

# A novel aspect of the structure of the avian thymic medulla

Ildikó Bódi · Krisztina Minkó · Dávid Molnár ·  
Nándor Nagy · Imre Oláh

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**Abstract** We provide evidence for the compartmentalization of the avian thymic medulla and identify the avian thymic dendritic cell. The thymic anlage develops from an epithelial cord of the branchial endoderm. Branches of the cord are separated by primary septae of neural crest origin. The dilation of the primary septae produces the keratin-negative area (KNA) of the thymic medulla and fills the gaps of the keratin-positive network (KPN). Morphometric analysis indicates that the KNA takes up about half of the volume of the thymic medulla, which has reticular connective tissue, like peripheral lymphoid organs. The KNA receives blood vessels and in addition to pericytes, the myoid cells of striated muscle structure occupy this area. The myoid cells are of branchial arch or prechordal plate origin providing indirect evidence for the neural crest origin of the KNA. The marginal epithelial cells of the KPN co-express keratin and vimentin intermediate filaments, which indicate their functional peculiarity. The basal lamina of the primary septum is discontinuous on the surface of the KPN providing histological evidence for the loss of the blood-thymus barrier in the medulla. In the center of the KNA, the dendritic cells lie in close association with blood vessels, whereas the B-cells accumulate along the KPN. The organization of the KPN and KNA increases the “surface” of the so-called cortico-medullary border, thereby contributing to the efficacy of central tolerance.

**Keywords** Thymic medulla · Keratin-negative area · Keratin-positive network · Cortico-medullary border · Thymic dendritic cell · Chicken

## Introduction

The hematoxylin-eosin (HE)-stained thymus is divided into a cortex of densely packed lymphocytes and a medulla with far fewer lymphocytes but with more epithelial cells having a bulky cytoplasm. The three-dimensional thymic epithelial reticulum develops from the third and fourth branchial pouch endoderm (Farley et al. 2013; Gill et al. 2003; Gordon and Manley 2011; Le Douarin 1967; Le Douarin et al. 1984; Neves et al. 2012; Rodewald 2008; Venzke 1952) and differentiates into cortical and medullary epithelial cells. Le Douarin (1967) isolated the branchial pouch endoderm, before fusion with the ectoderm of the branchial groove and co-cultured it with the mesenchyme; this resulted in the formation of functional thymic tissue providing evidence that (1) branchial endoderm without ectoderm was capable of thymic organogenesis and (2) thymic epithelial cell (TEC) differentiation required mesenchymal cell cooperation. The mesenchymal capsule surrounding the thymic rudiment, interlobular septae and the pericytes of the medulla develops from the cranial neural crest (Bockman and Kirby. 1984; Foster et al. 2008; Jenkinson et al. 2003; 2007; Le Lièvre and Le Douarin 1975; Müller et al. 2008; Noden 1978, 1983). Lack of a distinct mesenchymal cell population, which expresses platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ) results in diminished epithelial cell proliferation and subsequent hypoplastic thymus development (Jenkinson et al. 2007). Experiments of the last decade have confirmed not only the mesenchymal induction of TEC (Jenkinson et al. 2007; Itoi et al. 2007; Neves et al. 2012) but also that the epithelial-mesenchymal cross-talk is extended to epithelial–T-cell interaction (Klug et al. 2002; Rossi et al.

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I. Bódi · K. Minkó · D. Molnár · N. Nagy · I. Oláh (✉)  
Department of Human Morphology and Developmental Biology,  
Faculty of Medicine, Semmelweis University, Tüzoltó Str. 58,  
Budapest 1094, Hungary  
e-mail: olah.imre@med.semmelweis-univ.hu

2007). A common bipotent progenitor of TEC exists not only in the early embryo but also persists in the postnatal and possibly in adult thymus (Bennett et al. 2002; Bleul et al. 2006; Farr et al. 2002; Rossi et al. 2007).

Immature thymocytes colonize the subcapsular region of the thymus and during maturation, the single-positive CD4<sup>+</sup> and CD8<sup>+</sup> cells enter the medulla. Thus, the cortical TEC is responsible for T-cell receptor development, the positive selection of double-positive cells and the differentiation of the single-positive thymocytes, whereas medullary TEC and dendritic cells (DCs) at the cortico-medullary border regulate and/or execute negative selection by deleting the autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and subsequently generate central tolerance to self-antigen. The various functions of the cortical and medullary TECs possibly require different cytological phenotypes. At the 12th embryonic day, the putative progenitor marker, Mts24, is present in almost every epithelial cell (Rossi et al. 2007) and therefore, either the majority of the TECs can be considered to be bipotent progenitors or the Mts24 cannot be regarded as a specific progenitor marker. The latter statement seems to be true, because both Mts24-positive and -negative cells are capable of producing functional thymic tissue that supports T-cell development (Rossi et al. 2007). Klug et al. (1998) detected two distinct TEC populations. The major one expresses keratin (K) 8, whereas the minor subpopulation localizing at the cortico-medullary border produces both K8 and K5 intermediate filaments. The presence of the two intermediate filaments in TECs suggests two different functions and stages of differentiation. The relationship of the putative TEC progenitor marker, Mts24, to the keratin expression of cortical and cortico-medullary TEC in mammals remains unknown.

Previously, a keratin-negative area (KNA) has been known to occur in between the medullary TEC in mice (Van Ewijk et al. 1980) and in chicken (Boyd et al. 1992; Guillemot et al. 1984; Minkó and Oláh 1996). Although the cross-talk between the medullary TEC and mesenchyme has become generally accepted, the origin and role of the KNA of the thymic medulla remains unknown. The presence of KNA in the thymic medulla raises several questions that might be relevant for thymic function. (1) If the KNA is merely a dilation of the primary septum (PS) of neural crest origin, does it belong to the thymic medulla? (2) If so, is the medulla divided into a keratin-positive network (KPN) occupied by TEC and KNA, which develops from the neural crest cells, like the capsule and the septum themselves? (3) If the KNA is a part of the medulla, how can we determine the cortico-medullary border? (4) What is the function of the KPN and KNA? (5) Where are the DCs in the KPN and/or KNA? These questions are primarily related to anatomical or topographical problems but the lack of accurate morphological knowledge hinders the correct interpretation of the immunological function of the thymus.

The renaissance of thymic stromal cell research and our former observations (Minkó and Oláh 1996) have initiated the renewal of studying the thymic medulla. Our study indicates that, in the chicken, the volume of the KNA is close to 50 % of the medulla, which consists of connective tissue and produces extracellular matrix (ECM), unlike the KPN and cortex. The KNA of the medulla is much larger than the perivascular space, which is continuous with the PS. Hemopoietic cells of the KNA show a highly organized pattern that might contribute to the efficacy of negative selection.

## Materials and methods

### Animals

White Leghorn (*Gallus gallus*) eggs and chickens were obtained from a commercial breeder. Eggs were incubated at 38 °C in a humidified incubator. Embryos were staged according to the number of days of incubation. Tissue samples were taken from five chicken embryos at each of the sampling days at 11, 13, 15, 16, 17 and 20 days and from five 6-week-old adult chickens. Thymuses from 8-week-old rabbit and rat were used to compare the keratin staining pattern in the medulla of the bird with that of mammals. The experimental design and the conditions of the animals were approved by the Animal Ethical Committee of Semmelweis University (Budapest, Hungary).

### Silver impregnation

Silver impregnation was performed to demonstrate the reticular fibers. The thymic lobes from 6-week-old chickens were fixed in 4 % buffered paraformaldehyde, overnight. After being washed in phosphate-buffered saline (PBS, pH 7.4), tissue samples were embedded in paraffin and sectioned; the 8- $\mu$ m-thick sections were deparaffinized. Silver impregnation was carried out according to Gomori (Krutsay 1980).

### Immunohistochemistry and immunofluorescence

All the immunohistochemistry and immunofluorescence patterns presented here were obtained from at least five individual animals. For immunostaining, tissue samples were taken and completely covered by liver, from the same bird and the composite blocks were frozen in liquid nitrogen. Cryostat sections (10  $\mu$ m thick) were mounted onto poly-L-lysine (Sigma Aldrich)-coated slides and fixed in cold acetone for 10 min. The sections were rehydrated in PBS and the endogenous peroxidase activity was quenched with 3 % hydrogen peroxide (Sigma-Aldrich) for 10 min or developed with 3,3-diaminobenzidine (DAB, Sigma-Aldrich). Sections were washed in PBS and incubated with the primary antibodies

(Table 1) for 45 min at room temperature. Incubation of primary antibodies was followed by biotinylated horse anti-mouse IgG (Vector Laboratories) or anti-rabbit IgG (Vector Laboratories) for 45 min. The signal was enhanced by using the avidin-biotinylated peroxidase complex (ABC, Vector Laboratories) for 30 min. Peroxidase activity was visualized with 4-chloro-1-naphthol (Sigma-Aldrich). The staining patterns of the various antibodies (anti-CD4, anti-CD8 and anti-cytokeratin) were compared on serial sections.

For double-immunofluorescent staining, sections were incubated with primary antibodies, followed by labeling with AlexaFluor594-conjugated goat anti-mouse IgG1 secondary antibodies (Invitrogen). The ZenonFluor488 IgG1 antibody labeling kit (Invitrogen) was used to tag the anti-keratin antibody. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Sections were examined with fluorescence and confocal laser scanning microscopes (Zeiss Axiophot, Bio-Rad Radiance 2100 Rainbow, respectively). Digital images were captured with an AxioCam HRC camera and compiled by using Adobe Photoshop. Line drawings were made with Adobe Illustrator (Microsoft Office).

#### Morphometric measurement

The volumetric ratio of the thymic cortex and medulla and the ratio of the KPN and KNA of the medulla were studied by using the Cavalieri method and NeuroLucida software (MBF Biosciences). The Cavalieri method is a stereological probe for quantifying regional volumes (Tschanz et al. 2014). The volume of the thymus was estimated by summing the areas and multiplying the result by the slice thickness. Cryostat sections (12  $\mu\text{m}$  thick) from the thymic lobes of a 3- and a

6-week-old chicken were taken and every fiftieth and hundredth section, respectively, was immunostained with anti-cytokeratin. Seven and eight sections were morphometrically evaluated in 6- and 3-week-old chickens, respectively, representing the entire thickness of a thymic lobe.

#### Electron microscopy

Briefly, the tissue samples were fixed in 4 % buffered glutaraldehyde solution overnight and post-fixed in 1 % osmium tetroxide (Polysciences) for 2 h. After dehydration in graded ethanol, the tissue blocks were embedded in Polybed/Araldite 6500 (Polysciences) mixture. For transmission electron microscopy, ultrathin sections were contrasted with uranyl acetate and lead citrate and studied with a Hitachi electron microscope, type H-7600.

#### Dexamethasone treatment

Ten 6-week-old chickens were treated with dexamethasone (DM; Ratiopharm Hungaria) to induce thymic involution and to check the alteration of the medullary KPN and KNA. DM at 5 mg/1000 g body weight was administered intraperitoneally for 10 consecutive days. Thymic lobes were removed 2 h after the last injection of DM.

## Results

An epithelial cord grows out and divides from the third and fourth branchial pouch endoderm. Branches of the cord are

**Table 1** Primary antibodies used

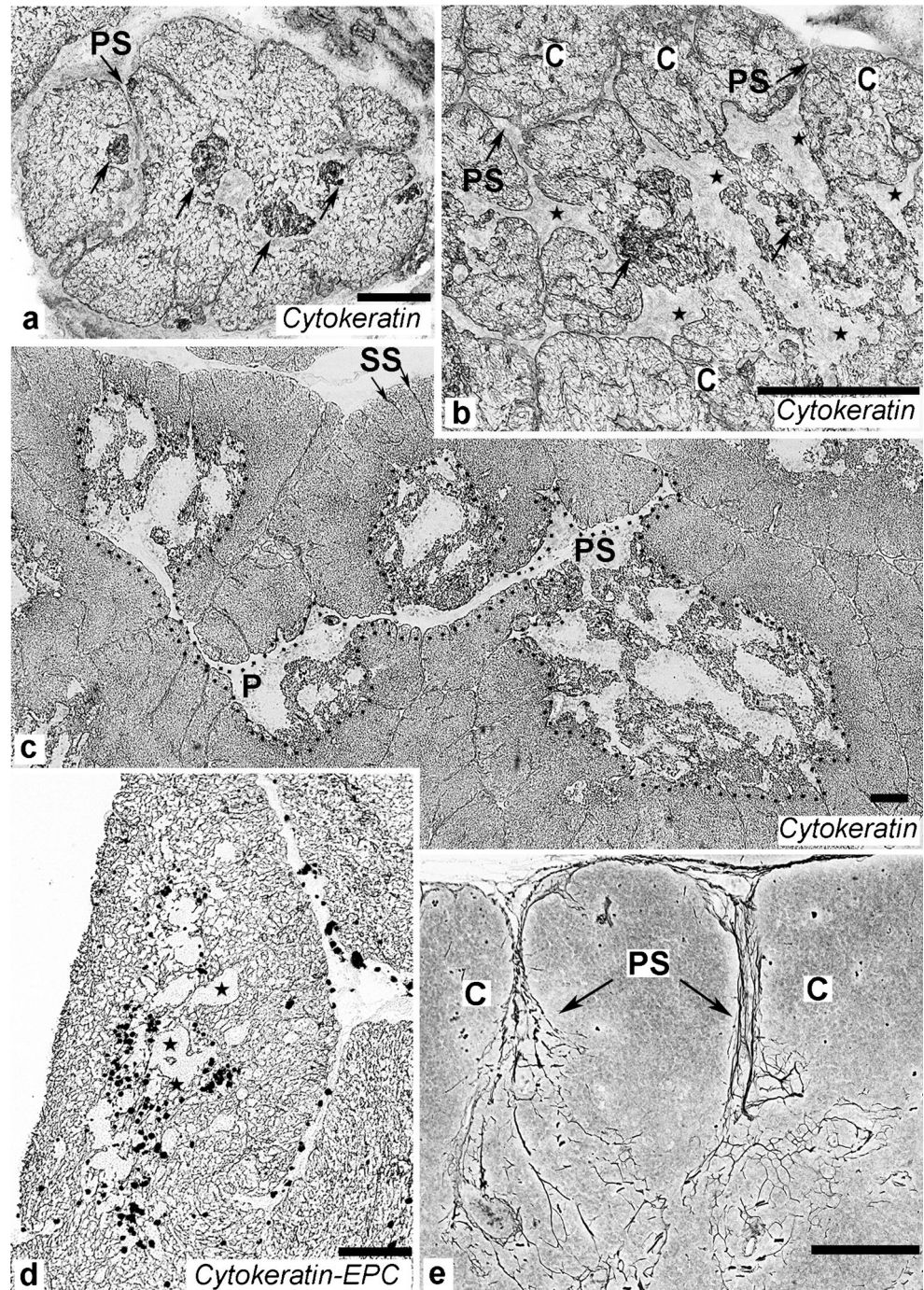
Antibody/clone	Host	Antibody specificity	Dilution	Source of antibody
Lu-5	Mouse IgG1	Pan-cytokeratin	1:100	BMA
Basal cell cytokeratin	Mouse IgG1	Cytokeratin5	1:200	Millipore
AMF-17b	Mouse IgG1	Vimentin	Supernatant	DSHB
3B4	Mouse IgG2a	Vimentin	1:200	Millipore
31	Mouse IgG1	Laminin	1:50	DSHB
3B2	Mouse IgG1	Collagen type III	1:1500	DSHB
B3/D6	Mouse IgG1	Fibronectin	1:5	DSHB
M1B4	Mouse IgG1	Tenascin	1:5	DSHB
D76	Mouse IgG1	Desmin	1:4	DSHB
CVI-ChNL-74.3	Mouse IgG1	Chicken dendritic cell marker	1:200	Prionics
BoA1	Mouse IgG1	chB6	Supernatant	Igyártó et al. (2008)
CT4	Mouse IgG1	Chicken CD4	1:200	Southern Biotech.
CT8	Mouse IgG1	Chicken CD8	1:200	Southern Biotech.
1A4	Mouse IgG2a	$\alpha$ -Smooth muscle actin	1:200	Dako
TAP1	Mouse IgG2a	Antigen-presenting cell	1:200	DSHB

separated from one another by PSs, which receive the thymic arteries and veins. The first histological sign of the formation of the thymic medulla appears on the 15th–16th days of incubation. Cytokeratin immunohistochemistry reveals slightly elevated keratin staining in close association with the PS, which serves as a stalk for the formation of KNA (Fig. 1a). These increased keratin staining spots indicate the anlage of the future KPN of the thymic medulla (Fig. 1a, b). Each branch of the epithelial cord ends in a club-shaped enlargement, which is the rudiment of the cortex (Fig. 1b). The

secondary septae (SSs) develop later in the club-shaped enlargement, they are narrower than the PSs, do not reach the medulla and may have only capillaries possibly originating from the cortex.

By embryonic day 20, a three-dimensional KPN develops from the elevated keratin-staining spots and the “dilation” of the PS forms the KNA. Thus, after hatching, the thymic medulla, recognized by HE staining, consists of the KPN connected to the cortical epithelial cells and of KNA multiply bound to the PSs (Fig. 1c).

**Fig. 1 a–d** Cytokeratin immunostaining. **a** Developing thymus of a 16-day-old chicken embryo. The areas of elevated cytokeratin staining (*arrows*) are in close association with the primary septae (PS). **b** Developing thymus of a 20-day-old chicken embryo. The epithelial branches and their club-shaped enlargements are separated by PS, which cannot be distinguished from the keratin-negative area (KNA; *stars*). The increased keratin staining (*arrows*) indicates the rudiment of the keratin-positive network (KPN; C cortex). **c** Developing thymus of a 6-week-old chicken. The PS is dilated (*dotted outline*) and continuous with the KNA of the thymic medulla. PS serves as a central stalk from which the four thymic medullary regions arise (*SS* secondary septae). **d** Thymus of an 8-week-old rabbit. KNA (*star*) and endogenous-peroxidase-positive cells (EPC; heavily stained deposits) are also present in mammals. **e** Thymus of a 6-week-old chicken, silver impregnation. The PSs at the cortico-medullary border spread and form reticular fibers in the KNA but not in the cortex (C). Bar 200  $\mu$ m



Morphometric analysis of thymuses of 3- and 6-week-old chickens revealed that the volumetric ratio of the cortex and medulla was approximately 3 to 2. In the 3-week-old chicken, the volume of the KPN and KNA was 57 % and 43 %, respectively, whereas these values in the 6-week-old chicken proved to be 53 % and 47 % (Fig. 2). In mammals, namely in rabbit and rat (data not shown), the KPN and the KNA also exist but the elevated keratin expression in the medulla cannot be seen (Fig. 1d).

Silver impregnation revealed reticular fibers in the PSs and KNA (Fig. 1e) but not in the cortex and KPN. The presence of type III collagen, which is the basic fibrillar motif of the reticular fiber, in the PS and KNA confirmed the result of silver impregnation (Fig. 3a). The existence of reticular fibers indicates that, in the PSs and KNA, reticular connective tissue is formed that produces other ECM molecules, like fibronectin (Fig. 3b) and tenascin (Fig. 3c). Reticular cells are modified fibroblasts, which are highly versatile cells, capable of ECM production, the isolation of reticular fibers from T-cells, B-cells and DCs, the phagocytosis of foreign substances and occasionally antigen presentation. Vimentin intermediate filament is produced by the fibroblasts and reticular cells. Anti-vimentin immunostaining shows the classic histological pattern of thymic cortex and medulla, without distinguishing the KPN and KNA (Fig. 3d). After hatching, an increasing number of B-cells enters the thymic medulla, which shows a similar staining pattern as the HE and anti-vimentin. The density of B-lymphocytes and vimentin-positive cells varies; in some areas, the cell density is high, whereas other areas are almost devoid of cells (Fig. 3d, e).

The surface epithelium of the thymic lobe facing towards the capsule and the septae is covered by basal lamina indicated by the presence of laminin (Fig. 3f). At the appearance of the KPN, the basal lamina becomes discontinuous (Fig. 3f, g). Thus, the KNA carrying the arteries and veins is separated by a discontinuous basal lamina from the KPN (Fig. 3f, g). The marginal epithelial cells of the KPN, in addition to keratin, express vimentin intermediate filament (see below). Cells of Hassall's bodies (not shown) do not express vimentin (Minkó and Oláh 1996), like the inner cells of the KPN.

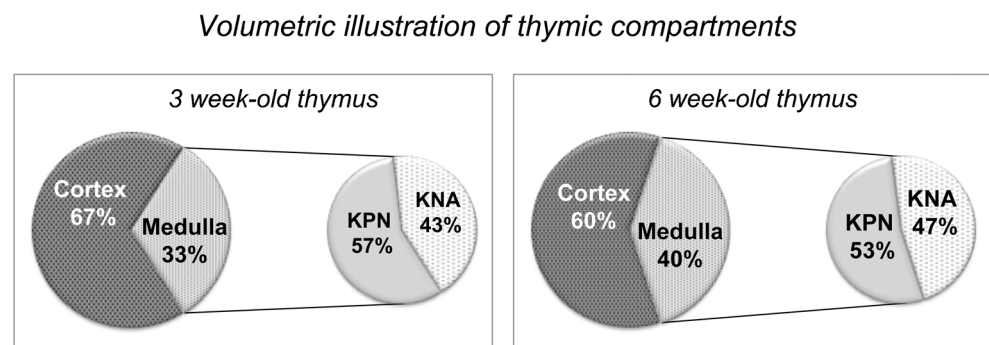
Administration of high-dose DM induces acute involution, which results in T-cell depletion and subsequently abrogation of the keratin pattern of the cortex (Fig. 3h). The entire lobe shows a medullary appearance and also elevated production of fibronectin (Fig. 3i) and tenascin (Fig. 3j).

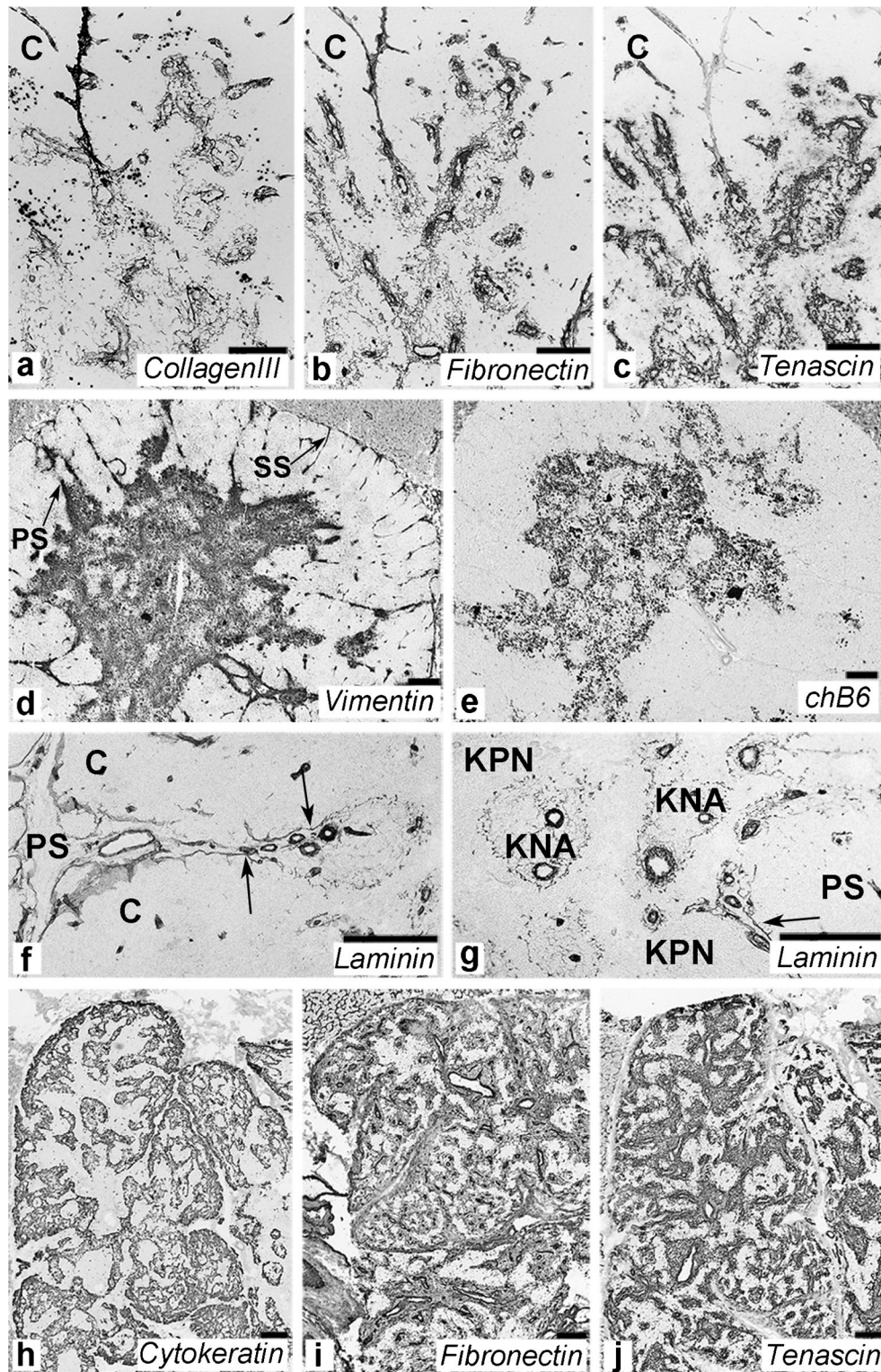
A unique cell type of the KNA is the myoid cell, which occurs with a round and/or elongated shape (Fig. 4a) and expresses desmin intermediate filament (Fig. 4b). Anti-desmin monoclonal antibody (mAb) is a convenient tool for the identification of myoid cells, which are single cells, like the smooth muscle cells. Myosin and actin filaments are occasionally organized in sarcomeres as in skeletal muscle, showing alternating light I and dark A bands (Fig. 4c).

The epithelial cyst is also a common structure of the KPN. The cyst is generally lined by microvilli that are highly regular, as in the small intestine and the cells secrete a mucus substance (Figs. 4d). The presence of microvilli, a terminal web, a cell junction at the lateral cell membrane (Fig. 4e) and the mucus substance inside the cyst indicate that the cyst-forming epithelial cells maintain their polarity, unlike the reticular epithelial cells.

We used chicken follicular dendritic cell (FDC)-specific mAb 74.3 and transmission electron microscopy for the *in vivo* search for thymic DCs. In the KNA of the thymic medulla, mAb 74.3 immunocytochemistry revealed grouped or confluent positive reaction and scattered individual cells (Fig. 5a). Endogenous-peroxidase-positive cells (EPC) also form groups in the medulla (Olah et al. 1991). However, EPC histochemistry and 74.3 mAb immunocytochemistry clearly indicated that the EPCs and 74.3-positive cells represented two different cell populations (Fig. 5a, inset). The EPCs occurred in both the KPN and KNA, unlike the 74.3-positive DCs, which were localized in the center of the KNA (Fig. 5b) in close association with the blood vessels (Fig. 5c) indicated by smooth muscle actin (SMA) immunostaining. These 74.3-positive DCs expressed MHC II antigen (Fig. 5d, inset). The distribution of chB6-positive B-lymphocytes and 74.3-positive DCs showed a topographical pattern, namely, DCs occupied the center of the KNA (Fig. 5b) and B-cells formed a dense layer close to the surface of the KPN

**Fig. 2** Volumetric illustration of thymic compartments

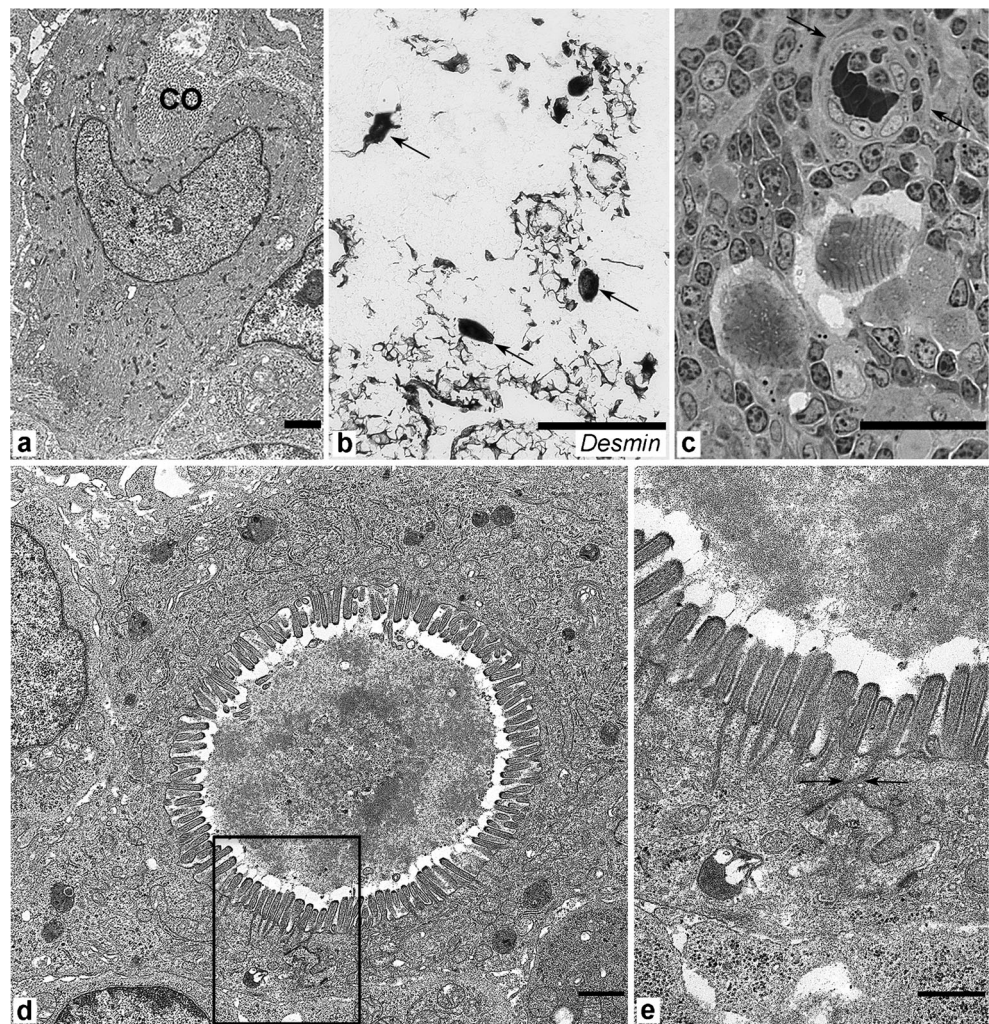




**Fig. 3** Chicken thymus at 6 weeks of age. **a** Collagen III is the basic fibrillar molecule of reticular fibers. **b** A fine fibronectin network is restricted to the KNA of the thymic medulla. **c** Tenascin is also located in the KNA. **d** Anti-vimentin monoclonal antibody recognizes the entire medulla but the density of vimentin-positive cells varies. Vimentin-positive cells outline both the primary septum (PS) and secondary septum (SS). **e** Anti-chB6 immunostaining. B-cells are located only in the medulla. The distribution of B-cell density is inhomogeneous. **f, g** Anti-laminin immunostaining. PS receives the thymic arteries and veins. The

surface of the cortex (C) is covered by a basal lamina that becomes discontinuous at the cortico-medullary border (arrows). **g** KNA with the blood vessels are outlined by the discontinuous basal lamina of the KPN (PS primary septum). **h–j** Dexamethasone-treated chickens. **h** Anti-cytokeratin immunostaining. **i** Anti-fibronectin immunostaining. **j** Anti-tenascin immunostaining. The cortical staining pattern completely disappears and the entire thymic lobe resembles the medulla. Both extracellular matrix (ECM) molecules show an increase in the KNA. Bar 200  $\mu$ m

**Fig. 4** **a** Transmission electron micrograph of a myoid cell embracing a bunch of collagen fibers (*CO*) in the KNA. **b** Myoid cells (*arrows*) are heavily stained by anti-desmin. **c** Semithin section stained by toluidin blue. Myosin and actin filaments are organized as in skeletal muscles. Migration of lymphocytes through the postcapillary (*arrow*). **d** Epithelial cells of a cyst are highly polarized. Apical surfaces of the cyst-forming epithelial cells are covered by microvilli and the lumen contains a medium-dense substance. **e** Higher magnification of the boxed area shown in **d**. The apical part of the lateral cell membrane displays a cell junction (*arrows*). Bar 200  $\mu$ m (**b, c**), 1  $\mu$ m (**a, d, e**)



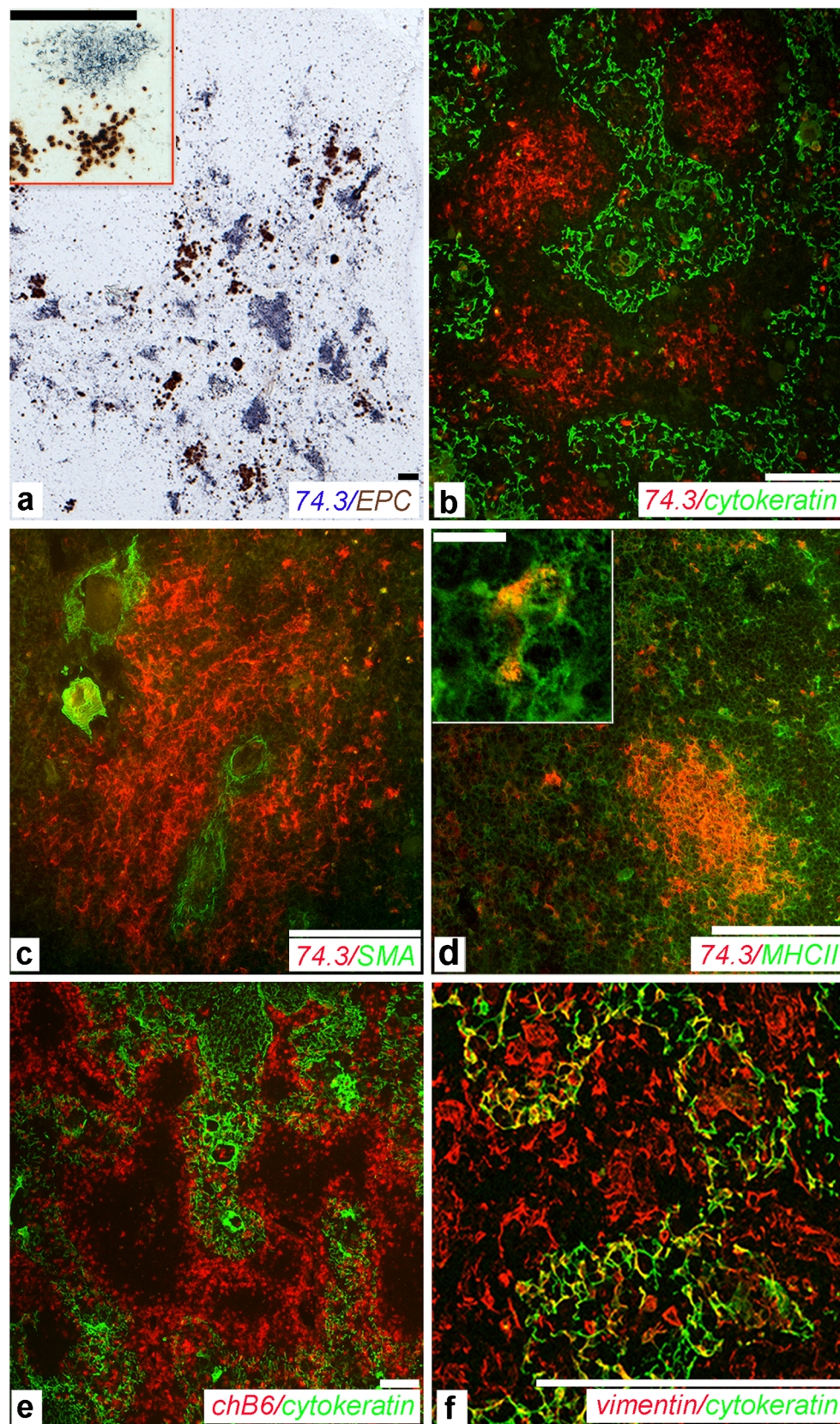
(Fig. 5e). The CD4 single-positive cells of the medulla were arranged mainly in the KNA (Fig. 6a, b), whereas the distribution of CD8 seemed to be the opposite (Fig. 6b, c).

Transmission electron microscopy of a thymic DC shows an excentrically located, irregularly shaped nucleus with nuclear-membrane-associated heterochromatin. The bulky cytoplasm produces a few short thick processes and highly electron dense, irregularly shaped and sized granules (Fig. 6d) that occasionally show a lamellated appearance (Fig. 6e).

## Discussion

The thymus is the only lymphoid organ that is lobulated and develops like a gland (Fig. 7a). In a cross-section, the epithelial cord and the club-shaped enlargements appear as islets embedded in mesenchyme (Rodewald 2008; Rodewald et al. 2001) of neural crest origin. These experimental observations confirm the early histological findings, which described the

thymic anlage as an epithelial island surrounded by mesenchyme (Maximov and Bloom 1938). The mesenchyme differentiates to PS separating the branches of the epithelial cord (Fig. 7a, b). One branch of the epithelial cord possibly develops from one epithelial progenitor cell. The club-shaped enlargements are the anlage of the future cortex, whereas the developing KNA seems to be dilations of the PSs. In this context, the PS can be regarded as a central stalk, from which the KNA of the medulla arises. Thus, the thymic medulla is divided for KPN and KNA. The close topographical connection between the PS and KNA suggests their common origin from cranial neural crest cells. In the KNA, the presence of myoid cells of skeletal muscle structure further supports the neural crest origin of KNA. Striated muscle normally can develop only from the myotomes of somites, the prechordal plate and cranial neural crest cells but not from the ectoderm and endoderm. The myogenic potency of the neural crest cells has been confirmed by several authors (Johnston et al. 1979; Nakano and Nakamura 1985; Yamashita and Sohal 1987). On the basis of experimental observations, the origin of the myoid

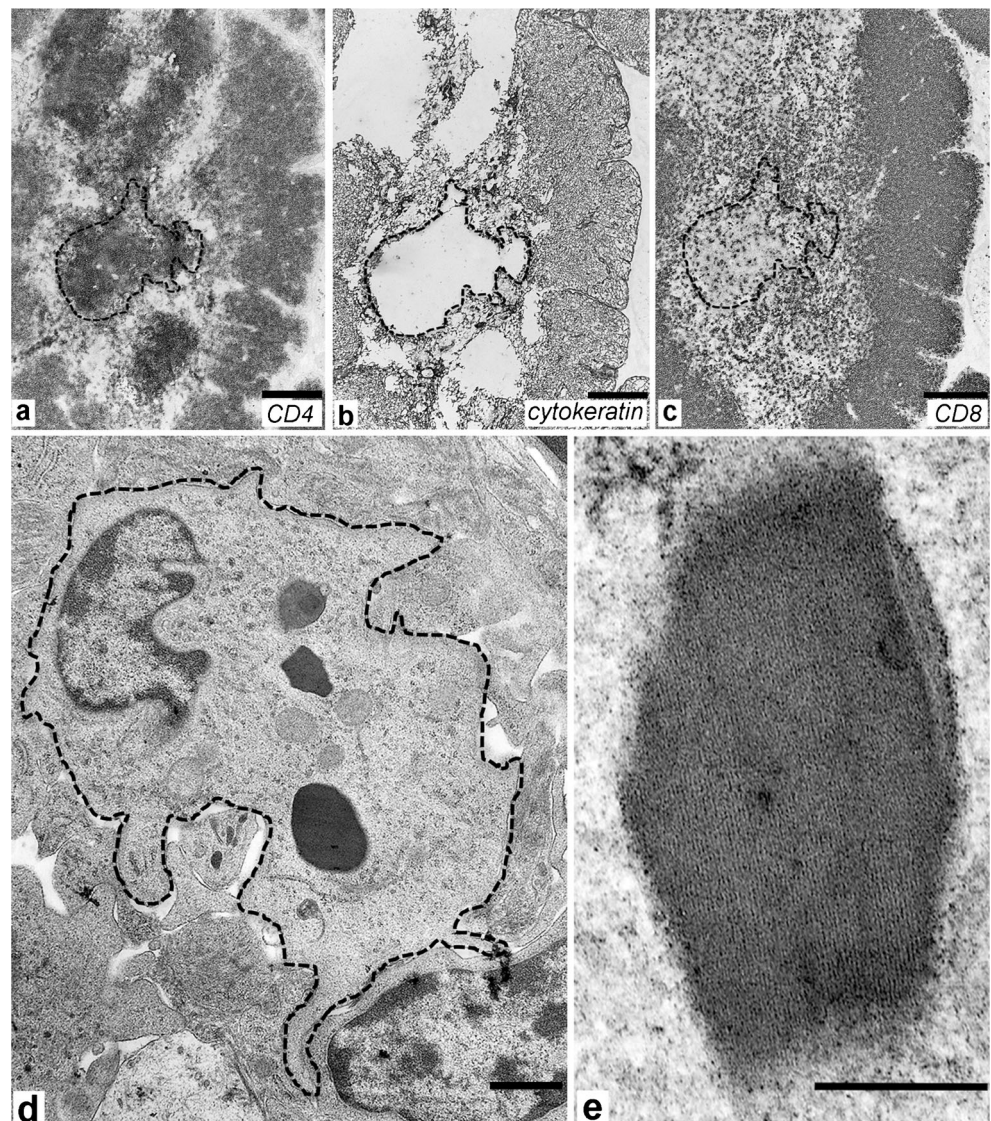


**Fig. 5** **a** Both EPCs (*brown*) and 74.3-positive cells (*blue*) are grouped but are not co-localized (*inset*). **b** The 74.3-positive dendritic cells (DCs; *red*) occupy the center of the KNA (KPN is *green*). **c** DCs (*red*) accumulate close to the blood vessels (*green*). **d** The 74.3-positive DCs (*red*) express

MHC II antigen (*inset* confocal micrograph) **e** The chB6 positive B-cells (*red*) accumulate in the KNA close to the surface of the KPN (*green*). **f** Confocal micrograph. Keratin (*green*) is co-localized with vimentin (*red*) in the marginal epithelial cells of the KPN (*yellow*). Bar 100  $\mu$ m



**Fig. 6** **a–c** Serial sections of a thymus with identical areas *outlined*. **a** CD4-positive cells accumulate in the KNA. **b** Anti-cytokeratin indicates the KNA. **c** CD8-positive cells occur in low numbers in the KNA. **d** Transmission electron micrograph of a thymic DC. The irregularly shaped nucleus, a few cytoplasmic granules and stocky processes of ectoplasm are characteristic features of thymic DC (*outlined*). **e** The granules of DC occasionally show lamellated structures. Bar 200  $\mu\text{m}$  (**a–c**), 1  $\mu\text{m}$  (**d, e**)

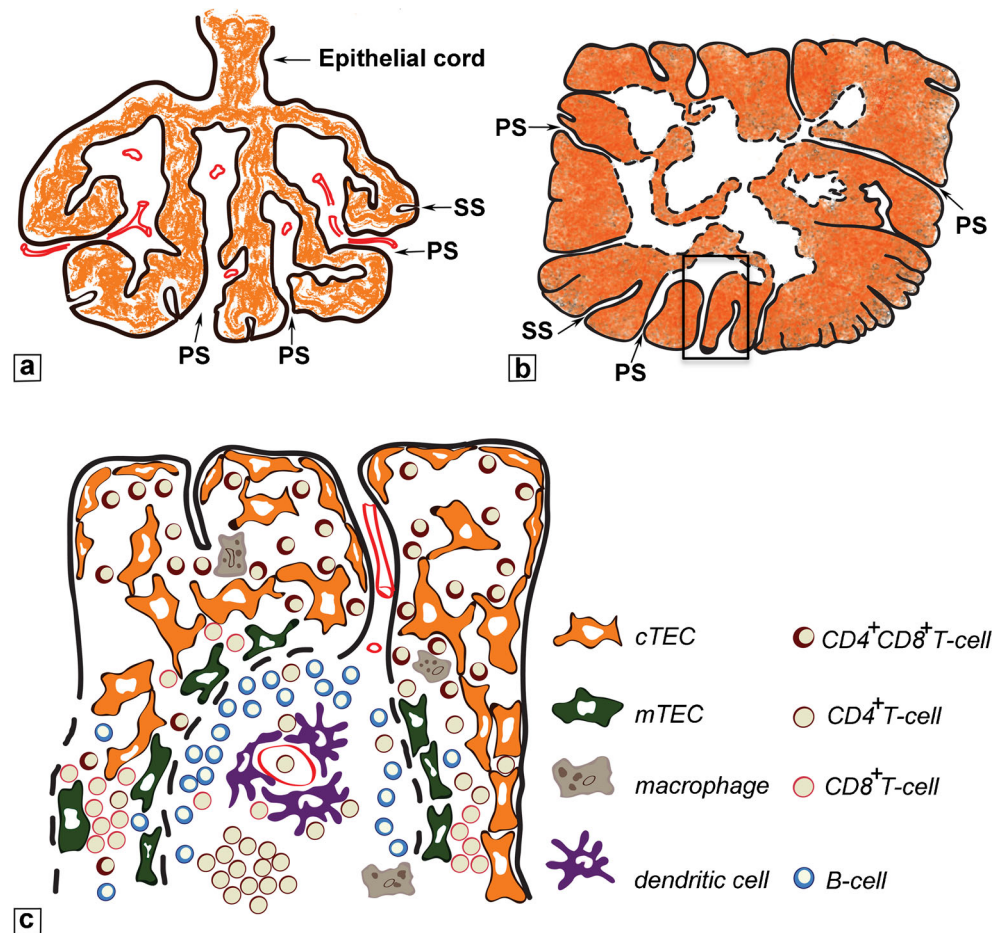


cells from neural crest cells has also been proposed (Nakamura and Ayer-Le Lièvre 1986) and might also indirectly confirm the neural crest origin of the KNA. However, Seifert and Christ (1990) reported that thymic myoid cells are derived from the axially located prechordal plate and not from mesencephalic or the rhombencephalic neural crest cells. These authors proposed that prechordal cells can contribute to the branchial arches from which the myoid cells can migrate into the thymic anlage. The presence of myoid cells, in addition to the pericytes in the KNA, indicates that the volume of KNA is much larger than the perivascular space. Morphometric analysis of keratin-stained serial sections confirmed that the KNA makes up about half the volume of the medulla, at least in the chicken (Fig. 2). Seifert and Christ (1990) suggested that the origin of the myoid cells is the prechordal plate. This theoretically means that the thymus develops not from three but from four different sources: (1) the branchial endodermal pouch, (2) the neural crest, (3)

hematopoietic progenitors and (4) the prechordal plate. This might explain the highly heterogenic cellular composition of the thymic medulla allowing the high number of tissue-specific antigens for the induction of central tolerance (Dooley et al. 2005).

The KNA contains reticular fibers, including type III collagen, plus fibronectin and tenascin. Both fibronectin and tenascin are capable of influencing cell migration (Savino et al. 2000; 2004). Various concentrations of tenascin can regulate neural crest cell migration (Akbareian et al. 2013) and subsequently influence the efficiency of the autoreactive T-cell selection. The presence of reticular fibers at the cortico-medullary border and in the inner medulla around the Hassall's bodies of human thymus has been shown by Yu and Lee (1993). The amount of reticular fibers and ECM components increases with age during physiological (Savino et al. 1993) and acute (Lannes-Vieira et al. 1991; Gameiro et al. 2010) involutions. Our experimental results, in which

**Fig. 7 a, b** Representation of the development of the thymic anlage. **a** Branches of the epithelial cord grow into the mesenchyme and form the PS. On the surface of the cortex and PS, the basal lamina is continuous (black line). **b** Relationship between the medulla and PS. The surface of the KPN is covered by a discontinuous basal lamina (dashed line). **c** Cellular organization of KNA. B-cells accumulate along the borderline of the KPN and KNA, whereas in the center of the KNA, the DCs are localized around the blood vessels



acute involution was induced by DM, confirmed the increase of the ECM of the KNA. In contrast to the cortex and the KPN, which have an epithelial reticulum of endodermal origin, the KNA is composed of reticular connective tissue, which is less sensitive to cortisone treatment than the central lymphoid organs. DM treatment results in severe T-cell depletion but the basic structural elements, namely, the endodermal epithelial cells, the thymic capsule and PSs of neural crest origin survive. After DM treatment, the entire thymic lobe becomes “medulla”, i.e., consists in the KPN and KNA. These thymic structural changes further support the neural crest origin of KNA.

In birds, the thymus and the bursa of Fabricius are the primary lymphoid organs contributing to the development of T- and B-lymphocytes, respectively. Not only the histological structure (reticular connective tissue) of the KNA is comparable to that of the cortex of the bursal follicle but functionally, both structures serve for cell migration between primary and secondary lymphoid organs. Therefore, both the KNA of the thymic medulla and the cortex of the bursal follicles can be regarded as a transit zone of primary lymphoid organs. Lymphocytes leaving these transit zones reach the gut-associated lymphoid tissues (the main peripheral lymphoid

organs of chickens) via high endothelial venules (HEV), which are regulated by  $CD11c^+$  DCs (Moussion and Girard 2011). The thymic postcapillaries are not HEV but an extensive cell migration takes place between the blood circulation and the KNA. Large numbers of immunologically matured T-cells enter the circulation and after hatching, a significant number of B-cells migrates into the thymic medulla. Double-immunolabeling of keratin and B-cells shows that the majority of the chB6-positive B-lymphocytes line up along the KPN, whereas the postcapillaries together with the 74.3 and MHC II double-positive DCs are localized in the center of the KNA (Fig. 7c). The topographical arrangement of B-cells and DCs indicates some cell-sorting mechanism in the KNA.

The blood-thymus barrier exists only in the cortex (Raviola and Karnovsky 1972) and therefore, thymocyte maturation takes place in an antigen-free microenvironment. The cortical barrier is formed by (1) endothelial cells and their basal lamina of the cortical capillaries and (2) the cortical surface epithelial cells and their basal lamina. However, there is neither a histological nor an anatomical explanation for the differences in the barrier between the cortex and medulla. The cortical epithelial cells produce only keratin filaments, unlike the KPN

surface epithelial cells, which express both keratin and vimentin intermediate filaments. The epithelial cells of the KPN have a discontinuous basal lamina, which breaks up the blood-thymus barrier. Thus, the anti-laminin staining pattern and the colocalization of keratin and vimentin in the surface cells of the KPN seem to be the first cytological sign and molecular marker for the difference of the blood-thymus barrier between the cortex and medulla and support epithelial plasticity (De Souza and Savino 1993).

After DM treatment, the cortex drastically shrinks and the entire lobe resembles the medulla. Subsequently, the classical cortico-medullary border alters, actually disappearing and the marginal epithelial cells of the KPN only occasionally express vimentin, unlike the untreated normal state. Therefore, the differentiation and/or plasticity of the marginal epithelial cells of the KPN are lost and the KNA is enlarged. Similar morphological events occur in mammals during age-related thymic involution, namely the expansion of the perivascular space (Gameiro et al. 2010). Infection by human immunodeficiency virus (HIV) is followed by thymic involution and histological disintegration of the cortico-medullary border (Napolitano et al. 2002). Similar experimental observations have been made after parasitic infection (Brito et al. 2003; Gameiro et al. 2010; Pérez et al. 2012) and malnutrition (Savino et al. 2007). These histological alterations are subsequently associated with increased susceptibility to autoimmune disease and cancer development (Lynch et al. 2009). Notably, once DM treatment ceases, the thymus soon regenerates, indicating that either cortical progenitor cells survive the treatment or the cortical and medullary epithelial cells have a common progenitor, as proposed earlier (Bennett et al. 2002; Farr et al. 2002; Bleul et al. 2006; Rossi et al. 2007).

The Hassall's corpuscles (not shown) and cysts (Vroegindewij et al. 2010) are other characteristic structures of the thymic medullary epithelium. Hassall's bodies are formed in the inner KPN and their cells do not express vimentin intermediate filament (Minkó and Oláh 1996), like the inner cells of the KPN. Possibly, the colocalization of keratin and vimentin in the marginal epithelial cells of the KPN represents epithelial plasticity (De Souza and Savino 1993) and the final differentiation stage, which is necessary for the function of the thymic medullary epithelial cells. The cells of the Hassall's body and the inner epithelial cells of the KPN possibly do not "reach" the final differentiation stage and therefore, these internal epithelial cells are aggregated (forming Hassall's bodies), keratinized and die. In this context, the Hassall's bodies and the vimentin-negative inner cells of the KPN have an identical structure and are functionally inert elements of the thymus. The "senescent" Hassall's bodies are invaded by vimentin-positive scavenger cells, such as heterophyl granulocytes and macrophages, which phagocytose, digest and eliminate the Hassall's bodies. The cysts develop from the epithelial

progenitors but their development is stopped at an early stage (Dooley et al. 2005) and therefore, the epithelial cells of the cysts maintain their polarized shape; this results in the absence of cross-talk between epithelial cells and T-cells (Klug et al. 2002; Rossi et al. 2007) and mesenchymal cells (Jenkinson et al. 2007; Itoi et al. 2007; Neves et al. 2012). We propose that the formation of both cysts and Hassall's bodies represents "developmental malformation" of the medullary epithelial cells, namely, the differentiation of epithelial progenitors is arrested in early and late stages, respectively.

The search for thymic DCs with a dendritic-specific monoclonal antibody (mAb 74.3) by immuno-electron microscopy was less successful but our previous studies have demonstrated irregular, highly electron dense granules with a lamellated inner structure in the FDCs of the germinal center of the caecal tonsil (Olah and Glick 1979). In the center of the KNA, mAb 74.3 immunocytochemistry visualized thymic DCs that express MHC II antigen. Thus, on the basis of the topographical location of mAb-74.3-positive cells, their MHC class II antigen expression and the presence of lamellated cytoplasmic granules, we identified the avian thymic DC *in vivo*. On the basis of these microscopical landmarks, conventional transmission electron microscopy revealed DC-like cells in the medulla. This is the first time that avian thymic DCs have been observed at the electron microscopic level.

In conclusion, anti-cytokeratin immunostaining shows that the thymic medulla consists in two regions. (1) The KPN possesses marginal epithelial cells that, in addition to keratin, express vimentin intermediate filaments and produce a discontinuous basal lamina. These cytological features might explain the absence of the blood-thymus barrier in the medulla. (2) The KNA is a dilation of the PS of cranial neural crest origin. The functional cortico-medullary border induces central tolerance and eliminates autoreactive T-cells in the border between the KPN and KNA. This study also indicates that the borderline of the KPN and KNA extends the functional cortico-medullary border. Early and late arrest of the differentiation of thymic progenitor cells results in medullary cysts and Hassall's body formation, respectively.

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