Tracheal epithelium and submucosal glands are predominant sites expressing Cytotoxic T Lymphocyte Antigen 2 alpha in the mouse

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SUMMARY

Cytotoxic T-lymphocyte antigen 2-alpha (CTLA 2-alpha) is cysteine proteinase inhibitor protein originally expressed in mouse activated T-cells and mast cells. Microarray, semi-quantitative PCR and Western blotting techniques identified CTLA 2-alpha as a novel lung tissue-specific secretory gene in the mouse. We also demonstrated the expression of CTLA 2-alpha in bronchiolar epithelial cells of the mouse lung. To extend these findings, we performed immunohistochemical analysis to determine the distribution of CTLA 2-alpha in the trachea. Results showed that CTLA 2-alpha is strongly expressed in pseaudostratified epithelium within columnar ciliated cells, and goblet cells that produce mucin. Staining was also evident in the submucosal glands within serous cells, and cuboidal cells lining ducts of submucosal glands. In the hyaline cartilage, CTLA 2-alpha was found in the cytoplasm of chondrocytes located in lacuna. The distribution pattern implicates an important role of CTLA 2-alpha in relation to immune defense of the trachea against infections and processing of secretions produced by the submucosal glands.

Keywords: CTLA 2-alpha, immunohistochemistry, distribution profile, mouse trachea

INTRODUCTION

The respiratory system evolved for efficient gas exchange with branching structures of trachea, bronchi, bronchioles and alveolar ducts that end as alveoli (Benkingsopp, 1967; Bowden, 1983). The trachea consists of pseudostratified epithelium with ciliated columnar cells, goblet, and basal cells. The epithelial cells interface directly with the environment and function in pathogen detection, fluid and electrolyte transport and mucus elevation. Underneath the epithelium is the lamina propria-submucosa which consists of simple coiled branched tubuloacinar glands (submucosal glands), predominantly serous with occasional mucous acini (Reznik, 1990). The submucosal glands are populated by two principal cells types: serous and mucous cells. Serous cells are the dominant cell type in the most distal, acinar portion of the secretory tubules (Meyrick, *et al*., 1969). They contain numerous apical electrondense secretory vesicles and are known to secrete a number of macromolecules including lysozyme, lactoferrin, secretory IgA, peroxidases, albumin and surface fluid that help protect the lungs from particles and infectious agents (Basbaum, *et al*., 1990; Finkbeiner, *et al*., 1999; Wang, *et al*., 2001; Dajani *et al*., 2005). Mucous cells are located more proximal within the tubules and ducts and secrete mucins that bind pathogens, inhibit their replication, and clear them from the airways (Wine, 2004). In human, the submucosal glands are found along the airways from the larynx down to the distal part of the main bronchi. However, in the mouse, they are restricted to the upper trachea, more specifically to the regions between the first and eighth cartilage rings (Treuting, *et al*., 2017) with the precise distribution depending on genetic background (Borthwick, *et al*., 1999; Innes, *et al*., 2001). Beneath the lamina propria is the hyaline cartilage covered by perichondrium. The cartilage forms the structural framework of the wall and prevents the trachea from collapsing.

Previous studies using microarray, semi-quantitative PCR and Western blotting techniques identified Cytotoxic T-lymphocyte antigen-2 alpha (CTLA 2-alpha) as a novel lung tissue-specific secretory gene in the mouse. Its expression was found to increase with lung development and during infections Zhang *et al*., 2015). We also reported the expression of CTLA 2-alpha in bronchiolar epithelial cells of the lung (Luziga, *et al*., 2018) demonstrating that, CTLA2-alpha is implicated in matrix remodeling, immune response and lung diseases. It was thought that if CTLA 2-alpha was expressed in the bronchioles; then, it would be interesting to find out whether or not CTLA 2-alpha is expressed in the trachea. Certainly, the trachea is known for its function as a defense barrier, anti-inflammatory and immune-modulating structure of respiratory tract (Bartlett, *et al*., 2008). So far, little information is available on the expression and physiological function of CTLA 2-alpha in biological tissues and it has never been demonstrated in the tracheal epithelium and submucosal glands. The objective of this study was therefore to evaluate by immunohistochemistry the distribution pattern and specific cell types expressing CTLA 2-alpha in the mouse trachea.

MATERIALS AND METHODS

Animals and tissue preparation

Experimental procedures were approved and performed in accordance to the guidelines for animal use of Sokoine University of Agriculture. A total of ten adult mice (five males and five females) were kept in animal laboratory house under controlled conditions of light (12-hour light-dark cycles) and temperature (20–25°C). They were fed standard laboratory chow and water ad libitum. The mice were anesthetized with sodium pentobarbital (60mg/kg body weight) by intraperitoneal injection. After sacrificing, tracheae were dissected, followed by fixation in 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO) in 0.1 phosphate buffer (PB; pH 7.4) for 2 hours at 4^{o}C.

Histological and immunohistochemical staining

The histological procedure was done as described previously (Slaoui and Fiette, 2011) with modifications. After fixation, the tissues were processed in ascending ethanol series to paraffin wax. Then, tissue blocks were made and cut at 5µm thick to produce tissue sections, which were deparaffinized in xylene followed by rehydration through a descending ethanol series to phosphate-buffered saline (0.01M PBS-pH7.4). Sections were prepared for Hematoxylin and Eosin (H&E) and immunohistochemical staining as described previously (Cuello, 1993).

To inhibit endogenous peroxidase activity, the sections were incubated for 30 min. at room temperature (RT) with a solution of 0.3% hydrogen peroxide (v/v) in distilled water, followed by washing (3×5 min.) in PBS. Sections were then incubated with 10% goat normal serum for 30 min. at RT to block non-specific binding followed by incubation with the CTLA 2-alpha antibody diluted at a ratio of 1:500 in PBS overnight in a dark, humid chamber at 4 °C. For negative controls, sections were treated as above except that 1% bovine albumin in PBS was applied in place of primary antibody. The sections were then washed (3X15 min.) in PBS followed by incubation for 30 min. at RT with biotinylated goat anti-rabbit IgG (MP Biomedicals, Inc, Germany). Sections were then washed (3×15 min.) in PBS before incubation with streptavidin-peroxidase conjugate for 20 min. at RT. The tissue section was then incubated for 3-5 min. with a solution containing 0.05 % 3,3′-diaminobenzidine tetra-hydrochloride (DAB), 0.01% hydrogen peroxide and 0.05 M Tris–HCl, pH 7.6 to visualize binding sites.

After incubation with DAB, the tissues were rinsed for 15 min. in water followed by dehydration through a graded ethanol series, clearance and mounting by a mixture of distyrene (a polystyrene), a plasticiser (Tricresyl phosphate), and xylene (DPX). Sections were examined using Olympus BH-2 microscope fitted with Olympus

camera for immunolabeling sites and image capturing.

Statistical analysis

Cell count immunohistochemical expression of CTLA 2-alpha in tracheal structures was performed using Image J bundled with 64-bit Java 8. Cell counts were recorded in Excel software and analysed for statistical significance using two-way ANOVA with the aid of R statistical software version 4.3.1. P-value < 0.05 was considered to be significant.

RESULTS

General histological overview of the mouse trachea

Hematoxylin-Eosin and immunohistochemical stained tracheal sections taken from upper part consisted of typical respiratory epithelium with several cells including the columnar ciliated cells, basal cells and mucus-secreting goblet cells resting on basement membrane. The ciliated columnar cells were the most abundant with cilia on each of their apical end which provide a lush cover on the luminal surface. In the lamina propria - submucosa, loose connective tissue arteries and veins as well as numerous simple coiled branched tubuloacinar submucosal glands, were observed. Underneath was the hyaline cartilage covered by perichondrium and to the outermost, the adventitia (Fig. 1).

Immunoreactivity of CTLA 2 alpha in various parts of the trachea

In both male and female mice, immunoreactivity for CTLA 2-alpha was seen in several layers of the trachea. In surface epithelial cells, strong staining was observed in the pseaudostratified columnar epithelium specifically in the cytoplasm of columnar ciliated cells, basal cells and in the cytoplasm of goblet cells but it was absent in the mucus secreted by the goblet cells. In the submucosa, intense staining was found in serous cells of secretory tubules and cuboidal cells lining the ducts of the glands but it was not observed in secretions. In the hyaline cartilage, strong staining was observed in chondrocytes (Fig. 2). Expression of CTLA 2-alpha for the first time was reported in mouse activated cytotoxic T lymphocytes and mast cells (Denizot, *et al*., 1989). Its expression was found to be closely related to immunological response and induced upon lymphocyte activation (Brunet, *et al*., 1988). The trachea is also known to harbor many T lymphocytes to regulate host defenses against viruses, fungal pathogens and bacteria due to its proximal location in the respiratory system and the continuous exposure to external environment (Chen, *et al*., 2013). Therefore, high expression of CTLA 2-alpha in the tracheal epithelium may be essential for normal T cell response and pulmonary host defense. Through a variety of receptor systems, the surface epithelial cells sense and respond to inhaled chemical, microbial, thermal, and particulate stimuli to maintain health. The trachea also consists of solitary neuroendocrine cells, dispersed in the epithelium (Cutz *et al*. 2013). Their distinctive structural feature is secretory of granules, which accumulate in the basal region of the cell. Secretory products of neuroendocrine cells are different amines, such as serotonin and γ-aminobutyric acid (GABA), and neuropeptides (Cutz *et al*. 2013). The observation of CTLA 2-alpha in the trachea is consistent with the localization of this protein in other secretory and immune privileged tissues such as the retina of the eyes, nerve fibers and neurons of the brain as well as in endocrine cells of the hypophysis and pancreas (Luziga *et al*., 2008; Sugita, *et al*., 2008; Luziga, et a., 2016; Luziga, 2018; Luziga, 2020) where it was implicated in immunity and proteolytic processing of secretory products of cells.

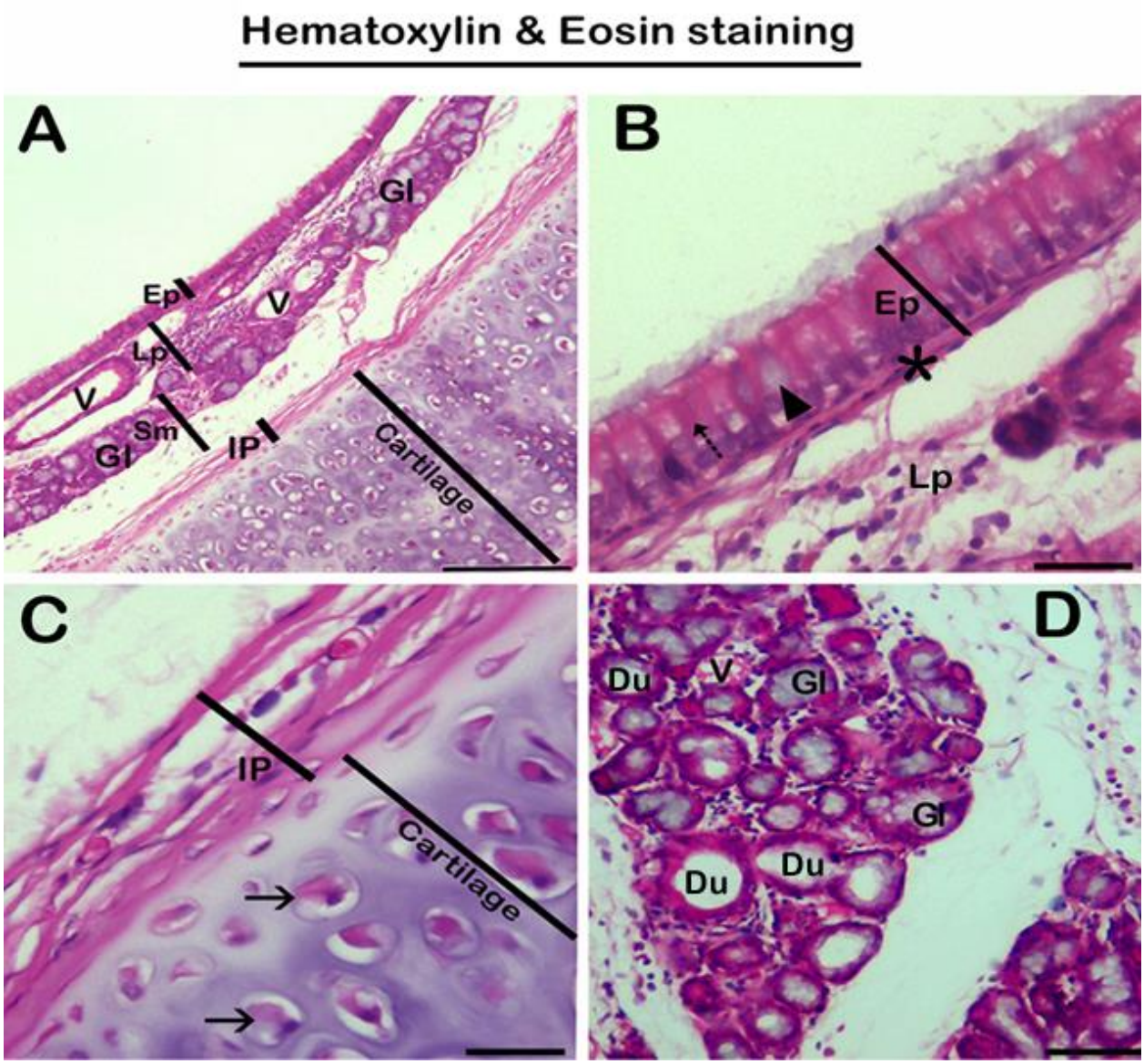

Figure 1. Figure 1. View of histological structures in the trachea. (**A**) Low and (**B**, **C** and **D**) high magnification images showing epithelium (Ep), lamina propria (Lp), submucosal glands (GI), inner perichondrium (IP) and hyaline cartilage. Epithelium (Ep) consisting of pseudostratified columnar epithelium, ciliated cell (dotted arrows) with goblet cells (arrow heads), resting on the basement membrane (Asterisk). Underneath is the lamina propria (Lp) with loose connective tissue and blood vessels. Inner perichondrium (IP) covering the hyaline cartilage and chondrocytes resting in lacuna (solid arrow heads); V: venous sinus; Sm: submucosa with loose connective tissue; Du: ducts of submucosal glands. Scale bar: A: 50 μm; B: 100 μm; C, D and E: 200 μm.

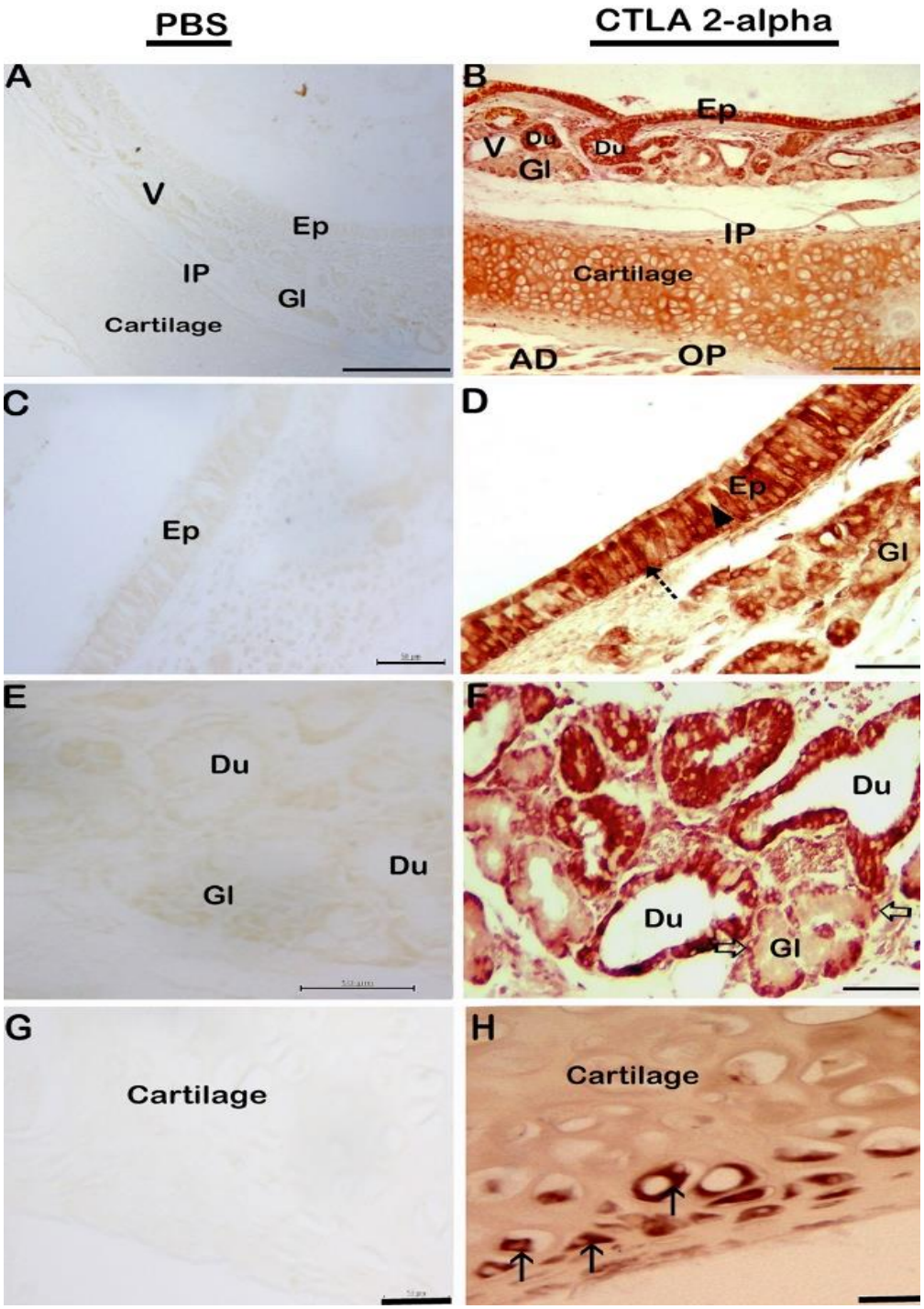

Figure 2. Immunohistochemical stained transverse sections of mouse trachea showing the distribution of CTLA 2-alpha in various tracheal cells. Tissue sections (**A**, **C**, **E**, **G**) incubated with PBS instead of CTLA 2-alpha antibody (control) showing absence of immunoreactivity for CTLA 2-alpha, and (**B**, **D**, **F**, **H**) incubated with CTLA 2-alpha antibody showing positive immunoreactivity for CTLA 2-alpha appearing as brown reaction product of DAB in epithelium (EP), submucosal glands (Gl) and glandular ducts (Du) and Hyaline cartilage. Higher magnification of positive immunoreactivity for CTLA 2-alpha is seen in (**D**) epithelium (Ep) within ciliated cells (dotted solid arrow heads) but is not seen in the mucus of goblet cells (arrows heads); **(F**) submucosal glands (GI) specifically in cells of serous acini (open arrow) and cells lining the ducts (Du) and (**H**) hyaline cartilage within cytoplasm of chondrocytes located in lacuna (arrows). EP: epithelium; Du: ducts of submucosal glands; V: venous sinus in the submucosa; IP: inner perichondrium and OP: outer perichondrium of the hyaline cartilage, and AD: adventitia. Scale bar: A and B: 100 μm; C – H: 200 μm.

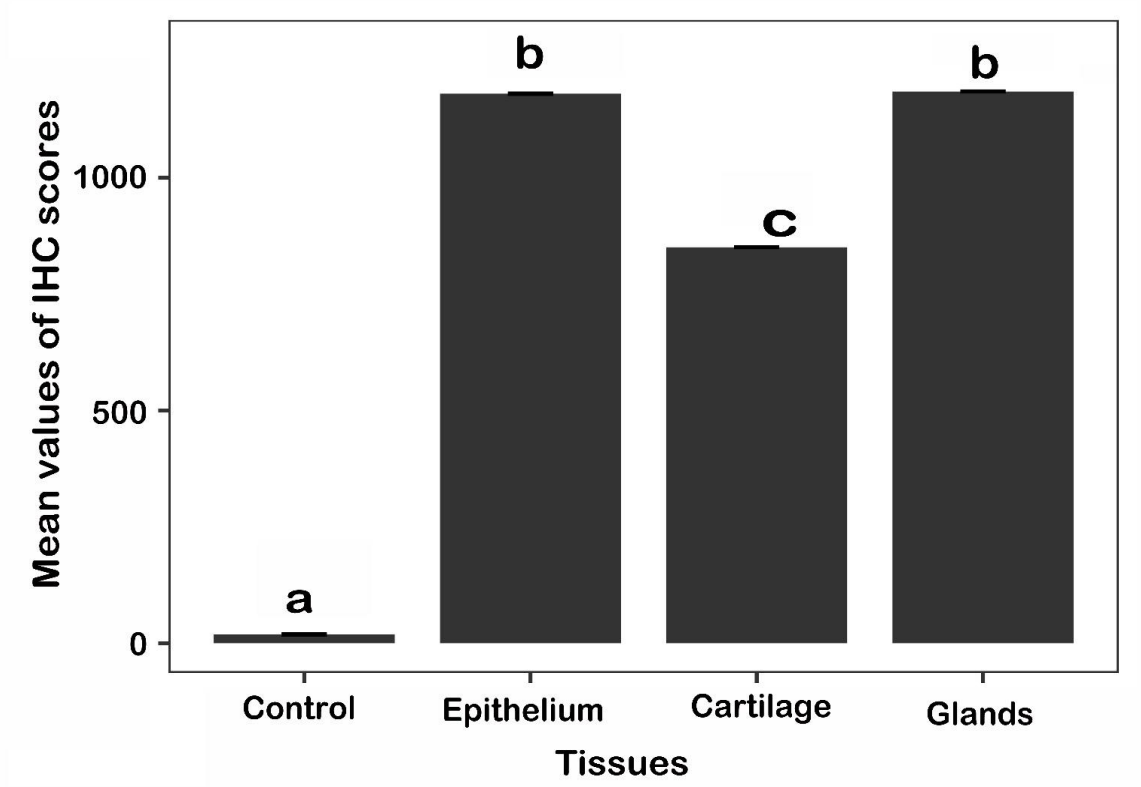

Figure 3. Cell count for CTLA 2-alpha immunohistochemistry (IHC) in tracheal structures using Image J. Cell counts was performed on control section, epithelium, cartilage and submucosal glands at 1000µm^2 area for each structure in ten microscopic fields in 5 slides. All data were averaged and analyzed for statistical significance of means by two-way ANOVA using R statistical software version 4.3.1. Bars with different letters were found to be statistically significantly different at $P < 0.05$.

DISCUSSION

In this study, results obtained on its distribution in the trachea shows that it is mainly localized in the pseaudostratified columnar epithelium in ciliated cells, goblet and basal cells. It is also expressed in the submucosal glands in serous acini cells and those lining the ducts as well as in the hyaline cartilage within chondrocytes in lacuna. Although the functional implications underlying the expression of CTLA 2-alpha in the trachea is not well known, it may be considered that CTLA 2-alpha localization in the trachea is related to inflammatory responses, considering the contact of epithelial cells with the extracorporeal space which is constantly exposed to microbes (Perl, *et al*., 2002). It may also be associated with processing of bioactive molecules and proteins contained in the secretions of submucosal glands or involved in the degradation of extracellular matrix proteins and remodeling of the hyaline cartilage. On the other hand, the submucosal glands are known for their role to secrete a wide variety of macromolecules including numerous mucins and antibacterial substances including lysozyme, lactoferrin, collectins, and defensins. In addition, the glands secrete liquid which serves both as the vehicle for carrying the macromolecules from the gland secretory tubules to the airway surface and providing critical volumes of fluid to the airway surface that are necessary for the support of mucociliary transport and hence help protect the lungs from particles and infectious agents (Finkbeiner, 1999; Wang, *et al*., 2001; Wine, *et al*., 2004; Dajani, *et al*., 2005). The localization of CTLA 2-alpha at high levels in cells of serous acini and cells lining the ducts and tubules is suggestive of its participation in the processing of secretions from the submucosal glands or possibly involved in some other novel biological functions that are yet to be identified. Other cell types that showed striking immunoreactivity for CTLA 2-alpha were the chondrocytes localized in the lacuna

within the matrix of hyaline cartilage. During its life cycle, each chondrocyte is responsible for the production and the continued maintenance of the matrix which surrounds it. If the chondrocytes are destroyed the matrix integrity breaks down (McCallion and Gilmore, 1987). Analysis of CTLA 2-alpha localization in the cartilage showed high level of expression in the cytoplasm of chondrocytes. This observation extends previous finding on the expression of CTLA 2-alpha in embryo in which intense labeling was detected within cranium, vertebrae of cervical and thoracic region and the sternabrae (Luziga, et l., 2015). It can be urged that these cells are metabolically active, producing matrix and being involved in remodeling. These findings are therefore suggestive of an important function of CTLA 2-alpha in relation to proper synthesis and deposition of extracellular matrix. Although the functional implications underlying the localization of CTLA 2-alpha in the trachea is still ambiguous, in light of these, it is reasonable to conclude that CTLA 2-alpha participate in the immune defense of the trachea against infections, processing of secretions produced by the submucosal glands and remodeling of extracellular matrix of the cartilage.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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