TLR4 inhibitor alleviates sepsis-induced organ failure by inhibiting platelet mtROS production, autophagy, and GPIIb/IIIa expression

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Abstract
Thrombocytopenia and impaired platelet function are associated with sepsis-induced organ failure. Numerous studies have shown that mitochondrial ROS (mtROS) and autophagy are related to organ injury in sepsis. However, the relationships between platelet mtROS, autophagy and sepsis organ failure remain unclear. Herein, we explored whether toll like receptor 4 (TLR4) inhibitor alleviates sepsis organ failure by inhibiting platelet mtROS production, autophagy, and GPIIb/IIIa expression.

Mice were administrated with LPS, LPS + TAK242 or vehicle. The lungs and kidneys were harvested and analyzed using hematoxylin and eosin staining assay. Platelet rich plasma (PRP) was isolated from blood and platelets aggregation and TLR4 expression were analyzed using flow cytometer and western blot. PRP from healthy volunteers was treated with saline, LPS, or LPS + TAK242, and then mitoSOX and calcium were detected using flow cytometer, and NOX2 and LC3B were tested using western blot.

Results showed that TAK242 effectively alleviated LPS-induced acute kidney and lung injury in mice, and decreased CD41 expression more significantly than CD62P.

In vitro, by inhibiting TLR4, TAK242 suppressed Ca2+, mitoSOX fluorescence, NOX2 expression and LC3BII/LC3BI ratio in LPS treated platelets.

TLR4 inhibitor TAK242 may effectively alleviate mouse lung and kidney injury by inhibition of mouse platelet GPIIb/IIIa, and reduce LPS-induced mtROS generation related to Ca2+ influx, thus reducing platelet activation.

Keywords Toll like Receptor 4 inhibitor · Sepsis · Platelet · Mitochondrial ROS · Autophagy

Introduction
Sepsis is a critical illness with extremely high mortality in intensive care units (ICUs), with an incidence of 6–30% (Martin 2012). The mortality from sepsis is as high as 28%-50% due to multiple organ failure such as sepsis-induced lung injury and kidney failure (Martin et al. 2003). Thrombocytopenia and impaired platelet aggregation are associated with sepsis-related mortality and sepsis-induced organ failure. The disorders of coagulation mechanism, microthrombosis, and vascular obstruction, and insufficient perfusion of tissues and organs caused by abnormal platelet function are the partial pathophysiological basis of organ dysfunction. Increased activation of the platelet surface marker P-selectin is related to acute lung injury (Baughman et al. 1993; Lundahl et al. 1998; Adamzik et al. 2012; Sakamaki et al. 1995; Russwurm et al. 2002; Gawaz et al. 1997) and platelet aggregation leads to acute kidney injury by enhancing microvesicle concentration (Zafrani et al. 2012). Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood (Clark et al. 2007), NETs mainly play a role in very small vessels, including lung capillar. Increasing levels of circulating NET biomarkers (free DNA/myeloperoxidase complexes) are correlated with multi-organ dysfunction (Czaikoski et al. 2016).

Liposaccharide (LPS) can induce production of intracellular reactive oxygen species (ROS) through Toll Like
Receptor 4 (TLR4), which can induce mitochondrial damage and activate platelets. However, the source of intracellular ROS and related molecular mechanisms remain unclear (Feng et al. 2018). Mitochondria are involved in pathophysiological processes such as energy production, intracellular signal transduction and cell death regulation, and play a key role in cell metabolism, survival and homeostasis. Many studies have shown that mitochondrial disorders are related to impaired organ function in sepsis.

ROS mainly come from the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) pathway and the mitochondrial electron transporter pathway. Organ failure in sepsis is mainly related to increase of mtROS. Reducing mtROS production can alleviate lung injury in sepsis (Wang et al. 2021). Autophagy is the upstream of mtROS production and is related to organ injury in sepsis. LPS can induce autophagy and mtROS production in macrophages, which further lead to acute liver injury (Unuma et al. 2015). Inhibiting autophagy in macrophages can suppress mtROS generation and reduce inflammation (Fei et al. 2020). Autophagy is related to septic kidney injury induced by LPS via the PI3K/AKT/mTOR pathway, and inhibition of autophagy can reduce septic kidney injury (Zhao et al. 2020).

Despite lacking a nucleus, platelets have substantial internal organelles such as mitochondria and lysosomes, and is a potential target for regulation of micro-thrombosis and inflammation in sepsis. LPS can induce release of mtROS via TLR4, leading to impairment of platelet function, which is a key cause of organ dysfunction in sepsis. However, the signal pathways through which platelet TLR4 is activated to mediate organ injury in sepsis remain unclear. In our previous studies, a TLR4 inhibitor (TAK242) was used to inhibit the expression of platelet TLR4, thereby reversing LPS-mediated septic organ injury in mice. Herein, we explore whether this TLR4 inhibitor can alleviate sepsis organ failure by inhibiting platelet mtROS production, autophagy, and GPIIb/IIIa expression.

**Methods and materials**

**Materials**

LPS was purchased from Sigma Chemicals (St. Louis, MO, USA). TAK242 (TLR4 receptor inhibitor) was purchased from MedChem Express (New Jersey, USA). H$_2$O$_2$ was purchased from Sinopharm Chemical Reagent Company (Shanghai, China). N-acetyl-L-cysteine (NAC) and Calcium Detection were purchased from Byotime (Shanghai, China). GAPDH, RIPA buffer, PMSF, protease, and phosphatase inhibitor cocktails were obtained from Cell Signaling Technology (Beverly, MA, USA). MitoSOX™ Red mitochondrial superoxide indicator (MitoSOX™) was obtained from Thermo Fisher (USA).

**Preparation of platelets**

Peripheral venous blood of healthy volunteers or blood (2–3 ml) collected from mice heart was treated with anticoagulant (0.38% sodium citrate), and then centrifuged at 150 g for 10 min. The platelet rich plasma (PRP) was collected, washed with Ca$^{2+}$ free-Tyrodes HEPES buffer, and then centrifuged at 850 g for 10 min. The supernatant was discarded, and the platelets were washed and suspended in Ca$^{2+}$ Free-Tyrodes HEPES buffer. The PRP was then randomly assigned to different groups with different treatments.

**Flow cytometric analysis**

PRP (2 × 10$^7$ platelets/ml) was treated with LPS (10 µg/ml), LPS (10 µg/ml) + TAK242 (132 nM), NAC (5 mM), H$_2$O$_2$ (100 µM), or vehicle, respectively at 37 ºC for 30 min. The 5 mM MitoSOX™ reagent stock solution was diluted with HBSS/Ca/Mg to make a 5 µM MitoSOX™ reagent working solution. 1.0–2.0 mL of 5 µM MitoSOX™ reagent working solution was used to cover the PRP adhered on coverslips. Cells were then incubated at 37 ºC for 10 min in the dark. The calcium probe was then incubated with PRP for 15 min in a thermostatic dark box at 37 ºC. After three washes, the fluorescence intensity of samples was examined by a flow cytometer (BD Calibur, USA).

**Western blot**

The platelet lysates (10$^9$ cells/ml) were incubated with LPS (10 µg/ml), LPS (10 µg/ml) + TAK242 (132 nM) or vehicle for 5 min at 37 ºC. The samples were then lysed and subjected to separation using 12% SDS-PAGE, and then transferred onto PVDF membrane. After a 1-h blockage with 5% bovine serum albumin (w/v) at room temperature, the membrane was incubated with anti-human LC3B, NOX2, or TLR4 primary antibodies (1:1000 dilution) at 4 ºC overnight. After incubation with secondary antibody (1:3000 dilution) at room temperature for 1 h, the membranes were developed with chemiluminescence ECL reagent (LumiGold, SignaGen Laboratories, Rockville, MD) and exposed to Hyperfilm MP (GE Healthcare Life Science, Pittsburgh, PA).

**Sepsis organ injury and analysis of the effect of TLR4 inhibitor in vivo**

Male BALB/c mice (20–25 g) purchased from SLAC Laboratory Animal Central (Changsha, China) were housed in an air-conditioned room with temperature of 25 ºC and...
relative humidity of 55% and acclimated for 7 days under a regular light–dark cycle. Mice were randomly divided into three groups (LPS, LPS + TAK242 and control groups). Mice in the LPS group received dorsal subcutaneous injection of LPS (9 µg) from day 2 to day 3. Mice in the TLR4 inhibition group received dorsal subcutaneous injection of TAK242 (5 mg/kg) from day 1 to day 3, and LPS (9 µg) was administered on day 2 and day 3. Mice in the vehicle group received dorsal subcutaneous injection of volume of saline solution on days 1–3. The animals were then sacrificed, and the lungs and kidneys were harvested for hematoxylin and eosin (H&E) staining and the pictures were taken (200× and 400×). The heart whole blood was collected, treated with anti-coagulant and then centrifuged at 150 g for 10 min. PRP was then isolated and analyzed using flow cytometer. The samples were then lysed and subjected to Western blot analysis.

Results

TAK242 alleviates LPS-induced acute kidney and lung injury and suppresses platelet activation in a sepsis mouse model

To investigate the effect of LPS and TLR4 inhibitor on sepsis in mice, histopathological studies were performed. H&E staining showed that LPS induced acute kidney and lung injury. Compared with the blank control group, LPS induced edema of renal tubular epithelial cells and hemorrhage (Fig. 1A), and increased pulmonary vascular permeability and pulmonary hemorrhage (Fig. 1B). While TAK242 (20 mg/kg) efficiently prevented kidney and lung injury, as well as inflammatory cell infiltration into kidney and lung tissue.

The flow cytometry analysis (Fig. 1C) showed that the activated platelet signal was mainly concentrated in the right region, especially the CD41(+)/CD62P(-) region. The proportions of CD41(+)/CD62P(-) and CD41(-)/CD62P(+) platelets in LPS treated mice was significantly increased compared with the control group (p < 0.05, and p < 0.001, respectively), while the proportion of CD41(-)/CD62P(-) platelets was slightly lower than that of the control group (p < 0.01). Interestingly, the proportion of CD41(+) CD62P(+) platelets in LPS treated mice was significantly lower than that of the control group (p < 0.001). The proportion of CD41(+) CD62P(-) platelets in the TAK242 intervention group was significantly lower than that in the LPS group (p < 0.01). The proportions of CD41(-) CD62P(+) and CD41(+) CD62P(+) platelets in the TAK242 intervention group were significantly lower than those of the LPS group, and the proportion of CD41(-) CD62P(-) platelets was slightly higher than that of the LPS group.

As shown in Fig. 1D, TAK242 inhibited TLR4 expression in sepsis mice platelets; the TLR4 grayscale value in LPS treated mice was significantly higher than those of control group (p < 0.001) and LPS + TAK242 group (p < 0.01).

TAK242 reduces inward flow of Ca²⁺ in LPS treated platelets

Our previous study showed that LPS can increase P-selectin expression on platelet surface and enhance platelet aggregation via releasing endogenous ROS, ultimately leading to platelet activation (Feng et al. 2018). Platelet activation is related to influx of calcium ions (Shiraiishi et al. 2010). Herein, using calcium ion probe Fluo-3 AM ester, we found that the calcium ion concentration in LPS treated platelets was significantly increased when compared with the control group (P < 0.001, LPS v.s. NC, Fig. 2). Similar change was observed in H₂O₂ group. While TAK242 decreased the intraplatelet calcium ion concentration induced by LPS (P < 0.001, LPS + TAK242 v.s. LPS, Fig. 2) and recovered it to the level similar with that of the control group (P > 0.05, LPS + TAK242 v.s. NC, Fig. 2). Similar change was observed in LPS + NAC group.

TAK242 decreases endogenous mtROS production in LPS treated platelets

Our previous study showed that LPS promoted the release of intracellular ROS through platelet TLR4 to activate platelets. In this study, we found that LPS stimulated platelets to produce a substantial amount of mtROS (P < 0.0001, LPS v.s. NC, Fig. 3), while TAK242 significantly reduced mtROS production by LPS-induced platelets (P < 0.001, LPS + TAK242 v.s. LPS, Fig. 3). Similar effect was observed using NAC.

TAK242 inhibits autophagy and NOX expression in LPS treated platelets

LC3B is widely used as an indicator of autophagy. The ratio of LC3BII to LC3BI can be used to estimate autophagic flux. In this study, Western blot analysis of LC3BII and LC3BI showed that LPS induced remarkable autophagic flux in platelets (P < 0.01, LPS v.s. NC, Fig. 4). Moreover, TAK242 significantly attenuated LPS-induced autophagy (P < 0.01, LPS + TAK242 v.s. LPS, Fig. 4).

Nicotinamide adenine dinucleotide (phosphate) (NAD(P) H) oxidase (NOX) isoforms are the main sources of ROS in platelets, followed by cyclooxygenase (COX), xanthine oxidase (XO), and mitochondrial respiration (Wachowicz et al. 2002). We found that LPS induced a significant increase in platelet NOX expression (P < 0.01, LPS v.s. NC, Fig. 4), while TAK242 decreased the expression of NOX.
A. Control, LPS, LPS+TAK242

B. Control, LPS, LPS+TAK242

C. Flow cytometry graphs showing CD4+CD25+ and CD4+CD25- cell populations in Negative Control, Blank Control, LPS, and LPS+TAK242.

D. Western blot analysis showing TLR4 and GAPDH expression in control, LPS, and LPS+TAK242 conditions. The graphs show the expression levels in different conditions.
in LPS-treated platelets ($P<0.05$, LPS + TAK242 v.s. LPS, Fig. 4). However, the expression of NOX in LPS + TAK242 group was still slightly higher than that in the control group ($P<0.05$, LPS + TAK242 v.s. NC, Fig. 4).

**Discussion**

P-selectin is helpful for preventing sepsis multiple organ failure (Shimizu et al. 2018), and GPIIb/IIIa might be the target to reverse sepsis organ failure (Sharron et al. 2012). Platelets are particles shed from the cytoplasm during the maturation of megakaryocytes. They are rich in organelles and contents but have no nucleus. These organelles and contents are involved in platelet activation. Platelet activation is also accompanied by the expression of multiple specific surface markers, including platelet granule membrane glycoprotein P-selectin (CD62P) and platelet plasma membrane glycoproteins (CD41 and GPIIb) (Metzelaar et al. 1992). During the resting period, one platelet contains 800–1120 CD62P molecules on membrane surface. When platelets are activated by thrombin, the alpha granules in the platelet fuse with the plasma membrane through the open canalicular system (OCS), increasing the number of plasma membrane CD62P to 1.0–1.3 $\times 10^3$ per platelet. Many bacterial proteins directly promote the expression of the GPIIb/IIIa complex via binding to the arginine-glycine-aspartic acid (RGD) sequence on the platelet surface, and thus activate the platelets (Brennan et al. 2009). In this study, we successfully constructed...
a mouse sepsis model by subcutaneous injection of LPS. We observed sepsis-induced pulmonary hemorrhage and kidney injury in mice by H&E staining. Platelet activation markers CD41 (representing GPIIb/IIIa) and CD62P (representing PS ectropion) were tested. The platelet-rich plasma of septic mice exhibited increased expression of platelet activation markers with CD41 increased more significantly than CD62P, similar finding was also reported previously (Brennan et al. 2009).

to make a 5 µM MitoSOX™ reagent working solution. 1.0–2.0 mL of 5 µM MitoSOX™ reagent working solution was used to cover the PRP adhered on coverslips. mtROS production was analyzed by flow cytometry (n = 3). The histogram displays the MFI of mtROS. Data are presented as mean ± SEM, ***p < .001 and ****p < .0001

Fig. 3 TAK242 decreases mtROS production induced by LPS in platelets. PRPs were isolated from peripheral venous blood of healthy volunteers. PRP (2 × 10⁷ platelets/ml) was treated with LPS (10 µg/ml), LPS (10 µg/ml) + TAK242 (132 nM), NAC (5 mM), H₂O₂ (100 µM), or vehicle, respectively at 37 °C for 30 min. The 5 mM MitoSOX™ reagent stock solution was diluted with HBSS/Ca/Mg

Fig. 4 TAK242 decreases autophagy and NOX2 expression induced by LPS in platelets. The platelet lysates (10⁹ platelets/ml) were incubated with LPS (10 µg/ml), LPS (10 µg/ml) + TAK242 (132 nM) or vehicle for 5 min at 37 °C. The expression of LC3B and NOX2 was detected by Western blotting. The representative blotting images (left) and densitometric analysis results (right) are shown (n = 3). Data are represented as mean ± SEM, *p < .05 and **p < .01
Ca\(^{2+}\) overload can act as a signal to open mitochondrial permeability transition pore (MPTP) (Fedotcheva et al. 2021). LPS binds to its receptor TLR4 on the surface of platelets to mediate micro thrombosis and organ injury led by insufficient perfusion. Furthermore, in response to Escherichia coli infection, platelet TLR4 contributes to bacterial trapping by supporting NET formation (Stark et al. 2012). Platelets promote leukocyte recruitment in LPS-induced lung injury. In contrast, low platelet counts are associated with increased secondary hemostasis, kidney damage, and exacerbated bacteremia and systemic bacterial dissemination in bacteria induced sepsis (Xiang et al. 2013; Stoppelaar et al. 2014; Powers et al. 2015). There are many ways to produce intracellular ROS. Bacterial invasion transfers TNF receptor associated factor 6 (TRAF6) to the outer mitochondrial membrane through TLR4, and regulates the process of oxidative phosphorylation via evolutionarily conserved signaling intermediate in toll pathways (ECSIT), ultimately leading to ubiquitination of TRAF6-dependent ECSIT and generation of mtROS (Vogel et al. 2007). When sepsis occurs, Ca\(^{2+}\) concentration in blood significantly reduces while Ca\(^{2+}\) overload appears in mitochondria (Bouillot et al. 2018; Stelzner et al. 2020; He et al. 2020).

In this study, we found that LPS stimulated Ca\(^{2+}\) influx in platelets. The effect of LPS on Ca\(^{2+}\) influx in platelets was similar with that of adding exogenous ROS (i.e. \(H\_2O_2\)). The inhibitory effect of TLR4 inhibitor TAK242 on LPS-induced Ca\(^{2+}\) in platelets was similar with that of the ROS scavenger NAC. This phenomenon is analogous to the effect of LPS on platelet-induced intracellular ROS production described in our previous study.

Mitochondria-targeted hydroethidine (Mito-SOX) is a redox probe in which the HE molecule is conjugated to a triphenyl phosphonium group (TPP\(^+\)) via an alkyl side chain. Mito-SOX is targeted to mitochondria because of the presence of the TPP\(^+\) moiety (Zielonka et al. 2008). It has excitation/emission maxima of approximately 510/580 nm. We therefore used the fluorescence intensity detected in the PE channel of the flow cytometer to determine mtROS levels in platelets.

Exogenous \(H\_2O_2\) can induce endothelial cell mitochondria to produce mtROS. We therefore used \(H_2O_2\) as a positive control (Kim et al. 2017). We found that both \(H\_2O_2\) and LPS induced production of a large amount of mtROS in mitochondria. The inhibitory effect of TAK242 on mtROS production was similar with that of NAC. Meanwhile, \textit{in vivo} experiments showed that TAK242 inhibited the aggregation of platelets in mice and reduced lung and kidney injury in septic mice. We speculate that TLR4 inhibitor can suppress platelet activation by decreasing platelet mtROS to alleviate organ injury in sepsis.

mtROS are involved in a series of signaling pathways, including those regulating immune responses and autophagy. NOX isoforms are the main sources of ROS in platelets, followed by cyclooxygenase (COX), xanthine oxidase (XO), and mitochondrial respiration (Wachowicz et al. 2002). mtROS and NOX-dependent ROS come from different pathways but are closely connected. In neutrophils and endothelial cells, translocation of mtROS to cytosol activates PKc and cSrc, and induces ROS production by NOX2 (Dikalov et al. 2014; Kröller-Schön et al. 2014). In our previous study, we found that LPS decreased p-PKC expression in platelets, but in this study, we found that LPS enhanced NOX2 expression in platelets with a concomitant increase in mtROS. The inhibition of PKc expression may be related to the increase of endogenous mtROS (Song et al. 2017).

mtROS are closely related to autophagy. Under physiological conditions, autophagy helps cells remove damaged mitochondria and peroxidized proteins. In most cases, autophagy promotes survival. Intracellular ROS cause mitochondrial damage, thereby initiating autophagy to clear damaged mitochondria (Feng et al. 2018). Therefore, autophagy has a protective effect against organ damage in sepsis. Blocking liver autophagy in a murine model of sepsis accelerates hepatocyte apoptosis and mitochondrial damage and decreases survival (Oami et al. 2018). Interestingly, previous report proposed that platelet activation can induce autophagy in platelets, which is closely related to thrombocytopenia and thrombosis (Ouseph et al. 2015). Consistent with this hypothesis, we found that LPS induces platelet aggregation through release of mtROS, accompanied by platelet autophagy.

**Conclusion**

1. Platelet TLR4 is associated with septic lung and kidney injury.
2. TLR4 inhibitor can effectively alleviate lung and kidney injury in a mouse sepsis model induced by LPS mainly through inhibition of mouse platelet GPIIb/IIIa.
3. TLR4 inhibitor can decrease LPS-induced mtROS generation related to Ca\(^{2+}\) influx, thus reducing platelet activation.
4. LPS can induce platelet autophagy by generating mtROS, which may be the pathophysiological mechanism of organ injury in sepsis.

**Author contribution** Guo Feng designed this study. Guo Feng and Ying Li performed the research, analyzed data, and wrote the paper. All authors read and approved the final version of this manuscript.

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Declarations

Ethics approval and consent to participate Volunteers were aware of this study and signed an informed consent form. All procedures were conducted in accordance with the protocol approved by the ethical committee of the Laboratory Animals Center of Central South University and all the procedures were under the guideline of the Third Xiangya Hospital of Central South University Human Research Committee.

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