

## **An Essential Role for Thymic Mesenchyme in Early T Cell Development**

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### **Abstract**

We show that the mesenchymal cells that surround the 12-d mouse embryo thymus are necessary for T cell differentiation. Thus, epithelial lobes with attached mesenchyme generate all T cell populations in vitro, whereas lobes from which mesenchyme has been removed show poor lymphopoiesis with few cells progressing beyond the CD4<sup>-</sup>CD8<sup>-</sup> stage of development. Interestingly, thymic mesenchyme is derived from neural crest cells, and extirpation of the region of the neural crest involved results in impaired thymic development and craniofacial abnormalities similar to the group of clinical defects found in the DiGeorge syndrome.

Previous studies have suggested an inductive effect of mesenchyme on thymic epithelial morphogenesis. However, we have found that mesenchyme-derived fibroblasts are still required for early T cell development in the presence of mature epithelial cells, and hence mesenchyme might have a direct role in lymphopoiesis. We provide an anatomical basis for the role of mesenchyme by showing that mesenchymal cells migrate into the epithelial thymus to establish a network of fibroblasts and associated extracellular matrix. We propose that the latter might be important for T cell development through integrin and/or cytokine interactions with immature thymocytes.

Key words: stem cells • migration • fibroblasts • fibronectin • extracellular matrix

### **Introduction**

The embryonic thymus arises as an epithelial bud from the third and fourth pharyngeal pouches and, as it descends in the neck, is surrounded by primitive connective tissue—the perithymic mesenchyme (1). By day 12 of gestation, the mouse thymus consists of a region of epithelium surrounded by a capsule, and the whole is embedded in a thick layer of mesenchyme. Lymphoid stem cells, which originate from hemopoietic foci in the fetal liver, are entering the thymus from the blood at this stage (2, 3). However, the epithelium is not yet vascularized, and stem cells migrate from blood vessels close to the thymus and traverse through the perithymic mesenchyme and thymic capsule before entering the epithelium.

The thymic epithelium is known to play an important role in T cell development (4) but less attention has been given to the thymic mesenchyme. Cell marker studies have shown that the mesenchyme surrounding the thymus is

derived from cells that migrate from the cephalic region of the neural crest (5). These experiments were performed in avian embryos but, recently, a novel transgenic technique has been used to confirm these findings in mice (6). There are several cogent reasons for focusing on the role of neural crest-derived mesenchymal cells in thymic development. For example, extirpation of the cephalic neural crest results in retarded lymphoid development in the thymus as well as producing craniofacial and cardiac defects (7). The DiGeorge syndrome, where there is also a defect in thymic development, provides a good clinical correlation for this group of abnormalities (8) and hence may result from a failure of neural crest migration to the pharyngeal region. Targeted disruption of the *rae28* gene, a mouse homologue of the *Drosophila* polyhomeotic gene, also results in defects in neural crest-related tissues including the thymus (9). This gene is thought to regulate Hox gene expression and, interestingly, *Hoxa-3* gene disruption itself affects neural crest cells and thymic development (10). In addition, these mice show reduced expression of *pax-1* in neural crest-derived mesenchyme, and both *pax-1* and *pax-3* mutant mice have thymic abnormalities related to defective neural crest function (11, 12).

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We have focused our studies on the 12-d mouse embryo thymus where mesenchyme surrounds, but has not penetrated, the epithelial thymic rudiment and so can be separated from the latter. It has been reported previously that the 12-d embryo thymus has limited developmental potential *in vitro* (13). It was not stated in that study whether mesenchyme was present in the cultures, but in a subsequent paper the authors showed that fibroblast cell lines could restore lymphopoiesis (14). Hence, the importance of perithymic mesenchyme in T cell development was not investigated. In other studies, enzymes were used to remove the thymic capsule and associated cells. It was reported that this procedure affected epithelial morphogenesis and MHC expression, but lymphopoiesis was not examined (15).

To test directly the notion that neural crest-derived mesenchyme plays an important role in T cell development, we have removed perithymic mesenchyme from 12-d mouse embryo lobes by microdissection. We have then studied the capacity of these lobes for lymphoid development in organ cultures compared with lobes with mesenchyme intact. We show that 12-d lobes cultured with attached mesenchyme are capable of generating all T cell subsets *in vitro*. However, lymphoid development is severely compromised in lobes cultured without mesenchyme, with most lymphocytes blocked at the CD4<sup>-</sup>CD8<sup>-</sup> stage of T cell development.

In earlier studies (13–15), it was suggested that mesenchyme influences thymic epithelial morphogenesis and hence, effects on lymphopoiesis are secondary to a defective epithelium. However, we have shown that mesenchyme-derived fibroblasts are still required for the early stages of T cell development, even when epithelial cells derived from 15-d lobes cultured in deoxyguanosine are used in reaggregate organ cultures (16). These epithelial cells are fully capable of supporting the later stages of T cell development, including positive selection (17). Hence, in addition to any effects on epithelial morphogenesis, mesenchymal cells might directly affect lymphopoiesis. To provide an anatomical basis for such a role, we have studied the distribution of mesenchymal cells during culture of thymus lobes. We show that mesenchymal cells migrate into the epithelial thymus and establish an intrathymic network of fibroblasts with an associated extracellular matrix (ECM). Our results suggest that it is the absence of this process in lobes deprived of mesenchyme that is responsible for poor lymphoid development, and implicate the ECM as necessary for integrin and/or cytokine interactions in thymopoiesis.

## Materials and Methods

**Mice.** Fetal thymic lobes were obtained from BALB/c and C57BL/10 mouse embryos at various stages of gestation, where day 0 is the day of vaginal plug detection. All studies were on BALB/c material except for NK1.1 analysis, where it was necessary to use C57BL/10.

**Microdissection and Organ Culture of 12-d Thymus Lobes.** Lobes were dissected as described previously (18). These lobes were then submerged in 200  $\mu$ l of DMEM in V-bottomed 96-well

plates (Becton Dickinson). Each well contained one lobe. These were kept in an enriched oxygen atmosphere and cultured for 10 d. 15-d lobes were cultured at an air/liquid interface on filters, with five lobes per filter for 7 d, as a comparison to 12-d lobes cultured for 10 d.

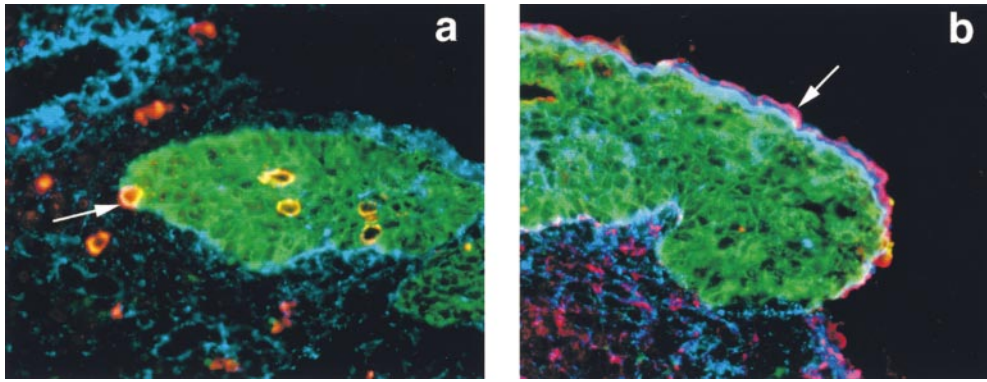
**Antibodies and Immunoconjugates.** For flow cytometric analysis, the following antibodies were used: PE-conjugated anti-CD4 (clone RM4-5; PharMingen), allophycocyanin-conjugated anti-CD8 (clone 53-6.7; PharMingen), FITC-conjugated anti-CD8 (clone 53-6.7; PharMingen), FITC-conjugated anti-TCR- $\alpha/\beta$  (clone H57-597; Sigma Chemical Co.), biotinylated anti-TCR- $\gamma/\delta$  (clone GL3; PharMingen), biotinylated anti-NK1.1 (clone PK136; PharMingen), streptavidin-PE (Becton Dickinson), streptavidin-FITC (Amersham Pharmacia Biotech), and streptavidin-allophycocyanin (PharMingen). For immunocytochemistry, the following antibodies were used: rat anti-CD45 (clone M1/9; American Type Culture Collection), mouse anti-pan cytokeratin (clones C-11, PCK-26, CY-90, Ks-1A3, M20, and A53-B/A2; Sigma Chemical Co.), rabbit antifibronectin (Sigma Chemical Co.), rat anti-ERTR7 (a gift from W. Van Ewijk, Erasmus University, Rotterdam, The Netherlands), sheep anti-rat Ig-biotin (Amersham Pharmacia Biotech), streptavidin-horseradish peroxidase (NEN Life Science Products), biotinyl tyramide (NEN Life Science Products), extravidin-tetramethyl rhodamine isothiocyanate (TRITC; Sigma Chemical Co.), goat anti-mouse Ig-FITC (Caltag), goat anti-mouse Ig-7-amino-4-methylcoumarin-3-acetic acid (AMCA; Chemicon), goat anti-rabbit Ig-AMCA (Dako), and goat anti-rabbit Ig-FITC (Sigma Chemical Co.). For labeling of mesenchymal cells, carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) was used.

**Frozen Sections.** After organ culture, lobes were embedded in OCT compound (Tissue Tek) on a microtome chuck and snap frozen in liquid nitrogen. Cryosections were cut 5- $\mu$ m thin onto Vectabond (Vector Laboratories)-coated multispot slides (C.A. Hendley-Essex, Loughton, Essex). Sections were air-dried for 1 h, fixed in ice-cold methanol for 1 h, and then stored at -20°C in sealed bags.

**Immunocytochemistry.** CD45 and the fibroblast marker ERTR7 were revealed using the tyramide amplification system (NEN Life Science Products) as described previously (18). The antifibronectin and anticytokeratin antibodies were recognized using anti-rabbit Ig-AMCA or -FITC, and anti-mouse Ig-FITC or -AMCA immunoconjugates, respectively.

**Surface Phenotyping for Analysis by Flow Cytometry.** Thymus lobes were removed from organ culture after 7 or 10 d. Thymocyte suspensions were then prepared by gently teasing the lobes with fine cataract knives in RPMI. Resulting cell suspensions were counted and then washed. These were then single, dual, or triple labeled with the antibodies mentioned. Primary antibodies were left on for 45 min on ice and then cells were washed in PBS. Secondary antibodies were incubated with cell suspensions for 30 min on ice and subsequently cells were washed in PBS. Cells were fixed in 1% paraformaldehyde in PBS, and analyzed using a Coulter Elite dual laser flow cytometer. Dead cells and debris were excluded by selective scatter gating.

**Fluorescein Labeling and Tracing of Mesenchymal Cells.** To investigate directly whether mesenchymal cells migrate into the epithelial thymus, thymic lobes with surrounding mesenchyme were removed from 12-d mouse embryos. Using a dissecting microscope and fine knives, mesenchyme was carefully separated from each epithelial lobe. The mesenchyme fragments were washed in calcium and magnesium-free PBS and trypsinized at 37°C for 20 min. A single cell suspension was obtained by gently



**Figure 1.** Frozen sections of 12-d embryo thymus with attached mesenchyme. (a) CD45<sup>+</sup> stem cells (red) migrating into the epithelial thymus labeled with antibodies to cytokeratin (green). A stem cell can be seen at the mesenchymal-epithelial border (arrow). Fibronectin (blue) is mainly seen over the mesenchymal cells that surround the epithelium, although strong staining is also seen over the endothelial cells of a blood vessel in the upper left corner of the photomicrograph (original magnification:  $\times 400$ ). (b) Staining with

the ERTR7 fibroblast marker (red) over mesenchymal cells and capsular material (arrow) surrounding the thymic epithelium (green). Again, fibronectin staining (blue) is mainly seen over mesenchymal cells (original magnification:  $\times 400$ ).

pipetting the fragments and, after inactivation of trypsin by addition of FCS, the cells were centrifuged and placed in PBS containing 1  $\mu\text{m}$  CFSE. Labeling of the cells was allowed to proceed for 30 min and then the cells were washed three times in fresh PBS. A small aliquot of cells was examined to ensure that adequate labeling of cells had been achieved.

32,000 labeled mesenchymal cells were reassociated with 4 epithelial lobes in 200  $\mu\text{l}$  of DMEM medium in V-bottomed 96-well plates. The plates were centrifuged at 1,000 rpm for 10 min and then 2,000 rpm for 5 min to ensure adequate reassociation of mesenchymal cells with epithelial lobes. The plates were incubated in an enriched oxygen atmosphere for 4–6 d, after which frozen sections were prepared from the cultures.

## Results and Discussion

*The 12-d Mouse Embryo Thymus Is Undergoing Colonization by Lymphoid Stem Cells and Consists of an Epithelial Primordium Surrounded by Mesenchyme.* When microdissected from mouse embryos, the 12-d thymus consists of an epithelial rudiment associated with mesenchyme. During dissection of the thymus from the embryo, there is a tendency for the mesenchyme to strip away from the epithelium, revealing a connective tissue capsule (Fig. 1 b). Note that there are no blood vessels within the epithelial primordium at this stage.

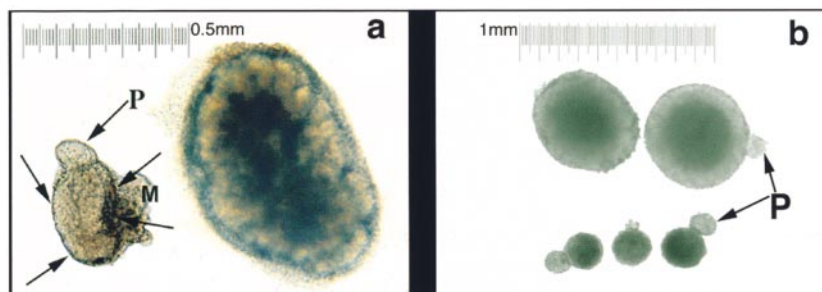
The thymus is undergoing active colonization by lymphoid stem cells, which migrate into the thymus from local blood vessels. CD45<sup>+</sup> stem cells can be seen both within the cytokeratin-positive epithelium and at the mesenchymal-epithelial boundary (Fig. 1 a). We have phenotyped

these cells in a previous study and shown that they are CD34<sup>+</sup>CD44<sup>+</sup> $\alpha_4$ -integrin<sup>+</sup>c-kit<sup>+</sup> (18).

The mesenchyme contains cells that express the ERTR7 fibroblast marker, and most of the fibronectin labeling is associated with these cells (Fig. 1 b); we show in Fig. 4 a that this fibronectin is in the form of an ECM. ERTR7 has been shown previously to mark intrathymic fibroblasts especially in the capsule and trabeculae (19).

*12-d Thymus Lobes Associated with Mesenchyme Generate All T Cell Subsets In Vitro whereas Lobes without Mesenchyme Show Limited T Cell Development.* We carefully microdissected lobes from 12-d embryos to ensure that mesenchyme remained attached. These lobes are small (Fig. 2 a shows a 15-d lobe for size comparison) and have an attached parathyroid. Because of the ease with which perithymic mesenchyme strips away from the thymic capsule, mesenchyme is often present on only one side of the thymus (Fig. 2 a). When cultured for 10 d, lobes with attached mesenchyme double in size (Fig. 2 b) and yield on average 65,000 cells/lobe. In contrast, lobes stripped of mesenchyme and cultured for the same time period remain small (Fig. 2 b) and yield on average 5,000 cells/lobe. In some cases, we left the parathyroid attached so as to provide a size comparison (Fig. 2 b).

We then studied the phenotypes of cells in lobes cultured with attached mesenchyme. There was a sufficient yield of cells from these lobes to allow analysis of CD4, CD8, TCR- $\alpha/\beta$ , TCR- $\gamma/\delta$ , and NK1.1 expression. Three-color flow cytometry for CD4, CD8, and  $\alpha/\beta$  ex-



**Figure 2.** Photograph of lobes cultured with or without mesenchyme. (a) Size comparison between a 12-d thymus lobe (left) with a 15-d lobe (right). The epithelial boundaries of the 12-d lobe (arrows), the attached mesenchyme (M), and a parathyroid gland (P) are indicated. (b) 12-d thymus lobes cultured for 10 d with mesenchyme (top two lobes) or without mesenchyme (bottom three lobes). The scale shows that b is at a lower magnification than a. The parathyroid gland (P) has been left attached to some lobes. There is an obvious size difference between 12-d lobes cultured with mesenchyme and those cultured without.

pression shows the presence of  $CD4^-CD8^-$ ,  $CD4^+CD8^+$ , and  $CD4^+$  or  $CD8^+$  populations, all of which contain  $\alpha/\beta^+$  cells (Fig. 3 a). The fact that  $\alpha/\beta^+CD4^+$  and  $\alpha/\beta^+CD8^+$  cells are found indicates that all stages of thymic maturation have been achieved. The proportions of the various populations are similar to those found after organ culture of 15-d thymus lobes for 7 d (Fig. 3 c), giving a comparable developmental stage.

In contrast to the results above, 12-d lobes cultured without mesenchyme show poor lymphoid development and yield so few cells that it is difficult to undertake an extensive phenotypic analysis. Fig. 3 b shows that few  $CD4^+CD8^+$  cells develop in these cultures and most cells are blocked at the  $CD4^-CD8^-$  stage of development. In addition to generating  $\alpha/\beta^+CD4^+$  and  $\alpha/\beta^+CD8^+$  cells, 12-d lobes associated with mesenchyme generate  $\gamma/\delta^+$  cells (Fig. 3 d) and  $NK1.1^+$  cells (Fig. 3 e). A small proportion of the latter is  $\alpha/\beta^+NK1.1^+$  T cells (Fig. 3 e). Most  $\gamma/\delta^+$  and  $NK1.1^+$  cells are  $CD4^-CD8^-$ .

In summary, the  $CD45^+$  lymphoid precursors in 12-d thymus are capable of generating all T cell populations in organ culture, provided that perithymic mesenchyme remains in close association with the epithelium.

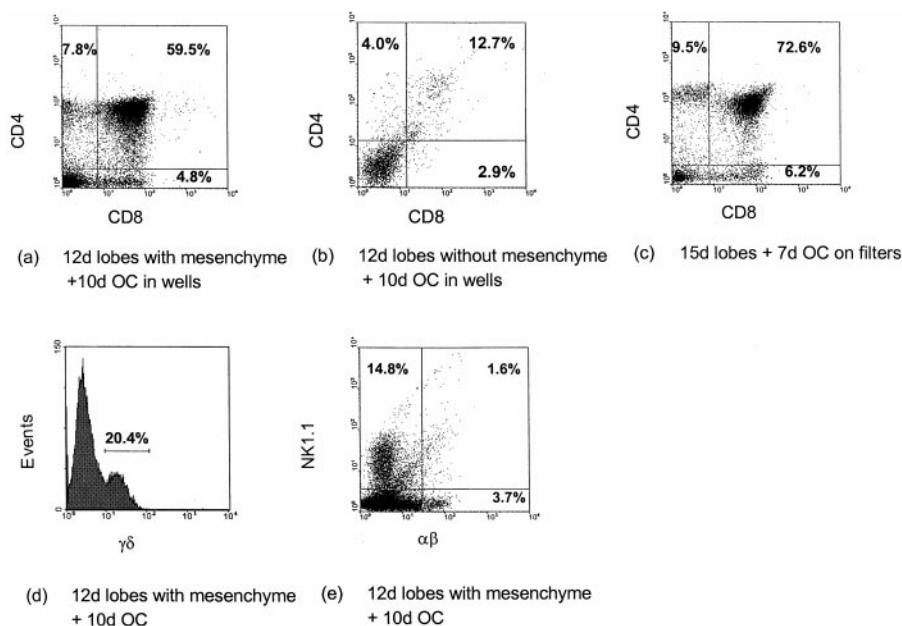
*After Lymphoid Stem Cell Colonization of the 12-d Thymus, Mesenchymal Cells Migrate into the Epithelial Primordium.* Using ERTR7 as a marker, mesenchymal cells can be seen surrounding the 12-d thymus, but they are not found within the epithelium itself (Fig. 1 b). However, in 14-d or 12-d lobes cultured with mesenchyme for 48 h, ERTR7<sup>+</sup> cells are found within the epithelium (Fig. 4, b and c). Some of these cells are present as individual cells or small clusters, and this pattern persists in the adult thymus (Fig. 4, d-f). As expected from a previous study (19), many ERTR7<sup>+</sup> cells are situated in the thymic capsule, septae, and blood vessels. ERTR7<sup>+</sup> cells are associated with fi-

bronectin staining (Figs. 1 b and 4 e). This could indicate that fibronectin is present within the cytoplasm of these cells or is present on their surfaces as a component of the ECM. Fig. 4 a shows that an ECM is indeed present on the surface of mesenchymal cells.

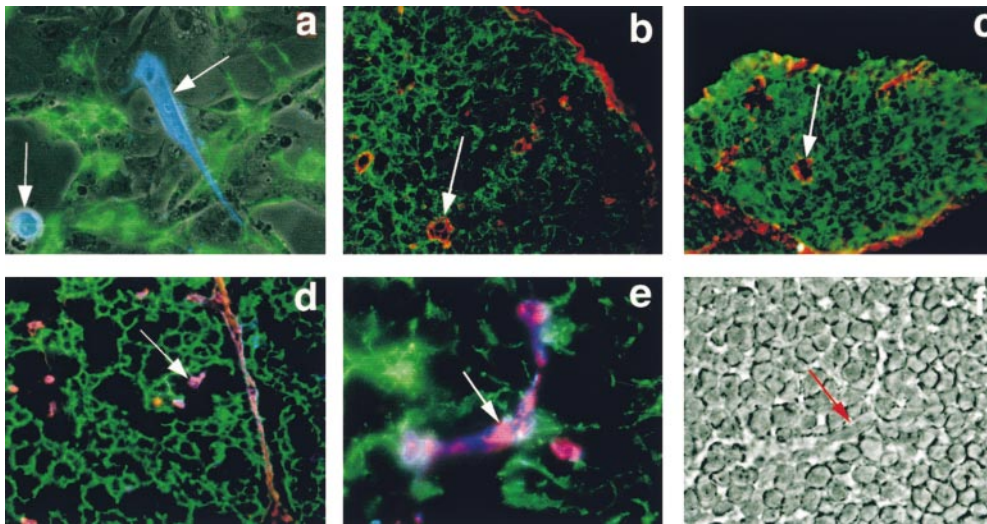
Although the sequence of events described above suggests a migration of mesenchymal cells into the thymic epithelium, we have sought to obtain more direct evidence by associating CFSE-labeled mesenchymal cells with 12-d thymic epithelial rudiments. After culture, we find CFSE-labeled cells in epithelial areas (Fig. 5 a). Fig. 5, b and c, shows that CFSE<sup>+</sup> cells in the epithelium are  $CD45^-$ , thus excluding the possibility that they are labeled lymphoid cells. We conclude that, shortly after day 12 of gestation, mesenchymal cells migrate into the epithelial thymus.

*The Effects of Mesenchyme on Thymic Lymphopoiesis.* Several mutations that affect the migration and/or function of cephalic neural crest-derived mesenchyme also result in thymic abnormalities (8-12), suggesting the importance of mesenchyme in thymic development. Auerbach (20) was the first to demonstrate experimentally that enzymic removal of the capsule and associated cells prevents lobulation of the 12-d epithelial rudiment. In a later study, enzymic removal of the capsule was shown to influence MHC expression in cultures of 12-d lobes (15). Neither study examined effects on lymphopoiesis. Amagai et al. (13) reported on the low level of lymphopoiesis in cultures of 12-d thymus lobes, but they did not comment on whether mesenchyme was present. Subsequently, they found that lymphopoiesis was restored by addition of fibroblasts but these were not thought to penetrate the epithelium (14). Our results show that addition of fibroblasts is unnecessary provided that perithymic mesenchyme is present.

In general, these studies have been used as evidence for the importance of an inductive effect of mesenchyme on

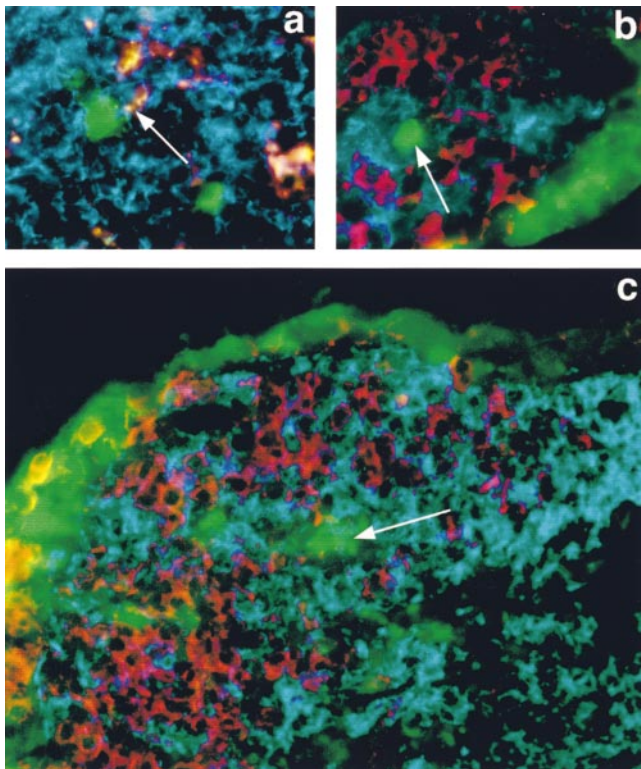


**Figure 3.** Flow cytometry of cells in lobes cultured with or without mesenchyme. (a) The proportions of cells expressing CD4 and CD8 in 12-d lobes that have been organ cultured (OC) for 10 d with mesenchyme. Note that the percentages of  $\alpha/\beta^+$  cells in each were as follows: 8.7% ( $CD4^-CD8^-$ ), 31.4% ( $CD4^+CD8^+$ ), 31.5% ( $CD4^+CD8^-$ ), and 12.2% ( $CD4^-CD8^+$ ). (b) The relatively few  $CD4^+CD8^+$  cells generated in 12-d lobes cultured without mesenchyme (these cultures yield only 7.7% of the total yield of cells found in cultures with mesenchyme). (c) The CD4 and CD8 phenotypes of cells generated from cultures of 15-d thymus lobes. (d) The development of  $\gamma/\delta^+$  cells in cultures of 12-d thymus with mesenchyme. (e)  $NK1.1^+$  cells are generated in thymic cultures with mesenchyme. A small proportion of the  $NK1.1^+$  cells are also  $\alpha/\beta^+$ .



**Figure 4.** Immunocytochemistry showing the presence of ERTR7<sup>+</sup> mesenchymal cells within the developing and adult thymus. (a) Cells from trypsinized 12-d thymic lobes (epithelium surrounded by mesenchyme). The cells were allowed to attach to wells, and after 48 h were stained for surface fibronectin (green) and, after fixation, for cytokeratin (blue). The image shows cells in phase-contrast with epithelial cells (arrows) and numerous mesenchymal cells. The matrix is associated with the mesenchymal cells. (b and c) Frozen sections of 14-d (not cultured) and 12-d thymus, cultured for 48 h, respectively. ERTR7<sup>+</sup> mesenchymal cells (red), some of which are indicated by arrows, can be

seen within the thymic epithelium (green). (d) ERTR7<sup>+</sup> cells (red) in the cortex of the adult thymus. Some of these cells are present in septae, one of which can be seen on the right of the photomicrograph, whereas others (one marked by an arrow) are present as single cells. Cytokeratin-positive epithelial cells are green and the ECM is blue. Original magnification: (a–d)  $\times 400$ . (e and f) A single ERTR7<sup>+</sup> fibroblast in the adult thymus at a higher magnification (original magnification:  $\times 1,000$ ). In e, the ERTR7 product (red) is indicated by an arrow, and there is a background of cytokeratin-positive epithelial cells (green). The ECM (blue) is associated with the fibroblast. f is a phase contrast view of the same cell (arrow) showing the intimate association with thymocytes.



**Figure 5.** CFSE-labeled mesenchymal cells migrate into the thymic epithelium of cultured 12-d lobes. (a) CFSE-labeled mesenchymal cells (green) inside the cytokeratin-positive epithelium (blue) of a cultured 12-d lobe. The ERTR7 product is red (arrow). (b and c) CFSE-labeled cells (green, arrow) are distinct from CD45<sup>+</sup> lymphoid cells (red). The cytokeratin-positive epithelium is blue. Original magnifications: (a and b)  $\times 1,000$ ; (c)  $\times 800$ .

thymic epithelial morphogenesis. However, even when epithelial cells have been allowed to mature, there may still be a requirement for mesenchyme-derived fibroblasts. Thus, epithelial cells derived from 15-d lobes cultured for 6 d in deoxyguanosine (giving a developmental age equivalent to birth), although sufficient for later stages of T cell development including positive selection (17), are not sufficient for early T cell development. Fibroblasts are also necessary at this stage (16). However, we cannot exclude the possibility that mesenchymal cells indirectly influence thymopoiesis by providing inductive signals to generate and maintain correctly organized epithelial microenvironments.

We have provided an anatomical basis for the role of fibroblasts by showing that mesenchymal cells migrate into the thymus and are present in lymphoid areas as well as in the capsule and trabeculae. Moreover, these cells are associated with fibronectin staining and, although fibronectin and other matrix molecules may be produced by many cell types, the formation of an ECM on the surface of cells is an active process requiring integrin receptors and cytoskeletal activity (21). By staining the surfaces of thymic stromal cells before fixation, we have noted an ECM on thymic fibroblasts in this study (Fig. 4 a) and in a previous study (16).

We cannot exclude the possibility that thymic epithelial cells might produce an ECM or, indeed, we cannot be sure that the ECM is the critical component of fibroblast function. However, there are several potential interactions between developing thymocytes and the ECM that could be of functional importance. The integrins  $\alpha_4\beta_1$  (22),  $\alpha_5\beta_1$  (23), and  $\alpha_6\beta_1$  (24) are expressed on thymocytes permitting interactions with fibronectin and laminin, as both are com-

ponents of the matrix (25). In addition, IL-7, which is known to be an important cytokine in T cell development, binds to sulfated glycosaminoglycans in the matrix from where it might be presented to thymocytes (26).

In summary, the elaboration of an ECM by mesenchyme-derived fibroblasts might explain the role of these cells in T cell development. We are attempting to confirm this proposition by inhibiting matrix assembly in cultures of 12-d thymus using fibronectin fragments and antifibronectin antibodies (27).

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