The Thai Journal of Veterinary Medicine

Volume 51
Issue 3 *September, 2021*

Article 14

9-1-2021

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Hong AN

Zhe SHI

Bofan FU

Xinxin YAO

Hemin NI

See next page for additional authors

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Original Article

The effects of endometrial epithelium-derived exosomes on the recruitment and activation of T Lymphocytes in the uteri of dairy cows

Hong AN[1] Zhe SHI[1] Bofan FU[1] Xinxin YAO[1] Hemin NI[1]

Yong GUO[1] Longfei XIAO[1*] Xiangguo WANG[1*]

Abstract

Endometritis is caused by endometrial cell injury and chronic inflammation of uteri infected by pathogens, leading to reproductive failure in dairy cows. Changes of the endometrial immune response regulates the development of endometritis. In a recent discovery, exosomes acted as a functional regulator secreted by a variety of cells, however, its regulatory mechanism in the local immune response of the uteri is still unclear. In this study, flow cytometry was performed to identify the increased number of T lymphocytes, namely Tc cells, in the uteri with endometritis while the Th and Treg cells decreased very significantly. Moreover, the functional marker factors FOXP3 of Treg cells and Th17 cells were significantly decreased and increased, respectively. The mRNA expression of immune tolerance regulators PD1, CTLA4 and Galectin-1 in the group of Treg cells which were co-incubated with LPS-stimulated endometrial epithelium-derived exosomes was recorded to be significantly lower than the Treg cells which were co-incubated with normal EEC-derived exosomes. Furthermore, the protein expression of PD1, CTLA4, Galectin-1, Foxp3 and IL-17 was consistent with the results in the Treg cells co-incubated with different source exosomes in CTLL-2 cells. It was demonstrated that endometrial epithelium-derived exosomes act as a vital regulator of changes in the composition and function of T lymphocyte subsets in the uteri of dairy cows.

Keywords: Endometritis, T lymphocytes, Exosomes, Treg

[1]*Animal Science and Technology College, Beijing University of Agriculture, Beijing 102206, China*

***Correspondence**: xiaolf1989@bua.edu.cn, xiangguo731@163.com* (L. Xiao, X. Wang)

Received February 22, 2021.

Accepted April 30, 2021.

doi: https://doi.org/10.14456/tjvm.2021.64

Introduction

As a common reproductive disease of dairy cows, endometritis can cause infertility in dairy cows and bring serious economic loss to dairy farming (Mounir *et al*., 2017). The occurrence of endometritis is closely related to immune status and the role of related immune cells is critical in the process of uterine involution in dairy cows (Brodzki *et al*., 2014[a]).

T lymphocytes play an essential role in protecting the body against pathogens, mainly by assisting other lymphocytes in playing their corresponding role(Kuwabara *et al*., 2018). Regulatory T (Treg)cells belong to the unique T cell subset which is a key regulator of inflammation and plays an important role in maintaining immune tolerance and regulating immune response (Göschl *et al*., 2019) and are mainly driven by the transcription factor Forkhead box p3（FOXP3） to enhance the lineage development and immunosuppressive function of immune cells (Deng *et al*., 2019). CD4+CD25+Foxp3+Treg cells belong to the CD4+T cell subgroup, which utilizes cell surface molecules such as CytotoxicT-lymphocyte-associated protein 4 (CTLA-4), IL-2Rα and glucocorticoid-induced tumor necrosis factor receptors (GITR) to exert immunosuppressive effects and can modulate the activity of antigen-presenting cells (APC) (Lu *et al*., 2017; Mahne *et al*., 2017; Mao *et al*., 2019; Zhao *et al*., 2020). PD-1 has been recognized as a sign of CD8+ T cell exhaustion, which plays an important role in the stability and inhibition of Treg cells (Bengsch *et al*., 2016; Ahn *et al*., 2018), that programmed cell death-1 (PD-1) and cytotoxicT-lymphocyte-associated protein 4 (CTLA4) jointly promote the inhibitory function of Treg PD-1. After being activated by binding to the corresponding ligand PD-L1, PD-1 can promote the development of tumors and thus inhibit the production of anti-tumor function factors IFN-γ and tumor necrosis factor α (TNF-α)by CD8+ T cells (Gao *et al*., 2019). CTLA-4, a competitive antagonist for CD28-CD80/86 binding, can further impede the priming and activation of T cells and belongs to the negative regulatory stimulation factor of T cell functions (Rowshanravan *et al*., 2018). CTLA-4 is associated with many autoimmune diseases and some researches have proved that CTLA-4 gene plays a key role in the immune tolerance of maternal–fetal interface and relates to the occurrence of abortion (Li *et al*., 2018). Galectin-1, as the galectin first discovered, is an anti-inflammatory mediator and also immunosuppressive factor. It can be expressed by Treg cells and has been proved to be associated with inflammatory response, cancer and autoimmune diseases (Brinchmann *et al*., 2018; Toscano *et al*., 2018; Nambiar *et al*., 2019).Recent studies have also indicated that Galectin-1 can be used for the treatment of cardiovascular disease and diabetes (Seropian *et al*., 2018; Al-Obaidi *et al*., 2019). Due to the presence of an abnormal number of six cysteine residues, Galectin-1 is prone to oxidative inactivation, which hinders its immunomodulatory activity (Guardia *et al*., 2014). Studies have confirmed that the number of Tregs increases significantly during normal pregnancy in humans and mice and the use of antibodies to antagonize CD25+Tregs can cause an increase in the number of mouse embryos absorbed. Furthermore, the number of CD4+CD25+Treg also shows a decreasing trend in human recurrent spontaneous abortion(Shima *et al*., 2010; Albonici *et al*., 2020; Cheng *et al*., 2020). However, the mechanism of local T lymphocyte and its immunoregulatory factor in the occurrence and development of endometritis in cows is still unclear.

As a collection of membrane-bound carriers containing complex contents, like proteins, lipids and nucleic acids (Barile and Vassalli, 2017), exosomes can be secreted by a variety of cells, such as nerve cells, epithelial cells, antigen presenting cells (APC) and tumor cells (Cordonnier *et al*., 2017). At present, exosomes are clinically used as biomarkers (Dixon *et al*., 2018; Ludwig *et al*., 2019; Sharma and Johnson, 2020), vaccines (He *et al*., 2018) and drug carriers (Alvarez-Erviti *et al*., 2011) for various diseases and can also be reasonably modified for therapeutic interventions (He *et al*., 2018), antigen presentation and immunomodulation (Zhu *et al*., 2019).

Therefore, this study analyzed the changes of local T lymphocyte subsets and the expression of immunoregulatory factors in the uteri of cows with endometritis, as well as the effect of LPS-stimulated endometrial epithelium-derived exosomes on the expression of immunoregulatory factors in Treg cells of healthy uteri and the induced Treg of mice, in order to elucidate the mechanism of endometrium epithelium-derived exosomes in regulating local T lymphocyte and its immunoregulatory factors in bovine uteri with endometritis and further provide a theoretical basis for the research and development of immunotherapeutic agents for cows with endometritis.

Materials and Methods

Acquisition, anatomical and cytological identification of bovine uteri: This study was performed in accordance with the guidelines of the Animal Ethics Committee of Beijing University of Agriculture (Permit number: SYXK(JING)2015-0004). All the experimental cows were Holstein cows from Beijing Shun Sunshine Farm. Before slaughter, the postpartum Holstein cows from Beijing Shun Sunshine Farm were monitored by rectal temperature measurement and rectal examination for uterine rejuvenation on Days 1, 7, 14, 21 and 30 after delivery, combined with the daily disease and medication conditions of dairy cows. The bovine (parity 2-4, body condition score 3.25-4.0) uterus without other diseases such as mastitis, hoof disease, dermatitis and postpartum paralysis (Archbold *et al*., 2012), whose body temperature was lower than 39.5°C and the mucopurulent or purulent secretions secreted through the vagina reaching the level 3 of vaginal mucus secretion in bovine endometritis were collected at 21-30 d postpartum as the clinical endometritis group (Williams *et al*., 2005). In addition, those healthy bovine uteri without disease (vaginal endoscopy without mucopurulent discharge) but with normal body temperature, 21-30 d postpartum served as the control group. The cows both in the healthy and in the endometritis group had no estrus and corpus luteum prominently protruding from the surface of the ovary within 21-30 days after delivery with rectal

examination. After being sent to the laboratory, the uterine horns (each group had 5 samples) were incised in the fresh uteri of cows in the healthy and diseased group, respectively, to observe endometrial morphology, color and inflammatory changes. Then mucus secreted from the uterine wall was scraped off and stained with the Diff-Quick staining kit in each group.

Identification of local lymphocyte subsets in bovine uteri and sorting of Treg cells: T lymphocytes in the uterine tissue were obtained according to the Bovine Organ Tissue Lymphocyte Extraction Kit (Beijing Solarbio Technology Co, Ltd.). Briefly, healthy and inflamed uterine local tissues were collected, the outer membrane removed, cut into small pieces and placed in a cell sieve. The bottom of the cell sieve was soaked in a plate containing lymphocyte separation liquid and ground; the ground cell mixture was collected and sieved. After that, the single cell suspension was carefully pipetted onto the surface of an equal amount of separation liquid and centrifuged at 500-900g at room temperature for 20-30 mins. After centrifugation, a pipette was used to carefully draw the second layer of ring-shaped milky lymphocytes into another clean 15 mL centrifuge tube, 10 ml of cell washing solution was added to the centrifuge tube to wash the buffy coat cells, 250 g, centrifuge for 10 mins. The supernatant was discarded and the cells resuspended in 5 mL of PBS and centrifuged at 250 g for 10 mins. The supernatant was discarded and the cells resuspended. Lymphocytes from healthy and endometritic bovine uteri were extracted and placed in RPMI Medium 1640 (containing 10% exosome-free fetal bovine serum (SBI, EXO-FBS-50A-1), penicillin 100 U/ml, and streptomycin 100 g/ml) for suspension culture in 5% CO_2 cell incubator at 37°C, and identified by flow cytometry the next day (Beijing Keyueda Biotechnology Co., Ltd.), respectively. At the same time, Treg cells were sorted from the lymphocytes isolated from the uteri of healthy cows (Beijing Boyunhui Biotechnology Co., Ltd.).

Identification of T lymphocyte subsets by flow cytometry: 100µl cell suspension was taken into flow tubes respectively, mixed with 2 ml Cell Staining Buffer and then centrifuged at 300 g for 5 minutes to discard the supernatant. Then, 10 µl CD4 FITC antibodies and 10 µl CD25 PE antibodies were added to the CD4/CD25 staining tube, 10 µl CD8 FITC antibody was added to the CD8 staining tube and 0.5 ug CD3 antibody was added to the CD3 tube; the blank control tubes and isotype control ones were set separately. After all tubes were incubated at room temperature in the dark for 20 minutes, 2 ml Cell Staining Buffer was added and mixed well and the mixture was centrifuged at 300 g for 5 minutes before the supernatant was discarded. Subsequently, 0.5 ug Goat anti-Mouse IgG-PE was added to the CD3 staining tube and its isotype control tube, mixed well and incubated at room temperature with the avoidance of light for 30 minutes. Then, 2 ml Cell Staining Buffer was added to mix well, the mixture was centrifuged at 300 g for 5 minutes and the supernatant was discarded. Lastly, 0.5 ml Cell Staining Buffer was added to each tube, mixed well and detected by flow cytometry.

Sorting of Treg cells by flow cytometry: The cow's lymphocytes were centrifuged in a 1.5 ml centrifuge tube for 5 mins. After the supernatant was absorbed, 10 µl cells were aspirated for cell counting. Then 10 µl of CD4 and CD25 antibodies were added and the mixture was left at 4°C for 30 mins. After centrifugation to retain the pellet, 1 ml PBS was added to re-suspend the cells. Subsequently, the cells were centrifuged at 200 g for 5 mins, the supernatant was discarded, then 500 µl M1640 was added to blow off the cells. Finally, the bovine Treg cells were sorted by flow cytometry, pipetted into 24-well plates, supplemented with RPMI Medium 1640 (without exosomes) and cultured in an incubator.

Isolation and identification of exosomes derived from endometrial epithelial cells: The cows' endometrial epithelial cell line (BEND, GDC-9643015) was subcultured and washed with DPBS containing 100U/mL penicillin and streptomycin. Both groups were added with DMEM/F12 culture solution (added serum without exosomes) and an additional 50 µg/ml LPS was added to the experimental group. After incubation for 24 h, the supernatant was stored by centrifugation. Then, exosomes from normal cultured endometrial epithelial cells and those from LPS-treated endometrial epithelial cells were extracted using the Exosomes Isolation Kit (Shanghai Bestbio Biotechnology Co., Ltd.). Briefly, 4 ml of cell supernatant were collected and centrifuged at 3000g for 15 minutes at 4°C and the pellet discarded. The supernatant was collected, centrifuged at 10000g for 20 minutes at 4°C, the precipitate discarded, 4 ml of the supernatant collected in another clean centrifuge tube, 1 ml of extract A added, mixed and let stand overnight at 2-8°C. Afterwards, the mixture was centrifuged at 10,000g for 60 minutes at 4°C, the precipitate collected and 50 microliters of exosomal preservation solution added to resuspend. The two groups of extracted exosomes were identified by electron microscopy and the expression of CD9 marker protein was detected with western blot.

Morphology of exosomes was examined by electron microscopy: The exosomes obtained from plasma and EEC cell culture solution were re-suspended in 30 µl PBS, 10 µl samples were added on the copper net for precipitate for 1 min and the floating liquid was absorbed with filter paper. And then, Uranyl acetate (phosphotungstic acid) 10 µl was added to copper net for precipitation for 1 min and the floating liquid was absorbed with filter paper. It was dried for a few minutes at room temperature. Electron microscopy imaging was performed at 80kv.

Incubation of exosomes with cows' Treg cells and mice's T lymphocyte CTLL-2: The Treg cells of bovine uteri sorted by flow cytometry were detected for the localization and expression of its functional marker FOXP3 by immunofluorescence to ensure the successful sorting of Treg cells. Then the Treg cells were transferred to 6 wells of a 24-well plate, 100 µl of normally cultured bovine endometrium epithelium-

derived exosomes were added to 3 wells and LPS-treated bovine endometrial epithelium-derived exosomes were added to the other 3 wells, before they were cultured for 24 h, respectively. Meanwhile, the mice's CTLL-2 T lymphocytes (purchased from ATCC cell bank) were subcultured, the prepared IL-2 M1640 was added to the mice's T lymphocytes and achieved a growth density of 80% to induce mice's T cells to generate Treg cells. Finally, two previously extracted exosomes were added, respectively and the mixtures were cultured in an incubator for 24 h.

Real-time detection of RT-PCR (qRT-PCR): Total RNA of each treated cell was extracted using the Trizol (Invitrogen, Inc., Carlsbad, CA, USA) and cDNA was synthesized using the PrimeScriptTM RT Reagent Kit (TaKaRa Bio, Inc., Dalian, China), according to the manufacturers' protocols. Real-time PCR was subsequently performed using an ABI 7500 Sequencing Detection System and SYBR Premix Ex Taq™. The GenBank accession numbers and primer sequences of CTLA4, PD1, Galectin1 and GAPDH are summarized in Tab. 1. All reactions were performed in at least three independent experiments and the calculated number of copies of target genes was normalized to the number of *GAPDH* mRNA copies in the same sample.

Table 1 CTLA4, PD1, Galectin1, GAPDH gene primers

Gene name	sequence
CTLA4-FO	TCTAAAGGGATGAATGTGACCCAG
CTLA4-RE	ACCTCGTCAGCTTTGCCTGAA
PD1-FO	CTGTCACAGTGGACTACGGG
PD1-RE	GAAGACGATGGTGGCGTACT
Galectin1-FO	AAGTGTCGTGGAGGTATGCA
Galectin1-RE	ACAGGTAGTTGATGGCCTCC
Bos GAPDH 1P1S	GGCGTGAACCACGAGAAGTA
Bos GAPDH 1P2A	GGCGTGGACATGGTCATAA

Western blot analysis: Cells and exosomes were harvested and lysed on ice for 30-45 mins in the lysis buffer. The supernatant was then collected in a new tube by centrifugation for 15 mins at 14,000 rpm at 4°C. After that, the protein concentration was calculated by the BCA assay. Total cellular protein of 40 µg was electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gradient (SDS-PAGE) gel and electro-transferred onto PVDF membranes. The membranes were then blocked for 1 h with 5-10% fatty acid-free milk in Tris-buffered saline that contained 0.5% Tween-X-100 (TBST) at room temperature and incubated at 4°C overnight in blocking solution containing primary antibody (Tab. 2). The next day the membranes were washed with TBST 5 times and incubated with the secondary antibody for 1 h at room temperature. Finally, the immunoreactive bands were visualized with the Gel Image System.

Immunofluorescent staining: Bovine Treg cells and exosome-stimulated CTLL2 cells were cultured in 24-well plates. Immunofluorescent staining of FOXP3, CTLA4, PD1 and Galectin1 was performed. Bovine Treg cells and exosome-stimulated CTLL2 cells were fixed in 4% paraformaldehyde for 30 mins and then permeabilized for 15 mins with 0.1% Triton X-100 in PBS, subsequently blocked for 1 h with 5% BSA in PBS at room temperature, and co-incubated with primary antibody at 37°C for 2 h (Tab. 2), respectively. Washing was followed by incubation with secondary antibody at 37°C for 1 h and the nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) for 3-5 mins. The fluorescent signals were examined under a Nikon A1R si confocal microscope system.

Statistical analysis: Data was analyzed by one-way ANOVA and the analysis was performed using SPSS software (Version 13.0, SPSS, Chicago, IL, USA) with Tukeys post hoc test. $p<0.05$ was regarded as statistically significant. All data is represented as the mean SEM of at least three separate experiments.

Table 2 Antibodies used in this study

Target(diluted)	Catalogue number	Company
CD8 Monoclonal Antibody (38.65)	MA1-80900	Invitrogen
CD4 Monoclonal Antibody (CC8)	MA1-80902	Invitrogen
CD25 Monoclonal Antibody (IL-A111)	MA5-28330	Invitrogen
Anti-BOVINE CD3	WS0561B-100	KingfisherBiotech
Goat Anti-Mouse IgG	1030-09S	SouthernBiotech
Anti-CD9 antibody	ab92726	Abcam
Rabbit Anti-beta-Actin (Loading Control) antibody(1:10000)	bs-0061R	Bioss
Rabbit Anti-IL-17 Polyclonal Antibody(1:1000)	bs-1183R	Bioss
Rabbit Anti-IL17E antibody(1:1000)	bs-10943R	Bioss
Rabbit Anti-FoxP3 antibody(1:1000)	bs-10211R	Bioss
Anti-CTLA4 antibody [CAL49](1:1000)	ab237712	Abcam
Anti-PD1 antibody [EPR20665](1:1000)	ab214421	Abcam
Anti-Galectin 1 antibody [6C8.4-1](1:1000)	ab205889	Abcam

Results

Anatomical and cytological identification of bovine uteri with endometritis: The results showed that the inside of the healthy uterus was flesh-colored with a smooth inner wall and a small amount of clear fluid (Fig. 1A) whereas the uterus of cows with endometritis showed dark red inflammatory tissue inside, with inflamed swelling and ulceration on the inner wall along with dark red fluid (Fig. 1B). Only exfoliated epithelial cells were presented in healthy uterine luminal fluid (Fig. 1C) in comparison to a large number of neutrophils in the uterine luminal fluid with endometritis (Fig. 1D).

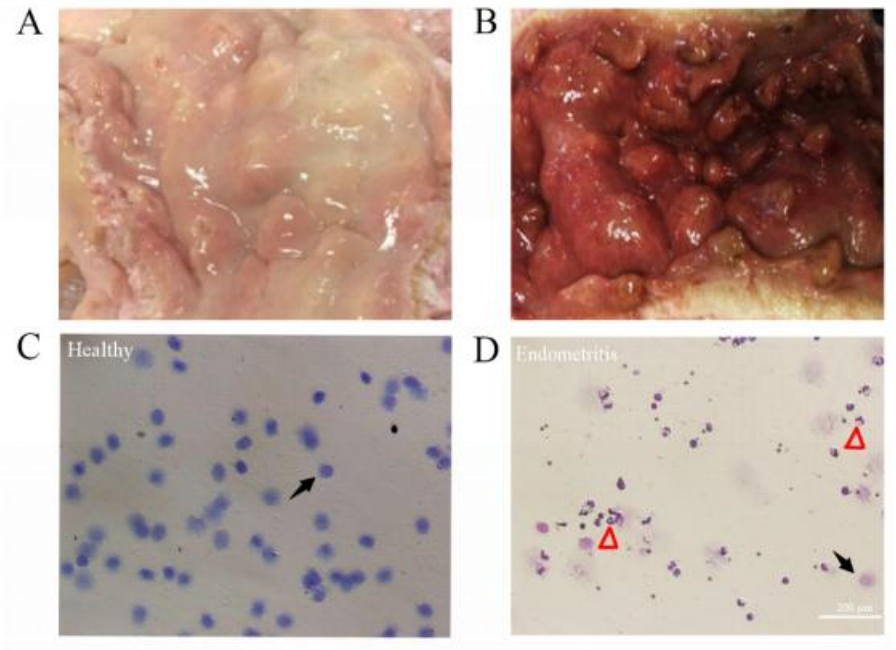

Figure 1 Anatomical and cytological identification of cow uterus. (A)Endometrial cavity of healthy cows. (B)Endometrial cavity of cows with endometritis. (C) Uterine lavage fluid cytology of healthy cows (D) Uterine lavage fluid cytology of cows with endometritis. The triangle indicates the shed epithelial cells of the uterus and the arrow points to a Neutrophil.

Changes of local T lymphocyte subsets and main regulatory factors in the uteri of cows with endometritis: Lymphocyte subpopulation analysis is an important indicator for detecting cellular immunity and humoral immune function. It generally reflects the body's current immune function, state and balance level, and can assist in the diagnosis of certain diseases and analyze their pathogenesis. The results of flow cytometry showed that the numbers of T cells (CD3+), Th cells (CD4+), Treg cells (CD4+ CD25+) and Tc cells (CD8+) in the uteri of the cows with endometritis were significantly higher than in healthy cows (Figure S1A and B, $p< 0.01$). Compared with healthy bovine uteri, the proportion of T cells (CD3+) did not change, whereas the proportion of Th (CD4+) and Treg cells (CD4+ CD25+) showed a significant decrease. However, the proportion of Tc cells (CD8+) in T cells increased extremely significantly (Fig. 2A, $p< 0.01$) in the uteri of cows with endometritis. At the same time, the western blot results also showed that the Treg cell (CD4+ CD25+) identification factor FOXP3 was significantly reduced (Fig. 2B and C, $p<0.01$), whereas, Th17 cell secreted factor IL-17 expression was significantly increased (Fig. 2B and C, $p<0.05$).

Isolation and identification of EECexo and LPSexo: The results show that both EECexo and LPSexo had a membrane vesicle structure with a diameter of between 30 and 150 nm (Fig. 3A) and the exosome marker factor CD9 was expressed in EECexo and LPSexo (Fig. 3B). In addition, in each treatment with the same number of cells group there was no significant difference in the amount of total RNA in exosomes derived from endometrial epithelial cells stimulated by LPS and exosomes derived from normal cultured endometrial epithelial cells (Fig. S2, $p>0.05$). Therefore, in our subsequent exosome stimulation experiments, we added exosomes derived from the same number of donor cells to the same number of recipient cell culture media.

Effect of EECexo and LPSexo on the expression of T cell regulatory factors in healthy bovine uteri: Related studies have found that CD4+CD25+Treg plays an important role in maintaining normal pregnancy in humans and mice(Shima *et al*., 2010; Albonici *et al*., 2020; Cheng *et al*., 2020). In this study, the results showed that Treg cells (CD4+ CD25+) were isolated from the uteri of the healthy cows by flow cytometry (Fig. 4A), so as to express the functional marker FOXP3 (Fig. 4B). Co-incubation of isolated Treg (CD4+ CD25+) and LPSexo significantly reduced the expression of the immune tolerance regulators PD1, CTLA4, and Galectin1 (Fig. 4C, $p< 0.01$).

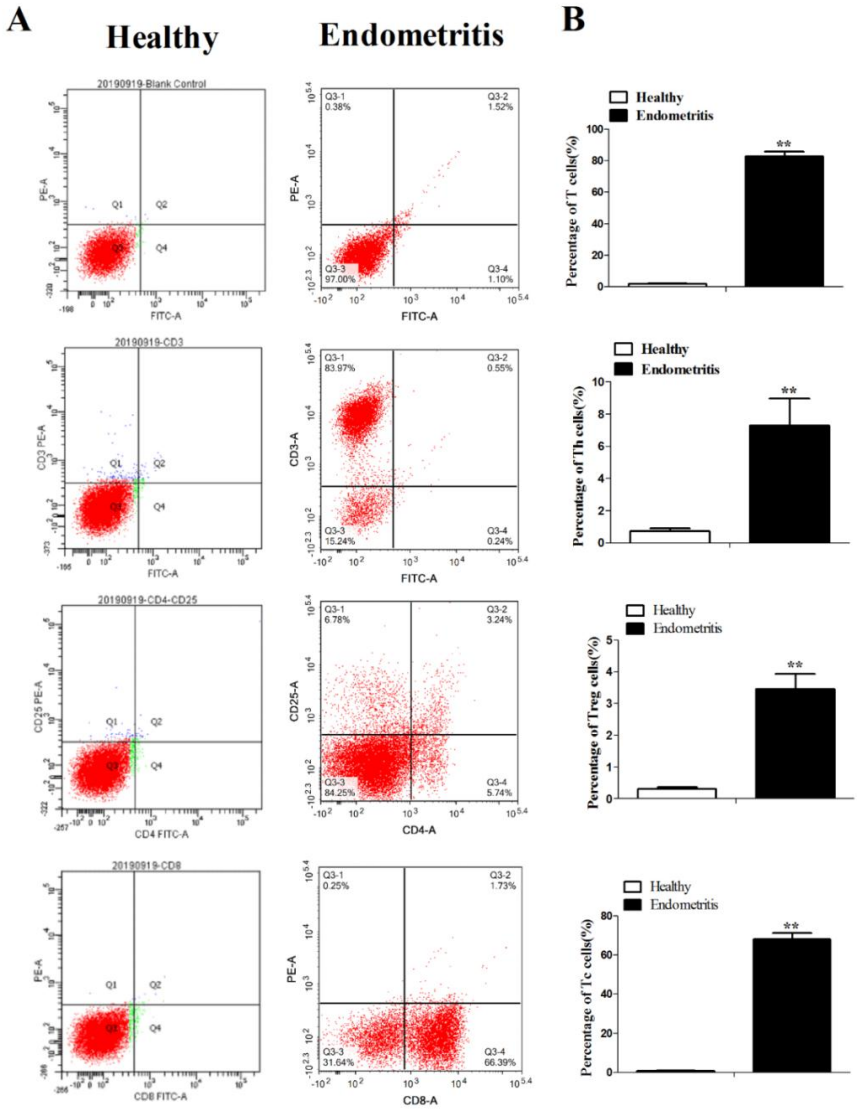

Figure S1 Comparison of the numbers of T lymphocyte subsets between healthy cows and those with endometritis. (A, B) Uteri were collected from the healthy cows and cows with endometritis, lymphocytes were isolated using lymphocyte separating medium, and the changes in the number of T cells (CD3 +), Th cells (CD4 +), Treg cells (CD4 + CD25 +) and Tc cells (CD8 +) were counted by flow cytometry. "** p"<0.01.

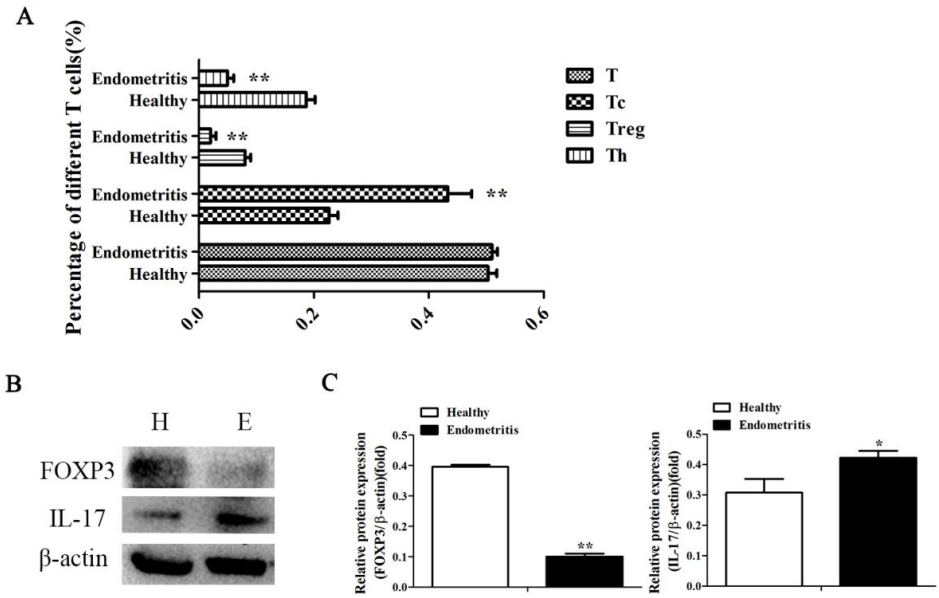

Figure 2 Analysis of the differentiation of T lymphocytes in the uteri of cows with endometritis. (A)The ratio of the number of each T lymphocyte subset to that of total T lymphocytes measured by flow cytometry; (B, C) Western blot detected the expression of Th17 cell-secreted factor IL-17 and Treg cell (CD4+CD25+) marker factor FOXP3. "*" p<0.05, "**" p<0.01.

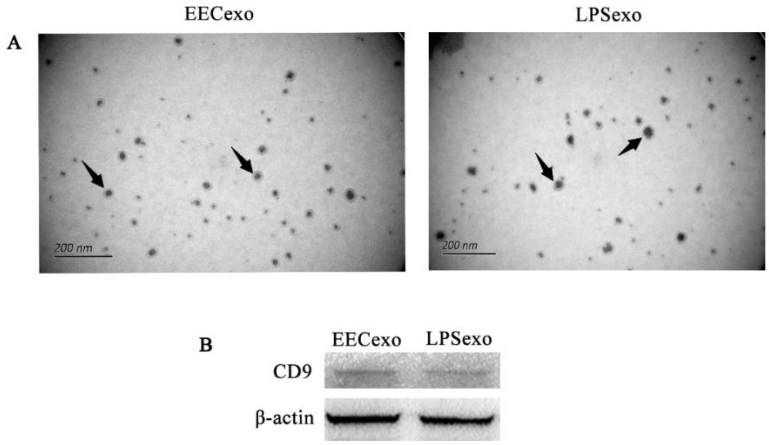

Figure 3 Isolation and identification of exosomes derived from endometrial epithelial cells. Exosomes (named EECexo) were isolated from the normal cultured endometrial epithelial cells (with the serum without exosomes added) using Bestbio Exosome Extraction Kit (for cell supernatant). Exosomes (named LPSexo) were isolated from the culture medium of bovine endometrial epithelial cells with 100 μg/mL LPS was added after 24 h. (A) Morphological analysis by transmission electron microscopy. (B) Western blot analysis of exosomal marker CD9 protein. Exosomes are indicated with black arrows.

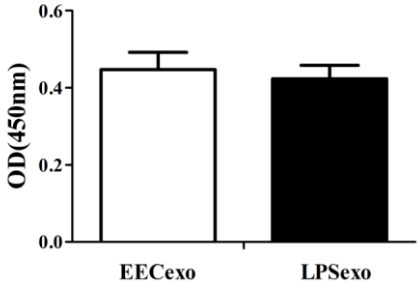

Figure S2 Total RNA in exosomes derived from normally cultured endometrial epithelial cells and LPS stimulated endometrial epithelial cells. Endometrial epithelial cells were seeded with the same number (1.0×10^6) in each well of a 6-well plate. The cells grew to occupy 80% of the bottom of the well. The control group was replaced with exosome-free serum medium, and the experimental group was additionally added with 50 μg/ml LPS at the same time. After continuing the cultivation for 24 hours, the supernatant was collected to and extract exosomes with the isolation kit. Total exosomes RNA was extracted with Trizol and the OD value was determined.

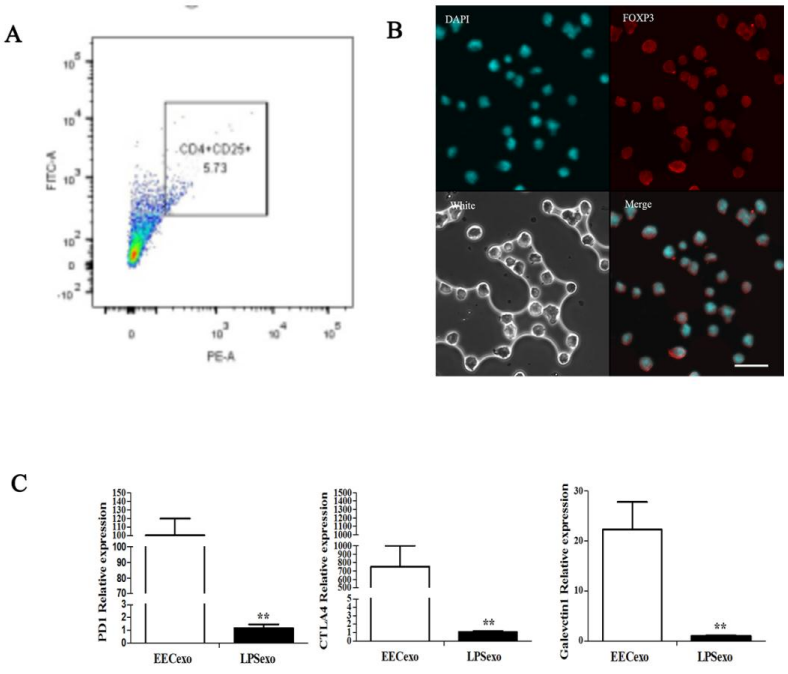

Figure 4 Effect of exosomes on the function of Treg cells (CD4+CD25+) derived from endometrium. (A) Treg cells (CD4 + CD25 +) derived from the uteri of healthy dairy cows as isolated by flow cytometry. (B) The localization and expression of FOXP3, a functional marker of isolated Treg cells (CD4 + CD25 +), as detected by immunofluorescence. (C) The isolated Treg cells (CD4+CD25+) were incubated with EECexo and LPSexo for 24 h respectively, and the expression changes of immune tolerance regulators PD1, CTLA4 and Galectin1 as detected with qRT-PCR. "**" $p<0.01$.

Effect of EECexo and LPSexo on the expression of T cell regulatory factors in mice: CTLL2 cells cultured *in vitro* were supplemented with IL-2 for growth promotion, then incubated with EECexo and LPSexo from the same number of cells, respectively. Co-incubation of CTLL2 and EECexo significantly increased the expressions of immune tolerance regulators PD1 and Galectin1 (Fig. 5A and 5B, p<0.01) and the expressions of FOXP3 and CTLA4 (Fig. 5A and 5B, p<0.05) and significantly decreased the expression of the immune proinflammatory factor IL17 (Fig. 5A and 5B, p< 0.05) in the meanwhile. Co-incubation of CTLL2 and LPSexo caused a significant decrease in PD1 and Galectin1 compared to the normal culture of CTLL2 and CTLL2-EECexo group (Fig. 5A and 5B, p<0.05). Similarly, the expressions of PD1, CTLA4, Galectin1 (Fig. 5A and 5B, p<0.01; Figure S3) and FOXP3 (Fig. 5A and 5B, p<0.05) were significantly reduced while the expression of the immune proinflammatory factor IL17, however, was significantly increased (Fig. 5A and 5B, p< 0.05).

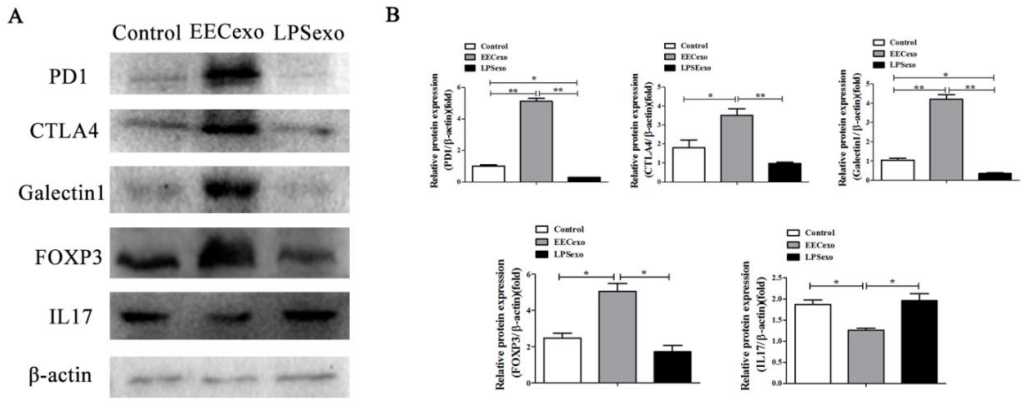

Figure 5 Effect of the exosomes on the differentiation of activated T Lymphocytes (CTLL2) of mice. CTLL2 cells cultured *in vitro* were supplemented with IL-2 to promote their growth for 24 h and then incubated with EECexo and LPSexo, respectively. (A) The expressions of the immune marker factors PD1, CTLA4, Galectin1, FOXP3, and IL-17 as detected by western blotting. "*" p<0.05, "**"p<0.01.

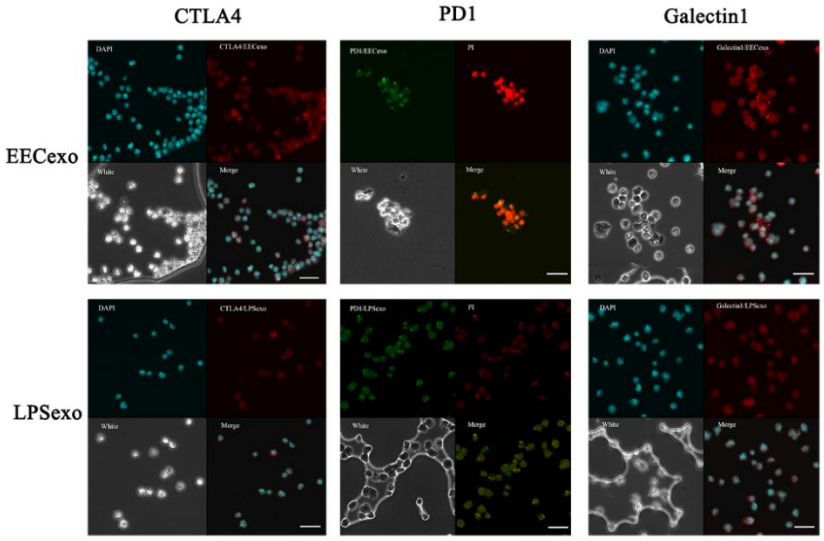

Figure S3 The effect of exosomes on the localization of immune tolerance factors in mice's T lymphocytes (CTLL2). After CTLL2 is incubated with EECexo and LPSexo respectively, the expression and localization of PD1, CTLA4 and Galectin1 as detected by immunofluorescence.

Discussion

The main leukocyte subtypes in the uteri of normal non-pregnant cows are T lymphocytes, macrophages and neutrophils (polymorphonuclear cells, PMNCs). In this study, a large number of exfoliated epithelial cells were predominant in the uterine luminal fluid of normal non-pregnant cows and a large number of PMNCs infiltrated in the uterine luminal fluid of cows with endometritis. The migration and distribution of T lymphocyte subsets plays an important role in the local immunity of the uteri of dairy cows. The study of the changes in numbers of CD4+ and CD8+ T cell subsets in the blood of cows will help to further understand the cellular immune defense mechanism of the uteri. Previous studies have found that the percentage of CD4+T cells showed an upward trend when compared to CD8+ T cells in the early postpartum period, while that of CD4+ T cells showed a rapid decrease and that of CD8+ T cells showed a rapid increase at the late postpartum period (Brodzki *et al*., 2014b) in the blood of cows diagnosed with clinical endometritis. It is well known that it takes 20–30 days for the uterus to recover and the constitution to recover after the calves are born.

Most dairy cows show their first noticeable estrus in 30–70 days after parturition. In this study, the uteri of cows with no estrus and rectal examination with corpus luteum prominently protruding from the surface of the ovary within 21–30 days after delivery were selected as the research subjects. Our results also confirmed that the occurrence of clinical endometritis is caused by the further deterioration of uterine inflammation and the massive activation of cytotoxic T lymphocytes, because the proliferation of helper T lymphocytes is inhibited, which further leads to low immunity of dairy cows. Meanwhile, the expression of Foxp3, which plays a key role in regulating immune homeostasis, was significantly decreased and that of IL-17, a soluble factor induced by T cells and promoting the development of inflammation, was significantly increased.

Treg has long been recognized as an effective inhibitor of the immune system and the normal number and function of Treg is an important prerequisite for maintaining homeostasis in the body, so their insufficient number may be a sign of immune dysregulation(Li *et al*., 2019). Treg cells can inhibit immune response by secreting a variety of cytokines, such as TGF-β (Zimmer *et al*., 2020). Under the co-induction of TGF-β and IL-6, it differentiates into Th17 and secretes IL-6 and IL-17. At the same time, related receptor molecules and transcripts on Treg cells also play an important role in immune regulation, such as CTLA-4, PD-1 and Galectin-1 (Tanaka and Sakaguchi, 2017; Gianchecchi and Fierabracci 2018; Seropian *et al*., 2018). In addition, Th17 can participate in the occurrence and development of various inflammations, autoimmune diseases and tumors (Asadzadeh *et al*., 2017). It has also been confirmed that Th17 and Treg have the opposite effect on each other under certain conditions and jointly participate in the mechanism of disease development (Yang *et al*., 2016). Lee (Lee, 2018) found that Treg cells can also secrete the cytokine IL-17, indicating that Treg cells not only inhibit the body's inflammatory state but may also be involved in the Th17-dominated proinflammatory response. In this study, it was found that the Th17/Treg in the uteri of cows with endometritis showed an imbalance compared with healthy cows. The imbalance of Th17/Treg was reflected in the increase in IL-17 secreted by proinflammatory Th17 cells, while the expression of Foxp3, CTLA4, PD-1 and galectin-1 protein in Treg cells was decreased, which is consistent with our results. In addition, Tc cells, belonging to CD8+ T cells, play an crucial role in anti-tumor (Maimela *et al*., 2019) and anti-virus behavior(Hickman, 2015). The cytotoxic effect of Tc cells can specifically kill those corresponding target cells. Therefore, the increased proportion of Tc cells may be a possible cause of embryo killing, leading to abortion of dairy cows suffering from endometritis. Therefore, controlling the proportion of Tc cells in the uteri and regulating the Th17/Treg balance may provide an effective target site of immune agents for cows with endometritis.

Endometrial epithelial cells, as the first barrier to contact with pathogens, can communicate with immune cells by releasing cytokines such as TNF-α, IL-1, IL-6, and IL-8, so that they exert an immune response function (Lyu *et al*., 2017; Yin *et al*., 2019). The accumulative evidence suggests that exosomes can act as new mediators of intercellular signal transmission between adjacent cells as well as between distant tissues (Wortzel *et al*., 2019) and work either independently or synergistically with soluble growth factors and hormones (Ng *et al*., 2013). Exosomes can transmit mRNAs, miRNAs, and etc. to other cells so that they can play further roles in recipient cells (Hergenreider *et al*., 2012; Zhang *et al*., 2015). In order to confirm the role of endometrial epithelium-derived exosomes in regulating the immune function in the uteri of cows with endometritis, we successfully isolated and identified endometrial epithelium-derived exosomes from the supernatant of the cultured endometrial epithelial cells and co-incubated them with the uterine-derived Treg cells from the healthy cows, respectively. The results showed that LPSexo decreased the expression of the immune homeostasis regulators CTLA4, PD-1 and Galectin-1, which is consistent with the local uterine changes in cows with endometritis. Furthermore, we incubated exosomes from different sources with the induced Treg cells from model animal mice. The data revealed that EECexo was inclined to promote the expressions of the immune homeostasis regulators Foxp3, CTLA4, PD-1 and galectin-1, inhibit the expression of the inflammatory cytokines IL17, maintain an immune tolerance homeostasis in the uteri and have an important regulatory role for embryonic survival. Conversely, LPSexo was inclined to inhibit the expressions of immune homeostasis regulators Foxp3, CTLA4, PD-1 and Galectin-1 and promote the expression of the inflammatory factor IL-17. Therefore, we conjecture that normal endometrial epithelial cell-derived exosomes have a natural function in regulating local uterine immunity and this function is mainly reflected in immune tolerance, that is, normal endometrial epithelium-derived exosomes may contain the related factors that can act on immune cells which can fuse with Treg cells to increase the secretion of Treg-associated immunosuppressive factors and thus inhibit the local uterine inflammatory response and ensure the survival of embryos in the uteri. However, the exosomes produced by epithelial cells in cases of endometritis can act on Treg cells to change their original immunosuppressive state from immune inhibition to immune activation. Moreover, uteri, as a special organ of an animal's body, are not only affected by microbial infection under the conditions of endometrium inflammation but also regulated by the hormone in different estrus cycles and pregnant stages. Further research is needed on the regulatory role of exosomes and their inclusion toward uterine function.

In summary, the imbalance of Th17/Treg and increased proportion of inflammatory cells in the uteri are important reasons for the occurrence and development of endometritis and affect the conception of dairy cows (Figure 6). In addition, the influencing mechanism of endometrial epithelium-derived exosomes on uterine immunoregulation is worthy of further exploration, so as to provide new ideas and methods in the clinical diagnosis of cows with endometritis and the research and development of immune agents.

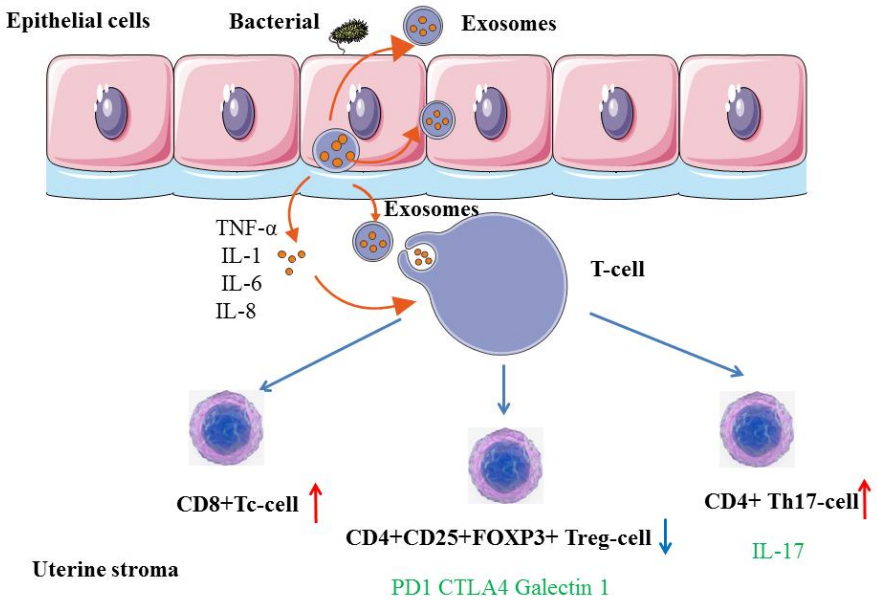

Figure 6 Schematic diagrams showing that endometrial epithelial cell-derived exosomes regulate the imbalance of Th17/Treg and the increased proportion of inflammatory cells in the uteri under healthy or endometritis inflammatory conditions.

Availability of data and materials: All data generated or analyzed during this study is available from the corresponding author on reasonable request.

Conflict of Interest Statement: The authors declare that they have no competing interests.

Authors' contributions: Xiangguo Wang and Hong An designed the experiments and interpreted the data and wrote the article. Hong An performed the experiments with assistance and advice from Bofan Fu, Xinxin Yao and Yong Guo. Hemin Ni conducted data analysis. Longfei Xiao and Zhe Shi revised the manuscript. All authors have read the manuscript and approved its submission to your journal.

Consent for publication: Not applicable.

Ethics approval and consent to participate: All experimental and surgical procedures involving animals were approved by the Beijing Laboratory Animal Management Committee.

Acknowledgements

This research was supported by the National Key RandD Program of China (Project No. 2017YFD0502200), National Natural Science Foundation of China (NO. 31802263), the Beijing dairy cattle industry innovation team project (NO. BAIC05-2017) and the Outstanding young talent project of Beijing municipal party committee organization department（No. 2018000020124G081.

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