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ORIGINAL ARTICLE

Cellular elements organization in the trachea of mallard (*Anas platyrhynchos*) with a special reference to its local immunological role

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Abstract

Many studies have been carried out to investigate the histological structure of the trachea in many species of birds. However, the cellular organization of the trachea in the mallard duck is still unclear. This study was performed on 12 sexually mature male Mallard duck to demonstrate the cellular organization of the trachea using light and electron microscopy. The tracheal epithelium is considered the first line of defense against airborne pathogens. The mallard trachea was lined by a pseudostratified ciliated columnar epithelium that contained many morphologically distinct cell types: ciliated, non-ciliated, basal cells that encircled by a population of sub-epithelial immune cells, fibroblasts, and telocytes (TCs). Telocytes were first recorded in duck trachea in this study and showed a wide variety of staining affinity. They presented two long telopodes that made up frequent close contacts with epithelium, tracheal cartilages, and other neighboring TCs, immune cells, blood capillaries, and nerve fibers. TCs express VEGF and S-100 protein. The immune cells include mast cells, eosinophils, basophils, lymphocytes, plasma cells, and dendritic reticular cells. The ciliated tracheal epithelium was interrupted by numerous intraepithelial mucous glands and solitary goblet cells. This mucociliary apparatus constitutes the major defense mechanism against inhaled foreign materials. The cellular organization of the duck trachea and its relation to the immunity was discussed.

Keywords Telocytes · Dendritic reticular cells · Immune cells · Mucous glands

Introduction

The mallard (*Anas platyrhynchos*) is the most common and widely distributed dabbling duck, having a widespread global distribution throughout Northern and Southern Hemispheres (Bentz 1985; Söderquist et al. 2013). Mallards have had a long relationship with humans. Almost all breeds of domestic duck are derived from the mallard, except for a few Muscovy breeds. It is considered an important source of meat and egg production that have a high nutritional value as human food (Pingel 2011). In North America, it generates millions of dollars per year in various ecosystem services (Green and Elmberg 2014). It is also one of the most important game species in the world (Elmberg 2009). Mallard is considered one of the most economically valuable bird species in many countries. However, recent studies recorded it as the main vector of the highly pathogenic avian influenza virus (HPAIV) (H5N1) (Keawcharoen et al. 2008).

Main functions of the respiratory system in birds are gas exchange, thermo-regulation, and contribution to voice production and local immunity (Lbe et al. 2008; Reece 2005; Nash 2007). The trachea provides a pathway for conducting air from the larynx to the lungs. Moreover, it is considered the main structure involved in local immunity of upper respiratory tract in birds so that the tracheal mucosa was used as a suitable experimental model for evaluating the possible changes after vaccination of avian species (Pourlis et al. 2018). Despite the importance of the avian trachea in normal and pathological conditions, there is no accurate morphological description of this organ in the domestic bird in general and no morphological description at all in mallard duck in particular.

Telocyte (TC) is a unique type of interstitial cells that has been recorded recently in various organs of humans and

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laboratory mammals (Hussein and Mokhtar 2018). However, ultrastructural characterization of TCs remains ill-defined in birds. Various functions of telocytes have been suggested. However, the main critical role of TCs is the stromal organization through modification of intercellular communication. Also, the tracheal TCs could be included in the regulation of the tracheal functions such as secretion and contractility (Rusu et al. 2012).

The present study aimed to provide a detailed description for the normal histological structure of trachea in mallard duck, focusing on the distribution and functional dependencies of telocytes and immunocompetent cells involved in the local immunity of the tracheal wall. This was achieved by investigating the trachea of mallard duck morphologically using light and electron microscopy, and immunohistochemistry.

Material and methods

Sample collection

This study was approved by the Ethics Committee of Assiut University, Egypt. The specimens were collected from 12 sexually mature male Mallard duck. Birds were killed by the intramuscular injection of a combination of ketamine HCl (Alfamine-Alsafan International, Holland) (20–60 mg/kg) and diazepam (Diazem-Deva) (2–4 mg/kg) (Thurmon et al. 1996). The whole trachea was exposed, extracted, and flushed with normal saline and taken immediately for fixation. Each trachea was divided into a proximal part (extends from the cricoid cartilage of the larynx to the thoracic inlet) and a distal part (extends from the thoracic inlet to the first syringe-tracheal cartilage).

Histological and histochemical preparation

Specimens for histological examination were taken from both proximal and distal parts. They were dissected at 1 × 1× 0.05 cm and immediately fixed in Bouin's fluid for 22 h. The fixed tissues were dehydrated in an ascending series of ethanol, cleared in methyl benzoate, and then embedded in paraffin wax. Serial transverse paraffin sections at 5–8 μ in thickness were cut. Histological sections were stained with Crossmon's trichrome, Gomori's silver method, PAS, Safranin O, Van Gieson-Alcian blue, Grimelius silver nitrate, and Weigert's elastica stain (Bancroft and Gamble 2002), and Giemsa stain (Giemsa 1904).

Immunohistochemistry

The immunohistochemical study was performed on formalin-fixed, paraffin-embedded tracheal tissues that cut at 4 μm thick. The sections were treated with 10 ml ML Tris buffer and 1 ml Ml ethylenediaminetetraacetic acid, pH 9.0 for 20 min at 90 °C. Endogenous peroxidase was inhibited by soaking the sections with 3% H_2O_2, and preincubation overnight at 4 °C in 1% bovine serum albumin in PBS. The sections were stained for 30 min at room temperature, using the following antibodies: rabbit polyclonal anti-vascular endothelial growth factor (VEGF) (1:300; Abcam, Cambridge, UK) and rabbit polyclonal anti-S100 protein antibody (1:200; Thermo Fisher Scientific, Cat. RB-044-A0) according to the avidin–biotin peroxidase complex method, as previously reported (Hsu et al. 1981). Sections were counterstained with hematoxylin, photographed by a Leica microscope (Germany).

Semi-thin and transmission electron microscopy

Small specimens of tracheal tissue taken from both proximal and distal parts were fixed in a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mL sodium-cacodylate buffer, pH 7.3, and left overnight at 4 ° C (Karnovsky 1965). They were washed and post-fixed in 1% osmic acid in 0.1 mL sodium-cacodylate buffer for 2 h at room temperature. The tissues were dehydrated in ethanol and embedded in Araldite-Epon mixture. Semithin sections (1 μm in thickness) were cut and stained using toluidine blue and examined under a light microscope. Ultrathin sections were stained with lead citrate and uranyl acetate (Reynolds 1963) and examined under the JEOL 100 II transmission electron microscope.

Scanning electron microscopy

Specimens of tracheal tissue were washed by 0.1 M sodium-cacodylate buffer. Then they were fixed in a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 ML sodium-cacodylate buffer, pH 7.3 at 4 °C for 4 h. Thereafter, they were washed and post-fixed in 1% osmic acid in 0.1 mL sodium-cacodylate buffer for 2 h at room temperature. The samples were dehydrated and critical point dried with a Polaron apparatus. Finally, they were coated with gold and photographed using the JEOL scanning electron microscope (JSM–5400 LV) at KV 10.

Results

Histological analysis

Cross-section in the trachea of mallard duck appeared oval in shape with closed cartilaginous rings partially overlapping each other (Fig. 1A). No considerable differences in the histological structure were observed between the proximal and

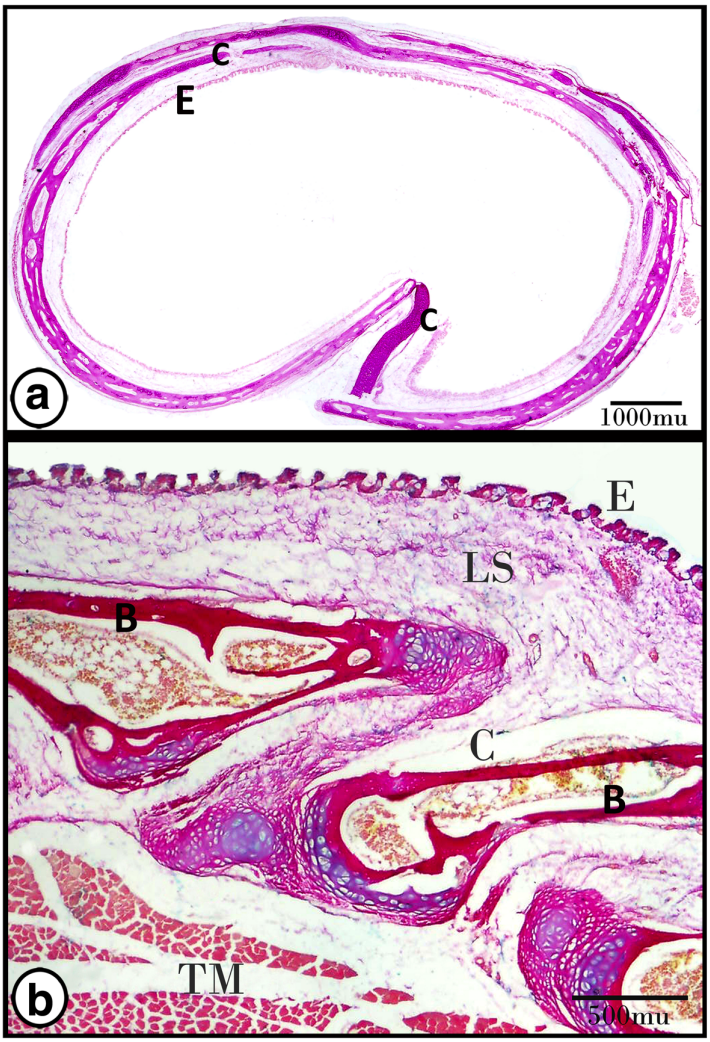

Fig. 1 General view of the trachea of mallard stained by Van-Gieson-Alcian blue. (**A**) Cross-section in the trachea with closed cartilaginous rings (C) partially overlapping each other. E: epithelium. (**B**) The tracheal wall was lined by respiratory epithelium (E), rested on lamina propria-submucosa (LS). Note the partially ossified tracheal cartilage (C), bone tissue (B), and tracheal muscle (TM)

distal part. The tracheal wall, in general, was lined by respiratory epithelium. The epithelium was rested on lamina propria-submucosa, represented by a layer of dense connective tissue that is surrounded by complete hyaline cartilaginous rings (partially ossified) and covered externally by tunica adventitia or serosa (Fig. 1B).

The mallard trachea was lined by a pseudostratified ciliated columnar epithelium that contained many morphologically distinct cell types. Ciliated cells were the main cell type. The ciliated cells appeared columnar in shape with vacuolated cytoplasm, contained large lightly stained nuclei, and possessed numerous long cilia. Basal cells appeared as relatively small oval to triangular cells. They were attached to the basement membrane and their apices did not reach the airway lumen (Fig. 2A). The tracheal epithelium was interrupted by numerous intraepithelial acinar glands that were formed by aggregated groups of mucous secreting cells producing exclusive amounts of mucus (Fig. 2B, C). Moreover, solitary goblet cells were also recognized within the tracheal epithelium (Fig. 2B, D). Some intraepithelial acinar glands showed a clear apoptotic activity and a complete sloughing of their secretory cells (Fig. 2E, F), while other glands showed a metachromatic reaction (Fig. 2G, H). Intraepithelial lymphocytes could be demonstrated in semithin sections as small darkly stained cells located mainly in the basal borders of the epithelium as well as some lymphocytes were demonstrated in the lamina propria-submucosa (Fig. 2G, H).

Histochemical analysis

Intraepithelial glands and solitary goblet cells exhibited positive reactions for both PAS and Alcian blue stains (Fig. 3A–C). Furthermore, they showed a metachromatic reaction to toluidine blue (Fig. 3D). In addition, lamina propria-submucosa showed a network of elastic, reticular, and collagenous fibers (Fig. 3E–G). The tracheal cartilage ring consisted

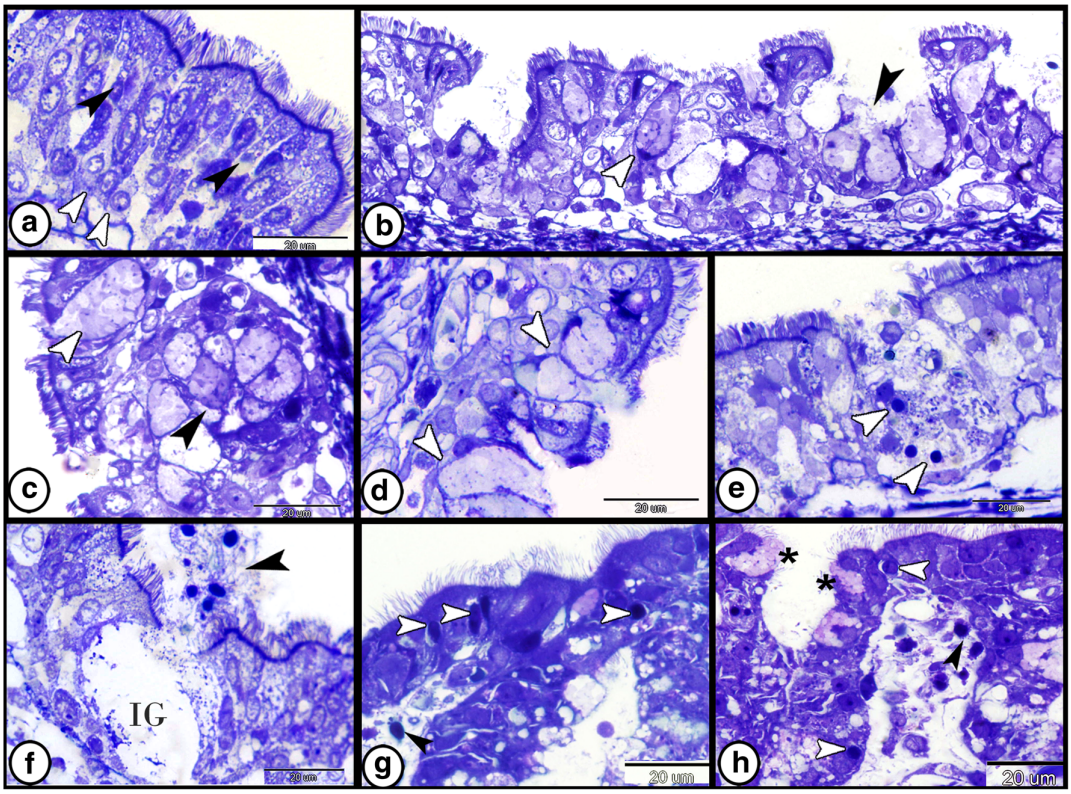

Fig. 2 Semi-thin section in the trachea of mallard stained by Toluidine blue. (**A**) Long ciliated cells (black arrowheads) and small basal cells (white arrowheads). (**B–D**) The tracheal epithelium was interrupted by intraepithelial acinar mucous glands (black arrowheads) and solitary goblet cells (white arrowheads) opening directly to the tracheal lumen. (**E**) The intraepithelial gland showed clear apoptotic figures (white arrowheads). (**F**) An intraepithelial gland (IG) showed a complete sloughing off its dead secretory cells in the form of apoptotic bodies (black arrowhead). (**G**, **H**) Intraepithelial lymphocytes (white arrowheads). Note, the metachromatic reaction of intraepithelial glands (asterisks) and the presence of lymphocytes (black arrowheads) in the lamina propria

of chondrocytes embedded in a large amount of extracellular matrix and surrounded by a zone of condensed supporting tissue (perichondrium) containing chondroblasts. The extracellular matrix was partially ossified containing several trabeculae of spongy bone enclosing numerous bone marrow foci (Fig. 3J). The calcifying cartilage showed a strong positive reaction to both PAS and Safranin O (Fig. 3H, I). The perichondrium of tracheal cartilage was surrounded by a network of elastic, reticular, and collagen fibers which merged with adventitial coat of the trachea (Fig. 3J–L).

Giemsa stain demonstrated mast cells invading the tracheal epithelium (Fig. 4A, B). In addition, mature lymphocytes, macrophages, and mast cells were recognized within the bone marrow foci of ossified tracheal cartilage (Fig. 4C). Moreover, the toluidine blue revealed the presence of a massive infiltration by mast cells and lymphocytes in tunica adventitia surrounding the blood capillaries (Fig. 4D).

The telocytes could be demonstrated within the epithelium and their long and thin processes (telopodes) extended around the intraepithelial acinar glands (Fig. 5A). They also were attached to the tracheal cartilages (Fig. 5B) and their telopodes established a network in the submucosa (Fig. 5C). Grimelius silver nitrate and Safranin O stains showed many telocytes with long telopods extending between the connective tissue fibers (Fig. 5D, E). Semithin sections demonstrated telocytes and a population of sub-epithelial immune cells includes dendritic reticular cells and lymphocytes in the lamina propria-submucosa. Telocytes with toluidine blue appeared as small dark cells that possessed small cell telopodes (Fig. 5F). Table 1 summarized the staining affinity of telocytes and other tracheal components.

Immunohistochemistry

Telocytes expressed vascular endothelial growth factor (VEGF) and could be observed within the epithelium and around the intraepithelial mucous glands or established a network in the lamina propria (Fig. 6A–D). VEGF-positive TCs were also located in close contact with ossified cartilages (Fig. 6E, F).

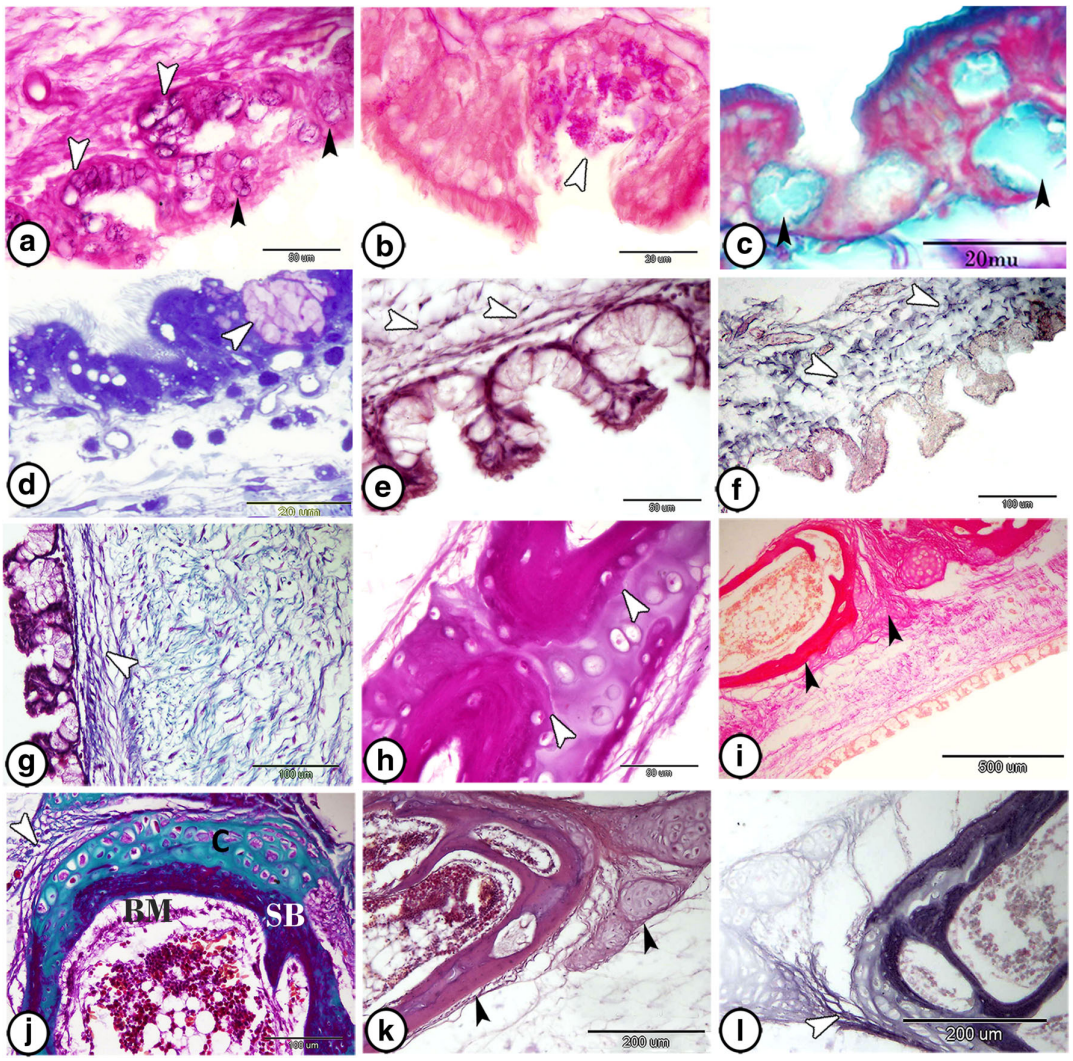

Fig. 3 Histochemical analysis of mallard trachea. (**A**, **B**) The lining cells of intraepithelial acinar glands (white arrowheads) and solitary goblet cells (black arrowheads) showed PAS-positive reaction. (**C**) The lining cells of intraepithelial glands stained by Van Gieson-Alcian blue showed Alcian blue positive reaction (black arrowheads). (**D**) The intraepithelial glands (arrowhead) showed metachromatic reaction by toluidine blue. (**E**) Well-distinct elastic fibers (white arrowheads) were recognized in lamina propria-submucosa by Weigert's Elastica. (**F**) A network of reticular fibers (white arrowheads) was also observed in lamina propria-submucosa by Gomori stain. (**G**) Bundles of collagenous fibers (white arrowheads) were demonstrated by Crossmon's trichrome in lamina propria-submucosa. (**H**) The calcifying tracheal cartilage showed a strong positive reaction to PAS (white arrowheads). (**I**) The calcifying tracheal cartilage showed a strong positive reaction to Safranin O (black arrowheads). (**J**) A part of tracheal cartilage (C) showed endochondral ossification by Crossmon's trichrome. Note the spongy bone (SB) enclosing a bone marrow area (BM). Fine bundles of collagen fibers (white arrowhead) surrounded the tracheal cartilage. (**K**, **L**) Networks of elastic (black arrowheads) and reticular fibers (white arrowhead) covered the perichondrium of calcified tracheal cartilage were observed by Weigert's elastica and Gomori stain respectively

The TCs also showed an intense expression of S-100 protein within the epithelium (Fig. 7A). Dendritic cells (DCs) with fine dendritic-like processes were also expressed S-100 protein. DCs were arranged in groups under the epithelium, in close contact with its basement membrane (Fig. 7B). In the lamina propria, the telopodes of TCs form a network (Fig. 7C). S-100 protein–positive TCs could be demonstrated in close contact with ossified cartilage (Fig. 7D).

Transmission electron microscopy

The tracheal epithelium

The lining epithelium consisted mainly of tall ciliated cells with large euchromatic nuclei. Their cytoplasm showed clear cytoplasmic vacuoles of different size and shapes (Fig. 8A). Numerous cilia emanated from the surface of each ciliated cell, as well as numerous shorter microvilli were

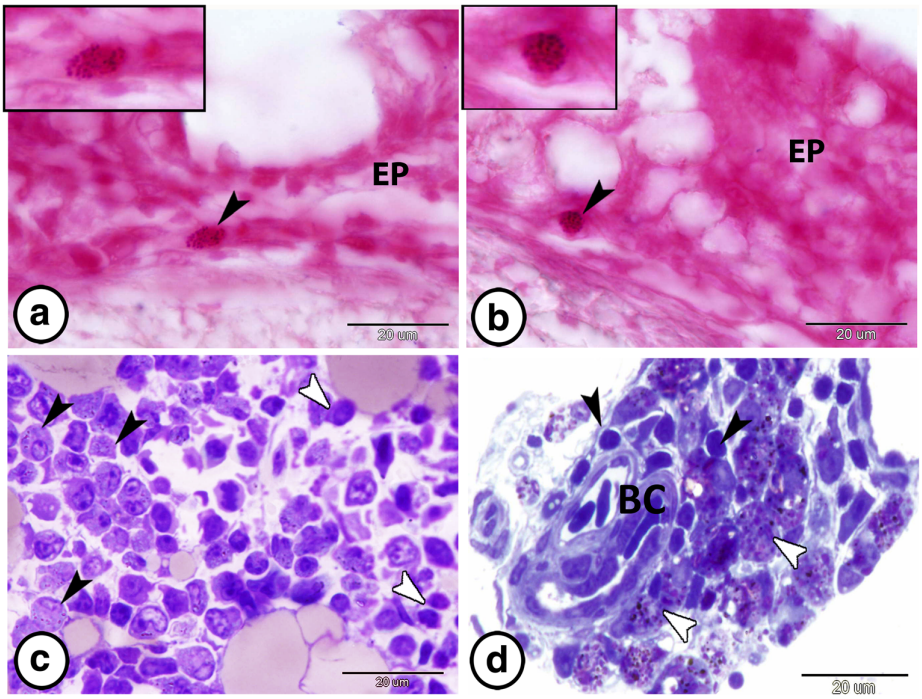

Fig. 4 The immune cells by Giemsa stain (**A**, **B**) and toluidine blue (**C**, **D**). (**A**, **B**) Mast cells (black arrowheads) were recognized within the tracheal epithelium (EP). (**C**) Mast cells (black arrowheads), lymphocytes (white arrowheads), and macrophage (arrow) were recognized within the bone marrow foci of ossified tracheal cartilage. (**D**) Massive infiltration by mast cells (white arrowheads) and lymphocytes (black arrowheads) was recognized in tunica adventitia surrounding the blood capillaries (BC)

demonstrated. The cells were connected to each other at their apical surfaces by tight junctions and laterally by desmosomes (Fig. 8B).

The mucous glands

Multicellular intraepithelial mucous glands were observed in association with solitary goblet cells within the tracheal epithelium. The cytoplasm of secretory cells was distended and filled by membrane-bound electron-lucent secretory granules (Fig. 8C, D).

The sub-epithelial immune cells

A population of active fibroblasts was observed immediately beneath the basement membrane. They showed many irregular cytoplasmic processes, large and euchromatic nuclei, and their cytoplasm contained numerous cytoplasmic vacuoles of different size (Fig. 9A). Plasma cell was also recognized with its characteristic eccentric nucleus and abundant rER (Fig. 9B). Dendritic cells were recorded underneath the epithelium. They displayed elongated to rounded cell bodies and possessed elongated cytoplasmic extensions or dendrites and showed an obvious high nuclear to cytoplasmic ratio. The nucleus was mainly heterochromatic and the cytoplasm contained mitochondria and few electron-dense secretory granules (Fig. 9A, C, D). A granular white blood cell was demonstrated in the lamina propria-submucosa in association with the dendritic cell (Fig. 9D).

Telocytes

Numerous TCs were observed along the lamina propria-submucosa. Telocyte presented two long telopodes that made up recurrent close contacts with other neighboring TCs, immune cells, blood capillaries, and nerve fibers (Figs. 9B and 10A–C). Telocyte showed a particular ultra-structural signature in the trachea of the mallard. It consisted of a small spindle-shaped cell body surrounded by a thin rim of cytoplasm containing few mitochondria and small secretory vesicles (Fig. 10D). Notably, the dendritic cells contributed in the formation of blood capillaries with endothelium and telocytes (Fig. 11A, B).

Scanning electron microscopy

Investigation of the luminal surface of mallard trachea revealed rows of ciliated cells interrupted by shallow narrow areas of non-ciliated cells (Fig. 12a, b). The ciliated cells were abundant without visible cell boundaries between them. However, the surface of non-ciliated cells exhibited numerous microvilli (Fig. 12d). Some non-ciliated cells possessed small protrusions that may represent the evidence of active merocrine secretory phase (Fig. 12c). Mucous occurred as discharged droplets which partially covered the luminal surface of some secretory cells and entangled in the cilia (Fig. 12d).

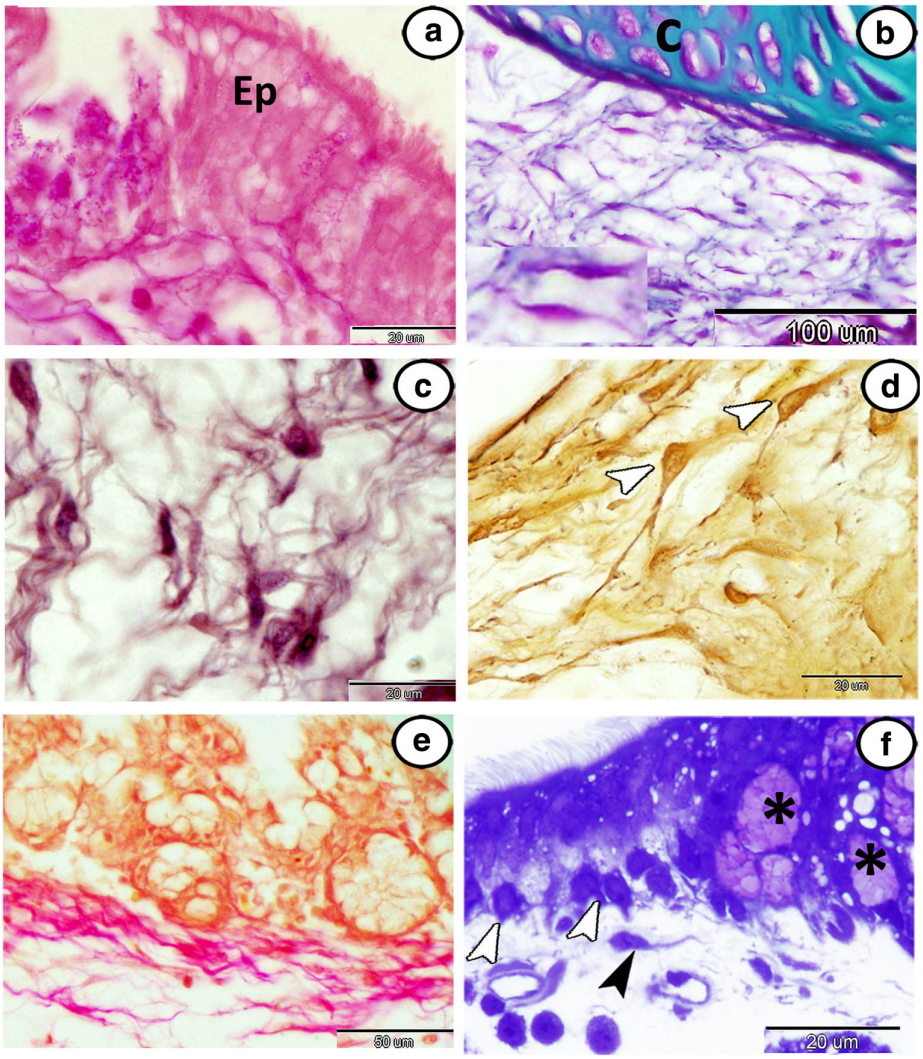

Fig. 5 The distribution of telocytes in the trachea with different stains. (**A**) Telocytes (boxed areas) within the epithelium (Ep) and around the glands (PAS/Van Gieson). (**B**) Telocytes (boxed areas) in the submucosa, attached to the cartilage (C) by Crossmon's trichrome stain. (**C**) By Weigert's/Van Gieson, TCs (boxed areas) form a network in the submucosa. (**D**) Telocytes (black arrowheads) stained dark brown by Grimelius silver nitrate method. Note that TCs showed long and thin telopods that connect to each other forming a network. (**E**) TCs (boxed areas) in the submucosa by Safranin O/Van Gieson. (**F**) Some intraepithelial glands showed metachromatic reaction (asterisks). A population of dendritic cells (white arrowheads), telocytes (black arrowheads), and lymphocytes (arrows) were demonstrated in lamina propria-submucosa

Discussion

The morphologic features of trachea had been investigated in some bird species (King and Mclelland 1984; Tasbas et al. 1986, 1994). However, this study offered a detailed description of the normal histo-morphological characteristics of the trachea in mallard duck for the first time.

The present investigation showed that the lining epithelium of mallard trachea was similar to that observed in turkey (AL-Mussawy et al. 2012) and quail (Pourlis et al. 2018). It is

Table 1 Histochemical staining reactions of the trachea

	Intraepithelial glands	Goblet cells	Telocytes	Calcifying cartilages	Mast cells
PAS	+	+	+	+	
Alcian blue	+	+			
Grimelius silver stain			+		
Safranin O			+	+	
Giemsa stain					+
Toluidine blue	+	+	+		+
Weigert's/Van Gieson			+		
Crossmon's trichrome			+	+	

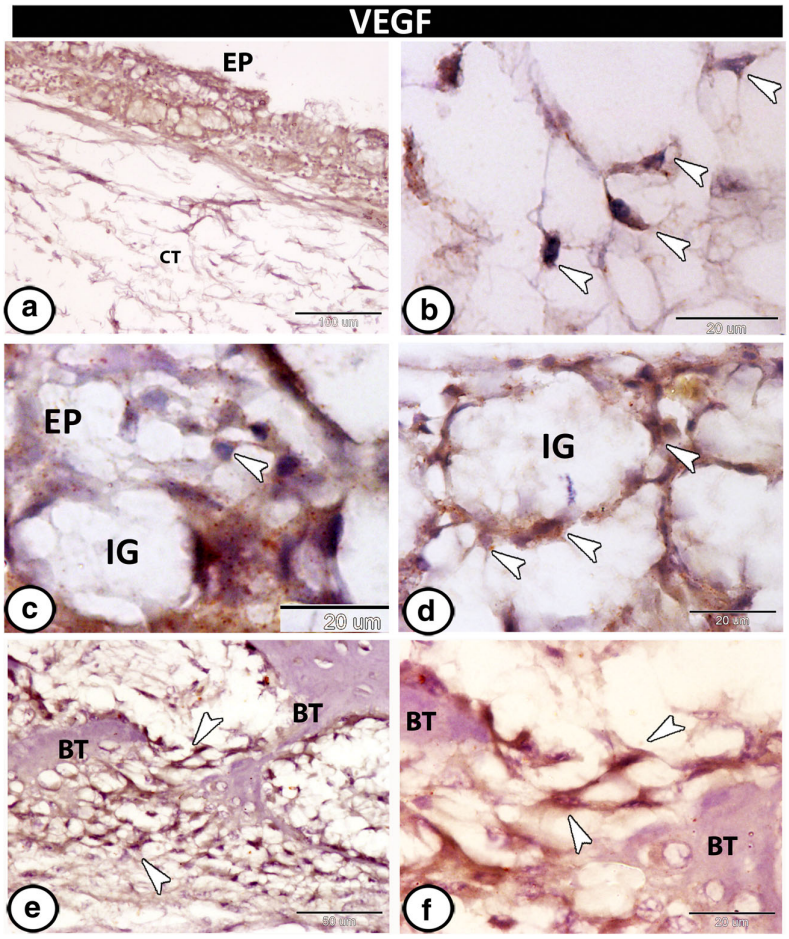

Fig. 6 VEGF immunohistochemistry. (A) An overview of mallard trachea showing the magnified areas; epithelium (EP) and connective tissue (CT). (B) TCs (arrowheads) established a network in the lamina propria. (C, D) VEGF-positive TCs (arrowheads) could be observed around intraepithelial mucus glands (IG). (E, F) Telocytes (arrowheads) expressed VEGF and were identified in close contact with bone trabeculae (BT)

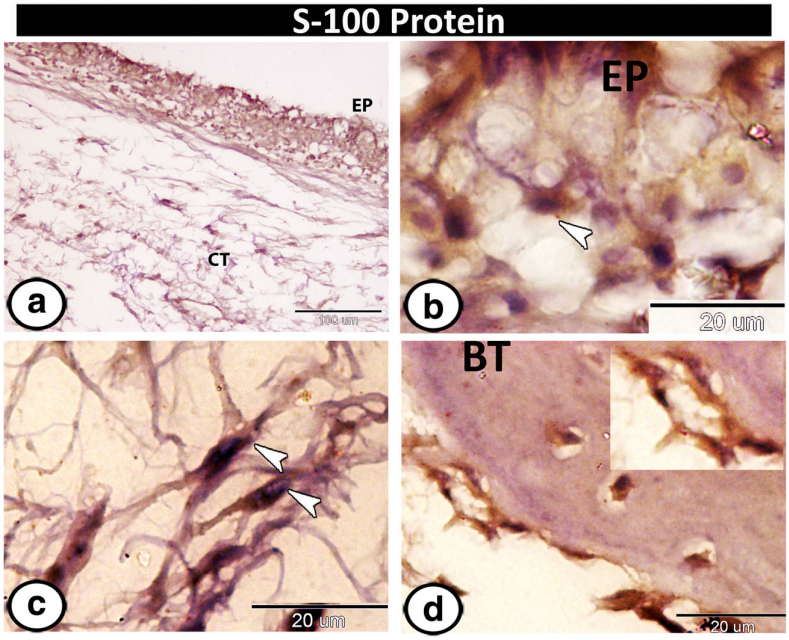

Fig. 7 S-100 protein immunohistochemistry. (A) An overview of mallard trachea showing the magnified areas; epithelium (EP) and connective tissue (CT). (B) TC (arrowhead) showed an intense expression of S-100 protein within the epithelium (EP). (C) The telopodes of TCs form a network (arrowheads) in the lamina propria. (D) S-100 protein-positive TCs (boxed areas) could be demonstrated in close contact with bone tissues (BT)

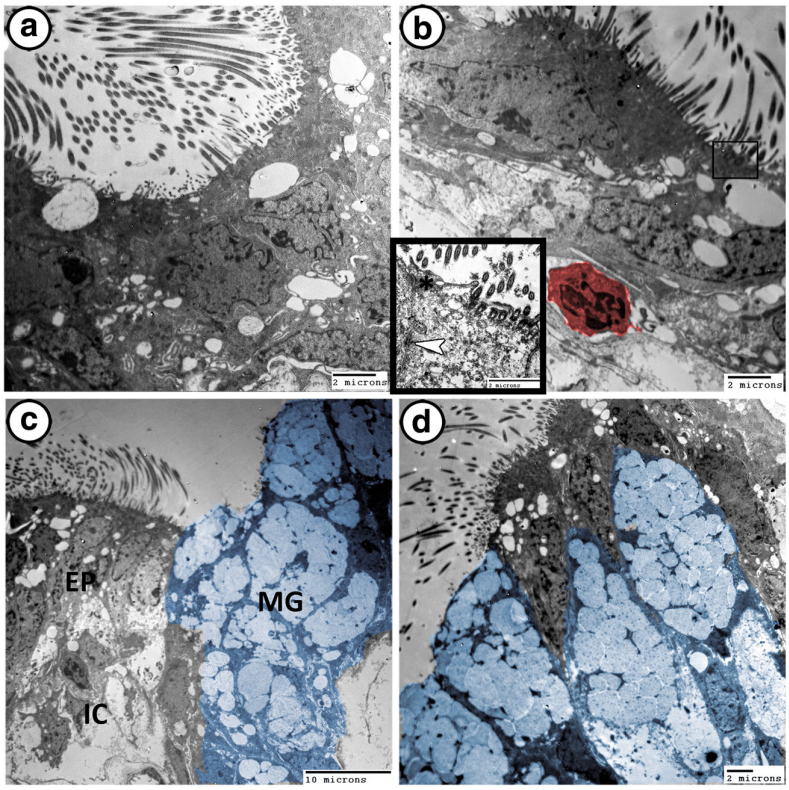

Fig. 8 Digital colored TEM images of the tracheal epithelium in mallard. (A) The tracheal epithelium consisted of ciliated and secretory cells. (B) A lymphocyte (red) was observed in the basal part of the epithelium. The magnified square showed the apical part of the ciliated cell that contained basal bodies for cilia. Note the epithelial cells were connected by an apical tight junction (asterisk) and desmosomes (arrowhead). (C) Intraepithelial mucous gland (MG, blue) was seen in association with ciliated epithelial cells (EP) and basally located intraepithelial immune cells (IC). (D) Solitary goblet cells (blue) filled by electron-lucent cytoplasmic granules were demonstrated within the epithelium

represented by ciliated epithelial cells interrupted with numerous intraepithelial acinar mucous glands and solitary goblet cells. The tracheal epithelium acts as the first line of defense against airborne pathogens. Similar to mammals (Godfrey et al. 1992; Herard et al. 1996) and other avian species (Lali and Ibrahim 1984), the tracheal epithelium in mallard trachea forms a strong physical barrier that is impermeable to most pathogenic agents. This barrier is achieved by the strong attachment of the epithelial cells with each other by tight junctions and desmosomes.

Vareille et al. (2011) reported that the airway epithelium is an effective strong impermeable mechanical barrier that is essential in the maintenance of a suitable ionic gradient for the secretion of many substances. Tight junctions ensure a professional impermeability of the epithelial barrier, enable adjacent cells communication, and regulate intercellular molecular exchange and transport (Roche et al. 1993). On the other hand, desmosomes form strong adhesive bonds in the form of a unique network that provide mechanical strength to tissues (Green and Simpson 2007). Cell–cell junctions act as a strong barrier that prevents the virus entrance into the airway submucosa. This is performed by hindering viral access to receptors in the epithelial plasma membrane, which is an important entry and interaction location for several viruses (Bergelson 2003; Bergelson 2009). It is interesting that recent studies have demonstrated a high focus on retinoic acid-inducible gene I (RIG-I) which is a key regulator of type I interferon (IFN) antiviral signaling at tight junctions. This suggests that cellular junctions between epithelial cells could also interact with antiviral innate responses (Mukherjee et al. 2009). Moreover, the mucous layer covering the airway epithelium provides a further protective mechanism by creating a semi-permeable barrier that allows the exchange of gases, water, and nutrients while being impermeable to most pathogens (Voynow and Rubin 2009).

Ciliated epithelial cells in mallard trachea showed relatively large cytoplasmic vacuoles and the non-ciliated cells exhibited microvilli and suggesting a secretory activity. A recent study by McConnell et al. (2009) indicated that microvilli may function as vesicle-generating organelles in vivo and containing several active catalytically enzymes. Hiemstra (2001) added that airway epithelial cells produce a variety of antiviral substances as well as pro-inflammatory cytokines, which are important in immediate innate immune responses and the initiation of mechanisms of adaptive immunity. The most important epithelium-derived antiviral host defense molecules are interferons (type I and III IFNs), lactoferrin (LF), β-defensins (BDs), and nitric oxide (NO). In addition, the airway epithelial cells play a critical role in immune responses to viral infection by secreting several chemokines and cytokines into the submucosa (McNamara et al. 2004, McNamara et al. 2005).

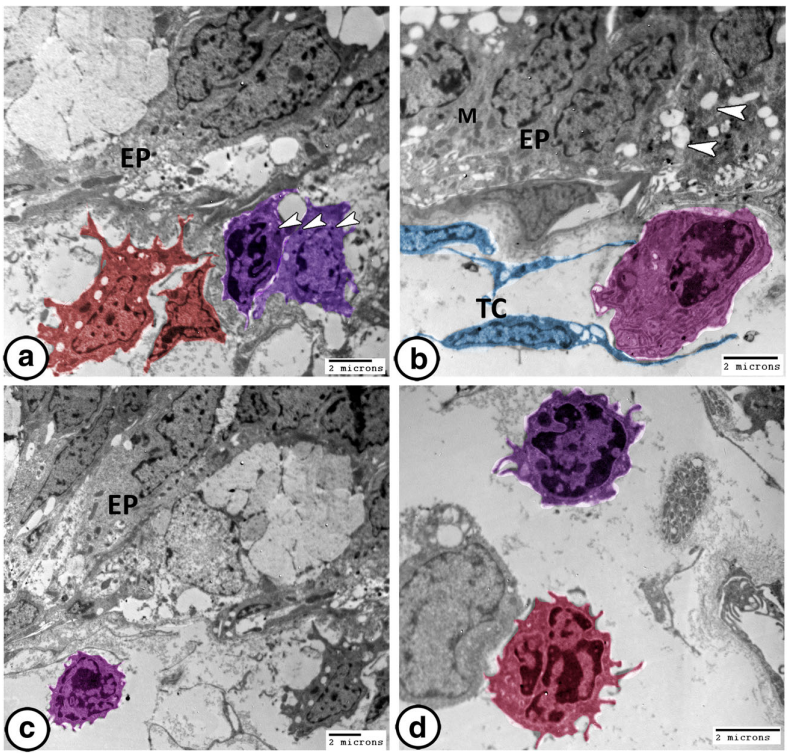

Fig. 9 Digital colored TEM images of the distribution of the immune cells in the tracheal wall. (A) Sub-epithelial fibroblasts (red) and dendritic reticular cells (violet) with few secretory granules (arrowheads) were seen underneath the basement membrane of the epithelium (EP). (B) Under the epithelium (EP), a plasma cell (pink) was connected with telopodes of telocytes (TC, blue). The plasma cell is characterized by an eccentric nucleus and abundant rER. Note the cytoplasm of the epithelium contained many mitochondria (M) and cytoplasmic vacuoles (arrowheads). (C, D) Dendritic cells (violet) were recorded underneath the epithelium in association with granulated white blood cell (red). The dendritic cells showed characteristic cell processes and an obvious high nuclear to cytoplasmic ratio. The nucleus is mainly heterochromatic and the cytoplasm contained mitochondria

Contrary to mammalian species and some other avian species, we did not recognize any glands in lamina propria-submucosa of mallard trachea. However, this is compensated by the presence of excessive multicellular intraepithelial mucous glands in association with numerous solitary goblet cells within the tracheal epithelium. They produce a considerable quantity of neutral and acidic mucus that forms a continuous coat covering the surface of mallard tracheal epithelium. The cilia and the mucous coat work in a coordinated manner to form a strong defense mechanism against large inspired particles and some airborne pathogenic agents. This defense mechanism is called mucociliary clearance. Ximena and Bustamante-Marin (2018) stated that mucociliary clearance is considered the primary innate defense mechanism in conducting airways of the mammalian respiratory system. This mechanism comprised some functional protective components, including the protective mucous coat, the airway surface liquid layer, and the cilia on the surface of ciliated cells. Cilia on the surface of ciliated cells play a critical protective role. Beats of these cilia can transport about 90% of the inhaled particles, including respiratory viruses from the trachea to the oropharynx. On the other hand, Nicholas et al. (2006) reported that the airway mucous coat has clear antiviral and anti-inflammatory properties. He added that the mucus secreted by respiratory epithelium is a visco-elastic gel that is composed of about 200 different proteins including antimicrobial substances (defensins and lysozyme), cytokines, and antioxidant proteins.

Ultrastructural examination of mallard trachea showed the presence of a population of fibroblasts located directly underneath the lining epithelium. This finding is matched with the attenuated fibroblast sheath in the mammalian airway that has been speculated to have the ability of interaction with adjacent epithelial cells and to be involved in the regulation of local inflammatory and regeneration processes (Evans et al. 1999).

Different varieties of immune cells were recognized in mallard trachea especially in lamina propria submucosa, tunica adventitia, and bone marrow foci of the ossified tracheal cartilage. Lymphocytes were recorded singly underneath the tracheal epithelium, throughout

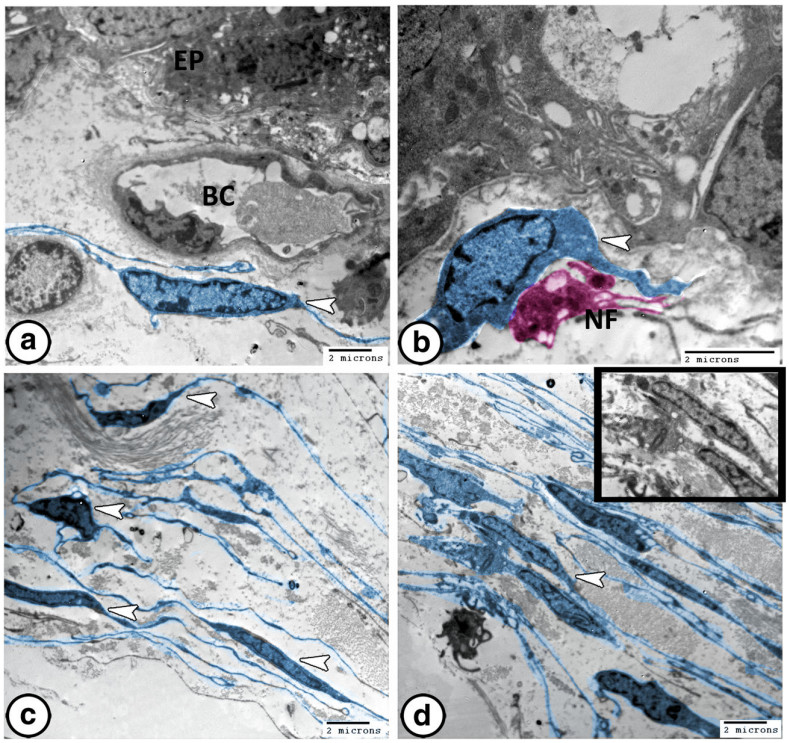

Fig. 10 Digital colored TEM images of the distribution of the telocytes (blue) in the tracheal wall. (A) Telocyte (arrowhead) with long and thin telopodes was recognized around the blood capillary (BC) underneath the epithelium (EP). (B) Telopode (arrowhead) is extended around the nerve fibers (NF) in the lamina propria. (C, D) Many telocytes with their telopodes (arrowheads) were distributed in the submucosa in association with collagen fibers. A magnified square showing a spindle-shaped cell body of the telocyte that contained euchromatic nucleus and the cytoplasm contained small secretory vesicles and mitochondria

the lamina propria, and surrounding the blood capillaries in tunica adventitia. Moreover, intraepithelial lymphocytes (IELs) were identified within the tracheal epithelium. The lymphocytes are implicated in reacting to inhaled antigens as well as they are involved in the modification of functions of the airway epithelial cells (Erle and Pabst 2000). Intraepithelial lymphocytes (IELs) are a heterogeneous population of T lymphocytes, which are recognized within the lining epithelium of the mucosal organs including the intestine, the oral cavity, the reproductive tract, the upper respiratory tract, and the lungs (Lap ae Silva et al. 1989). The majority of IEL in the upper respiratory tract and bronchi are T lymphocytes. The CD81 cytotoxic/suppressor T cells are usually the predominant over CD41 T-helper cells IELs. Their main function is to maintain the integrity of the mucosal barrier by the protection of the epithelium against pathogenic agents or immune-induced pathology (Goto et al. 2000; Erle and Pabst 2000).

Mast cells (MCs) are the key inflammatory cells. They are highly distributed at sites that are exposed to the external environment, such as the skin, airways, and gastrointestinal tract (Galli et al. 1999). In our study, MCs were recognized in tracheal mucosa invading the tracheal epithelium. Moreover, they appeared massively infiltrated around the blood capillaries especially in tunica adventitia. Dawicki and Marshall (2007) found that MCs have a critical effect on the tissue microenvironment as they release several mediators. Mast cells are involved in the mechanisms of anti-virus defense and viral disease pathomechanisms. MCs express receptors responsible for the identification of virus-derived PAMP molecules, mainly Toll-like receptors. Furthermore, MCs generate many mediators, chemokines, and cytokines, which control the intensity of inflammation and modulate the course of innate and adaptive antiviral immunity (Witczak and Brzezińska-Błaszczyk 2012). Interestingly, St John et al. (2011) reported that MCs are capable for recognition of dengue virus (DENV) that results in MC degranulation and activation. Sun et al. (2009) suggested that mast cells are implicated in New-Castle Disease–induced mucosal injury in chicken and suppression of mast cell mediators release may represent a new strategy to control this problem.

The present study demonstrated the dendritic cells (DCs) in mallard trachea for the first time. They form a densely interconnected network underneath the basement membrane of the tracheal epithelium. We suggest that they induce the immune response by tracheal mucosa. The main function of these DCs is the processing of antigens and presenting them to the T cells (Mokhtar and Hussein 2019). In human, the airway mucosal DCs capture the airborne antigens by extending their

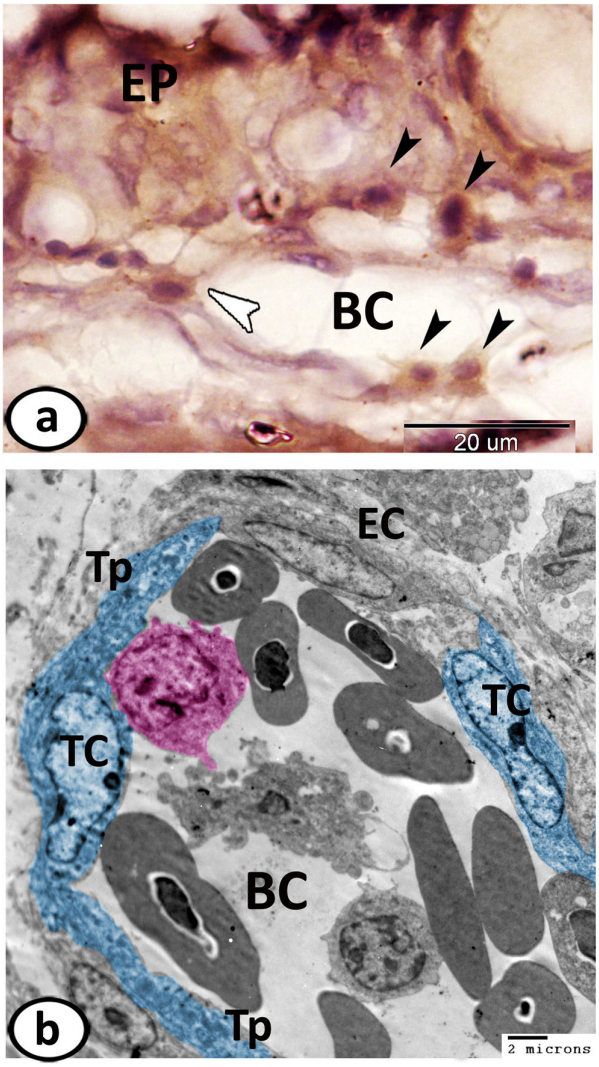

Fig. 11 The role of telocytes and dendritic cells in angiogenesis. (A) The dendritic cells (black arrowheads) expressed S100-protein, distributed under the epithelium (EP), and form the wall of blood capillary (BC) with TC (white arrowhead). (B) Digital colored TEM of the wall of the blood capillary (BC) that consisted of endothelial cell (EC), dendritic cell (pink), and two telocytes (TC, blue) with their telopodes (Tp)

cytoplasmic processes or dendrites through the intact epithelium into the airway lumen (Von Garnier and Nicod 2009). Similar to mammals, there are different subtypes of DCs that have been identified in birds. The dendritic cells in birds show some distinct and unique characteristics. It presents a highly elongated cell body and short dendrite-like processes. Moreover, its cytoplasm contains numerous and prominent secretory granules (Olah and Nagy 2013).

Our study revealed a true ossification of tracheal cartilage of mallard with the presence of numerous bone marrow foci that contained numerous granulocytes. The tendency of tracheal cartilage to ossify is well known in avian species, and was reported as a distinguishing feature of the class Aves by the early comparative vertebrate anatomists (Huxley 1971). However, the timing of tracheal ossification in bird compared to mammals is interesting. In birds, ossification of tracheal cartilage begins well and reaches its final extent before the maturity age. In mammals, tracheal ossification is considered a degenerative process that occurs in older ages; this led us to ask the question of whether tracheal ossification has an important functional significance in birds. We suggest that tracheal ossification in mallard can help in the prevention of tracheal collapse. In addition, the bone marrow foci seem to play a significant role as a local factory for the production of the immune cell.

Light and electron microscopy images revealed the evidence of stromal synapses between TCs and other immune cells such as plasma cells and lymphocytes. This indicates that TCs are involved in an immune surveillance role (Cretoiu and Popescu 2014). In addition, the direct contact of TCs with the nerve endings suggests their active function in the neurotransmission or motor activity (Popescu et al. 2005). The present study indicated that TCs contribute to the formation of the blood capillaries and their processes connected with the endothelial cells. Therefore, they have a critical role in angiogenesis as they expressed VEGF and our study supports the findings of Hussein and Mokhtar (2018). Zheng et al. (2014) reported that TCs produce angiogenic factors as VEGF that activates the formation and proliferation of endothelial cells and promotes the vascular permeability. VEGF is also considered a maintenance factor for newly formed blood vessels (Alon et al. 1995). S-100 is a calcium-binding protein, which is excessively expressed in various cells, such as Schwann cells, dendritic cells, and neural cells, and also telocytes in the human fallopian tube (Popescu et al. 2005). In the current study, S-100 protein expressed in TCs of lamina propria, suggesting the neuronal property of these cells (Donato et al. 2013). The present study was the first to identify the TCs within the epithelium of avian species. However, a recent study by Kaestner (2019) revealed that foxl-1-expressing telocytes were found in the basolateral membranes of the intestinal columnar epithelial cells and the author suggested that TCs may have a role in epithelium proliferation and are considered a critical source of Wnt signals that maintain the function of stem cells. In addition, these cells could mediate the luminal antigens' interaction with the immune system. Furthermore, we suggest that the TCs have a contractility role based on their location around the intraepithelial glands.

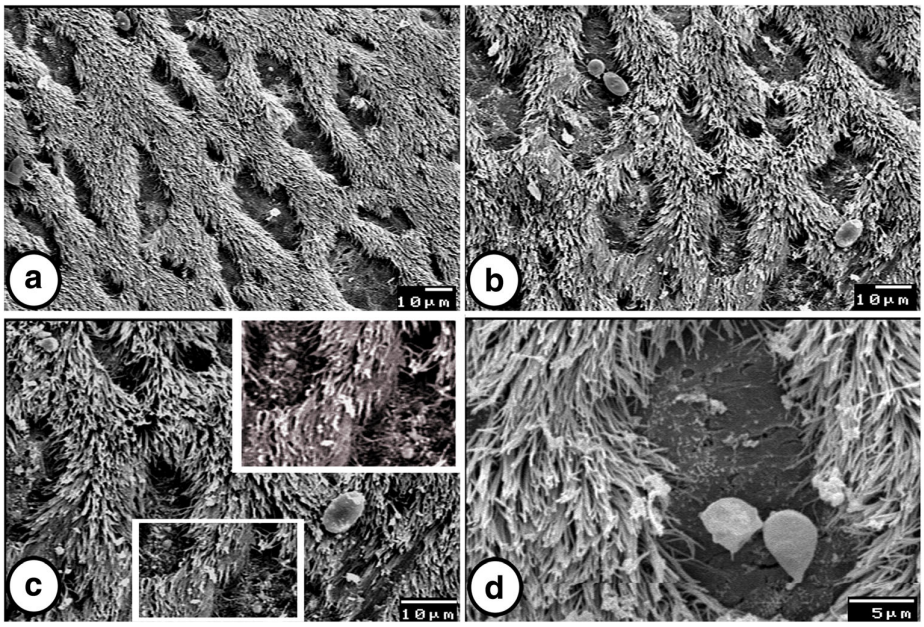

Fig. 12 Scanning electron micrograph of the luminal surface of the trachea. (**a**, **b**) Rows of ciliated cells (yellow arrows) interrupted by shallow narrow areas of non-ciliated cells (red arrows). (**c**) Magnified square showing some non-ciliated cells possessed small protrusions (yellow arrows) that may represent the evidence of active merocrine secretory phase. (**d**) The ciliated cells were covered by long cilia (yellow arrow); however, the luminal surface of non-ciliated cells exhibited numerous microvilli (red arrow). Note the discharged droplets of mucous (white arrow)

Conclusion

This study demonstrates the cellular element organization in the trachea of the mallard duck. Both the light and electron microscopic studies identified many cell types in the trachea based on their morphological features. The mast cells, plasma cells, and dendritic reticular cells as well as basophils, eosinophils, and lymphocytes were recognized in the tracheal epithelium and connective tissues. The distribution of the telocytes was demonstrated for the first time in the duck trachea in this study. The trachea of the mallard duck is considered the main structure involved in local immunity so that the tracheal mucosa is a suitable experimental model for evaluating the possible changes after vaccination of avian species.

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Author contributions D. M. Mokhtar performed the electron-microscopical study, immunohistochemistry, analyzed the results, and contributed to preparing and reviewing the paper. M. M. Hussein collected the samples, performed the histological and histochemical study, analyzed the results, and contributed in preparing and reviewing the paper. The authors contributed equally to this work.

Compliance with ethical standards

Ethical approval and consent to participate The study was approved by the Ethics Committee of Assiut University, Egypt.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

Abbreviations DC, dendritic cell; IEL, intraepithelial lymphocyte; MC, mast cells; TCs, telocytes; TEM, transmission electron microscopy; Tps, telopodes; VEGF, vascular endothelial growth factor

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