In vivo identification of avian thymic dendritic cells and the compartmentalized thymic medulla

PhD thesis

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1. INTRODUCTION

Well-known, or official historical story of lymphoid dendritic cells dated to 1973 when Steinman and Cohn published a paper of an unknown cell type, which was isolated from mouse spleen. The cell revealed an irregular shaped nucleus around which the cytoplasm contained few granules and well-developed cytoplasmic processes. The cell was called on the basis of shape, dendritic cell (DC). Later works provided information about the phenotype (MHC class II antigen is constituently expressed), non-phagocytic and capable of antigen presentation. The immature form of DC is phagocytic and the mature one is a potent accessory cell for adaptive immune response. Dendritic cells are essential stromal cells for both central and peripheral lymphoid organs. In the central or primary lymphoid organs the DC possible contributes to the maturation and/or selection of T- and B-cells, while in the peripheral lymphoid organs (spleen, lymph node, gut,-bronchus,-skin-associated lymphoid tissue) their location follows the T- and B-cell areas. Interdigitating dendritic cell (IDC) locates at the T dependent and the follicular dendritic cell (FDC) occupies the B dependent follicules. IDC presents antigen for T-cell, while FDC binding immuncomplexes, stimulates follicular B-cells. The lymphoid DCs express hemopoietic marker (CD45) providing evidence for the hemopoietic origin.

After the general survey of DC, it is worthy commemorating for the recognition of the avian DC. “A further immunofluorescence study (White 1963) of the fate of human serum albumin (HSA) injected intravenously into the chicken revealed that this immunogen localized to certain cells, designated “dendritic cells, probably a type of macrophage”, in the germinal centres

within the white pulp of the spleen and persisted at these sites for at least 2 week" (White et al 1969). Upto now there is a lot of debates if the DC and the macrophage represent one cell lineage i.e. they have a common progenitor "monocyte" or they have different maturation states or they belong separate sublineages. In the publication (White et al 1969) the authors confirmed their earlier statement "antigen-bearing cells appear to migrate through the white pulp and subsequently to appear (88 hr-6 days) as dendritic cells within germinal centres". This scientific achievement far preceded 1973, the beginning of triumphal march of DC, which was awarded by Nobel price.

In vivo FDC and bursal secretory dendritic cells (BSDC) were identified in an few years after Steinman and Cohn (1973) historical publication. During the last 40 years the study of avian dendritic cells in peripheral and central lymphoid organs is tradition in our laboratory.

Avian DCs have been studied in detail: light and transmission electron microscopical structure have been described, phenotypic characterization was carried out by a panel of monoclonal antibodies, the origin was determined by chick-quail chimeric experiments. However, the thymic dendritic cells (TDC) in chicken upto now was unknown, therefore my major topic was the identify chicken TDC, in vivo. According to my knowledge there is only one in vitro paper, which suggested, that an MHC class II positive, ruffled surfaced cell (dendritic appearance) would be dendritic cell (Oliver and Le Douarin 1984).

2. THE AIM OF THIS STUDY

The avian DCs have been identified and characterized in the peripheral lymphoid organs like the spleen, cecal, -pyloric- and esophageal tonsils even in the pineal gland too, which is temporary lymphoid organ. In case of the central lymphoid organs; bursa of Fabricius (responsible for B cell maturation) the DCs, called bursal secretory dendritic cell (BSDC) has been recognized since 1978 (Oláh and Glick, 1978), unlike the DCs in the thymus. In spite of notwithstanding, that the thymic DCs play an essential role in the negative selection of T-lymphocytes and central tolerance, the thymic DCs did not get satisfactory attention. The reason possibly is that the thymic cytological structure is much more sophisticated than that of the bursa of Fabricius.

Almost 40 years after the discovery of BSDC my **goal** was to **recognize** and **describe** the **thymic DC in vivo**. The **topographical relationship** between **DC** and the **thymic epithelial reticular cell** is fundamental for thymic function. Therefore the recognition of DC was followed by searching of their **intrathymic location**. These works indicated that the thymic **medulla is compartmentalized**: **keratin positive network** (KPN) intermingled by **keratin negative area** (KNA). **Morphometric analysis** was made **to measure the volume of KPN and KNA**, and **the cellular composition** of the two compartment was analyzed.

3. MATERIALS AND METHODS

3.1 Experimental animals:

White Leghorn SPF chicken (Gallus gallus), quail (Coturnix coturnix japonica) and guinea fowl (Numida meleagris) were used at age 4 to 8 weeks old. Chicken and quail embryos were incubated in our laboratory and 3, 6, 11, 13, 17, 20 days old chicken were involved into the experiments. In each groups of age minimum four embryos or birds were used. To provide evidence, that the compartmentalization is an avian peculiarity or it is present in mammals young adult rabbit thymuses were also stained by anti-cytokeratin antibody.

The animal experiments were made by the permission (No. 22.1/10032-4/2010) of Scientific Ethical Committee.

3.2 Immunocytochemistry

A panel of monoclonal antibodies was used to determine and characterize the phenotype of a unique cell type, which fulfilled the dendritic cell feature. The immunocytochemical studies involved chromogenic and immunofluorescence detection of antigen. Occasionally, confocal microscope helped to clarify critical co-localization of antigens.

3.3 Electron microscopy

The tissue samples were fixed in 4% glutaraldehyde and embedded into Araldite. Semithin sections (1,5μm) were stained with 1% toluidine blue, and the ultrathin sections were contrasted with uranyl acetate and lead citrate.

3.4 Pre-embedding immunocytochemistry

74.3 monoclonal antibody (mAb) stained 50μm thick sections, which were embedded into Araldite and the semithin and ultrathin section were prepared as indicated above.

3.5 Dexamethasone (DM) treatment

Acut thymic involution was induced by DM treatment of young (6 weeks old) chickens. 5mg DM /kg body weight was injected 10 times before sampling. The aim of this treatment was to determine the alteration of KNA and KPN.

3.6 Thymic epithelial cell proliferation

Regeneration of thymic epithelial cells was studied by using bromo-deoxyuridin (BrdU). Three days after completing the DM-treatment-when the regeneration of thymic epithelial cells is expected – 100mg/kg BrdU was intraperitoneally injected, and on the tissue samples anti-BrdU immunocytochemistry was made.

3.7 Morphometric measurment

Stereological analysis was made according to Cavalieri method for the volumetric measurement of cortex and medulla as well as KPN and KNA. 10μm thick serial sections were made from one thymic lobe of three controls and three DM treated birds. On every 25th sections anti-cytokeratin immunostaining was made for morphometry.

3.8 Quail-chicken and opposite chicken-quail chimeric experiments

In order to determine the origin of DC 3 days old quail embryo's branchial pouches were transplanted into the coelom of 3 days old chicken and incubated for 2 weeks. The transplanted branchial pouches were removed and histological and immunocytochemical demonstration was made.

Chicken-quail chimeras were made to determine the origin of 74.3 positive cortical cells.

3.9 Quail-chicken chorioallantois membrane chimera

In order to exclude the possible error from the contact of donor transplant and host.

3.10 Silver impregnatiom

Assuming, that the basic supporting tissue of KNA is reticular connective tissue, like in the peripheral lymphoid organs silver impregnation has been made according to Krutsay (1980).

4. RESULTS

4.1 Inquiring after avian thymic dendritic cells

The 74.3 mAb recognizes BSDCs, follicular dendritic cells (FDCs) in the germinal centers. Our work provided evidence, that both BSDCs and FDCs expressed vimentin intermediate filament and the 74.3 positive antigen and vimentin are colocalized. The search for DC started with these two mAb(s), which was complemented by MHC class II.

The cell type, which expressed 74.3 positive intracytoplasmic and MHC class II membrane antigens, appeared in clusters in the medulla and as single cell in the cortex. Question raised if the cells in clusters and single one represent one cell lineage or two different ones? Mammalian DC homolog markers CD83 and DEC205 helped to clearify this problem, In the cell clusters the 74.3 and CD83 antigens colocalize, and the cells express CD45 hemopoietic marker. DEC205 did not recognized cells in the medulla, but single cells were positive in the cortex, which also expressed 74.3 positive antigen, but not CD45, suggesting that they are not of hemopoietic origin. In the medullary clusters the cells are $74.3^{+}CD83^{+}$MHC class $II^{+}CD45^{+}$. These markers suggest, that the majority of the clusters consist of DC. In order to determine the cytology of cells in the clusters, I studied the thymic medulla by transmission electron microscope, which indicated a “unique” nuclear and cytoplasmic structure; different from epithelial reticular cells, lymphocytes and macrophages. The cytological structure and phenotype of these cells supported that they are the thymic dendritic cells (TDCs). TDCs locate in the KNA around the blood vessels.

The cortical DEC205^{+}74.3^{+}MHC class II^{+}CD45^{-} cells are cortical reticular epithelial cells because these cells express cytokeratin intermediate filament.

Chicken-quail and quail-chicken chimeric experiments confirmed the results of immunocytochemistry, i.e. the DC are of hemopoietic origin and the cortical DEC205^{+}74.3^{+} and MHC class II^{+} cells express cytokeratin are epithelial cells of entodermal origin.

4.2 The avian thymic medulla is compartmentalized into keratin positive network and keratin negative areas

Hematoxylin-eosin (HE) and anti-vimentin immunostaining reveal cortex and medulla in the thymus. However, the anti-cytokeratin clearly indicates that the thymic medulla consists of keratin positive network (KPN) and keratin negative areas (KNA). Discrepancy between the results of HE and anti-cytokeratin staining indicates the selection of a suitable method for research is essential.

Thymus is a complex organ. The fine supporting element; epithelial reticulum develops from the third, possibly fourth pharyngeal pouches of foregut entoderm. The connective tissue of the capsule and septae develop from the cardiac neural crest cells; and the lymphocytes, macrophages and dendritic cells are of hemopoietic origin, immigrate into the developing anlage.

Epithelial cord of the foregut entoderm is surrounded by mesenchyme of neural crest origin, which receives the blood vessels. The epithelial cord is dividing in the mesenchyme, wich forms between the branches of the epithelial cord, so-called, “primary septae” with the blood vessels. The club-shaped ends of the epithelial branches will develop to the thymic cortex, while the area close to the branching of epithelial cord develops to medulla. The epithelial cells on the surface of the club-shaped ends of epithelial branches and primary septae are covered by continuous basal-lamina. However at the bottom of the primary septae -which is the cortico-medullary border- the basal lamina becomes discontinuous. This is the first histological mark, which may explain the absence of blood-thymus barrier in the medulla.

The anti-cytokeratin immunostaining indicates that the KNA seems to be dilations of the primary septae. The connective tissue of primary septae is continuous with the KNA, which suggests that the area consists of same tissue.

Silver impregnation justified this hypothesis and it was further confirmed by anti-fibronectin and –tenascin immunostaining.

Unique cellular element of the KNA is the myoid cell, which appears in elongated and/or avoid shape. The myosin and actin filaments form regular sarcomers, like in the skeletal musle. Skeletal muscle can develop only from somites and cranial neural crest cells. Therefore, I suppose that the KNA of the medulla –like the thymic capsule and septae- also is of neural crest origin.

Blood vessels in the medulla locate exclusively in the KNA. Around the blood vessels the DCs accumulate in clusters, while B-lymphocytes line up at the margin of KNA. It may be reasonable to note that the CD4 and CD8 positive cells mainly accumulate in the KNA and KPN, respectively.

The KPN shows cysts, Hassall's bodies and the marginal cells express vimentin beside the keratin intermediate filament. The vimentin –as a member of cytoskeleton- provides more plasticity for the marginal cells, through which the T-cell traffic takes place. It seems to be that these vimentin-expressing marginal cells completed their differentiation unlike the cysts and Hassall's bodies. According to the opinion of Dooley et al (2005a), the epithelial cells of the thymic cysts maintain their polarity –which is present in the epithelial cord- therefore their further differentiation stopped in early stage of thymus development, and they lost the communication with the lymphocytes. The epithelial cells of cyst express MHC class II antigen, like thymic epithelial reticular cells and produce surfactant protein – B like type II pneumocytes.

Hassall's bodies locate inside the KPN and do not express vimentin as the marginal cells do it. I propose, that the differentiation of the cells of Hassall's bodies stopped in late development of thymus, therefore the cells are keratinized. I propose, that the surface epithelial cells of KPN reach the

destination i.e. final differentiation, which locate on the margin of KPN and express vimentin in addition to keratin.

The thymic epithelial anlage develops from the foregut entoderm, like the lung. The cells of the thymic cysts contain lamellar bodies like the type II pneumocytes and the anti-surfactant protein B (SP-B) indicates, that the cyst epithelium is capable of surfactant production.

Finally, the question has been raised: what is the functional cortico-medullary border? The border shows as the HE stained section or the much larger KPN-KNA border?

5. CONCLUSION

1) The TDCs have been identified in vivo.

2) At electron microscopic level the cytological structure of dendritic cells has been described.

3) Avian thymic medulla is compartmentalized: keratin positive network (KPN) and keratin negative areas (KNA), which are sharply separated.

4) TDCs are clustered around blood vessels in the KNA of the medulla.

5) Chimeric experiments (chicken-quail and quail-chicken) provided evidence, that the $74.3^{+}DEC205^{+}CD45^{+}$ dendritic cell are clustered in the medulla, while the cortical $74.3^{+}DEC205^{+}CD45^{-}$ single cells are epithelial cells.

6) Marginal cells of the KPN, besides the keratin intermediate filament, also express vimentin intermediate filament, which may provide high plasticity for the cells. The marginal cells did reach the terminal differentiation of thymic epithelial reticular cells.

7) The cyst epithelial cells may be produced surfactant, like type II pneumocytes and express MHC class II antigen like thymic reticular epithelial cells.

8) The cells of Hassall's bodies do not express vimentin, their differentiation stopped in the late thymic development; therefore the cells keratinized.

9) The thymic epithelial cells locating on the surface of the capsule and along the primary septae are covered by continuous basal lamina, which becomes discontinuous at the bottom of primary septum. This is the first sign, which may explain the lack of blood-thymus barrier in the medulla.

10) Presence of myoid cells in the KNA –which is directly connected with the primary septae- indicates, that the KNA may be developed from cardiac neural crest cells.

11) Finally, the question has been raised, that what is the functional cortico-medullary border? Does the HE stained section show the border or the border of KPN-KNA, which is much larger?

6. LIST OF PUBLICATIONS

List of publications related to the theme of this PhD

Nagy N, **Bódi I**, Oláh I (2015) Avian dendritic cells: phenotype and ontogeny in lymphoid organs Dev Comp Immunol **IF: 2, 815**

Bódi I, Minkó K, Molnár D, Nagy N, Oláh I (2015) A novel aspect of the structure of the avian thymic medulla. Cell Tissue Res 359:489-501. **IF: 3,565**

Kocsis K, Benyeda Z, **Bódi I**, Molnár D, Nagy N, Fejszák N, Palya V, Oláh I (2012) Chicken dendritic cells and type II pneumocytes express a common intracellular epitope. Br Poult Sci 53:397-400. **IF: 1,005**

List of publications not related to the theme of this PhD

N. Nuthalapati, N. Alugubelly, B. Felfoldi, **I. Bódi**, N. Fejszak, G.T. Pharr and I. Olah (2015) Monoclonal Antibody EIV-E12 Recognizes a Glycoprotein Antigen, Which Differs from the B-Cell-Specific chB6 (Bu-1) Antigen. Int J Poult Sci 4 (8): 479-484.

Bódi I, Nagy N, Sinka L, Igyártó BZ, Oláh I (2009) Novel monoclonal antibodies recognise guinea fowl thrombocytes. Acta Vet Hung 57:239-246. **IF: 0,624**