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In vivo dynamic distribution of multivesicular bodies and exosomes in spleen of DTMUV infected duck

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ABSTRACT

Exosomes are vesicles secreted by the multivesicular bodies (MVBs), which have been shown to mediate immunity regulation and virus transmission. In this study, the dynamic distribution and function of the MVBs and their exosomes was investigated through morphological characterization and molecular analyses in duck spleens infected with duck Tembusu virus (DTMUV) at different times post infection (1hpi, 2hpi, 12hpi, 24hpi). CD63, the marker of MVBs and exosomes, was distributed in the sheathed capillaries and the periellipsoidal lymphatic sheaths (PELS) of the white pulp. The numbers of MVBs and their exosomes were dramatically increased at 2 hpi, and with the increasing infection time, the numbers of MVBs and their exosomes were gradually decreased. DTMUV proteins were associated with exosomes according to double label immunofluorescence results. Ultrastructural characterization by transmission electron microscopy revealed four developing stages of MVBs containing exosomes were detected in high endothelial cells of the sheathed capillaries, lymphocytes and the ellipsoid-associated macrophages in PELS. Free exosomes were observed in the extracellular matrix and the blood vessels. Genes and proteins related to the endocytosis pathway were obviously up-regulated at 2 hpi as confirmed by RT-qPCR and western blotting. We speculated that DTMUV mediates host invasion through the endocytosis pathway by utilizing MVBs and their exosomes. The in vivo distribution pattern of MVBs and their exosomes in DTMUV infected spleens is shown for the first time in this study. This report could lay the foundations for understanding the infection mechanism of DTMUV.

1. Introduction

Duck Tembusu virus (DTMUV) is a positive single-stranded RNA virus, classified in the family of Flaviviridae, genus Flavivirus. It has been reported that ducks (Yan et al., 2011), chickens (Liu et al., 2012), geese (Yun et al., 2012) and other poultry (Chen et al., 2014; Ti et al., 2016) could be infected by DTMUV. Most importantly, a recent study has shown that humans could also be infected by DTMUV (Tang et al., 2013). DTMUV is seriously threatening poultry farming and human health, therefore elucidating its pathogenic mechanism is imperative.

Exosomes are vesicles with a diameter of 50–150 nm that are secreted by almost all types of cells (van Niel et al., 2018) and exist in various body fluids (Crawford, 1971; Thery et al., 2002). Exosomes originate as intraluminal vesicles (ILVs), which are budded out from early endosomes (EEs) in a manner of "reverse sprouting". Next, EEs develop into multivesicular bodies (MVBs) in four developing stages; first as endocytic vesicular structures, then as pre-multivesicular bodies, followed by pale multivesicular bodies (pM) and finally as dense multivesicular bodies (dM) (Tang and Zhang, 1990). Mature MVBs have two kinds of fate, either they are combined with lysosomes and eventually get degraded, or they are fused with the plasma membrane and release the ILVs into the extracellular space. After they release from the MVBs, the ILVs are called exosomes (Kowal et al., 2014).

Flavivirus enters the host cells through the clathrin-mediated endocytic pathway (CME) (Chu and Ng, 2004; van der Schaar et al., 2008). CME is the principal entry pathway for large cargo and pathogens, which involves a wide variety of vesicular structures including clathrin-coated vesicle, EEs, late endosomes (LEs), ILVs, MVBs, exosomes, and others (McMahon and Boucrot, 2011; Ungewickell and Hinrichsen, 2007). Some endosomal vesicles including MVBs and exosomes, have been shown to transfer a variety of proteins, lipids and nucleic acids from the cytoplasm and play an important role in *cell-to-*

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cell communication, antigen presentation, viral pathogenesis and *modulation of immunity* (Alenquer and Amorim, 2015; Schwab et al., 2015). Interestingly, some in vitro studies have proven that exosomes have the function of delivering virus-associated proteins and genes or virus particles between virally infected and uninfected cells (Lagana et al., 2013; Nour and Modis, 2014; Zuo et al., 2017).

Up to present, research on the development and the role of MVBs and their exosomes in viral infection and disease is still in its early stages. Many studies on exosomes related to human viruses (Arakelyan et al., 2017; Bukong et al., 2014; Jaworski et al., 2014; Zhu et al., 2015) have been carried out. However, studies on exosomes concerning animal viruses have rarely been performed. So far, no study has reported the formation and the role of MVBs and their exosomes after DTMUV infection. Previous reports have shown that the spleen is the main organ with the earliest obvious pathological changes and the highest virus titer after DTMUV infection (Wu et al., 2014). Therefore, in this study, the dynamic distribution of MVBs and their exosomes in the duck spleen at different time points after DTMUV infection were analyzed in vivo. Our findings could lay the foundation for the study on exosomes related to poultry virus disease.

2. Materials and methods

2.1. Virus strain

The purified DTMUV strain XZ-2012, provided by the College of Veterinary Medicine in Nanjing Agricultural University, was propagated in 10-day-old specific pathogen-free (SPF) duck embryos (200 μL of each). Allantoic fluids were collected at three to four days after injection and packed frozen at −80 °C for further use.

2.2. Virus titration

Virus Titration was performed by the $TCID_{50}$ method. The virus allantoic fluids were serially diluted 10-fold from 10^{-1} to 10^{-10}. BHK-21 cells were grown in 96-well plates with DMEM (10% FBS, 1% penicillin-streptomycin) and infected with 100 μL of the diluted virus allantoic fluids (each dilution was tested in 8 replicas). At 1 h post infection, cell maintenance fluid (2% FBS, 1% penicillin-streptomycin) was added, and the cells were cultured for 72 h at 37 °C. Samples were then observed under an inverted microscope TS100 (Nikon, Japan), and the results were recorded. The $TCID_{50}$ was calculated by the Reed-Muench method.

2.3. Animals

Eighty healthy, six months old, egg-laying shelducks, negative for any DTMUV and their antibody in vivo, were used in this experiment and divided into eight groups (ten ducks in each group). The ducks in the four of the groups were intramuscularly injected with DTMUV at the dose of $10^4 \times (TCID_{50})$ in 0.9% NaCl and euthanized at 1 h, 2 h, 12 h and 24 h post infection (pi). The ducks of the four remaining groups served as controls and were intramuscularly injected with the same volume of 0.9% NaCl and were euthanized at the same four time points. The spleen samples were harvested and packed frozen at −80 °C for the subsequent RT-PCR and Western blotting experiments. For the transmission electron microscopy (TEM), the spleen samples were fixed in 2.5% glutaraldehyde. For the immunohistochemical and immunofluorescent analyses, the spleen samples were fixed in 4% paraformaldehyde-PBS. The sampling procedures were approved by the College of Veterinary Medicine in Nanjing Agricultural University. The protocol was approved by the Science and Technology Agency of Jiangsu Province (SYXK (SU) 2010-0005).

2.4. RT-PCR

RNA from the dissected spleens was extracted using the Trizol method (TransGen Biotech, China) and its purity and quality was determined using the ND-1000 spectrophotometer (Thermo, USA). The RNA samples were reverse transcribed into cDNA according to the manufacturer's instructions (abm, China). 20 μL reaction volume of the Reverse transcription mix: Mix (4 ×) 2 μL, RNA 6 μL, Stopper (5 ×) 2 μL, MasterMix (5 ×) 4 μL, RNase free H_2O 6 μL. The reverse transcription conditions: 25℃ 10 min; 42℃ 50 min; 85℃ 5 min. Primers were designed based on the sequences of the *E* gene of the virus strain XZ-2012 reported in GenBank (Accession No.: KM188953), which the sequence of the forward primer was 5′-GAAGCGAGCACCTACCACA-3′, and the sequence of the reverse primer was 5′-CGCTGATGACCCTGTCCAT-3′. The amplified fragment size was expected to be 249bp.

2.5. Immunohistochemical analysis

The spleen tissues were embedded in paraffin and sectioned at a 6 μm thickness using a Leica microtome (Germany). The sections were dehydrated, and then inactivated with 3% hydrogen peroxide for 10 min at room temperature. After being washed with distilled water, the antigen was exposed to citric acid buffer at 121°C for 15 min. The sections were blocked with blocking solution (5% bovine serum albumin) at 37 °C for 30 min and incubated with CD63 antibody (Boster Biological Technology, Wuhan, China; Catalog #PB0236), a marker of MVBs and exosomes, at a 1:100 dilution, at 4 °C overnight. After being washed with 0.02 × PBS, the sections were incubated in a wet box with the secondary antibody (HRP-Polymer anti-Rabbit IHC Kit) at a 1:100 dilution, at 37 °C for 30 min. Following washing with 0.02 × PBS, the sections were developed with DAB (DAB-kit-Pale-brown-20) for 5 min and were counterstained with hematoxylin, and then dehydrated and sealed with neutral balsam. The sections were examined and images were acquired using an Olympus DP73 microscope. The integral optical density of the CD63 positive reaction was calculated by the Image-Pro Plus 6.0 software.

2.6. Double label immunofluorescence analysis

Tissues were sectioned at a 3 μm thickness, the sections were dehydrated, and then the antigen was exposed with citric acid buffer at 121 °C for 10–15 min. The sections were blocked with blocking solution at 37 °C for 30 min and were incubated with the rat anti-CD63 and the rabbit anti-DTMUV E protein polyclonal antibody at a 1:100 dilution at 4 °C overnight. After being washed with 0.02 × PBS, the sections were incubated with the secondary antibody Alexa Fluor 488 affinipure goat anti-mouse IgG (H + L) (Fcmacs Biotech, China) and Alexa Fluor 647 affinipure goat anti-rabbit IgG (H + L) (Fcmacs Biotech, China) a 1:100 dilution at 37 °C for 1 h. Next, the sections were washed with 0.02 × PBS, and then were developed with DAPI (Boster Biotech, China), under dark conditions, for 5 min and sealed with glycerol. The sections were then observed under a fluorescence microscope (Olympus, Japan).

2.7. Transmission electron microscopy (TEM)

The spleens were trimmed into 1 mm^3 blocks, and then were dehydrated in ethyl alcohol, infiltrated with a propylene oxide-Araldite mixture, and embedded in Araldite. Then the ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate for 20 min each. The samples were visualized under a H-7650 transmission electron microscope (Hitachi).

2.8. RT-qPCR

Seven genes related to endocytosis were analyzed by RT-qPCR.

Table 1
Primers used in the RT-qPCR.

Target genes	Primer sequences (5'-3')	Amplicon length (bp)
ARPC4	F: ATTTTCATCCCCAGGGCTAC R: GCTTCATCTCGCTGATCTCC	156
ARR3	F: TACCACGGAGACCCCATCAA R: TTTGGAGAAGGTGGAGTTGG	179
CHMP4C	F: GTGTAAATTCGCGGTGGTCT R: GGAACTCGATGGTTGAGAGC	140
CLTA	F: AGGCAAGATGAAAAGCTCCA R: CTCAGTGCCCGGAAATACAT	149
IST1	F: GGCAGAAGCGGTCTCTACAC R: CAGTTTGTGCATCAGCCTGT	169
VPS26B	F: GGACTGGACCCAACGTGTAT R: GTTGGAGTCAGCTCGTAGCC	127
CD63	F: TATGCCATGTCTCAGGACGA R: TCGGCTTCTATCAGCTCCTC	158
TSG101	F: CAACTGGCCCACCAACTACT R: GTCACAGGAGGCTGAGAAGG	169
β-actin	F: GATCTGGCACCACACCTTCT R: GCTACATACATGGCTGGGGT	153

Primers were designed using the Primer 3.0 software and shown in Table 1. The *β-actin* gene of ducks was used as the internal reference. The procedure of relative qPCR was performed by Applied Biosystems 7500 Real-Time PCR Systems (ABI, US). The relative quantities of genes were calculated with the $2^{-\Delta\Delta Ct}$ method.

2.9. Western blotting

The primary antibodies that recognize seven proteins related to endocytosis (ARR3, CHMP4C, CLTA, IST1, VPS26B, CD63 and TSG101) (ABclonal Technology, China) were used for western blotting verification. The HRP signal was detected by an enhanced luminol-based chemiluminescent substrate (Tanon Science & Technology, China). The protein expression differences were analyzed by calculating the gray value using the Image J software.

2.10. Data analysis

The corresponding histogram was plotted in the GraphPad Prism 5 software. All data were presented as mean ± standard error of the mean (SEM).

3. Results

3.1. DTMUV RT-PCR

RT-PCR results from DTMUV infected duck spleens were detected by agarose gel electrophoresis. The positive band size was observed at approximately 250bp, which was in accordance with the expected size (249bp) of the designed PCR amplified product (Fig. 1). The positive band could be seen from 2 hpi.

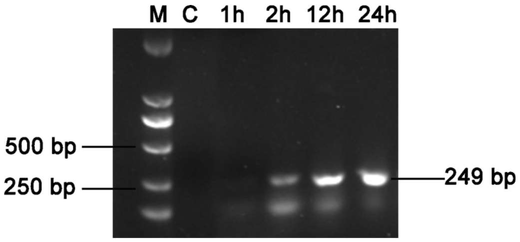

Fig. 1. RT-PCR results of duck spleens infected with DTMUV.
M: DL2000bp DNA marker; C: control group.

3.2. CD63 immunohistochemistry on DTMUV infected duck spleens

The immunohistochemical results can reveal the dynamic distribution of MVBs and their exosomes in spleens infected with DTMUV at different times post infection. A weakly positive signal was detected in the control spleen. Notable positively stained areas were mainly distributed in high endothelial cells of the sheathed capillaries, lymphocytes and the ellipsoid-associated macrophages within PELS of the white pulp at 2 hpi. The levels of positive staining in PELS were significantly reduced at 12 hpi relative to 2 hpi. At 24 hpi, there was a small amount of positive staining in the red pulp, and this was relatively lower than the staining at 12 hpi (Fig. 2).

3.3. CD63 and DTMUV-E protein double label immunofluorescence in duck spleens

The MVBs and their exosomes were detected as green fluorescent particles under the fluorescence microscope. At 1 hpi, few of fluorescent particles were observed in the PELS of the spleen white pulp. At 2 hpi, the number of fluorescent particles and the intensity of fluorescence were significantly increased, and the particles were mainly distributed in the high endothelial cells of the sheathed capillaries, lymphocytes and the ellipsoid-associated macrophages within the PELS. With the increasing infection time, the number of fluorescence particles and the intensity of fluorescence were gradually decreased. At 24 hpi, few fluorescence particles were detected (Fig. 3).

The DTMUV-E were detected as red fluorescent particles. The fluorescence particles were detected in the PELS (Fig. 3).

The colocalization of CD63 and DTMUV-E protein in the infected duck spleens was examined at 2 hpi by the emission of yellow fluorescence and observed in the PELS of the white pulp. The colocalization of CD63 and DTMUV-E protein was not observed in other infected duck spleens time points (Fig. 3).

3.4. Ultrastructural characteristics of MVBs and their exosomes

Ultrastructurally, different morphological features of MVBs containing exosomes were clearly observed in lymphocytes (Fig. 4a–c), the ellipsoid-associated macrophages of PELS (Fig. 4d), and the high endothelial cells of the sheathed capillaries (Fig. 5a–f) related to the blood-spleen barrier (BSB) and lymphocyte homing. Free exosomes were observed in blood vessels (Fig. 4g) and the extracellular matrix (Fig. 4f). The virus particles were detected in MVBs and cytoplasm (Fig. 5g–j). Typical structures of the four developing stages of MVBs, such as endocytic vesicular structures (Fig. 5b), pre-multivesicular bodies, pale multivesicular bodies (pM) and dense multivesicular bodies (dM), were detected. The pre-multivesicular bodies were characterized by their larger size, low electron density, and contained 1–3 vesicles (Fig. 4c, 5 d). There were often some vesicular structures observed around the pre-multivesicular bodies, and this vesicular structure could be fused with pre-multivesicular bodies. The pMs were characterized by their larger size, low electron density, and sparse arrangement of the contained vesicles (Fig. 4a–f). Some of the pMs' surfaces included microvilli-like structures, which were sequentially, broken into small pieces and entered the cytoplasm, resulting in the formation of smaller dMs (Fig. 4d). The dMs were characterized by their small size, high electron density, and the inner vesicles were closely arranged (Fig. 5b, d). Some MVBs were fused with the cell membrane at 24 hpi (Fig. 4e), indicating exosome release from MVBs to the adjacent lymphocytes. Compared to the control duck spleens, the number of MVBs and their exosomes were significantly increased at 2hpi in the duck spleens.

3.5. RT-qPCR analysis of genes related to endocytosis

Seven genes related to endocytosis (ARR3, CHMP4C, CLTA, IST1,

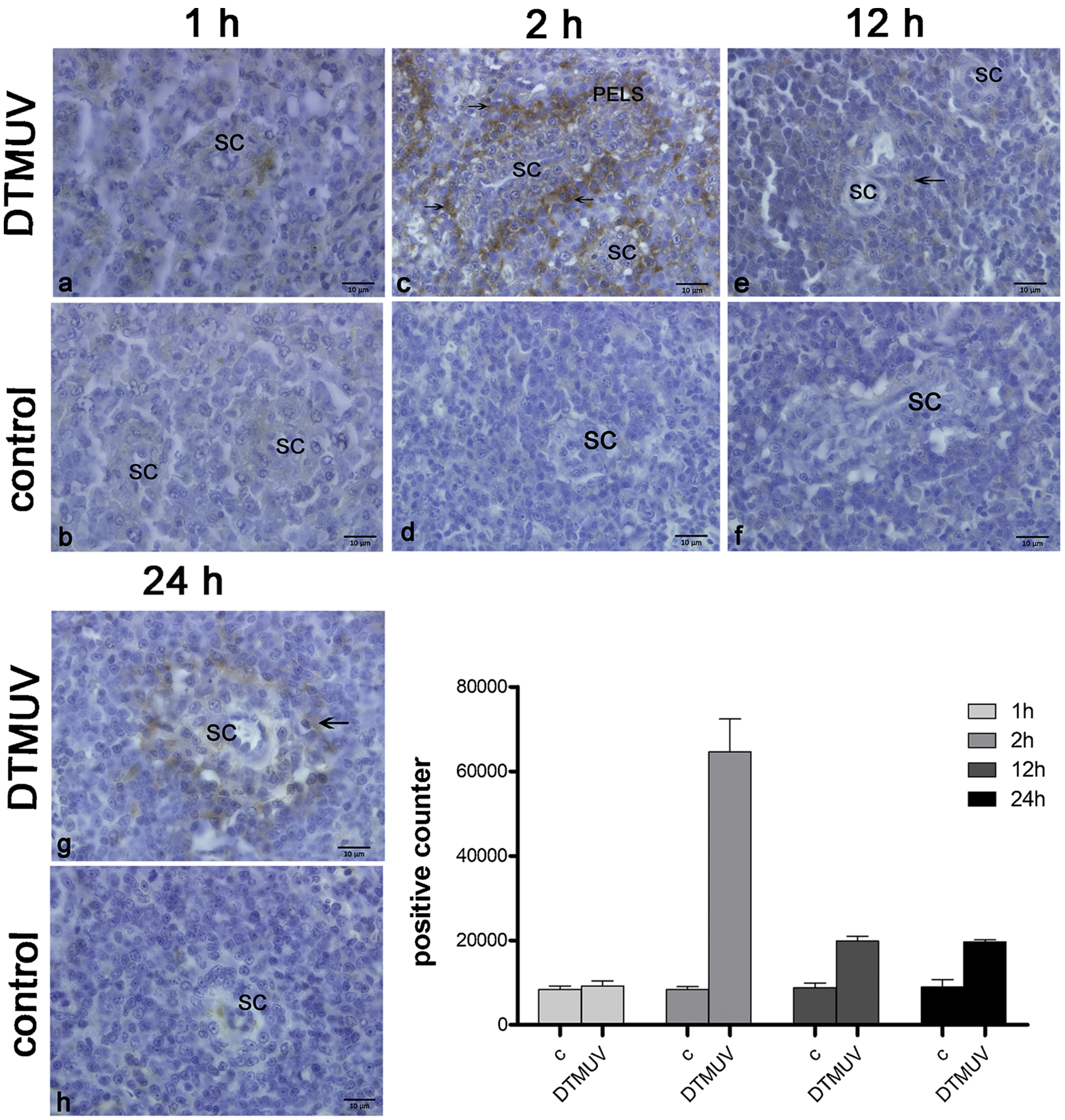

Fig. 2. CD63 immunohistochemistry on spleen slices of DTMUV infected ducks.
CD63 (arrows) mostly localized in the PELS of the white pulp in the infected spleens. SC: sheathed capillary; PELS: periellipsoidal lymphatic sheaths. Results are presented as means ± SEM.

VPS26B, CD63 and TSG101) were analyzed by RT-qPCR. The results revealed that the expression of these genes was significantly up-regulated at 2 hpi, and then down-regulated at 12 hpi and 24 hpi (Fig. 6).

3.6. Western blotting detection of endocytosis related proteins

The expression of proteins related to endocytosis was assessed by western blotting, using β-actin as an internal reference. The results revealed that the expression levels of these proteins were significantly increased at 2 hpi, which was consisted with the results obtained by RT-qPCR (Fig. 7).

4. Discussion

In our study, the DTMUV strain XZ-2012 was detected in the spleen of infected ducks from 2 hpi to 24 hpi by RT-PCR, which indicated that the invaded DTMUV was amplified in the spleen at 2 hpi. Meanwhile, the location and distribution dynamics of MVBs and their exosomes in the DTMUV infected duck spleens were determined by immunohistochemistry and immunofluorescence labeling. It was found that the numbers of MVBs and their exosomes were dramatically increased at 2 hpi, but with the increasing infection time, the positive staining within the PELS was significantly reduced. The RT-PCR, immunohistochemistry and immunofluorescence results suggested that a large number of MVBs and their exosomes were secreted in the duck spleen when DTMUV first invaded the host.

Spleen is the largest secondary lymphoid organ in ducks, and has a special tissue structure, which is characterized by alternating red and white pulp, with no marginal zone between them. High endothelial cells, lymphocytes, ellipsoid-associated macrophages, reticular cells, and reticular fibers form the duck's blood-spleen barrier (BSB) within the periellipsoidal lymphatic sheaths (PELS) of the white pulp are required for lymphocyte homing and immune defense functions (Boehm et al., 2012; Sun et al., 2018). Electron microscopy results revealed different morphological features and four developing stages of MVBs containing exosomes in high endothelial cells, lymphocytes, and the ellipsoid-associated macrophages. This finding was consistent with the results of immunohistochemistry and immunofluorescence, in which

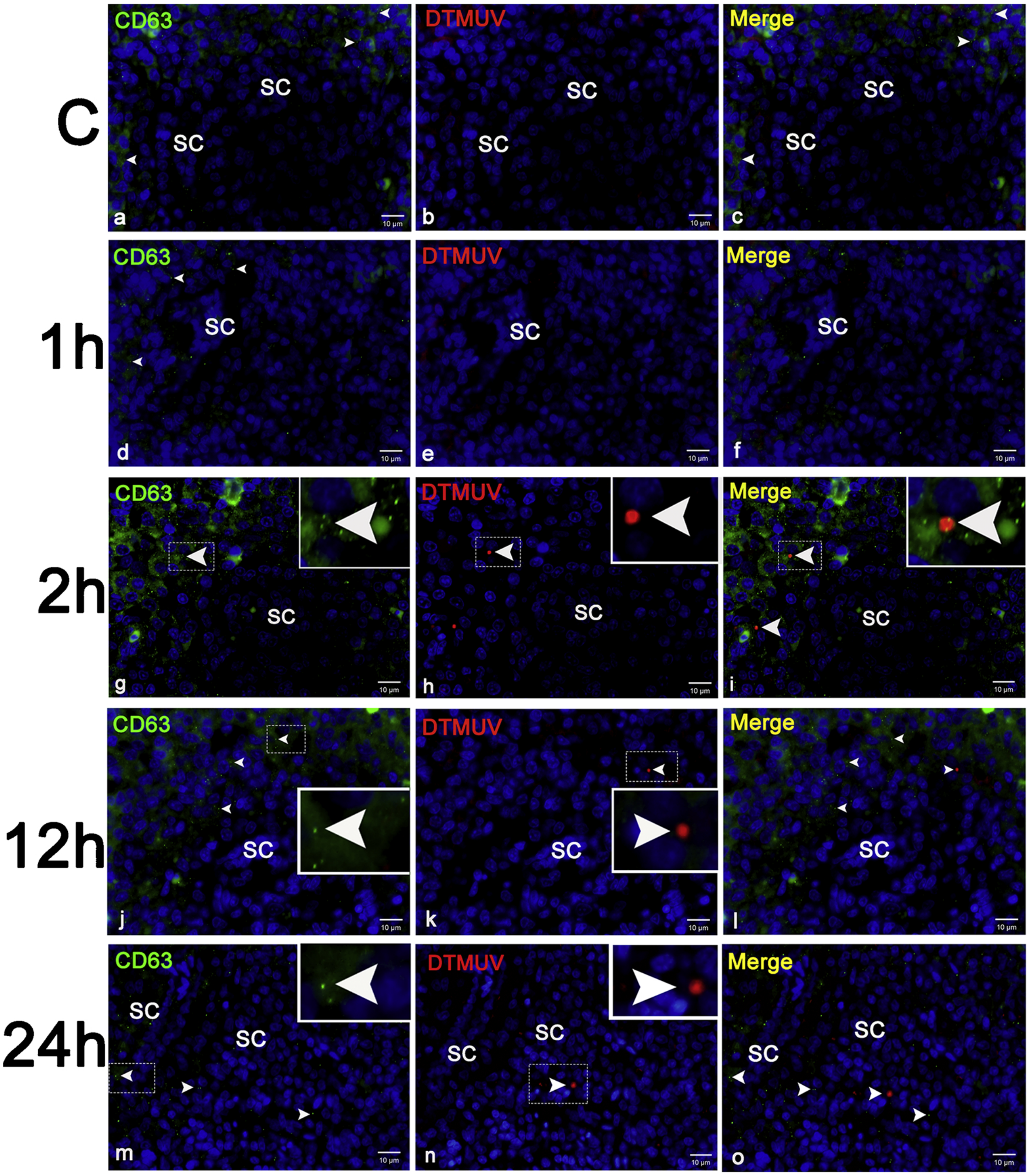

Fig. 3. CD63 and DTMUV-E double label immunofluorescence on spleen slices of DTMUV infected ducks.
CD63 single label, bar: 10 μm; DTMUV single label, bar: 10 μm; CD63 and DTMUV-E double labeled, bar: 10 μm. White arrow heads illustrate the positive in the upper right images, SC: sheathed capillary.

positive signals were mainly detected in the high endothelial cells of the sheathed capillaries, B lymphocytes (Olah and Glick, 1982) and the ellipsoid-associated macrophages within PELS (Figs. 8 and 9). It is possible that DTMUV have developed tissue tropism of lymphocytes, ellipsoid-associated macrophages and high endothelial cells, when they invade the duck spleen.

Studies have shown that viruses enter the host cells through the clathrin-mediated endocytosis (CME) (Chu and Ng, 2004; van der Schaar et al., 2008). The virus first binds to clathrin via receptors, then is fused with plasma membranes and finally gets incorporated into the newly formed endocytic vesicles. Endocytic vesicles containing the viral particles, are then fused with early endosomes (EEs). Simultaneously, a large number of intraluminal vesicles (ILVs) are formed by the invagination of endosomal membrane in the EEs, which sequentially mature to MVBs. During this process, the virus delivers its genome into ILVs, which serve as vehicles to transport the genetic material; ILVs are the origin vesicles of exosomes that are released from MVBs into extracellular matrix (Nour and Modis, 2014; Schorey et al., 2015). The clathrin-mediated endocytosis pathway involves the regulation of multiple genes (ARR3, CHMP4C, CLTA, IST1, VPS26B, CD63 and TSG101). CLTA participates in the formation of coated vesicles. ARR3 binding targets receptors to the internalization machinery. VPS26B and IST1 participate in protein sorting activities. TSG101, CHMP4C and CD63 label in MVBs and exosomes respectively. In our study, morphologically different vesicles, typical of the endocytic system, such as endocytic vesicles, MVBs and exosomes, were detected under electron microscopy, and the virus particles were detected in the MVBs during DTMUV infection. Meanwhile, the RT-qPCR and Western blotting results further confirmed the implication of the endocytic system since the expression of endocytosis-related genes and proteins were obviously

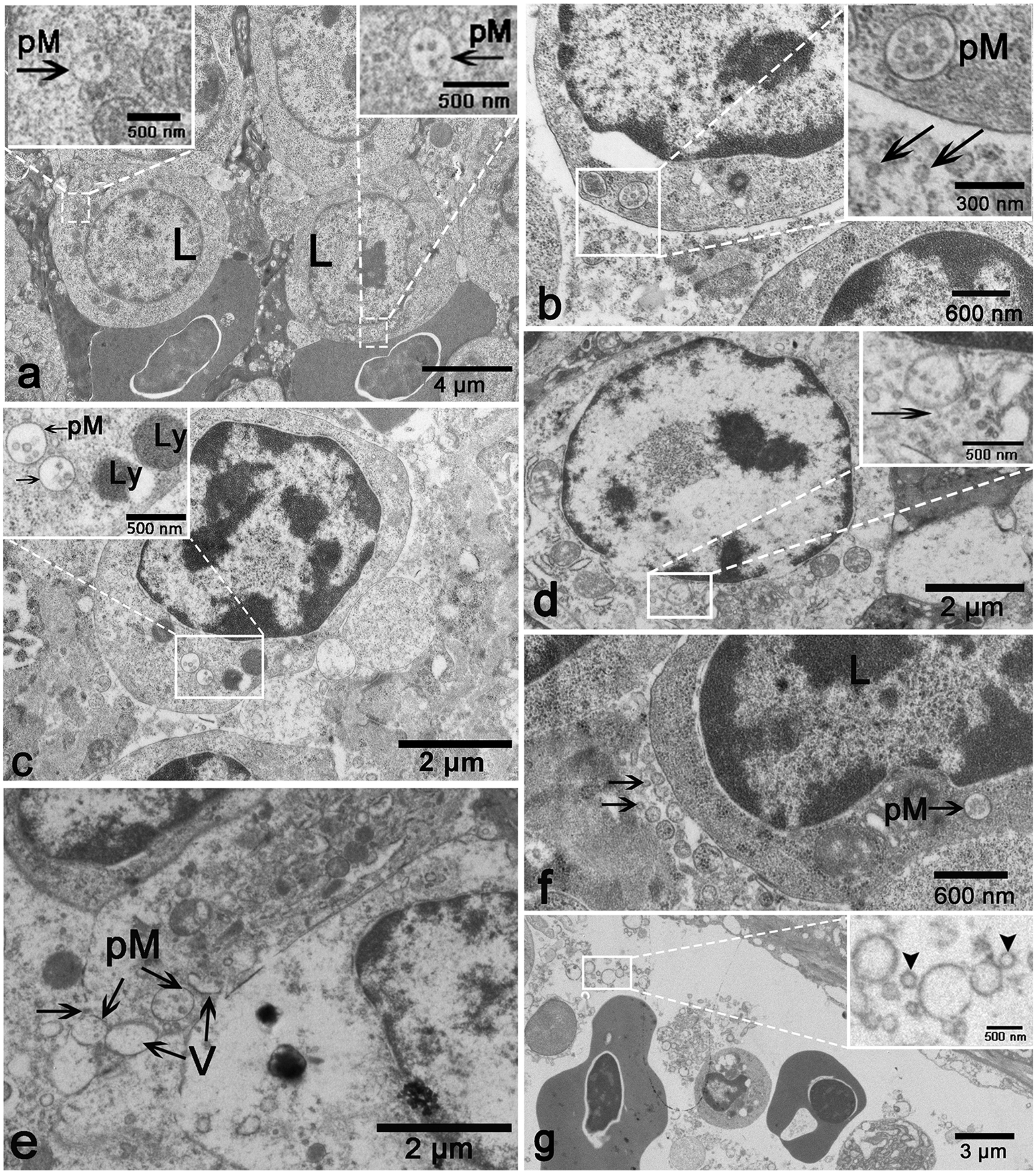

Fig. 4. MVBs and their exosomes of DTMUV infected duck spleen under TEM.
(a):The pMs in lymphocytes of red pulp of control spleen; (b): A pM in the lymphocytes at 2 hpi, exosomes in extracellular matrix (lower bar: 600 nm, upper bar: 300 nm); (c): The premultivesicular body and a pM surrounded by lysosomes in the lymphocytes at 12 hpi (lower bar: 2 μm, upper bar: 500 nm); (d): A pM with microvilli structure on its enveloping membrane in macrophages at 2 hpi. (lower bar: 2 μm, upper bar: 500 nm); (e): two pMs surrounded by some vesicular structures at 24 hpi, which are fused to the cell membrane, while the exosome is released (→) (bar: 2 μm); (f): exosomes (→) in extracellular matrix of lymphocytes at 2 hpi (bar: 600 nm); (g): exosomes (▲) in the intravascular at 24 hpi (lower bar: 3 μm, upper bar: 500 nm). pM: pale multivesicular body; V: vesicular structure; Ly: lysosome; L: lymphocyte.

up-regulated at 2 hpi compared to the control. At the same time, double-label immunofluorescence findings showed that DTMUV proteins were associated with exosomes. Therefore, it is probable that DTMUV enters the spleen cells via the clathrin-mediated endocytic pathway.

A report by Gould (Gould et al., 2003) indicated that retroviruses could “catch” exosomes and utilize them as a means of extracellular communication to transfer viral proteins, genes and whole virus particles between host and target cells, hence enabling them to escape host immune surveillance. Some enveloped viruses, such as the human immunodeficiency virus, the dengue virus and the Japanese encephalitis viruses were shown to utilize exosomes from host cells as transport vehicles to deliver their genetic material into neighboring cells (Arakelyan et al., 2017; Chahar et al., 2015). The results of double-label immunofluorescence revealed that the DTMUV proteins are associated with exosomes, thus it is presumed that MVBs and their exosomes in the high endothelial cells, lymphocytes, the ellipsoid-associated macrophages and the extracellular matrix contain DTMUV genes or proteins, could help the virus to infect cells and escape the host’s immune surveillance.

In summary, this study presents for the first time the localization and dynamics distribution of MVBs and their exosomes in the duck

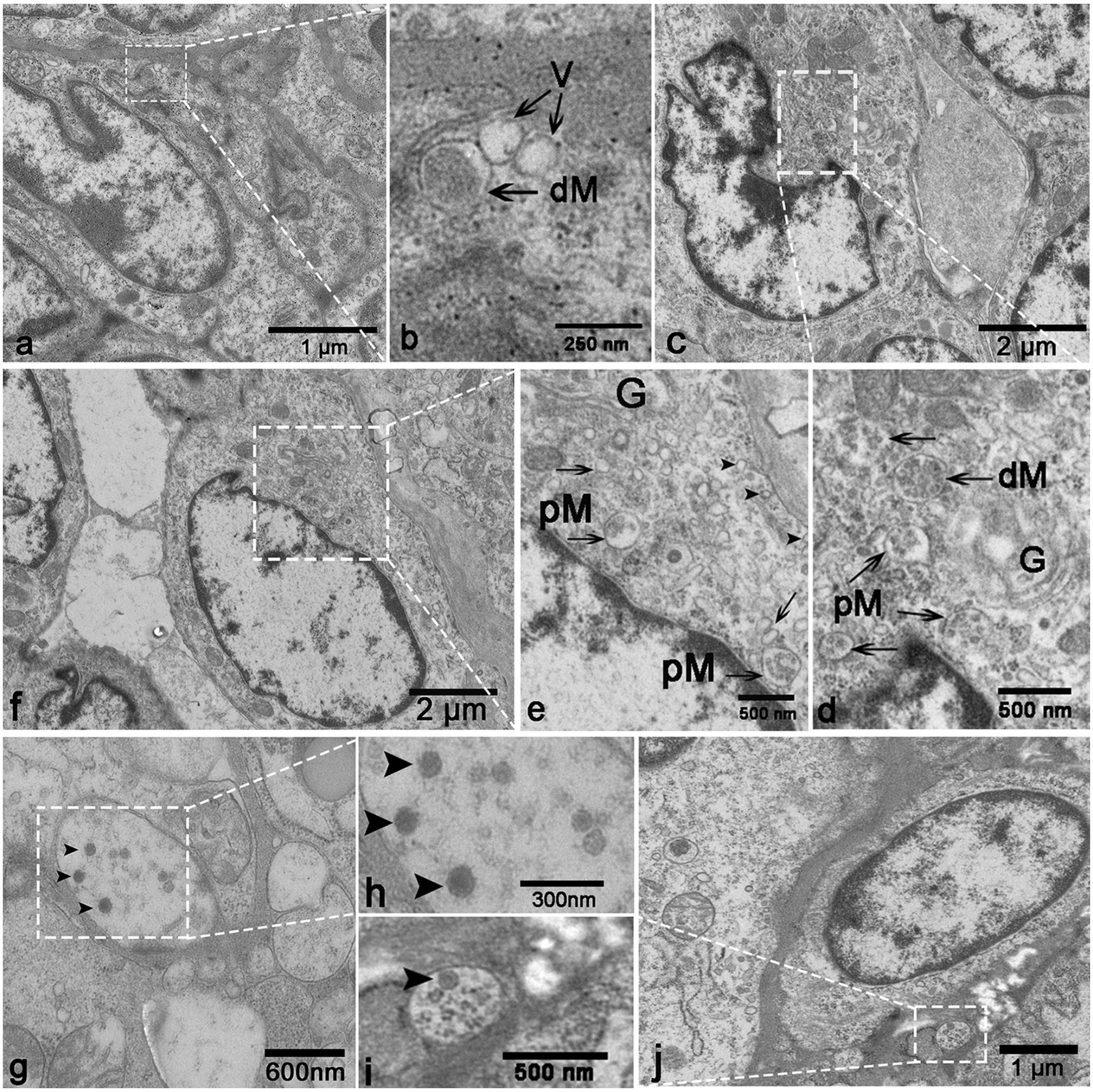

Fig. 5. MVBs and their exosomes of DTMUV infected duck spleens under TEM.
(a): The high endothelial cells in the control spleen (bar: 1 μm); (b): A dM in the basement membrane of the sheath capillaries, near the two vesicular structures (V) (bar: 250 nm); (c): The high endothelial cells at 2 hpi (bar: 2 μm); (d): The pM, dM and a premultivesicular body (→) in Golgi region(G) (bar: 500 nm); (e): At 2 hpi, two pMs in Golgi region(G) are surrounded by some vesicular structures (→) and some endocytic vesicular structures (▲) near the plasma membrane (bar: 500 nm); (f): The high endothelial cells at 2 hpi (bar: 2 μm); (g): The virus particles in cytoplasm at 24 hpi (bar: 600 nm); (h): The magnification of Fig(g) (bar: 300 nm); (i): A virus particle (▲) in MVB at 2 hpi (bar: 500 nm); (j): A MVB at 2 hpi (bar: 2 μm). pM: pale multivesicular body; dM: dense multivesicular body; G: Golgi region; V: vesicular structure; MVB: multivesicular body.

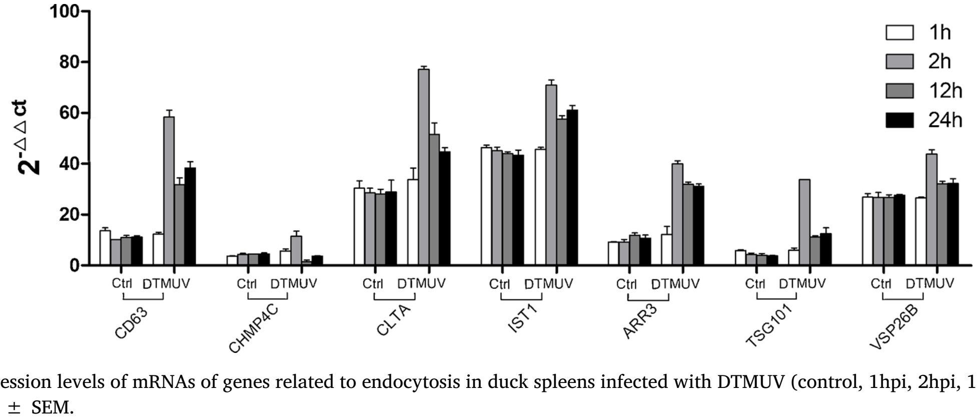

Fig. 6. Differential expression levels of mRNAs of genes related to endocytosis in duck spleens infected with DTMUV (control, 1hpi, 2hpi, 12hpi, 24hpi). The results are presented as means ± SEM.

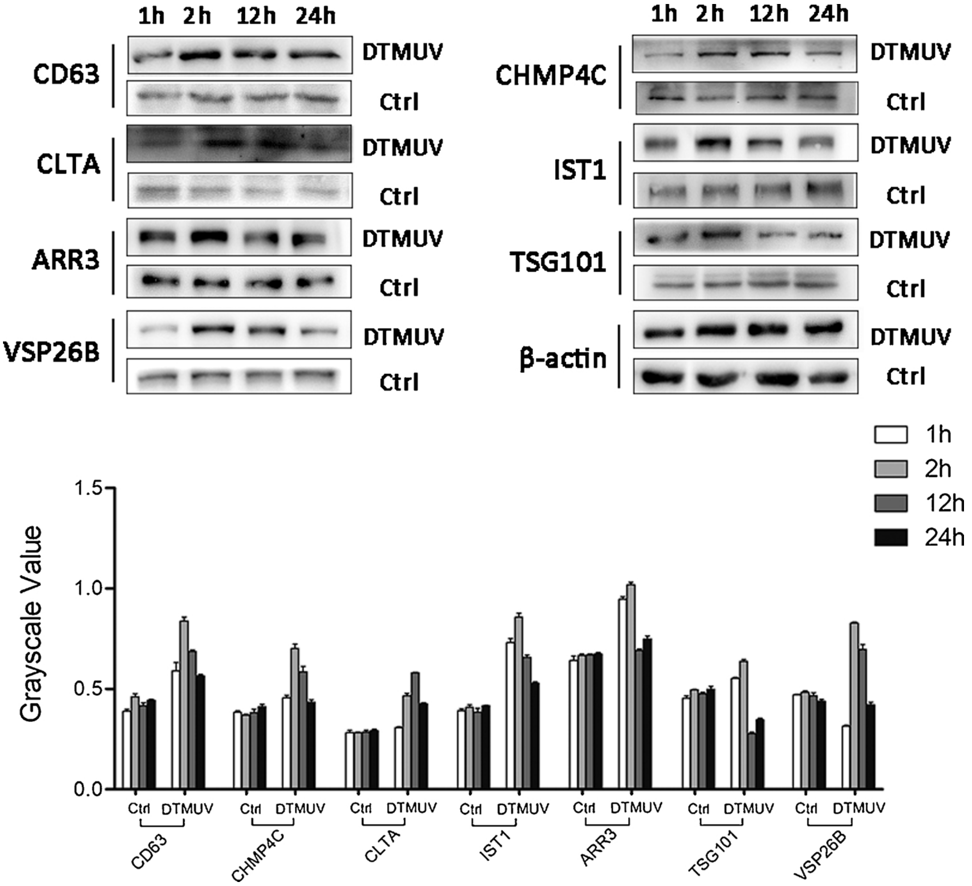

Fig. 7. Western blotting results (A) and grayscale value (B) of proteins related to endocytosis (ARR3, CHMP4C, CLTA, IST1, VPS26B, CD63 and TSG101) in duck spleens infected by DTMUV. β-actin served as the internal reference. Results are presented as means ± SEM.

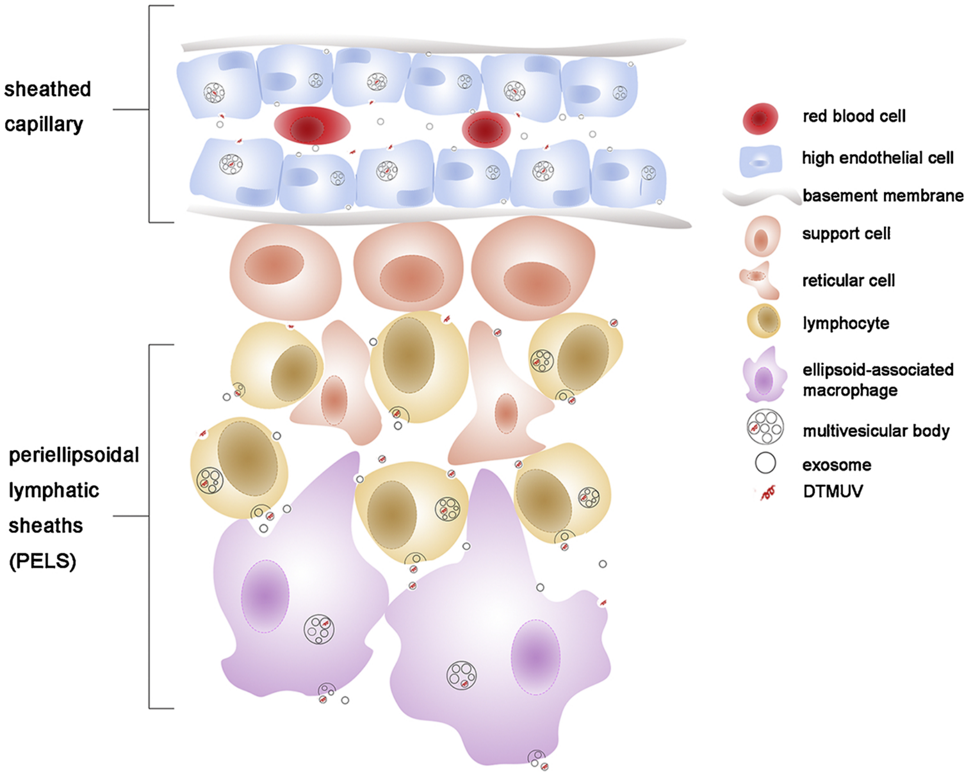

Fig. 8. Schematic representation of the release of MVBs and their exosomes from DTMUV-infected cells in duck spleens. The virus enters into spleen cells by utilizing the endocytosis pathway, and then enters into MVBs which contain a large number of intraluminal vesicles (ILVs). These MVBs get fused with the plasma membrane and release their contents, including exosomes, and these exosomes may communicate information between infected and uninfected cells.

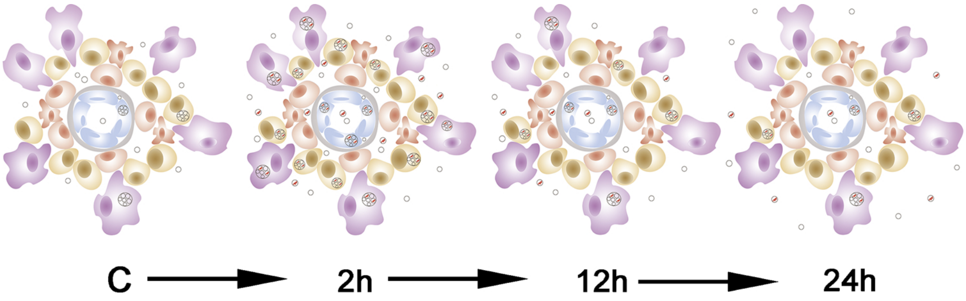

Fig. 9. Schematic representation of the dynamic distribution of MVBs and their exosomes in PELS of duck spleens infected by DTMUV. DTMUV infected duck spleens stimulate spleen cells especially cells around PELS form MVBs and exosomes. With the increasing infection time, the number of MVBs and their exosomes is reduced and has a tendency spread to red pulp.

spleen infected with DTMUV. When DTMUV first invaded into the duck spleen, a large number of MVBs and their exosomes were secreted in the lymphocytes, the ellipsoid-associated macrophages and the high endothelial cells. Simultaneously, as the strategy of viruses is to invade cells and escape host immune responses, the clathrin-mediated endocytic pathways were exploited by the virus to entry into the spleen cells, and the exosomes were "hijacked" by the virus to infect other uninfected neighboring cells.

Authors' contributions

The authors have made the following declarations about their contributions: Enxue Liu, Xuejing Sun and Qiusheng Chen designed the experiments. Enxue Liu performed the experimental work about MVBs and exsosomes, analyzed data with the assistance of Xuejing Sun, Xindong Wang and Wenqian Li. Xuejing Sun performed the experimental work about virus culture, titration and detection. Enxue Liu wrote the manuscript with the assistance of Xuejing Sun. Taozhi Wang helped with the animal experiment. Imran Tarique and Ping Yang gave advice of the manuscript writing. Qiusheng Chen revised the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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