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ORIGINAL PAPER

Treponema pallidum induces the activation of endothelial cells via macrophage-derived exosomes

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Abstract

Recent studies have shown that exosomes play a role in pathogenesis and in the treatment of inflammatory diseases and tumours. We explored the effects of *Treponema pallidum*-induced macrophage-derived exosomes on vascular endothelial cells to determine whether they are involved in the pathogenesis of syphilis. A syphilis infection model was established using rabbits to harvest *T. pallidum* at the peak of proliferation. Exosomes derived from macrophages were extracted using commercial kits and characterized by transmission electron microscopy, western blot assays, and nanoparticle tracking analysis. Secreted cytokine levels and the adhesion and permeability of human umbilical vein endothelial cells were evaluated in a co-culture model using the extracted exosomes. The results of this study revealed that exosomes derived from *T. pallidum*-infected macrophages enhanced cell adhesion and permeability. The levels of the secreted cytokines, including ICAM-1, VCAM-1, VEGF, and IL-8 were higher in the experimental group than in the control group. Our findings suggest that exosomes derived from *T. pallidum*-infected macrophages affect the cell adhesion and permeability of vascular endothelial cells. These changes may play important roles in syphilis pathogenesis. This study is the first to reveal the effects of exosomes derived from *T. pallidum*-infected macrophages on the adhesion, permeability, and secreted cytokines of human umbilical vein endothelial cells.

Keywords Syphilis · *Treponema pallidum* · Macrophage · Exosome · Endothelial cells

Introduction

Syphilis, which is a sexually transmitted disease caused by *Treponema pallidum* subspecies pallidum (*T. pallidum*) [1], is still a serious public health problem in the world, including China [2, 3]. Endarteritis of the vasa vasorum, the adventitia, and the media of large- and medium-sized arteries is the most common inflammatory change, which may result in vascular wall thickening, thrombosis, and vascular occlusion [4]. However, little is known regarding how *T. pallidum* gives rise to this vessel endarteritis.

Previous studies showed that *T. pallidum* membrane lipoprotein 0751(Tp0751) functions as a protein hydrolase by adhering and degrading laminin and fibrinogen from chancres through blood circulation to everywhere in the body [5]. Our previous research predicted that recombinant *T. pallidum* membrane lipoprotein 17 and protein 0965 (rTp0965) upregulated the expression of intercellular cell adhesion molecule 1 (ICAM-1), E-selectin, and monocyte chemoattractant protein 1 in human umbilical vein endothelial cells (HUVECs) [6, 7]. Those cytokines enhanced the adhesion of monocytes to endothelial cells and increased the transendothelial migration of monocytes [6, 7]. The above demonstrated that *T. pallidum* membrane proteins may induce endothelial cell dysfunction and consequently affect the progress of vessel inflammatory response. However, the molecular mechanism remains unclear.

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Recent studies have shown that exosomes are an important medium of communication between cells involved in the immune response, antigen presentation, cellular migration, cellular differentiation, and tumour metastasis, among others [8–10]. A previous study has suggested that pro-inflammatory exosomes produced by macrophages infected with *Salmonella typhimurium* trigger the TLR4-dependent release of TNF-α from naïve macrophages and stimulate the secretion of various cytokines [11]. Osada demonstrated that macrophage-derived exosomes induced inflammatory factors in coronary endothelial cells by upregulating the expression levels of ICAM-1 and plasminogen activator inhibitor-1 under hypertensive conditions [10]. Exosomes secreted by monocytes under the stimulation of IFN-α or lipopolysaccharide activated nuclear factor-κB and recruited monocyte to HUVECs and human brain microvascular endothelial cells in vitro [12].

Given these findings on exosomes in pathogens and endothelial cells, we assume that *T. pallidum* may induce the adhesion and permeability of endothelial cells via exosome secretion to provide a basis for endovasculitis.

Materials and methods

Ethics statement

This study was ethically conducted under protocol number 2017-KY-010 approved by the Ethics Committee of Institute of Dermatology and Skin Hospital, Chinese Academy of Medical Sciences.

Cell lines and cell culture

HUVECs were purchased from the American Type Culture Collection (ATCC, USA, CRL-1730, Lot 63781508) and were cultured in F-12K (Gibco Life Technologies, New York, USA) supplemented with 10% foetal bovine serum (FBS) (Sciencell, Los Angeles, USA), 1% endothelial cell growth supplement, and 10 mg/ml stock heparin solution (Sciencell, Los Angeles, USA). THP-1 monocytes were cultured in RPMI-1640 (Gibco Life Technologies, New York, USA) supplemented with 10% FBS and 0.05% β-mercaptoethanol (Sigma-Aldrich, Missouri, USA). Macrophages were obtained by the differentiation of THP-1 monocytes stimulated with 50 ng/ml PMA (Sigma-Aldrich, Missouri, USA) and cultured in RPMI-1640 supplemented with 10% exosome-free FBS (Vivacell, Shanghai, China) [10]. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Experiments were conducted using cells in a logarithmic growth phase.

T. pallidum harvesting

T. pallidum (Nichols strain) was propagated by intratesticular inoculation of New Zealand male rabbits approximately 3 months of age and 2.5–3.0 kg in weight with negative RPR. *T. pallidum* was harvested in saline supplemented with 20% heat-inactivated (56 °C for 30 min) normal rabbit serum (Biological Industries, Beit Haemek, Israel) at peak orchitis, and centrifuged at 700×*g* for 5 min and 12,000×*g* for 30 min at 4 °C. Finally, these bacteria were resuspended in PBS and counted under dark-field microscopy.

Exosome preparation and purification

Exosomes acquired from the equivalent amount of macrophage (2×10^6 particles) supernatant with or without treatment by *T. pallidum* at a multiplicity of infection (MOI) of 30:1 for 12 h were prepared and isolated [4, 13]. Macrophages were washed in PBS twice gently and continuously cultured in fresh medium for an additional 48 h to collect exosomes. Macrophage supernatant was collected and centrifuged at 300×*g* for 10 min and 2000×*g* for 10 min at 4 °C to remove cellular debris. Then, the supernatant was filtered by 0.22-μm sterilized filter (Millipore, Billerica, USA) to remove impurities including particles larger than 0.22 μm. Then, exosomes were isolated from 24 ml of macrophage supernatant using the exoEasy Maxi Kit (Qiagen, Frankfurt, Germany) according to supplier's protocol. Exosomes were stored at − 80 °C until subsequent use for following experiments [14].

Transmission electron microscopy (TEM)

We viewed exosomes by TEM, which show a fine structure of less than 0.2 μm. The prepared exosome eluate was stained with 3% phosphotungstic acid solution for 3–5 min and viewed on a JEOL-Jem-1230EX (JEOL, Tokyo, Japan) transmission electron microscope [15].

Nanoparticle tracking analysis (NTA)

NTA was recently applied to determine both the concentration and diameter of exosomes [16]. In this study, all measurements were conducted using the qNano system (Izon Sciences Ltd., NZ) combining tuneable nanopores with proprietary data capture and analysis software (Izon Control Suite v.3.3.2.2000) [15, 17–19].

Western blotting analysis

We used western blot analysis to determine the expression of membrane proteins on the exosomes. First, we loaded appropriate levels of exosomes into 10% SDS–polyacrylamide gels and transferred them onto a PVDF membrane (Millipore, Billerica, USA). Then, the PVDF membrane was blocked with 5% bovine serum albumin (BSA) (Double-Helix, Shanghai, China) and incubated in primary antibodies against CD63, CD9, CD81, β-tubulin (Abcam, Cambridge, England) and goat anti-mouse IgG secondary antibodies (Fcmacs, Shanghai, China). The protein levels were measured by a Molecular Imager Gel DocTM system (Bio-Rad, California, USA) after exposure to chemiluminescence reagents (Cell Signaling Technology, Boston, USA).

Immunohistochemistry

To explore whether exosomes could be internalized by HUVECs that provide a basis for delivering material, we labelled exosomes (approximately 10^{10} particles/ml) with PKH67 membrane dye (Sigma-Aldrich, Missouri, USA). Then, stained exosomes were cocultured with HUVECs, which were plated in 300 µl of RPMI supplemented with 10% exosome-free FBS in a confocal laser dish (1×10^4 cells/dish). After incubation for 8 h, HUVECs were washed and photographed under a laser focus microscope (OLYMPUS IX81, Tokyo, Japan).

Enzyme-linked immune sorbent assay (ELISA)

We measured adhesion molecules including VCAM-1 and ICAM-1 secreted from HUVECs after treatment with exosomes. HUVECs (200,000/well in 12-well plates) were cultured with exosome-depleted FBS in 1.5 ml of DMEM medium for 24 h, followed by incubation with 4.5×10^5 particles/well normal macrophage-derived exosomes or macrophage-derived exosomes stimulated by *T. pallidum*, denoting them as EXO and EXOTp, for 12 h, 24 h, and 48 h. Levels of ICAM-1 and VCAM-1 were analysed using ELISA kits (Becton, Dickinson and Company, New Jersey, America) according to the manufacturer's instructions.

Some cytokines, such as VEGF and IL-8, are related to HUVEC permeability. Thus, we measured cytokine levels to understand the permeability of HUVECs co-cultured with exosomes. HUVECs (150,000 cells/well seeded in the upper chambers of 12-transwell plates) were cultured with exosome-depleted FBS in 400 µl of DMEM, and the lower compartments were filled with 1.0 ml of DMEM containing 10% exosome-depleted FBS for 4 days. HUVECs were incubated with 2×10^{10} particles/well EXO or EXOTp for 12 h, 24 h, and 48 h. We collected the supernatants from the upper chambers to measure the concentrations of VEGF and IL-8 by specific ELISA kits (Becton, Dickinson and Company, New Jersey, USA).

Final data were collected on a microplate spectrophotometer (Thermo Fisher Scientific, New York, USA) at a wavelength of 450 nm.

Reverse transcription quantitative real-time PCR (RT-qPCR)

In addition to measuring the levels of adhesion molecules in HUVEC serum, we explored the VCAM-1 and ICAM-1 that from HUVECs membrane by RT-qPCR to elucidate HUVEC's adhesion more properly. Total RNA (over 1 µg) was isolated from HUVECs treated with 4.5×10^{10} particles of EXO and 4.5×10^{10} particles of EXOTp for 48 h. The RNeasy® Mini Kit (Qiagen, Frankfurt, Germany) was used according to the manufacturer's instructions.

Affinity Script Reverse Transcriptase was used to generate cDNA from 1 µg of RNA in 20 µl of water following the manufacturer's instructions. RT-qPCR was performed from 2 µl of cDNA in Applied Biosystems (Thermo Fisher Scientific, New York, USA) equipment using SYBR Green assays for ICAM-1 and VCAM-1 mRNA. Primer sequence sets (ShengGong, Shanghai, China) used for RT-qPCR experiments are supplied below: ICAM-1 F-primer GTC ACCTATGGCAACGACTCCTTC,R-primer GTGTCT CCTGGCTCTGGTTCC; VCAM-1 F-primer TCATTG ACTTGCAGCACCACAGG, R-primer TGCACAGGT AAGAGTGTTCGTTCC; GAPDH F-primer CCAAGG TCACCATGACAAC, R-primer CCATCACGCCACAGT TTC.

Adherence assay

HUVECs were seeded at a density of 2×10^5 cells per well with DMEM containing 10% exosome-depleted FBS for 24 h and were treated with EXOTp at 4.5×10^{10} particles (experimental group) or equal levels of EXO (control group) at 37 °C in 5% CO_2 for 48 h. THP-1 cells were stained with 5 µmol/l calcein AM (Sigma-Aldrich, Missouri, USA) for 30 min and incubated for additional 6 h at 37 °C in 5% CO_2 at a density of 1×10^5 cells per well. Then, THP-1 cells in the supernatants of both groups were counted under optical microscope.

THP-1 cells adhering to endothelial cells were visualized under confocal laser scanning microscopy and quantified as follows: percentage of THP-1 cells binding = (number of cells added per well − number of non-adherent cells) / (number of cells added per well) × 100% [6, 7].

Permeability assay

A 400-µl HUVECs suspension at a concentration of 1.5×10^5 cells/ml was placed into the upper chamber of transwell inserts with a 0.4-µm pore size. The lower chambers were filled with 1.0 ml of DMEM containing 10% exosome-depleted FBS. All chambers were incubated at 37 °C for 24 h. The transepithelial electrical resistance between endothelial cells (TEER) was measured daily by Millicell ERS-2 (Millipore, Billerica, USA). The TEER of HUVECs was calculated as (measured resistance − blank resistance) × available membrane area ($\Omega \times cm^2$) [20, 21]. After 4 days, the TEER was at its peak, indicating that HUVECs had formed a confluent monolayer.

HUVECs were supplemented with fresh medium and stimulated with the same amounts of EXO or EXOTp (2.0×10^{10} particles) for 24 h. Permeability was measured by adding 100 µl of HRP (Solarbio, Beijing, China) to upper chamber. After 30 min, 100 µl of fluid from the lower chamber was sampled and then supplemented with 100 µl tetramethylbenzidine substrate and stop solution (Solarbio, Beijing, China). HRP flux was determined using a microplate spectrophotometer (Thermo Fisher Scientific, New York, USA) based on the oxidation of TMB at OD450nm [21].

Statistical analysis

Statistical analyses were performed using SPSS statistical software package version 22 (SPSS Inc., USA). Either a paired or an unpaired Student's *t* test was used to compare the adhesion, permeability, and cytokine concentrations of HUVECs across the two groups. The results are expressed as mean values ± standard deviations. *P* values less than 0.05 were considered statistically significant. All experiments were performed using three biological replicates.

Results

Identification of exosomes derived from macrophages

T. pallidum was harvested from an induced animal model of syphilis. Dark-field microscopy revealed the slender spiral morphology of *T. pallidum* (Fig. 1a). Macrophages were successfully differentiated from THP-1 cells in suspension and in an adherent state with a polygonal shape under a microscope. TEM images showed that exosomes exhibited vesicle morphology and ranged in size from 30 to 100 nm (Fig. 1b). The results of western blotting confirmed the expression of CD9, CD63, and CD81 in exosomes (Fig. 1c).

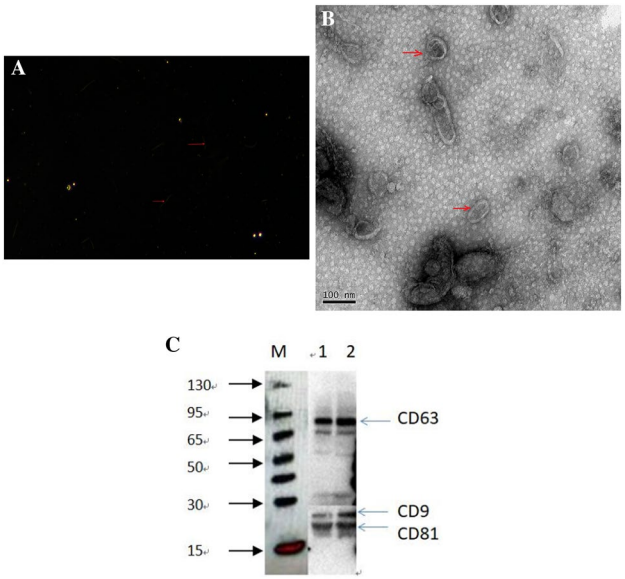


Fig. 1 Identification of exosomes derived from macrophages. **a** Tp in dark-field microscopy images (400X). **b** The morphology of EXOTp, as shown by transmission electron microscopy. **c** Expression levels of CD9, CD63, and CD81 in macrophage-derived exosomes stimulated by Tp (EXOTp), as shown by western blotting

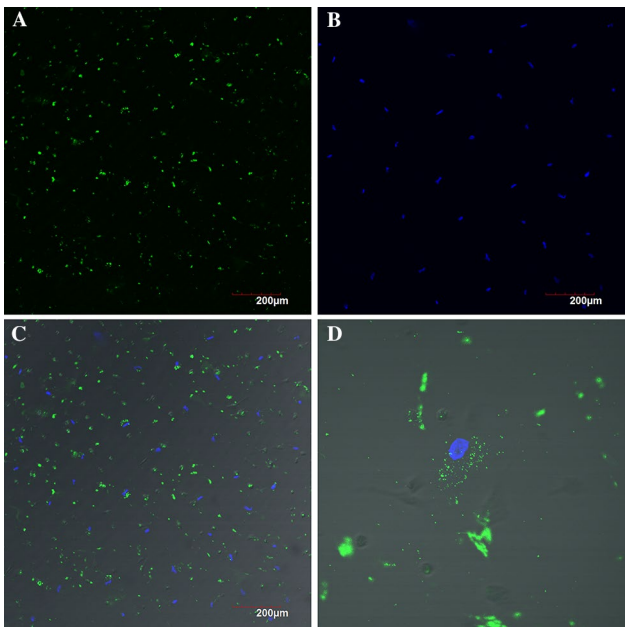


Fig. 2 Macrophage–exosome coculture with HUVECs. Fluorescent images revealed that PKH67-labelled macrophage–exosomes (green) (**a**) were located around the nucleus of DAPI-labelled HUVECs (blue) (**b**). Immunofluorescence staining demonstrated that exosome could be internalized by HUVECs (**c, d**)

HUVECs endocytose exosomes

After exosomes were co-cultured with HUVECs for 8 h, the results were analysed by fluorescence microscopy. PKH67-labelled exosomes (green) (Fig. 2a) were located around the nuclei of DAPI-labelled HUVECs (blue) (Fig. 2b).This result is indicative of phagocytosis and provides a biological basis for the potential functional influence of exosomes on HUVECs. (Fig. 2c, d).

Exosomes activate HUVECs and increase cell adhesion

After HUVECs were co-cultured with EXOTp or EXO for 12 h, 24 h, and 48 h, the results of ELISA showed that the concentrations of ICAM-1 and VCAM-1 secreted by HUVECs in the EXOTp group were higher than those secreted by HUVECs in the EXO group, particularly after 48 h (Fig. 3a, b). RT-qPCR analysis also showed that ICAM-1 and VCAM-1 were highly expressed in the EXOTp group after 48 h (Fig. 3c).

The adherence of THP-1 and HUVECs was observed using fluorescence microscopy (Fig. 3d, e). We found that the adhesion rate of HUVECs and THP-1 in the EXOTp group was significantly higher than that in the EXO group, and the differences were statistically significant ($t = 4.293$ and $P = 0.014 < 0.05$) (Fig. 3f). The results of adhesion assays showed that EXOTp promoted the adhesion of THP-1 and HUVECs.

EXOTp enhances the permeability of HUVECs

HUVECs formed a confluent monolayer in 4 days (Fig. 4a). Confluent primary cultures of HUVECs were treated with EXO or EXOTp for 12 h, 24 h, and 48 h. The levels of VEGF and IL-8 in supernatant of HUVECs co-cultured with EXOTp were significantly higher than those co-cultured with EXO, reaching a peak at 24 h (Fig. 4b, c). These results suggest that EXOTp can stimulate endothelial growth factors to facilitate the self-repair ability of damaged HUVECs. Above all, we showed that EXOTp increased the HRP permeability of the HUVEC monolayer at 24 h. The results of HRP flux showed that the permeability of the HUVECs was greater in the EXOTp group than in the EXO group (Fig. 4d).

Discussion

T. pallidum is very difficult to maintain in vitro for a long time unless it is in the testis of male rabbits [22]. While *T. pallidum* degrades the connective matrix of the capillary and the supporting polysaccharide matrix around the blood vessels through the hyaluronic acid enzyme, which ultimately induces tissue necrosis and ulceration, it can also be eliminated by macrophages via antibody-mediated phagocytosis [13]. After *T. pallidum* invades, local monocytes/macrophages can be activated and release IL-1β, IL-6, IL-12, IL-8, TNF, CXCL, NO, adhesion molecules, and other cytokines that not only promote inflammatory response but also result in vasculitis in histology [23, 24]. Hence, we choose macrophage-derived exosomes to explore exosome–HUVEC interactions ex vivo.

Initially, polymorphonuclear heterophilic cells infiltrated the primary site after *T. pallidum* infection and gradually replaced mononuclear cells (T-lymphocytes), which disseminated throughout the body [22, 25]. The leukocytes adhere to vascular endothelial cells (VECs) through adhesion molecules and can roll across vessel walls, leading to perivasculitis once these immune cells penetrate into the aorta and meningeal vessels [7, 22, 25].

The high purity of secreted exosomes is a crucial factor in research on cell functions, which are influenced by EXOTp. There are many exosome extraction methods, such as ultracentrifugation, immunoaffinity column chromatography, size-based techniques, precipitation, and microfluidics-based techniques [26]. After a series of experiments, we finally selected the exoEasy Maxi Kit, which uses a membrane-based affinity binding step to isolate exosomes and other extracellular vesicles from serum, plasma or cell culture supernatants. In addition, the purity of *T. pallidum* may be a concern since it was mixed with rabbit testicular tissue, although the samples were extracted by differential centrifugation. Thus, we added exosome-free FBS to the medium and tried to ensure that the two groups were under the same conditions.

Cell adhesion molecules located on the cell surface are involved in binding with other cells or with the extracellular matrix. Adhesion molecules affect receptor–ligand binding and cell–cell, cell–matrix, or cell–matrix–cell adhesion and in the processes of cell activation, signal transduction, migration, proliferation and differentiation. Among many diseases, adhesion molecules are the basis of physiological and pathological processes that include the immune response, inflammation, coagulation, tumour metastasis, and cellular repair.

In this study, we stimulated HUVECs with macrophage-derived exosomes under the stimulation of *T. pallidum*. Then, we measured the concentrations of ICAM-1 and VCAM-1 of HUVECs in serum and on the membrane by ELISA and qRT-PCR, respectively. Afterwards, we estimated the adhesion rate of HUVECs and THP-1 by laser confocal microscopy and cytometry. Our results showed that EXOTp upregulated the expression of ICAM-1 and VCAM-1 in HUVECs and promoted the adhesion of THP-1 to HUVECs in vitro, providing a basis for the perivasculitis caused by *T. pallidum*.

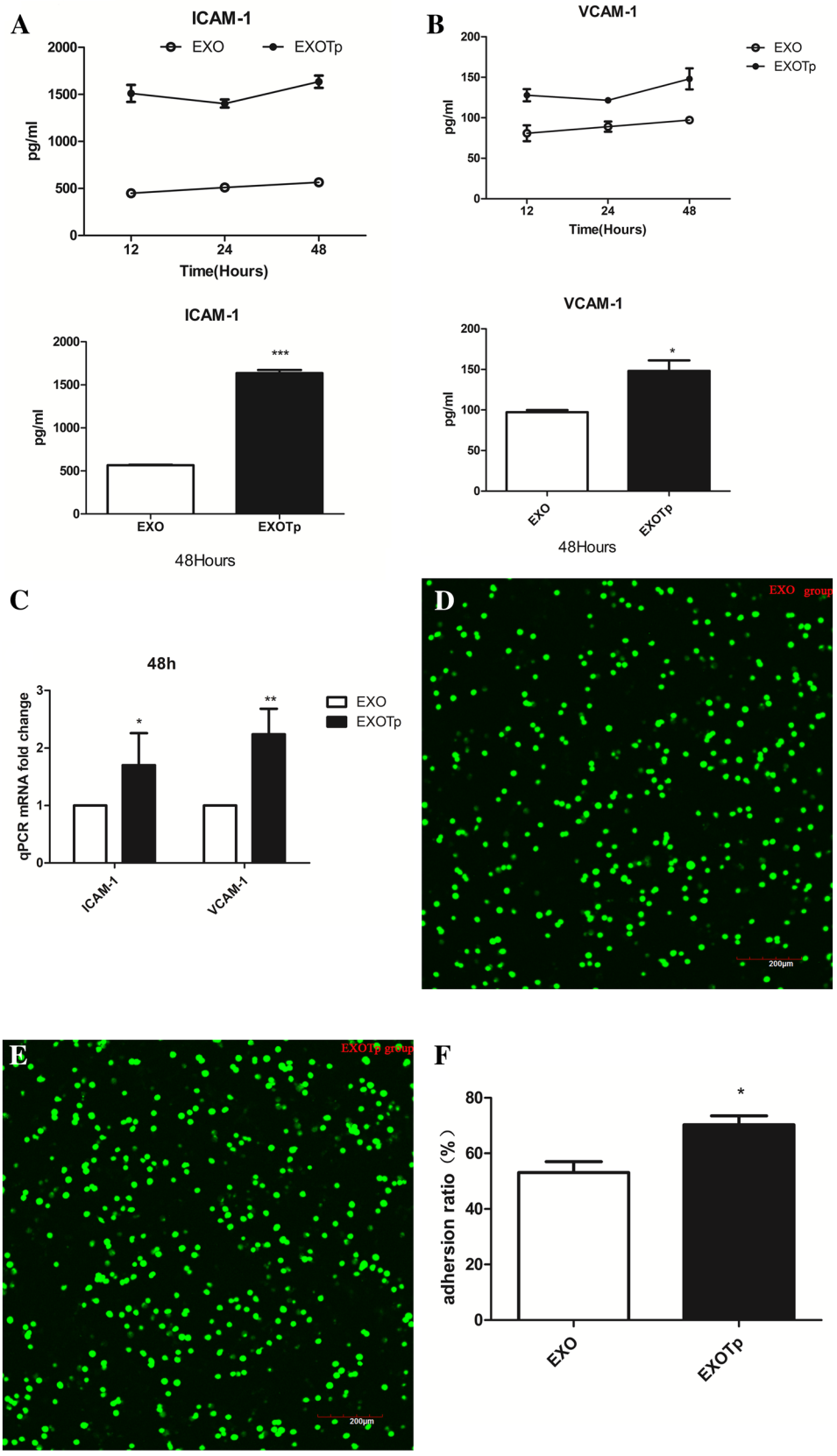


Fig. 3 Exosomes activate HUVECs and increase cell adhesion. The concentrations of ICAM-1 (**a**) and VCAM-1 (**b**) secreted by HUVECs in the EXOTp group were higher than those in the EXO group, particularly at 48 h ($n=3$). **c** RT-qPCR analysis also showed that ICAM-1 and VCAM-1 were highly expressed in HUVECs in 48 h ($n=3$). Fluorescence microscopy results of the EXO (**d**) and EXOTp (**e**) groups are shown. **f** EXOTp increased the binding of calcein AM-labelled THP-1 cells to HUVECs ($n=9$). ($^{*}P<0.05$, $^{***}P<0.001$)

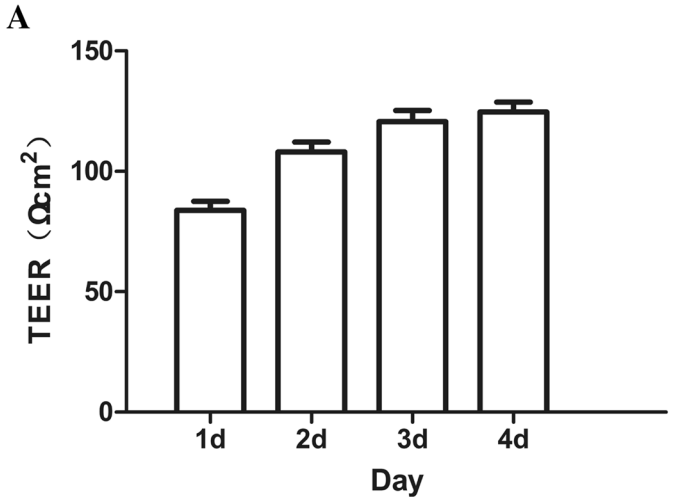
The vascular endothelium, composed of vascular monolayer cells to cover the surface and extracellular matrix, can regulate vascular smooth muscle contractility, host defence, vascular angiogenesis, and the homeostatic balance of interstitial fluid [27]. The semi-permeable barrier of VECs is important for regulating the flow of biomacromolecules and body fluids between blood vessels and tissues. The endothelial barrier also regulates the migration and adhesion of many types of leukocytes to tissues, which is the basis of inflammatory and immune responses [21].

VEC's permeability is maintained by the dynamic balance of the adhesion of intercellular junctions, including adhesion connections and tight junctions, and cytoskeleton contractility [28]. There are many reasons for changes in VEC permeability, including endothelial cell contraction, endothelial cytoskeleton reconstruction, destruction of adhesion connections and tight junctions, and immune cells migrate to vascular walls. We found Tp0965-activated HUVECs reconstructed actin micro-filaments and increased the permeability of cell monolayers. Our results indicate that macrophage-derived exosomes induced by *T. pallidum* infection increase the permeability of VECs in vitro. However, additional studies are needed in the future to determine whether exosomes package the related genetic information of *T. pallidum* membrane proteins (such as Tp0965) and increase the permeability of VECs through actin filament reconstruction.

VEGF is a homologous, dimer, heparin-binding glycoprotein. VEGF not only stimulates vasculogenesis and angiogenesis by promoting the division and proliferation of VECs but also increases the permeability of VECs because of the gaps between endothelial cells and the degeneration of the basement membrane [29]. IL-8, a chemokine in the CXC family, is overexpressed in most inflammatory reactions. IL-8 induces the chemotaxis of granulocytes and leukocytes to acute inflammatory sites and influences the permeability of endothelial cells by destroying cytoskeleton components [30].

In this study, THP-1-differentiated macrophages and HUVECs in this study had certain differences from natural macrophages and other VECs. The limitation of exosomes secreted by single immune cells cannot mimic the infection process in the syphilitic host. Therefore, it is necessary to perform animal experiments to comprehensively explain the role of EXOTp in VEC function and the pathogenesis of syphilis. Adhesion, permeability and relative cytokines in the EXOTp group were higher than those in the EXO group, indicating that EXOTp might mediate inflammatory responses and promote leukocyte and monocyte adhesion to HUVECs. However, the contribution of EXOTp in mediating the higher permeability of HUVEC needs to be verified in further studies.

Inflammation and adaptive immune responses are able to clear microorganisms and cooperate in damaged tissues. Several mechanisms have been suggested to ensure the continued existence of microorganism in the host. To better understand how *T. pallidum* leads to perivasculitis, we investigated the secreted cytokine levels, adhesion, and permeability of HUVECs after stimulation by EXOTp in vitro. In brief, exosomes were found to be involved in changes in HUVEC functionality, which may provide a basis for future research.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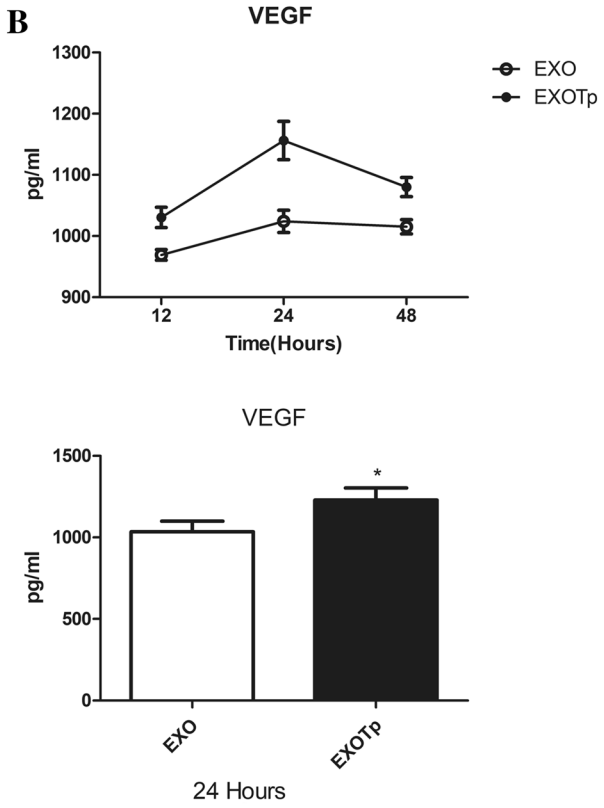
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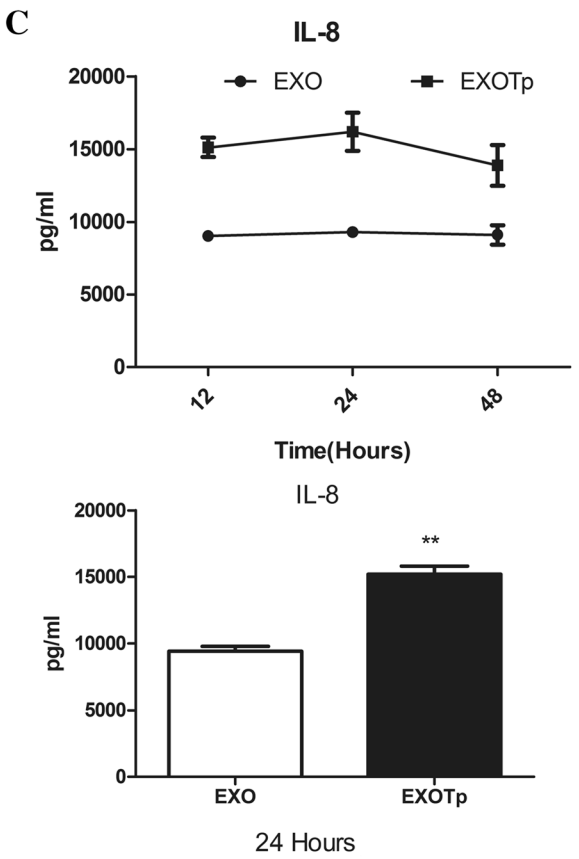
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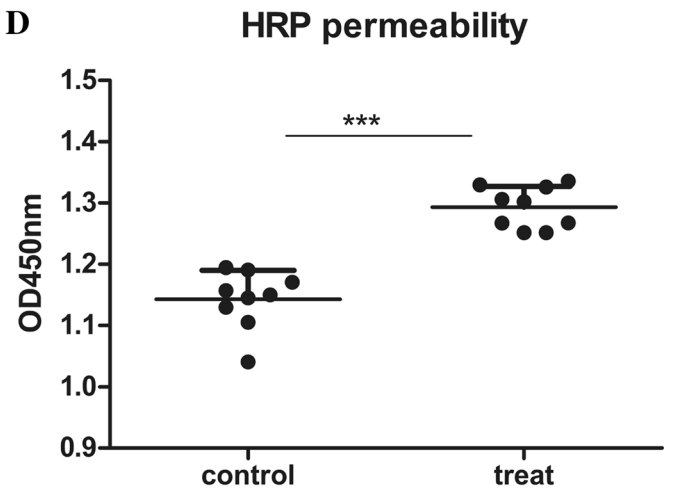
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◂**Fig. 4** EXOTp enhances permeability of HUVECs. **a** The TEER of confluent monolayers was monitored for 4 days to establish the model monolayer of HUVECs. The concentrations of VEGF (**b**) and IL-8 (**c**) secreted by HUVECs in the EXOTp group were higher than those in the EXO group, particularly at 24 h ($n=3$). **d** EXOTp increased the permeability of the HUVEC monolayer from HRP flux at 24 h ($n=9$). (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

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Author contributions Q-QW, R-LZ, and B-FX conceived the experiments. B-FX conducted the experiments. Q-QW, B-FX, and W-LH analysed the results. B-FX and Q-QW wrote the manuscript. All authors contributed to the review of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

Ethics approval All animal experimentation was conducted following the Guide for the Care and Use of Laboratory Animals and according to protocols reviewed and approved by the Nanjing Military Region CDC in China. The experimental rabbits were anaesthetized with urethane and killed under air embolism by euthanasia. The project licence number (2017-KY-010) was assigned by the ethics committee that approved our animal experiments.

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