Exosomal PGE2 from M2 macrophage inhibits neutrophil recruitment and NET formation through lipid mediator class switching in sepsis

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Article

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**Abstract**

Excess polymorphonuclear neutrophil (PMN) recruitment or overzealous neutrophil extracellular trap (NET) formation could lead to the development of multiple organ dysfunction during sepsis. M2 macrophage-derived exosomes (M2-Exo) have exhibited anti-inflammatory activities in some inflammatory diseases to mediate organ functional protection. However, whether M2-Exo could modulate PMN abnormal behaviors to prevent potentially deleterious inflammatory effects during sepsis is poorly understood. Here, we report that M2-Exo inhibited PMN migration and NET formation, alleviated lung injury and reduced the mortality without significantly affecting bacterial load in the lungs in cecal ligation and puncture (CLP)-induced sepsis model. In vitro co-culture experiments using PMNs isolated from both healthy volunteers and septic patients further demonstrated that M2-Exo significantly decreased PMN migration and NET formation capacity, leading to lipid mediator class switching from pro-inflammatory leukotriene B4 (LTB4) to anti-inflammatory lipoxin A4 (LXA4) through upregulating 15-lipoxygenase (15-LO) expression in PMNs. Treatment with LXA4 receptor inhibitor attenuated the function of M2-Exo on PMNs and lung injury. Mechanistically, LXA4 increased by M2-Exo inhibited PMN function through downregulating chemokine (C-X-C motif) receptor 2 (CXCR2) and reactive oxygen species (ROS) expressions in PMNs. In addition, prostaglandin E2 (PGE2) was highly expressed in M2-Exo. By deleting PGE2 in M2-Exo, we found that exosomal PGE2 from M2 macrophage was necessary for 15-LO upregulation and PMN inhibition through functioning on EP4 receptor. Our findings reveal a previously unknown role of M2-Exo in regulating PMN migration and NET formation, thus highlighting the potential application of M2-Exo in controlling PMN-mediated tissue injury in patients with sepsis.

**Introduction**

Sepsis is a prevalent disease worldwide and one of the leading causes of hospital death, characterized by infection-triggered immune hyperactivation and cytokine storms, which cause tissue damage and eventually lead to multiple organ dysfunction syndrome (MODS)\(^1\). Acute respiratory distress syndrome (ARDS) is the most common severe manifestation of MODS and an important factor contributing to the morbidity and mortality of sepsis\(^2\).

Polymorphonuclear neutrophils (PMNs), the most abundant leukocytes in mammals, reach the inflammatory site in a cascade-like manner, where they activate specific effector functions such as release of reactive oxygen species (ROS), degranulation, formation of neutrophil extracellular traps (NETs), and phagocytosis\(^3,4\). After fulfilling the appropriate effector functions, dampening PMN activation and infiltration is crucial to prevent damage to the host\(^5\). However, the aberrant recruitment or activation of PMNs is one of the hallmarks of ARDS\(^5\). Therefore, modulation of PMN recruitment and function during sepsis to justify between the beneficial antimicrobial function and the potentially deleterious inflammatory effect has gained increasing interest over the years.

Macrophages are also known as key mediators in determining the outcome of inflammatory responses. Macrophages are divided into two phenotypically distinct populations: a pro-inflammatory or an anti-
inflammation/pro-resolving phenotype, named M1 and M2 macrophages respectively. In general, M2 macrophages appear at later stages of infection to control and resolve inflammation and repair tissues\(^7\). The crosstalk between PMNs and macrophages in regulating inflammation during sepsis-related ARDS has been documented\(^8\). M2 macrophages function to clear PMNs accumulated in the inflammatory sites and NETs could be removed by macrophages via phagocytosis\(^9,10\).

Exosomes, an important form of extracellular vesicles, have been demonstrated as the intercellular communication mediators in various physical processes\(^11\). Recently, M2 macrophage-derived exosomes (M2-Exo) have been revealed to play an immunoprotective role in inflammatory disease and mediating organ functional protection\(^12,13\). We and others have also demonstrated that exosomes are novel mediators between PMNs and macrophages during sepsis\(^8,14\). However, the roles of M2-Exo macrophage-derived exosomes on PMN recruitment and function during sepsis remain unclear.

In addition, previous studies have demonstrated that prostaglandin E2 (PGE2) produced by local macrophages switches lipid mediator biosynthesis from predominantly proinflammatory leukotriene B4 [LTB4; 5-lipoxygenase (5-LO)-initiated pathway] to anti-inflammatory lipoxin A4 (LXA4), a 15-LO product in PMNs\(^15,16\). LTB4 initiates and amplifies PMN chemotaxis and NET formation, and these effects can be reversed by LXA4, which is considered as an endogenous “stop signal” in inflammation\(^17,18\). Switching from LTB4 to LXA4 production marks the resolution phase\(^19\). However, whether intrinsic lipid mediator class switching controls neutrophil phenotype alteration in sepsis needs to be further addressed.

In this study, we firstly identified that M2-Exo could alleviate lung injury, and reduce mortality by inhibiting PMN migration and NET formation during sepsis. Mechanistic study revealed that M2-Exo-PGE2 switched lipid mediator biosynthesis from LTB4 to LXA4 in PMNs through functioning on its receptor EP4. These findings suggest a previously unidentified role of M2-Exo in sepsis, and may help us better understand the endogenous mechanisms for resolution of inflammation and lead to the development of novel therapeutic approaches.

**Materials And Methods**

**Patient samples and ethics statement**

EDTA-anticoagulated venous blood (20 mL) was collected from patients with early (less than 24 h) diagnosis of sepsis who were admitted to the ICU of Nanjing Drum Tower Hospital (Nanjing, China) between January to November 2022. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) were used to diagnose sepsis. Pregnant women and patients under 18 years of age and those with severe anemia, active bleeding, or chemotherapy were excluded. Blood samples from healthy volunteers were used as control. Then, plasma and neutrophils were isolated from healthy donors and septic patients. All plasma samples were aliquoted and stored at -80°C. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the institutional ethics
and review board of Nanjing Drum Tower Hospital (Approval No. 2022-257-01). Informed consent was obtained from the participating volunteers, patients or their representatives.

**Macrophage cell culture**

Human peripheral blood was obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll 1.077 density gradient (Solarbio, Beijing, China) as described previously. PBMCs were differentiated into macrophages by incubation with Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 20 ng/mL recombinant human macrophage-colony stimulating factor (rhM-CSF, PeproTech, Suzhou, China). Mature M0 macrophages were induced after 7-day culture. M2 macrophages were induced by adding 20 ng/mL IL-4 (PeproTech) for additional 48 h. To inhibit PGE2 expression, celecoxib (20 µM; #HY-14398, MCE corporation), a selective COX-2 inhibitor, was added into the culture medium.

In addition, we also polarized mouse Raw264.7 macrophages to M2-type macrophages by adding 20 ng/mL mouse IL-4 and IL-13 (PeproTech) to complete culture medium (DMEM containing 10% FBS, supplemented with 50 mg/ml penicillin/streptomycin) for 48 h. Then, RT-qPCR was performed to identify the M2 polarization of the macrophages. To inhibit PGE2 expression, celecoxib (20 µM) was also added into the culture medium.

**Exosome isolation and characterization**

After the induction of M0/M2 macrophages, the culture medium was renewed to RPMI 1640 containing 10% exosome-free FBS (#EXO-FBS-50A-1; System Biosciences, Palo Alto, CA, USA) for 24 h. Then, exosomes were isolated from the supernatant of M0 macrophages (M0-Exo) and M2 macrophages (M2-Exo) using ExoQuick-TC (#EXOTC10A-1; System Biosciences) according to the manuscript. The detailed methods used to determine exosomal morphology, size distribution and surface marker expression are described in supplementary Methods.

**PMN isolation and in vitro co-culture experiments**

PMNs were isolated from the venous blood of healthy volunteers or septic patients by polymorphprep™ isolation reagent (#1114742; Axis-Shield, Norway), according to the manufacturer’s instructions. Ex vivo neutrophil function analysis was performed on ICU admission samples using septic plasma. PMNs were activated upon adding 20% septic plasma (SP) sterilized through a 0.22-mm filter (Millipore) to the culture medium, and 20% plasma from healthy volunteers (HP) was used as negative control. After 1 h, PMNs were co-cultured with M0/M2-Exo (100 µg/mL) derived from PBMC-differentiated macrophages for 5 h at 37 °C. For migration capacity analysis of neutrophils from septic patients, PMNs were directly co-cultured with M0/M2-Exo (100 µg/mL) for 5 h after isolation.

In addition, part of PMNs were cultured with or without 10 µM BOC-2 (LXA4 receptor antagonist; #HY-P1795, MCE corporation), 1 µM PD146176 (15-LO inhibitor; #HY-103157, MCE corporation), 1 µM E7046
(EP4 receptor antagonist; #HY-103088, MCE corporation), 100 nM LXA4 (#90410, Cayman Chemical, Michigan, USA), and 100 nM PGE2 (#HY-101952, MCE corporation).

**Transwell assay**

After treatment with M0/M2-Exo for 5 h, neutrophils (2 x 10^5) were collected and plated in the upper insert (polycarbonate filter 5 µm) of the transwell kits (#3415, Corning, USA). The medium containing 20 ng/mL recombinant human IL-8 (Sino Biological, Beijing, China) was placed in the lower well as a chemotactic stimulus. After 2-h incubation, cells in the lower chamber were counted under a microscope.

**NET quantification assay**

NETs in the cell culture supernatant, plasma and mouse peritoneal lavage fluid (PLF) were quantified using the PicoGreen dsDNA Quantiﬁcation Kit (Invitrogen, Carlsbad, CA, USA) according to the instructions.

NET formation was also quantified by confocal microscopy. PMNs were allowed to settle on glass coverslips precoated with poly-L-lysine (#354085; Corning, NY, USA) for 30 min prior to be treated for a specific period of time. PMNs were incubated with 1 µM SYTOX Green reagent (#S7020; Invitrogen) at 37°C for 10 min. Nuclei were counterstained using DAPI, and the cells were mounted in Antifade Mounting Medium (#P0126; Beyotime Biotechnology, Shanghai, China) for imaging with the confocal microscope. For each slice, 5 random ﬁelds were captured and analyzed. NET-positive cells and NET area were quantified using ImageJ software v.1.3.7. Only structures depicting NET morphology and positive for SYTOX Green were selected for area quantiﬁcation and intact granulocyte nuclei were excluded from the analysis.

**Animals**

Wild type (WT) male C57BL/6J mice aged 6-8 weeks (Vital River Laboratories, Zhejiang, China) were fed under a speciﬁc pathogen-free environment in the Laboratory Animal Center of Nanjing Drum Tower Hospital. All animal experiments were conducted under the rules approved by the Ethics Committee of Nanjing Drum Tower Hospital (Approval No. 2021AE01055).

**Establishment of the mouse model of cecal ligation and puncture (CLP) and in vivo exosome administration**

The CLP mouse model was prepared as previously described. The mice were sedated with an intraperitoneal injection of pentobarbital (60 mg/kg). After disinfection, a 1 cm midline laparotomy was made in the abdomen. The cecum was then exteriorized, and ligated below the cecal valve, and punctured with an 18-gauge needle to induce sepsis. A small drop of cecal content was extruded. The cecum was then returned to the peritoneal cavity and the abdominal incision was closed with sutures. The animals were resuscitated with (5 ml/100 g) saline. Sham animals underwent the same surgical procedures without cecum ligation and puncture. To explore exosome function in vivo, mice were treated with
M0/M2-Exo (300 μg/mouse) derived from mouse Raw264.7 macrophages through intraperitoneally (i.p.) injection using 31-gauge insulin syringes 1 h after CLP surgery. After 24 h of CLP, blood, PLF and lung tissues were harvested as described previously. The detailed methods for histopathological evaluation, bacterial load quantification, neutrophils and NET formation quantification in the lung tissues are described in the supplementary Methods. The methods to quantify neutrophils in the peripheral blood are also described in the supplementary Methods.

Statistical analysis

Normally distributed data were tested using the Shapiro-Wilk test and presented as means ± standard deviations. Comparisons between two groups were performed by the 2-tailed Student’s t test. Multiple group comparisons were performed by one-way ANOVA followed by Tukey’s multiple comparisons test with GraphPad Prism 8 software. Comparison of survival rates between groups was performed using Log-rank test. A value of $P < 0.05$ was considered statistically significant.

Results

M2-Exo reduces lung injury and improves survival in septic mice

The characterization of M0/M2-Exo isolated from the supernatant of polarized mouse Raw264.7 macrophages were shown in Fig. 1A-C. To investigate the function of M2-Exo in the biology of sepsis-related ALI/ARDS, we set up a CLP-induced sepsis model that was clinically consistent with human septic peritonitis, and either M0-Exo or M2-Exo was administered i.p. 1 h after surgery (Fig. 1D). Ex vivo fluorescence imaging showed that Dil-labeled exosomes accumulated in the lung tissue 24 h after i.p. injection, and co-localized with Ly6G (neutrophil-specific marker) (Fig. 1E-F). The histopathological appearance of the lung tissue showed marked accumulation of neutrophils and alveolar septal thickening following CLP surgery, and the lung injury was attenuated after M2-Exo treatment (Fig. 1G). Consistent with the histological evaluation, M2-Exo i.p. injection significantly inhibited the expression of proinflammatory mediators (IL-6, IL-1β and TNF-α) in the lung tissue following CLP (Fig. 1H). Furthermore, compared with M0-Exo-treated group, M2-Exo treatment increased the survival of the animals significantly (Fig. 1I), while the bacterial load in the lungs was not significantly different between the two groups (Fig. 1J). All these data indicate that M2-Exo could alleviate lung injury and improve the survival rate of the septic mice in vivo.

M2-Exo inhibits PMN recruitment and NET formation during sepsis in vivo

The high lethality of sepsis is associated with dysregulation of the host inflammatory response, including the detrimental effect of aberrant activation of PMNs21,22. Therefore, we decided to test whether M2-Exo could inhibit the overactivation of PMNs in sepsis. It was found that M2-Exo i.p. injection significantly reduced the number of PMNs in peripheral blood (Fig. 2A-B) and lung tissue (Fig. 2C-D) following CLP, indicating impaired recruitment of PMNs occurred from bone marrow to the circulation and eventually to the organ. In addition, IF staining with anti-CitH3 and anti-MPO showed that NETs deposited in the CLP...
model but were reduced after treatment with M2-Exo (Fig. 2E). The amount of NET component (dsDNA) in plasma and PLF was significantly lower in M2-Exo-treated mice as compared with those in M0-Exo-treated controls (Fig. 2F-G). However, the inhibited PMN recruitment into the lung tissue could also result in less NET formation. Therefore, whether the capacity of NET formation is influenced by M2-Exo still needs to be further addressed.

**M2-Exo inhibits PMN recruitment and NET formation during sepsis in vitro**

To further determine whether M2-Exo could limit the chemotaxis and NET formation capacity of PMNs, we established an in vitro co-culture system. As illustrated in Fig. 3A, PMNs from healthy volunteers were activated by septic plasma, and then co-cultured with 100μg/mL M0/M2-Exo derived from PBMC-differentiated macrophages. The characterization of exosomes is shown in Supplementary Fig. 1. PMN migration towards IL-8 was decreased significantly after M2-Exo treatment compared with that in M0-treated group (Fig. 3B). In addition, the recruitment of PMNs isolated from septic patients towards IL-8 was also inhibited after M2-Exo treatment (Fig. 3C-D).

Subsequently, we observed typical NET structure formation in PMNs isolated from both healthy volunteers and septic patients after incubation with plasma from septic patients (SP), but not with plasma from healthy control (HP). Upon culturing PMNs with M2-Exo, we found that NET formation induced by septic plasma was significantly reduced compared with that in M0-Exo group (Fig. 3E-H). Besides, treatment with M2-Exo showed no effect on the apoptosis rate of PMNs (Supplementary Fig. 2). All these data suggest that M2-Exo possessed the ability of inhibiting PMN chemotaxis and NET production during sepsis.

**M2-Exo leads to lipid mediator class switching of PMNs during sepsis**

Next, we went further to investigate how M2-Exo inhibited the migratory and NET formation ability of PMNs. Emerging evidence indicate switching of eicosanoid biosynthesis from predominantly pro-inflammatory lipid mediator LTB4 to anti-inflammatory lipid LXA4 during acute exudate formation could “reprogram” the exudate neutrophils to promote resolution-a process termed “lipid mediator class switching”15. However, whether this process also played a role in inflammation resolution of sepsis remained unclear. ELISA assay of the present study showed that M2-Exo significantly increased the LXA4 concentration in the supernatant of PMNs isolated from both healthy volunteers and septic patients (Fig. 4A and 4C), while the LTB4 concentration was decreased after M2-Exo treatment (Fig. 4B and 4D). Besides, no significant difference of M0 or M2-Exo-carried LTB4 was found (Fig. 4E), as well as the LXA4 concentration in exosomes which was below the detection limit of the ELISA kit (Data not shown). All these results indicate that M2-Exo switched LTB4 to LXA4 production in PMNs during sepsis.

**M2-Exo inhibits PMN recruitment and NET formation through LXA4 upregulation**

LXA4 is an important endogenous lipid that mediates resolution of inflammation through functioning on the LXA4 receptor/formyl peptide receptor 2 (ALX/FPR2)23. To test whether upregulated LXA4 production
mediated the inhibitory function of M2-Exo on PMNs, we chose BOC-2 (a LXA4 receptor inhibitor) to abrogate the effect of LXA4. First, we performed an in vitro co-culture experiment and found that the migration and NET formation capacity of PMNs isolated from both healthy volunteers and septic patients inhibited by M2-Exo was reversed by BOC-2 treatment (Fig. 5A-C). To further investigate the M2-Exo’s LXA4-dependent action in vivo, we co-administered M2-Exo and BOC-2 via i.p. injection in the CLP model, and found that BOC-2 increased dsDNA concentration in plasma and PLF (Fig. 5D-E) and NET deposition in the lung (Fig. 5F) compared with M2-Exo group. We also observed a significant increase in PMN recruitment to peripheral blood (Fig. 5G-H) and lung tissue (Fig. 5I-J) when septic mice were treated with M2-Exo+BOC-2 compared with that in M2-Exo group. In addition, consistent with the increased PMN recruitment and NET formation in the lung, the protective effect of M2-Exo against morphological changes and proinflammatory mediator production in the lung tissue was abolished after BOC-2 treatment (Fig. 5K-L). These data demonstrate that M2-Exo-mediated reduction of CLP-induced PMN migration and NET formation was dependent on LXA4. However, it remained unclear how upregulated LXA4 altered the PMN behavior.

LXA4 increased by M2-Exo downregulates CXCR2 and ROS expression in PMNs

Chemokine (C-X-C motif) receptor 2 (CXCR2) signaling-induced and reactive oxygen species (ROS)-dependent NET formation has been demonstrated to be a therapeutic target in sepsis. Therefore, we postulated that upregulated LXA4 by M2-Exo could inhibit PMN activity through regulating CXCR2 and/or ROS expression. As expected, CXCR2 and ROS expression in PMNs isolated from both healthy volunteers and septic patients was downregulated by M2-Exo compared with M0-Exo, which was reversed by BOC-2 (Supplementary Fig. 3A-D). In addition, M2-Exo induced CXCR2 and ROS downregulation in peripheral blood neutrophils was abrogated by BOC-2 in the CLP model (Supplementary Fig. 3E-F), suggesting that the effect of LXA4 upregulated by M2-Exo might be mediated via downregulating CXCR2 and ROS expressions.

M2-Exo promotes LXA4 production in PMNs by increasing 15-LO expression

We next investigated how M2-Exo increased LXA4 production in PMNs. Previous studies have shown that arachidonate 15-lipoxygenase (15-LO) activity, in combination with arachidonate 5-lipoxygenase (5-LO), can produce lipoxins, and 5-LO activity alone forms leukotrienes. We found that M2-Exo increased 15-LO expression in PMNs isolated from both healthy volunteers and septic patients, while the expression of 5-LO remained unchanged significantly (Fig. 6A-B). Treatment with PD146176, a specific 15-LO inhibitor, significantly decreased the LXA4 concentration and increased the LTB4 concentration in the supernatant of M2-Exo-treated PMNs (Fig. 6C-D). PD146176 also increased migration and NET formation capacity of M2-Exo-treated PMNs, and addition of exogenous LXA4 following 15-LO inhibition abrogated the effect of PD146176 (Fig. 6E-G). Flow cytometry showed that the CXCR2 and ROS expressions in M2-Exo-treated PMNs were increased after PD146176 treatment, which was reversed by LXA4 addition (Fig. 6H-I). All these results indicate that M2-Exo promoted LXA4 production through increasing the 15-LO expression in PMNs.
Exosomal PGE2 from M2 macrophage is necessary for 15-LO upregulation and PMN inhibition

A recent study reported that PGE2 could promote resolution of inflammation by switching lipid mediator biosynthesis and guiding neutrophil phenotype alteration\textsuperscript{27}. We therefore sought to determine whether PGE2 could be transferred through M2-Exo and whether PGE2 in M2-Exo mediated its inhibitory effect on PMNs. As shown in Fig. 7A, PGE2 level in M2-Exo was significantly higher than that in M0-Exo. To delete PGE2 in M2-Exo, we blocked PGE2 production by celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor. It was found that the PGE2 level in exosomes from celecoxib-treated M2 macrophages (Cel-M2-Exo) was significantly lower than that in M2-Exo (Fig. 7A). As expected, 15-LO expression in Cel-M2-Exo-treated PMNs was lower than that in M2-Exo-treated PMNs, and addition of exogenous PGE2 with Cel-M2-Exo increased the 15-LO expression in PMNs (Fig. 7B), indicating that 15-LO upregulation in PMNs following M2-Exo treatment was dependent on PGE2. As well, Cel-M2-Exo showed impaired ability on lipid mediator class switching, while addition of PGE2 mimic the effects of M2-Exo on LXA4 and LTB4 alteration (Fig. 7C-D).

Additionally, the in vitro co-culture experiment showed that NET formation (Fig. 7E-F), neutrophil migration capacity (Fig. 7G), CXCR2 (Fig. 7H) and ROS expressions (Fig. 7I) of PMNs were all increased in Cel-M2-Exo group compared with M2-Exo group, while supplementary of PGE2 in the culture medium reversed the effects of Cel-M2-Exo on PMNs.

To further confirm the results obtained from the in vitro experiment, we administered M2-Exo or Cel-M2-Exo \textit{i.p.} 1 h following CLP, finding an increasement of NET formation (Fig. 7J-L), trafficking of neutrophils from bone marrow to the circulation (Fig. 7M-N) and lung tissues (Fig. 7O-P), and increased expression of CXCR2 (Fig. 7Q) and ROS (Fig. 7R) in peripheral blood neutrophils following Cel-M2-Exo administration compared with M2-Exo. In addition, the lung injury score (Fig. 7S), proinflammatory mediator production in the lung tissue (Fig. 7T) and the mortality rate (Fig. 7U) in Cel-M2-Exo group were all significantly higher than those in M2-Exo group.

Collectively, these data suggest that M2 macrophage secreted PGE2 through exosomes, increased 15-LO expression in PMNs to upregulate LXA4 production, and then modulated the neutrophil migratory ability and NET formation capacity.

Exosomal PGE2 from M2 macrophage functions on EP4 receptor of PMNs

PGE2 has proved to signal in vivo through EP4 receptor of PMNs and then increase 15-LO expression\textsuperscript{16}. To further testify whether exosomal PGE2 from M2 macrophage modulated the neutrophil phenotype through functioning on EP4 receptor, we used a specific antagonist (E7046) to block the binding of PGE2 to its receptor EP4. As shown in Supplementary Fig. 4A, the 15-LO expression was significantly lower in M2-Exo+E7046 group than that in M2-Exo group. The M2-Exo-induced alteration of LXA4 and LTB4 was blocked following E7046 treatment (Supplementary Fig. 4B-C). Treatment with E7046 also increased the NET formation (Supplementary Fig. 4D-E), the neutrophil migration capacity ((Supplementary Fig. 4F),
CXCR2 (Supplementary Fig. 4G) and ROS expressions (Supplementary Fig. 4H) of PMNs, while LXA4 addition abrogated the effect of E7046 on PMNs.

Discussion

In this study, we investigated the effect of exosomes secreted by M2 macrophage on PMNs in sepsis-induced acute lung injury (ALI) by using a CLP murine model and in vitro co-culture experiments. We demonstrated that M2-Exo could alleviate lung injury and reduce the mortality during sepsis. We further suggested the underlying mechanism was that exosomal PGE2 from M2 macrophage upregulated LXA4 production in PMNs by increasing 15-LO expression to modulate the capacity of PMN migration and NET formation possibly through downregulating PMN CXCR2 and ROS expressions (Fig. 8).

M2-Exo has been studied in different disease settings, such as spinal cord damage, myocardial ischemia-reperfusion, atherosclerosis, and inflammatory pain, all showing promising therapeutic effects. However, it is unknown whether M2-Exo could induce the anti-inflammatory activities in sepsis-associated tissue injury. Our study firstly showed that M2-Exo could clearly alleviate sepsis-induced lung injury and improve survival without significantly affecting bacterial load in the lung, suggesting M2-Exo had minimal impact on beneficial antimicrobial activities. Further in vitro co-culture experiments were conducted using plasma from septic patients to activate PMNs according to the previous studies, and we found an immunosuppressive effect of M2-Exo on PMNs from both healthy volunteers and septic patients ex vivo. Combined with our previous studies showing that alveolar macrophage-derived exosomes induced PMNs undergoing proinflammatory cell death, and PMN-derived exosomes promoted macrophage M1 polarization and pyroptosis during sepsis, we proposed the significant role of the cross-talk between macrophages and PMNs by exosomes in sepsis.

A growing body of evidence indicates that resolution of inflammation is an active process involving the production of lipid-derived specialized pro-resolving mediators (SPMs), which include lipoxins, resolvins, protectins, and maresins. SPMs actively stimulate cardinal signs of resolution, namely, cessation of leukocytic infiltration, counterregulation of proinflammatory mediators, and the uptake of apoptotic PMNs and cellular debris. The biosynthesis of these resolution-phase mediators in sensu stricto is initiated during lipid mediator class switching, in which the classic initiators of acute inflammation, prostaglandins and leukotrienes, switch to produce SPMs. Previous studies have proved that lipid mediator class switching played a critical role in determining neutrophil phenotype, altering neutrophil migratory behavior. Our data showed that M2-Exo promoted lipid mediator class switching from LTB4 to LXA4 in PMNs, and blocking LXA4 receptor ALX/FPR2 abrogated M2-Exo's effects on PMN inhibition, lung injury improvement. LTB4 is a product of the 5-LO pathway, known to strongly initiate and amplify PMN chemotaxis as well as release of granule products and superoxide anion. While LTB4-stimulated PMN functional responses are opposed by LXA4, a distinct class of 15-LO-derived eicosanoids, signals through ALX/FPR2 to limit neutrophil trafficking and lifespan and to promote efferocytosis, emerged as master regulator of neutrophil responses and fates. Therefore, the use of LXA4...
to treat sepsis has gained great attention, and consistent with our results, mounting evidence showed that LXA4 limited leukocyte recruitment and relieved sepsis-related ALI\textsuperscript{37,38}.

Our study showed that M2-Exo didn’t influence bacterial load in lung tissues, even with the downregulation of PMN migration and NET formation. A previous study\textsuperscript{39} demonstrated that LXA4 treatment decreased PMN migration to the peritoneum but augmented blood and peritoneal PMN phagocytic ability in a CLP-induced model of sepsis. Further ex vivo experiments also proved that LXA4 (1 nM) increased phagocytosis in blood PMNs without affecting apoptosis. This phenotype may partly explain our result, suggesting that M2-Exo-induced LXA4 upregulation could reduce PMN migration and increase their bacterial clearance function.

PMN recruitment from bone marrow to infectious tissues is critical for early innate responses, which is mainly triggered by interaction between chemokines and chemokine receptors\textsuperscript{40}. The chemokine receptor CXCR2 is largely responsible for driving PMN migration during bacterial infection and inflammation\textsuperscript{41}. In addition, a recent study\textsuperscript{24} showed that inhibition of CXCR1/2 by reparixin reduced NET formation, multi-organ injury, and mortality in septic mice, without impairing bacterial clearance, which highlights that CXCR1/2 signaling-induced NET formation is a therapeutic target in sepsis. In accordance with our result, LXA4 was shown to inhibit chemokine signaling in PMNs in vitro, and chemokine receptor desensitization would be a strong candidate for explaining how LXA4 altered the PMN migration pattern, leading to inflammation resolution\textsuperscript{42}.

Neutrophil ROS production plays a critical role in antibacterial host defense, although an uncontrolled continuous oxidative burst response can be detrimental\textsuperscript{43}. In this study, we also demonstrated that treating PMNs from both healthy volunteers and septic patients with M2-Exo reduced the excessive ROS production elicited by septic plasma. These findings are consistent with others previous studies\textsuperscript{44}, which demonstrated that LXA4 treatment reduced \textit{Porphyromonas gingivalis}-induced leukocyte ROS production in human whole blood. As elevated ROS production may promote leukocyte infiltration and NET formation, M2-Exo-mediated attenuation of PMN function may be partly due to the reduced ROS production.

Previously, prostaglandin E2 (PGE2) was a well-known pro-inflammatory lipid mediator produced by prostaglandin E synthase in leukocytes. Nowadays, accumulating evidence indicates that PGE2 has both anti- and proinflammatory effects dependent on the timing of its production and concentration\textsuperscript{45}. Previous study suggested that PMN exposure to PGE2 induced the phenotype switch from LTB4 production to lipoxin production, which marks the resolution phase\textsuperscript{16}. Our data also showed that the level of PGE2 in M2-Exo was significantly higher than that in M0-Exo, which begs a question of whether exosomal PGE2 from M2 macrophages could alter the lipid mediator class in PMNs. Cyclooxygenase-2 (COX-2) is an inducible enzyme that can convert arachidonic acid (AA) into PGE, the level of PGE2 is typically used as an indicator of COX2 activity\textsuperscript{46}. Therefore, we used the COX-2 inhibitor celecoxib to delete PGE2 expression in M2-Exo according to a previously published paper\textsuperscript{47}. The result showed that
M2 macrophage secreted PGE2 through exosomes, increased 15-LO expression in PMNs to upregulate LXA4 production, and then modulated the PMN migratory ability and NET formation capacity. It has been shown that exosomes expressing PGE2 can interact with PGE2 receptors (EP2, EP4) on dendritic cells, leading to CD73 production by the latter\textsuperscript{48}, which is consistent with our result that inhibition of EP4 receptor significantly abrogated the effect of M2-Exo on PMNs.

In conclusion, this study reveals a hitherto unknown role of exosomal PGE2 derived from M2 macrophage to increase 15-LO expression in PMNs, and then upregulate LXA4 production to downregulate PMN CXCR2 and ROS expressions, and finally inhibit the capacity of PMN migration and NET formation, alleviate lung injury, and reduce the mortality in sepsis. These findings may add a new element to macrophage exosome-PMN cross-talk, and M2-Exo may therefore be a better treatment to target PMN-mediated tissue injury in patients with sepsis.

**Declarations**

**Acknowledgments**

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**Authorship**

Y.J., T.Z. and M.L. contributed equally to this work. Z.L.M., X.P.G. and X.Y.S. contributed to the conception and design. Y.J., M.L., R.X. and Y.L.H. performed the experiments, analyzed data, and wrote the manuscript. M.Z.Q. collected the blood sample from septic patients. X.X. participated in the animal experiments. T.Z. supervised the work and helped write the manuscript. L.Y.Z. helped to revise the manuscript. All authors read and approved the final manuscript.

**Conflict-of-interest disclosure**

The authors declare no competing financial interests.

**Competing interests:** The authors declare that they have no competing interests.

**References**


**Figures**
Figure 1

**M2-Exo reduces lung injury and improves survival in septic mice.** (A) Electron micrograph of exosomes derived from the supernatant of polarized mouse Raw264.7 macrophages. Scale bar, 100 nm. (B) Exosome size distribution was measured by NanoSight tracking analysis. (C) CD63, CD9 and TSG101 protein expression in exosomes were quantified by Western blot loaded with equal amounts of exosome protein (40 μg). (D-J) WT C57BL/6 mice were administered with M0-Exo or M2-Exo (300 μg/mouse) derived from mouse Raw264.7 macrophages intraperitoneally 1 hour after CLP. (D) Schematic design for...
M0-Exo/M2-Exo treatment in the CLP model. (E) Representative images of direct immunofluorescence staining of DNA (blue), Ly6G (green), Dil (red) in the lung sections following i.p. injection with Dil-labeled exosomes, and white arrows indicate Dil-positive neutrophils. Scale bar, 50 μm. (F) Ex vivo fluorescent signals in the lungs of mice injected i.p. with Dil-labeled exosomes. (G) Evaluation of lung histology by H&E staining (magnification×400). Red arrows indicate neutrophils in the alveolar and interstitial space, blue arrows indicate alveolar macrophages, green arrows indicate proteinaceous debris filling, and black arrows indicate thickening of the alveolar walls. Scale bar, 40 μm. Lung injury scores were assessed. (H) Detection of inflammatory cytokine mRNA (IL-1β, IL-6, TNF-α) expression in the lung tissues by RT-qPCR. (I) Survival rate of CLP mice with M0-Exo or M2-Exo treatment (n = 8) and log-rank test was used for the analysis. (J) Lung tissues were harvested at 24 h after CLP. The supernatant was made after homogenation and centrifugation. An equal amount of the supernatant was spread on agar plates for colony formation. The number of bacterial colonies was assessed. CFU, colony-forming unit. One-way analysis of variance with Tukey’s multiple comparisons test was used for the analysis. Graphs represent means ± standard deviations, n ≥ 3; *P < 0.05, **P < 0.01 compared within two groups; NS, not significant.
M2-Exo inhibits PMN recruitment and NET formation during sepsis in vivo. WT C57BL/6 mice were administered with M0-Exo or M2-Exo (300 μg/mouse) derived from mouse Raw264.7 macrophages through i.p. injection 1 hour after surgery. 24 hours after CLP, venous blood, PLF and lung tissues were harvested. (A) Flow cytometry detection of percentage of systemic circulating PMNs by staining with CD11b and Gr-1. (B) Absolute neutrophil number in peripheral blood. (C and D) Ly6G+ cells in the lung.
tissues were detected by immunohistochemistry and immunofluorescence. Scale bar, 40 μm. (E) Representative images showing the presence of NETs (MPO, red; citrullinated H3, green) in the lung tissues, as indicated by white arrows. Nuclei were counterstained with DAPI (blue). Scale bar, 40 μm. (F and G) Quantification of dsDNA in the plasma and peritoneal lavage fluids (PLF) of mice using PicoGreen fluorescent dye. One-way analysis of variance with Tukey’s multiple comparisons test was used for the analysis. Graphs represent means ± standard deviations, n = 5-6; *P < 0.05, **P < 0.01 compared within two groups.
Figure 3

**M2-Exo inhibits PMN recruitment and NET formation during sepsis in vitro.** (A and B) PMNs from healthy volunteers were activated by septic plasma, and then co-cultured with M0/M2-Exo (100 μg/mL) derived from PBMC-differentiated macrophages. After 5 h, PMNs were collected for migration capacity analysis with IL-8 as a chemokine. After 2-h incubation, cells in the lower chamber were collected and counted under a microscope. (C and D) PMNs isolated from septic patients were directly co-cultured with M0/M2-Exo (100 μg/mL) derived from PBMC-differentiated macrophages for 5 h after isolation. Then, PMNs were transferred for transwell assay. (E-H) Ex vivo NET formation assay with neutrophils isolated from healthy volunteers or septic patients activated by septic plasma (SP). Plasma from healthy volunteers (HP) was used as negative control. Typical images of NET formation are presented in (E) and (G) using SYTOX Green (green), where white arrows indicate NETs. Scale bar, 50 μm. NET formation was quantified as the percentage of neutrophils forming NETs and NET area per microscopic field. (F and H) Quantification of dsDNA in the supernatant of cultured PMNs using PicoGreen fluorescent dye. One-way analysis of variance with Tukey’s multiple comparisons test was used for the analysis. Graphs represent means ± standard deviations; *P < 0.05, **P < 0.01 compared within two groups.
**Figure 4**

**M2-Exo leads to lipid mediator class switching of PMNs during sepsis.** (A and B) PMNs isolated from healthy volunteers were activated upon adding 20% septic plasma (SP) to the culture medium, and 20% plasma from healthy volunteers (HP) was used as negative control. After 1 h, PMNs were co-cultured with M0/M2-Exo (100 μg/mL) derived from PBMC-differentiated macrophages for 5 h. (C and D) PMNs from septic patients were directly co-cultured with M0/M2-Exo (100 μg/mL) for 5 h after isolation. LXA4 (A and
C) and LTB4 (B and D) concentration in the supernatant of co-cultured PMNs were detected by ELISA kit. LTB4 level was compared in M0-Exo and M2-Exo from PBMC-differentiated macrophages by ELISA. One-way analysis of variance with Tukey's multiple comparisons test (A-D) or student's t test (E) was used for the analysis. Graphs represent means ± standard deviations, n = 4-5; *P < 0.05, **P < 0.01 compared within two groups.

Figure 5
M2-Exo inhibits PMN recruitment and NET formation through LXA4 upregulation. (A) Ex vivo NET formation assay with PMNs isolated from healthy volunteers or septic patients activated by septic plasma (SP), and then co-cultured with M0/M2-Exo (100 μg/mL) derived from PBMC-differentiated macrophages for 5 h with or without BOC-2 (10 μM). Quantification of dsDNA in the supernatant of cultured PMNs using PicoGreen fluorescent dye. (B) Typical images of NET formation using SYTOX Green (green), where white arrows indicate NETs. Scale bar, 50 μm. NET formation was quantified as the percentage of neutrophils forming NETs and NET area per microscopic field. (C) Transwell analysis of PMN migration capacity isolated from healthy volunteers or septic patients. One-way analysis of variance with Tukey’s multiple comparisons test was used for the analysis. n = 4. (D-L) WT C57BL/6 mice were administered with M2-Exo (300 μg/mouse) derived from mouse Raw264.7 macrophages through i.p. injection 1 hour after CLP. To block LXA4 receptor, mice were treated with 50 μg/kg BOC-2 i.p. 30 min before CLP. (D and E) Quantification of dsDNA in the plasma and peritoneal lavage fluids (PLF) of mice using PicoGreen fluorescent dye. (F) Representative images showing the presence of NETs (MPO, red; citrullinated H3, green) in the lung tissues, as indicated by white arrows. Nuclei were counterstained with DAPI (blue). Scale bar, 40 μm. (G) Flow cytometry detection of percentage of systemic circulating PMNs by staining with CD11b and Gr-1. (H) Absolute neutrophil number in peripheral blood. (I and J) Ly6G+ cells in the lung tissues were detected by immunofluorescence and immunohistochemistry. Scale bar, 40 μm. (K) Evaluation of lung histology by H&E staining (magnification×400). Red arrows indicate neutrophils in the alveolar and interstitial space, blue arrows indicate alveolar macrophages, green arrows indicate proteinaceous debris filling. Scale bar, 50 μm. Lung injury scores were assessed. (L) Detection of inflammatory cytokine mRNA (IL-1β, IL-6, TNF-α) expression in the lung tissues by RT-qPCR. Student’s t test was used for the analysis. Graphs represent means ± standard deviations, n = 6; *P < 0.05, **P < 0.01 compared within two groups.
Figure 6

M2-Exo promotes LXA4 production in PMNs by increasing 15-LO expression. (A-I) PMNs isolated from healthy volunteers were activated by septic plasma (SP), and then co-cultured with M2-Exo (100 μg/mL) derived from PBMC-differentiated macrophages for 5 h with or without PD146176 (1 µM), LXA4 (100 nM), as indicated in the figures. DMSO was used as negative control. (A) Representative images of 15-LO in PMNs detected by immunofluorescence. (B, left panel) 15-LO and 5-LO expressions in PMNs were detected by Western blot. (B, right panel) PMNs isolated from septic patients were co-cultured with...
M0/M2-Exo (100 μg/mL) derived from PBMC-differentiated macrophages for 5 h. 15-LO and 5-LO expressions in PMNs were detected by Western blot. (C and D) LXA4 and LTB4 concentrations in the supernatant of PMNs were detected by ELISA. (E) Transwell analysis of neutrophil chemotaxis towards IL-8. (F) Typical images of NET formation using SYTOX Green (green), where white arrows indicate NETs. Scale bar, 50 μm. NET formation was quantified as the percentage of neutrophils forming NETs and NET area per microscopic field. (G) Quantification of dsDNA in the supernatant of cultured PMNs using PicoGreen fluorescent dye. Flow cytometry detection of CXCR2 (H) and ROS (I) expressions in co-cultured PMNs. Student’s t test (C and D) or one-way analysis of variance with Tukey’s multiple comparisons test (E-I) was used for the analysis. Graphs represent means ± standard deviations, n = 4-6; *P < 0.05, **P < 0.01 compared within two groups.
Figure 7

**Exosomal PGE2 from M2 macrophage is necessary for 15-LO upregulation and PMN inhibition.** (A) PGE2 level in M0-Exo/M2-Exo/exosomes from celecoxib (20 µM)-treated PBMC-differentiated M2 macrophages (Cel-M2-Exo) was examined by ELISA. PMNs isolated from healthy volunteers were activated by septic plasma (SP), and then co-cultured with M2-Exo/Cel-M2-Exo/Cel-M2-Exo+PGE2 (100 nM) for 5 h. (B) Immunoblot analysis of 15-LO in PMNs. (C and D) LXA4 and LTB4 concentrations in the supernatant of
PMNs were detected by ELISA. (E) Typical images of NET formation using SYTOX Green (green), where white arrows indicate NETs. Scale bar, 50 μm. NET formation was quantified as the percentage of neutrophils forming NETs and NET area per microscopic field. (F) Quantification of dsDNA in the supernatant of cultured PMNs using PicoGreen fluorescent dye. (G) Transwell analysis of neutrophil chemotaxis towards IL-8. Flow cytometry detection of CXCR2 (H) and ROS (I) expressions in co-cultured PMNs. One-way analysis of variance with Tukey’s multiple comparisons test was used for the analysis. n = 4-6. (J-U) WT C57BL/6 mice were administered with M2-Exo/Cel-M2-Exo (300 μg/mouse) derived from mouse Raw264.7 macrophages through i.p. injection 1 hour after CLP. (J) Representative images showing the presence of NETs (MPO, red; citrullinated H3, green) in the lung tissues, as indicated by white arrows. Nuclei were counterstained with DAPI (blue). Scale bar, 40 μm. (K and L) Quantification of dsDNA in the plasma and peritoneal lavage fluids (PLF) of mice using PicoGreen fluorescent dye. (M) Flow cytometry detection of percentage of systemic circulating neutrophils by staining with CD11b and Gr-1. (N) Absolute neutrophil number in peripheral blood. (O and P) Ly6G+ cells in the lung tissues were detected by immunohistochemistry and immunofluorescence. Scale bar, 40 μm. CXCR2 (Q) and ROS (R) expressions in peripheral blood neutrophils were detected by flow cytometry. (S) Evaluation of lung histology by H&E staining (magnification×400). Red arrows indicate neutrophils in the alveolar and interstitial space and black arrows indicate thickening of the alveolar walls. Scale bar, 50 μm. Lung injury scores were assessed. (T) Detection of inflammatory cytokine mRNA (IL-1β, IL-6, TNF-α) expression in the lung tissues by RT-qPCR Student’s t test was used for the analysis. Graphs represent means ± standard deviations, n = 6. (U) Survival rate of CLP mice with M2-Exo or Cel-M2-Exo treatment (n = 8) and log-rank test was used for the analysis. *P < 0.05, **P < 0.01 compared within two groups.
Figure 8

**Working model.** Our data demonstrated that exosomal PGE2 from M2 macrophage functions on EP4 receptor to increase 15-LO expression in PMNs, and then upregulates LXA4 production to downregulate PMN CXCR2 and ROS expressions, and finally inhibits the capacity of PMN migration and NET formation, alleviates lung injury, and reduces the mortality in sepsis. The graph was drawn on Figdraw online website (Export ID: RIOIT0bb56).

**Supplementary Files**

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- Supplementaryresultsandmethods.pdf