Early Human T Cell Development: Analysis of the Human Thymus at the Time of Initial Entry of Hematopoietic Stem Cells into the Fetal Thymic Microenvironment

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Summary

To determine events that transpire during the earliest stages of human T cell development, we have studied fetal tissues before (7 wk), during (8.2 wk), and after (9.5 wk to birth) colonization of the fetal thymic rudiment with hematopoietic stem cells. Calculation of the approximate volumes of the 7- and 8.2-wk thymuses revealed a 35-fold increase in thymic volumes during this time, with 7-wk thymus height of 160 μM and volume of 0.008 mm^3, and 8.2-wk thymus height of 1044 μM and volume of 0.296 mm^3. Human thymocytes in the 8.2-wk thymus were $CD4^+CD8\alpha^+$ and cytoplasmic $CD3\epsilon^+cCD3\delta^+CD8\beta^-$ and $CD3\zeta^-$. Only 5% of 8-wk thymocytes were T cell receptor $(TCR)-\beta^+$, <0.1% were $TCR-\gamma^+$, and none reacted with monoclonal antibodies against $TCR-\delta$. During the first 16 wk of gestation, we observed developmentally regulated expression of CD2 and $CD8\beta$ (appearing at 9.5 wk), CD1a,b, and c molecules (CD1b, then CD1c, then CD1a), TCR molecules ($TCR-\beta$, then $TCR-\delta$), CD45RA and CD45RO isoforms, CD28 (10 wk), $CD3\zeta$ (12–13 wk), and CD6 (12.75 wk). Whereas CD2 was not expressed at the time of initiation of thymic lymphopoiesis, a second CD58 ligand, CD48, was expressed at 8.2 wk, suggesting a role for CD48 early in thymic development. Taken together, these data define sequential phenotypic and morphologic changes that occur in human thymus coincident with thymus colonization by hematopoietic stem cells and provide insight into the molecules that are involved in the earliest stages of human T cell development.

Study of the earliest stages of human T cell development provides important information regarding the molecules involved in the complex series of events that transpire during establishment of the T cell repertoire. Knowledge of the sequence of expression of developmentally regulated T cell functional molecules can provide insight into mechanisms of normal T cell maturation, and can provide information necessary to form hypotheses regarding molecular mechanisms of thymus growth. We must understand the stages of normal T cell development to devise successful strategies for immune reconstitution of a variety of acquired and congenital T cell immune deficiency syndromes.

Whereas enormous progress has been made in our understanding of the earliest stages of murine (for review see reference 1) and avian (for review see reference 2) thymus development, the sequence of events at the time of colonization of the human thymus with hematopoietic stem cells has not been studied. We have previously studied human fetal thymuses at 7 wk, before lymphoid colonization of the thymus (3), and at 9.5 wk of gestation, 1.5 wk after hematopoietic stem cell migration to the thymus (3–5). Our prior efforts to study lymphoid fetal thymus at earlier than 9.5 wk of gestational age were thwarted by lack of availability of thymus tissues at the time of colonization of the thymus, the small size of the human thymus at 8 wk ($\sim$1 mm $\times$ 1 mm $\times$ 0.5 mm), and the frequent contamination of first trimester fetal tissue with placental tissue containing $CD7^+$, decidual granular lymphocytes of NK lineage (6, 7). Thus, the phenotypic and morphologic events that occur at the initiation of lymphopoiesis in the human thymus have not been observed. In this study, we have definitively identified the human thymus in fetal thorax at 8.2 wk of gestation, defined its three-dimensional structure, and determined the reactivity of 64 mAbs with this tissue, as well as with fetal thymuses at 9.5, 10, 12.75, 13, and 16 wk of gestational age. Our data document the extraordinary thymus growth that occurs at the time of stem cell colonization of the thymus and demonstrate developmentally regulated expression of a large series of lymphoid and thymic microenvironment molecules.

Materials and Methods

Thymic Tissue. Fetal tissues were obtained as discarded material from the Pathology Department, Duke University Medical

Center, from either elective first trimester abortions or at the time of surgery for ectopic pregnancy as described (3–5). Gestational age, determined by crown-rump length, menstrual records when available, and fetal part morphology, ranged from 7 to 34 wk of gestation (3–5). Fetal tissues 50 (7 wk; reference 3) and 58 (8.2 wk) were serially sectioned through the thorax and neck area in 4-μM sections and hematoxylin and eosin (H&E)[1]-stained sections were prepared every 40 μM (50; reference 3) or 80 μM (58). In these tissues, the thymus was identified as a keratin-containing structure in the neck lateral to the trachea and esophagus (7 wk; reference 3) or in the upper mediastinum anterior to the trachea and great vessels (8.2 wk; reference 8).

Computer Reconstruction of 7- and 8.2-wk Fetal Thymuses. Three-dimensional reconstruction of thymus 50 (7 wk) and thymus 58 (8.2 wk) were performed using the PC3D three-dimensional reconstruction program, version 5.0 (Jandel Scientific, Corte Madera, CA). For 8.2-wk fetal thymus 58, thymus perimeters were traced from 14 sections.

Monoclonal and Polyclonal Antibodies. Monoclonal and polyclonal antibodies used in this study and their sources are listed in Table 1. Each antibody was used at saturating concentrations.

Immunofluorescence Assays. Indirect immunofluorescence (IF) assays were performed on acetone-fixed 4-μM tissue sections as described (3, 4) by use of FITC-conjugated goat anti–mouse Ig (Kirkegaard and Perry Labs., Inc., Gaithersburg, MD). Indirect IF assays on cell suspensions were performed as described (3–5) and analyzed on either a FACS® Star Plus (Becton Dickinson & Co., Mountain View, CA) or profile flow cytometer (Coulter Corp., Hialeah, FL). IF assays were usually performed in duplicate, and select mAb IF assays were repeated three times.

Results

Computer-assisted Three-dimensional Reconstruction of Fetal Thymus 50 Before Thymic Colonization and Fetal Thymus 58 at the Time of Thymic Colonization. Fetal thymus 50 at 7 wk of gestation (before lymphoid colonization) was devoid of CD45+ cells and consisted of only two spherical thymic rudiments, each ∼160 × 180 × 200 μM, in the lateral neck areas (3). Only four H&E-stained sections were available for this tissue; thus, a fully interpretable computerized image could not be generated. The calculated total volume (both rudiments) of nonlymphoid thymus 50 at 7 wk was 0.008 mm³.

In contrast, 8.2-wk thymus 58 was filled with CD45+ hematopoietic cells, and was 1,044 μM in length, 1,200 μM wide, and 480 μM deep. The 8.2-wk right and left thymic rudiments were located anterior to the esophagus, trachea, and great vessels in the upper thorax (Fig. 1). Computer-assisted reconstruction of the 8.2-wk fetal thymus demonstrated that the lower lobes of the thymus had fused, whereas the right and left upper thymic rudiments remained separate. The right thymic rudiment was a nonlobulated cylinder, whereas the left thymic rudiment was just beginning to become lobulated. Most published images of fetal thymus at this stage of development show two elongated cylindrical tubes

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[1] *Abbreviations used in this paper:* c, cytoplasmic; H&E, hematoxylin and eosin; ICAM, intercellular adhesion molecule; IF, immunofluorescence; s, surface; TdT, terminal deoxynucleotidyl transferase; TE, thymic epithelial; VLA, very late antigen of activation.

on each side of the neck, meeting in the middle (9). However, analysis of the actual three dimensional structure of the 8.2-wk thymus demonstrated a globular structure, with the right and left lobes beginning to rotate around a central axis and intertwine. The calculated volume of 8.2-wk thymus 58 was 0.296 mm³. Thus, we estimated that the thymus increases in volume ∼35-fold from 7 wk (nonlymphoid stage) to 8.2 wk (after lymphoid colonization).

Expression of Human T Cell Lineage Molecules During Early Thymus Development. The expression of a panel of T cell lineage molecules (Table 1) was studied in 8.2-, 9.5-, 10-, 12.75-, 13-, and 16-wk fetal thymuses (Table 2). Studies in thymuses 48 (9.5 wk), 22 (10 wk) and 40 (12.75 wk) have been reported previously for some of the molecules studied (3–5) and are included in Table 2 for completeness. The new and striking findings in this study were (a) lack of expression of CD2 at the time of thymic colonization at 8.2 wk; (b) lack of expression at 8.2 wk of CD8β with expression of CD8α; and (c) sequential expression of CD1b, then CD1c, then CD1a molecules from 8.2 to 13 wk. Fig. 2 shows lack of CD2 and CD8β expression at 8.2 wk, with all 8.2-wk thymocytes CD4+CD8α+CD7+, and CD5+. The finding that most thymocytes at this stage were CD4+CD8+ (double positive) was also surprising, since most murine thymocytes at the stage analogous to the 8.2-wk human thymus development (11 d) are CD4−CD8− (10, 11). By 10 wk, all thymocytes expressed CD8β, followed by appearance of CD8α/β+ and CD8α/β− subsets in the thymic medulla at 12.75 wk (Table 2). By 12.75 wk, CD1a, -b, and -c expression was similar to that seen in postnatal thymus, with the typical CD1 pattern of all cortex and few medullary cells being CD1+.

Expression of CD45 Molecule Isoforms During Early Human T Cell Development. It has been suggested that CD45RA+ human thymocytes represent the generative thymocyte lineage destined for positive selection and thymic export, whereas CD45RO+ thymocytes are destined to undergo negative or no selection and die intrathymically (12). We found few CD45RA+ cells in 8.2- and 9.5-wk thymuses (Fig. 3, B and E), whereas in 12.75-wk thymus, a distribution of CD45RA+ cells similar to postnatal thymus was seen, that is, scattered cortical thymocytes were CD45RA+, with large clusters of CD45RA+ thymocytes in the thymic medulla (Fig. 3 H). Similarly, by 10–12.75 wk, most thymocytes were CD45RO+ (Fig. 3 I), raising the possibility of onset of thymocyte-negative selection during this period (13, 14).

Expression of TCR-associated Molecules During Early T Cell Development. Data for percentages of cells positive for TCR-β, TCR-δ, and CD3ε in thymuses 48, 22, 5, and 40 have been previously reported (3–5). Whereas CD3ε and CD3δ were present in 8.2-wk thymus in most thymocytes (Fig. 4, D and E), expression of CD3ζ chain was not observed until 10 wk (Fig. 4 F and Table 3). By 12.75 wk, most medullary thymocytes were CD3ζ+. CD3ζ expression gradually increased in cortical thymocytes until birth, when CD3ζ mAb 2H2 reacted with a pattern (cortical thymocytes low positive, medullary thymocytes high positive) similar to a reactivity pattern of CD3ε mAb, Leu 4 (not shown).

Table 1. *mAbs Used to Study Fetal Tissues*

mAb	Specificity	Source
NA1/34	CD1a	A. McMichael, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK
10DR2	CD1a	2nd IWLDA
NUT	CD1b	2nd IWLDA
4A76	CD1b	2nd IWLDA
M241	CD1c	2nd IWLDA
35.1	CD2	J. Hansen, University of Washington, Seattle, WA
OKT11	CD2	American Type Culture Collection, Rockville, MD
Leu 4	CD3ϵ	R. Evans, University of Rochester, Rochester, NY
SP34	CD3δ	C. Terhorst, Harvard University, Boston, MA
2H2	CDζ	P. Anderson, Harvard University, Boston, MA
Leu 3a	CD4	R. Evans, University of Rochester, Rochester, NY
T101	CD5	2nd IWLDA
12.1	CD6	P. Martin, University of Washington, Seattle, WA
3A1e	CD7	B. Haynes, Duke University, Durham, NC
Leu 2a	CD8α	R. Evans, University of Rochester, Rochester, NY
25T8-5H7	CD8β	E. Reinherz, Harvard University, Boston, MA
TS1/22	CD11a/LFA-1α	T. Springer, Harvard University, Boston, MA
MO-1	CD11b/MO-1	R. Todd, University of Michigan, Ann Arbor, MI
Leu M3	CD14	R. Winchester, Columbia University, NY
4F7	CD16	P. Anderson, Harvard University, Boston, MA
TAC	CD25	T. Waldmann, National Institutes of Health, Bethesda, MD
9.3	CD28	P. Martin, University of Washington, Seattle, WA
KOLT2	CD28	2nd IWLDA
MY9	CD33	Coulter Corp., Hialeah, FL
HPCA-1	CD34	Becton Dickinson & Co., Mountain View, CA
F10-89-4	CD45	J. Fabre, R. Dalchau, University of London, London, UK
F8-11-13	CD45RA	J. Fabre, R. Dalchau, University of London, London, UK
UCHL-1	CD45RO	P. Beverly, University College, Middlesex School of Medicine, London, UK
6.28	CD48	5th IWLDA
Mo2PT501	CD48	5th IWLDA
TS2/7	CD49a/VLA-1	M. Hemler, Harvard University, Boston, MA
12F1	CD49b/VLA-2	M. Hemler, Harvard University, Boston, MA
A3-IIF5	CD49c/VLA-3	5th IWLDA
B5G10	CD49d/VLA-4	M. Hemler, Harvard University, Boston, MA
K20	CD29/VLA-β1	A. Bernard, University of Nice, Nice, France
BQ16	CD49f/VLA-6	5th IWLDA
RR-1	CD54/ICAM-1	R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT
TS2/9	CD58/LFA-3	T. Springer, Harvard University, Boston, MA
H19	CD59	A. Bernard, University of Nice, Nice, France
5E9	CD71/transferrin receptor	B. Haynes, Duke University, Durham, NC
10F9	CD99/E2-MIC2	B. Haynes, Duke University, Durham, NC
βF1	TCR-β	M. Brenner, Harvard University, Boston, MA

Table 1. *(continued)*

mAb	Specificity	Source
Polyclonal	TCR-γ	M. Brenner, Harvard University, Boston, MA
TIgammaA	TCR Vγ9	T. Hercend, Gustave Roussy Institute, Villejiuf, France
TCRδ1	TCR-δ	M. Brenner, Harvard University, Boston, MA
A13	TCR Vδ1	L. Moretta, University of Genoa, Genoa, Italy
BB3	TCR Vδ2	L. Moretta, University of Genoa, Genoa, Italy
TCSδ1	TCR Vδ1-Jδ1	T Cell Sciences Inc., Cambridge, MA
HAE2a	VCAM-1	T. Tedder, Duke University, Durham, NC
ELAM-1	E-selectin	T. Tedder, Duke University, Durham, NC
LAM1.3	L-selectin	T. Tedder, Duke University, Durham, NC
αVWF	VWF	C. Greenberg, Duke University, Durham, NC
133	B7-1	L. Nadler, Harvard University, Boston, MA
TE-4	TE-4	B. Haynes, Duke University, Durham, NC
TE-3	TE-3	B. Haynes, Duke University, Durham, NC
TE-8	TE-8	B. Haynes, Duke University, Durham, NC
AE-1	Keratin	H. Sun, New York University, New York
12-1/2	HTLV-I p19 and 46KD keratin	M. Robert-Guroff, National Institutes of Health, Bethesda, MD
TE-19	A blood group	B. Haynes, Duke University, Durham, NC
A2B5	GQ gangliosides	G. Eisenbarth, University of Colorado, Denver, CO
6D5(V2)	Vessels	B. Haynes, Duke University, Durham, NC
TE-7	TE-7	B. Haynes, Duke University, Durham, NC
7401	Tissue transglutaminase	C. Greenberg, Duke University, Durham, NC
HB15a	Dendritic cells	T. Tedder, Duke University, Durham, NC

IWLDA, International Workshop on Leukocyte Differentiation Antigen.
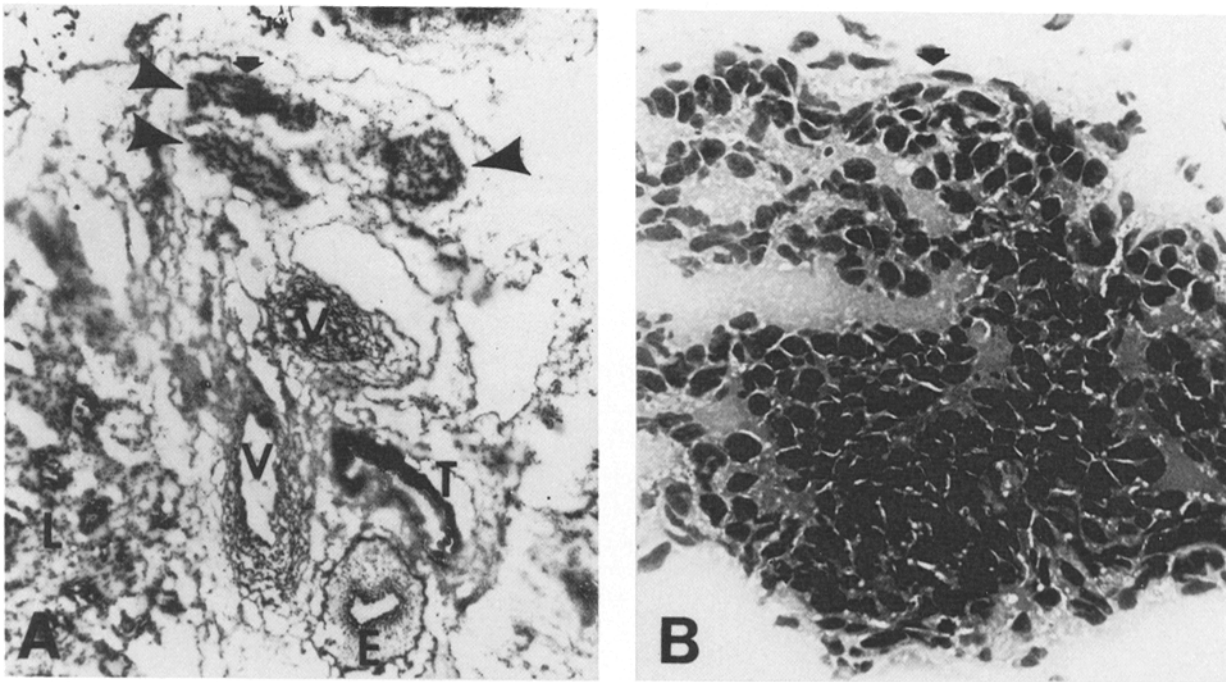
Figure 1.   H&E-stained sections of fetal tissue 58. *A* shows single right and double left thymus lobes (*arrowheads*), trachea (*T*), esophagus (*E*), and left lung (*L*). ×50. *B* shows left thymus lobe. Small arrowhead on upper thymus left lobe in *A* shows same point at small arrowhead of magnified left thymus lobe in *B*. ×800.

Table 2. *Expression of Thymocyte Lineage Molecules During Early Thymus Development*

Fetal thymus no.	Age	Molecule analyzed											
		CD34	CD7	CD1a	CD1b	CD1c	CD2	CD4	CD8α	CD8β	CD5	CD6	CD28
	wk												
58	8.2	C,V+	+	–*	+‡	+/–	–	+	+	–	+	–	–
48	9.5	C,V+	+	+/–	+/–	+/–	+	+	+	+/–	ND	ND	–
22	10.0	C,V+	+	+/–	+/–	+/–	+	+	+	+	ND	–§	+/–
40	12.75	C,V+	+	+/–	+/–	+/–	+	+	+/–	+/–	+	+	+
56	13.0	C,V+	+	+/–	+/–	+/–	+	+	+/–	+/–	+	+	+
57	16.0	C,V+	+	+/–	+/–	+/–	+	+	+/–	+/–	+	+	+

* mAb nonreactive or rare cell⁺ (2–4/slide); +/–, subset of cells reactive.
‡ All cells reactive in the appropriate thymus zone (e.g., for CD1, all cortical thymocytes reactive; for CD5, all thymocytes reactive; for CD6, all medullary thymocytes reactive, etc.). For CD1b at 8.2-wk and CD8α and CD8β at 10 wk, all thymocytes were +. For CD8β at 12.75, 13, and 16 wk, +/– signifies cortex⁺ and a subset of medullary thymocytes⁺; at 9.5 wk, +/– for CD8β signifies that only a subset of total thymocytes were reactive.
§ With mAb T12, thymocytes at 10 wk were very dim +.
C,V+, capsule and thymic vessels +. Beginning at 9.5 wk, rare CD34⁺ mononuclear cells also were seen scattered in thymus, but from 8.2 to 16 wk, most thymocytes were CD34⁻. ND, not done.

TCR-β expression was acquired in increasing percentages of thymocytes during the first trimester, with only rare (~5%) 8.2-wk thymocytes being TCR-β⁺ (Fig. 4 A). Only very rare TCR-γ cells were found to be present at 8.2 wk by use of a polyclonal anti-TCR-γ antibody or the anti-Vγ9 mAb, T1γA (not shown). No thymocytes at 8.2 wk expressed

TCR-δ as determined by mAbs TCR-δ1 (anti-TCR-δ), BB3 (anti-Vδ2) (Fig. 4 B) or A13 (anti-Vδ1). Thus, in the human fetal thymus, TCR-β expression was first observed coincident with the appearance of rare TCR-γ⁺ cells.

As previously reported (4), TCR-δ expression peaked (11% of T cells) at 9.5 wk, the time of first appearance of TCR-δ
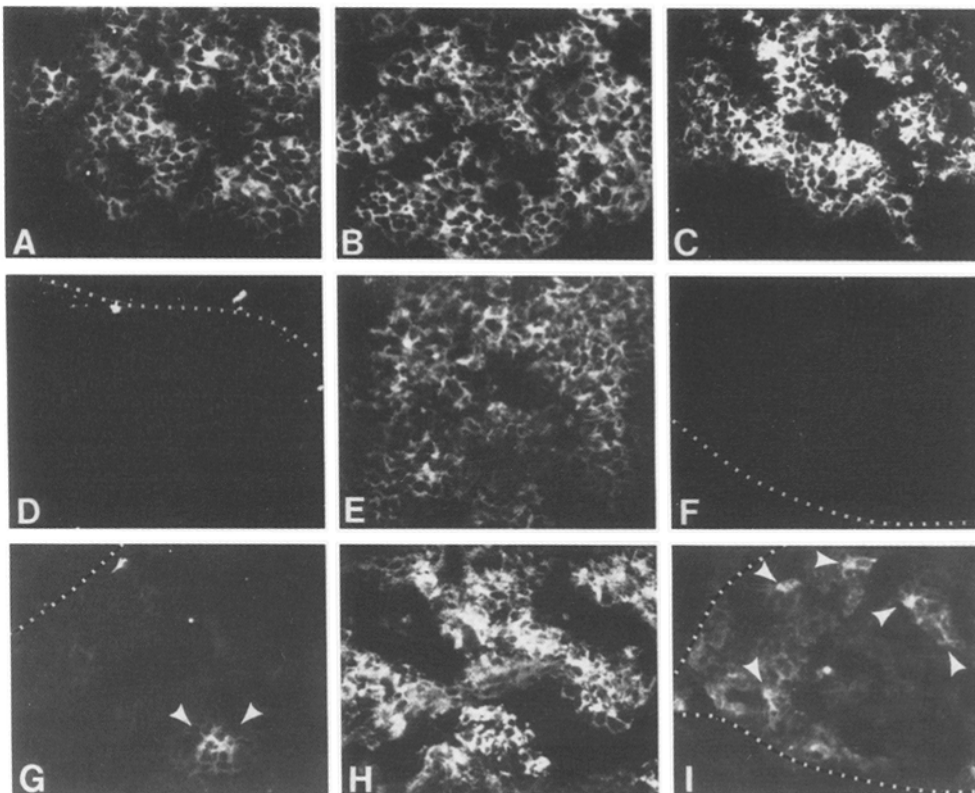
Figure 2. Analysis of thymocyte phenotype at the time of initial colonization of the human thymus. Reactivity of human 8.2-wk thymocytes are shown in A with CD7 mAb 3A1e, in B with CD4 mAb Leu 3a, and in C with CD8α mAb Leu 2a. However, no thymocytes were reactive with the CD8β mab 25T8-5H7 (D). All thymocytes were CD5⁺ with mAb T101 (E). Surprisingly, no thymocytes reacted with either CD2 mAb 35.1 (F) or OKT11 (not shown). There was also asynchronous expression of CD1a, CD1b, and CD1c molecules. CD1a mAb NA1/34 only reacted with rare scattered macrophage-like cells (G), whereas CD1b mAb NUT2 reacted with all 8.2-wk thymocytes (H). CD1c mAb M241 only reacted with ~1% of 8.2-wk thymocytes (I). Dotted line shows borders of thymus. ×400.
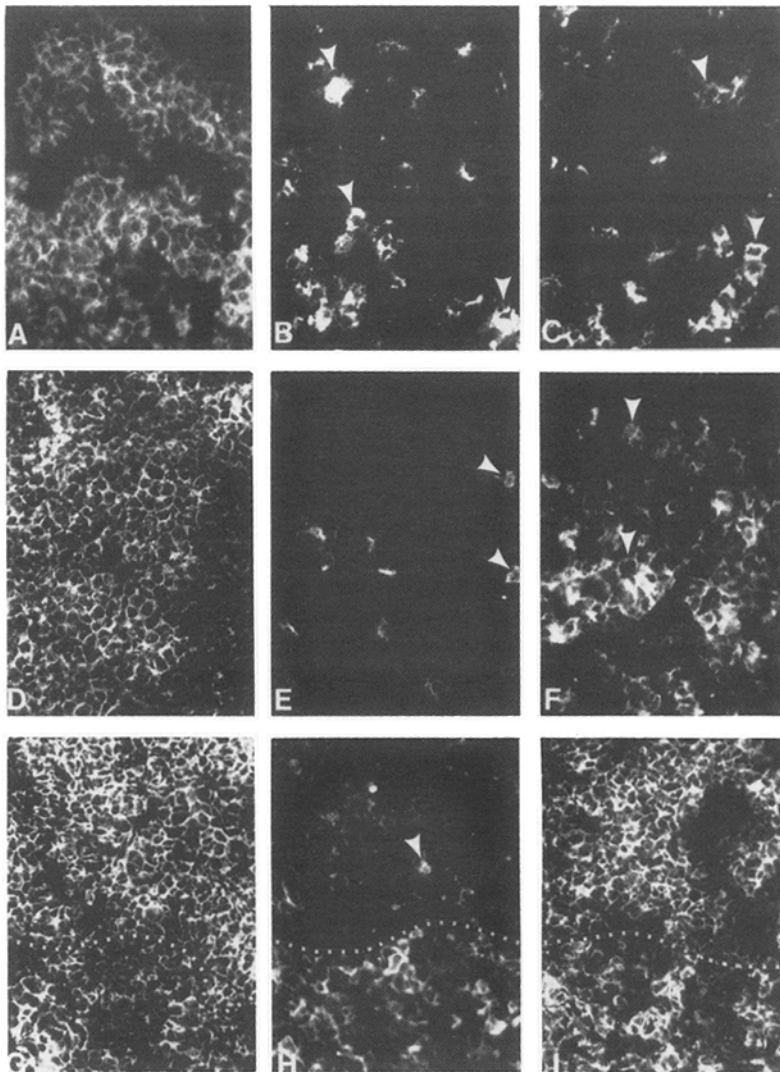
Figure 3. Ontogeny of expression of CD45 isoforms during the earliest stages of human thymus development. (*A–C*) 8.2-wk fetal thymus 58; (*D–F*) 9.5-wk fetal thymus 48; (*G–I*) 12.75-wk fetal thymus 40. (*A, D,* and *G*) Reactivity of the pan-CD45 mAb F-10-89-4; (*B, E,* and *H*) reactivity of the CD45RA mAb F-8-11-13; and (*C, F,* and *I*) reactivity of the CD45RO mAb UCHL-1. ×400.

(Table 3). In this study with anti-Vδ1 and -Vδ2 mAbs, we found that the ratio of Vδ2/Vδ1 T cells varied over time of gestation (Fig. 5). There were two general peaks of Vδ2 predominance over Vδ1 TCR-δ⁺ cells, initially at 9.5 wk and later beginning at 16 wk and continuing through the last trimester. Even though the ratio of Vδ2/Vδ1 remained high until birth, the precentage of total TCR-δ⁺ thymocytes remained low during the second and third trimesters.

Expression of Adhesion Molecules During Early Human T Cell Development. In contrast to lack of reactivity of CD2 mAbs with the 8.2-wk thymus, LFA-3 (CD58) mAb TS2/9 reacted with 8.2-wk thymic epithelium, raising the question of the presence of a second intrathymic CD58 ligand at 8.2 wk (Fig. 6 *B*). Recently, the CD48 molecule in mouse and humans has been shown to bind to CD58, although the significance of CD58–CD48 interactions in humans has been questioned (15). Using two CD48 mAbs (6.28 and MO2PT501), we found that thymocytes from 8.2 wk through birth were CD48⁺ (Fig. 6 *B*). Two additional putative CD2 ligands, CD59 (H19/DAF) (16) and CD99 (E2/MIC2) (17), were

found to be present in 8.2-wk thymus (Fig. 6, *C* and *D*). Intercellular adhesion molecule 1 (ICAM-1) (CD54)–LFA-1 (CD11a/CD18) interactions are also important in thymic epithelial (TE)–thymocyte interactions (18, 19). At 8.2 wk (Fig. 7, *E* and *F*), and throughout gestation (18, 20), thymocytes were CD11a⁺, and TE cells were ICAM-1⁺.

CD49 (very late antigen of activation [VLA] CD49/CD29) molecules are a family of integrins that mediate cell binding to a variety of extracellular matrix and cell surface molecules (21). In the 8.2-wk thymus, VLA-1 mAbs reacted with thymic vessels, VLA-2 mAb reacted throughout with thymic stromal cells (fibroblasts and TE cells), VLA-3 mAb A3-IIF5 reacted with TE cells and thymocytes in a surface and antinuclear antibody pattern, VLA-4 reacted with 8.2-wk thymocytes (although thymic vessels did not express V-CAM, a ligand for VLA-4, until 13 wk), and VLA-6 mAb BQ16 reacted with thymic stroma throughout the 8.2-wk thymus (Fig. 7). VLA-β1 (CD29) mAb K20 reacted with all thymus components throughout ontogeny (Fig. 7 *D*).

Because it is often difficult to distinguish between thymic

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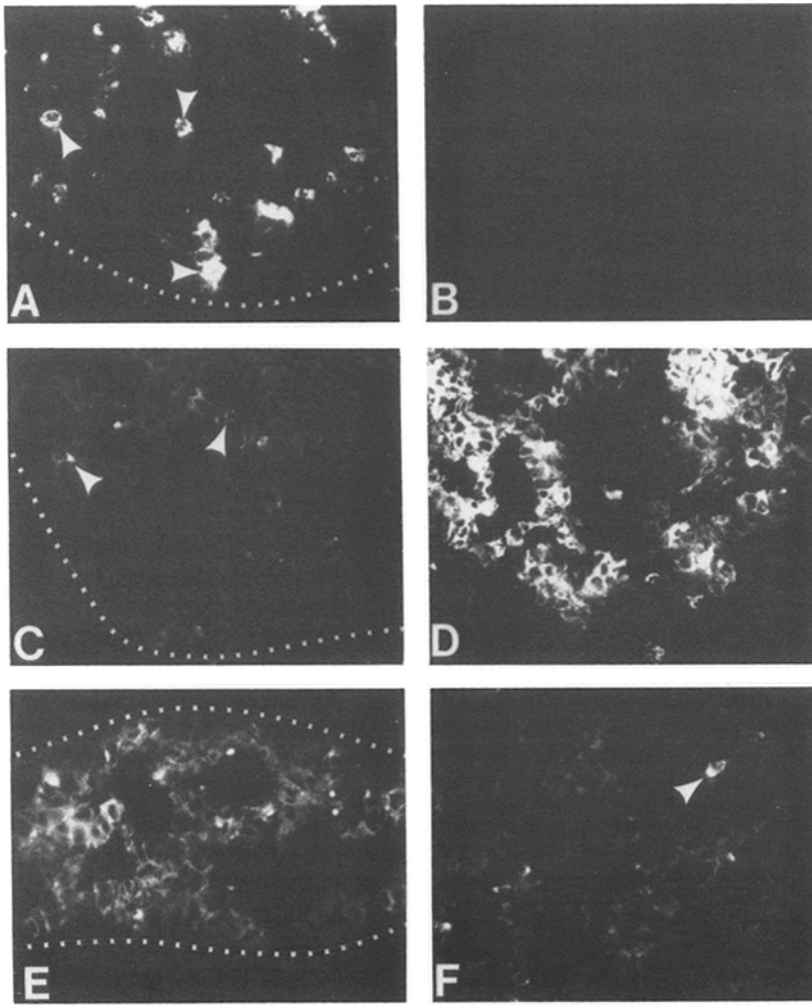
Figure 4. TCR-related molecule expression in 8.2-wk human thymus. (*A*) cTCR-β expression with mAb βF1. (*B*) No reactivity with TCR-δ mAb BB3, which is specific for Vδ2. The pan-TCR-δ mAb TCR-δ1 was also nonreactive (not shown). (*C*) Rare Vγ9+ cells in thymus with mAb T1γA. (*D*) All thymocytes were CD3ε+. The dark area in the center of the thymus is tissue artifact. (*E*) Most thymocytes were also weakly CD3δ+. (*F*) A rare 8.2-wk cell (*arrowhead*) that was CD3ζ+ with mAb 2H2 with most thymocytes at 8.2 wk CD3ζ−. ×400.

**Table 3.** *Expression of TCR-associated Molecules During Early Human T Cell Development*

Fetal thymus no.	Age	Molecule analyzed									
		CD3ε	CD3δ	CD3ζ	TCR-β	TCR-γ	TCR-Vγ9	TCR-δ	TCR-Vδ1	TCR-Vδ2	Vδ2/Vδ1
	wk										
58	8.2	100	100	0	5	0*	0*	0	0	0	0
48	9.5	96	100	0	32	ND	ND	11.0	0.4	9.0	18
22	10.0	100	100	0*	47	ND	0.1	4.0	0.4	6.0	15
40	12.75	100	100	c+/−,m+	69	ND	ND	1.0	1.0	8.0	8
56	13.0	100	100	c+/−,m+	60	ND	1.0	0.6	0.5	1.0	2
5	15.0	99	100	ND	83	ND	ND	1.0	0.7	1.5	2
57	16.0	100	100	c+/−,m+	80	ND	1.0	1.3	0.1	1.4	5

* Rare cells +; c+/−, subset of cortical thymocytes +; m+, most medullary thymocytes +.
Data for CD3ε, TCR-β, and TCR-δ are from reference 8. *ND*, not done.
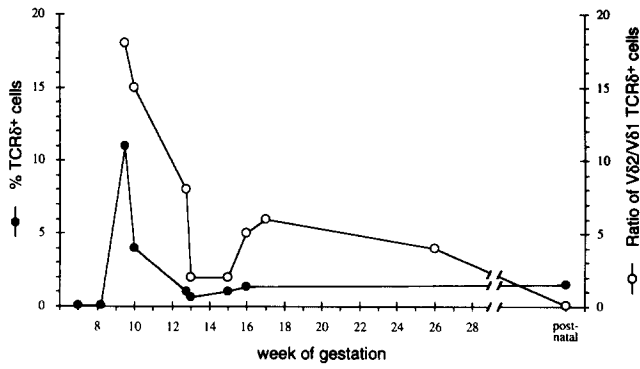
Figure 5. TCR-δ cell development in human fetal thymus. The figure shows the percentage of TCR-δ+ cells in fetal thymuses (*solid circles*) (data are from reference 8 and this study) and an analysis of the ratio of the number of Vδ2+ (mAb BB3) to Vδ1+ (mAb A13) cells (*open circles*). Analysis shows an initial peak in the percentage of TCR-δ cells at 9.5 wk of gestation (8) with most of the TCR-δ cells Vδ2+. By week 12, the percentage of TCR-δ cells decreased to 1–2% of total thymocytes, with Vδ2+ cells predominating throughout fetal thymus development.
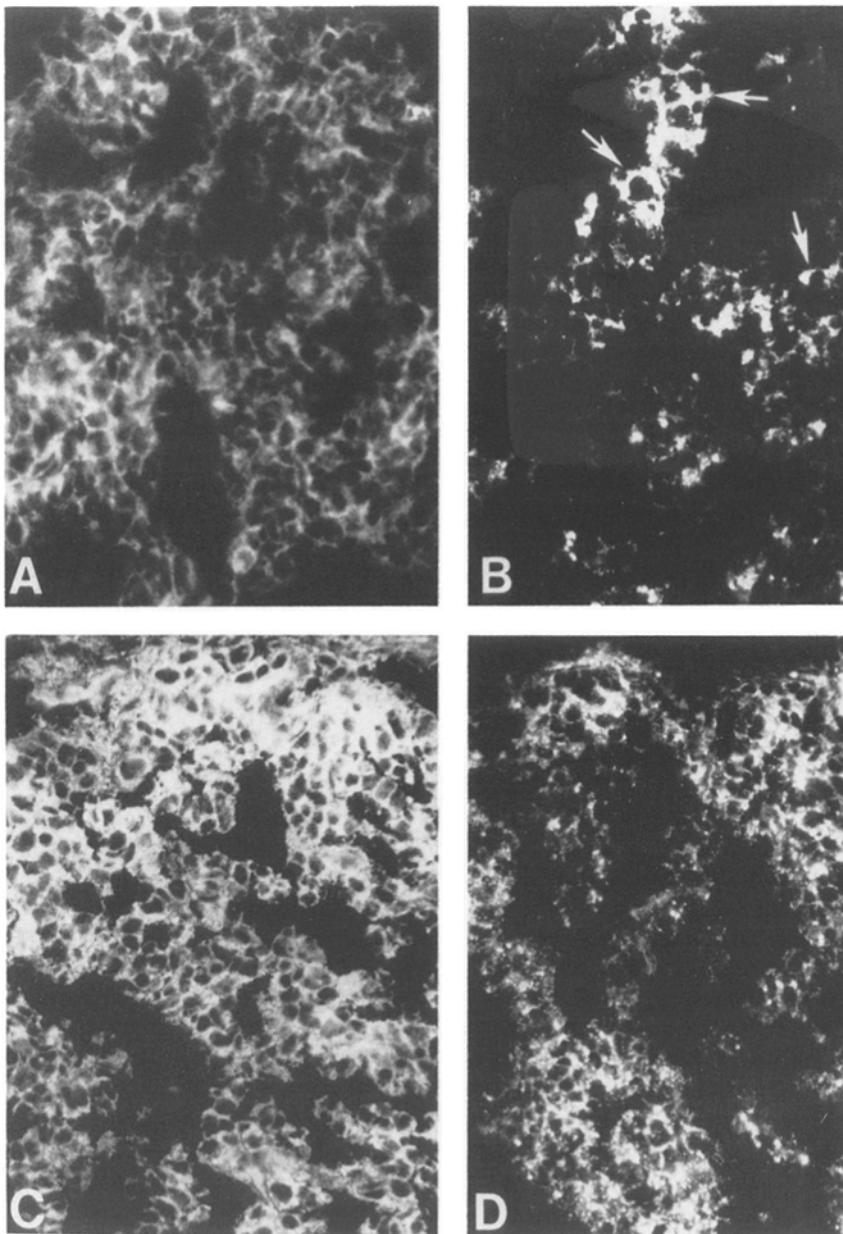
Figure 6. Expression of CD58, CD48, CD99 and 59 antigens in 8.2-wk fetal thymus. The figure shows analysis of 8.2-wk fetal thymus in indirect IF assay with mAbs against CD58 (TS2/9, *A*), CD48 (6.28, *B*) CD99 (10F9, *C*), and CD59 (H19, *D*). Whereas CD2 was not expressed by thymocytes in 8.2-wk thymus (Fig. 3 *F*), a second ligand for CD58, CD48, was expressed (*A*). ×400.
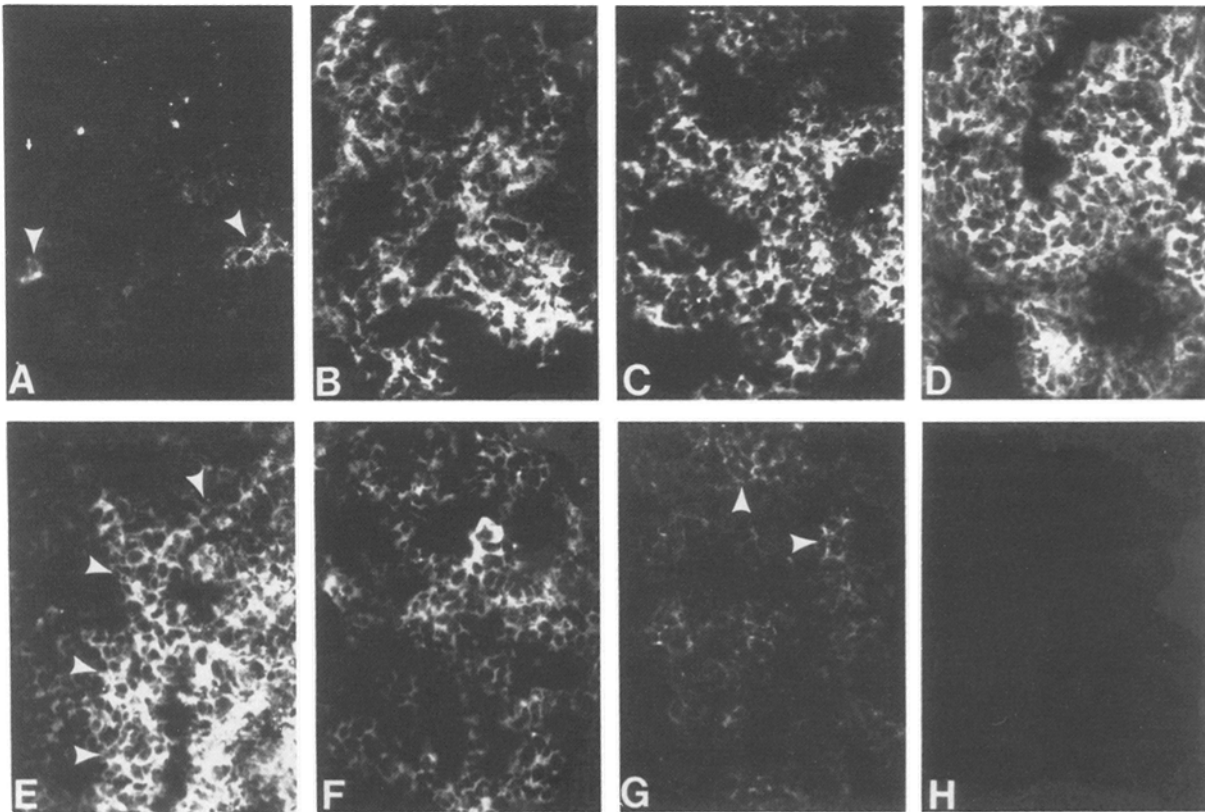
Figure 7. Expression of adhesion molecules in 8.2-wk fetal thymus. (*A*) CD49a (VLA-1) mAb reactivity with vessels within the thymus (*right arrowhead*) and with scattered thymic microenvironment cells (*left arrowhead*). VLA-2 mAb 12F1 reacted with thymic stromal elements throughout the 8.2-wk thymus (*B*), whereas VLA-4 mAb B5G10 reacted with most thymocytes (*C*). (*D*) Reactivity of CD29 (VLA-β1) (mAb K20) with both thymic stromal and thymocyte thymus components. (*E* and *F*) Reactivity of CD54 mAb (RR.1) and CD11a/CD18 LFA-1 (TS1/22) with 8.2-wk thymus. CD54 expression was on central epithelium only (*E*), LFA-1 mAb TS1/22 reacted with mononuclear cells throughout the thymus (*F*). (*G*) Faint scattered cell reactivity with L-selectin mAb, LAM1.3; (*H*) lack of reactivity of 8.2-wk thymic vessels with VCAM-1 mAb HAE2a. ×400.

stromal and thymocyte reactivity in tissue section IF assays, the reactivity of VLA-1, -2, -3, -4, -6, VLA-β1, CD59, CD99, and CD48 mAbs with postnatal thymocytes and cultured TE cells was assayed by use of flow cytometry on single-cell suspensions. VLA-2, VLA-3, and VLA-6 mAbs reacted with TE cells, and VLA-4 mAbs reacted only with thymocytes (21). VLA-1 mAb TS2/7 reacted weakly with TE cells grown in culture, while in tissue sections, only thymic vessels were VLA-1$^+$ (this study and Patel, D. D., and B. F. Haynes, unpublished observations). VLA-β1 mAb K20 reacted with both TE cells and thymocytes. In cell suspensions, CD48 was thymocyte specific, while CD59 and CD99 mAbs reacted with both TE cells and thymocytes (not shown).

From 9.5 wk through birth, VLA-2 mAb 12F1 reacted primarily with subcapsular cortical thymic epithelium, and VLA-4 mAb B5G10 remained thymocyte specific (21). VLA-1 mAb TS2/7 reacted with vessels and scattered stromal cells, and VLA-β1 K20 mAb reacted with all thymus cell types. VLA-3 mAb A3-IIF5 reacted with thymic fibroblasts, vessels, and epithelium. VLA-6 mAb BQ16 reacted with TE cells, fibrous capsule, and vessels in fetal and postnatal thymuses (not shown).

Analysis of Thymic Microenvironment Components of 8.2-wk Fetal Thymus 58. We have previously reported the analysis

of thymic microenvironment components of thymuses of 9.5 wk of gestation and older with many of the reagents listed in Table 1 (3, 22–26). The key issues with the 8.2-wk fetal thymus were (*a*) Could separate endodermal (TE-3$^+$) (early cortical) and ectodermal (TE-4$^+$, p19$^+$) (early medullary) zones of TE cells be visualized? (*b*) Were vessels present within the parenchyma of the right and left 8.2-wk thymus? (*c*) Were macrophages and dendritic cells present in the thymus at 8.2 wk? and (*d*) Was there any evidence of myelopoiesis in the 8.2-wk thymus?

First, all 8.2-wk thymic epithelium was TE-3$^+$, whereas only the central TE zone was TE-4$^+$, directly demonstrating that TE-4 medullary epithelium was located in an inner core of the thymus cylinder, and demonstrating that the TE-3 antigen, though preferentially expressed in cortical TE in postnatal thymus (23), was equally expressed at 8.2 wk in both cortical and medullary TE cells (Fig. 8, *C* and *D*). Thus, these data suggested that before thymus lobulation, there was a TE-4$^-$ outer cortical (endodermal) epithelial zone surrounding a TE-4$^+$ inner medullary (ectodermal) epithelial core.

There were no vessels revealed by morphology or antivessel mAb reactivity in the nonlobulated right thymus, but in the lobulated left thymus, there were vessels approaching (Fig.

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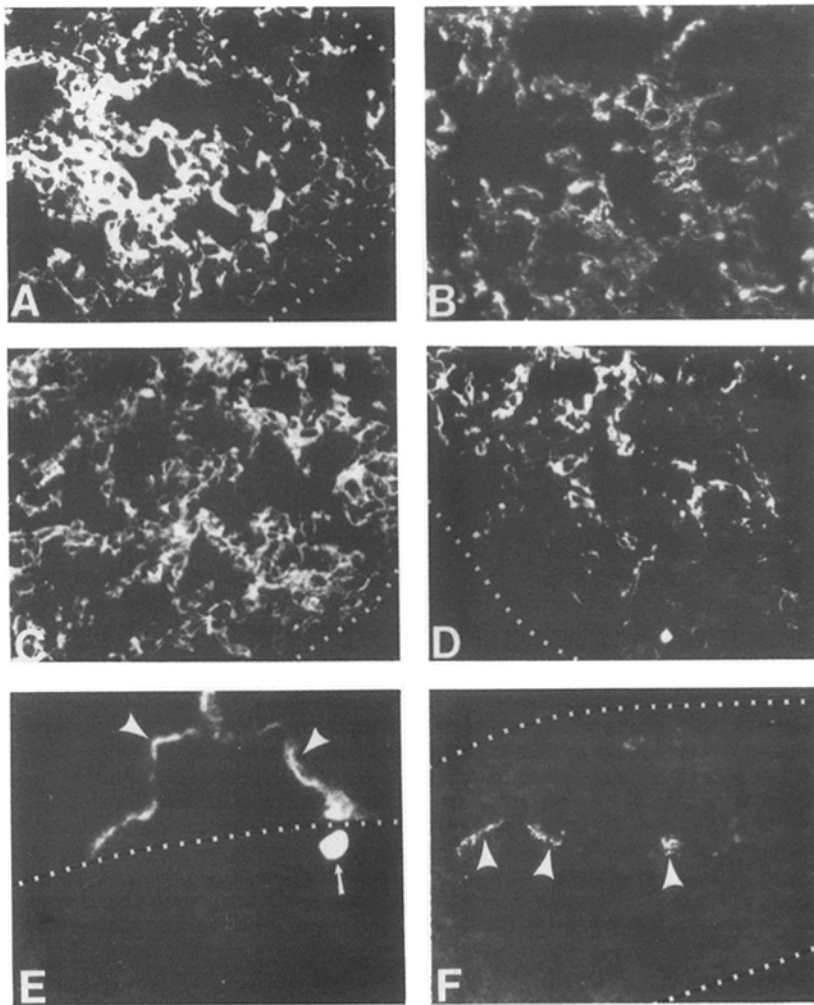
Figure 8. Analysis of human thymic microenvironment components of human 8.2-wk fetal thymus. In all panels, the dotted line shows the outer border of the thymus. (*A*) Keratin-positive epithelium in the right thymus with anti-keratin mAb AE1. (*B*) The right thymus expressed the endocrine epithelial ganglioside antigen A2B5. (*C*) Reactivity of all thymic epithelium with mAb TE-3, whereas (*D*) only central TE cells of the right thymus were TE4+. (*E*) An extrathymic bifurcating vessel (mAb V2) (*arrowheads*) just approaching the left thymus capsule (*dotted line*) with the thymus below the dotted line. The arrow points out autofluorescent artifact. (*F*) Scattered Von Willebrand factor–positive endothelial cells within the left thymus. ×400.

8 *E*) and within (Fig. 8 *F*) the thymus. In addition, thymus 58 epithelium was keratin+p19− (mAb that binds a developmentally regulated keratin [22, 24, Palker, T. J., and B. F. Haynes, unpublished observations]), TE-8+TE19+ (medullary TE only; 25), B7− and A2B5+ (a marker of medullary TE; 26). Connective tissue around 8.2-wk TE cells was TE-7+ and tissue transglutaminase+.

Papiernik et al. (27) have suggested that hematopoietic stem cells, upon entering the thymus, are driven to differentiate into thymic macrophages and dendritic cells as well as T cells. Kurtzberg et al. showed CD7+CD3−CD4−CD8− thymocyte differentiation into myeloid as well as lymphoid lineages in vitro (28). In 8.2-wk thymus 58, no immature myeloid cells were detected with CD33 mAb MY9 (not shown). CD14+ and CD11b+ macrophages were present in 8.2-wk thymus, as were CD16+ cells with a similar morphology (not shown). No HB15a+ dendritic cells were found in 8.2-wk thymus. Whereas intrathymic lymphocytes were CD34−, scattered large cells with long cytoplasmic processes were CD34+, and similar cells were seen around the spinal cord, aorta, fetal liver, and esophagus (not shown). CD34+ cells around the esophagus were CD4−CD8−CD7−CD44+

MO-1+HB15a−CD33−. Thus, cells of monocyte/macrophage lineage were present within human thymus coincident with colonization of the thymus by stem cells, and CD34+ CD44+MO-1+ cells were scattered around the esophagus, aorta, and spinal cord. However, no evidence of active intrathymic myelopoiesis was seen.

Discussion

In this study, we have used a large panel of anti–human mAbs to define phenotypic and morphologic events that transpire during the earliest stages of human thymus development, and have outlined the developmentally regulated expression of a number of functional T cell molecules.

Auerbach (29) has proposed that TE cell–fibroblast interactions occur during thymus development that provide potent stimulatory signals to TE cell growth. Nonlymphoid thymuses in human SCID are dysplastic due to lack of colonization of the thymus by stem cells (30). Early work with bone marrow reconstitution in human SCID suggested an inductive effect of hematopoietic stem cells on thymic epithelial proliferation (31). Recent work with transgenic mice

has directly shown the dependence of murine medullary TE cell growth on the presence of thymocytes in the thymus (for review see reference 32). Thus, the striking 35-fold increase in volume of the 8.2-wk (colonized) thymus compared with the 7-wk nonlymphoid thymus was expected. What was unexpected was the globular shape and the intertwined upper poles of the 8.2-wk right and left thymus lobes.

We have previously shown that one adhesion ligand pair involved in TE cell ligation of thymocytes is CD2 on thymocytes and CD58 (LFA-3) on TE cells (33, 34). Although CD2-LFA-3 interactions have been postulated to be important in triggering TCR$^-$ early T cells (35), CD2-homologous recombinant mice show no defect in T cell development (36), and treatment of mice with anti-CD2 mAbs early in life does not interfere with thymopoiesis (37). Nonetheless, we and others (3–5) have generally regarded CD2 among the earliest T cell molecules expressed on developing T cells in the thymus. Thus, it was surprising that CD2 expression was not detected in 8.2-wk thymus with two CD2 mAbs (35.1 and OKT11) (Fig. 2 F) that reacted well with thymuses from other gestational ages. Importantly, CD2$^-$ surface (s)CD3ϵ^+ fetal thymocytes have been identified in 20–24-wk thymuses, demonstrating that during human fetal thymus development, it is not obligatory for CD2 to be expressed surface sCD3/TCR expression (38). Our study shows that after stem cell colonization of the human thymus, CD3, CD8, CD3ϵ, CD3δ, CD48, and TCR-β expression all precede CD2 expression. Arulanandam et al. (15) demonstrated that human CD48 is a weak ligand for CD58, and suggested that CD48–CD58 interactions in man are vestigial events left over from the primary role CD48 plays as a CD58 ligand in other species such as mice. The demonstration that CD2 was not expressed in human thymus at the initiation of thymopoiesis provides evidence that, if CD58 ligation is important at this stage of thymus development, CD48–CD58 interactions may be involved in thymocyte–thymic stromal interactions.

The sequential acquisition of CD8α (8.2 wk) followed by CD8β (9.5–10 wk) is consistent with the work of Galy et al. (39), who described an early T cell maturation pathway in sCD3$^-$ postnatal thymocytes of CD8$^-$ to CD8α^+ to CD8α/β^+ thymocytes. CD8α associates with p56-lck and is critical for CD8$^+$ thymic-positive selection on MHC class I molecules (40). CD8β expression augments TCR–MHC class I interactions above the effects of CD8α (41), but the role of CD8β in thymocyte-positive selection is not known. If CD8β is required for CD8$^+$ T cell selection, then the acquisition of CD8β expression at 9.5–10 wk implies that MHC class I–mediated selection does not occur until this time. Similarly, CD28 expression first occured at 10 wk, and CD28 has been postulated to be required for thymocyte-positive selection (42), though not for thymocyte-negative selection (43).

The gradual acquisition of medullary thymocyte CD45RA expression at 10–12.75 wk also implies the gradual onset of positive selection. CD45RA$^+$ thymocytes have been suggested to be those destined to survive intrathymic selection events (12). Recently, studies in transgenic mice demonstrated that CD45RA expression is induced on thymocytes that have

undergone positive selection (44). Conversely, expression of CD45RO by thymocytes increases the efficacy of TCR-mediated thymocyte apoptosis and MHC-restricted negative selection (14, 45). Thus, CD45RO expression at 9.5–10 wk suggests that thymocyte negative selection could begin at the same time as positive selection or just before. TCR-mediated signaling not only requires CD45 tyrosine phosphatase expression but also requires expression of CD3ζ (46). Acquisition of CD3ζ between 10 and 12.75 wk also implies that thymocyte selection does not begin to occur until this time.

The sequence of expression of TCR-β, TCR-γ, and then TCR-δ is similar to that seen in mice, with two important differences. First, in the mouse there is a wave of TCR-γ/δ cell development before TCR-α/β cell development (for reviews see references 1, 47). In human thymus, TCR-δ cells peak in percentage at 9.5 wk of fetal development (4). However, in humans, appearance of TCR-δ cells in thymus coincides with increasing TCR-β and sTCR-α/β expression (3–5, 48). Thus, in humans there is near-coincident appearance of TCR-δ and -α/β thymocytes. Our observations of TCR Vδ2$^+$ cell predominance over TCR Vδ1$^+$ cells throughout fetal thymic development are compatible with the observations of others (49–51), who found that TCR Vδ2$^+$ expression in T cell clones from 11–22-wk fetal thymuses predominated over TCR Vδ1. Parker et al. (52) proposed that TCR Vδ2$^+$ cells develop in the thymus during fetal gestation, seed the periphery, and clonally expand upon contact with antigen.

Second, in the 8.2-wk human thymus, TCR-β synthesis begins simultaneously with T cell precursor colonization of the thymus, whereas in the mouse, TCR-β synthesis begins around day 15, 5 d after mouse thymus colonization by stem cells (53). It has recently been demonstrated in mice that TCR-β can be expressed on the developing thymocyte surface either as a dimer or associated with a novel protein, gp33, as well as expressed in an α/β heterodimer (for review see reference 54). Due to the small size of the 8.2-wk thymus (<1 mm^3), we were limited to phenotypic analysis of the thymus in situ via IF assays on tissue sections, thus negating our ability to definitively distinguish surface from cytoplasmic(c) mAb reactivity. In the mouse, all thymocytes are sCD4$^-$sCD8$^-$ from the time of colonization of the murine thymic rudiment on day 10–11 until day 16–17 (10, 11). Levelt et al. (53) demonstrated that cytoplasmic expression of murine TCR-β begins on day 15, with 3.5% of thymocytes cTCR-β^+ before expression of CD4 or CD8. Thus, the 8.2-wk human thymus is similar to murine day 15 thymus in that 8.2-wk thymus TCR-β expression (5%) is likely cytoplasmic, but differs from mouse thymus in that most human thymocytes just after thymus colonization express CD4 and CD8, whereas day 15 mouse thymocytes are CD4$^-$CD8$^-$ (10, 11, 53).

Our previous work has shown that by 16 wk of gestation, CD4,CD8,CD3ϵ and TCR-β were expressed in fetal thymus in patterns similar to postnatal thymus, and gave rise to the prediction that the T cell repertoire is established in humans by the end of the first trimester (22). Recent molecular anal-

yses of the human thymus TCR Vβ repertoire at 15–17 wk of gestation have shown no difference from the newborn thymus Vβ repertoire (55–57).

Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase that functions in the absence of a template to act as a somatic mutagen by the insertion of extra random nucleotides (N regions) at the D-J joining region during TCR-β rearrangements, contributing to TCR-β diversity. TdT expression was not found by immunohistology in human thymocytes until 20 wk of gestation (48). However, George and Schroeder (57) found TCR N region additions as early as 8 wk of gestation with PCR identification of TdT transcripts, and demonstrated progressive increases in N additions in TCR-β, CDR3, VD, and VDJ transcripts during fetal development. Whether the 8-wk tissue in this latter study was as early as tissue 58 in our study (i.e., was a nonlobulated thymic rudiment) is unknown since morphologic data were not reported (57). Nonetheless, all data point to initiation of diversification of the TCR repertoire very early on in human T cell ontogeny.

Investigators have isolated CD4⁻CD8⁻sCD3ε⁻ cells from human fetal liver and postnatal thymus and shown that they give rise to CD3ε⁺CD3δ⁺ NK cells, suggesting a close lineage relationship between T and NK cells (58, 59). We did not observe a stage of human thymic development wherein a majority of thymocytes expressed NK markers such as CD16 or CD56, although scattered CD16⁺ cells were present in thymus at 8.2 wk.

The expression of VLA 1-6 in thymus development beginning at 15 wk has been previously reported (21). New in our study is the demonstration of reactivity of VLA-1, -2, -3, -4, -6, and β1 mAbs on 8.2-wk thymus. Sanchez et al. (60) have demonstrated VLA-4 expression on T cell progenitors in human fetal liver, and TE cells may bind certain thymocyte subsets via VLA-4 (Le, P. T., personal communi-

cation). A number of integrin molecules have been postulated to be involved in stem cell migration to the human thymic rudiment, but this question is difficult to directly address experimentally.

Our study identified the organization of TE-4⁺ central medullary thymic epithelium surrounded by TE-4⁻ cortical epithelium in the 8.2-wk thymus. The gradual appearance of CD45RA⁺ cells in central thymic areas, and the expression of CD6 and CD28 in thymocytes in central TE areas, also suggested that the primordial thymic medulla is the central core of the 8.2-wk thymic rudiment that has begun to organize immediately subsequent to thymus colonization. In a separate study, we have found that MHC class I and II allotypic determinants are also expressed brightly in the central 8.2-wk thymus area—a pattern typical of medullary TE cell MHC expression (Haynes, B. F., unpublished observations).

Finally, it was of interest to observe CD34⁺ cells around the aorta, spinal cord, and esophagus at 8.2 wk. Recently, two groups have suggested that mesoderm around the dorsal aorta, gonads, and mesonephros (AGM) areas of mice, and not yolk sac, provide the source of stem cells before fetal liver development (61). Whereas no functional data are possible from our study, one explanation for the presence of CD34⁺ cells in the dorsal thorax of 8.2-wk fetal tissue is that this area may be analogous to the AGM mesoderm in mice (61).

Thus, our study has defined phenotypic and morphologic changes that occur in and around the human thymus at the time of colonization of the thymus by hematopoietic stem cells. This study raises a number of important questions regarding human thymus function, including the nature of the role of CD48–CD58 interactions in early T cell development, the significance of developmentally regulated thymocyte CD1b, -c, -a expression, the regulatory mechanisms involved in CD8β, CD28, and CD6 expression, and the functional roles integrins play in early intrathymic maturation.

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