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Circulating Exosomes Isolated from Septic Mice Induce Cardiovascular Hyperpermeability through Promoting Podosome Cluster Formation

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Abstract

Septic shock increases vascular permeability, leading to multiple organ failure including cardiac dysfunction, a major contributor to septic death. Podosome, an actin-based dynamic membrane structure, plays critical roles in extracellular matrix degradation and angiogenesis. However, whether podosome contributes to endothelial barrier dysfunction during septic shock remains unknown. In this study, we found that the endothelial hyperpermeability, stimulated by PMA and thrombin, was accompanied by increased formation of podosome clusters at the cell periphery, indicating a positive correlation between podosome clusters and endothelial leakage. Interestingly, we observed that circulating exosomes collected from septic mice were able to stimulate podosome cluster formation in cardiac endothelial cells, together with increased permeability *in vitro/vivo* and cardiac dysfunction. Mechanistically, we identified that septic exosomes contained higher levels of reactive oxygen species (ROS) than normal ones, which were effectively transported to endothelial cells (ECs). Depletion of ROS in septic exosomes significantly reduced their capacity for promoting podosome cluster formation and thereby, dampened vascular leakage. Finally, we elucidated that podosome cluster-induced endothelial hyperpermeability was

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associated with fragmentation/depletion of zonula occludens-1 (ZO-1) at the cell periphery. Our results demonstrate that septic exosomes were enriched with high amounts of ROS, which can be transported to ECs, leading to the generation of podosome clusters in target ECs and thereby, causing ZO-1 relocation, vascular leakage and cardiac dysfunction.

Keywords

Sepsis; Exosomes; Podosomes; ROS; Vascular Permeability; ZO-1

Introduction

Sepsis is initiated by an uncontrolled immune response following a local severe infection (1). Despite recent advances in antibiotic therapy and supportive critical care, sepsis remains a leading cause of death in intensive care units (1). Endothelial barrier disruption is well recognized to be a major complication in sepsis, as it can induce microvascular hyperpermeability, leading to cardiac dysfunction and eventually heart failure (2). Notably, septic patients with myocardial dysfunction have a much higher mortality rate (70%) than those without cardiac abnormalities (20%) (3). At present, most prior studies have focused on the sepsis-triggered endothelial barrier dysfunction in the lungs that results in lung edema and acute respiratory distress syndrome (ARDS) (4). However, sepsis-caused cardiac vascular hyperpermeability and its underlying mechanisms have been less investigated.

Podosome is an actin-based cytoskeletal structure containing abundant F-actin, integrin, and actin regulators such as cortactin, Arp2/3 complex, and adhesion mediators (*i.e.*, paxillin and vinculin) (5). It is predominately found at the outer surface of plasma membrane in animal cells (*i.e.*, monocytic lineage, smooth muscle cells, epithelial cells, and endothelial cells) (5). Numerous studies have pointed out several pivotal roles of podosomes in extracellular matrix degradation, invasive cell motility, and sprouting angiogenesis (6, 7). Particularly, a recent study by Kremerskothen *et al.* demonstrated that ZO-1, a central regulator of intercellular junctions, is involved in the formation of podosomes through relocation from cytosol and cell cortex to newly formed podosomes (8, 9). Therefore, we speculated that podosomes might function in breaking cardiac endothelial integrity through targeting ZO-1.

Exosomes are membrane-bound nano-vesicles naturally released from living cells. Prior studies had indicated that exosomes, collected from platelets of sepsis patients, contained NADPH oxidase subunits: p22 and gp91 and exhibited intrinsic reactive oxygen species (ROS) production and induces vascular cell apoptosis (10, 11). We recently made novel findings that global blockade of exosome generation in mice could attenuate sepsis-triggered inflammatory response and thereby improve cardiac function (12). Nonetheless, whether circulating exosomes released under septic conditions affect podosome formation in cardiac vascular cells and further lead to vascular hyperpermeability has not been investigated.

In this study, we first tested whether 1) podosome formation stimulated by Phorbol 12-myristate 13-acetate (PMA) in mouse cardiac endothelial cells (MCECs) could affect their permeability; and 2) the thrombin-induced endothelial barrier disruption was accompanied by the formation of podosome. Next, we examined the effects of circulating exosomes

collected from septic mice on the podosome formation in MCECs and endothelial permeability. Lastly, the possible mechanisms were identified. We found that, during septic shock, circulating exosomal ROS could be transported to endothelial cells, leading to the formation of podosome clusters which, in turn, caused the fragmentation/depletion of ZO-1, a controller of endothelial adherent junctions and barrier formation.

Materials and Methods

Animals and MCEC cell line

Male C57BL/6 mice (8-week old) were obtained from Jackson Laboratory (Indianapolis, IN). A mouse cardiac endothelial cell line (MCEC), initially developed in Dr. Weksler's laboratory, was purchased from CELLutions Biosystems Inc. (Burlington, ON, Canada) (13). Details about animal care and cell culture conditions are described in Supplemental Methods.

Isolation and characterizations of circulating exosomes

Circulating exosomes were collected at various time points from mice subjected to LPS injection or cecal ligation and puncture (CLP) surgery (septic exosomes). The CLP surgeries were conducted according to previous descriptions by other authors (14, 15, 16). Circulating exosomes isolated from PBS-treated or sham-operated mice were used as controls (non-septic exosomes). Details are described in supplemental Methods. The size and quantity of exosomes were determined by NanoSight NS300 (Malvern Instruments Ltd) as reported (17). Data for each sample were collected for 60 s and analyzed using NanoSight NTA 3.1 software. All samples were measured in triplicates. Purified exosomes were labeled with SYTO RNA Select green fluorescent stain (Molecular Probes) following the manufacturer's instructions. MCECs were stained with Wheat Germ Agglutinin, Texas Red®-X Conjugate (Molecular Probes) at 0.5 h post administration of labeled exosomes.

In vitro endothelial permeability assay

MCECs (8×10^4 cells/well) were seeded onto 12-well Transwell with 0.4- μ m pore-size culture inserts (Corning Life Sciences, Lowell, MA) and cultured for 2-3 days to form a monolayer. MCECs were starved with 0.5% FBS or exosome free FBS overnight before experiments. Experimental treatments with PMA (Sigma) (80 ng/ml or 160 ng/ml), thrombin (Sigma) (5 U/ml), Mn (III) tetrakis (4-benzoic acid) porphyrin Chloride (MnTBPA, Merck Millipore) (40 μ M) (a scavenger of ROS), exosomes (1.2×10^{10} particles/ml) or relative vehicles were added to the upper compartment in order according to different experimental requirements. Endothelial permeability assay was conducted following a protocol described by Monaghan-Benson and Wittchen (18). Details are described in supplemental Methods.

Western blot analysis

Total protein was extracted from exosomes or PMA-treated endothelial cells with procedures described previously (16). Equal amounts of protein were subjected to SDS-PAGE and gel electrophoresis as described in detail elsewhere (19). The following antibodies were used: rabbit anti-CD63 (1:500, Santa Cruz), rabbit anti-ZO-1 (1:500, Invitrogen), rabbit anti-

cortactin (1:1000; Abcam); rabbit anti-paxillin (1:2000; Abcam) and rabbit anti-GAPDH (1:1000, Cell Signaling) used as an internal control.

Immunofluorescence microscopy

Immunofluorescence staining was performed by standard methods and is described in supplemental Methods. Cells were imaged with a confocal LSM 710 (Carl Zeiss Microimaging, Jena, Germany). Images were recorded with ZEN (Black) and analyzed with ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Quantitation of cells showing podosome cluster on cell edge was assessed in three independent experiments. At least 250 cells were counted in each experiment.

To obtain live images of endothelial cells, MCECs were transiently co-transfected with Cortactin-pmCherryC1 (a gift from Christien Merrifield, Addgene plasmid # 27676) (20) and mEGFP-Lifeact-7 (a gift from Michael Davidson, Addgene plasmid # 54610) with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. MCECs were imaged with Nikon A1R LUN-V Inverted TIRF Microscope at 100 ×/ 1.5 NA oil objective lense.

Measurement of ROS and lactate dehydrogenase (LDH) release assay

The ROS levels in exosomes or MCECs were measured using ROS-Glo H₂O₂ Assay kit (G8820, Promega) according to the manufacturer's instructions. The luminescence was measured with a Tecan Microplate Reader (Tecan, Durham, USA). Background of baseline obtained from the absorption of PBS or medium was subtracted. The ROS levels in heart tissues were determined by using an oxidation-sensitive fluorescent probe, CM-H₂DCFDA (Invitrogen) according to the manufacturer's instructions and procedures described previously (21). The ROS level was measured by the fluorescence intensity in each well at an excitation wavelength of 495 nm and an emission wavelength of 530 nm.

Cell culture medium were subjected to LDH release assay with an *in vitro* Toxicology Assay Kit (Sigma, TOX7) following the manufacturer's instructions. The values were expressed in units per milliliter (U/ml).

In vivo measurements of cardiac vascular permeability and cardiac function

Cardiac vascular permeability was assessed by using Evans blue dye (EBD) leakage index as a marker according to the method described by Castanares-Zapatero et al (22). Cardiac function was assessed *in vivo* using transthoracic echocardiography (iE33 Ultrasound System, Phillips) with a 40-MHz probe (19). For additional details, see supplemental Methods.

Statistics

Data were expressed as means ± standard deviations of the means (SD). Significance was determined by Student t test and one way or two way analysis of variance where appropriate to determine the differences within groups. Statistical significance was considered when P value was less than 0.05.

Results

PMA and thrombin both promote the generation of podosome clusters and the increase of MCECs permeability

Podosomes are usually organized as two different structures (individual podosomes or podosome clusters) in arterial, venous, and microvascular ECs (7). Both structures are rarely presented in cultured ECs, but can be formed through PMA induction (23). To explore the possible correlation between the podosome formation and vascular permeability, mouse cardiac endothelial cells (MCECs) were treated with PMA. We observed that most vehicle-treated ECs displayed thin stress fibers, polymerized F-actin along the cell cortex and concentrated cortactin at perinuclear sites (Fig. 1A, supplementary video 1). However, in PMA-treated ECs, cortactin relocated from the perinuclear region to the cell periphery where it colocalized with F-actin to form podosomes or podosome clusters (Fig. 1A; supplementary video 2). In addition, the PMA-induced podosome clusters were further confirmed by double immunofluorescence staining of F-actin and paxillin (Supplemental Fig.S1). Furthermore, the percentage of podosome cluster-containing MCECs was significantly increased in the PMA-treated group ($34.0 \pm 5.2\%$) compared to that in the control group ($9.8 \pm 4.4\%$) (Fig.1B). Consistently, the protein levels of cortactin and paxillin, two markers of podosome formation, were significantly increased in MCECs after PMA treatment according to our western blot analysis (Fig. 1C and D). In parallel, MCECs were seeded on Transwell permeable membrane, a model of barrier function *in vitro*, and treated with PMA (80 or 160 ng/ml) or PBS vehicle. Following treatment, EC permeability was monitored by determining values of trans-endothelial electrical resistance (TEER). Electrophysiological measurements showed that there were no significant changes in the TEER values for vehicle-treated MCECs over 1.5-h incubation period (Fig. 1H). However, under PMA treatment, the resistance of monolayers was decreased by around 30% at 0.5 hours post-treatment (hpt), and further decreased (55.6%, PMA 80 ng/ml; 74.6%, PMA 160 ng/ml) at 1 hpt ($P < 0.001$) (Fig. 1H). In addition, the PMA-triggered disturbance on the monolayer integrity was further verified by fluorescein isothiocyanate (FITC)-dextran flux assay (Fig. 1I).

On the other hand, thrombin is well known to induce endothelial cell monolayer permeability *in vitro* and *in vivo* via reorganization of cell-to-cell junctions (24, 25). However, whether thrombin can promote the generation of podosome clusters in ECs remains unknown. To this end, we treated MCECs with thrombin (5 U/ml) for 30min, followed by co-immunostaining for F-actin and cortactin. As presented in Fig. 1A, thrombin promoted the generation of podosome clusters at the boundary of endothelial cells similar to PMA treatment, and the percentage podosome clusters presented in MCECs was significantly raised by almost 3-fold ($31.7 \pm 6.3\%$), compared to that in vehicle controls ($12.2 \pm 2.9\%$) (Fig. 1E). In addition, both protein levels of cortactin and paxillin were markedly upregulated in MCECs under thrombin stimulation, similar to the PMA treatment group (Fig. 1, F and G). Meanwhile, EC monolayer permeability was elevated by thrombin treatment, evidenced by significant reductions (21.8%, 0.5 hpt and 33.8%, 1 hpt) in TEER values versus vehicle controls (Fig. 1J). Collectively, all these observations indicate that

there is a positive correlation between the generation of podosome clusters and the development of hyperpermeability in MCECs.

Septic exosomes increase permeability of MCECs

To determine the functional role of circulating septic exosomes, we generated a septic mouse model by cecal ligation and puncture (CLP) surgery and a mouse model of systemic endotoxemia by intraperitoneal injection of *Escherichia coli* lipopolysaccharides (LPS), then isolated exosomes from such mouse serum. Before applying them into cultured MCECs, we characterized exosomes by nanoparticle tracking assay and western-blot analysis. The nanoparticle tracking assay results showed that the average size of septic and non-septic exosomes was similar (~150 nm) (Fig. 2, A and B; Supplemental Fig.S2). The western-blot analysis showed the presence of CD63, an exosomal marker, in both septic exosomes and non-septic exosomes (Fig. 2C). As shown in Fig. 2D, stained exosomes (Green) were internalized into MCECs (Red).

Next, we examined whether circulating septic exosomes are capable of increasing the permeability of cardiac ECs. Firstly, MCECs were stimulated with circulating exosomes collected at four different time points post-injection of LPS/PBS and the dynamic changes of endothelial permeability were monitored by determining the cell monolayer TEER. Intriguingly, amongst four different groups of septic exosomes, only exosomes collected at 3 or 6 h post-LPS injection significantly reduced TEER values, compared to PBS exosome and no exosome controls (Fig. 2E), suggesting that the function of circulating exosomes may vary during the development of systemic endotoxemia. Septic exosomes isolated at 3 h post-LPS injection (LPS 3 h Exo) caused the greatest decrease (29%) of TEER at 3 h post-incubation with MCECs (Fig. 2E). Similarly, our FITC-dextran permeability assay showed that septic exosomes isolated at 3 h post-LPS injection markedly stimulated emigrations of the FITC-dextran to the lower chamber evidenced by 2-fold increase in fluorescence signal, compared with PBS exosomes treatment and vehicle controls (Fig. 2F). Similar findings were observed in MCECs treated with circulating exosomes isolated from mice at 3 h post-CLP surgery (Fig. 2G). Therefore, in the subsequent experiments, we used circulating exosomes collected at 3 h post-LPS injection and post-CLP to treat MCECs.

Septic exosomes induce the formation of podosome clusters in MCECs without affecting cell viability

Considering that there is a positive correlation between the generation of podosome clusters and development of hyperpermeability in cardiac ECs, it is highly possible that septic exosomes may also stimulate the formation of podosome clusters. Thus, experiments were conducted to determine the effect of exosomes on the podosome generation. After incubating MCECs with 1.2×10^{10} exosomes/ml for 1.5 h, higher numbers of podosome clusters arose at the cell periphery (Fig. 3 A and C, supplementary video 3), and the percentage of ECs carrying podosome clusters presented around 2-fold increases in septic exosome-stimulated groups (LPS, $20.2 \pm 4.6\%$; CLP, $18.7 \pm 3.8\%$), compared to that in non-septic exosome control groups (PBS, $9.9 \pm 3.0\%$; Sham, $11.4 \pm 3.3\%$) (Fig. 3, B and D). In addition, there were slight but significant elevations in protein levels of cortactin and paxillin in MCECs at 2 h post septic exosome stimulation, compared to control groups (Fig. 3, E-H).

To determine whether septic exosome-induced permeability is due to cytotoxicity-related cell damage, LDH release assay was performed. No significant change was observed in the amount of LDH released from different groups except for the Triton $\times$ -100 treatment control (Supplemental Fig.S3) ($P > 0.1$), suggesting that the incubation of MCECs with septic exosomes (1.2×10^{10} exosomes/ml) or PMA (80 ng/ml) for 2 h did not cause cell damage. Collectively, these results demonstrate that the generation of podosome clusters could play an important role in septic exosome-triggered cardiovascular hyperpermeability.

Septic exosomes deliver ROS to MCECs which contributes to the increase of podosome clusters and endothelial permeability

Considering that part of circulating exosomes are derived from inflammatory cells (neutrophils and macrophages) which contribute to oxidative burst and endothelial dysfunction by producing large amounts of ROS during septic shock (26), we sought to determine the ROS levels in circulating exosomes. Given that hydrogen peroxide (H_2O_2) has a relatively longer half-life compared to other kinds of ROS, which can be quickly converted to H_2O_2 within cells, the change in H_2O_2 can reflect the general alterations in intracellular ROS levels (27). Therefore, in the present study, we measured the levels of ROS within exosomes by using ROS-Glo H_2O_2 Assay kit (G8820, Promega). As shown in Fig. 4A, all septic exosomes presented markedly higher luminescent signals, indicating higher ROS levels in septic exosomes than non-septic exosomes ($P < 0.01$). Next, we determined whether exosomal ROS can be delivered to ECs. 1.2×10^{10} exosomes/ml were added to cultured ECs for 1 h, (PMA treatment was used as a positive control), followed by measurement of ROS levels in ECs. Compared with control groups, luminescent signals in ECs exposed to PMA/septic exosomes were respectively increased by 2.2-fold/1.5-fold (Fig. 4, B-D, $P < 0.01$). To verify that ROS in septic exosomes is responsible for the septic exosome-induced increases of ROS in ECs, we pretreated septic exosomes with Mn (III) tetrakis (4-benzoic acid) porphyrin Chloride, MnTBAP, (MnT) at 40 μ M for 1 h to scavenge exosomal ROS prior to stimulation. MnTBAP (MnT), as a cell permeable scavenger of ROS, possesses SOD and catalase-like activity, which not only converts superoxide to hyperoxide, but also catalyzes dissociation of H_2O_2 to water (28, 29). After 1 h stimulation with MnT-pretreated septic exosomes, no increase in luminescent signals was observed in ECs, indicating that the ROS-elevating effect of septic exosomes was abolished by MnT (Fig. 4, C and D). Thus, these observations suggest that septic exosomes are able to increase intracellular ROS levels in ECs through transferring exosomal ROS to target cells.

To determine whether PMA-induced or exosomal ROS-induced intracellular ROS in ECs are causative for the podosome cluster assembly, MCECs were 1) pretreated with MnT (40 μ M, 1 h) before addition of PMA (80 ng/ml) or 2) incubated with septic exosomes pretreated by MnT (40 μ M, 1 h). As shown in Figure 4E, the presence of MnT in ECs attenuated PMA-induced accumulation and co-localization of cortactin and F-actin at the cell periphery. In the same vein, MnT pretreatment abolished the capability of septic exosomes in inducing podosome cluster formation (Fig. 4E). Furthermore, our quantification results showed that, upon MnT pretreatment, the percentage of podosome cluster-containing cells was decreased by 30% in PMA-treated groups (Fig. 4F), and the percentage in septic exosome-treated groups was reduced from $20.2 \pm 4.5\%$ to $11.0 \pm 2.6\%$ (basal level) (Fig. 4G) ($P < .01$).

These results indicate that septic exosomes triggered-podosome cluster generation is ROS dependent.

Since there is a positive correlation between the generation of podosome clusters and development of hyperpermeability in MCECs, we speculated that the increase of podosome clusters contributes to PMA/septic exosome-triggered hyperpermeability of MCECs. To verify this, we performed TEER and FITC-dextran flux assays on MCECs which were pretreated with MnT or incubated with MnT-pretreated exosomes as mentioned above. As shown in Fig. 4H, PMA treatment resulted in 62% decrease of TEER value in ECs without MnT pre-treatment. However, the TEER value was only decreased by 25% in MnT-pretreated ECs after PMA stimulation (Fig. 4H). Further, in the FITC-dextran flux assay, the presence of MnT in ECs prevented the reduction of permeability of EC monolayer by 68% at 1 hpt and 65% at 1.5 hpt, compared to that (100%) in the absence of MnT under PMA stimulation (Fig. 4J). Notably, scavenging exosomal ROS by applying MnT successfully prevented septic exosome-induced drop in TEER value (Fig. 4I). Consistently, our FITC-dextran flux assay results showed that the reduction of monolayer permeability was significantly decreased by around 65% in ECs exposed to MnT-pretreated septic exosomes, compared with ECs subjected to un-pretreated septic exosomes (100%) (Fig. 4K). All together, these data suggest that septic exosomes contain high levels of ROS which can be delivered to endothelial cells, leading to activation of podosome cluster formation and thereby, vascular leakage.

Podosome clusters promote endothelial dysfunction via targeting ZO-1 at the cell periphery

Zonula occludens-1 (ZO-1, 225kDa) is regarded as a controller of endothelial adherent junctions and barrier formation (8). Moreover, ZO-1 has been demonstrated to be involved in the formation of podosomes (9). Therefore, we speculated that the podosome cluster promoted-EC dysfunction may be associated with ZO-1. To test this speculation, MCECs were incubated with PMA (80 ng/ml, 0.5 h) or 1.2×10^{10} exosome /ml for 1.5 h and then, subjected to immunofluorescence staining with the ZO-1 antibody. Under control conditions (MCECs incubated with PBS or non-septic exosomes), a near continuous immunofluorescent signal was observed at the cell border (Fig. 5, A-C). In contrast, pronounced fragmentation and depletion of ZO-1 were observed at 0.5 h post-PMA stimulation (Fig. 5A). Interestingly, the ZO-1 linear pattern was partially restored by pretreating ECs with MnT for 1 h (Fig. 5A). Similarly, addition of septic exosomes also significantly fragmented and depleted ZO-1 but not as remarkably as PMA treatment (Fig. 5, B and C). However, application of MnT into septic exosomes abolished the fragmentation/depletion effect of septic exosomes on ZO-1 (Fig. 5, B and C). To determine whether this fragmentation and depletion was due to the down-regulation of expression or relocation of ZO-1, MCECs were exposed to 80 ng/ml PMA for 0.5 h. Then, the protein content of ZO-1 in MCECs was determined by Western blotting. Interestingly, the protein levels of ZO-1 were unchanged after PMA stimulation, compared to control conditions (Fig. 5, D and E), suggesting that the newly formed podosome clusters may cause fragmentation/depletion of ZO-1 without affecting total expression in ECs.

Septic exosomes induce cardiovascular hyperpermeability and cardiac dysfunction *in vivo*

To explore whether septic exosomes are capable of inducing cardiovascular permeability and cardiac dysfunction *in vivo*, we determined ROS levels, expression of cortactin and paxillin, Evans blue dye (EBD) leakage and cardiac function in mice exposed to septic exosomes or non-septic exosomes (3.6×10^{12} exosomes/kg). At 1 hpt, ROS levels in heart tissue were remarkably increased in septic exosome-stimulated mice, compared to non-septic exosome-stimulated mice, indicating the important role of septic exosomes in ROS delivery (Fig. 6 A). Consistent with our *in vitro* results, western blotting analysis showed upregulation of cortactin and paxillin in hearts of mice exposed to LPS Exo ($P < 0.05$) (Fig. 6, B and C), suggesting that LPS Exo injection may promote podosome cluster formation in cardiac vascular endothelial cells of heart. Furthermore, the microvascular permeability experiment showed that the EBD extravasation was elevated by 1.8-fold at 3 hpt in the heart of mice exposed to septic exosomes, demonstrating a significant increase of cardiovascular permeability in relation to control groups (Fig. 6, D-F).

Next, considering that vascular leakage is a major contributor to cardiac dysfunction in sepsis (2), we evaluated the effect of septic exosome on cardiac function. Cardiac function was assessed by echocardiography in mice injected with PBS, PBS Exo or LPS Exo. Septic exosome injection markedly increased left ventricular end systolic dimension (LVESd) (Fig. 6, G and H), as well as decreased left ventricular ejection fraction (LVEF %) (Fig. 6 I) and left ventricular fractional shortening (FS) (data not shown), compared with non-septic exosome injection and PBS injection controls. In addition, CLP Exo stimulation exhibited a similar effect on cardiac function as we observed above with LPS Exo treatment (data not shown).

Discussion

Vascular leakage is an important hallmark of septic shock. Given that all blood vessels are lined with endothelial cells, the loss of endothelial integrity can account for the vascular leakage and tissue edema in sepsis. Therefore, it is important to understand both cellular and molecular mechanisms behind the endothelial barrier dysfunction. Such studies might lead to new therapies for sepsis. In this study, we discovered that PMA, thrombin, LPS-stimulated and septic circulating exosomes (mainly originate from macrophages, neutrophils, monocytes and platelets) could promote the generation of podosome clusters at the EC periphery and consequently, induce endothelial hyperpermeability. Mechanistically, we found that septic exosomes contained high amounts of ROS which can be delivered to target ECs leading to the elevation of intracellular ROS (Fig. 7). By contrast, scavenging ROS in septic exosomes abolished the effects of septic exosomes on promoting the formation of podosome clusters and development of endothelial hyperpermeability. Furthermore, we recognized that the generation of podosome clusters could form inter-endothelial gaps by relocating ZO-1 at the cell periphery (Fig. 7).

Exosomes contain many unique features like RNAs/DNAs, proteins and lipids, and are perceived to be carriers of these cargoes to diverse locations in the body. After fusion with a recipient cell, exosomes transport their contents, such as microRNA (miRNA) and proteins, into the cytoplasm to perform various biological functions (30). Over the past decade,

exosomes have been extensively studied as miRNA and protein carriers in the cardiovascular disease research field (31). However, in this study, we observed, for the first time, that circulating exosomes could transport active ROS to ECs, resulting in increased podosome clusters and vascular leakage. During septic shock, stimulated inflammatory cells, such as neutrophils and macrophages, produce large amounts of ROS which contribute in part to the host defence against bacterial spread but also play crucial roles in initiating intercellular junctional disassembly and vascular hyperpermeability (26, 32). Notwithstanding, the mechanism of transportation of the ROS during sepsis from their site of origin to the place of action (such as ECs) has not been investigated yet. Although the plasma membrane has been assumed to be freely permeable to ROS, current evidences suggest that passive diffusion of ROS across cell membranes is limited due to poor permeability ROS across the plasma membrane (33-35). Furthermore, the rapid release and ingress of ROS into the cell are essential for effective signal transduction processes during septic shock. In this study, we found that septic exosome carrying abundant ROS could enter MCECs within 0.5 h *in vitro* and induce ROS elevation within 1 h both *in vitro* and *in vivo*, indicating that exosomes possibly work as highly efficient transporters for ROS during septic shock. Our findings may establish a better insight into the nature and physical transport mechanisms of ROS into cells and reveal a novel function of exosomes during septic shock.

One important mechanism behind ROS-promoted endothelial permeability is that ROS are able to work as critical inducers of actin cytoskeleton reorganization in endothelial cells, leading to junctional opening and intercellular gap formation (26). In addition, it has been shown that the generation of podosomes, as a result of actin cytoskeleton reorganization, is dependent on ROS (6). Furthermore, ROS-activated PKC, a small G protein Rho, Src tyrosine kinase, MLCK, ERK1/2 and p38 MAPK have been demonstrated to be crucial positive regulators of podosome assembly (6, 36-39). Therefore, we speculated that septic exosome-caused elevation of ROS in ECs can activate PKC, a small G protein Rho or Src tyrosine kinase which, in turn, promote the generation of podosome clusters. In the present study, we provide direct evidence showing that septic exosome-induced generation of podosome clusters in MCECs is dependent on the high amounts of ROS encased.

ZO-1, a central regulator of intercellular junctions and a major cytoskeletal organizer in ECs, not only regulates the overall distribution of F-actin but also interacts with tight junctions and adherent junctions to keep the endothelial integrity (8). Recently, ZO-1 has been shown to be involved in podosome formation via binding cortactin, a key scaffold protein which controls actin assembly at the core region of podosomes (9). Moreover, after PMA treatment, ZO-1 was observed to be rapidly relocated from the cell cortex toward newly formed podosomes in smooth muscle cells (9). In the present study, we discovered that PMA treatment not only induced the generation of podosome clusters, but also caused ZO-1 fragmentation and deletion at the cell periphery without down-regulating of total ZO-1 protein levels in MCECs. Similar results were observed in septic exosome-treated MCECs. More interestingly, in the presence of MnT, septic exosomes failed to induce: 1) the formation of podosome clusters, 2) fragmentation/deletion of ZO-1, and 3) the increase of monolayer permeability in ECs. Likewise, pre-treatment of MCECs with MnT dramatically attenuated the PMA-mediated effects on these three phenomena mentioned above. Collectively, our data presented in this study, for the first time, demonstrate that septic

exosomes stimulate endothelial cells to assemble podosome clusters through the transferring of exosomal ROS to the target ECs, leading to relocation of ZO-1 from cell periphery to newly formed podosomes which, in turn, break the endothelial integrity (Fig. 7).

It is important to mention here that platelet-derived exosomes from septic patients were previously reported to directly trigger EC apoptosis (10, 11). However, whether such platelet-derived exosomes can induce podosome formation in ECs and eventual hyperpermeability were not examined in the present study. The exosomes used in this study were isolated from the serum of septic mice, and these septic exosomes did not cause EC damage based on our LDH assay results. This difference may be ascribed to different amounts of exosomes, different cellular origins of exosomes, and/or different type of ECs used in these experiments. A recent study by Zhou et al showed that exosomes derived from cancer cells can transfer miR-105 to endothelial cells and increase vascular permeability by targeting the tight junction protein ZO-1, which may support our present findings (40). In addition, while this study focused on the role of septic exosomal ROS in the podosome formation and vascular hyperpermeability, we cannot exclude the contributions of other exosomal contents (*i.e.*, miRNAs and cytokines).

In summary, we demonstrated that septic exosomes were enriched with high amounts of ROS, which can be transferred to ECs, leading to the generation of podosome clusters in the target ECs and thereby, causing the relocation of ZO-1 and the formation of inter-endothelial gaps (Fig. 7). This study may present a new vascular redox signaling pathway related to cardiovascular dysfunction in sepsis. It also uncovers a novel functional role of endothelial podosomes in vascular dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PMA and Thrombin promote the generation of podosome clusters and hyperpermeability of MCECs

(A) MCECs were spread on 1 mg/mL collagen-coated coverslips for 12 hours and then treated with control vehicle, PMA (80 ng/ml) or thrombin (5 U/ml) for 0.5 h. After that, cells were fixed and stained for F-actin (Green), Cortactin (Red), and nuclei (Blue, DAPI). Images were captured with confocal LSM 710 (Carl Zeiss Microimaging, Jena, Germany). White square indicates magnified area of the cell. Red arrows represent podosome clusters at the cell periphery. White arrows indicate individual podosomes in cell. Scale bar: 20 μ m. (B and E) The percentage of ECs carrying podosome clusters were calculated after treatment with PBS vehicle, PMA or thrombin for the indicated time period. Results are shown as

means $\pm$ SD of three independent experiments in which 250 cells were analyzed per experiment ($*P < 0.001$). **(C, D, F, G)** Representative western blots of cortactin and paxillin in MCECs with different treatments. Values are presented as means $\pm$ SD; $n = 4$, $*P < 0.05$. **(H and J)** MCECs were seeded onto 12 mm diameter 'Transwell Clear' filter inserts (0.4 μ m pore size) at a density of 8×10^4 cells/well and allowed to grow until a monolayer was formed. Cells were exposed to different concentrations (80 ng/ml or 160 ng/ml) of PMA, thrombin (5 U/ml) or vehicle. Monolayer TEER were measured at different time points. Values are presented as means $\pm$ SD; $n = 4$, $*P < 0.001$. **(I)** Monolayer permeability was assayed by adding FITC-dextran (1 mg/ml) to the upper chamber in the presence or absence of PMA and evaluating the emigration of the FITC-dextran to the lower chamber by fluorimetry at different time points. Fluorescence was read with a 485 nm and 530 nm filter set. Values are presented as means $\pm$ SD; $n = 4$, $*P < 0.001$.

Figure 2. Effects of circulating exosomes on permeability of MCECs

Exosomes were isolated with Total Exosome Isolation Reagent (Invitrogen). (A) The size of exosomes was determined using a NanoSight NS300 (Malvern Instruments Ltd). (B) The averaged sizes of septic exosomes and un-septic exosomes were calculated. Values are presented as means $\pm$ SD. NS: not significant ($n=3$, $P>0.1$). (C) Exosomal marker (CD63) was identified in both septic exosomes and non-septic exosomes. Heart-derived protein was used as positive control. (LPS 100/PBS 100: exosomes isolated from 100 μ l serum; LPS 50/PBS 50: exosomes isolated from 50 μ l serum). (D) SYTO without Exo: No exosomes, only SYTO Dye in PBS was filtered and added to MCECs. SYTO with Exo: Labelled exosomes were filtered and added to MCECs. White arrows indicate exosomes. Exosomes (Green); MCEC membranes (Red); cell nuclei (Blue). Scale bars: 20 μ m. (E) Serum-derived exosomes were collected at 3, 6, 12, and 24 h post LPS (15 μ g/g) injection (LPS Exo) and equivalent volume PBS injection (PBS Exo). MCECs grown to a confluent monolayer on transwell were exposed to LPS Exo or PBS Exo (1.2×10^{10} exosomes/ml) collected at different time points. Then, TEER were measured at indicated time intervals over 10 h stimulation. Values are presented as means $\pm$ SD; ($n=3$, $*P<0.01$). (F and G) MCECs monolayers grown on 0.4 μ m filters were stimulated with exosomes (PBS/LPS Exo, Sham/CLP Exo) that were collected at 3 h post septic shock or sham treatment. The

permeability of MCECs monolayer was monitored by measuring fluorescence signal in lower chamber during a 1.5 h time course. Values are presented as means $\pm$ SD; (n = 6, * P < 0.01).

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Figure 3. Septic exosomes induce the formation of podosome clusters in MCECs without affecting cell viability

(A and C) MCECs were incubated with septic exosomes or non-septic exosomes (1.2×10^{10} exosomes/ml) for 1.5 h. After that, cells were fixed and stained for F-actin (Green), Cortactin (Red), and nuclei (Blue, DAPI). Red arrows indicate podosome clusters on the cell edge. Scale bar: 20 μ m. (B and D) Percentages of ECs carrying podosome clusters were quantified in both septic exosome and non-septic exosome treatment groups. Results are shown in means $\pm$ SD of three independent experiments in which 250 cells were analyzed per experiment (* $P < 0.01$). (E-H) Representative western blots of cortactin and paxillin in MCECs stimulated with septic exosomes or non-septic exosomes. Values are presented as means $\pm$ SD; n = 4, * $P < 0.05$.

Figure 4. Septic exosome-induced elevation of intracellular ROS in MCECs contributes to the increase of podosome clusters and EC hyperpermeability

Septic exosomes were able to increase the intracellular ROS levels in MCECs. (A) The ROS levels in septic and non-septic exosomes were measured with ROS-Glo H₂O₂ Assay Kit. The relative light units (RLU) of luminescence was recorded by microplate reader. (B) MCECs were incubated with PMA (80 ng/ml) or equivalent volume of PBS for 0.5 h before conducting ROS assay. (C and D) To investigate the role of exosomal ROS, MnT (a scavenger of ROS) was added into septic exosomes to a final concentration of 40 μ M at 1 h before applying into MCECs. ECs were then incubated with non-septic exosomes or septic exosomes (with/without MnT) for 1 h. After that, ECs were washed with PBS, and ROS assays were performed. Values are presented as means $\pm$ SD (n=6, **P* < 0.01). The increase of ROS in ECs contributed to the generation of podosome clusters. (E) PMA: MCECs were exposed to PMA (80 ng/ml) for 0.5 h. MnT PMA: MCECs were incubated with MnT (40 μ M) for 1h before 0.5 h PMA (80 ng/ml) stimulation. LPS Exo: MCECs were cultured with LPS Exo (1.2×10^{10} exosomes/ml) for 1.5 h. LPS Exo MnT: LPS Exo was incubated with MnT (40 μ M) for 1 h, and then applied into MCECs for 1.5 h. After these treatments, MCECs were fixed and stained with F-actin (Green), Cortactin (Red), and nuclei (Blue, DAPI). White arrows indicate podosome clusters at the cell periphery. Scale bar: 20 μ m. (F and G) The percentages of ECs containing podosome clusters were calculated in different

treatment groups. Results are shown as means $\pm$ SD of three independent experiments in which 250 cells were analyzed per experiment (* $P < 0.01$). **(H-J)** The hyperpermeability of ECs induced by PMA and LPS Exo were attenuated by inhibiting the increase of intracellular ROS with MnT. MCECs were grown to a confluent monolayer on 12 mm diameter 'Transwell Clear' filter inserts (0.4 μ m pore size). Cells and LPS Exo were pretreated with MnT in the same way as in panels **E**. **(H and I)** After incubating with PMA or LPS Exo, the TEER values were collected at indicated time points in the presence or absence of MnT. **(J and K)** The permeability of MCECs monolayers under different treatments was measured by monitoring the appearance of FITC-dextran (which was added to the top chamber at the beginning of the experiment) in the bottom well during a 1.5 h time course. Values are shown as means $\pm$ SD (n=3, * $P < 0.01$).

Figure 5. Podosome clusters relocate ZO-1 and lead to the formation of intercellular gaps
MCECs were seeded on collagen coating coverslips until reaching monolayers. **(A)** ECs were pretreated with MnT (40 μ M) for 1 h. ECs pretreated or un-pretreated with MnT were then exposed to PMA (80 ng/ml) for 0.5 h. For control, ECs were exposed to PBS vehicle. **(B and C)** MnT was added into septic exosomes (LPS/CLP Exo) to a final concentration of 40 μ M at 1 h prior to applying into MCECs. ECs were then incubated with septic exosomes with or without MnT for 1.5 h. ECs exposed to PBS Exo were used as control. After that, ECs were analyzed by immunofluorescence staining for ZO-1 (green) and DAPI (blue, cell nuclei). White arrows indicate the integrity linear pattern of ZO-1. Yellow arrows present the fragmentations and depletions of ZO-1. Scale bar: 20 μ m. **(D and E)** MCECs were exposed to PMA (80 ng/ml) or PBS vehicle for 0.5 h. The protein level of ZO-1 in MCECs post treatment was analyzed by immunoblotting. Values are presented as means $\pm$ SD (n=3, * P > 0.1).

Figure 6. Septic exosomes induce cardiovascular hyperpermeability and cardiac dysfunction *in vivo*

(A) The ROS levels were determined in hearts of mice exposed to LPS Exo or PBS Exo (3.6×10^{12} exosomes/kg) for 1 h ($n=4$, $*P < 0.05$). (B, C) Protein levels of cortactin and paxillin in hearts were evaluated at 3 h post exosome injections ($n=6$, $*P < 0.05$). (D-F) Cardiovascular permeability was assessed in mice exposed to exosomes for 3 h. Frozen sections were observed under a confocal LSM 710 microscope. Scale bar: 30 μ M. The intensity of relative fluorescence in different treatment groups was quantified with Image J software. Values are presented as means $\pm$ SD ($n=6$, $*P < 0.01$). (G-I) At 6 h post injection of exosomes or PBS, the cardiac function was determined by echocardiography. The values of the left ventricular end systolic dimension (LVESd) were markedly increased, and the values of left ventricular ejection fraction (EF%) were significantly decreased in LPS Exo injected mice ($n = 6$), compared with controls ($n = 4$) ($*P < 0.05$).

Figure 7.

A work model elucidating that circulating exosomes derived from septic mice are enriched with high amounts of ROS, which can be transported to ECs, leading to the generation of podosome clusters in target ECs and thereby, causing ZO-1 relocation, endothelial hyperpermeability and cardiac dysfunction.