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CD34⁺CD38⁻lin⁻ Cord Blood Cells Develop into Dendritic Cells in Human Thymic Stromal Monolayers and Thymic Nodules

G. Diego Miralles,* Clayton A. Smith,* Leona P. Whichard,* Michael A. Morse,* Barton F. Haynes,† and Dhavalkumar D. Patel*†

Thymic dendritic cells (DCs) appear to have distinct biologic and functional properties compared with DCs in other tissues. Currently, little is known about human thymic DCs because they have been difficult to isolate and culture in vitro. Here, we report that human thymic stroma can support the development of primitive human hemopoietic stem cells into mature DCs without cytokine or serum supplementation. Coculture of CD34⁺CD38⁻ lineage (lin⁻) and CD34⁺CD38⁻lin⁻ umbilical cord blood cells with thymic stromal monolayers induced 43 ± 17-fold and 32 ± 16-fold expansions, respectively, of umbilical cord blood progenitors and also generated large numbers of cells with the morphologic, phenotypic, and functional characteristics of mature DCs. These cells expressed class I and class II MHC, CD1a, CD2, CD4, CD11c, CD40, CD45, CD80, CD83, and CD86 and were potent stimulators of allogeneic T cell activation. Primitive hemopoietic progenitors also developed into mature DCs in a novel tissue culture system of thymic nodules wherein thymic epithelial cells and fibroblasts were grown in nodular aggregates in vitro. These results demonstrate that human thymic stroma efficiently supports the development of CD34⁺CD38⁻lin⁻cord blood cells into mature DCs. In addition, the culture conditions described in this report are useful systems for studying the ontogeny of human DCs in thymic microenvironments. The Journal of Immunology, 1998, 160: 3290–3298.

Dendritic cells are APC distributed widely in lymphoid and nonlymphoid tissues (1–4). Several subsets of DCs have been demonstrated in peripheral blood, skin, lymphoid organs, and thymus (5–8). DCs possess a distinct morphologic appearance, express high levels of MHC class I and II, and have a potent ability to process Ags and activate T cells (9–15). They also appear to play a critical role in mounting effective immune responses against microorganisms, neoplasms, and transplanted organs and may play a vital role in the induction of tolerance (1, 16).

DCs developing within the thymus appear to be biologically distinct from extrathymic DCs (17–24). Bone marrow, peripheral blood, and umbilical cord blood (UCB) hemopoietic progenitors cultured with granulocyte-macrophage (GM)-CSF, TNF-α, and other cytokines in vitro generate mixed colonies containing both monocytes and DCs that typically express primarily myeloid cell markers (5–7, 25–28). In contrast, thymic DCs express molecules normally considered as markers of lymphoid cells (4, 18–22, 24, 29–33). In addition, thymic DC progenitors have been reported to generate both lymphoid cells and thymic DCs (19, 20, 29, 34). Shortman and his colleagues (19, 20, 29, 34) have shown that murine “low CD4” precursors isolated from the thymus can develop into DCs and lymphoid cells, but not myeloid cells, following in vivo injection into the thymus or in vivo culture under certain conditions. These observations indicated that thymic DCs may be more closely related to lymphoid cells than extrathyrmic DCs. The growth and development of intrathymic DCs may also be governed by cytokines different than those important in the development of extrathymic DCs. Saunders et al. (19) demonstrated that murine “low CD4” thymic precursor developed into thymic-type DCs in vitro with a combination of TNF-α, IL-1β, IL-3, IL-7, and stem cell factor (SCF). In contrast, the generation of DCs from peripheral blood and bone marrow progenitors required GM-CSF (5, 25, 35–37). Thymic DCs may also have functional properties different than those of extrathymic DCs. In particular, thymic DCs may participate in the process of T cell-negative selection and tolerance induction within the thymus (4, 18, 38–40). Taken together, these observations suggest that thymic DCs constitute a subset of DCs with distinct developmental, immunophenotypic, and functional properties.

Currently, most studies evaluating thymic DC biology have utilized murine models, in part because human thymic DCs have been difficult to isolate and culture efficiently. For example, while Barcena et al. (41) demonstrated that human fetal thymic organ cultures (FTOC) could support the development of human fetal liver CD34⁺lineage (lin⁻) cells into monocytoïd cells that displayed DC morphology, too few putative DCs were recovered to be fully characterized. In another study, Res et al. (42) demonstrated that individual human CD34⁺CD38dim thymocytes could differentiate into both T and NK cells in FTOC and develop into DCs when cultured in vitro with GM-CSF and TNF-α. However, the role that the thymus played in mediating the development of DCs from intrathymic or extrathymic progenitors remained unclear because

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3 Abbreviations used in this paper: DC, dendritic cell; FTOC, fetal thymic organ culture; TE, thymic epithelial; TF, thymic fibroblasts; UCB, umbilical cord blood; GM, granulocyte-macrophage; lin, lineage; SCF, stem cell factor; PE, phycoerythrin; Cy, cychrome.
extrathymic culture of CD34⁺CD38dim cells was required to generate DCs. Consequently, major aspects of human thymic DC biology remain uncharacterized, including the developmental pathways of thymic DCs, the cytokines that govern DC differentiation and proliferation, and the mechanisms through which thymic DCs mediate negative selection of developing thymocytes.

Since no experimental systems currently exist other than FTOC that generate DCs in vitro in a thymic microenvironment, we sought to develop in vitro systems using purified thymic stromal cells for generating thymic DCs from hemopoietic progenitors. In this article, we report that human thymic stroma can fully and efficiently support the generation and expansion of DCs from primitive human extrathymic hemopoietic CD34⁺CD38⁻ progenitors in the absence of any exogenous cytokines or serum. While culture of CD34⁺CD38⁻ human cord blood cells in serum-free medium had no effect, coculture on thymic stromal monolayers in serum-free medium resulted in progenitor cell expansion and generation of cells with morphologic, phenotypic, and functional characteristics of mature DC. In addition, we observed that CD34⁺CD38⁻ cord blood cells can migrate into nodules of thymic epithelial (TE) and thymic fibroblast (TF) cells grown in vitro and differentiate into DCs in the context of a three-dimensional thymic stromal matrix.

Materials and Methods

Thymic stromal cultures

TE cells and TF were cultured by an explant technique and propagated in enriched medium containing 67% DMEM (Life Technologies, Grand Island, NY), 22% F-12 (Life Technologies), 5% Fetal Clone II serum (HyClone, Logan, UT), 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 11 ng/ml recombinant human epidermal growth factor (Collaborative Biomedical, Bedford, MA), 0.18 µM adenosine, 10⁻⁹ M cholera toxin (ICN Biomedicals, Aurora, OH), 0.25 µg/ml Fungizone, and 50 µg/ml gentamicin (TE medium). On irradiated NIH 3T3 fibroblast feeder layers as described (43, 44), human thymus tissue was obtained from the Department of Pathology, Duke University Medical Center, as discarded tissue from children undergoing corrective cardiovascular surgery according to a Duke Institutional Review Board-approved protocol. Thymic stromal cells (TF and TE cells) were depleted of T cells by culture in TE medium and extensive washing. Cells were either used immediately or stored frozen in 7.5% DMSO-containing medium before expansion and use for reconstruction of the thymic microenvironment. Thymic stromal cells were passaged 1 to 3 times (3 to 8 wk) before coculture with CD34⁺CD38⁻ cord blood cells. Contaminating TF were removed from TE cell monolayers by treatment with 0.02% EDTA in PBS followed by complement-mediated lysis with mAb IB10, which binds to a cell surface Ag on human fibroblasts (43). TE cell preparations were >95% positive for the keratin marker AE-3 and negative for CD1a, CD7, and CD14. For coculture with sorted cord blood CD34⁺CD38⁻ cells, 2.5 × 10⁵ TE cells were plated in 24-well plates on irradiated NIH 3T3 fibroblast feeder layers and irradiated with 2500 cGy once cells became confluent. TF were obtained by an explant technique and grown in TE medium without an NIH 3T3 feeder layer. Typically, TF outgrew TE cells and were ~98% pure by the first passage. The TF cultures used in this study were >98% positive for M38 (procollagen), ≥2% positive for AE3, and negative for CD1a, CD7, and CD14.

Lineage depletion and stem cell isolation by FACS

UCB was obtained as discarded material from the Department of Obstetrics and Gynecology of Duke University Medical Center through an Institutional Review Board-approved protocol for the use of discarded material. The UCB used in these studies was collected in sterile bottles containing an anticoagulant citrate buffer and processed within 18 h of collection. The blood was diluted 1:2 with Dulbecco’s PBS, and RBC were agglutinated at room temperature using 1% Hespan (DuPont Pharma, Wilmington, DE). Nonagglutinated white blood cells were harvested, and residual red cells were hemolyzed at 37°C in 0.17 M NH₄Cl containing 10 mM Tris-HCl, pH 7.2, and 200 mM EDTA. For lineage depletions, the white blood cell fractions were brought to 6 to 8 × 10⁵ cells/ml in PBS containing 4% FCS and were depleted through the addition of a commercial Ab mixture and magnetic colloid as per the manufacturer’s instructions (CD34⁺ StemSep enrichment mixture, StemCell Technologies, Vancouver, Canada). The mixtures of cells, Abs, and magnetic colloid were cleared of lineage-marked cells over a column held in a wraparound magnet. The cells that passed through the column (lin⁻ cells) were collected, washed in DMEM with 10% FCS and stored on ice.

For FACS, lin⁻ cells were pelleted, resuspended in 100 µl of PBS/2% FCS, and incubated with anti-CD34 and anti-CD38 for 20 to 30 min. After three washes in PBS/2% FCS, cells were sorted on a FACStarPlus cell sorter (Becton Dickinson, Mountain View, CA) and collected in sterile polystyrene tubes containing 100% FCS. Postsort analysis was performed on the last 10% of cells that were collected in separate tubes and reanalyzed on the flow cytometer.

Coculture of sorted stem cells and thymic stromal monolayers

Sorted CD34⁺CD38⁻ lin⁻ or CD34⁺CD38⁻ lin⁻ cells were added onto irradiated confluent thymic stromal monolayers at 10⁵ to 10⁶ cells/well and cultured in 1 ml of serum-free medium. This medium was made with 80% Iscove’s modified Dulbecco’s medium (Life Technologies) 20% BIT 9500 (StemCell Technologies), 1 mg/ml glutamine, 40 µg/ml lipoprotein (Sigma), and 0.1% mercaptoethanol. Cells were fed three times weekly by carefully removing 0.5 ml of supernatant and replacing it with fresh medium.

![Figure 1](http://www.jimmunol.org/DownloadedFrom/3291/TheJournalOfImmunology.png)
stromal cell monolayers have the morphology of monocytes. Data are representative of two experiments. The bars represent 10 μm.

Antibody reagents

mAbs to the following Ags were used for indirect immunofluorescence staining: P3x63Ag8 (IgG1, from American Type Culture Collection (ATCC), Rockville, MD); CD1a (Na1/34, from Andrew McMichael) (45); CD2 (35.1, ATCC), CD3 (Leu-4, Becton Dickinson), CD4 (Leu-3a, ATCC), CD7 (3A1e) (46), CD14 (Leu-M3) (47), AE3 (keratin from T.T. Sun) (48), 1B10 (fibroblasts) (43); M38 (C-terminal region of type I procollagen) (49), and fluorescein-conjugated goat anti-mouse Ig (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Directly conjugated Abs to the following Ags were also used for multicolor analyses of cell surface Ags: CD2 (Leu-5, FITC), CD3 (Leu-4, peridinin chlorophyll protein (PerCP), CD5 (Leu-1, phycoerythrin (PE)), CD7 (Leu-9, FITC), CD8 (SK1, FITC), CD10 (Leu-11, PE), CD14 (Leu-12, FITC), CD25 (2A3, FITC), CD33 (Leu-M9, PE), CD34 (HPCA2, FITC, PE, and cychrome (Cy)), CD38 (Leu-17, PE), CD56 (Leu-19, PE), CD80 (L307.4, PE) HLA-DR (L243, FITC), IgG1 (3A1e) (46), CD1a (T6, PE), CD4 (T4, PE), and CD83 (HB15a, PE) from Coulter (Hialeah, FL); CD3 (UCHT1, Cy) from Immunotech (Westbrook, ME); CD1a (HI49, FITC), CD2 (RPA-2.10, Cy), CD40 (5C3, FITC), CD86 (231461(FUN-1), FITC), CD95 (DX2, FITC), HLA A,B,C (G46-2.6, FITC), ad IgG1 (MOPC-21, Cy) from PharMingen (San Diego, CA); and CD8 (DK25, R-PE-Cy5) and CD13 (F0831, FITC) from Dako (Carpinteria, CA).

Phenotypic analysis using flow cytometry

For FACS analysis of cultured cells, cells were gently resuspended to leave thymic monolayers undisturbed, pelleted, and resuspended in 100 μl of PBS/4% FCS and held on ice. Fluorescence-conjugated Abs were added directly to the cell suspensions. Following incubations for 20 to 30 min at 4°C, the cells were washed three times in PBS/4% FCS. Where necessary, the cells were fixed in 1% formaldehyde in PBS/2% FCS. Quantitation of the surface staining was performed on a FACScan and a FACScalibur (Becton Dickinson) using a 488 argon laser for fluorescence excitation. Data were analyzed using CellQuest software (Becton Dickinson). In all experiments, cells stained with isotype-matched control Abs were used to set cursors so that <1% of the cells were considered positive.

Microscopy

Sorted cells were centrifuged onto glass slides using a Shandon cytocentrifuge (Shandon Southern Instrument Co., Sewickley, PA) at 1000 rpm for 3 min. Cytospins were air-dried and stained with Wright-Giemsa stain and examined by light microscopy. For transmission electron microscopy, thymic nodules and sorted cells were fixed with 2% glutaraldehyde in 150 mM sodium cacodylate buffer plus 2.5 mM CaCl2, pH 7.2, washed, and embedded in 1% agar. After postfixation for 1 h on ice with 2% osmium tetroxide plus 1% potassium ferrocyanide, blocks were washed with cacodylate buffer followed by 200 mM sodium acetate, pH 5.2. Samples were stained en bloc for 1 h with 1% uranyl acetate in sodium acetate buffer. After dehydration with ethanol, the pellet was infiltrated with and embedded in EMBED 812 (EM Sciences, Fort Washington, PA). Sections of 90 nm were cut on a Reichert-Jung (Wien, Austria) Ultracut E microtome and stained en bloc for 1 h with 1% uranyl acetate in sodium acetate buffer. Sections were counterstained with tetroxide plus 1% potassium ferrocyanide, blocks were washed with cacodylate buffer followed by 200 mM sodium acetate, pH 5.2. Samples were stained en bloc for 1 h with 1% uranyl acetate in sodium acetate buffer. After dehydration with ethanol, the pellet was infiltrated with and embedded in EMBED 812 (EM Sciences, Fort Washington, PA). Sections of 90 nm were cut on a Reichert-Jung (Wien, Austria) Ultracut E microtome and stained with uranyl acetate, followed by Sato lead, washed, and examined with a Philips EM300 electron microscope (Philips, Eindhoven, The Netherlands).

Mixed lymphocyte reactions

Allogeneic responder PBMCs (1.5 × 10^5) obtained from healthy donors were cultured in RPMI 1640 supplemented with 10% FCS or 10% human AB serum in 96-well U-bottom tissue culture plates. Irradiated (3500 rads) sorted CD1aCD14 and CD1aCD14 cells were added in graded doses of 1.5 × 10^3 (1:1,000) to 1.5 × 10^4 (1:10) cells in a total volume of 200 μl. Cell proliferation after 96 h was quantified by adding 1 μCi (37 kBq) of [methyl-3H]TdR (NEN-DuPont, Boston, MA) to each well. After 16 h, the cells were harvested onto filters, and radioactivity was measured in a scintillation counter with results presented as the mean counts per minute for triplicate cultures.

Development of human thymic stromal microenvironment nodules

Cultured thymic stromal cells were cocultured in an artificial capillary sytem (Cellmax, Cellico, Germantown, MD) with a coating of ProNectin F to promote adhesion of stromal cells to the capillaries. Thymic stromal cells (20–100 × 10^4) (95% TE cells by reactivity with anti-keratin Ab AE-3 and 5% TF by reactivity with anti-procollagen Ab M38) were seeded per capillary module with an extracapillary space of 12 ml. TE medium (44) was pumped through the capillaries at a rate of 10 ml/min. Within 2 to 6 wk, 1- to 2-mm nodules were readily apparent by visual inspection in the extracapillary space of the capillary modules. Nodules were harvested by cutting the module with a sterile pipe cutter. The nodules were separated from the capillaries by scraping with a sterile rubber policeman and washed with DME containing 5% FCS. Sorted CD34cord blood cells differentiate into CD1a+ cells on thymic stromal cell monolayers

To determine whether human thymic stroma could support the development of DCs from hemopoietic progenitors, CD34cord blood cells differentiated into CD1a+. Data are representative of two experiments. The bars represent 10 μm.

CD34 cord blood cells differentiate into CD1a+ cells on thymic stromal cell monolayers
and CD34<sup>−</sup>CD38<sup>−</sup>lin<sub>2</sub> UBC cells (R1 in Fig. 1A) were isolated by sterile cell sorting and coculture with preestablished irradiated human thymic stromal monolayers (50, 51). Before coculture, the sorted populations had >98% purity (Fig. 1, B and C) and were >98% CD1a<sup>−</sup> (Fig. 1D). Following coculture with thymic stromal monolayers in serum-free medium for 21 days, CD34<sup>+</sup>CD38<sup>−</sup> cells expanded 43 ± 17-fold (n = 3), and the CD34<sup>−</sup>CD38<sup>−</sup> cells expanded 32 ± 16-fold (n = 3). UCB progenitors cultured in serum-free medium alone did not expand or change in morphology. Immunophenotypic analysis of cocultured cells revealed the presence of a number of CD1a<sup>−</sup>CD14<sup>−</sup>HLA-DR<sup>−</sup> cells (Figs. 1 and 2) similar to previous descriptions of human DCs (5). The percentage of CD1a<sup>−</sup>CD14<sup>−</sup> cells generated from CD34<sup>+</sup>CD38<sup>−</sup> cells ranged from 5 to 15% (mean, 8.2%; n = 3) and that from CD34<sup>−</sup>CD38<sup>−</sup> cells ranged from 2 to 10% (mean, 4.8%; n = 3). While the percentage and number of CD1a<sup>−</sup> cells progressively increased with time in coculture, experiments were terminated at 21 days because the integrity of the thymic stromal monolayers became compromised beyond this time point. The observation that CD1a<sup>−</sup>CD14<sup>−</sup> cells could be generated from both the CD34<sup>+</sup>CD38<sup>−</sup>lin<sub>2</sub> and CD34<sup>−</sup>CD38<sup>−</sup>lin<sub>2</sub> populations suggested that both of these cell types could develop into DCs in the thymic stromal monolayers.

**Morphology of CD34<sup>+</sup> cells expanded in thymic stroma**

To confirm that the CD1a<sup>−</sup> cells grown in thymic monolayers were DCs, CD1a<sup>−</sup>CD14<sup>−</sup> cells generated after 21 days of culture from both CD34<sup>+</sup>CD38<sup>−</sup>lin<sub>2</sub> and CD34<sup>−</sup>CD38<sup>−</sup>lin<sub>2</sub> umbilical cord cells were isolated by FACS and examined by light and electron microscopy (Fig. 2). CD1a<sup>−</sup>CD14<sup>−</sup> cells were also sorted from both cultures to serve as controls. Analysis of the sorted cells revealed a purity >97%. By light microscopy, CD1a<sup>−</sup>CD14<sup>−</sup> cells possessed a DC morphology with an irregular shape and multiple dendritic processes. Examination of the ultrastructure by EM showed that CD1a<sup>−</sup>CD14<sup>−</sup> cells had euchromatic, lobulated, or indented nuclei and a clear cytoplasm with rough endoplasmic reticulum and well-developed Golgi apparatus. These cells did not contain Birbeck granules. In contrast, the control CD1a<sup>−</sup>CD14<sup>−</sup> cells from both precursor types had the morphologic appearance of macrophages, with indented nuclei, foamy cytoplasm, and no evidence of cytoplasmic dendritic projections.

**Immunophenotype of CD1a<sup>+</sup> cells expanded in thymic stroma**

To better characterize the DCs generated from UCB progenitors on thymic monolayers, we performed extensive phenotypic evaluations using multiparameter FACS analysis (Fig. 3). CD1a<sup>−</sup> cells were processed for two- or three-color staining with CD1a conjugated to FITC or PE and mAbs labeled with complementary fluorescent molecules (FITC, PE, or Cy) as indicated. Histograms show fluorescent intensity of test (thick line) and isotype-matched control (thin line) antibodies gated on CD1a<sup>−</sup> cells. Results are representative of more than three experiments for each Ab tested.
generated on thymic stroma from CD34⁺CD38⁻ lin⁻ UCB cells were negative for surface CD3, CD8, CD19, CD25, CD34, and CD95, and expressed CD2, CD4, CD11c, CD13, CD16, CD33, CD38, CD40, CD45, CD49e, CD80, CD83, CD86, MHC class I, and MHC class II.

**Ability of CD1a⁺CD14⁻** and **CD1a⁻CD14⁻** cells generated in thymic stroma to act as APC

To determine whether the putative DCs generated on thymic stroma were able to activate T cells, CD1a⁺CD14⁻ and CD1a⁻CD14⁻ cells were sorted by FACS and tested in allogeneic MLRs. CD1a⁺CD14⁻ cells were much more potent stimulators in the MLRs than CD1a⁻CD14⁻ cells (Fig. 4). Further, CD1a⁺CD14⁻ cells generated from CD34⁺CD38⁻ lin⁻ UCB cells were more potent stimulators of the MLR on a per cell basis than the CD1a⁻CD14⁻ cells generated from CD34⁺CD38⁻ lin⁻ cells (Fig. 4). This suggests not only that more primitive progenitors may generate larger numbers of DCs but also that these DCs may be qualitatively different from DCs generated from more mature progenitors.

**Effect of TNF-α on thymic DC**

Analysis of CD1a and CD14 expression on CD34⁺CD38⁻ lin⁻ UCB progenitors cocultured with thymic stroma revealed the presence of several phenotypically distinct populations of cells (Fig. 2). One possible explanation for this observation is that the cocultures contained DCs at multiple stages of development. To test this hypothesis, the cocultures were treated for 48 h with TNF-α (10 ng/ml), a previously described DC maturation factor (5, 6, 25). TNF-α treatment increased the expression of CD1a, CD83, CD80, and CD86 on large numbers of cells derived from CD34⁺CD38⁻ lin⁻ progenitors (Fig. 5). In addition, most of these cells displayed a DC morphology (Fig. 6). While TNF-α treatment of cocultures established with CD34⁺CD38⁻ lin⁻ cells caused an increase in the fraction of cells with mature DC markers, not all cells expressed DC markers, and a significant number of CD1a⁺CD33⁺ cells were also observed (not shown). This suggested that these cultures may have contained a significant fraction of non-DC myeloid cells. This was confirmed by light microscopic examination that revealed a number of myeloid lineage cells including neutrophils and macrophages at different stages of maturation in the CD34⁺CD38⁻ lin⁻ cocultures treated with TNF-α (Fig. 6).

**Formation of thymic microenvironment nodules from cultured TE cells and TF**

Since thymic stromal monolayers do not have the full differentiation capacity of reaggregation cultures such as that seen with FTOC (41, 42, 52), and due to the difficulties of obtaining sufficient human fetal thymus for studies, we developed a culture system to form three-dimensional aggregates of cultured postnatal TE cells and TF. After 2 to 6 wk of coculture in an artificial capillary system, human TF and TE cells aggregated to form 1- to 2-mlm nodules with a morphology and phenotype consistent with a thymic stromal microenvironment devoid of hemopoietic cells (n = 10) (Fig. 7). The nodules contained TE cells (keratin positive) in a fibroblast matrix (identified by TE7) that was encapsulated by a layer of procollagen-positive fibroblasts. By transmission electron microscopy, the thymic stromal nodules contained numerous desmosomes and hemidesmosomes (Fig. 8), indicating that the epithelial cells within the nodules are able to interconnect and form a network similar to that seen in normal thymus (53, 54).

TE cells in nodules did not terminally differentiate as determined by lack of reactivity with mAbs STE1, STE2, and 11.24 (CD44v9) (55), nor did they form Hassall’s bodies. This pattern is similar to that seen in the thymic stroma of patients with SCID (reviewed in Refs. 53 and 56).

**CD34⁺ cord blood cells differentiate in thymic nodules into CD1a⁺ cells with DC morphology**

To test the functional status of the thymic nodules, we evaluated whether UCB hemopoietic cell progenitors migrate into and differentiate in the nodules in vitro. lin⁻ UCB cells were incubated

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**FIGURE 4.** CD1a⁺CD14⁻ cells generated in vitro from CD34⁺CD38⁻ lin⁻ or CD34⁺CD38⁻ lin⁻ UCB cells on thymic stromal cell monolayers are good stimulators in allogeneic MLRs. Irradiated CD1a⁺CD14⁻ and CD1a⁻CD14⁻ cells grown on thymic stromal cell monolayers for 21 days and separated by FACS were used to stimulate 1.5 x 10³ allogeneic monocyte-depleted PBMCs at different responder-stimulator ratios. Shown is a representative experiment of two performed. Error bars are mean ± SD of triplicate wells in the experiment shown. A, TdR uptake (in counts per minute less background of CD4⁺ responder T cells alone) induced by CD1a⁺CD14⁻ and CD1a⁻CD14⁻ cells derived from CD34⁺CD38⁻ lin⁻ UCB progenitors. CD1a⁻CD14⁻ cells induce significantly more proliferation in MLR than CD1a⁺CD14⁻ cells (p < 0.001 at all stimulator-responder ratios). B, TdR uptake induced by CD1a⁺CD14⁻ and CD1a⁻CD14⁻ cells derived from more mature CD34⁺CD38⁻ lin⁻ UCB progenitors. CD1a⁻CD14⁻ cells generated from CD34⁺CD38⁻ lin⁻ UCB are also good stimulators in MLR but are less potent that those generated from CD34⁺CD38⁻ lin⁻ progenitors (p = 0.001). CD1a⁻CD14⁻ cells generated from CD34⁺CD38⁻ lin⁻ progenitors are not potent stimulators in MLR.
with thymic nodules in a 24-well flat-bottom plate in serum-free medium at 37°C. After 28 days of coculture with thymic nodules, the nodules were analyzed for markers of T and NK cells (CD1a, CD3, CD7), progenitor cells (CD33, CD34), myeloid cells (CD14), and DC (CD1a, CD83). Nodules cultured in the absence of UCB progenitor cells were also analyzed. No CD3 or CD7 expressing cells were detected in the nodules by indirect immunofluorescence. However, there were numerous CD1abright cells with dendritic morphology in the nodules seeded with lin^2 UCB cells (Fig. 9B), but not in the nodules cultured without UCB cells (Fig. 9A). The CD1a bright cells were CD33 low and CD83^2. Further, at day 0, lin^2 cells did not express CD1a (data not shown), suggesting that CD1a^+ cells resulted from the differentiation of progenitor cells within the nodule and not from spontaneous expansion of DCs contaminating the lin^- population. Taken together, these findings suggested that progenitor cells migrated into the thymic nodules, and that thymic stromal nodules were able to support CD34^-lin^- UCB cell development into DCs.

**Discussion**

In this report, we have shown that UCB CD34^+CD38^-lin^- and CD34^-CD38^-lin^- extrathymic hemopoietic progenitor cells cocultured on neonatal thymic stroma in serum-free medium developed into cells with the phenotypic, morphologic, and functional characteristics of DCs. These findings suggest that human thymic stroma can fully support the differentiation of primitive hemopoietic stem cells into functional DCs without exogenous cytokines or other growth factors.

Although murine thymic DCs have been extensively characterized, few reports have described the in vitro generation of human DCs in thymic microenvironments (41, 42). Since in vitro culture systems that generate large numbers of human thymic DCs are not available, most studies have analyzed human thymic DCs obtained from thymic suspensions followed by enrichment of DCs by removal of cells expressing CD1a and CD2 (24, 31, 33). The phenotype of the DCs observed in the thymic stromal cocultures has some similarity to human thymic DCs enriched in this fashion (24, 31, 33). Both express high levels of MHC class I and II, CD11c, and CD40 and lack expression of CD34, CD14, and CD19. In addition, DCs generated on the human thymic stroma did not contain Birbeck granules which are characteristic of myeloid lineage Langerhans-type DC and not found in mature thymic DCs (5, 6, 31).

From the studies reported here, it is clear that the human thymic stroma can support development of DCs from early hemopoietic...
cells. Data are representative of two experiments. The bars depict the magnification of desmosomes between neighboring TE cells, and the arrowheads point to the fibrous capsule surrounding the nodule, and the arrows point to a nest of thymic epithelium forming a rosette in sequential sections. Data are representative of 10 experiments.

progenitors. We did, however, observe some phenotypic differences between previously isolated human thymic DCs and those generated in thymic stroma. It appears from different studies that both murine and human DCs undergo phenotypic and functional changes during short term culture, suggesting that a particular isolation technique could influence the cell phenotype (33). It is also possible that thymic stromal cells can support development of different subsets of DCs or that the source of the hemopoietic precursors may affect the resulting phenotype. Ongoing experiments are addressing these questions.

One function of thymic stroma-derived DCs is the ability to serve as stimulators in allogeneic MLRs. This observation implies that these cells can function as potent APCs and efficiently activate T cells. While little is known about the function of human thymic DCs, murine thymic DCs have been shown to activate T cells in a variety of systems and may be the most effective APCs for a variety of Ags (57, 58). Further analyses of the DCs generated in the thymic stromal cocultures for their ability to eliminate self-reactive thymocytes will provide insights into whether these cells may function in negative selection.

The simple culture systems described in this report could allow detailed studies of human DC ontogeny in thymic microenvironments. We observed multiple phenotypically defined populations in the thymic stroma cocultures at different time points based on expression of CD1a, CD14, and HLA-DR as well as other markers. Morphologic analysis and function in MLRs indicate that the CD1a+CD14− cells were relatively mature DCs, while the CD1a−CD14+ cells were monocytes. The presence of cells that coexpressed both CD1a and CD14 (Fig. 3) indicates that there may be a thymic progenitor common to both populations. The maturation of cells in the thymic cocultures to mature DCs following exposure to TNF-α for 48 h (see Figs. 5 and 6) suggests that the CD1a+CD14+ cells may also be DC progenitors. In the peripheral blood, CD14+ progenitors have been identified that can yield both monocyte/macrophages and DCs under the appropriate conditions (59, 60). This is in contrast to the murine system where it appears that thymic DCs are closely related to the lymphoid lineage and do not develop from a progenitor capable of generating myeloid progeny (4, 20, 21, 32). Common lymphoid/DC progenitors have also been identified in the human thymus in CD34+CD10−lin− and CD34brightCD1+ cells (61, 62). These findings, while suggesting that at least some thymic DCs may be of lymphoid origin, do not exclude the possibility that the thymus also contains myeloid lineage DCs. Our observations that human DCs generated in thymic microenvironments can express myeloid markers including CD14 support the possibility that at least some thymic DCs may be of lymphoid origin, do not exclude the possibility that the thymus also contains myeloid lineage DCs. One possibility that unifies these disparate observations is that the thymic stromal cocultures may support the generation of multiple types of DCs. Systemic studies designed to follow the developmental status of discrete cell subsets isolated from the thymic stromal cocultures may help confirm or disprove this possibility.

In the thymic stromal cocultures, DCs were generated without cytokine or serum supplementation. Consequently, this system may not only allow the study of DC ontogeny in the thymus but...
also improve our understanding of the cytokines involved in intrathymic DC maturation. Previous studies have shown that although CD34+ cells cultured with GM-CSF and TNF-α develop into DC, GM-CSF in the absence of TNF-α does not support growth of DC clones (6, 7, 63). Since TNF-α is not produced by the thymic stroma (not shown), these observations suggest that DC generation cannot be solely explained by production of GM-CSF by the thymic stroma. Furthermore, it has been demonstrated that although GM-CSF appears to be required for DC generation from peripheral blood, bone marrow, and UBC progenitors, thymic DCs may be generated without GM-CSF (19). Unlike most studies where DCs have been generated in vitro, our cultures were performed in serum-free medium. Strobl et al. (64) have shown that a combination of GM-CSF, TNF-α, and SCF was unable to induce DC development from bulk CD34+ cells in the absence of serum supplementation. However, the addition of TGF-β to these cytokines appeared to partially overcome the absence of serum and allow development of DC (64). Since it has been shown that TE can produce TGF-β (65), it is possible that the generation of DCs in the thymic stromal cocultures was at least partially due to TGF-β. However, DCs generated in defined cultures with GM-CSF, TNF-α, SCF, and TGF-β differed from those we observed in the thymic stromal cocultures in that the former demonstrated the presence of Birbeck granules in large numbers and expressed a different surface phenotype (64). This suggests that alternative or additional growth factors generated by the thymic stroma played a role in governing DC development relative to those used to generate DCs with defined cytokines.

Despite showing that hemopoietic progenitors acquired lymphoid cell markers by coculture with human thymic stroma, we were unable to observe T cell development, as reported previously on thymic stroma (66, 67). It is possible that the use of serum-free medium, the techniques by which thymic stromal monolayers were established, the use of UCB cells, the stem cell isolation procedures, or other technical factors prevented development of T cells in our system.

The systems that have been capable of inducing the development of T cells in vitro have primarily been based on reaggregation of fetal thymic stromal cells, devoid of contaminating lymphoid cells, into three-dimensional structures reproducing the thymic microenvironment (41, 52). This has been difficult to do in the human system. We have devised a method by which we can induce the aggregation of purified TE cells and TF, devoid of any hemopoietic cells, into three-dimensional structures resembling the nonlymphoid thymic microenvironment. These nodules were functional in inducing development of DCs from UCB progenitors, but we did not detect T cell development in these colonized nodules. Further studies will need to be performed to determine whether the three-component thymic microenvironment containing TE cells, TF, and DCs can function in T cell development.

In conclusion, we have shown that human thymic stroma without exogenous cytokines or serum addition supports the differentiation and expansion of CD34+CD38lin- cord blood progenitors into large numbers of DCs with many of the properties of authentic thymic DCs. This argues that generation of DCs may be one of the primary functions of the thymus. The thymic stroma culture systems described in this report may facilitate the study of human DC ontogeny in the thymic microenvironment. In addition, these systems may ultimately provide means for generating DCs with enhanced or novel therapeutic properties.

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References