Cutting Edge: Thymocyte-Independent and Thymocyte-Dependent Phases of Epithelial Patterning in the Fetal Thymus

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Thymic epithelial cells (TECs) in adult mice have been classified into distinct subsets based on keratin expression profiles. To explore the emergence of TEC subsets during ontogeny, we analyzed keratin 8 and keratin 5 expression at several stages of fetal development in normal C57BL/6J mice. In addition, thymic epithelial development and compartmentalization were explored in recombination-activating gene 2/common cytokine receptor γ-chain-deficient and Ikaros-null mice that sustain early and profound blocks in thymocyte differentiation. The results demonstrate that initial patterning of the thymic epithelial compartment as defined by differential keratin expression does not depend on inductive signals from hematopoietic cells. However, thymocyte-derived signals are required during late fetal stages for continued development and maintenance of TEC subsets in the neonate and adult.

The epithelial compartment in the thymus is unique in that it cannot be classified strictly as simple or stratified epithelium. Thymic epithelial cells (TECs) are organized into a three-dimensional network rather than forming epithelial sheets arranged on a basement membrane as is characteristic of epithelial organization in other organs (11). The mesh-like arrangement of TECs facilitates thymocyte migration among and interaction with thymocyte subsets located in the subcapsular, cortex, and medullary regions (11, 12). TECs have been characterized according to location, morphology, and function (1, 13). As in other epithelial tissues, keratins serve as differentiation markers that distinguish thymic epithelial subsets. We have demonstrated that the thymic cortex contains a predominant subset of K8⁺K18⁺K5⁻K14⁻ cells and a minor subset of K8⁺K18⁺K5⁺K14⁻ cells (7, 14). TECs in the latter population (hereafter referred to as K8⁺K5⁺) are concentrated at the corticomедullary junction and scattered throughout the cortical and subcapsular regions. The medulla contains a major K8⁻K18⁻K5⁺K14⁻ subset and a minor K8⁻K18⁺K5⁻K14⁻ population that is distinguished from the cortical subset by globular morphology and Ulex europaeus agglutinin lectin binding properties. Previous studies of the adult murine thymus revealed that K8⁺K5⁺ precursors generate the major cortical K8⁺K5⁺ TEC subset in a process dependent on signals from T lineage-committed thymocytes (7, 15). Two recent reports have shown that progenitor activity is restricted to a subset of K8⁺K5⁺ TECs that expresses MTS24 cell surface glycoprotein (16, 17). Ectopic grafts of isolated and reaggregated MTS24⁺ TECs can differentiate into cortical and medullary TEC subsets that support thymocyte development. Although it is well established that proper differentiation of the thymic epithelial compartment requires signals from mesenchymal...
cells and thymocytes, it was not known whether thymocyte-derived signals were necessary to establish initial patterning of TEC subsets defined by keratin expression in the thymic anlage. Therefore, we compared TEC development and compartmentalization in wild-type mice to that which occurs in the recombination-activating gene (RAG)2/common γ-chain (γc)-deficient and Ikaros-null mice that sustain early and profound blocks in thymocyte differentiation. Our results demonstrate that there is an early developmental window within which thymic epithelial subsets defined by keratin expression patterns develop independently of thymocyte-mediated signals during thymic organogenesis. However, thymocyte-derived signals are required during late fetal development to generate and sustain a normal thymic epithelial compartment in the neonate and adult.

**Materials and Methods**

**Mice**

C57BL/6J and human (h)CD3ε-transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). RAG2/γc mice were purchased from Taconic Farms (Germantown, NY). Ikaros-null mice were the generous gift of Dr. K. Georgopoulos (Harvard Medical School, Charlestown, MA) (18).

**Antibodies**

Polyclonal anti-mouse K5 was obtained from Covance Research (Richmond, CA). Troma-1 mAb (anti-K8) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA) (19). MTS10, c-kit, and CD25 mAbs were purchased from BD PharMingen (San Diego, CA). Immunoreactivity detected with fluorochrome-conjugated anti-Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) was enhanced as indicated by tyramide amplification (PerkinElmer Life Sciences, Boston, MA).

**Immunohistology**

Serial sections (5 μm) from OCT-embedded frozen tissue were fixed in acetone and incubated overnight at 4°C with optimal dilutions of anti-K8 and/or anti-K5 Abs before washing and incubation with fluorochrome-conjugated secondary reagents. Control slides were incubated with non-immune serum or isotype-matched Ig. Analysis was performed with an Olympus ProVis AX70 microscope (Olympus, Melville, NY).

**Results and Discussion**

To investigate the emergence of TEC subsets during thymic organogenesis, we analyzed keratin expression patterns in murine fetal thymi obtained at various gestational stages. Fig. 1 shows that the epithelium of the early thymic anlage at E11.5 is arranged in a two-dimensional bilayer consisting of cells that express K8 but not K5. By E12.5, the epithelial compartment assumes a clustered organization, and a prominent K8/K5+ subcompartment emerges that is centrally located and surrounded by K8/K5− TECs. The centralized clusters of K8/K5+ TECs persist in E13.5 fetal thymi. By E15.5 smaller clusters of K8+K5+ TECs are observed emanating toward the periphery of the thymus interspersed among the K8+K5− subset. K5 expression is generally highest in the innermost cells of the K8+K5+ clusters, whereas cells at the boundary express less K5, suggesting a transitional population. Some TECs in the central clusters were K8−/K5+, consistent with later development of a K8+K5− phenotype characteristic of the predominant medullary subset (7). There is a notable change by E17.5, when the cortex becomes well organized and consists predominantly of K8+K5+ TEC similar to the adult thymus. Although an abundant subset of TECs that coexpress K8 and K5 is still apparent, they are no longer organized into central cores. We also observed incipient medullary regions that contain K8+K5+ TECs, similar to the predominant medullary TEC subset in the adult (7).

Development of the thymic epithelial compartment depends on inductive signals from hematopoietic as well as mesenchymal cells. The migration of hematopoietic progenitors into the thymic primordium is initiated between E11 and E12, which corresponds temporally to the appearance of K8+K5+ TEC clusters (20, 21). This correlation suggests that interactions with differentiating thymocytes might induce K5 up-regulation in fetal TECs. Alternatively, the appearance of K8+K5+ TEC clusters by E12.5 might occur independently of thymocyte-derived signals. To explore these possibilities, we examined keratin expression patterns in fetal thymi from RAG2/γc-deficient mice and Ikaros mutant mice. The absence of a functional γc gene in RAG2/γc-deficient mice precludes IL-7/IL-7R interactions, and the lack of RAG2 prevents TCR gene rearrangement. Consequently, thymocyte cellularity is drastically reduced (<105 cells) and there is a severe block in T, B, and NK cell development in RAG2/γc-deficient mice (22). The Ikaros transcription factor is indispensable for commitment of hematopoietic stem cells to the lymphoid lineage. Targeted deletion of the carboxyl-terminal region of the Ikaros gene results in a null phenotype characterized by failure of B cell development and an absence of T cell precursors during the fetal period (18). Fig. 2 shows that, despite these early T cell developmental blocks, the epithelial compartment in E13.5 and E15.5 RAG2/γc-deficient and Ikaros thymi organizes into a three-dimensional structure containing a predominant K8+K5+ TEC subset and centralized K8+K5+ TEC clusters. Thus, thymocyte-derived signals are not required to generate the K8+K5+ clusters in early fetal thymic development.

It has been reported that IL-7 is not as essential for thymopoiesis in fetal compared with adult thymi (23). This may account for the small but detectable population of CD25+ double negative thymocytes in E13.5 RAG2/γc-deficient thymi. However, it is unlikely that these cells are responsible for up-regulating K5 expression because CD25+ thymocytes are not detectable in E13.5 Ikaros-null thymi that contain K8+K5+ central clusters (Fig. 3A). Moreover, although a few c-kit+ thymocytes are observed in Ikaros-null thymi at E15.5, these early progenitors are not found at

![FIGURE 1. K8 and K5 expression in TECs during ontogeny in C57BL/6J mice. Cryostat sections of embryos (E11.5–E13.5) or dissected thymic lobes (E15.5 and E17.5) were stained with Abs to K8 and K5 followed by incubation with FITC- or Texas Red-conjugated anti-Ig secondary reagents. The original magnification was ×200.](http://www.jimmunol.org/Download/FIG1/FIG1.png)
The central region of K8/H11001 and RAG2/H9253 in RAG2/H9253 present in the E13.5 normal thymus and the developmental blocks are required for medullary region organization (5, 24), are not yet expression. Cryostat sections of E13.5 normal C57BL/6J, RAG2/H9253 interning of the E13.5 fetal TEC subsets defined by K8, K5, and MTS10 fii

**FIGURE 3.** Thymocyte-derived signals are not necessary for early pat-tering of the thymic epithelium into K8⁻K5⁻ and K8⁺K5⁺ TEC subsets occurs independently of thymocyte-derived signals.

Interestingly, the K8⁺K5⁺ clusters in E13.5 thymi from normal, RAG2/γc, and Ikaros-null mice express the medullary marker MTS10 (Fig. 3C). The brightest MTS10⁺ cells are found within the central region of K8⁺K5⁺ clusters, whereas the surrounding K8⁺K5⁻ fetal TECs are MTS10 negative. Mature T cells, which are required for medullary region organization (5, 24), are not yet present in the E13.5 normal thymus and the developmental blocks in RAG2/γc-deficient and Ikaros mice preclude or delay their appearance. Thus, the MTS10 expression pattern is consistent with the notion that the K8⁺K5⁺ subset contains progenitors of medullary as well as cortical epithelium.

Although hematopoietic precursors do not determine initial pat-tering of the fetal thymic rudiment, thymocyte/TEC interactions are indispensable for maintaining TEC differentiation and organization in the adult thymus (5, 7, 25). This is apparent in adult mice that sustain a T cell developmental arrest at the CD4⁻CD8⁻ CD44⁻CD25⁻ precursor stage due to expression of a hCD3e transgene. The severely hypoplastic thymi in hCD3e mice have a disorganized TEC compartment that reverts to a two-dimensional organization and consists almost entirely of K8⁺K5⁺ TECs (6, 7, 11). Not surprisingly, we found a similar TEC phenotype in adult RAG2/γc-deficient thymi, which also have a profound block in early T cell development (data not shown). Given that thymocyte-derived signals are required to maintain compartmentalization and architecture of the adult thymic epithelium but are not involved in establishing the fetal thymic epithelial network, we examined the duration of the developmental window within which TEC differenti-ent proceeds independently of thymocyte/TEC interactions. As shown in Fig. 4, the newborn C57BL/6J thymus has a well-developed cortex with K8⁺K5⁺ TECs that are oriented perpen dicuilar to the capsule. Small medullary regions are forming that contain K8⁺K5⁺ TECs surrounded by K8⁺K5⁻ TECs at the corticomedullary junction. Although epithelial organization is similar in newborn and E17.5 thymi, the K8⁺K5⁻ subset is more prominent at E17.5. In striking contrast, newborn RAG2/γc-deficient and hCD3e-transgenic thymi are not hypoplastic, with a keratin expression pattern similar to that observed at E13.5–E15.5 (i.e., prominent centralized clusters of K8⁺K5⁺ TECs). Well-or-organized mature medullary regions containing K8⁺K5⁻ TECs are absent. The early fetal-like keratin expression pattern persists until ~1 wk of age, after which the majority of TECs assume the aberrant K8⁺K5⁻ phenotype characteristic of adult RAG2/γc-defi-cient and hCD3e-transgenic thymi (data not shown and Ref. 7).

Thus, thymocyte-derived signals impinge upon TEC development by E15.5, a time frame that is coincident with the appearance of CD25⁺ immature thymocytes.

In conclusion, this study demonstrates that regional differences in keratin expression patterns are established early in the genesis of the thymic rudiment. We find that the epithelial cells of the E11.5 thymic primordium express K8 before up-regulating K5 expression. At this developmental stage, K5 is expressed in pharyngeal ectoderm but not in pharyngeal endoderm (data not shown). The K8⁺K5⁻ phenotype of early TECs is consistent with other studies that maintain the thymus is derived exclusively from endoderm (3, 4). Similarly, Gill et al. (17) reported that K8 is widely expressed in the E11.5 thymic primordium, whereas only rare K5⁺ cells are present. In contrast, Bennett et al. (16) found K5 expression to be more generalized throughout the E11.5 anlage. Discrepancies in the appearance of K5⁺ TECs during the earliest stage of thymic organogenesis may be due to differences in mouse strains and/or staging criteria.

**FIGURE 4.** Thymocyte-derived signals are required to sustain normal TEC differentiation in the neonate. Cryostat sections of newborn thymic lobes from C57BL/6J, RAG2/γc-deficient, and Ikaros-null mice were stained with Abs to K8 and K5 as described for Fig. 1.
It is not yet clear whether the K8\(^{+}\)K5\(^{-}\) epithelial cells in the developing thymic rudiment are equivalent to K8\(^{+}\)K5\(^{-}\) cortical TECs in the adult. The K8\(^{+}\)K5\(^{-}\) epithelial cells in the early thymic primordium may be lost during fetal development, similar to the developmental fate of the K8\(^{+}\)K5\(^{-}\) periderm, the transient outermost layer of embryonic epidermis that disappears before birth (26). Regardless, by approximately E12.5, K5 is up-regulated in a discrete subset of TECs that are localized toward the central region of the developing thymus. Thus, heterogeneity within the epithelial compartment is established early during thymic organogenesis.

Itoi et al. (20) reported that the thymic epithelium converts from a stratified bilayered epithelium at E11 to a clustered organization of the developing thymus. Thus, heterogeneity within the epithelial compartment may be lost during fetal development, similar to the periderm, the transient outermost layer of embryonic epidermis that disappears before birth. However, it is not clear whether K5 expression is independent of hematopoietic-derived signals. Mesenchyme-derived inductive signals may be responsible for early patterning of the thymic epithelial compartment. Byrne et al. (27) found that K5 expression in the developing epithelium does not correlate with morphogenesis per se, but rather with changes in the embryonic origin of underlying mesenchyme. Earlier studies demonstrated that neural crest-derived mesenchymal cells play a crucial role in thymic development (8, 9, 28). Fibroblast growth factor (Fgfi7) and Fgfl0 produced by mesenchymal cells surrounding the thymic primordium activate proliferation of FgFR-IIb-expressing TECs (29). Mesenchymal cells may also impart cues that induce differentiation and initial patterning of the thymic rudiment. In any case, we demonstrate that there is a discrete developmental window beyond which thymocyte-derived signals are required to sustain TEC organization and differentiation as defined by keratin expression patterns. Further studies are needed to define the various signaling pathways that induce thymocyte-independent and -dependent phases of TEC differentiation.

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References