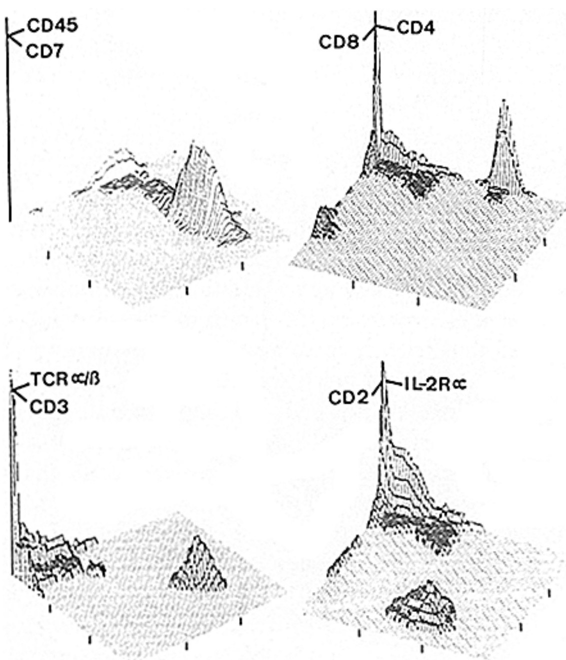

Figure 8. Human fetal liver hematopoietic precursors differentiate in vitro into CD3-TCR α/β^+ mature T cells in the presence of thymic epithelial cells. Freshly isolated 8-wk human fetal liver cells were cocultured with monolayers of the ET-VII thymic epithelial cells in medium containing 10 nM rIL-2. After 20 d of culture, two-color flow cytometry analyses were performed using the following combinations of mAbs: FITC-GAP8.3 (anti-CD45) and biotinilated 3A1 (anti-CD7) plus PE-streptavidin; FITC-OKT4 (anti-CD4) and PE-Leu-2a (anti-CD8); BMA031 (anti-TCR α/β) plus FITC-goat anti-mouse F(ab')$_2$ IgG and PE-Leu-4 (anti-CD3); and FITC-MAR-108 (anti-IL-2Rα) and T11-RD1 (anti-CD2). Similar results were obtained in three independent experiments.

More importantly, all these phenotypic changes were shown to precede the appearance of CD3$^+$ mature T cells in the cultures (12 and 31% of cells at days 13 and 20, respectively) (Table 3). Most CD3$^+$ cells coexpressed the TCR α/β (Fig. 8), as assessed by staining with the BMA031 mAb (67), while few, if any, TCR γ/δ^+ cells were detected throughout culture (data not shown). Acquisition of the CD3-associated TCR α/β complex correlated with the differentiation of fetal liver cells into single-positive cells expressing high levels of CD4 (CD4^{bright}) (Table 3). In fact, most CD4^{bright} cells (81%), but not the small fraction of CD4^{dull} cells detected in these cultures, coexpressed CD3 (Table 3, and data not shown). As expected from the observed CD3 vs. CD4 distribution, few CD8$^+$ cells (<2%) were detected within the CD3$^+$ subset. It is also noteworthy that no expansion of CD8$^+$CD3$^-$ immature T cells was observed throughout culture in the presence of epithelial cells, although a small cell fraction (12%) displaying this phenotype was observed at day 20 (Table 3). Two-color flow cytometry analyses showed a mutually exclusive distribution of CD4 and CD8 differentiation antigens (Fig. 8). Coexpression of both CD4 and CD8 molecules was never detected at any tested culture times (days 7–20), suggesting that CD4$^+$CD8$^+$ double-positive cells were not generated throughout culture. Accordingly, CD1a expression, generally associated with the double-positive phenotype, was not found in our cultures (Table 3). Finally, CD14 and CD4^{dull} expression was observed in a small subset (9%) of CD3$^-$ fetal liver cells. This suggests that, in addition to T cell differentiation, myeloid differentiation was also driven in cultures where epithelial cells were present.

Discussion

In the present study, phenotypic, molecular, and functional approaches have been used to investigate maturation events that occur at very early stages in human fetal T cell ontogeny. Flow cytometry analyses of hematopoietic cells arising in the liver during human embryogenesis showed that neither T cell–, B cell–, nor myeloid lineage–restricted markers were expressed by fetal liver cells before 8 wk of gestation. However, a surprisingly high expression of the 4F2 molecule, as well as of certain cell adhesion receptors (including LFA-3 and VLA-4), was detected on the surface of virtually all fetal liver cells at this early embryonic stage. LFA-3$^+$VLA-4$^+$4F2$^+$ fetal liver cells may contain multipotent hematopoietic progenitors, as those molecules are also detected on CD34$^+$ hematopoietic stem cells from the human bone

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marrow (our unpublished results). In addition, expression of VLA-4 molecules has also been shown in murine bone marrow lymphomyeloid precursors (68). Last, Tfr molecules were detected on a significant fraction of fetal liver cells, suggesting that they may include Tfr-bearing erythroid precursors and, to a lesser degree, CFU-GM progenitors (69, 70). Since Tfr are also expressed on the most immature human thymocytes (14), early T cell precursors, lacking lineage-restricted markers, may reside within the Tfr^+ fetal liver cell subset.

Despite the lack of T lineage–restricted markers on fetal liver cells from early (7–8-wk) embryos, our molecular studies provided evidence that T cell–specific differentiation events were initiated in the human fetal liver before the time of intrathymic colonization. RNA transcripts for the γ, δ, ϵ, and ζ chains of the CD3-TCR complex were consistently detected in variable numbers of 8-wk-old fetal liver cells. This finding could suggest that transcription of genes encoding all four invariant CD3-TCR components may occur within a discrete subset of putative T cell precursors. However, the possibility that they are independently expressed by different cell types cannot be formally excluded. Fetal liver prethymic T cell precursors have been previously identified as a minor subset of $CD7^+$ cells expressing cytoplasmic CD3-ϵ (cCD3-ϵ) molecules (10). However, cCD3-ϵ expression does not prove T cell lineage commitment, since it can also occur in $CD7^+$ immature thymocytes that display phenotypic and functional features of NK cells, but lack CD3-γ and CD3-δ mRNA (71, and our unpublished results). Our finding in fetal liver of CD3-γ and CD3-δ transcripts further supported the fact that certain fetal liver hematopoietic progenitors represent an early $CD7^-$ maturational step towards mature T cells. Indeed, $CD7^-$ stem cells containing T cell precursors may exist in vivo, since CD7-depleted cells from human bone marrow are still able to reconstitute all hematopoietic lineages (72). Our results also showed that γ, δ, ϵ, and ζ gene transcription in fetal liver cells preceded the expression of TCR-α- and TCR-β-rearranged genes. This gene expression pattern closely resembles that of intrathymic T cell precursors, which contain mature CD3 transcripts but not TCR-α or TCR-β ones (14). Taken together, these data indicate that the transcription of genes coding for the invariant CD3-TCR complex components occurs early in human ontogeny in certain fetal liver cells, which we believe constitute prethymic T cell precursors. In contrast, the expression of genes encoding the α and β polymorphic subunits of the TCR appears as a thymus-restricted T cell maturation event.

Parallel molecular analysis of the transcription of genes encoding IL-2, IL-4, and their corresponding receptors allowed us to investigate the developmental regulation of the activation of these cytokine pathways. As previously observed in the mouse (6, 9), our studies revealed that IL-2 and IL-2Rα transcripts were absent in the human fetal liver. In contrast, both genes were expressed in the fetal thymus, but not in mature thymocytes, suggesting that they are specifically induced intrathymically in early T cell precursors. An interesting finding was the presence of IL-2Rβ transcripts in all fetal tissues analyzed, including the earliest fetal liver samples. In fact, expression of the IL-2Rβ gene was detected in most (70%) fetal liver cells from 8-wk-old embryos. We believe that these cells transcribing the IL-2Rβ gene contain the proposed subset of fetal liver T cell precursors, and functional data using IL-2 lend further support to this notion (see below). However, the quantitative results on the transcription of CD3-TCR genes do not allow us to make absolute correlations, since only ~40, 35, 15, and 8% of fetal liver cells transcribed the ϵ, δ, γ, and ζ genes, respectively. These results also suggest that IL-2Rβ gene expression in fetal liver cells may reflect a common developmental feature of hematopoietic progenitors for different cellular lineages.

Molecular studies also revealed that activation of the IL-4 pathway was induced in fetal liver at 8 wk of gestation. Since IL-4 transcripts were detected in a low fraction (15–20%) of fetal liver cells (data not shown), these analyses did not reveal the cellular source of IL-4 gene expression within the human fetal liver. Therefore, primitive T cell precursors, but also other hematopoietic cells shown to transcribe the IL-4 gene (such as basophils and mast cells; 73), may be included in the IL-4-producing fetal liver cell subset.

Additional evidence that primitive T cell precursors were indeed present in the human fetal liver as early as 8 wk of gestation was provided by functional in vitro studies showing that fetal liver hematopoietic cells were able to proliferate and to differentiate along the T cell lineage. Cell proliferation was sustained by IL-2, as well as IL-4, for at least 5 wk. These studies, however, did not address whether a single fetal liver subpopulation is responding to both cytokines or, alternatively, different hematopoietic precursors selectively respond to particular soluble mediators through the corresponding specific receptors. In favor of the last possibility, we have observed that phenotypically distinct fetal liver cells proliferated in response to IL-2 or IL-4. IL-4-induced growth paralleled the appearance in vitro of cells expressing myeloid-associated antigens. This finding supports the proposed role of IL-4 as a stimulatory factor for myeloid precursors (74). IL-2-proliferating cells, however, mostly acquired CD7 and CD8 T cell markers, and some of them also expressed CD2 molecules. Interestingly, similar $CD8^+$ single-positive immature T cells have been characterized in the rat and mouse thymus as the immediate precursors of $CD4^+CD8^+$ double-positive cortical thymocytes (75). The immature T cell phenotype of the fetal liver cells proliferating to IL-2, together with the finding that most of them, as shown in intrathymic T cell precursors (15), expressed the IL-2Rβ, further suggests that precursors of the T cell lineage are included in the IL-2Rβ^+ subpopulation detected in the early human fetal liver.

The preferential differentiation of $CD7^+$ cells at the expense of $CD14^+$ and $CD4^{dull}$ cells observed in IL-2-driven cultures could indicate that, in vivo, availability of IL-2 by primitive fetal liver T cell precursors may be important in tilting the T lymphoid vs. myeloid differentiation balance. However, terminal differentiation of fetal liver precursors into mature T cells was never observed in vitro upon culture with IL-2 and, therefore, other signals provided by the thymic microenvironment may be involved in the process. Accordingly, mature CD3-TCR α/β^+ T cells appeared in our cultures when thymic epithelial cells (ET-VII) were provided.

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The lack of both TCR α and TCR β transcripts in 8-wk fetal liver cells directly analyzed ex vivo by PCR provided evidence that new genetic programs were initiated in vitro in fetal liver precursors after coculture with thymic epithelial cells, and formally ruled out a possible outgrowth of a small contaminating subset of mature T cells. In addition, they could suggest the functional implication of the IL-2 pathway in the proliferation and the initiation of the T cell differentiation program in early thymic immigrants coming from the liver. However, the recent observation of phenotypically mature T cell populations in IL-2-deficient mice (76) indicates that IL-2 is neither sufficient, nor essential, for T lymphocyte development and, therefore, other cytokines produced in the thymus may functionally replace the missing IL-2. The inductive signals involved in the differentiation program observed in vitro appeared to be delivered upon close cell-to-cell interactions, rather than by epithelium-derived soluble mediators, since cytokines produced by the ET-VII epithelial cells, including IL-3, IL-6, and IL-7, failed to induce the differentiation of fetal liver cells into mature CD3-TCR$^+$ lymphocytes. Neither did those cytokines induce differentiation of CD3-TCR$^+$ mature T cells in the presence of thymic epithelial cells, although acquisition of some T cell markers, such as CD7 and IL-2Rα, was detected in those cultures, as well as in cultures set up in the presence of thymic epithelium without exogenous cytokine supplementation (our unpublished results). These data are in agreement with previously reported data on the important role that interaction with the thymic epithelium plays in the induction of the IL-2Rα gene (7).

Finally, the absence of detectable CD8$^+$CD4$^+$ double-positive cells at different time points from the onset of our cultures would suggest that this subset does not significantly contribute to the generation of TCR α/β–expressing mature T cells from fetal liver precursors. However, the possibility that a small number of double-positive cells, not detected in our study, do serve as T cell progenitors cannot be excluded considering that most cells within the double-positive subset die rapidly in culture, as they do in vivo (77). Another important question is whether the requirements for the generation of CD4$^+$ or CD8$^+$ single-positive mature T cells are different. This seems very likely in view of our results showing that differentiation of CD4$^+$ cells is preferentially driven in our culture conditions. As previously proposed (47, 49), this could reflect the existence of functionally distinct types of epithelial cells within the thymic microenvironment, influencing the differentiation of T cell precursors into a given T cell subset. Establishment of phenotypically and functionally distinct subpopulations of thymic epithelial cells may provide a useful experimental system to approach this question in the near future.

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