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Multivesicular bodies containing exosomes in immune-related cells of the intestine in zebrafish (*Danio rerio*): Ultrastructural evidence

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ABSTRACT

Exosomes are secreted from various cells by multivesicular bodies (MVBs) that fuse with the plasma membrane and are involved in the intestinal immune response to maintain intestinal homeostasis. Here, we demonstrate the ultrastructural characteristics of MVBs and their exosomes in immune-related cells of the zebrafish intestine, including goblet cells (GCs), mitochondria-rich cells (MRCs), high endothelial cells (HECs) and lymphocytes. In GCs, MVBs with a low electron density were present under the nucleus. MVBs with exosomes were observed among mucin granules. "Heterogeneous" MVBs were identified within the cytoplasm around mucin granules. MRCs were observed in the intestinal mucosa epithelium, including "open-type" MRCs and "close-type" MRCs. Typical MVBs were identified in these MRCs. MVBs with a variety of exosomes were observed in the HECs of the capillary located in the lamina propria (LP). The HEC basement membrane budded outward to LP cells to form a plurality of basal blebs, later containing a large number of exosomes. MVBs also existed in the LP lymphocytes. A schematic diagram of the ultrastructural distribution of MVBs and their exosomes in the intestinal mucosal immune-related cells was created. Our findings provide cytological evidence for the source and ultrastructural distribution of exosomes within the different intestine cells of zebrafish. Component analysis and immunological functions of exosomes require future study.

1. Introduction

The intestine is not only a place for nutrient digestion and absorption, but also the most important part of the mucosal immune system [1]. Intestinal lymphocytes pass through the vascular wall surrounded by high endothelial cells to reach the mucosal epithelium, which is important for intestine immune balance and lymphocyte homing [2]. Mucin and exosomes derived from the intestine form a tight biological barrier to provide defence from harmful pathogens [3,4]. Upon inhibition of mucin loss or the secretion of exosomes, immune dysfunction or inflammatory bowel disease (IBD) occurs [5,6]. The intestine has a variety of functions that depend on its complex barrier structure. The barriers are composed of the intestinal mucosa, muscle layer and serosa [7]. The intestine mucosa is the first defence of the body's contact with the external environment and relies on intercellular communication to resist the invasion of harmful substances and maintain the dynamic balance of the intestinal microenvironment. The mucosal cells consist of absorptive cells, goblet cells, enteroendocrine cells, Paneth cells, high endothelial cells and lymphocytes [8]. However, the ultrastructural distribution and functions of multivesicular bodies (MVBs) and their exosomes in intestinal mucosal immune-related cells remain unclear.

Exosomes are secreted membranous vesicles that occur when MVBs fuse with the plasma membrane (PM) and are found in a wide variety of living mammalian cells [9], including intestinal epithelial cells (IECs) [10], endothelial cells [11] and lymphocytes [12]. Previous studies have shown that IECs secrete different exosomes from the apical and basolateral aspects [13]. The apical exosomes are involved in host responses caused by parasite infection of IECs [14]. The basolateral exosomes regulate the lamina propria T cells through dendritic cells and participate in intestinal immune responses [15]. Exosomes derived from endothelial progenitor cells promote the migration and angiogenesis of endothelial cells [16]. Lymphocyte-derived exosomes carry an antigen peptide that improves the response of effector lymphocytes

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[17]. Although the intestine secretes exosomes under physiological or pathological conditions, the source of exosomes in the intestine remains unclear.

Zebrafish (*Danio rerio*) have a short breeding cycle, a complete genetic background, and clear intestine development [18,19]. Additionally, zebrafish are a valuable IBD model [20]. The aim of this study was to explore the ultrastructure of MVBs and their exosomes in the intestinal mucosal immune-related cells of zebrafish. The ultrastructural distribution of MVBs and their exosomes was identified in goblet cells, mitochondria-rich cells, high endothelial cells and lymphocytes by transmission electron microscope. These findings provide the cytological evidence for the immune functions of intestinal exosomes in vertebrates.

2. Materials and methods

2.1. Experimental animals

Zebrafish were raised in the Shanghai Experimental Animal Research Centre. Standard feeding conditions were used, and the water was dechlorinated in advance with a temperature of 26 ± 0.5 °C and a pH of 6.9. Mature wild type AB female zebrafish (*Danio rerio*) were used. Zebrafish were anaesthetized using MS-222 (150 mg/L) (A5040, Sigma, Saint Louis, Missouri, USA). All tasks were executed to minimize animal suffering and experiments were approved by the Experimental Animal Ethics Committee of Nanjing Agricultural Veterinary College (The approval ID is SYXK (SU) 2010–0005). After fasting for 24 h before taking the material, the contents of the intestine were drained.

2.2. Transmission electron microscope sample preparation and observation

After anaesthesia, the zebrafish were cut from the fan-shaped eye through the vent hole along the abdomen to fully expose the abdominal visceral mass. The intact visceral mass was carefully collected and infiltrated with Phosphate Buffer solution (PBS), and the tissue around the intestine was removed. The complete intestine was fixed in 2.5% glutaraldehyde in PBS (4 °C, pH 7.4, 0.1 M), fixed in 1% hungry acid, dehydrated using an alcohol gradient, treated with acetone, embedded in Epon 812, and double stained with uranyl acetate citrate. The samples were observed using an HITA H-7650 transmission electron microscope (TEM).

3. Results

3.1. The ultrastructural distribution of MVBs and their exosomes in intestinal goblet cells

Goblet cells were scattered between the intestinal villus absorptive epithelium (Fig. 1a). Developed endoplasmic reticulum and immature mucin granules were located in the basal cytoplasm of the goblet cells. MVBs were observed under the nucleus. These MVBs have low electron density and few exosomes (Fig. 1b). Other MVBs were found among mature mucin granules (Fig. 1c). Importantly, “heterogeneous” MVBs were identified in the cytoplasm around mature mucin granules (Fig. 1d). These MVBs have two different electron densities; one has lower electron density and less exosomes, and the others have a higher electron density.

3.2. MVBs and their exosomes in mitochondria-rich cells were identified by TEM

Abundant mitochondria and a developed tubular system were observed in mitochondria-rich cells (MRCs) (Fig. 2a). In the zebrafish intestinal epithelium, clustered MRCs were identified (Fig. 2a). The apical plasma membrane of these MRCs was connected to the intestinal lumen, so called “open-type” MRCs. MVBs containing a substantial number of exosomeswere located in the apical cytoplasm of these MRCs (Fig. 2b). Meanwhile, single “close-type” MRC were found between the intestinal absorptive epithelium (Fig. 2c). Typical MVBs were identified in these MRCs. Exosomes and tubules were present in MVBs (Fig. 2d).

3.3. The ultrastructural identification of MVBs and their exosomes in high endothelial cells and lamina propria lymphocytes

Many blood vessels were located in the connective tissue of the lamina propria (Fig. 3a). High endothelial cells (HECs) are the main barrier structure of blood vessels and can induce gut lymphocyte homing. Tight junctions were identified among these HECs (Fig. 3c). MVB with many exosomes and other membranous vesicles were detected in the HECs (Fig. 3d). Many exosomes were secreted by IECs through the formation of a basal bleb from the basal plasma membrane, suggesting that this is a new method of communication to exchange information between HECs and lamina propria cells (Fig. 3d). In addition to blood vessels, some lymphocytes were observed in the lamina propria. MVBs with low electron density were observed around the nucleus of these lymphocytes (Fig. 4a and b). However, no MVBs were found in the mucosal intraepithelial lymphocytes (Fig. 3a).

4. Discussion

The dynamic balance of the intestinal mucosa depends on precise communication among mucosal immune-associated cells in the intestine. Exosomes are critical regulators of intestinal absorptive cells to the external environment [21], yet their roles in intestinal immune-related cells have been largely unexplored. In the present study, the distribution of MVBs and their exosomes in goblet cells, HECs, and lamina propria lymphocytes was observed by TEM. Interestingly, mitochondria-rich cells (MRCs) were identified between the zebrafish intestinal absorptive epithelium. MVBs were located in the apical cytoplasm of MRCs.

Absorptive cells and goblet cells constitute the intestinal epithelium. Previous studies have demonstrated that exosomes are released by intestinal epithelial cells, especially intestine absorptive cells, of mammals [22] and zebrafish [21]. However, the TEM characteristics of exosomes in the intestinal goblet cells remain unclear. In this study, the distribution of MVBs and their exosomes in goblet cells was observed by TEM. Developed endoplasmic reticulum (ER) in the basal cytoplasm of goblet cells is a place for mucin synthesis [23]. In the present study, MVBs were detected in the basal cytoplasm of the goblet cells. The MVBs were surrounded by immature mucin granules. To investigate whether MVBs and their exosomes are associated with the mucin maturation process, we observed that MVBs with a small number of exosomes were located among many mature mucin granules in the apical cytoplasm of goblet cells. Two types of MVBs have been identified in professional antigen presenting cells (APCs), such as dendritic cells [24], oligodendroglia cells [25] and absorptive cells [14]. We identified that two-types of MVBs exist in the cytoplasm around mature mucin granules. MVBs with a high electron density were observed in the apical cytoplasm of the goblet cells. Meanwhile, MVBs with low electron density and less exosomes were found in the same location. In the present study, two types of MVBs were called “heterogeneous” MVBs. MUC2 is an important mucin that is involved in the construction of the mucin barrier [26]. Our findings are consistent with a role for the MUC family in exosomes [27]. Whether MVBs and the exosome pathways are directly associated with the mucin granule maturation process is uncear; however, we demonstrated the distribution of MVBs and their exosomes in goblet cells at the ultrastructural level.

High endothelial cells (HECs) are important barrier structures for lymphocyte homing [28]. In the present study, high endothelial venules were located in the lamina propria of the zebrafish intestine. Meanwhile, typical tight junction structures were observed between HECs, which is consistent with the barrier structures that prevent harmful

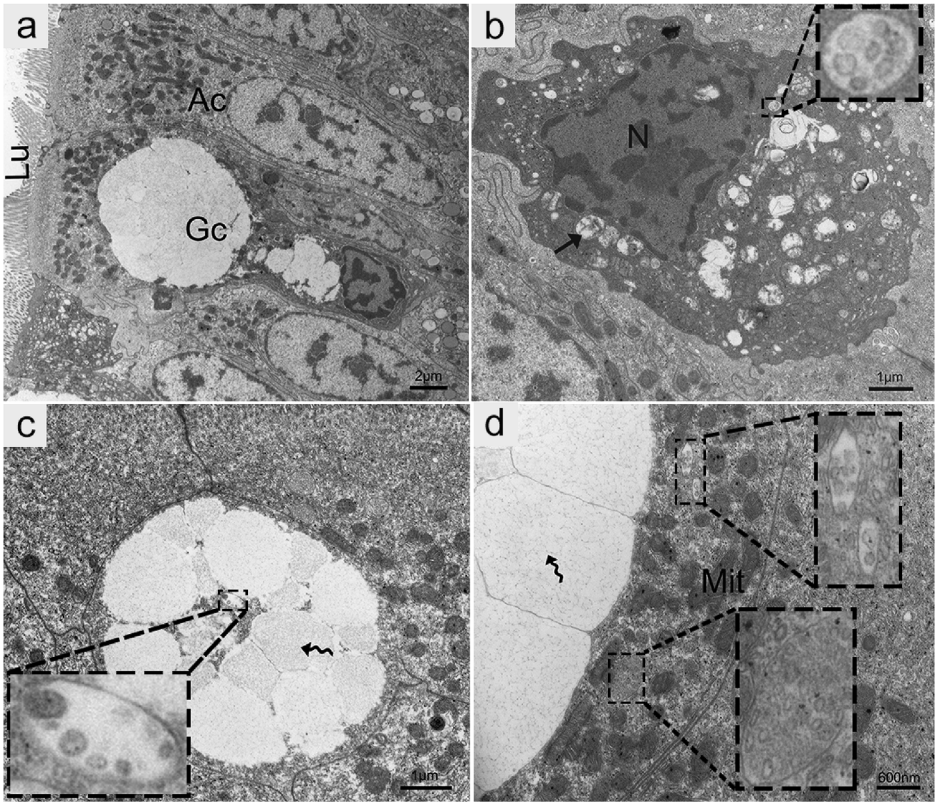

Fig. 1. The ultrastructural characteristics of MVBs and their exosomes in goblet cells. Ac: absorptive cell; Gc: goblet cell; Lu: lumen; N: nucleus; immature mucin granules (arrow); mature mucin granules (curved arrow); Mit: mitochondria; multivesicular body (black box); scare bars: a = 2 μm; b = 1 μm; c = 4 μm; d = 600 nm.

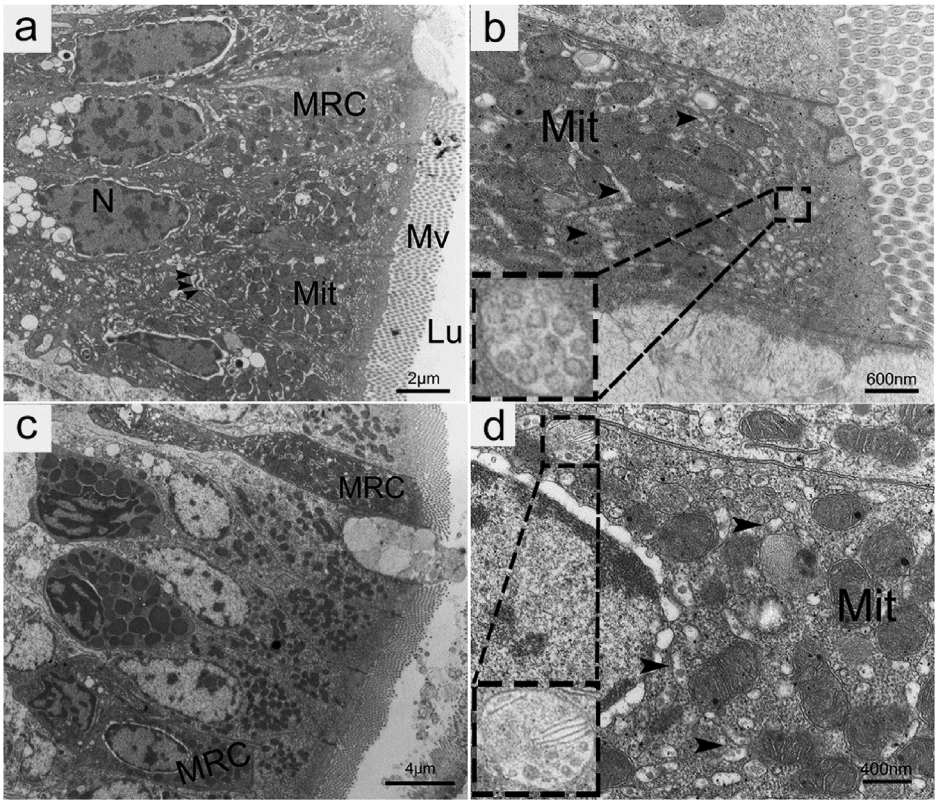

Fig. 2. MVBs and their exosomes in "open-type" mitochondria-rich cells and "close-type" MRCs. MRC: mitochondria-rich cell; Lu: lumen; N: nucleus; Mv: microvilli; Mit: mitochondria; MVB: multivesicular body (black box); developed tubular system (arrowhead); scare bars: a = 2 μm; b = 600 nm; c = 4 μm; d = 400 nm.

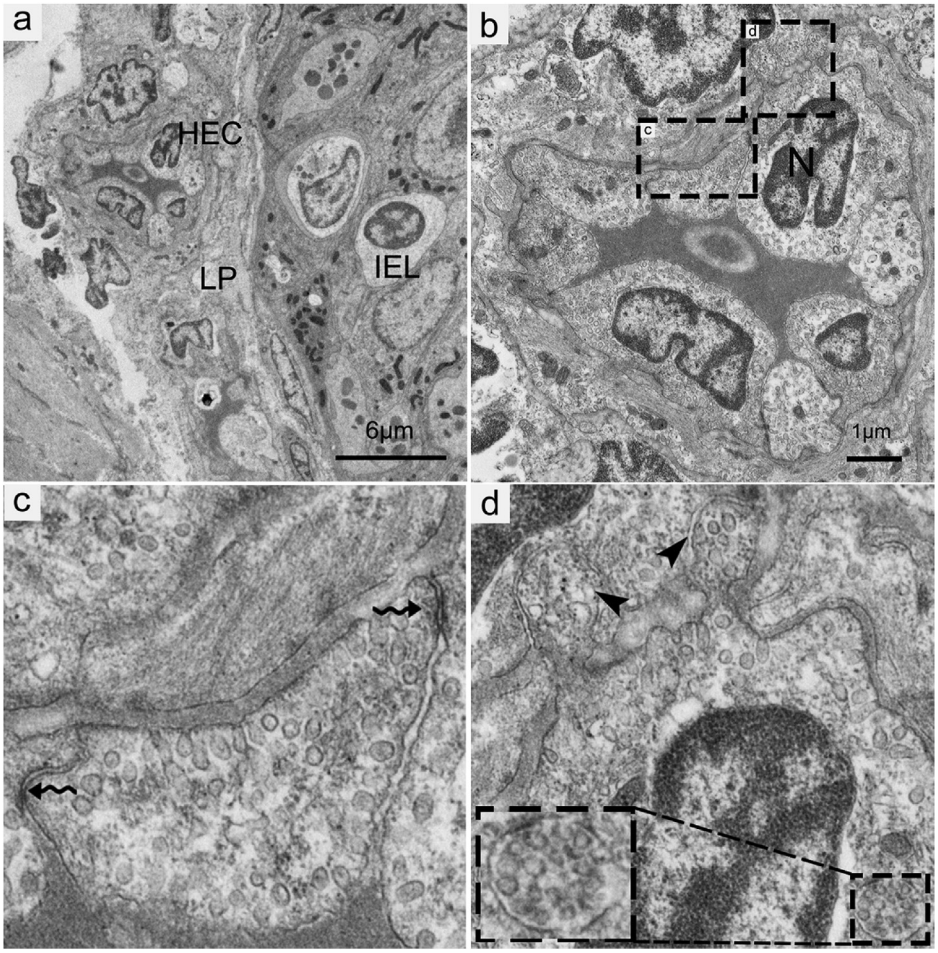

Fig. 3. The ultrastructural distribution of MVBs and their exosomes in high endothelial cells. IEL: intraepithelial lymphocyte; LP: lamina propria; HEC: high endothelial cell; N: nucleus; MVB: multivesicular body (black box); tight junction (curved arrow); scare bars: a = 6 μm; d = 1 μm.

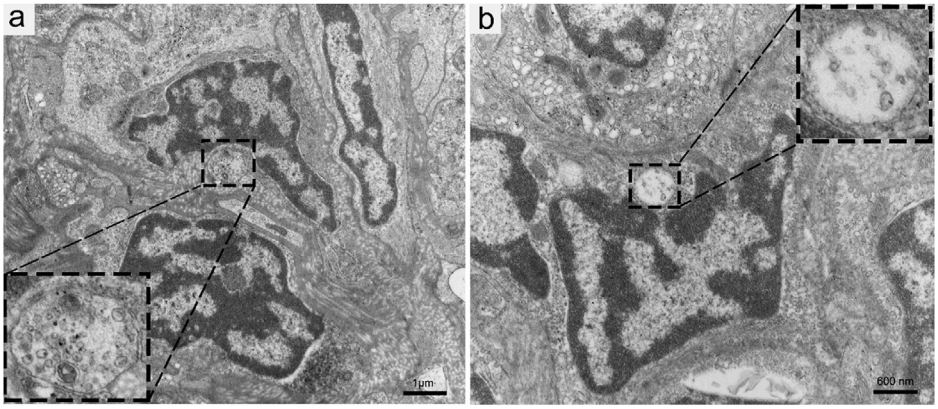

Fig. 4. MVBs containing exosomes in lamina propria lymphocytes. MVB: multivesicular body (black box); scare bars: a = 1 μm; b = 600 nm.

substance from entering intestinal tissue [29]. Previous studies have demonstrated that membrane vesicles and mitochondria are present in the cytoplasm of spleen HECs [30], which is consistent with our findings in intestinal HECs. Importantly, typical MVBs containing a large number of exosomes were found in HECs. Recent studies have confirmed that exosomes are secreted to the epididymal intraluminal compartment by the formation of blebs in the apical pole of epithelial cells [31]. In the present study, HEC communication with the lamina propria cells was observed through the formation of basal blebs of the basal plasma membrane (PM) from HECs, which is similar to the process by which exosomes are released when MVBs fuse with the PM in cells [32]. However, our results could not distinguish the lamina propria cells from lamina propria lymphocytes, and this question requires further study. To investigate whether MVBs and their exosomes are associated with lymphocytes, we demonstrated that they are mainly located in the pit of nuclei in lymphocytes. These MVBs displayed low electron density and contained many exosomes. However, no MVBs were observed in the mucosal epithelial lymphocytes, which is consistent with MVBs and exosomes that promote naïve lymphocytes into effector lymphocytes [33].

In the present study, we first demonstrated that mitochondria-rich cells (MRCs) are present among the absorptive epithelial cells of the

zebrafish intestine, and they are classified into “open-type” and “close-type” MRCs based on whether they are directly connected to intestinal lumen. MRCs were originally called chlorine-secreting cells due to their function of secreting Cl^- [34]. With more research, they have been found to have a bidirectional transport function for various ions [35]. MRCs are widely distributed between the gill epithelium of fish [36]. In this study, TEM showed clusters of “open-type” MRCs in the intestinal epithelium. These cells have features of well-arranged microvilli, a developed tubular system and abundant mitochondria, which is consistent with a role for MRCs in the gill [36]. Importantly, typical MVBs were present in the apical cytoplasm of “open-type” MRCs, which have features of low electron density and abundant exosomes. In contrast, “close-type” MRCs with no microvilli were observed. MVBs and their exosomes were distributed in the vicinity of the nucleus for “close-type” MRCs, which were typically characterized by a small number of exosomes and tubular structures in the cavity. However, the function of these subcellular organelles in MRCs remains unknown.

Previous studies have shown that intestinal absorptive cells and dendritic cells secrete exosomes [6]. In this study, MVBs and their exosomes were identified in intestinal goblet cells (GCs), high endothelial cells (HECs), lamina propria lymphocytes (LPLs) and mitochondria-rich cells (MRCs). With mature mucin granules, small MVBs and “heterogeneous” MVBs were confirmed in goblet cells at the ultrastructural level. HECs and lamina propria cells exchange information through the outside bud of the basal cytoplasmic membrane of HECs. The bud structures contain many exosomes. A schematic model of the ultrastructural distribution characterization of MVBs and their exosomes in intestinal mucosal immune-related cell was drawn (Fig. 5). Importantly, these findings provide cytological evidences for the source and ultrastructural distribution of intestinal exosomes in vertebrates.

Author contributions

Xuebing Bai and Qiusheng Chen conceived and designed the experiments. Xuebing Bai performed most of the experimental task and organized figures. Xuebing Bai and Yonghong Shi together drafted the manuscript with assistance and advice from Imran Tarique and Waseem Ali Vistro. Yufei Huang, Hong Chen, Abdul Haseeb and Noor Samad Gandahi participated in the study design and performed data analysis. All zebrafish raised by Jinxing Lin. The samples and figures of all zebrafish TEM were completed by Yonghong Shi. Qiusheng Chen, Jinxing Lin and Ping Yang revised the paper. All authors read and approved the final manuscript.

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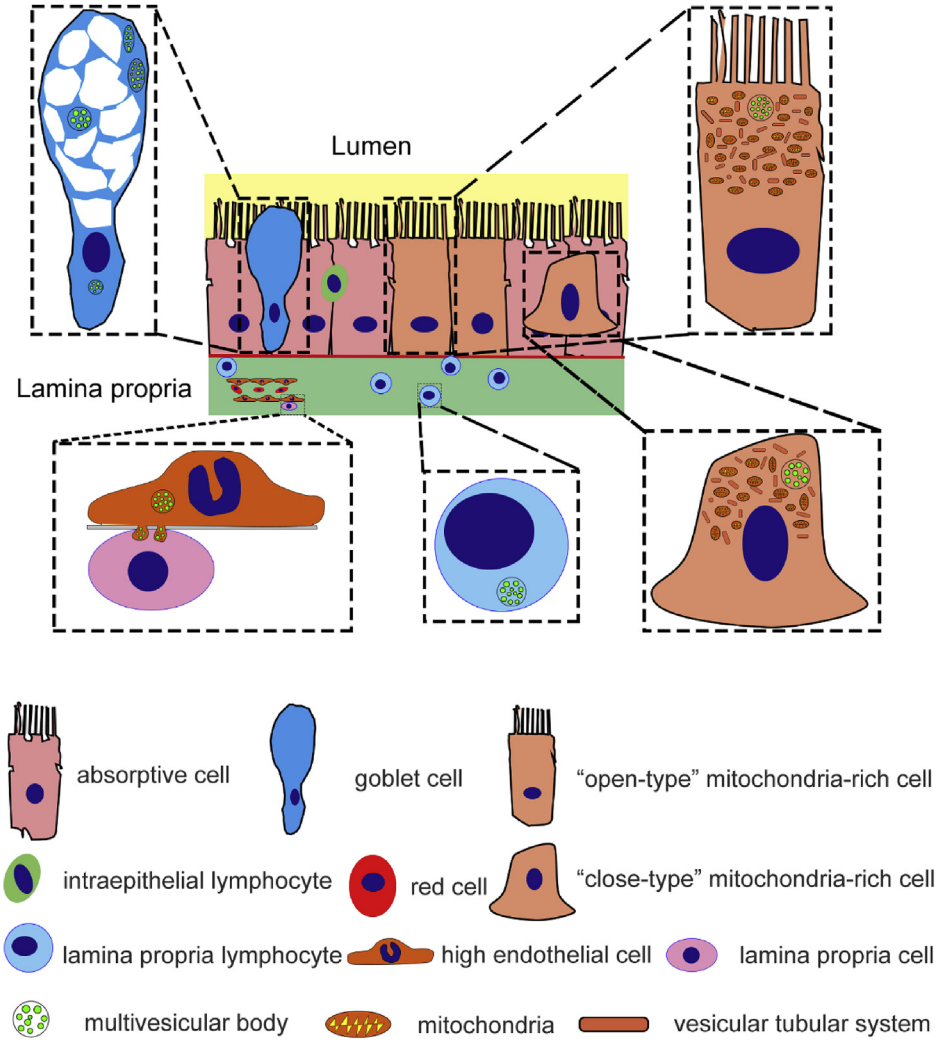

Fig. 5. Schematic diagrams of the ultrastructural characteristics of MVBs and their exosomes in intestinal mucosal immune-associated cell. Mitochondria-rich cells (MRCs) with an abundance of mitochondria and an extensive tubular system were observed in the intestinal epithelium. MVBs and their exosomes were found in goblet cells, MRCs, high endothelial cells (HECs) and lamina propria lymphocytes (LPLs).

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Declaration of competing interest

The authors declare no competing financial interests.

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