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Blockade of exosome generation with GW4869 dampens the sepsis-induced inflammation and cardiac dysfunction

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ABSTRACT

Sepsis is an infection-induced severe inflammatory disorder that leads to multiple organ failure. Amongst organs affected, myocardial depression is believed to be a major contributor to septic death. While it has been identified that large amounts of circulating pro-inflammatory cytokines are culprit for triggering cardiac dysfunction in sepsis, the underlying mechanisms remain obscure. Additionally, recent studies have shown that exosomes released from bacteria-infected macrophages are pro-inflammatory. Hence, we examined in this study whether blocking the generation of exosomes would be protective against sepsis-induced inflammatory response and cardiac dysfunction. To this end, we pre-treated RAW264.7 macrophages with GW4869, an inhibitor of exosome biogenesis/release, followed by endotoxin (LPS) challenge. *In vivo*, we injected wild-type (WT) mice with GW4869 for 1 h prior to endotoxin treatment or cecal ligation/puncture (CLP) surgery. We observed that pre-treatment with GW4869 significantly impaired release of both exosomes and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in RAW264.7 macrophages. At 12 h after LPS treatment or CLP surgery, WT mice pre-treated with GW4869 displayed lower amounts of exosomes and pro-inflammatory cytokines in the serum than control PBS-injected mice. Accordingly, GW4869 treatment diminished the sepsis-induced cardiac inflammation, attenuated myocardial depression and prolonged survival. Together, our findings indicate that blockade of exosome generation in sepsis dampens the sepsis-triggered inflammatory response and thereby, improves cardiac function and survival.

1. Introduction

Sepsis, a leading cause of death in the intensive care unit (ICU), is a severe inflammatory response syndrome caused by infection [1,2]. Multiple organ dysfunction is a hallmark of this syndrome, with sepsis-induced cardiomyopathy responsible for a large number of death cases. Indeed, septic patients with cardiac abnormalities have a 70% chance of mortality compared to 20% mortality rate for patients without myocardial dysfunction [3,4]. While the underlying mechanism leading to septic cardiomyopathy remains largely ambiguous, it is well accepted that circulating pro-inflammatory cytokines (*i.e.*, TNF-α, IL-1β, IL-6) are the myocardial depressive factors (MDFs) [5–8]. These cytokines are mainly produced by macrophages and other leukocytes through activation of toll-like receptors (TLRs) and subsequent NF-κB signaling cascades upon septic shock [9–14]. In addition, other inflammatory mediators such as nitric oxide may stimulate synthesis of pro-inflammatory cytokines in macrophages and cardiomyocytes, and thus contribute to myocardial depression [15–17].

Recently, exosomes, a type of membrane-bound nanoparticle naturally released from living cells, have been defined as novel factors to mediate the pathogenesis of sepsis [18–20]. Particularly, exosomes isolated from platelets of septic patients induce myocardial depression in animal models by promoting the expression of pro-inflammatory proteins such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) [19,20]. Furthermore, these platelet-derived exosomes promote vascular dysfunction by inducing the NAD(P)H oxidase-mediated generation of reactive oxygen species in endothelial cells [21,22]. Therefore, it would be beneficial for septic patients if exosome secretion from platelets were inhibited. Nevertheless, macrophages are the first-line to fight against sepsis since multiple cardiac depressors (*i.e.*, inflammatory

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cytokines) are produced by macrophages. As a matter of fact, macrophage-deficient mice appeared to attenuate LPS-caused myocardial depression [23]. Additionally, rat cardiomyocytes incubated with LPS-treated macrophages exhibited a significant reduction in contractility, suggesting a direct cross-talk between macrophages and cardiomyocytes [24]. Several studies have also implicated macrophages as autocrine and paracrine signaling mediators that initiate further inflammatory responses in both neighboring macrophages and other types of cells [25,26]. Recently, exosomes isolated from mycobacterium-treated macrophages were found to be pro-inflammatory in naive macrophages [27,28]. We thus questioned whether blockade of exosome release from macrophages and other cell types (*i.e.*, platelets) would be able to attenuate sepsis-triggered inflammation and thereby, improve cardiac function.

Currently, GW4869, a neutral sphingomyelinase inhibitor, is the most widely used pharmacological agent for blocking exosome generation [29–32]. GW4869 inhibits the ceramide-mediated inward budding of multivesicular bodies (MVBs) and release of mature exosomes from MVBs [30]. It was first utilized by Kosaka et al. to successfully inhibit exosome release in HEK293 cells [29]. In the present study, we determined the pro-inflammatory effects of exosomes derived from LPS-treated macrophages, and then examined the consequence of GW4869-mediated inhibition of exosomes on cytokine production in LPS-treated macrophages *in vitro*. We next investigated the *in vivo* effects of GW4869 on pro-inflammatory cytokine production, cardiac function and mortality in endotoxin-challenged mice and a more clinically relevant sepsis model, induced by cecal ligation and puncture (CLP) surgery. Our results indicate that the global blockade of exosome production with GW4869 attenuates sepsis-induced inflammation, improves cardiac function and prolongs animal survival.

2. Methods and materials

2.1. Animals and macrophage cell line

Male wild-type C57BL/6 mice were purchased from Jackson Laboratory (Indianapolis, IN). The mice were maintained and bred in the Division of Laboratory Animal Resources at the University of Cincinnati Medical Center. All the animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences, published by the National Institutes of Health, and approved by the University of Cincinnati Animal Care and Use Committee (Animal Welfare Assurance Number: A3295-01). The mouse macrophage cell line RAW264.7 was purchased from American Type Culture Collection (ATCC), Rockville, MD. The macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 15% of fetal bovine serum (FBS; Sigma), 2 mm L-glutamine (Gibco, USA), 100 u/ml penicillin and 100 u/ml streptomycin (Sigma). The macrophages were grown at 37 °C with 5% CO_2 in fully humidified air. Culture medium was changed every 1–2 days. Subsequent passages were performed with a 0.025% Trypsin (Sigma) containing 0.02% EDTA for 10 min at 37 °C. The fourth passage macrophages were used for experiments in this study.

2.2. Treatment of macrophage cell line with LPS

RAW 264.7 macrophages were plated in 100 mm petri dishes at 1.2×10^6 cells/dish. Macrophages were allowed to adhere for 24 h before any treatments. Macrophages were treated with culture media in the presence or absence of 1 μg/ml *Escherichia coli* LPS (Sigma, 0111:B4) and incubated at 37 °C and 5% CO_2 for 24 h. Culture supernatants were then collected for exosome isolation, acetylcholinesterase (AChE) activity assay, and cytokine measurement. For the exosome collection and function assays, exosome-depleted FBS (System Biosciences Inc.) was used in the cell culture.

2.3. Isolation and characterizations of exosomes

Supernatants from cultured RAW264.7 macrophages were collected on ice and centrifuged at 2000 ×*g* for 30 min to remove any cells and cellular debris, and then supernatants were transferred to a fresh tube and centrifuged at 10,000 ×*g* for 30 min at 4 °C. Subsequently, supernatants were transferred to a fresh tube and centrifuged at 100,000 ×*g* (Ti-45 rotor) for 10 h at 4 °C. The exosomal pellet was then washed once with sterile PBS to remove any secreted proteins and re-suspended in 500 μl of PBS. The quality of exosomes was confirmed by dynamic light scattering using a particle and molecular size analyzer (Zetasizer Nano ZS, Malvern Instruments) according to the manufacturer's instructions. The quantity of exosomes was determined by the Micro-BCA assay (Pierce, Rockford, IL) for measurement of total protein. The pro-inflammatory cytokine content of the isolated exosomes was determined by ELISA assays. Endotoxin levels in isolated exosomes were measured to determine possible endotoxin contamination, using the ToxinSensor Chromogenic LAL Endotoxin kit (Genscript) per the manufacturer's protocol.

2.4. Western blot analysis

Equal amounts of protein were subjected to SDS-PAGE. Binding of the primary antibody was detected by peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Pharmacia), and bands were quantified with densitometry. The sources of antibodies and dilutions used were as follows: rabbit anti-CD63 (sc-15363, 1:1000 dilution) and rabbit anti-CD81 (sc-9158, 1:1000 dilution). GAPDH (1:1000 dilution, GeneTex) was used as an internal control.

2.5. Treatment of macrophages with exosomes or GW4869

Fresh RAW264.7 macrophages were plated in 100 mm petri dishes at 1.2×10^6 cells/dish, and treated with either culture media containing 20 μg of exosomes isolated from non-LPS treated macrophages (non-LPS exosomes) or 20 μg of exosomes isolated from LPS treated macrophages (LPS exosomes), or exosome-free media. The culture supernatants were harvested for cytokine ELISA assays at 10 min and 24 h. For inhibition of exosome generation, macrophages were pre-treated with either culture media containing 10 μM or 20 μM GW4869 (Sigma) for 2 h prior to treatment with 1 μg/ml LPS incubation, Culture supernatants were collected after 24 h for AChE activity assay and cytokine measurement. GW4869 was initially dissolved in DMSO (Fisher Scientific) into a stock solution of 5 mM GW4869 before dilution in culture supernatant to achieve 10 μM or 20 μM GW4869 concentration in cell culture condition (note: final DMSO concentration is 0.005%). To determine the possible toxicity of DMSO or GW4869, fresh RAW 264.7 macrophages (1.2×10^6 cells/dish) were incubated in culture media containing 0.005% DMSO, 10 μM GW4869 and 20 μM GW4869 for 24 h. Cell injury was determined by measuring the release of lactase dehydrogenase (LDH) into the culture media, using a LDH detection kit (Sigma) according to the manufacturer's protocol.

2.6. GW4869 treatment in endotoxin-challenged mice

Wild-type male C57BL/6 mice were studied at 10 to 12 weeks old. The mice were randomly assigned to four groups: PBS, GW4869, PBS + LPS and GW4869 + LPS (n = 5 per group). GW4869, dissolved in DMSO (0.005%), was intraperitoneally (*i.p.*) injected at one dose of 2.5 μg/g. Mice in the PBS + LPS group were pre-injected *i.p.* with PBS 1 h prior to an *i.p.* injection of LPS (25 μg/g). Mice in the group of GW4869 + LPS were pre-injected *i.p.* with GW4869 (2.5 μg/g) for 1 h, followed by an *i.p.* injection of LPS (25 μg/g, 100 μl). Mice received injections of PBS to a comparable volume (100 μl) as controls. The survival

rate of the PBS + LPS and GW4869 + LPS groups was monitored every 6 h for a 36 h period.

2.7. Pre-treatment with GW4869 in CLP polymicrobial sepsis model

Polymicrobial sepsis was surgically induced by cecal ligation and puncture (CLP) as previously described [33]. Wild-type C57BL/6 mice (10–12 weeks, male) were randomly assigned to four groups: PBS + Sham, GW4869 + Sham, PBS + CLP and GW4869 + CLP. Before sham or CLP surgery, mice were administered by intraperitoneal (*i.p.*) injection at one dose of GW4869 (2.5 μg/g). Same volume of PBS was injected as controls. For CLP surgery, mice were anesthetized by isoflurane inhalation and ventilated with room air using a rodent ventilator. A 1- to 2-cm midline incision was made below the diaphragm to expose the cecum. The cecum was ligated at 1.0 cm from the tip with a 5-0 sterile silk suture. A single through and through puncture was made at the middle between the ligation and the tip of the cecum with an 18-gauge to induce severe septic injury. After puncturing, the cecum was gently squeezed to extrude a small amount of feces and returned to the abdominal cavity. The abdominal wall incision was closed in layers. After surgery, pre-warmed normal saline (0.05–0.1 ml/g body weight) was administered subcutaneously. Post-operative pain control was managed with subcutaneous injection of bupivacaine and buprenorphine. Sham controls were exposed to the same surgery; however, their cecum was neither ligated nor punctured. The survival rate of the PBS + CLP and the GW4869 + CLP groups was monitored every 6 h for 2 days.

2.8. Assessment of AChE activity, cytokine and myeloperoxidase (MPO) Activity

AChE activity was used to indirectly determine the quantity of exosomes in culture supernatants or sera, because acetylcholinesterases are localized to the membrane of exosomes [34]. The AChE activity was determined using a commercially available AChE activity assay kit (Sigma). Culture supernatants and serum samples were incubated in the working reagent in 96-well plates. After incubation, the colometric product was read at 412 nm on a microplate reader and expressed as AChE activity (units/l) per the manufacturers' instructions. The levels of tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin 1β (IL-1β) in the sera and culture supernatants were determined, using commercially available ELISA kits (Bio-legend). Cytokine levels were established by comparison to a standard curve per the manufacturer's instructions. Myeloperoxidase (MPO) activity was measured using commercial MPO Elisa kit (Abnova). MPO, which is released upon neutrophil infiltration, enhances tissue inflammation through the release of cytotoxic oxidants [35]. Heart samples were homogenized in ice-cold lysis buffer and centrifuged at 12,000 ×*g* for 10 min, after which supernatants were used for the MPO activity assay. The MPO kit utilizes a sandwich enzyme-linked immune-sorbent assay method, with MPO activity measured at 450 nm on a microplate reader in comparison to a standard curve.

2.9. Assessment of cardiac function in vivo

Cardiac function was assessed *in vivo* using trans-thoracic echocardiography (Vevo®2100 Imaging System, Visualsonics) with a 15-MHz probe. Left ventricular (LV) end-diastolic (LVDd) and end-systolic diameters (LVDs) were measured from M-mode recordings. LV function variables were assessed at 12 h after LPS administration and 12 h after sham/CLP surgery. LV ejection fraction (EF) was calculated as: $[(LVDd^3 - LVDs^3) / LVDd^3] \times 100$. LV fractional shortening (FS%) was determined as $[(LVDd - LVDs) / LVDd] \times 100$. All measurements were performed according to the American Society for Echocardiology leading-edge technique standards, and average over three consecutive cardiac cycles.

2.10. Statistical analysis

Data were expressed as means ± SEM. Significance was determined by Student *t* test, and one- or two-way analysis of variance was appropriate to determine differences within groups, with a Bonferroni post hoc analysis. The survival rates were constructed using the Kaplan–Meier method, and differences in mortality were compared using the log-rank-test. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. LPS treatment increases exosome release and pro-inflammatory cytokine production in macrophages in vitro

A number of studies have indicated that bacterial infection can stimulate exosomes and pro-inflammatory cytokine release from macrophages [27,28]. To validate these observations, we treated RAW264.7 macrophages with *E. coli* LPS, and measured the amount of exosomes and pro-inflammatory cytokines in the culture supernatant. We observed a significantly higher activity of the AChE, a specific enzyme associated with exosome membrane, in the supernatants of LPS-treated macrophages compared to control macrophages (Fig. 1A). Further, the protein concentration in exosomes isolated from LPS treated macrophages (LPS exosomes) was significantly higher than protein quantities in 'non-LPS exosomes' (Fig. 1B). Results of ELISA analysis showed that LPS treatment caused a 7-fold increase in TNF-α, 4.5-fold increase in IL-1β and 616-fold increase in IL-6 levels compared to non-treated macrophages (Fig. 1C–E). These data indicate that endotoxin can stimulate macrophages to release exosomes, together with the secretion of pro-inflammatory cytokines.

3.2. Exosomes derived from LPS treated macrophages are pro-inflammatory

To explore the possible properties of 'LPS exosomes', we first characterized exosomes isolated from LPS-treated macrophages and non-LPS-treated macrophages. Using dynamic light scattering, we observed that the average size of exosomes derived from LPS-treated macrophages (43.82 nm) was similar to the size of exosomes derived from non-LPS-treated macrophages (43.88 nm) (Fig. 2A). Moreover, western blot analysis of exosomal proteins revealed the presence of CD63 and CD81, two exosomal markers, in both LPS exosomes and non-LPS exosomes (Fig. 2B). Endotoxin levels in non-LPS and LPS exosomes were extremely low and negligible (<0.1 EU/ml, <0.0002 EU/μg of exosomal protein) compared to levels in culture medium from LPS-treated macrophages (Supplemental Fig. 1a/b). Interestingly, the content of TNF-α and IL-6 in LPS exosomes was significantly higher than non-LPS exosomes (Fig. 2C/E), although levels of IL-1β were similar in both exosomes (Fig. 2D). To elucidate whether LPS exosomes are pro-inflammatory, we treated fresh RAW264.7 macrophages with 20 μg of respective exosomes for 24 h, and then performed cytokine analysis in the culture supernatant. To eliminate the possibility that the pro-inflammatory cytokines in the culture supernatant after 24 h might be attributed to the initial cytokine content of exosomes, culture supernatant was also collected 10 min after treatment with both sets of exosomes. We observed that, while TNF-α and IL-6 levels were significantly higher even after 10 min incubation of LPS exosomes compared to non-LPS exosomes, they were much higher (0.37-fold increase in TNF-α and 10-fold increase in IL-6 levels) in the supernatants of macrophages incubated with LPS exosomes after 24 h compared to levels after 10 min (Fig. 2F/G). Of note, IL-1β levels were too low to detect in these sets of experiments, and non-LPS exosomes also induced a smaller increase in TNF-α (but not IL-6) after 24 h incubation compared to levels after 10 min incubation (Fig. 2F). Put together, these results affirm that exosomes derived from LPS-treated macrophages are pro-inflammatory.

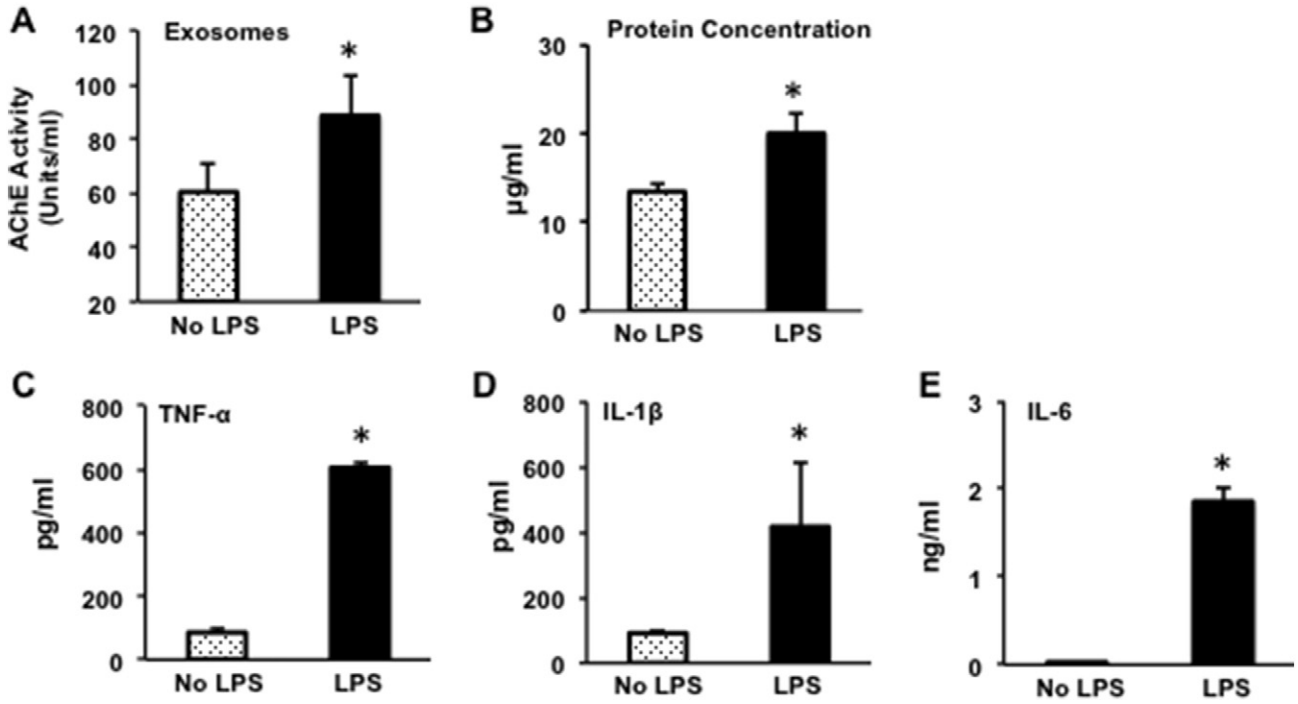

Fig. 1. LPS treatment enhances the release of exosomes and pro-inflammatory cytokines from RAW264 macrophages. (A) The amount of exosomes was significantly increased in macrophages (RAW264.7 cells) upon LPS stimulation, evidenced by the increase in acetylcholinesterase (AChE) activity. (B) Protein concentration in exosomes derived from LPS stimulated macrophages was significantly greater than exosomes derived from non-LPS treated macrophages. (C–E) LPS stimulation significantly elevated levels of inflammatory cytokines TNF-α (C), IL-1β (D) and IL-6 (E) in RAW264.7 macrophages. n = 3; *, $P < 0.05$ *vs.* non-LPS.

3.3. GW4869 inhibits both exosome release and pro-inflammatory cytokine production in macrophages

Next, we sought to determine if blockade of exosome generation would diminish the LPS-mediated pro-inflammatory responses in RAW264.7 macrophages. GW4869 has been successfully used to block the secretion of exosomes from HEK293 cells, cardiomyocytes, liver cells and lung epithelial cells [29–32]. In this study, we treated RAW264.7 macrophages with two different doses of GW4869 (10 μM and 20 μM). We observed that LPS-triggered exosome generation was remarkably attenuated in macrophages upon pre-treatment of macrophages with 10 μM GW4869, as evidenced by a 22% reduction in the activity of AChE (Fig. 3A). Such attenuation was further enhanced by treatment with 20 μM GW4869 (Fig. 3A). To test the possible toxicity of GW4869, we measured the levels of lactase dehydrogenase (LDH), a marker of cell injury, in the supernatants of macrophages upon exposure to 10 μM and 20 μM GW4869, as well as 0.005% DMSO (solution used to dissolve GW4869) for 24 h. Our results showed that LDH levels were similar in 0.005% DMSO, 10 μM GW4869, 20 μM GW4869 and non-treated groups (Fig. 3B). This suggests that 0.005% DMSO and GW4869 do not have toxic effects on macrophages. Assessment of pro-inflammatory cytokines revealed a significant decrease in the levels of TNF-α, IL-1β and IL-6 by 16.4%, 62%, and 15.6% respectively, in the supernatants of GW4869-treated macrophages upon LPS challenge, compared to control macrophages treated with LPS only (Fig. 3C–E). Collectively, these data indicate that GW4869 suppresses the endotoxin-triggered generation of exosomes in RAW264.7 macrophages and subsequently, decreases the production of pro-inflammatory cytokines.

3.4. Inhibition of exosome release blocks LPS-stimulated pro-inflammatory cytokine production and cardiac inflammation in mice

To determine the *in vivo* effects of GW4869, WT mice were injected *i.p.* with either PBS or GW4869 (2.5 μg/g) for 1 h, followed by LPS injection at one dose of 25 μg/g. 12 h later, levels of serum exosomes and pro-inflammatory cytokines, as well as myocardial inflammation were assessed. We noticed that, under basal conditions, GW4869 treatment significantly decreased exosome levels by 37% in sera, compared to levels collected from control mice (Fig. 4A). At 12 h after LPS injection, the levels of circulating exosomes were increased significantly compared to PBS-controls, as evidenced by a 1.7-fold elevation in the AChE activity (Fig. 4A). However, such LPS-induced elevation of serum exosomes was significantly inhibited in mice subjected to pre-treatment with GW4869, compared to the PBS + LPS group (Fig. 4A). Accordingly, pre-treatment with GW4869 resulted in a significant decrease in endotoxin-triggered production of pro-inflammatory cytokines (TNF- α, IL-1β and IL-6) in the serum, as evidenced by a 17% reduction of TNF-α (Fig. 4B), 11% decrease of IL-1β (Fig. 4C), and 28% reduction of IL-6 (Fig. 4D), compared to the PBS + LPS group. Also, we observed that MPO activity was significantly reduced by 12% in the hearts of mice pre-treated with GW4869 upon LPS challenge, compared to mice pre-treated with PBS. This indicates that LPS-caused infiltration of neutrophils into the myocardium is suppressed by GW4869 (Fig. 4E). Taken together, our results suggest that pre-treatment of WT mice with GW4869 could attenuate LPS-triggered production of exosomes and pro-inflammatory cytokines in the blood which subsequently reduces myocardial inflammation.

3.5. GW4869 mitigates LPS-caused myocardial dysfunction and improves survival in mice

It is well recognized that the production of circulating pro-inflammatory cytokines mediates myocardial dysfunction during septic shock [5–8]. Given the inhibitory effects of GW4869 on production of pro-inflammatory cytokines and cardiac inflammation, we next evaluated the effects of GW4869 on endotoxin-induced cardiac dysfunction. WT mice were treated with either PBS or GW4869 (2.5 μg/g) for 1 h, followed by LPS injection at one does of 25 μg/g. Cardiac function was assessed by echocardiography. Consistent with previous observations,

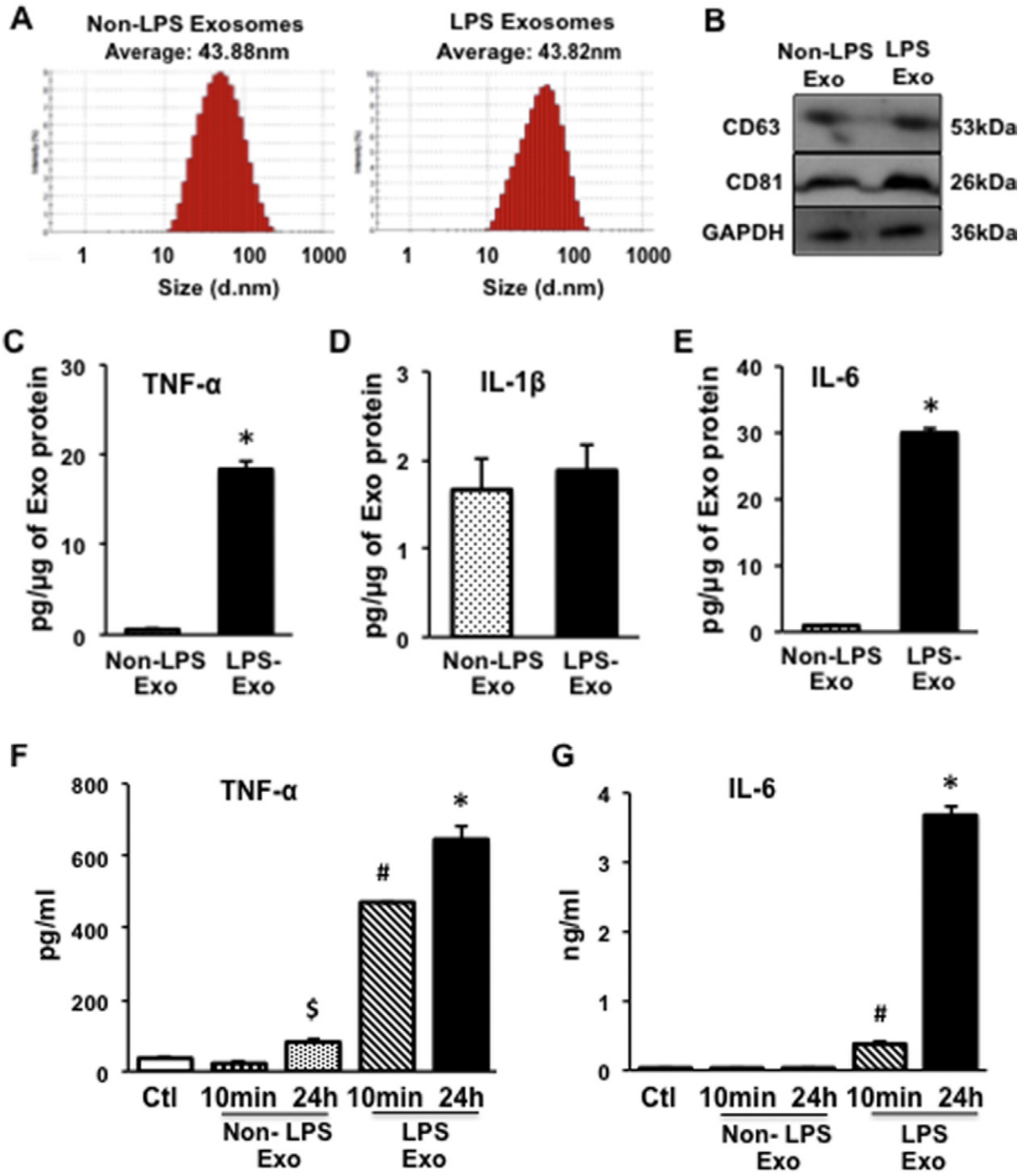

Fig. 2. Effects of exosomes derived from LPS-treated macrophages on naïve RAW264.7 macrophages. Exosomes were isolated from the culture supernatants of RAW264.7 cells in the presence or absence of LPS, using differential centrifugation. (A) The size of exosomes was measured by dynamic light scattering, using a particle and molecular size analyzer (Zetasizer Nano ZS, Malvern Instruments). (B) Exosomal markers (CD63 and CD81) were expressed in both non-LPS exosomes and LPS exosomes. Levels of TNF-α (C) and IL-6 (E) in LPS exosomes were significantly higher than levels in non-LPS exosomes while levels of IL-1β (D) were similar in both exosomes. n = 4; *, $P < 0.05$ *vs.* non-LPS exosomes. (F/G) LPS exosomes stimulated significantly higher levels of TNF-α (F) and IL-6 (G) after 24 h than 10 min incubation. Notably, non-LPS exosomes also stimulated a small increase in TNF-α levels after 24 h compared to 10 min incubation. n = 4; $, $P < 0.05$ *vs.* 10 min (non-LPS Exo); #, $P < 0.05$ *vs.* Ctl; *, $P < 0.05$ *vs.* 10 min (LPS Exo).

cardiac function was depressed in our endotoxemic mice compared to controls (Fig. 5A–C). However, pre-treatment of mice with GW4869 evoked an improvement of cardiac function, evidenced by a significant increase in left ventricular ejection fraction (EF%) and fractional shortening (FS%), compared to LPS-injected mice pre-treated with PBS controls (Fig. 5A–C, and Supplemental Table 1). At 36 h post-LPS injection, only 16.7% of mice pre-treated with PBS (n = 12) survived whereas 66.7% of mice pre-treated with GW4869 (n = 9) survived (Fig. 5D). Collectively, our data indicate that blockade of exosome generation with GW4869 diminishes the endotoxin-caused myocardial dysfunction and decreases mortality in mice.

3.6. GW4869 blocks the production of pro-inflammatory cytokines and cardiac inflammation in CLP mice

We next examined whether the GW4869-mediated protective effects can be replicated in a clinical-relevant septic model, induced by

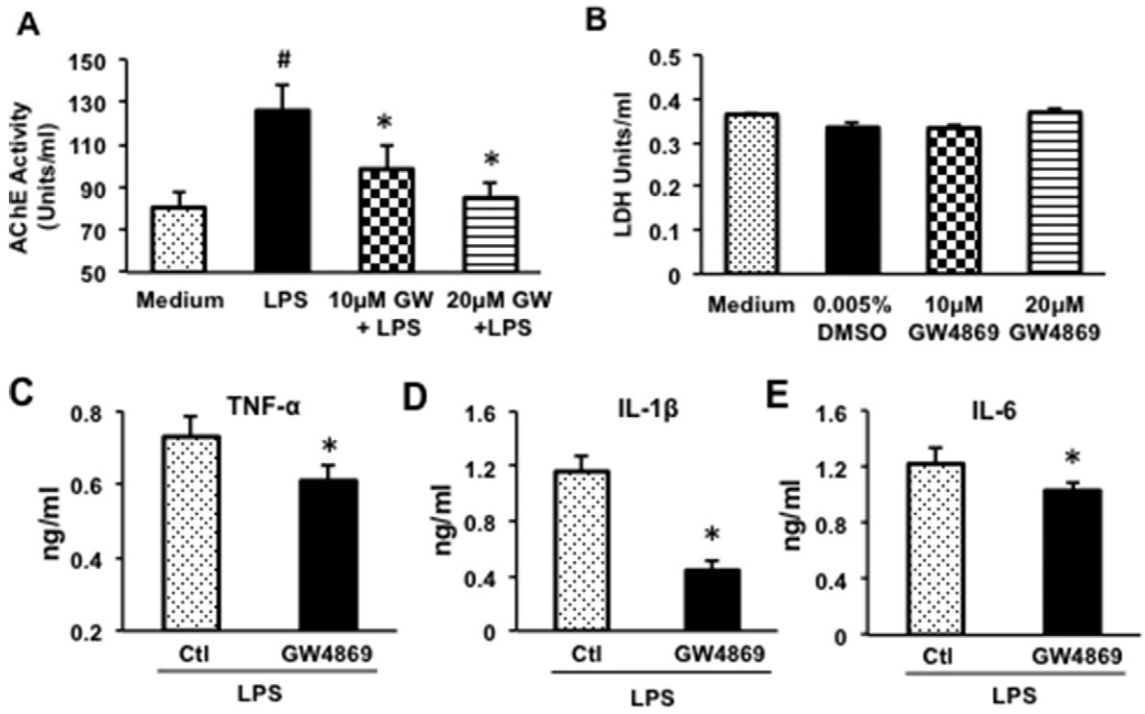

Fig. 3. Effects of GW4869 on exosome release and pro-inflammation cytokine production in LPS-treated RAW264.7 macrophages. (A) LPS-stimulated exosome generation was significantly inhibited by addition of 10 μM and 20 μM GW4869 to cultured RAW264.7 cells. n = 3; #, $P < 0.05$ *vs.* Medium; *, $P < 0.05$ *vs.* LPS. (B) 0.005% DMSO, 10 μM and 20 μM GW4869 had no cytotoxic effects on RAW 264.7 cells, shown by similar levels of lactase dehydrogenase (LDH) in all treatments compared to non-treated group. (C–E) Pre-treatment with GW4869 significantly reduced levels of (C) TNF-α, (D) IL-1β and (E) IL-6 from LPS-treated macrophages. Control macrophages were pre-treated with 0.005% DMSO. n = 3 wells; *, $P < 0.05$. Similar results were observed from three independent experiments.

cecal ligation and puncture (CLP). WT mice were injected with PBS or GW4869 (2.5 μg/g), followed by CLP or sham surgery. The levels of exosomes and pro-inflammatory cytokines in the serum, as well as myocardial inflammation, were determined at 12 h after the surgery. In sham-surgery controls, pre-treatment with GW4869 decreased exosome concentration by 33% compared to mice injected with PBS (Fig. 6A). Similar to endotoxin challenge, CLP surgery caused a significant elevation in the levels of serum exosomes, as measured by AChE activity (Fig. 6A). Importantly, such CLP-stimulated exosome release was significantly inhibited by pre-treatment of CLP mice with GW4869, compared to CLP mice pre-treated with PBS (Fig. 6A). The results of pro-inflammatory cytokine analysis in the serum revealed a significant decrease in the levels of TNF-α by 85% (Fig. 6B) and IL-6 by 46% (Fig. 6C) in CLP-mice pre-treated

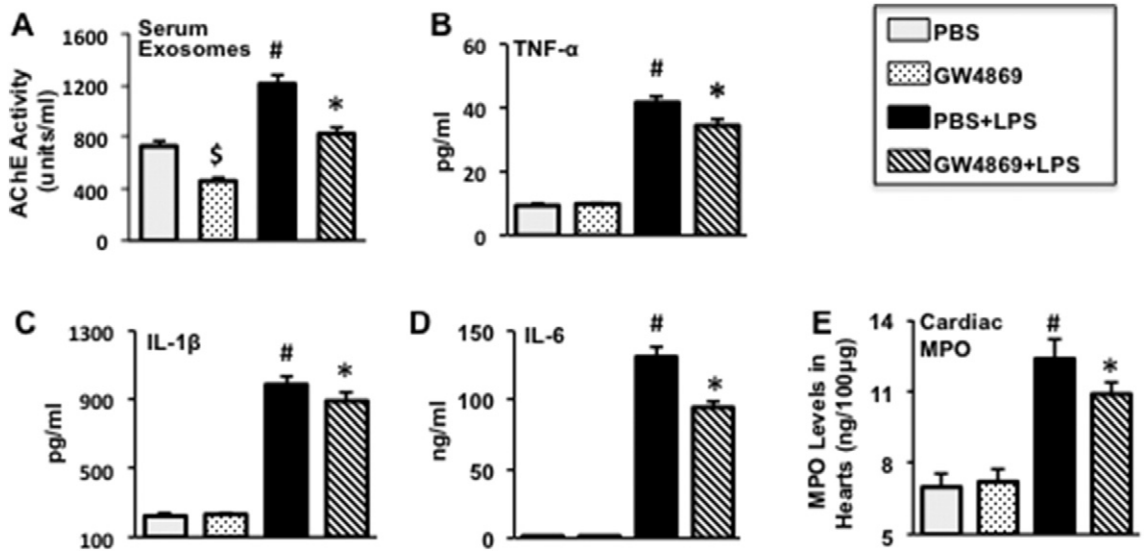

Fig. 4. Effects of GW4869 pre-treatment on exosome release, pro-inflammatory cytokine production in the serum and myocardial inflammation in LPS-treated WT mice. (A) Pre-treatment with GW4869 (2.5 μg/g) significantly inhibited serum exosome concentration in PBS-treated mice and LPS-treated mice. Accordingly, LPS-stimulated inflammatory response and cardiac inflammation were dampened by blockade of exosome release, as evidenced by reduced serum levels of TNF-α (B), IL-1β (C), IL-6 (D) and cardiac MPO activity (E). n = 5 for ELISA and MPO assays; $, $P < 0.05$ *vs.* PBS; #, $P < 0.05$ *vs.* PBS; *, $P < 0.05$ *vs.* PBS + LPS.

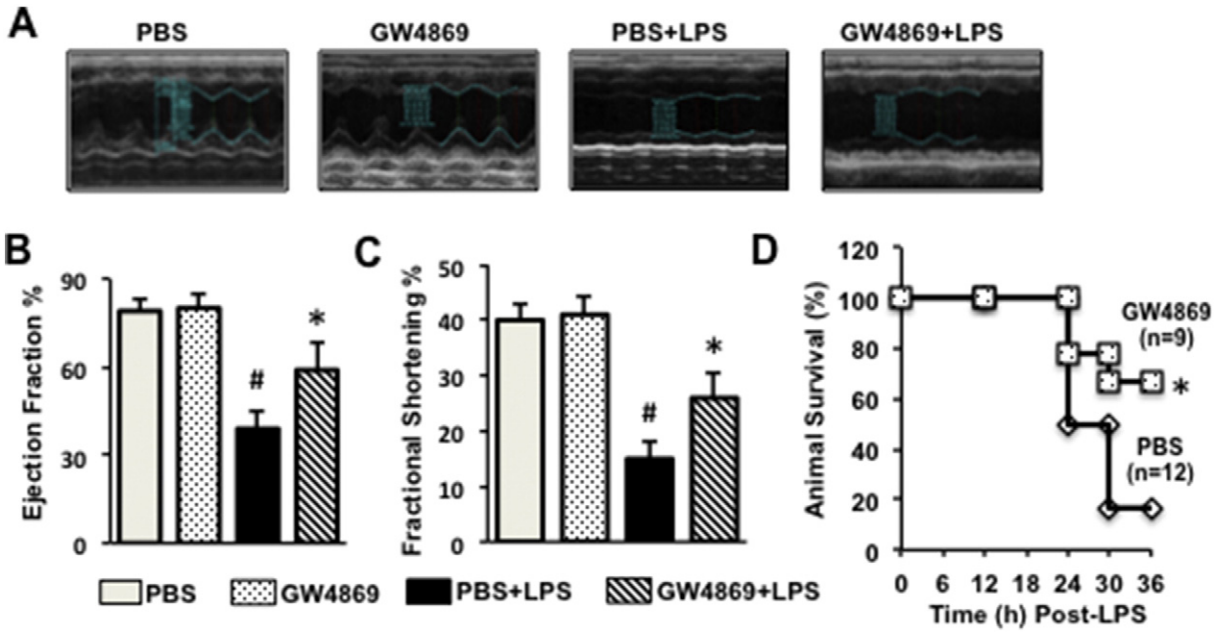

Fig. 5. Effects of GW4869 pre-treatment on endotoxin-induced cardiac dysfunction and survival. (A) Representative images of echocardiography showed that LPS-triggered cardiac dysfunction was attenuated in GW4869-pre-treated mice, as measured by left ventricular ejection fraction (EF%) (B), and fractional shortening (FS%) (C). n = 6–11 for echocardiography; #, $P < 0.05$ *vs.* PBS; *, $P < 0.05$ *vs.* PBS + LPS. (D) Pre-treatment of mice with GW4869 significantly improved animal survival after LPS injection, compared with PBS-pre-treated mice; *, $P < 0.05$, n = 9–12.

with GW4869, compared to the PBS + CLP group. CLP-induced cardiac inflammation, assessed by MPO activity, was greatly alleviated in the GW4869 + CLP group, compared to the PBS + CLP samples (Fig. 6D). Hence, our data suggest that GW4869 could inhibit the CLP-triggered production of pro-inflammatory cytokines in serum and myocardial inflammation.

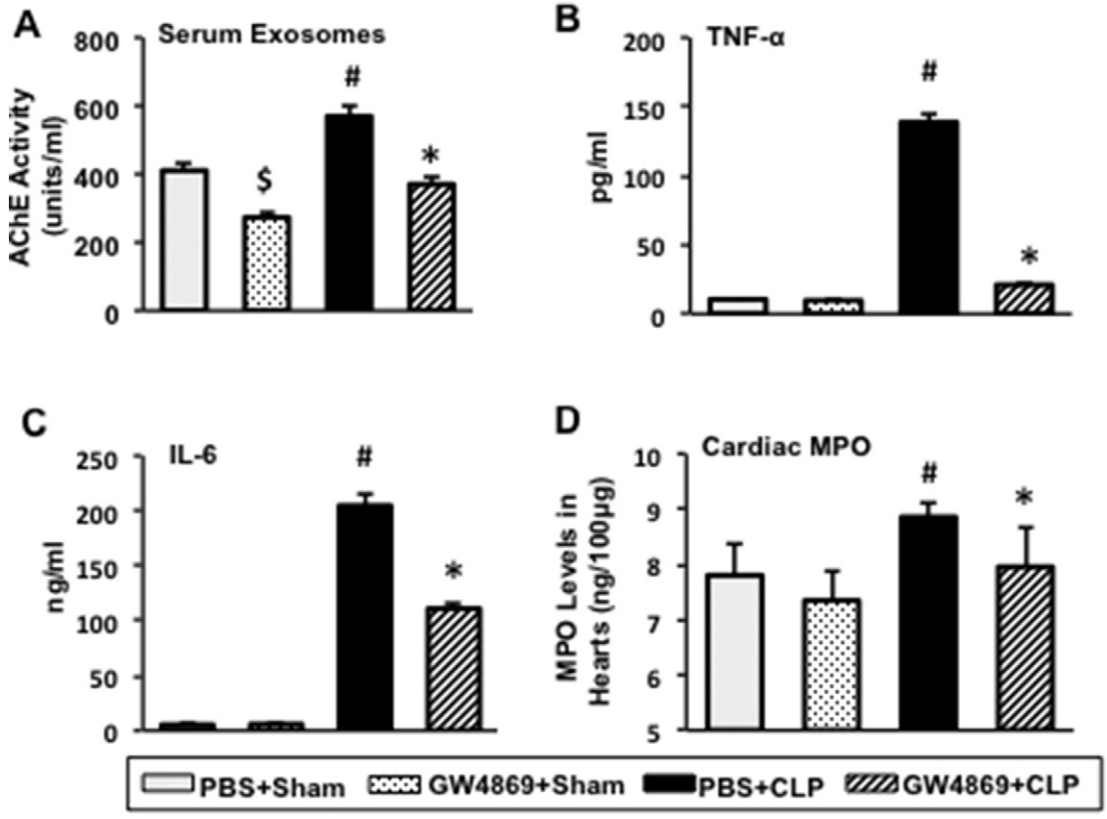

Fig. 6. Effects of GW4869 pre-treatment on exosome release, pro-inflammatory cytokine production in the serum and cardiac inflammation in CLP mice.(A) Pre-treatment with GW4869 (2.5 μg/g) significantly inhibited serum exosome concentration in sham- and CLP-mice. Accordingly, CLP-induced inflammatory response and cardiac inflammation were dampened by blockade of exosome release, as evidenced by reduced serum levels of TNF-α (B) and IL-6 (C) and myocardial MPO activity (D). n = 5; $, $P < 0.05$ *vs.* PBS + Sham; #, $P < 0.05$ *vs.* PBS + Sham; *, $P < 0.05$ *vs.* PBS + CLP.

3.7. Inhibition of exosome release blocks CLP-induced cardiac dysfunction and improves survival

To determine the effects of GW4869-mediated blockade of exosome generation on myocardial dysfunction in CLP mice, we pre-treated WT mice with either PBS or GW4869 prior to CLP surgery, with sham operation used as controls. At 12 h post-CLP surgery, myocardial function was depressed, as evidenced by a remarkable reduction of left ventricular ejection fraction (EF%) and fractional shortening (FS%) in PBS + CLP mice, compared to sham controls (Fig. 7A–C and Supplemental Table 2). However, such CLP-caused myocardial depression was attenuated by GW4869 pre-treatment (Fig. 7A–C and Supplemental Table 2). Accordingly, pre-treatment with GW4869 had a beneficial impact on mortality of the mice that underwent CLP surgery. At 48 h post-CLP surgery, only 6.25% of mice pre-treated with PBS (n = 16) survived, whereas 66.7% of mice pre-treated with GW4869 (n = 12) survived (Fig. 7D). Taken together, these data indicate that GW4869-mediated blockade of exosome generation alleviates the CLP-induced myocardial dysfunction and improves survival.

4. Discussion

The present study was conceptualized to determine the effects of blocking exosome release on the production of pro-inflammatory cytokines and sepsis-induced myocardial dysfunction. We demonstrated that GW4869 could effectively inhibit the generation of exosomes in macrophages and *in vivo*. Importantly, we discovered that GW4869 suppressed the production of pro-inflammatory cytokines in LPS-treated RAW264.7 macrophages *in vitro* and sepsis-caused inflammatory response *in vivo*. As a result, sepsis-induced cardiac dysfunction and mortality were attenuated by GW4869 pre-treatment. Therefore, our study presented here may provide a novel approach to the treatment of sepsis.

Sepsis is characterized by a severe cytokine storm where high amounts of pro-inflammatory cytokines can be detected in circulation [13]. It is well accepted that sepsis-caused multiple organ failure is attributed to the prolonged cytokine storm [1,2]. Especially, these circulating pro-inflammatory cytokines could result in the development of myocardial dysfunction, which contributes to about 250,000 deaths annually [36,37]. However, clinical trials using anti-inflammatory agents (*i.e.*, TNF inhibitors) have largely been unsuccessful in improving the survival rate of sepsis patients [2]. Hence, there might be other factors contributing to the exaggeration of the cytokine storm. Indeed, we observed in this study that there was a remarkable increase in circulating exosomes besides the elevation of pro-inflammatory cytokine levels. Our data also showed that exosomes derived from LPS-treated macrophages are pro-inflammatory and could stimulate naïve macrophages to release much more inflammatory cytokines, leading to a positive feed-back mechanism and exaggerated inflammatory response. Therefore, septic exosomes may be culpable for enabling the cytokine storm during severe sepsis.

Recently, exosomes have been receiving enormous attention due to their involvement in the progression of pathological/physiological conditions such as inflammation, tumorigenesis, angiogenesis and coagulation [18,30,38–40]. In the sepsis setting, high amounts of exosomes have been reported in the plasma of septic patients compared to healthy individuals [20]. Importantly, exosomes derived from platelets of septic patients caused depression of rat cardiomyocyte contractility [20]. Nevertheless, it remains unclear whether blocking the generation of exosomes could offer protection against sepsis injury. Considering that macrophages are a major source of pro-inflammatory cytokines and play a critical role in the pathogenesis of sepsis, we thus selected macrophages as the model cell to investigate the possible effects of reduced exosome secretion on cytokine production. In this study, we employed GW4869 as an inhibitor of exosome generation, since it has been utilized to prevent the production of exosomes in a variety of cells [29–32]. Consistently, we observed that GW4869 could effectively suppress the secretion of exosomes from LPS-challenged macrophages, which was accompanied by reduction of pro-inflammatory cytokines. As such, we propose an autocrine signaling of macrophage-derived exosomes on neighboring macrophages, which magnifies the amount of pro-inflammatory cytokines produced by the initial bacterial LPS stimulation (Fig. 8). Similarly, pre-treatment of endotoxemic and polymicrobial septic mice with GW4869 caused a reduction in the levels of circulating exosomes and pro-inflammatory cytokines. As a consequence, sepsis-induced cardiomyopathy was mitigated and animal survival was improved in GW4869-pre-treated mice. Therefore, our data may provide the first evidence that blockade of exosome generation

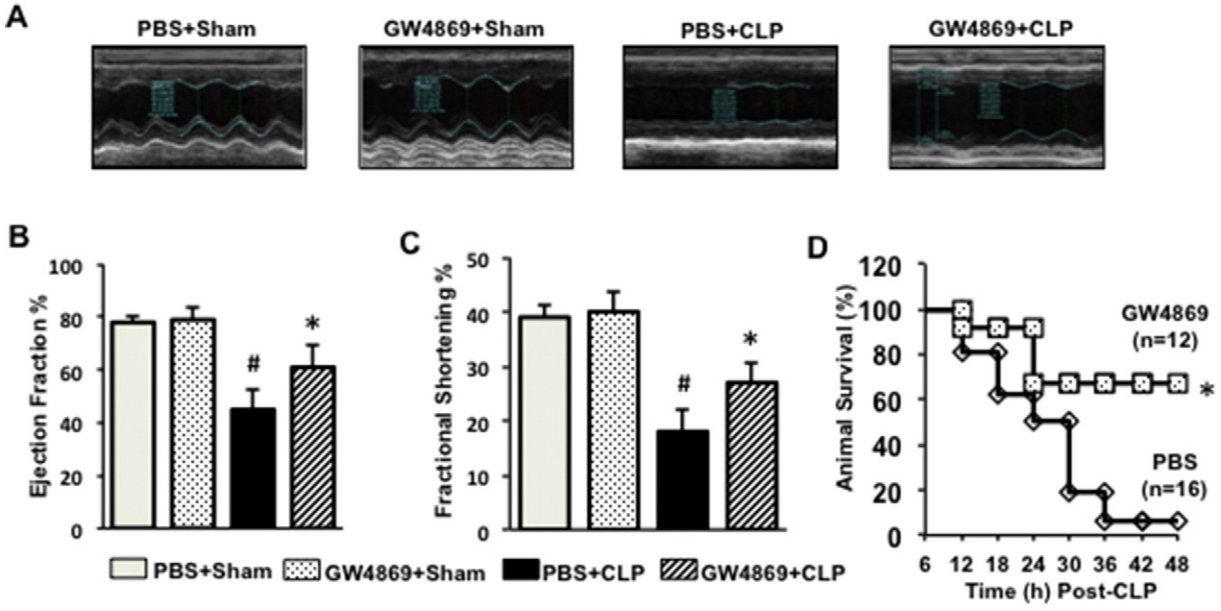

Fig. 7. Effects of GW4869 pre-treatment on CLP-induced cardiac dysfunction and survival. (A) CLP-induced cardiac dysfunction was attenuated in GW4869-treated mice, as evidenced by a significant improvement of LVEF% (B) and LVFS% (C), compared to PBS-treated controls; n = 6–11 for echocardiography; #, $P < 0.05$ *vs.* PBS + Sham; *, $P < 0.05$ *vs.* PBS + CLP. (D) Pre-treatment of mice with GW4869 significantly improved animal survival after CLP surgery, compared with PBS-pre-treated mice; *, $P < 0.05$, n = 12–16.

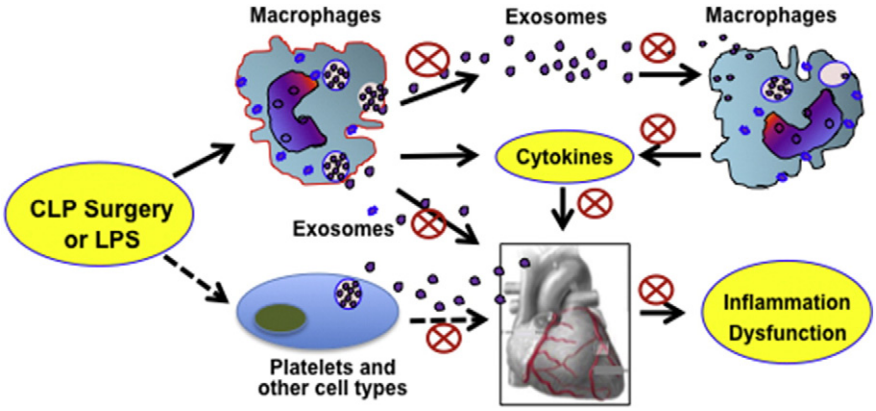

Fig. 8. Proposed scheme depicting the role of exosomes in sepsis. CLP surgery or bacterial LPS infection can induce the release of exosomes and pro-inflammatory cytokines from macrophages and other cell types. The macrophage-derived exosomes act as autocrine and paracrine signal mediators by inducing further synthesis of pro-inflammatory cytokines in the original macrophages and neighboring macrophages. Such over-production of inflammatory cytokines may partially impair cardiac function. Exosomes derived from macrophages and other cell types (*i.e.*, platelets) may also directly depress myocardial contraction during sepsis. All these communication lines could be cut off by GW4869-mediated blockade of exosome generation, leading to attenuation of sepsis-induced cardiac inflammation and dysfunction.

could inhibit inflammatory response, improve cardiac function and prolong animal survival in sepsis.

There is growing evidence to support exosomes as vehicles to release cell-associated cytokines [41–43]. In the present study, LPS exosomes possessed higher quantities of TNF-α and IL-6 compared to control exosomes. Notwithstanding, LPS exosomes appeared to trigger further production of pro-inflammatory cytokines in fresh macrophages (Fig. 2F/G). Mechanistically, the higher pro-inflammatory cytokine content of LPS exosomes may act as signaling molecules to evoke production of more cytokines in neighboring macrophages, or stimulate the transcription of inflammatory mediators [44]. In addition, LPS exosomes has been shown to stimulate a pro-inflammatory response through TLR/MyD88-dependent manner [27]. Of course, LPS exosomes may incorporate a specific set of pro-inflammatory miRNAs and proteins, although such analysis may fall outside the scope and intent of this report. Nonetheless, all these observations consistently suggest that exosomes derived from LPS-treated cells are pro-inflammatory. Another fascinating observation from our study was the ability of non-LPS exosomes to induce a significant increase in TNF-α levels after 24 hour incubation (Fig. 2F/G). Regardless, the amount of TNF-α produced by the non-LPS exosome-treated macrophages was minuscule compared to the effects of LPS exosomes (Fig. 2F/G). This observation suggests that macrophages may maintain their basic inflammatory property through exosomes.

It is worth mentioning that, although GW4869 caused a modest reduction of circulating pro-inflammatory cytokines and cardiac inflammation, there was a remarkable rescue of cardiac function in both endotoxemic and CLP septic models. This indicates that there are additional effects other than reduction of inflammatory response contributing to GW4869-induced benefits *in vivo*. Indeed, other cardiac depressors (miRNAs and proteins) carried with exosomes might have been suppressed by GW4869. Furthermore, *in vivo* global treatment of mice with GW4869 may not only inhibit exosome release from macrophages, but also from neutrophils, platelets, endothelial cells and other cells (Fig. 8). As a matter of fact, it has been observed that platelet-derived exosomes from septic patients directly impaired cardiac contractile function in animal models *via* the transfer of exosomal contents (NADPH, NOS, PDI, ICAM-1 and others) to cardiomyocytes [18–23]. Therefore, the beneficial effects of GW4869 on cardiac function, as presented in this study, may be ascribed to not only the reduction of pro-inflammatory exosomes, but also the reduction of cardiac-depressing exosomes. Additionally, effects of GW4869 beyond blockade of exosomes generation cannot be excluded. Finally, hemodynamic changes play a major role in sepsis pathophysiology, and thus may impact cardiac performance, and eventually cardiac function parameters, ejection fraction and fractional shortening. As such, it can be difficult to ascertain whether sepsis-induced cardiac dysfunction is a consequence of systemic hemodynamic changes or intrinsic load-independent myocardial depression. Nevertheless, septic mice pre-treated with GW4869 had higher preload and stroke volumes (Supplemental Tables 1 and 2), suggesting the beneficial effects of GW4869 on both load-independent cardiac contractility and systemic hemodynamic changes, possibly due to reduction of both systemic and cardiac inflammation.

In conclusion, we demonstrate in this study that macrophage-derived exosomes are pro-inflammatory which may partially contribute to myocardial inflammation and depression during septic shock. Preventing the generation of these 'harmful exosomes' has the capability to dampen systemic inflammation, attenuate sepsis-induced cardiac dysfunction and improve survival (Fig. 8). Our study suggests that interrupting such cell-to-cell communication by either blockade of exosome generation or with engineered/artificial exosomes could be valuable therapeutic strategies in patients with septic shock.

Transparency document

The Transparency document associated with this article can be found, in the version.

Funding sources

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.08.010.

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