

Histochemical and Ultrastructural Studies on Melanomacrophage Centre (MMC) within the Thymus Gland of Climbing Perch, *Anabas testudineus* (Bloch, 1792) with Special Reference to Thymic Defense

Sk Samim Hossin*¹ and Subrata Kumar De²

¹ Centre for Life Sciences, Vidyasagar University, Midnapore (West) - 721 102, West Bengal, India

² Department of Zoology, Vidyasagar University, Midnapore (West) - 721 102, West Bengal, India

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Abstract

Melanomacrophage centers (MMC) within the thymus gland of air-breathing Climbing perch [*Anabas testudineus* (Bloch, 1792)] are critically examined under light and electron microscope. The distinct distributions of melanomacrophages (MM) within the primary lymphoid organ of climbing perch are strongly providing their structural and functional aspect within thymic stroma. The predominated thymocytes, epithelial cells, granulocytes, macrophages, plasma cells and integrated vascular networking system are very significant for lymphoid cell proliferation, maturation as well as thymocyte selection in fish. The large PAS (Periodic acid-Schiff) positive pigmented melanomacrophages centers are observed with cellular or sub-cellular debris. This frequent accumulation of melanomacrophages and cellular inter-connection within the thymus gland under high resolution electron microscope are significantly influence the cell-mediated thymic defense in a fragile aquatic ecosystem of fish (*Anabas testudineus*).

Key words: *Anabas testudineus*, Thymus gland, Melanomacrophage centers, Thymocytes, Plasma cells, Thymic defense

The thymus is a key lymphoid organ significant for the development and maturation of lymphoid cells that plays a principal role in cell-mediated immunological defense in vertebrate systems [1]. In fish, this gland does not remain functional all over the developmental stages of fish species [1-2]. The imperative phenomena called 'Involution' occur due to several physiological factors (nutritional, hormonal, ageing, etc.) or environmental factors (seasonal, aquatic pollution, etc.) that affect the overall health of teleost species [1]. Ronald Roberts (1975) first used the phrase "melano-macrophage centre" in the vertebrate system [3]. These tiny pigmented melanomacrophage (MM) accumulations, which form large pigmented center, are found in teleost haematopoietic organs called melanomacrophage centers (MMC), predominated within the spleen and kidney (the spleen is only a secondary lymphoid organ, while the kidney is both a primary and secondary lymphoid organ in the vertebrate system), that also occasionally observed within the thymus gland, a primary lymphoid organ of fish [4, 5]. They are also present in the liver in some primitive fish species [4-5]. A central element of the vertebrates immune system is macrophages, which have several important intercellular phagocytic activities and process heterogeneous materials like cellular debris, melanin pigment (mostly of ectodermal origin), hemosiderin granules, lipofuscin residues, lipid droplets, mucopolysaccharides, and protein aggregates densely packed and frequently form clusters within the melanomacrophage centres (MMCs) after the systemic process of the phagocytic activity [6-8]. The appearance and

morphofunctional distribution of melanomacrophages differ at the species level of teleosts [3], [10] as well as among the organs level [10], [21] or within the same species under various physiological or pathological circumstances, i.e., age [11-14], nutritional status [15], [6], tissue type [10], and iron, are some of these factors [16]. Apart from that, significant study on the lymphoid organs has increased in recent years, particularly regarding pigmented phagocytes such as melanomacrophages (MMs). The primary function of MMC is to detoxify, eliminate, or recycle both endogenous and exogenous biomolecules and their derivatives [17], but their role in thymic defense and cellular homeostasis has not been clearly established in teleost species or in *A. testudineus*. Although it's functional significance, and detailed cytological characterization of the lymphoid tissue in *Anabas testudineus* remains sparse. To elucidate the structural and biochemical features of the MMC within the thymic stroma, a conventional histochemical and ultrastructural techniques are essential for clarifying morphofunctional characterization of the melanomacrophage centers (MMC) and the significant interaction of immune cells that are associated with it.

MATERIALS AND METHODS

Specimen

Anabas testudineus (Bloch, 1792) (Order: Perciformes; Family: Anabantidae) is a freshwater air-breathing fish of South-East Asia. The amphibious nature and unique opercular

*Correspondence to: Sk Samim Hossin, E-mail: samimhossin03@gmail.com; Tel: +91 7908124732

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walking behaviour over the land famous them as climbing perch around the world. According to IUCN Red list category this species categorized under Least Concern (LC) Ver. 3.1 [24]. This is extremely delicious, nutrient rich and popular table food in West Bengal, India and Bangladesh. In India this omnivorous species mostly found in lowland ponds, canals, swamps, lakes, estuaries, rivers and paddy fields. This species can tolerate extremely turbid, heavily polluted fragile stagnant water bodies and buried live under mud during the dry season [18-19].

Morphoanatomical study

Anabas testudineus is a fresh water climbing perch bears visible spiny double dorsal fin with a blunt snout. Live, healthy *Anabas testudineus* of variable total body length (9 cm. - 21 cm.) and of variable body weight (25gm. - 60gm.) were collected from the channels and ponds of Purba Medinipur district, West Bengal, India. The collected specimens were brought to the laboratory and acclimatized in a laboratory conditions at room temperature for 24 hours. The acclimatized specimens were critically anaesthetized using buffered tricaine methanesulfonate (MS-222 dose: 100mg/L-1 - 200mg/L-1) [20] for morphoanatomical observation. The thymus glands were dissected out from the dorso-lateral position of opercular cavity of the specimen and fixed in standard aqueous Bouin's solution. The morpho-anatomical structure was viewed under stereo zoom light microscope (Carl Zeiss: Stemi Doc 508).

Scanning electron microscope (SEM) study

Thymus glands were dissected out from the dorso-lateral position of opercular cavity from the anaesthetized specimen *A. testudineus* and immediately fixed in 2.5% glutaraldehyde (Sigma-Aldrich, EM Grade) in 0.1(M) phosphate buffer (pH 7.2-7.4) at 4°C for 2 hours. After completion of fixation, the thymic tissues were washed (2 to 3 changes) in the same buffer. Then the thymic tissues were dehydrated with graded chilled acetone (ascending chilled acetone, 30% - 50% - 70% - 90% - 100%) with a regular interval of 15 minutes each and then the tissue rinsed in 100% acetone over 30 minutes followed by isoamyl acetate. After dehydrated with dry acetone, tissue was critically dried with critical point drying (CPD) using liquid carbon dioxide (Hitachi 8CP2) and immediately placed on a metal stub for sputter coating with pure gold (16nm thick) using sputter coater (Quorum Q150TES) and examined under Scanning electron microscope (SEM: Zeiss EVO18) operated at 20 kV [21].

Histochemical study

For the histochemical study dissected glands were directly fixed in 4% paraformaldehyde in 0.1 (M) phosphate buffer (pH- 7.2-7.4) for 2 hrs. The fixed tissues were then washed in the same buffer 2 changes for 30 minutes interval. Washed tissue was cryoprotected within gradually 15%, 20% & 30% graded sucrose solution in 0.1 (M) phosphate buffers at 4°C for 30 minutes each. Then the cryoprotected tissues were carefully sectioned using cryostat (LEICA CM 1850); operated at -19°C to -23°C having thickness 3µm - 5µm and placed on clean gelatin coated glass slides. Prepared semi-thin slides were stained using Hematoxylin - Eosin (H-E) and Alcian-PAS stain (1% Alcian Blue in 3% aquas acetic acid, 0.5% aquas periodic acid and Schiff reagent) followed by dehydration with graded ethanol and covering the DPX mounted stained tissue sections with square shaped clean cover glass. Therefore, the semi-thin thymic tissue was examined under trinocular light microscope [Primo Star; Carl Zeiss, GmbH].

Transmission electron microscope (TEM) study

The dissected thymus gland of *Anabas testudineus* primarily fixed in 2.5% glutaraldehyde (Sigma-Aldrich, EM Grade) in 0.1 (M) phosphate buffer (pH 7.2-7.4) for 2 hours at 4°C, and the secondary fixation was done using 1% osmium tetroxide (OSO₄) dissolved in the same buffer (0.1 M phosphate buffer, pH 7.2-7.4) at room temperature for 1 hour, followed by dehydration through graded chilled acetone. Dehydrated tissue was polymerized within the bubble free resin (Araldite CY 212), and embedded in an embedding mold at 60°C for 48 hours under an incubator. The tissue blocks were then cut into ultrathin sections (60-90 nm) using an ultramicrotome (Leica UC7), double stained with 1% uranyl acetate and 1% lead citrate, and finally examined under a Transmission electron microscope [TEM: TALOS F200X G2, THERMO SCIENTIFIC] operated at 120 kV [SAIF, AIIMS, New Delhi].

RESULTS AND DISCUSSION

The thymus gland of *Anabas testudineus* (Bloch, 1792) (total body length 9±2 cm and operculum height 12±2 mm) located within the anterior-dorsolateral position of the opercular cavity (Fig 1C). Under stereo zoom light microscope Bouin's fixed thymus gland slightly triangular in shape, covered with fibromuscular cell sheath and associated developing gill lamellae (Fig 1D). High resolution surface topography structures depict the surface topography of thymus gland (*) with fibromuscular structure (arrows) that was always expose to external aquatic environment (Fig 2C). These structures demonstrate the reticular epithelial cells closely associated with macrophage cells on the ventral surface of the thymus gland of *Anabas testudineus* (Fig 2A-B). The cytoplasmic protrusion of macrophage cells clump with tinny lymphoid cells, these phenomena assume to that lymphoid cells are undergoes clonal selection process and failed to select (Fig 2A-B). Under light microscope, Alcian-PAS method recognized neutral glycoprotein's or polysaccharides are stained magenta colour by PAS namely large granulocyte containing pigmented macrophages centre called Melanomacrophage centre (MMC) and acidic mucosubstances of cytoplasmic matrix of the thymic stromal cells stained blue colour by Alcian Blue stain (Fig 3A). Hematoxylin and Eosin staining method also depicts deep brown color of MMC and characteristic blue colour of lymphoid and non-lymphoid cell nucleus (Fig 3B). The thymic stroma, which facilitates the proliferation, migration and interaction of thymocyte cells interact with macrophages, plasma cells (solid arrows) and other essential secretory epithelial cells that are involved in immune cell regulation are observed under light microscope (Fig 3A-B). Transmission Electron Microscopy (TEM), depict the thymic ultrastructure and sub-cellular componential interaction, including the identification of significant basal lamina region (BL) associated with vacuolar phagocytic cell (PC), dendritic epithelial cells (dEC) and secretory granulocyte cells (cGC)/ mast cells within the thymic stroma of *Anabas testudineus* (Fig 3A, 4A-D). Melanomacrophages centre exhibit intracytoplasmic granules containing electron-dense spine-like structures and round electron dense granules. Numerous large nucleated thymocytes (Th) interacted with electron-dense lysosomal bodies (LB), dilated mitochondria (dMt), cytoplasmic vacuoles, sub cellular debris into the macrophage centre and reticular epithelial cells are critically examined within the thymus gland of *Anabas testudineus* (Fig 2A-B, 4B-D). The macrophage delimited by a membrane is also observed throughout the Melanomacrophage centre of thymus gland in fish (*Anabas testudineus*) (Fig 4B). Apart from that, large endometabolic deposition

(glycogen/lipid-like storage materials) seen in perivascular region of basal lamina with a few numbers of larger vacuolar structure under transmission electron microscope (Fig 4D). These types of accumulation of melanomacrophage centers and

endometabolic deposition within the thymic stroma of *Anabas testudineus* is very much significant indicator for cell mediated immune regulation as well overall health status of this teleost species in a hypoxic aquatic condition.

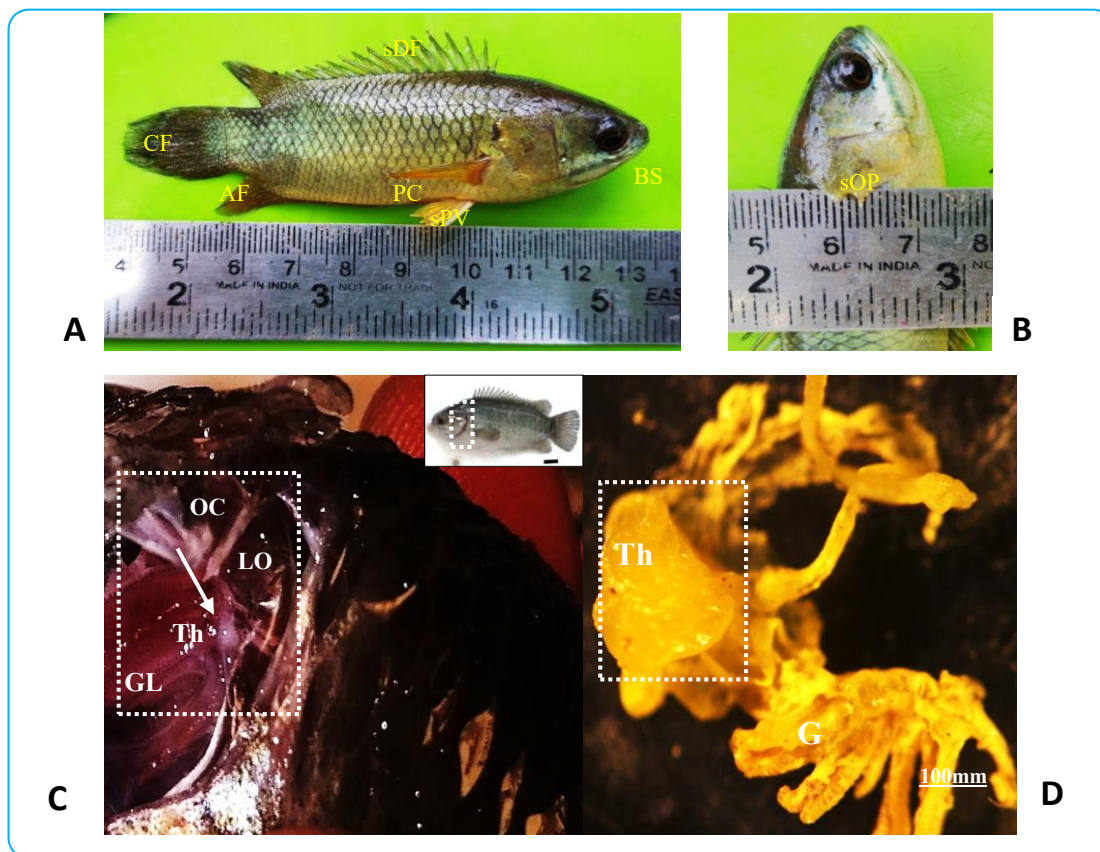


Fig 1 [A] Photograph shows morphoanatomy of the species *Anabas testudineus* (Bloch, 1792) (total body length 9 cm) marked with spiny double dorsal fin (SDF), blunt snout at anterior head region, spiny pelvic fin (sPV) close to soft ray's pectoral fin (PC), rounded caudal fin (CF) and anal fin (AF) at posterior region. [B] Photograph shows spiny operculum (SOP) of *A. testudineus* (12mm height). [C] Photograph shows location of thymus gland within the *A. testudineus* noted with opercular cavity (OC), gill lamellae (GL), accessory respiratory Labyrinth organ (LO) and position of thymus gland (solid arrow) [Not to scale]. [D] Bouin's fixed light microscope (stereo zoom) picture shows shape of thymus gland (Th) with developing gill lamellae (GL)

The functional origin of Melanomacrophages (MMC) were first explored by Agius [6], who illustrate pigmented macrophage-like cells in a developing rainbow trout (*Oncorhynchus mykiss*), appear after two weeks onset of feeding but the developmental origin of these center's was unclear. Zuasti *et al.* [23] provided further insight by signifying melanin-synthesis activity within the renal MMCs of gilthead sea bream (*Sparus auratus*), indicated functional role for these structures outside the inactive pigment accumulation. Later, Meseguer *et al.* [24] identified a subpopulation of mononuclear phagocytes in the head kidney of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*) to facilitate engulfment of melanin-laden cellular debris, suggesting to active phagocytic contribution in fish. Melanomacrophage centers (MMC) in lymphoid organs like, spleen, kidney and thymus (in *A. testudineus*) are potential for significant role in clearance of apoptotic cell organelles and antigen presentation. The Alcian Blue-Periodic Acid-Schiff (Alcian-PAS) stain differentiates the acidic muco-substances and neutral glycoproteins or polysaccharides within the thymic tissues (Fig 3 A-B). These metabolic components are critical for forming the thymic extracellular matrix, which supports lymphoid cell migration, interaction, and lymphoid cell selection for a haematopoietic stem cell of teleost species [22]. Alcian-PAS staining can also highlight the presence of secretory rich epithelial cells/ mast

cells and pigment-associated macrophages critically involved in immune regulation. The scanning ultrastructure discloses remarkable phagocytic activities of macrophages on the distinct outer surface of the thymus gland in *Anabas testudineus*. These macrophages interact directly with lymphoid cells that may employ in the phagocytosis of apoptotic cells and potentially detrimental for external biological or chemical milieus (Fig 2A–B). This process triggers first line of cell-mediated thymic defense critically for maintaining the tissue homeostasis and immune surveillance of fish. The presence of electron dense granules like, lipofuscin or melanin (Fig 4B) along with lysosomal bodies, distorted mitochondria (Fig 4C-D) and frequent appearance of plasma cells close to melanomacrophage centers (Fig 3A) within the thymic stroma strongly suggests a significant influence of the antigen presenting functions that regulate the overall immune system and thymic homeostasis in climbing perch (*Anabas testudineus*).

CONCLUSION

The thymus gland of *Anabas testudineus* situated on either side of the opercular cavity, it is always exposed to aquatic or moist atmospheric environmental conditions and resembles a surprising adaptive immune response against the

biological or chemical milieu in a fragile or degraded habitat of the species. This study emphasizes the distinct morphofunctional distribution and immunological impact of melanomacrophage centers (MMCs) within the thymic microenvironment of *Anabas testudineus*. The previous observations from several studies establish the crucial understanding of melanomacrophage centers (MMCs) mainly on the basis of pigment accumulation. But this finding demonstrated that thymic melanomacrophage centers (MMCs) exhibit active phagocytic properties that may engage in apoptotic cell clearance and potentially participate in antigen presentation of fish. Light and ultrastructural observations further reveal the presence of endometabolically active epithelial cells, macrophages, and plasma cells in close association with melanomacrophage centers (MMCs), which strongly trigger a provocative role in immune surveillance and tissue homeostasis in *Anabas testudineus*. This pertinent

evidence not only supports the definite function of thymic MMCs but also nurtures a significant role in cell-mediated thymic defense of fish (*Anabas testudineus*).

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Conflict of interest

Authors declare no conflict of interest that could affect this study.

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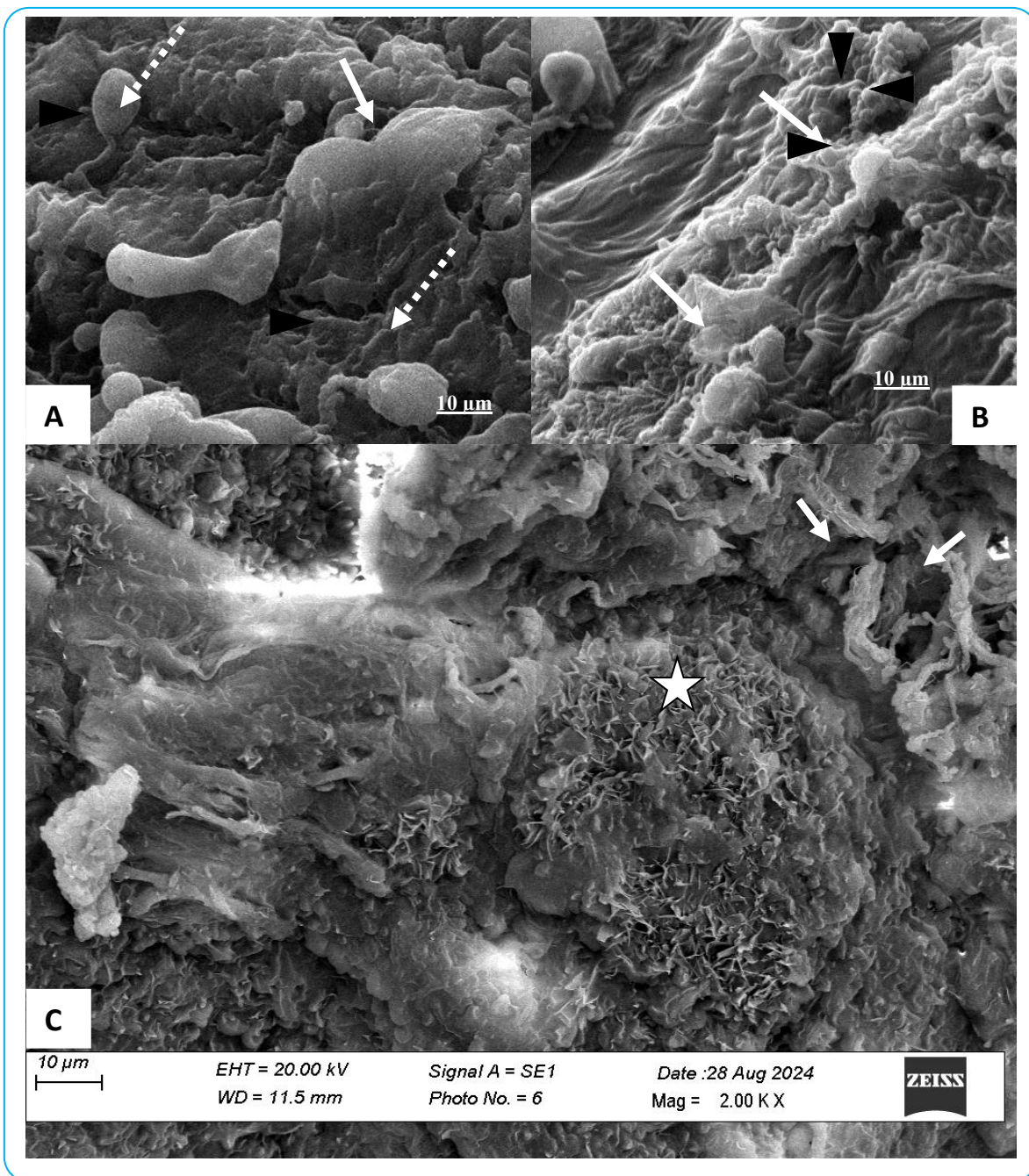


Fig 2 [A] Surface topography ultrastructure shows reticular epithelial cells (dashed arrow) closely associated with macrophage cells (solid arrow) on the ventral surface of the thymus gland of *A. testudineus*. [B] Scanning micrographs shows cytoplasmic protrusion of macrophage (solid arrows) and clump with tinny lymphoid cells (arrow head). [C] Ultrastructure depict the surface topography of thymus (*) with fibromuscular structure (arrows) that was always expose to external aquatic environment

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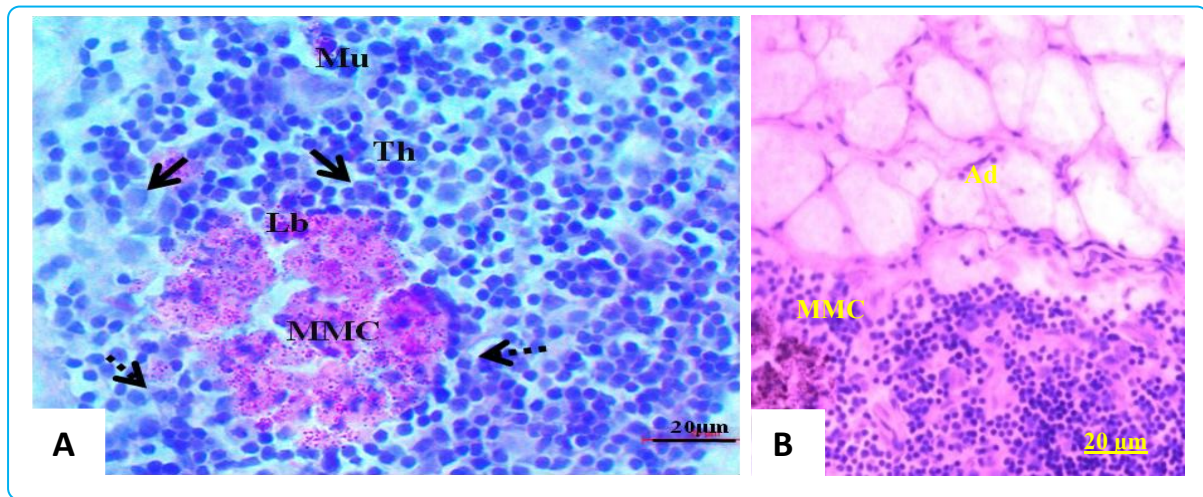


Fig 3 **[A]** PAS positive Melanomacrophages centre (MMC) with plasma cells (arrows), round nucleated numerous thymocytes (Th), lysosomal bodies (Lb), mucin deposition (Mu) and reticular epithelial cells (dashed arrows) within the thymus gland of *A. testudineus* are characterized under trinocular light microscope. **[B]** The LM photograph shows melanomacrophages center (MMC) within the thymic stroma of *A. testudineus* through H-E stained associated with adipose layer (Ad)

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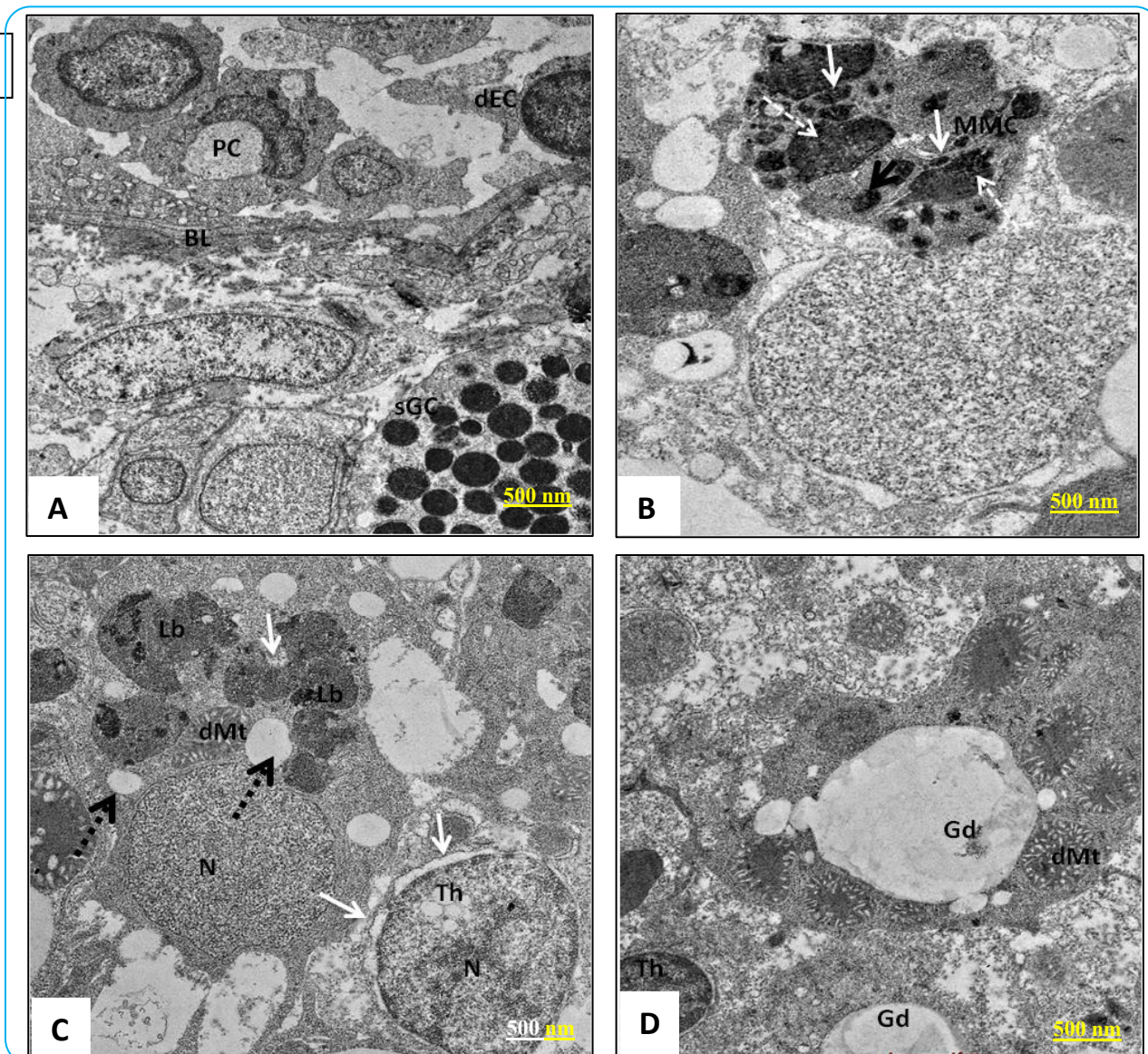


Fig 4 **[A]** Electron micrograph shows basal lamina region (BL) associated with vacuolar phagocytic cell (PC), dendritic epithelial cells (dEC) and secretory granulocyte cells (cGC)/ mast cells within the thymic stroma of *A. testudineus*. **[B]** EM photograph depict Melanomacrophages centre (MMC) displaying intracytoplasmic granules containing with electron-dense spine-like structures (white arrows) and round electron dense granules (dashed white arrows). **[C]** Photomicrograph shows large nucleated thymocytes (Th) interacted with electron-dense lysosomal bodies (LB), dilated mitochondria (dMt), cytoplasmic vacuoles, debris of macrophage centre and reticular epithelial cells (dashed arrows) within the thymus gland of *A. testudineus*. [Note: the macrophage delimited by a membrane (white arrows)]. **[D]** Large cytoplasmic deposition (glycogen like storage materials) shows within the large vacuolar structure under TEM

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