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MORPHOLOGICAL CHARACTERISATION OF THE VESTIBULAR FOLDS AS LARYNX-ASSOCIATED LYMPHOID TISSUE IN THE ADULT BACTRIAN CAMEL (*Camelus bactrianus*)

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ABSTRACT

Ultrastructure and composition of the lymphoid follicles (LF) found in the vestibular folds (FVC) of the bactrian camel were examined by immunohistochemistry, scanning and transmission electron microscopy. Immunohistochemical examination revealed that CD^3 and CD^{20} positive lymphocytes, CD^{68} positive macrophages were distributed in the different areas of follicles at different ratio. The positive follicular dendritic cells (FDC) were only found in germinal centres. Electron microscopy revealed that non ciliated microvillous cells with irregular microvilli, displaying features characteristic of microvillous cells (M cells), were present in the follicle-associated epithelium (FAE) that covered the lymphoid follicles. The bright germinal centre (GC) contained lymphoblasts, follicular dendritic cells, apoptotic lymphocytes and macrophages. The distribution of high endothelial venules (HEVs) was observed in the parafollicular area (PFA). The morphological findings of LF suggested that the presence of lymphoid follicles and inflammatory cells in the FVC was common, and was part of the normal histological morphology of larynx in the camel. As an active component of the larynx associated lymphoid tissue (LALT) in the camel, the FVC were inductive sites for the common mucosal immune system as well as important components in larynx defense.

Key words: *Camelus bactrianus*, histology, lymphoid tissue

The larynx is a mucosal organ positioned at the divergence of the respiratory and digestive tracts (Barker *et al,* 2006), which can be divided into the supraglottic, glottic and infraglottic region, each serving different functions. Clinical evidence shows that larynx diseases present a great specificity for glottic cavity regions. Organised lymphoid tissue in the wall of mucosa containing organs plays a central role in the induction of immune reactions. The distribution of organised larynx associated (LALT) lymphoid tissue on the epiglottis was based on its equivalence with gut-associated lymphoid tissue (GALT) (Kracke *et al,* 1997). FVC are located in the supraglottic region, while the true vocal cords are found in the glottic region. In studies of human, the LF found in the FVC protect the upper air tracts, similar to the lymphoid tissue associated to the respiratory mucosa (Rossi and Silva *et al,* 2009).

In the bactrian camel (*Camelus bactrianus*), LALT composes the integrated mucosal immune system as essential component of defense in the upper respiratory tract. The morphological findings of LF with germinal centre suggest that FVC may participate in the protection of the larynx of the camel (Yang *et al,* 2010). However, studies that characterise the phenotype of the cells that compose this lymphoid follicle and its characterisation as LALT are lacking in the camel. The objective of the present study was to provide detailed information on the ultrastructure and immunohistochemical criteria of LF in the FVC.

Materials and Methods

Ten larynges of the adult bactrian camels of both sexes without symptoms of clinical disease (based on daily inspection by a veterinarian) were obtained from the slaughterhouse of the Right Alasan Banner Food Company in Inner Mongolia Autonomous Region, China, after the animals had been killed by exsanguination for human consumption. The larynges were dissected free and flushed with normal saline.

Immunohistochemistry

The larynges were transversally sectioned at points above and below the glottic cavity at a

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distance of 3 cm between each point. The fragment from the FVC was fixed in 10% formaldehyde, dehydrated, cleared and embedded in paraffin for immunohistochemical process. Serial tissue sections (8 µm thick) with a 100 µm interval were made from all tissues. The sections were dewaxed and rehydrated, and antigen retrieval was performed by microwaving the sections for 20 min (4X5 min) at medium power in 0.01 M sodium citrate buffer (pH 6.0) and were allowed to cool for 10 min. Then, the endogenous peroxidase was inhibited with 3% H_2O_2 in PBS for 30 min. After non-specific binding was blocked with 10% normal goat serum in PBS, the sections were rinsed three times in PBS containing 0.02% Triton X-100. Then the sections were incubated separately with monoclonal mouse anti-human antibodies, i.e. CD20 (DAKO®), CD3 (DAKO®), CD68 (DAKO®), and FDC (DAKO®) overnight at 4°C. Again, sections were rinsed with PBS and then were covered with the biotinylated goat anti-mouse IgG. After washing in PBS, sections were incubated with streptavidin-labeled peroxidase complex for 3 h at room temperature. After two further washes with PBS, the immunolabeling was visualised using diaminobenzidine (DAB) with haematoxylin counterstaining. Negative control sections were performed in a similar manner, except the primary antibody was substituted with PBS. Images were captured using a digital camera (Leica DFC320). The positively reacting cells in different areas were counted with X 400 magnification in three different fields. Relative number of positive cells was assessed as follows: +++, >50% of cells strongly stained; ++, 10–50% of cells distinctly stained; +, <10% were stained; -, negative.

Transmission electron microscopy (TEM)

Small pieces of the FVC for TEM were pre-fixed in 3% glutaraldehyde buffer (pH=7.2) and fixed for 3 h. The tissues were then washed thrice in 0.1 M phosphate buffer for 30 min before being cut into 1 mm^3 pieces and post-fixed with osmium tetroxide for 1 h. The samples were washed thrice in 0.1 M phosphate buffer and then dehydrated in ascending grades of ethanol before being embedded in epon 812. Semi-thin sections of each tissue were collected and stained with toluidine blue. Ultra-thin sections (50–70 nm) were then collected on copper grids. The ultra-thin sections were stained with a saturated solution of uranyl acetate for 30min, followed by lead citrate for 7 min in a carbon dioxide-free environment. Sections were then washed in CO_2 free water, dried and examined under a transmission electron microscope (JEOL, JEM-1230).

Scanning electron microscopy (SEM)

Tissue samples of the FVC for SEM were fixed as described above. After dehydration in a series of ascending grades of alcohol, samples were freeze-dried at vacuum and coated with platinum. The mounted specimens were observed under a scanning electron microscope (JEOL, JSM-680LA).

Results

Organised lymphoid tissue with the cytomorphological and immunophenotypic features of MALT was identified in the FVC specimens from 10 camels. The CD^{20+} B lymphocytes, CD3+ T lymphocytes, CD^{68+}, and FDC^{+} were indicated by the presence of cytoplasmic and/or membrane brown staining. CD^{20+} B lymphocytes and CD^{3+} T lymphocytes were detected inside the epithelium, although their occurrence was rare (Fig 1-A, B). In germinal centre containing CD^{3+} T lymphocytes, we also observed a small number of follicular dendritic cells (Fig 1-E) and CD^{68} macrophages (Fig 1-C, D). Macrophages were mainly localised in the parafollicular and inter-follicular areas (Fig 1-C). In the parafollicular area, mainly CD^{3+} T lymphocytes, some CD^{20+} B lymphocytes and macrophages were present. CD^{68+} positive immunocytes were similarly found between the secretory acini. The distributions of subsets of lymphoid cells, follicular dendritic cells and macrophages in different lymphoid compartments were summarised in Table 1.

The surface of the FVC as viewed by SEM appeared irregular with circular and polygonal markings, which was composed of ciliated and non-ciliated cells. Most of non-ciliated cells, usually the

Table 1. Relative numbers of lymphoid cells, follicular dendritic cells and macrophages in the FVC of the camel.

Phenotype	Epithelium	Laminapropria mucosae	Inter-follicular area	Parafollicular area	Germinal centre
CD^{20+}	+	++	+	+	++
CD^{3+}	++	+	+	++	+++
CD^{68+}	+	++	++	+	+
FDC^{+}	–	–	–	–	++

–, not detected; +, few; ++, moderately frequent; +++, very frequent.

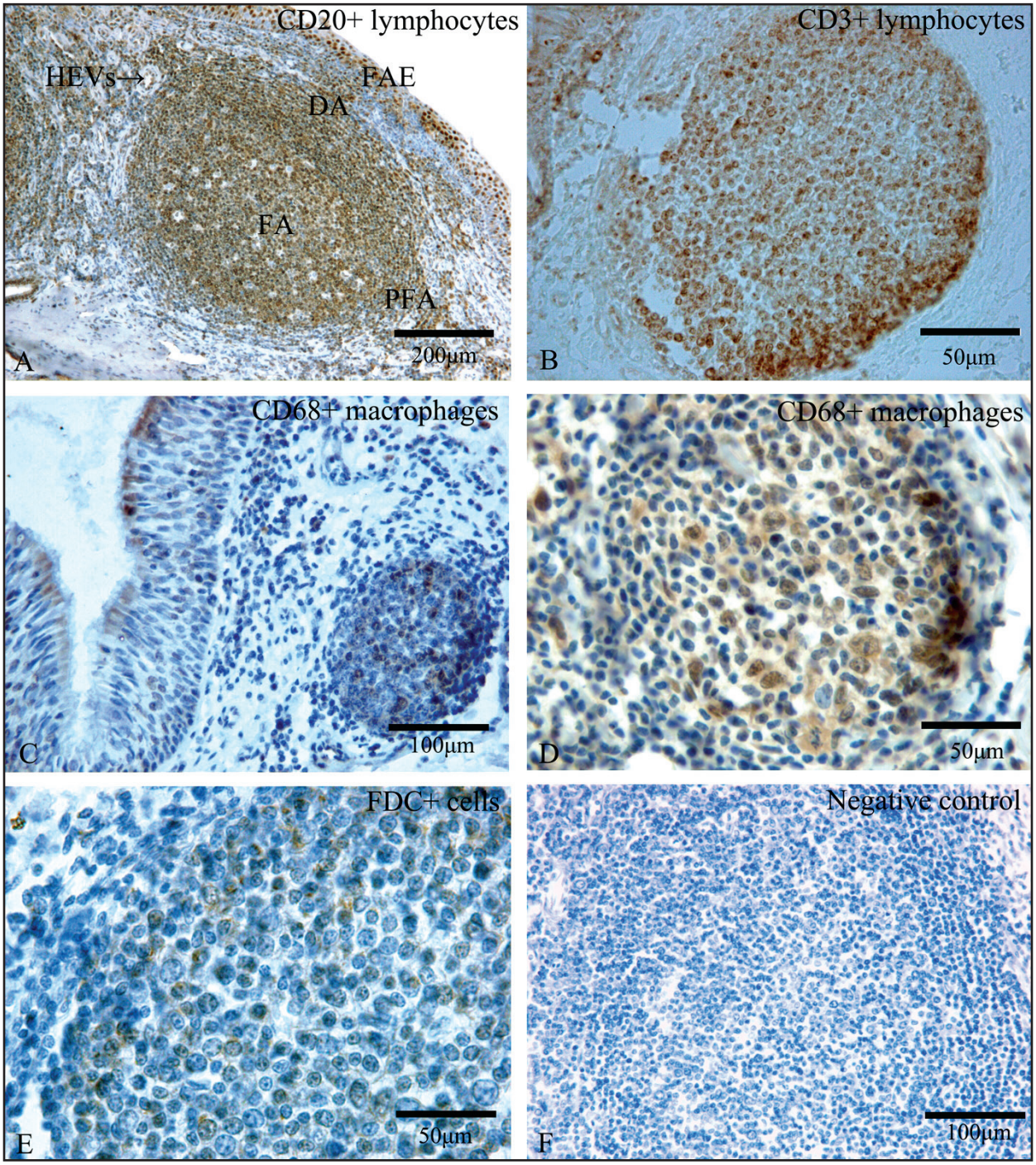

Fig 1. Immunohistochemical staining for A: CD^{20+} B lymphocytes; B: CD^{3+} T lymphocytes; C and D: CD^{68+} macrophages; E: FDC^{+} cells; and F: Negative control in the organised lymphoid tissue of the FVC in the camel. FAE: follicle-associated epithelium; DA: dome area; FA: follicular area; PFA: parafollicular area.

larger ones, had a smooth membranous surface with very few short microvilli and microridges which were mainly located along the cell borders (Fig 2-A). The lymphoid follicles were very prominent and closely aggregated hemispheres when viewed by SEM (Fig 2-B). They had a roundish to oval shape and a size ranging from about 0.2 to 0.8 mm in the longest axis. At the follicular flanks the epithelium showed a rough and uneven surface, whereas the FAE at the apex was relatively smooth blunted ridges.

TEM showed that the FAE overlying FVC demonstrated morphology characteristic of M cells, including attenuated apical cell surface with blunted microvilli and microfolds (Fig 3-A, B). The bright

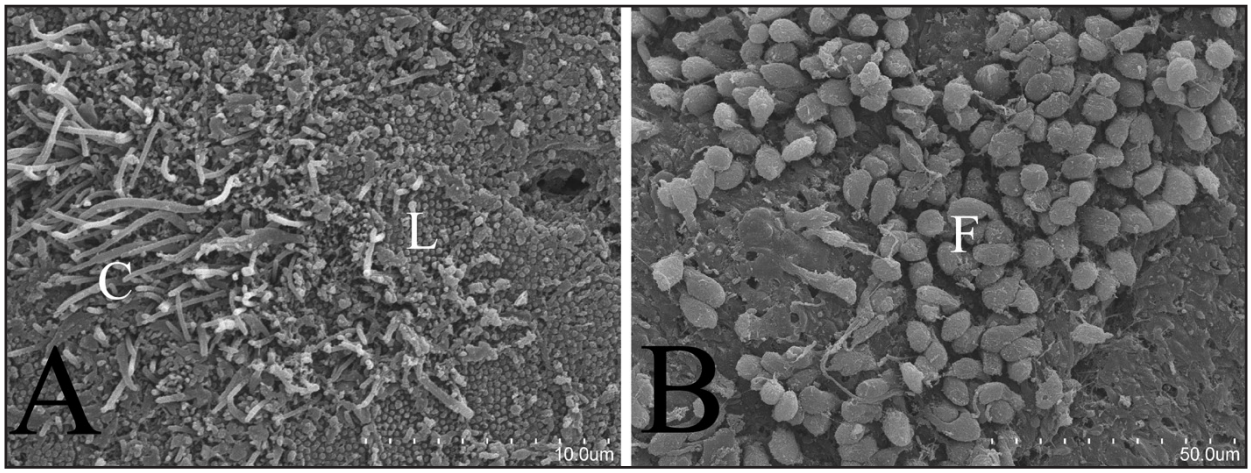

Fig 2. SEM images showing cilia (C) and microvillus cells (L) on the surface of the FVC (A). Follicles (F) aggregated prominent round to oval hemispheres in non-ciliated regions of the vestibulum FVC (B).

GC of the lymphoid follicles appeared less electron dense than the rest of the follicle because densely packed cells with large cytoplasm occupied the GC. Lymphoblasts with a larger cytoplasm were abound in organelles, including several mitochondria, and had euchromatic nucleus of roundish or irregular shape (Fig 3-C). The nucleus of apoptotic lymphocytes was small and dark, which was filled by peripheral dark masses of interspersed heterochromatin (Fig 3-D). Large FDC with a highly euchromatic nucleus of roundish or oval shape sent long cytoplasmic processes between the lymphoid cells. The cytoplasm of these cells contained the usual organelles (Fig 4-A). Plasma cells with rough endoplasmic reticulum were found in the PFA (Fig 4-B). Macrophages with an irregularly shaped or indented nucleus were detected by numerous lysosomes and phagolysosomes in the cytoplasm (Fig 4-C). In the parafollicular zone beneath the follicles, distinct, occasionally large, lymphatic vessels with lymphocytes in the lumen were regularly found lying in a lymphoid tissue. HEVs were numerous in the parafollicular zone and also extended into the underlying dense stroma (Fig 4-D).

Discussion

LALT could serve as a connection between tonsils and bronchus-associated lymphoid tissue (BALT) (Hiller *et al,* 1997). There are differences in the age-dependent distribution pattern of LALT between the supraglottic and infraglottic regions in humans. The FVC are located in the supraglottic region. Inflammatory cells are common in the human FVC, whether in the form of LF or cellular aggregates. Lymphoid follicle development or acquisition occurs on the FVC because of infectious agents (Kutta *et al,* 2003). They diminish with age, and are part of the localised inflammatory process in the respiratory tract (Rossi-and-Silva *et al,* 2009). In the previous study, aggregated follicles have been observed in all specimens of the adult camel FVC (Yang *et al,* 2010). The general composition of the lymphoid follicles resembles that of a predetermined lymphoid organ, demonstrating that LALT in camel is a highly structured and organised secondary lymphoid tissue that has specific functions within the mucosal immune system. Secondary lymphoid follicles consist of four areas: FAE, DA, FA and PFA (Yang *et al,* 2010). The morphological findings of LF suggest a probable participation of the FVC in the protection of the larynx and lungs (Rossi-and-Silva *et al,* 2009). Consequently, LALT could be important components in larynx defense in the adult camels. Application of immunogens to inductive sites in the upper respiratory tract may be most effective for generating protective responses against organisms responsible for upper airway infections (Russell *et al,* 2000).

In the camel, obvious LF were observed in the FVC. These aggregations met the criteria for designation as LALT, which contains all the elements necessary to function as a potent antigen sampling site. The organised lymphoid tissue comprises a B-cell and a T-cell component in the human LALT (Kracke *et al,* 1997; Hiller *et al,* 1997). Immunohistochemical staining revealed inflammatory cell phenotypes in the FVC of the camel. CD^{20+} B lymphocytes and CD^{3+} T lymphocytes were distributed in the LF. The germinal centre presented FDC^{+} cells, which are in accordance with the observation of FDC by TEM. Large cytoplasmic processes on FDC possessed large numbers of filaments. Few CD^{20} and CD^{3} positive

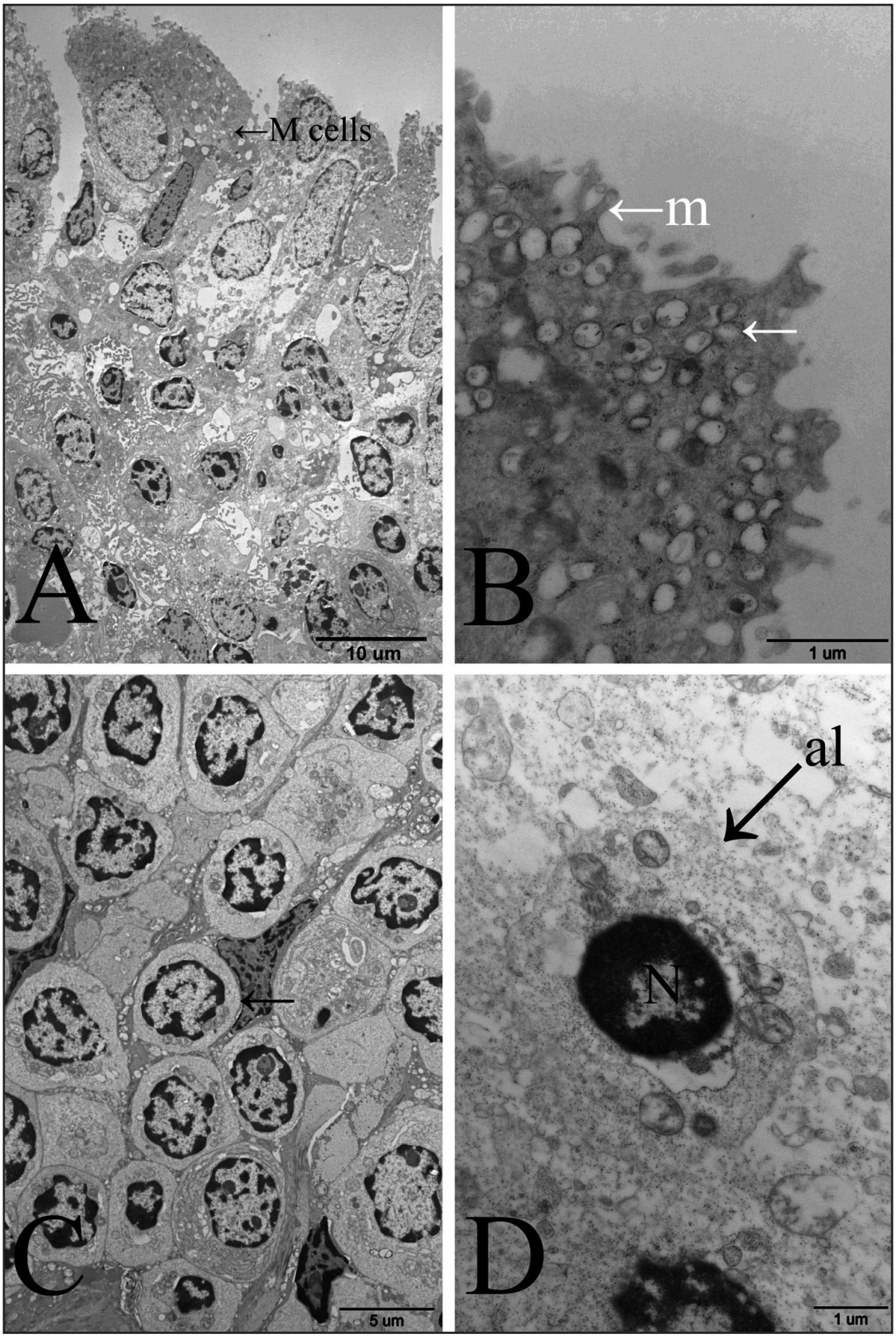

Fig 3. TEM images of FAE showing small vesicles (→) beneath the outer cell membrane and inside the M cell with short microvilli (m) (A and B). Lymphoblasts (←) with a larger cytoplasm (C) and apoptotic lymphocytes (al) with a small dark nucleus and in the bright GC (D).

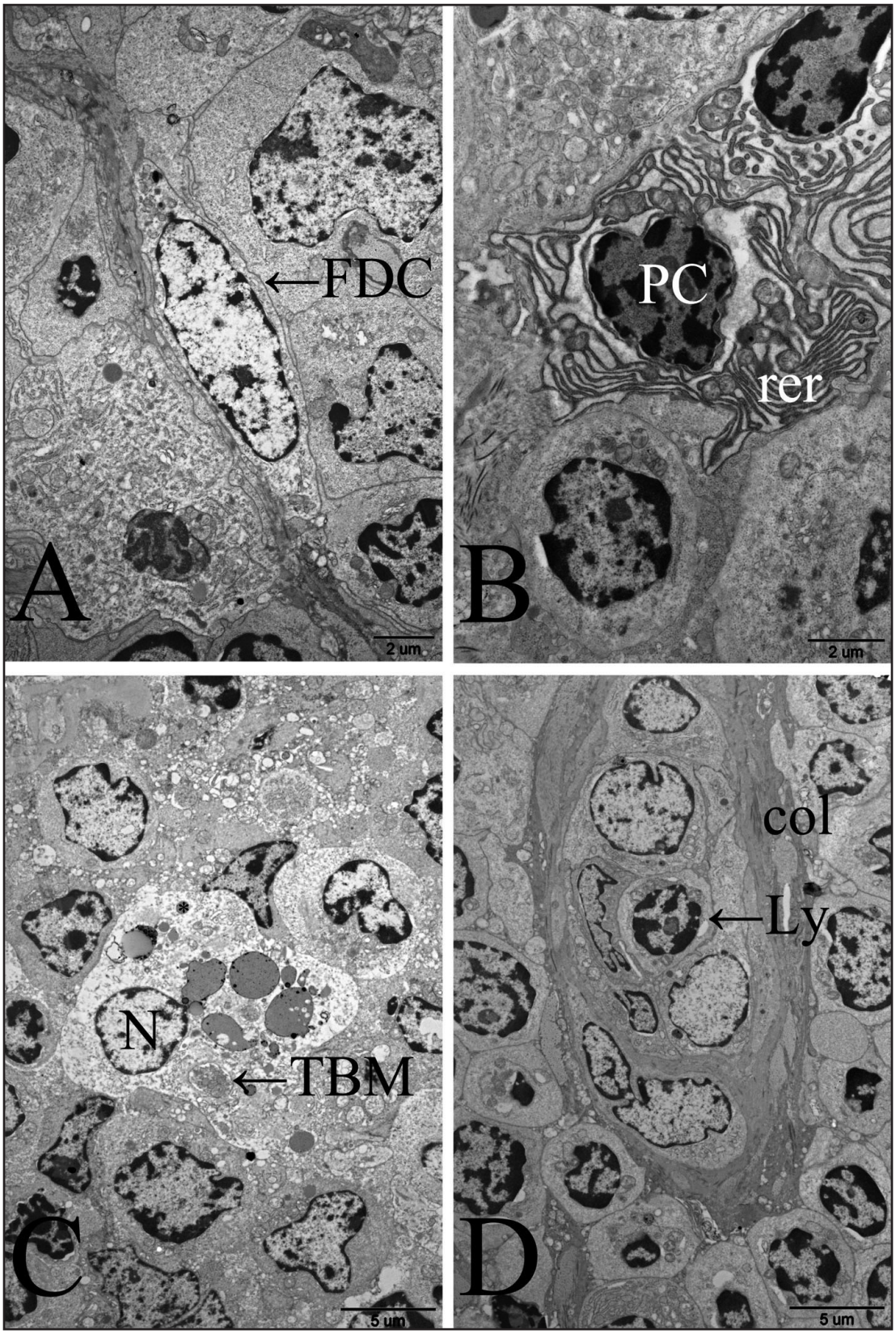

Fig 4. TEM images of FDC with a highly euchromatic nucleus of oval shape sent long cytoplasmic processes between the lymphoid cells (A). Plasma cell (PC) with rough endoplasmic reticulum (rer), lymphoid cells (Ly) in the PFA (B). Tingible body of macrophages (TBM), vacuole (v), lysosome (*) and macrophage (m) in the germinal centre. A lymphoid cells (Ly) in HEVs, located among bundles of collagen (col) fibrils (D).

immunocytes were similarly detected inside the epithelium and the connective tissue between the secretory acini, which further proved the fact that lymphocytes distributed in these areas by histology. CD68 macrophages localised in the germinal centre, parafollicular and inter-follicular as in the human FVC (Kutta *et al,* 2003; Rossi-and-Silva *et al,* 2009). The results revealed that the presence of lymphoid follicle and inflammatory cells in the FVC was common, and was part of the normal histological morphology of larynx in the camel. These results suggested that FVC in the camel has the cytologic basis of normal mucosa reaction. As an active component of the LALT in the camel, the FVC were inductive sites for the common mucosal immune system as well as important components in larynx defense.

Based on their morphological features and relationship to underlying lymphoid tissue, specialised follicle-associated epithelium consisting of ciliated and non-ciliated cells can be easily distinguished from the adjacent epithelium in the FVC of the camel, both topographically and ultrastructurally. These non ciliated microvillous cells resemble the antigen sampling cells occurring in MALT, which are commonly known as M cells (Stanley *et al,* 2001). It seems probable that the microvillous cells in FAE of camel are fully capable of taking up particulate antigens. Whether the microvillous cells are functionally similar to M cells described in other species and in other locations needs to be investigated in the camel. A bright germinal centre of proliferating lymphocytes was found in the camel FVC follicles. FDC develop through transformation of fibroblastic reticulum cells during germinal centre formation (Heusermann *et al,* 1980). FDC with typical morphological features and cytochemical and immunological phenotypes occur in the dynamic germinal centres of lymphoid organs including the equine lingual tonsil and tubal tonsil (Kumar and Timoney, 2005 a,b; Kutta *et al,* 2003). We found evidence for proliferating lymphoblasts and for FDC that continuously provide antigens on their surface related to the presence of prominent vesicles in their cytoplasm, as observed here and in other lymphoid tissues (Szakal *et al,* 1983). FDC bind antigen-antibody complexes to their surface for long periods and are essential for generation of effective humoral antibody responses (Heinen *et al,* 1995). HEVs are the dominant vessel type in the thymus dependent paracortical zones of lymph nodes, tonsils and Peyer's patches. HEVs support active lymphocyte migration from peripheral blood into MALT (Kracke *et al,* 1997) and also into LALT as seen here.

In conclusion, the presence of follicles in the camel FVC with follicular M-cells, GC and para-follicular HEVs further supports the concept of LALT (Kutta *et al,* 2003; Kutta *et al,* 2004; Yang *et al,* 2010) as important components in larynx defense.

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