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Compartmentalization of Human Thymic Medulla: Facts and Hypotheses

Ildiko Bodi, Krisztina H.-Minko, Zsolt Prodan and Imre Olah

Abstract

The thymus function was discovered in the middle of the last century. The role of the thymus in the adaptive immune system facilitated its histological and experimental studies. Before the role of the thymus was discovered, the thymus was called as a gland on the basis of lobulation; even some histological textbook listed it up among the endocrine glands. In addition to the cortex and medulla, the immunohistochemical studies revealed a further compartmentalization in the thymic medulla, which is related to the epithelium-free areas (EFA) and keratin-positive network (KPN). The two medullary compartments have different cellularity that determines their role. This chapter would concentrate on the medullary compartmentalization and their cellularity. Furthermore, this chapter discusses the relationship of thymic septae with the perivascular space, the vascular embedding and thymic dendritic cells.

Keywords: medulla, epithelium-free area, keratin-positive network, dendritic cells

1. Introduction

In the histological textbooks, the main morphological landmarks of the thymus are (1) the lobulation—like the glands; (2) the darkly and lightly stained cortex and medulla, respectively; and (3) the Hassall's bodies. The relatively simple structure of the thymus did not help to "decode" the enigmatic thymic function until the middle of the last century. The three morphological landmarks may be supplemented by the keratin-negative area (KNA) [1, 2] or "epithelium-free area" (EFA) [3–6]. EFA can be found in both the cortex and medulla [6–8]. In the cortex, the EFA is a nest of double-positive (CD4+, CD8+) T lymphocytes [4] and various macrophages [7]. Others suggest that the cortical EFA is a pathological phenomenon [3, 6].

If the KNA/EFA is a permanent compartment of the human thymic medulla, then it should be added to the general histological features of the thymus of the warm-blooded vertebrates.

2. Relationship between perivascular space (PVS) and keratin-negative and/or epithelium-free areas

In the medulla, the perivascular space (PVS) [7, 9–12]—that is, the dilated primary septum (PS)—carries the blood vessels, which locate "outside" the basal lamina of the thymus [1, 2, 12, 13], but Foxn-1, which is a thymic epithelial cell-specific
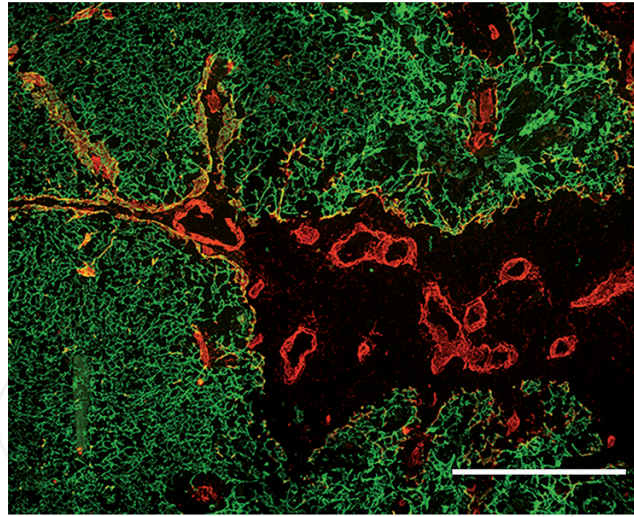
Figure 1.
Double staining of chicken thymus: cytokeratin (green) and laminin (red). At the PS, the laminin shows a continuous basal lamina, which becomes discontinuous in the EFA. The KNA/PVS receives the blood vessels.

transcription factor [14], regulates thymic vascularization [15]. At the cortico-medullary junction, PS merges to the PVS of the medulla [9, 10]. A continuous basal lamina covers the thymic parenchyma toward the capsule and PS, and it becomes discontinuous at the end of the PS (**Figure 1**) [2, 7] where it turns to be the PVS or KNA/EFA. In the thymic medulla, the lack of blood-thymus barrier [16] may be explained by the discontinuity of the basal lamina on the border of the KNA/EFA [2]. The discontinuity of the basal lamina suggests that the microenvironment of the KNA/EFA and PVS is identical. Silver impregnation shows that both the PS and the KNA/EFA consist of reticular connective tissue [2, 9, 17] and have common extracellular matrix [11]. Secondary septae appear after formation of the cortex and medulla, and they are just small invaginations of the capsule and usually do not reach the medulla and do not receive blood vessels. The medullary EFA occupies about one-fifth of the rat thymus [18]. In chicken our morphometric studies confirm the considerable size of the KNA, that is, close to 50% of the medulla [2]. The border of the keratin-positive network (KPN) and KNA is an epithelial-mesenchymal border that could be the functional cortico-medullary (CM) border of the thymus [2]. The KPN-KNA/EFA border is supported by cellular background, unlike the hematoxylin-eosin-stained, classical CM border, which is based only on lymphocyte density and subsequently stainability. The mesenchymal tissue of the PVS develops from neural crest cells [19–22]. The PVS is a transit zone of migratory cells between the thymus and circulation [12, 23].

Anti-cytokeratin immunostaining identifies the KPN and KNA/EFA in both embryonic and postembryonic chicken thymuses. In an 11-day-old chicken embryo, the thymic epithelial anlage shows a starfish-shaped form (**Figure 2**). Between the 5–6 secondary epithelial cords, the unstained PS(s) consist of mesenchyme. During the next two ED (11 and 13), the cortical epithelial cells rapidly proliferate resulting in enlarged thymic rudiment (**Figure 3**) which is colonized by hematopoietic cells. In 11-ED-old birds, the wide PS became narrow, and the bottom of the PS is involved into the medulla as the KNA/EFA.

The PS is going on as the KNA/EFA, and both regions consist of reticular connective tissue stained with silver impregnation [2, 6, 17, 24]. Mesenchymal markers desmin and ER-TR7 [6, 25] revealed specific staining in the capsule, septae, and medullary PVS. In the PVS, the neural crest cells differentiate into smooth muscle cells of thymic blood vessels and pericytes of thymic capillaries [21]. These histological findings suggest the common origin of the PS and KNA/EFA: namely, the KNA/EFA develops from the cranial neural crest cells [19, 21, 26].
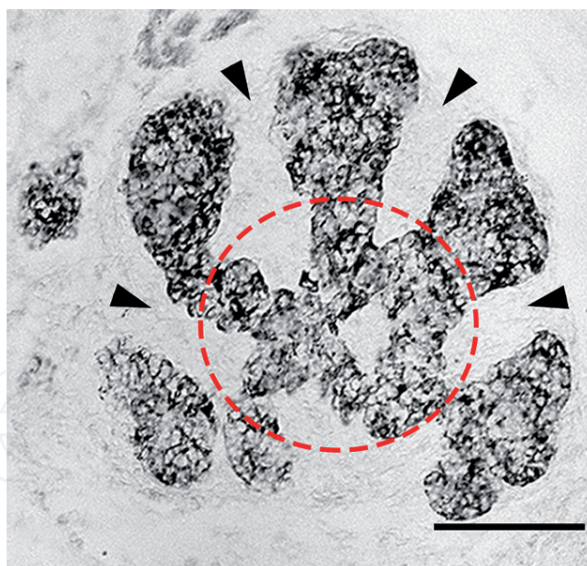
Figure 2.
11 ED chicken thymus: anti-cytokeratin. The starfish-shaped epithelial thymic anlage shows the branching of the primary epithelial cord to 5–6 secondary cords. The dashed circle outlines the future medulla.
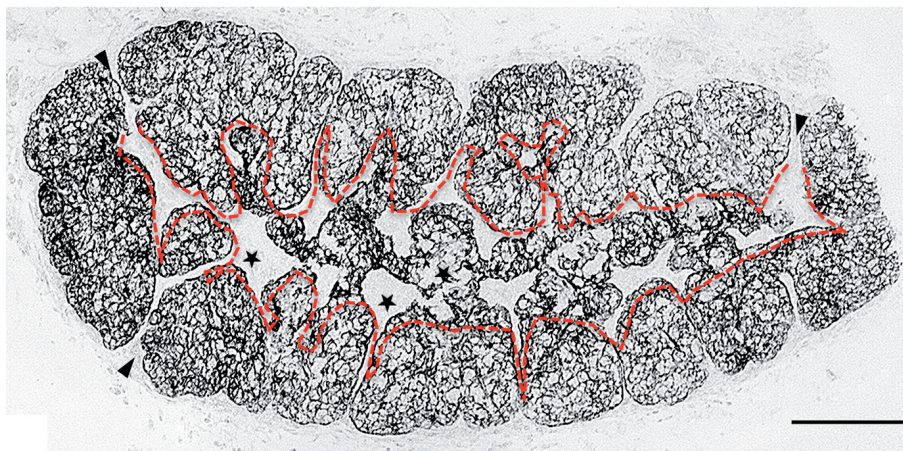

Figure 3.
13 ED chicken thymus: the rapid proliferation of epithelial cells enlarged the thymic cortical epithelium. The bottom of the PS (arrowhead) dilates and becomes the KNA/EFA of medulla (star).

3. Keratin-negative area/epithelium-free area

Mesenchymal cells and fibroblasts express vimentin intermediate filament. Cortical thymocytes and epithelial cells are vimentin negative (**Figure 4**), but thymic medulla shows homogenous staining pattern, indicating that keratin-positive and keratin-negative compartments cannot be distinguished (**Figure 5**). The homogenous vimentin staining of the medulla may indicate that the medullary epithelial cells express vimentin intermediate filament. Hassall's bodies are vimentin negative (**Figure 5**), like cortical epithelial cells. In the majority of vimentin-positive cells, the immunoreaction appears in the periphery of the cell cytoplasm. The nature of the medullary vimentin-positive cells is not clear, because vimentin can colocalize with other intermediate filaments, like neurofilament, cytokeratin, and desmin; therefore the anti-vimentin immunostaining used for identification of mesenchymal cells is limited [25]. Blood vessels and dendritic cells are the most significant structures of the KNA/EFA. Anti-von Willebrand factor identifies endothelial cells (**Figure 6**). Transmission electron microscopy shows the organelle-rich cytoplasm of the interdigitating dendritic cell (IDC) (**Figure 7**). The IDC is located in close association with the blood vessels [2].
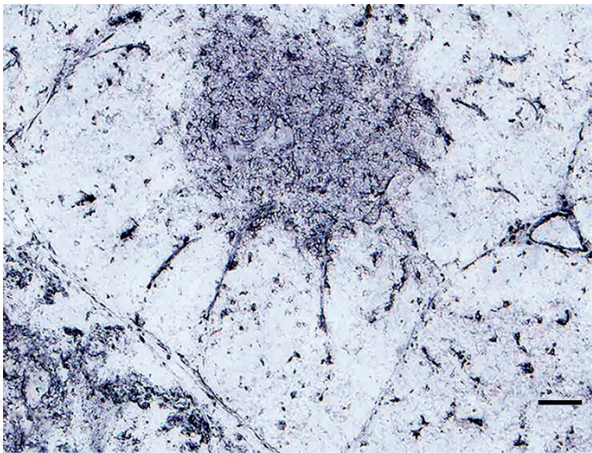
Figure 4.
Anti-vimentin antibody outlines the capsule and PS. The cortical epithelial cells and T cells are negative, while all medullary cells express vimentin. C, cortex; M, medulla.
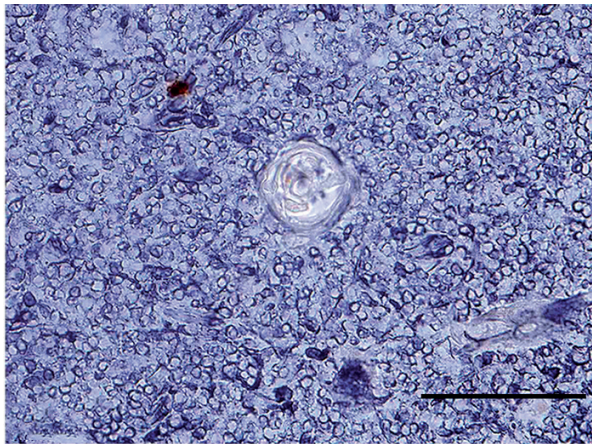

Figure 5.
Higher magnification of anti-vimentin-stained thymic medulla. The round-shaped Hassall's body is negative, while the medullary cells show a round-shaped-positive reaction.
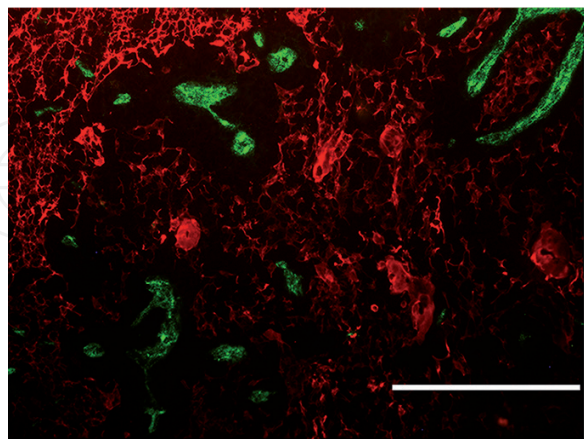

Figure 6.
Double staining: cytokeratin (red) and von Willebrand factor (green). Blood vessels exclusively locate in the KNA/EFA.

As mentioned above, the PVS consists of mesenchymal cells of neural crest origin; therefore, the supporting tissue of the KNA/EFA is also mesenchyme. The thickness of the PVS is used to be in one or two cell layers, but in chicken thymus, the morphometric studies show that the ratio between keratin-positive and keratin-negative fields is close to one to one [2]. Considerable size of the KNA/EFA
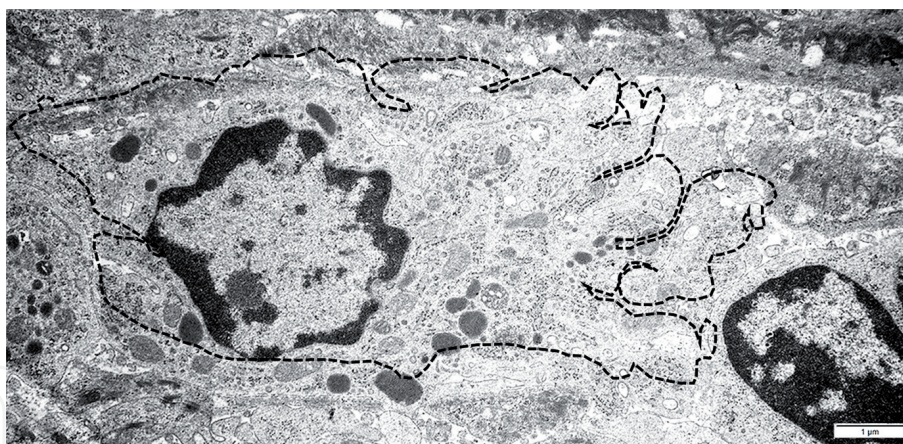
Figure 7.
Transmission electron micrograph of a thymic dendritic cell. The cell membrane is outlined. The cytoplasm is rich in organelles and electron-dense granules.

(**Figure 6**) suggests that its functional significance must be more than a passive transit zone for cell migration between circulation and the thymus [4, 10–12]. Thymic IDC [9] is located in the KNA/EFA [2] and contributes to T-cell selection. In nonobese mouse, autoimmune type I diabetes develops, resulting in abnormal distribution of epithelial cells and consequently giant PVS [11, 17]. After cyclosporin A (CyA) treatment of the rats, the thymic medulla disappeared, and 2 weeks after CyA treatment, the recovery of the medulla took place, but the "holes" were epithelial cell-free [5]. The occurrence and size of the KNA/EFA may be varied from species to species [2] even among the strains of rats [3]. In the CM region of BB rat thymus, the EFA has been reported, but this KNA/EFA is not as complete as in man. In the thymic medulla, the frequency of the KNA/EFA alters by age: in young Wistar rats, the occurrence of the KNA/EFA is higher than in old animals [7]. These changes may be related to acute [5] and/or physiological thymic involution.

The thymic stromal elements develop from the endodermal epithelium and neural crest mesenchyme. Hematopoietic cells colonize the epithelial-mesenchymal anlage. In chicken embryo the KNA/EFA appears when the medulla and cortex differentiate; therefore the epithelial-mesenchymal transition [6] would create a "second" mesenchyme, besides the mesenchyme of cranial neural crest origin. Therefore, in the thymus the epithelial-mesenchymal transition may be redundant.

It was difficult to identify large epithelium-free areas by transmission electron microscopy, and Foxn-1 thymic epithelial cell-specific transcription factor showed
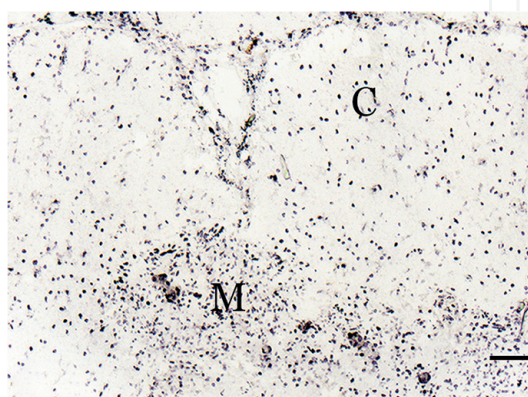

Figure 8.
Thymus 5. Age 16 months: Foxn-1 antibody recognizes epithelial nuclei over the entire thymus. Density of Foxn-1-positive cells is higher in the medulla (M) than in the cortex (C). The Foxn-1-positive knots are possibly Hassall's bodies.

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positive cells in the KNA/EFA (**Figure 8**). Foxn-1 expression in the medullary KNA/EFA is a puzzle. In early embryogenesis, Foxn-1 expresses in several mesenchymal and epithelial cells [27]; therefore one of the possibilities for solving the puzzle is that Foxn-1 is maintained in the KNA/EFA after thymus development. The other possibility is that the thymic epithelial cells induce Foxn-1 expression in mesenchymal cells of cranial neural crest origin [28]. Removal of perithymic mesenchyme at ED12 or culture of purified ED14 epithelial cells alone resulted in a threefold reduction in the bromodeoxyuridine incorporation by keratin-positive cells. Proliferation of thymic epithelial cells in the early thymus is regulated by signals from mesenchyme [29]. These mesenchymal cells produce fibroblast growth factors 7 and 10, which stimulate epithelial cell proliferation [20, 30, 31], but differentiation requires Foxn-1 [32]. The KNA/EFA [2, 9, 11] consists of mesenchyme; therefore the term EFA seems to be more appropriate than the KNA that we used [2].

4. Keratin-positive network (KPN)

The human thymic cortex shows a fine, dense keratin network that sharply differs from that of the medulla. The medulla has a loose epithelial lattice, with small, irregular-shaped EFA. A ring-shaped, anti-cytokeratin-negative "gap" of the EFA is found between the cortex and medulla (**Figures 9** and **10**) that seems to be a unique feature for the human thymus. The chicken's thymic medullary epithelial cells form a 3D network (**Figure 11**). In some places the ring-shaped "cortico-medullary gap" has small "outpocketing" toward the medulla that shows the connection of medullary EFA with the cortico-medullary gap (CMG). The medullary and cortical epithelial cells are connected, through the CMG, with epithelial bridges of medullary-type epithelial cells. In several places the CMG is not covered by the
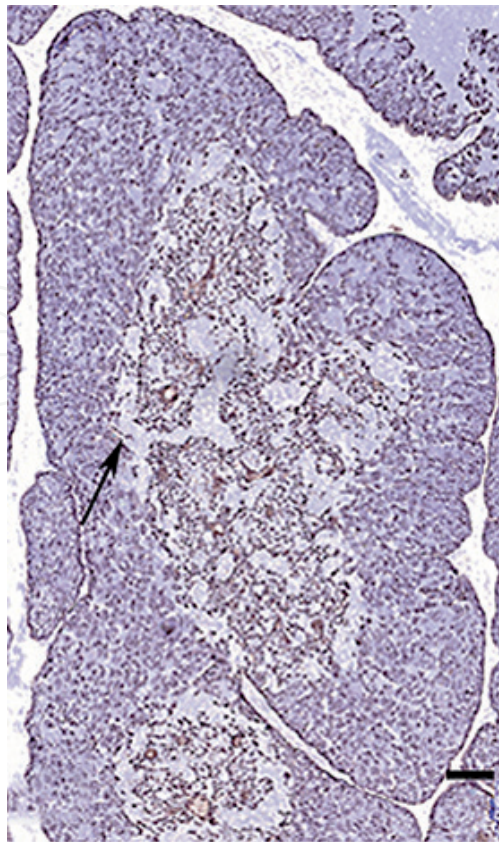

Figure 9.
Thymus 1. Age 5 months. Cortical EFA is significant compared with that of the medulla. Medullary EFA is connected with the CMG (arrow).
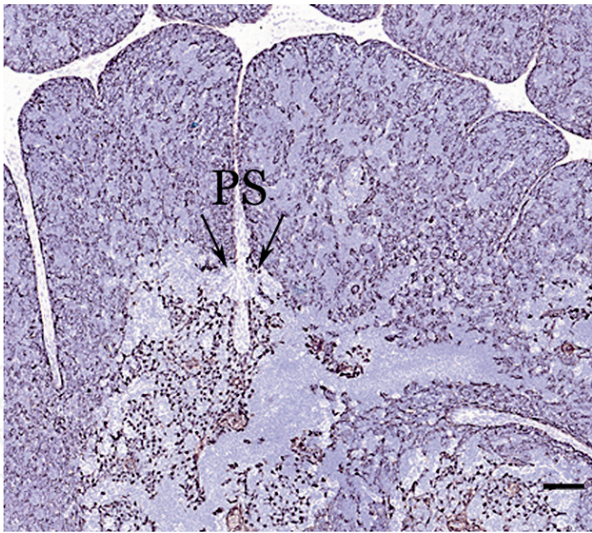
Figure 10.
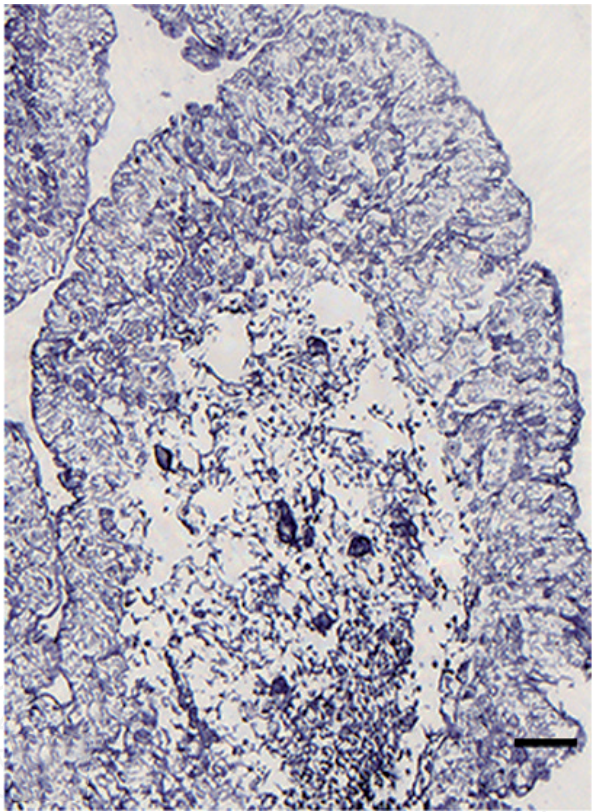*Thymus 1. Age 5 months. The PS continues with the medullary EFA (arrow).*

Figure 11.
Thymus 5. Age 16 months: at several places, the medullary epithelial cells, through the CMG, connect the cortex and medulla. Keratin accumulations in the medullary epithelial lattice represent Hassall's bodies.

cortex; therefore the CMG is in contact with the end of the PS. The PS reaches the CM border, widens, and become part of the medulla. Therefore, the human thymic medulla also consists of two sharply separated compartments: a keratin-positive network or lattice and an epithelium-free area. Inside the keratin-positive medullary area, few Hassall's bodies could be seen as aggregated keratin expression (**Figures 9** and **10**).

In the early embryonic life of chicken, the thymic anlage appears as a primary epithelial cord, which starts to develop from the third branchial pouch of the foregut endoderm. The epithelial cord ramifies, and this ramification area develops to medullary region of the thymus [2]. Between the offshooted secondary cords,
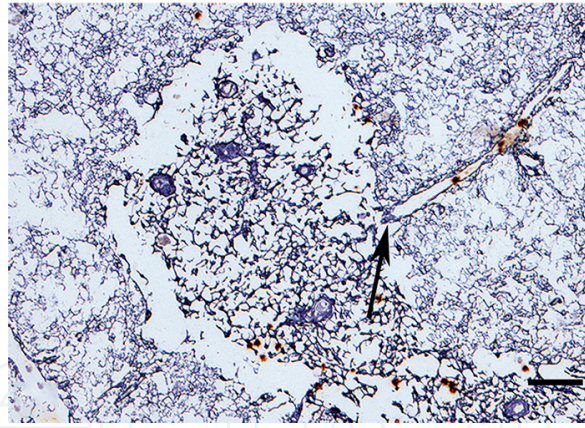
Figure 12.
Thymus 2. Age 18 months: the PS ends at the CMG (arrow). In the medulla, Hassall's bodies appear as solid, keratin-positive knots. Small keratin negative areas are also present in the cortex. The cortical surface of the gap is sharp.

the mesenchyme forms the PS and capsule (**Figure 2**). The cells at the end of the secondary cords rapidly proliferate, and by day 13, the thymic tissue is histologically recognizable (**Figure 3**). In the KPN of the medulla, several epithelial cells make large surface contact, excluding lymphocytes, from Hassall's corpuscle. It is surprising that few cells of Hassall's bodies show surfactant protein B (SPB) immunoreactivity (**Figure 12**). In the medulla, scattered SPB-positive cells also occur, which like type II pneumocytes might be developed from the foregut epithelium, that is, respiratory diverticulum.

During the last century, the origin of the thymic epithelial anlage created a hot debate: namely, the epithelial rudiment develops either from the epithelium of the endodermal pouch and ectodermal cleft or solely from the endodermal pouch. At the beginning of this century, the debate seemed to be settled: in chicken-quail chimeric experiments [26] and in mouse, transplantation of the third branchial pouch epithelium under the kidney capsule [33] proved that the pouch epithelium developed to functional thymus. Furthermore, mouse chimeras with different haplotypes of class II MHC proved that only one haplotype contributed to thymic epithelial anlage [34]. However, in human thymus the presence of a sharp CMG, among the cortex and medulla, raises again the possibility of double-germ layer origin of thymic epithelial rudiment. Bargman [35], Norris [36], and von Gaudecker [9] studied the development of human thymus and came to the conclusion that the corresponding ectodermal cleft epithelium attaches and unites with the descending third pouch epithelium (**Figure 13**). Cordier and Hamount [37] compared the thymus development of NMRI and nude mice and studied that either the lack of ectodermal cleft epithelium, which surrounded the endodermal rudiment, or the absence of a secreted substance from cleft epithelium [20] resulted in the dysgenesis of nude mouse thymus. In human thymus the major EFA is represented by the "gap" (**Figures 9** and **10**).

The debate is going on, but the subject changed over the thymic epithelial stem cell, which may be also connected to the endo- and ectodermal origin. Namely, one epithelial stem cell develops to cortical and medullary progenitors (single-germ layer origin), or there are, *sui generis*, cortical and medullary epithelial stem cells (ectodermal cleft and endodermal pouch will give raise to cortical and medullary progenitors, respectively). Between cortical and medullary microenvironment, there are many differences that also may be related to the ectodermal and endodermal origin of thymic epithelial rudiment.

The double-germ layer origin of human thymic epithelial cells is supported only by reliable histological studies and functional differences: (1) cortical epithelial
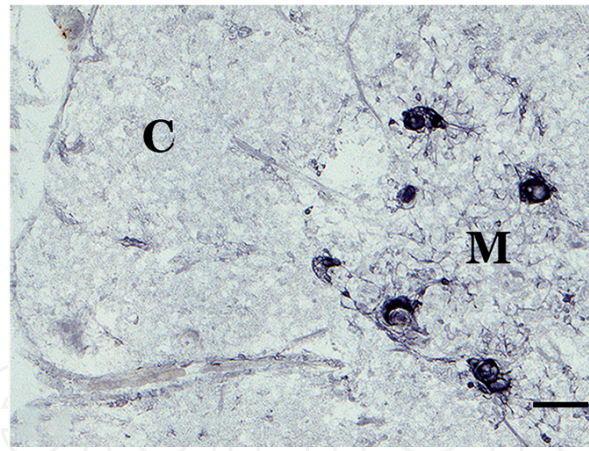
Figure 13.
Thymus 2. Age 18 months: anti-surfactant protein B (SPB) immunostaining recognizes Hassall's bodies and scattered positive cells in the medulla (M). The cortex (C) is free of SPB.

cells contribute to T-cell maturation, and the medullary ones participate in T-cell selection. (2) CMG is present in man, but not in birds and mammals studied up to now. (3) Hassall's bodies and SPB-producing cells are found only in the medulla. (4) Thymus-blood barrier exists only in the cortex [16]. (5) In the rat and mouse thymus, the cortical epithelial cells are keratin $K5^-$ $K8^+$ $CD205^-$, and the medullary epithelial cells are $K5^+$ $K8^{null}$ $CD205^-$ (in chicken the cortical thymic epithelial cells are $CD205^+$ and the medullary thymic epithelial cells are $CD205^-$). (6) As mentioned above thymic dysgenesis in nude mice is related with the absence of the cleft epithelium and/or cleft-derived biological active substance, which induces branchial pouch epithelial cell proliferation [31]. In mouse and chicken, the experimental data proved that the thymic epithelial anlage develops solely from the third branchial pouch [31, 33, 38–40]. The differences in the origin of epithelial anlage between man and mouse may be traced back to evolution. In marsupials there is cervical thymus, which is purely of ectodermal origin, while the cervicothoracic thymuses have mixed ecto-endodermal [37]. Evolutionary differences in organ development between man and mouse also occur: in man the allantois has rest of urachus beyond umbilicus, while in mouse the urachus is a small rudiment, and the allantois consists of pure mesenchyme [28, 41]. The relationship between thymic epithelial cells and skin keratinocytes has been supported by serological and immunofluorescence studies in normal [29, 42] and pathological conditions [3]. These investigations provide solid evidence for cross-reactive antigens among some thymic epithelial cells, cells of Hassall's corpuscles, and some subpopulation of skin keratinocytes [4].

Gupta et al. [43] studied the cytokeratin (CK5 and CK8) expression in human embryos. Before 16 weeks of gestation, the two cytokeratins are homogenously expressed in both the cortex and medulla, but after 16 weeks of gestation, the cortex and medulla show $CK8^+$ and $CK5^+$ staining, respectively. Double-positive ($CK5^+$ and $CK8^+$) epithelial progenitor cells were present only in the cortex at all gestational stages. This finding indirectly suggests that the cortex is the source of the epithelial progenitor cells. Norris [36] was able to show that the branchial cleft epithelium (cervical sinus) rapidly proliferates and surrounds the endodermal thymic rudiment. Thus, the presence of double-positive progenitor cells in the cortex and the rapid proliferation of cleft epithelium support the contribution of ectodermal component to human thymic epithelial anlage.

Hassall's bodies built up from epithelial cells. The centrally locating cells of Hassall's bodies gradually keratinized, like the epidermal cells of the skin. Neutrophil granulocytes and macrophages enter the corpuscle and digest the keratinized cells [44]. Norris [36] studied the human fetal thymuses and described migration of ectodermal cells

into the medulla. This finding may be confirmed by monoclonal antibodies (mAb(s)) (RCK 105 and RGE5) which recognize cortical epithelial cells and some medullary ones [7]. These experiments may show that cortical epithelial cells enter the medulla. Furthermore, MTS29 mAb stains isolated in medullary epithelial cells. The antigen was also present in the epidermal epithelium [4]. The marginal cells of the corpuscle are alive and perhaps temporarily capable of producing SBP (**Figure 12**) and/or other biological active substances. If we adopt the double-germ layer origin of thymic epithelial cells, then both type II pneumocytes (SPB-producing cells) and the cortical stellate cells and cells of Hassall's body are in "foreign environment" of the medulla. The surface of the cell provides important information for the neighboring cell to form tissue and organs. According to the law of thermodynamic stability, if *in vitro* two types of cells are mixed and the bond among different cells is weaker than among homotypic cells, then the cells are sorting out and the homotypic cells aggregate [45]. Possibly, this is the situation *in vivo*, in case of Hassall's body formation. Several cortical cells enter the medulla and sort out, aggregating in the form of Hassall's body. The SPB-producing type II pneumocytes have got a similar situation as cortical epithelial cells; therefore the SPB-producing cells also sorting out "join" to the Hassall's bodies, resulting in SPB-positive Hassall's corpuscles [46].

5. Foxn-1 expression

Thymic epithelial cell-specific transcription factor, Foxn-1, shows scattered positive cells in both the cortex and medulla and line up along the thymic capsule and PS (**Figures 8** and **14**). The density of Foxn-1-positive cells seems to be higher in the medulla than in the cortex. Double staining with anti-cytokeratin and anti-Foxn-1 antibodies shows that Foxn-1 is expressed in both medullary compartments; namely, Foxn-1 positive cells are present in the EFA (**Figure 15**).

Acute thymic atrophy can be induced by Foxn-1 disruption [29]. Foxn-1 is necessary for the differentiation of both cortical and medullary epithelial cells [27, 47–49]. By age the number of Foxn-1-expressing epithelial cells seems to decrease [49], and this change may be paralleled with the diminished occurrence of the EFA. In elderly people, the risk of autoimmune disease is increased that may be in connection with the accumulation of Foxn-1-negative epithelial cells [49, 50] or the increased number of Foxn-1-expressing mesenchymal cells and decreased volume of EFA. However, in addition to the increasing number of Foxn-1-negative
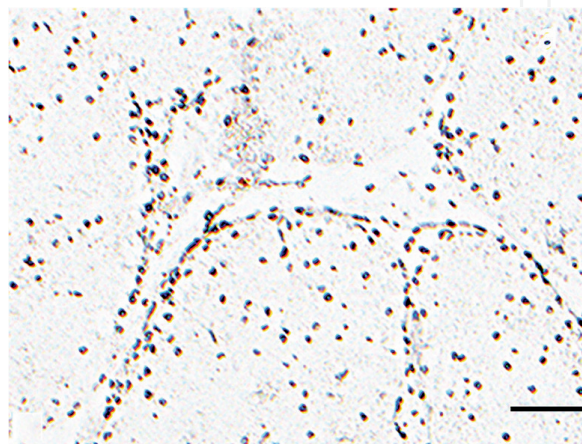

Figure 14.
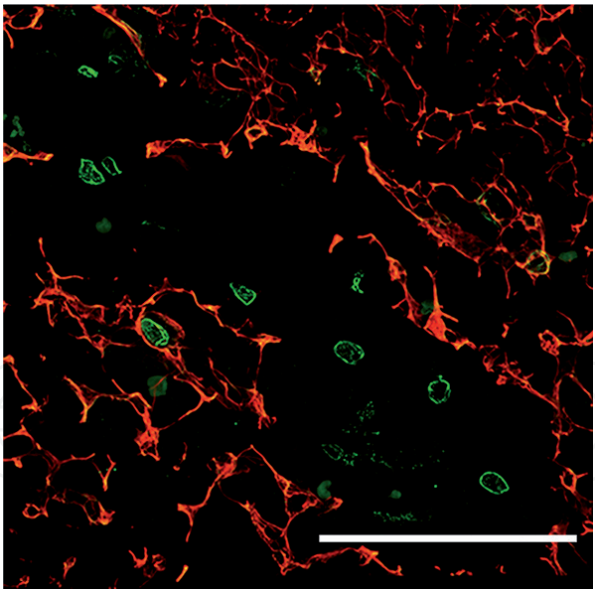*Thymus 5. Age 16 months: Foxn-1-positive cells outline the thymic lobuli.*

Figure 15.
Double staining: cytokeratin (red) and Foxn-1 (green). Foxn-1-positive cells are present in both the KPN and EFA of the thymic medulla.

epithelial cells, Foxn-1 is expressed in the KNA, that is, in non-epithelial cells with unknown consequences (**Figure 15**).

6. Conclusions

In the epithelium-free areas, several vessel-associated cells like pericyte and smooth-even-striated muscle cells develop from neural crest cells. It is reasonable to assume that the reticular tissue of epithelium-free area also develops from neural crest cells. In addition to this hypothesis, it is remained also unsolved if the mesenchymal cells or abnormal (keratin-free) epithelial cells express Foxn-1 transcription factor. In mouse and chicken, where the thymus develops solely from the endodermal pouch epithelium, the cortical cells enter the medulla, sort out, and
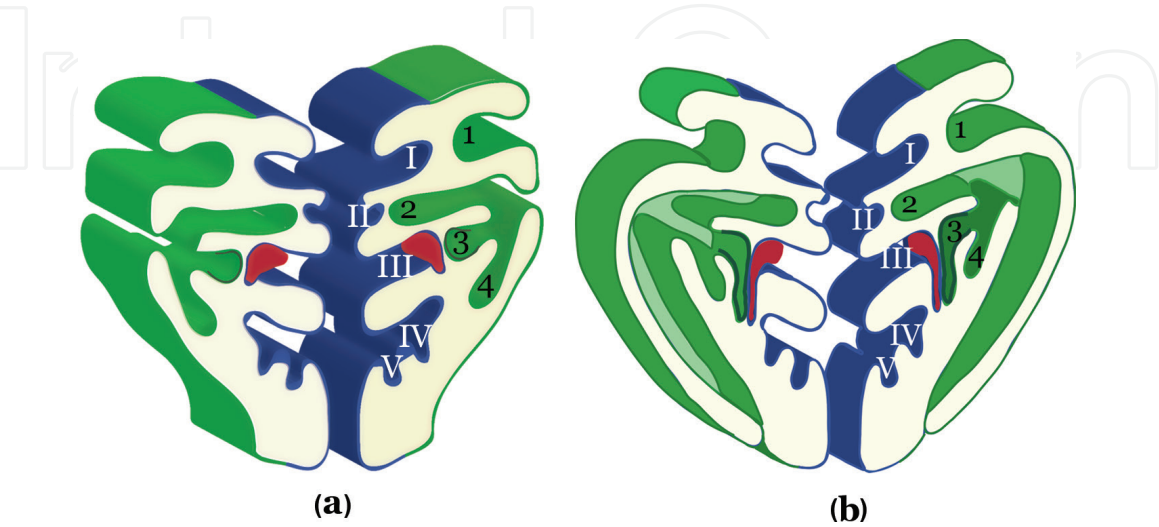

(a)   (b)

Figure 16.
Scheme: (a) from the ectodermal cervical sinus, the cervical vesicle (dark green) separates and attaches to the corresponding third branchial pouch (red), which descends into the upper mediastinum (b). (I–V, pharyngeal pouches; 1–4, pharyngeal groves). Ventral region of the third pharyngeal pouch (red) gives the endodermal part of the thymus. Part of the cervical vesicle (ectoderm, green) contributes to the thymic anlage.

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form Hassall's bodies. In human thymus Hassall's corpuscles are large (compound structures), while in mouse and chicken, Hassall's bodies are small. The differences in Hassall's bodies may be related with the double- and/or single-germ layer origin of the thymic epithelial anlage (**Figure 16**).

Conflict of interest

The authors declare no conflict of interest and confirm that all these figures are original.

Abbreviations

CyA	cyclosporin A
CK	cytokeratin
CM	cortico-medullary
CMG	cortico-medullary gap
EFA	epithelium-free area
Foxn-1	Forkhead box N1
IDC	interdigitating cell
KNA	keratin negative area
KPN	keratin positive network
MHC	major histocompatibility complex
mAb	monoclonal antibody
PS	primary septum
PVS	perivascular space
SPB	surfactant protein B

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