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Immunohistological Characterization of Thymic Dendritic Cells

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Abstract. *Background: Dendritic cells play key roles in thymic histophysiology and histopathology. Therefore, we analyzed the immunotopographical distribution of cells expressing markers of dendritic cells and macrophages in postnatal human thymus. Materials and Methods: The streptavidin-biotin peroxidise-labeled (LSAB) and the double-LSAB/alkaline phosphatase/anti-alkaline phosphatase (APAAP) immunohistochemical procedures were used. Results: S100 protein-, Cluster of designation 1a (CD1a)-, CD207-, CD11c- and CD123-positive cells, many of them exhibiting the morphology of dendritic cells, were detected in the cortex but mainly in the medulla. These markers, except CD123, were also detected in cells of juvenile and immature Hassall bodies. CD68- and CD163-positive cells were detected in the cortex and the medulla but not in Hassall bodies. Conclusion: The immunohistological detection of S100-, CD1a-, CD207- and CD11c-positive dendritic cells in juvenile and immature Hassall bodies may reflect an important role of these structures in the cooperation of epithelial and dendritic cells in the process of T-cell differentiation.*

The human thymus is a lymphoepithelial organ which supports the production of self-tolerant T-cells with both competent and regulatory functions and their migration to the peripheral blood circulation (1-6). In this multistep process of T-cell maturation and differentiation the cellular components of the thymic microenvironment [thymic epithelial cells (TECs), thymic dendritic cells (TDCs), macrophages, fibroblasts] play an essential role (7-56). Among these, TDCs play an important role in the generation of T-cell tolerance through the negative-selection of auto-reactive thymocytes and in the development of thymic T-regulatory cells (7-31, 36-44). TDC are bone marrow-derived dendritic cells (DCs), which are professional antigen-presenting cells (APC) involved in immunity and tolerance and characterized by differences in their anatomic distribution, cell surface marker expression and function (57-62).

Human TDCs are typically large cells (20-30 μm) that express Human leukocyte antigen –DR (HLA-DR) and Clusters of designation 4 (CD4) and by using flow cytometry, these cells can be subdivided on the basis of their immunophenotypic features into plasmacytoid TDCs and classical TDCs (reviewed in 7-11). The plasmacytoid TDCs exhibit the immunophenotype HLA-DR intermediate/CD11c-negative/CD123^{high}, whereas the classical TDCs can be further subdivided into two different immunophenotypic subsets: a) a population of immature HLA-DR intermediate/CD11c-positive/CD123-negative TDCs, and b) a population of more mature HLA-DR^{high}/CD123-negative TDCs, which can be further differentiated into b1) HLA-DR^{high}/CD11c^{high}/CD123-negative/CD11b-positive and b2) HLA-DR^{high}/CD11c-positive/CD123-negative/CD11b-negative cells (11, 14-16, 22-24).

HLA-DR intermediate/CD11c-negative/CD123^{high} plasmacytoid TDCs express high levels of CD4 and CD45RA, but not CD45RO, and secrete interleukin-12 (IL-12) following stimulation with IL-3 and CD40 ligand (CD40L); a subset of plasmacytoid TDCs express CD2, CD5 and CD7 (14, 22, 23, 36). It was suggested that plasmacytoid TDCs may protect the thymus against viral infection since they produce high levels of interferon-alpha (IFN-α) in response to some viruses, such as HIV-1 and influenza virus (23, 37). Furthermore, there is evidence that plasmacytoid TDCs may also affect the positive selection of thymocytes through IFN-induced up-regulation of Major histocompatibility complex-I (MHC-I) on thymic stromal cells (24, 36, 37).

HLA-DR intermediate/CD11c-positive/CD123-negative immature TDCs express the myeloid-related marker CD33, as well as high levels of CD4 and low levels of CD2, CD7, CD45RO and CD45RA (23).

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HLA-DR^{high}/CD11c^{high}/CD11b-negative/CD123-negative mature TDCs express high levels of CD40, CD83 and CD86 proteins and secrete high levels of IL-12 following stimulation with IL-3 and CD40L; they also express DC maturation marker DC-lysosome-associated membrane glycoprotein (*DC-LAMP*) mRNA, thymus- and activation-regulated chemokine (TARC, CCL19) and thymus-expressed chemokine (*TECK, CCL25*) mRNA which both regulate thymocyte homing/trafficking, but they do not express macrophage inflammatory protein-1α (*MIP-1α*) mRNA (14, 17, 23, 39, 40). In contrast, the HLA-DR^{high}/CD11c^{high}/CD11b-positive/CD123-negative mature TDCs are CD45RO^{high}, express granulocyte macrophage-colony stimulating factor (*GM-CSF*) receptor and *MIP-1α* mRNA but they do not express TARC or TECK mRNA, and secrete low levels of IL-12 following stimulation (14).

Although some authors reported that various cytokines and growth factors, such as tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), IL-7, IL-6, IL-4, IL-3. Stromal Cell Factor (SCF)(c-KIT ligand) and Flt-3 ligand (FL) regulate the differentiation and the survival of peripheral DCs (reviewed in 57-61), only one study focused on human TDCs, reporting that GM-CSF is an anti-apoptotic cytokine for human TDCs and a significant modulator of their accessory function (43). Moreover, evidence has been provided that the survival and function of human TDCs are regulated by an autocrine Hedgehog signaling, which plays critical roles in the development of numerous tissues in embryogenesis (44).

Various lines of evidence indicate either a common origin for some TDCs and thymocytes, or the existence of separate intrathymic T-cell lineage and DC precursors, but recent evidence supports the notion that intrathymic DCs and T-cells arise from different precursors (10). Indeed, Luche *et al*., analyzed a major subset of TDCs expressing CD8a and Langerin (CD207), which were thought to derive from progenitors with lymphoid potential, and demonstrated that these TDCs do not share a common origin with T-cells, but originate from intrathymic precursors that express markers that are normally present on all (CD11c and MHC-II molecules), or on some [CD207, CD135, CD8a, chemokine (C-X3-C motif) receptor 1 (CX3CR1)] DC subsets (12). They demonstrated that the earliest intrathymic precursors of CD8-positive TDCs correspond to myeloid-type CD44-positive/CD25-negative double-negative 1c (DN1c) cells and support the view that under physiological conditions, myeloid-restricted progenitors generate the whole constellation of DCs present in the body, including the thymus (12). The results of Luche *et al*., are consistent with the recent findings of Schlenner *et al*., who generated IL-7 receptor-α (*IL7r*) Cre recombinase knock- in mice and found that more than 85% of T-cell progenitors were *IL7r* reporter-positive, whereas most myeloid cells in the thymus were derived from *IL7r* reporter-negative cells (13). Schlenner *et al*., showed that thymic myeloid cells and DCs (except plasmacytoid dendritic cells) were mostly of non-lymphoid origin and concluded that lymphoid-restricted progenitors are the major route to T-cells and distinct origins of lymphoid and myeloid lineages represent a hallmark of hematopoiesis.

Although age-dependent alterations during thymic involution affect TECs and thymocytes, there is evidence that thymic macrophages and TDCs are only slightly affected by age (52-56). Indeed, Varas *et al*., (52, 53) analyzed the immunophenotype of purified human TDCs by flow cytometry using antibodies to MHC-II, CD80, CD86, CD40 and CD54, and investigated the T-cell stimulatory capacity of TDCs from young and elderly donors by *in vitro* studies. Despite the slight decrease of the number and the thymocyte-stimulatory capacity of TDCs in elderly donors, the proportion of TDCs remain constant with age and are still able to induce the proliferation of alloreactive T-cells, suggesting that TDC functions remain quite unaltered in the aged thymus (52, 53).

Immunohistological studies on thymic tissue sections are important for gaining insight into thymic histology and histopathology (8, 14-16, 20-23, 31-35, 62-71). However, there is paucity of multiparametric immunohistological information regarding the topographical distribution of human thymic cells expressing markers of DCs in comparison to cells expressing markers of macrophages in paraffin sections, which permit reliable morphological evaluation of immunopositive cells. Therefore, by double-immunostaining, we analyzed the immunotopographical distribution of human thymic cells expressing S100, CD1a, CD207, CD11c, CD123, CD68 and CD163 in paraffin sections of postnatal human thymuses.

Materials and Methods

Materials. Paraffin sections from normal human postnatal thymuses (from individuals aged 14 days to 14 years) previously analyzed for the immunohistological expression of cytokeratins, neural/neuroendocrine proteins and beta-tubulin isotypes (63), were included in the present study.

Immunohistochemistry. The immunostainings were performed using the streptavidin-biotin peroxidase-labeled (LSAB) procedure and by the double-LSAB/alkaline phosphatase/anti-alkaline phosphatase (APAAP) immunohistochemical procedures (63, 64). The immunostainings were performed, in most cases, using a Ventana autoimmunostainer, according to the manufacturer's protocols and instructions. The antibodies used are presented in Table I. S100, CD1a and CD68 immunostainings were used in our previous study as markers for the identification of DCs and macrophages in normal thymic tissue but the immunotopographical distribution of cells expressing these markers was not analyzed in detail (63). Positive control slides (reactive lymph nodes) and negative controls with

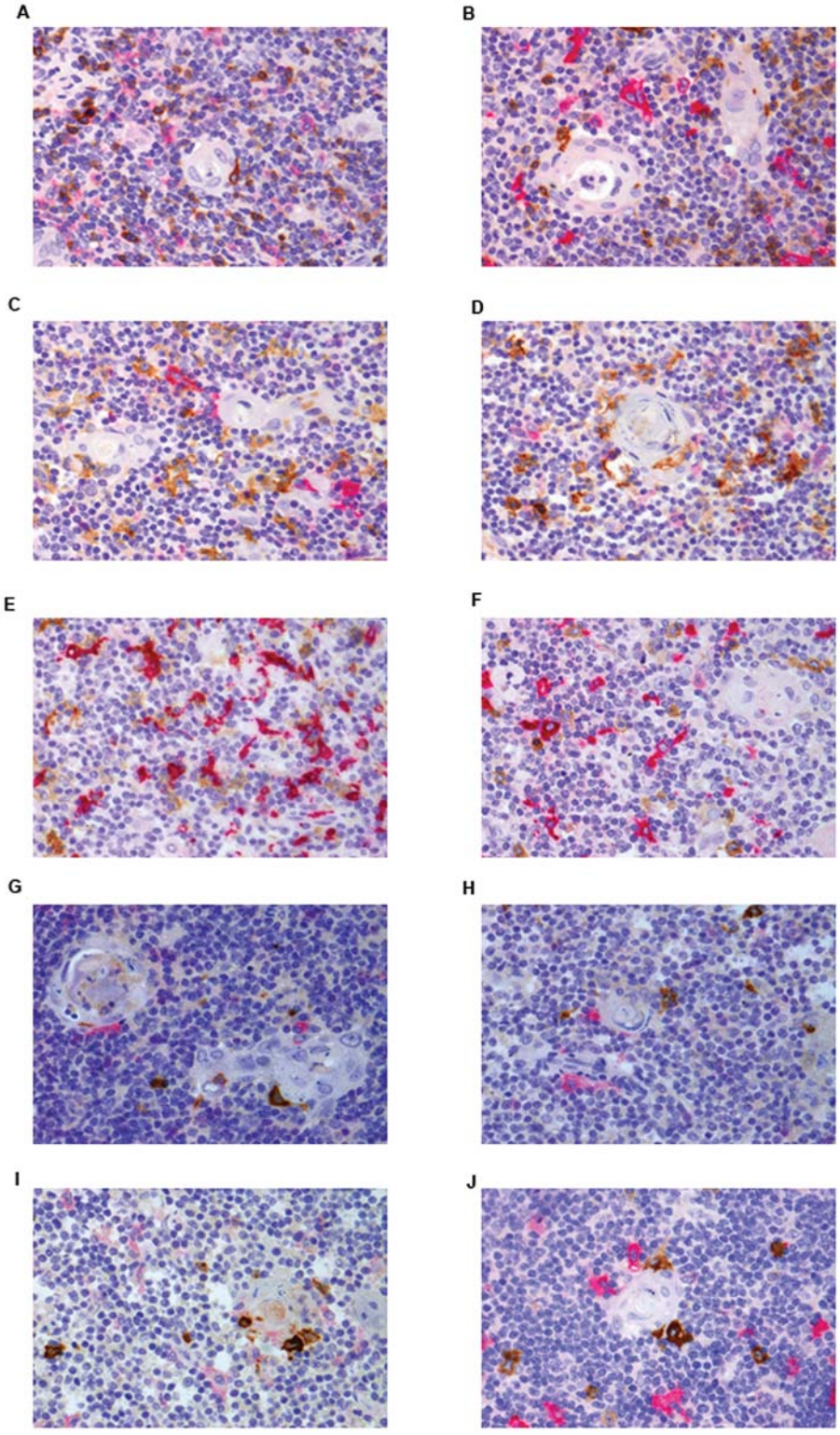

Figure 1. *Immunohistological analysis of thymic medulla using the double streptavidin-biotin peroxidase-labeled (brown staining)/alkaline phosphatase anti-alkaline phosphatase (red staining) immunohistochemical procedures. Double immunostaining for CD1a (brown)/CD123 (red) (A), CD1a (brown)/CD163 (red) (B), CD11c (brown)/CD1a (red) (C), CD11c (brown)/CD123 (red) (D), CD11c (brown)/CD163 (red) (E), CD123 (brown)/CD 163 (red) (F), CD207 (brown)/CD 1a (red) (G), CD207 (brown)/CD 1a (red) (H), CD207 (brown)/CD11c (red) (I), CD207 (brown)/CD 163 (red) (J) (magnification ×400).*

Table I. *Antibodies used for immunohistochemical analysis.*

Antibody	Clone	Dilution	Source
CD1a	CD1a007	Ready-to-Use	Biocare Medical Concord, CA, USA
CD68	KP1	1:50	Dako Glostrup, Danmark
CD207	SNCL-Langerin	1:100	Novocastra Newcastle, UK
CD123	7G3	1:10	BD Pharmingen San Diego, CA, USA
CD11c	S-HCL-3	1:100	R&D Systems Abingdon, England
CD163	SNCL-CD163	1:100	Novocastra Newcastle, UK

omission of the primary antibody were also included and the antibodies used in the present study were also tested in various types of lymphoid malignancies (Hodgkin's lymphomas and B- and T-cell non-Hodgkin's lymphomas) from our previous studies (72-76).

The LSAB and double-LSAB/APAAP immunostainings were analyzed qualitatively by light microscopy using a Nikon eclipse 50i microscope. Cells showing positive immunostaining with LSAB and APAAP were labeled brown and red, respectively (Figure 1). Then the images from the sections with the double-LSAB/APAAP immunostainings were converted from RGB (Red Green Blue) color space to L*a*b* color space, a perceptually uniform color space which is able to quantify qualitative color differences that the human eye perceives (77). The L*a*b* space consists of a luminosity layer "L*", a chromaticity layer "a*" indicating where color falls along the red-green axis, and a chromaticity layer "b*" indicating where the color falls along the blue-yellow axis. Both, a* and b* layers contain all required color information needed for the identification of stained tissue areas. Images were subjected to color segmentation analysis using L*a*b* (CIELAB) color space and K-means clustering algorithm (77, 78). The difference between two colors was estimated using the Euclidean distance metric and image pixels were classified into "a*b*" space, using K-means clustering (78). Following the clustering process, clusters characterized as brown areas (representing cells showing positive immunostaining with LSAB procedure), were clearly separated from red areas (representing cells showing positive immunostaining with APAAP procedure), thereby validating the results obtained by qualitative optical microscopy analysis.

Results

The Hassall bodies (HBs) in the present study were classified as juvenile, immature, mature, senescent and lymphocyte-rich according to previous studies (32, 33). Briefly, juvenile HBs were small- or medium-sized, ovoid or irregular formations of epithelial cells with unaltered morphology, without necrosis or cellular debris. Immature HBs were round or oval formations, consisting of squamous epithelial cells with prominent cytoplasmic acidophilia, but without degenerative changes. Mature HBs were medium- or large-sized formations, with necrotic or cystic degenerative changes in the central area, but presenting squamous epithelial cells at their periphery. Senescent HBs were of very large size, without any epithelial cells, but with calcified, necrotic material, cellular debris or cystic dilatation. Lymphocyte-rich HBs were medium- or large-sized formations, containing compact islands or small groups of lymphocytes. S100-, CD1a-, CD207- and CD11c-positive cells were detected in the cortex and mainly in the medulla, and in juvenile and immature HBs but not in mature and senescent HBs. Most of these S100-, CD1a-, CD207- and CD11c-positive cells were large, with irregularly-shaped nuclei and abundant cytoplasm, consistent with DCs morphology (Table II, Figure 1). CD123-positive cells were detected in the cortex and mainly in the medulla, but not in HB. Most CD123-positive cells were of medium and large size, having ovoid or irregularly-shaped nuclei, with some of them exhibiting visible nucleoli (Table II, Figure 1). S100-positive cells outnumbered CD1a-, CD207-, CD11c- and CD123-positive cells. CD1a also stained most cortical and some medullary thymocytes. CD68- and CD163-positive cells were detected in the cortex and the medulla but not in HB. Double-immunostainings showed clearly distinct populations expressing CD123, CD11c, CD1a, CD207, or CD163. Double-immunostainings did not reveal expression of chromogranin and synaptophysin by cells expressing CD1a, CD207, CD11c, CD123, CD163 and CD68.

Table II. *Expression of dendritic cell and macrophage markers in thymus.*

	Cortex	Medulla	Hassall bodies
S-100	+	+	+
CD1a	+	+	+
CD207	+	+	+
CD123	+	+	–
CD11c	+	+	+
CD68	+	+	–
CD163	+	+	–

Discussion

Immunohistology is important for gaining insight into thymic histology and histopathology (8, 14-16, 20-23, 31-35, 62-71). Indeed, immunohistology showed the various subsets of

normal human TDCs to be localized mainly at the cortico-medullary junction and the medulla but also in the cortex (8, 14-16, 21-23). In fact, using frozen-section immunofluorescence, Paessens *et al*., recently reported, the identification of CD209-positive APCs in the human thymic cortex which exhibit both immunophenotypic features of macrophages and immature TDCs (HLA-DM-positive/CD83-negative/CD86low/CD68-positive) (21). It was suggested that these cortical CD209-positive TDCs appear to function in thymocyte selection and/or removal of apoptotic thymocytes from the thymic cortex (21). Moreover, paraffin-section immunohistology allowed for analysis of the development and maturation of TDCs during human ontogeny. Indeed, Savchenko *et al*., showed that during the early period of thymic ontogeny (at 4 months), S100-positive TDC precursors appear at the corticomedullary border and in the medullary region (15). All of the TDC and macrophage markers examined (CD1a, CD207, CD163) were subsequently expressed from 5 months of age and together with the development of HB, GM-CSF was observed in TECs of HB (at 5–6 months) (15). Furthermore, paraffin-section immunohistology detected alterations of TDCs in patients with early defects in T-cell development. Indeed, Poliani *et al*., using S-100, CD208/DCLAMP, CD11c, and CD303/BDCA2 as markers of TDC and claudin-4, Ulex europaeus agglutinin-1 ligand and Aire as markers of TECs, identified severe reduction of TDCs and profound abnormalities of TEC differentiation in nine infants with various genetic defects leading to complete or partial block in T-cell development (T-negative severe combined-immunodeficiency, reticular dysgenesis, and Omenn syndrome) (20).

Prompted by the above data, we used double-immunostainings to analyze the immunotopographical distribution of human TDCs in paraffin sections from thymuses which have been previously analyzed for the expression of cytokeratins, neural/neuroendocrine markers and beta-tubulin isotypes (63).

In the present study, double immunostainings revealed S100-, CD1a-, CD207- and CD11c-positive cells in the medulla and in juvenile and immature HBs, but not in mature and senescent HBs. This is supported by previous studies which detected S100-, CD1a- and CD207-positive cells with dendritic morphology, dispersed among the epithelial cells of juvenile and immature HBs (15, 33). The detection of S100-, CD1a-, CD207- and CD11c-positive DCs in the medulla and in juvenile and immature HBs may reflect an important role of HBs in the cooperation between epithelial and DCs in the process of T-cell differentiation. There is accumulating evidence that HBs may play an important role in thymic histophysiology. Indeed, the epithelial cells of HBs secrete cytokines and growth factors and express IL-7, TGF-α, CD30 ligand, stromal cell-derived factor-1 (SDF-1), macrophage-derived chemokine (MDC), thymic stromal lymphopoietin (TSLP), its receptor, GM-CSF and IL-7 (2, 3, 34, 35). There is evidence that TSLP secreted by human HBs may activate immature CD11c-positive TDCs to up-regulate HLA-DR and DC-LAMP and to express high levels of the co-stimulatory molecules CD80 and CD86 (14, 31). These DCs are then able to induce the proliferation and differentiation of CD4-positive/CD8-negative/CD25-negative thymic T-cells into CD4-positive/CD8-negative/CD25-positive/FOXP3-positive T-regulatory cells (31). Moreover, it has been shown that co-cultivation of human thymocytes with DCs in the presence of TSLP induced a significant increase of CD4-positive/CD25-positive T-cells (31). In addition, immunohistology revealed that CD25-positive/CTLA4-positive regulatory T-cells associate in the thymic medulla with activated (mature) TDCs and TSLP-expressing HBs (31). On the basis of these findings Watanabe *et al*., suggested that HBs have a critical role in TDC-mediated positive selection of medium-to high-affinity self-reactive T-cells, leading to the generation of CD4-positive/CD25-positive T-regulatory cells within the thymus (31).

In the present study, CD123-positive plasmacytoid TDCs were not detected in HBs. These CD123-positive/CD11c-negative cells are phenotypically and functionally different from the CD123-negative/CD11c-positive TDCs (11) that we observed among epithelial cells of HBs. It is possible that CD123-positive plasmacytoid TDCs are not directly involved in the functional network, implicating the communication of HBs with developing T-cells and antigen-presenting CD11c-positive TDCs.

In the present study, double immunostainings showed clearly distinct populations expressing CD123, CD11c, CD1a, CD207, or CD163. Although the CD123 immunostaining pattern has not been previously analyzed by paraffin section immunohistology on human thymus, our CD123 results are consistent with the flow cytometric findings of Smith *et al*., who found that human TDCs, highly expressing CD123 were CD11c- and CD14-negative (16). Moreover, our CD11c, CD163, CD1a and CD207 results are consistent with the paraffin section immunohistological findings of Savchenko *et al*., (15) and Poliani *et al*., (20) on human thymus. In these studies, double immunostainings showed some CD11c/CD163 and CD1a/CD207 double-positive cells (15, 20). The detection of these double-positive cells, considered together with the identification of clearly distinct populations expressing CD123, CD11c, CD1a or CD207, may indicate different stages of human TDC differentiation.

In conclusion, the diversity of the immunohistological profiles and the immunotopographical distibution of human TDCs may reflect the diversity of their biological functions and/or their different stages of differentiation. The present results provide further immunohistological evidence that DC-associated proteins may be required for the development and function of the human thymic microenvironment. The

detection of S100-, CD1a-, CD207- and CD11c-positive human TDCs in the medulla and in juvenile and immature HBs may reflect an important role of HB in the co-operation between epithelial and DCs in the process of T-cell differentiation. The absence of S100-, CD1a-, CD207- and CD11c-positive cells in mature and senescent HBs may reflect the involution of these forms of HB. Moreover, our present and previous findings in normal human thymic tissues (63, 64) provide a detailed immunohistological mapping of proteins associated with the cell-cycle, apoptosis, and epithelial and DC differentiation, which may be helpful for the further understanding of thymic histology and histopathology.

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