Toll-like receptor 4 agonist-based nanoparticles orchestrate protection against sepsis

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ABSTRACT

Sepsis, a life-threatening organ dysfunction induced by severe infection and uncontrolled host immune response, threatens the health of people all over the world. Herein, a type of nanoparticle formulation with simple components is synthesized by encapsulating monophosphoryl lipid A (MPLA), a TLR4 agonist, with poly(lactic-co-glycolic acid) (PLGA) nanoparticle. The obtained nanoparticles (MPLA@PLGA) could provide *Escherichia coli* (*E. coli*)-induced sepsis protection by regulating the immune system after sepsis challenge, including promoting the levels of various cytokines, boosting the percentage of natural killer cells and accelerating bacterial clearance. Notably, the survival mice pre-treated with these nanoparticles could resist repeated *E. coli*-induced sepsis. Our work therefore provides the great promise of MPLA@PLGA nanoparticles as a simple yet effective nano-drug for prevention and protection against *E. coli*-induced sepsis.

Introduction

The increasing misuse of antibiotics has led to an increasing rate of drug resistance in various infectious diseases, such as pneumonia and urinary tract infection [1, 2]. As one of the most common causes of death in hospitalized patients, sepsis, defined as life-threatening organ dysfunction induced by severe infection and uncontrolled host immune response, threatens the health of people all over the world. It

has been reported that more than 19 million people suffer from sepsis each year, as well as a death rate of more than 40% [3, 4]. Furthermore, about 3 million surviving patients have organ dysfunction, such as infective cardiomyopathy, or cognitive impairment [5–8]. It was also found in clinical studies that the pre-infection immune status of clinical patients would directly determine the prognosis of infected patients [9]. And immunomodulators can regulate the in vivo release of cytokines, improve the immune function of sepsis patients, effectively maintain the

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in vivo normal immune homeostasis in sepsis patients, reduce injuries of important organs and decrease the mortality of sepsis [10–12]. Therefore, it is important to use immunomodulators for preventing and treatment of sepsis induced by severe infection, which may provide new treatment options for sepsis patients.

The interactions between pathogen-associated molecular patterns (PAMPs), defined as highly conserved molecular structure in pathogenic microorganism, and pattern recognition receptors (PRRs) play an important role in the prevention and treatment of sepsis, including the recognition of infectious pathogens, the initiation of host inflammatory response and the activation of host immune defense [13–15]. Toll-like receptor (TLR) 4 activator, a typical PAMP with powerful biological functions, can regulate host inflammation, recruit granulocytes and macrophages to the infected sites and effectively promote pathogen clearance [16]. Furthermore, the insufficient activation of TLR4 signaling pathway in the host can promote the occurrence of infection and aggravate the progress of infection [1, 17, 18]. And it was found that the anti-infective capability was enhanced in clinical patients pre-treated with TLR4-related immunoregulators [19–21]. Therefore, it is important for the prevention and treatment of various infections by using TLR4 immunoregulators, especially sepsis.

Monophosphoryl lipid A (MPLA), a TLR4 agonist used as vaccine adjuvant in clinic, can enhance the in vivo bactericidal efficacy of granulocytes and macrophages without the release of cytotoxic products [22–24]. However, MPLA is a hydrophobic molecule with poor water solubility. Herein, based on the above findings, MPLA loaded into poly(lactic-co-glycolic acid) (PLGA) nanoparticles, a polymeric carrier that has been widely used as drug delivery and approved by FDA for clinical use, is developed [25]. We hypothesized that these nanoparticles (MPLA@PLGA) could continuously release MPLA post-intradermal injection to provoke immunological effect for *Escherichia coli* (*E. coli*)-induced sepsis protection by stimulating intradermal immune cells and host immune system (Fig. 1a). The rate of survivors in the MPLA@PLGA-treated group was significantly increased. Excitingly, the survival mice pre-injected with MPLA@PLGA could resist to the repeated *E. coli*-induced sepsis challenge. Therefore, this nanoparticle could be used as a simple yet effective

preventive nano-drug for bacteria-induced sepsis protection.

Materials and methods

Materials

Raw 264.7 cells, a kind of murine cell line, were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL of penicillin and 100 µg/mL of streptomycin in a humidified atmosphere incubator with 5% CO₂ at 37 °C. Dimethyl sulfoxide (DMSO) and dichloromethane (CH₂Cl₂) were purchased from Chinasun Specialty Products Co., Ltd. The antibodies for flow cytometry assay were obtained from BioLegend. Limulus amoebocyte lysate (LAL) endotoxin assay kit was obtained from GeneScript. Other unmentioned chemicals were purchased from Sigma–Aldrich.

All the C57BL/6 mice (6–8 weeks, female) in the experiments were fed under standard laboratory conditions (ventilated room, 25 ± 1 °C, 60 ± 5% humidity, 12-h light/dark cycle) and had free access to standard water and food. All procedures were conducted in accordance with the “Guiding Principles in the Care and Use of Animals” (China) and were approved by Soochow University Laboratory Animal Center (approval number: SYXK(Su) 2017-0043).

Fabrication of MPLA@PLGA nanoparticle

MPLA@PLGA nanoparticle was produced by using a previously reported O/W single-emulsion method [27]. Briefly, monophosphoryl lipid A (MPLA) was dissolved in DMSO solution. Ten milligrams of PLGA was added into 0.4 mL CH₂Cl₂ solution and then added to PVA solution (0.8 mL, 50 mg/mL). MPLA solution (1 mg/mL, 20 µL) was mixed with the above solution and homogenized by ultrasonic at 200 w for 30 min after adding 2.2 mL deionized water. Then, the emulsion was added into PVA solution (50 mg/mL, 1 mL) and stirred overnight at room temperature. The MPLA@PLGA nanoparticles were obtained by centrifuging (30,000 rpm, 15 min) and washed by deionized water.

Fluorescent dye DID encapsulated into PLGA nanoparticles (DID@PLGA) was synthesized by the same method by using DID instead of MPLA.

Figure 1 Synthesis and characterization of MPLA@PLGA nanoparticles. **a** The schematic illustration of the synthesis and function of MPLA@PLGA nanoparticles. **b** A TEM image of MPLA@PLGA nanoparticles. **c** The size distribution of MPLA@PLGA nanoparticles. **d** The diameter profiles of

MPLA@PLGA nanoparticles in water, PBS or DMEM at different time points. **e** The MPLA release of MPLA@PLGA nanoparticles in water at various time points. Data are presented as mean $\pm$ the standard errors of the mean (SEM).

Characterization and drug release of MPLA@PLGA

The diameter and stability in various solutions of MPLA@PLGA were determined by dynamic light scattering (Zetasizer Nano-ZS). The transmission electron microscopy (TEM) image of the nanoparticles was obtained by JEM-1230. The release and encapsulation efficiency of MPLA encapsulated into the nanoparticles were determined by the LAL endotoxin assay kit.

In vitro bacterial uptake

E. coli was cultured at 37 °C in Trypticase soy broth, harvested at the mid-logarithmic growth phase, washed three times and resuspended in PBS for the in vitro experiments. The collected *E. coli*, heat-killed for 30 min at 95 °C, was labeled by Cy5.5 (*E. coli*-cy5.5). The RAW 264.7 cells, cultured in 24-pore plate, were then stimulated with MPLA@PLGA (0.2 μ g/mL MPLA) for 6 h. The stimulated raw 264.7 cells were incubated with *E. coli*-cy5.5 at 37 °C for 30 min (the ratio of cell/bacteria = 1/20). The *E. coli* uptake was analyzed by flow cytometry and confocal fluorescence microscope (OLYMPUS).

In vivo subcutaneous fluorescence imaging

DID@PLGA nanoparticles were intradermally injected into the skin of healthy mice. The DID signal in the back skin was detected at different time points by PE Lumina III.

In vivo *E. coli* challenge and analysis of immunocyte and cytokine

C57BL/6 mice (6–8 weeks, female) were randomly distributed in the PBS or MPLA@PLGA group. All the mice were challenged by *E. coli* (5×10^6 CFU) at 24 h after intradermal injection of MPLA@PLGA (0.1 mg/kg MPLA). Survival was monitored for at least 14 days.

At 6 h after *E. coli* challenge, the main organs from the mice, killed by cervical dislocation, were acquired and determined for *E. coli* counts.

The expression levels of the markers in different immune cells in blood and peritoneal lavage fluids were measured by a flow cytometer at 6 h post-sepsis challenge. The concentration of cytokines was analyzed by ELISA.

Statistical analysis

All results were expressed as means $\pm$ SEM. Student's *t*-test was performed when two groups were compared. Statistical differences in survival were determined by the log-rank test. The statistical analyses were performed with GraphPad Prism and Origin. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

Results and discussion

PLGA is a kind of clinically approved biomedical polymer and widely used as a drug carrier for drug delivery [26]. MPLA, a kind of TLR4 agonist, was encapsulated into PLGA nanoparticles (MPLA@PLGA) through a previously reported method [27]. As revealed by transmission electron microscopy (TEM) image, it was shown that the synthesized MPLA@PLGA had a uniform spherical structure (Fig. 1b). The average diameter of MPLA@PLGA nanoparticles, exhibited by dynamic light scattering data, was about 110 nm (Fig. 1c). The encapsulation efficiency of MPLA loaded into PLGA nanoparticles, measured by the LAL endotoxin assay kit, was about 49.43%. Furthermore, it was shown that the MPLA@PLGA nanoparticles could be stable in deionized water, phosphate buffer saline (PBS) solution and DMEM cell culture medium (Fig. 1d), indicating the good stability in various solutions. The release profile of MPLA was then studied by using the LAL endotoxin assay kit to measure the released MPLA (Fig. 1e). It was found that the slow release of MPLA encapsulated with PLGA nanoparticles could be observed.

Next, DID fluorescent dye loaded into PLGA nanoparticles (DID@PLGA), the surrogates for MPLA@PLGA, was used to mimic and evaluate the in vivo retention of the MPLA (Fig. 2b and c), which was intradermally injected into the healthy C57/BL6 mice. As shown by fluorescence imaging, the DID@PLGA nanoparticles showed prolonged retention in vivo.

Furthermore, the anti-infective effect enhanced by the MPLA@PLGA nanoparticles was detailed studied in vivo (Fig. 2a). Firstly, the PBS-treated or MPLA@PLGA-treated mice were given intradermal injection of PBS or MPLA@PLGA (0.1 mg/kg MPLA), respectively. At different time points (6 h, 24 h and 48 h) post-intradermal injection, all the mice

Figure 2 In vivo protective effect against *E. coli*-induced sepsis induced by the MPLA@PLGA nanoparticles. **a** Schematic illustration to exhibit the protection of the MPLA@PLGA nanoparticles after different time points in *E. coli*-induced sepsis. **b** and **c** In vivo fluorescence imaging photographs **b** and quantification (**c**) of release profiles of DID encapsulated with PLGA nanoparticles (DID@PLGA). **d** and **e** Survival rate (**d**) and individual body weights (**e**) of the mice challenged by *E. coli*-induced sepsis (*n* = 5 per group) at different time points after MPLA@PLGA nanoparticles intradermal injection. (**f** and **g**) Survival rate (**f**) and individual body weights (**g**) of the mice 24 h after different treatments challenged by *E. coli*-induced sepsis (*n* = 12 per group). S, survival rate. (**h** and **i**) Representative images (**h**) and the statistic data (**i**) of *E. coli* colonization in the major organs of the mice at 6 h after sepsis challenge (*n* = 3 per group). Black and red points mean PBS-treated and MPLA@PLGA-treated group, respectively. (**j**) **h** and **e** staining images of the major organs of the mice at 16 h after sepsis challenge. Data are presented as means $\pm$ SEM. Survival curves were compared by log-rank test. Statistical significance was calculated by Student's *t*-test.

were acquired by lethal intraperitoneal injection (200 μ L, 5×10^6 CFU, *E. coli*). It is exhibited in Fig. 2d that all the mice treated by PBS died within 25 h, while the mice treated by MPLA@PLGA nanoparticles could achieve 60% for 6-h treatment, 80% for 24-h treatment and 48-h treatment, respectively. The survival data demonstrated that sepsis protection induced by the MPLA@PLGA nanoparticles could be enhanced by the in vivo increased release of MPLA. The body weight of the living mice turned to the healthy weight within 5 days after sepsis challenge (Fig. 2e).

Based on the in vivo efficiency of sepsis protection, we chose to evaluate and analyze the in vivo antibacterial effect of MPLA@PLGA nanoparticles about 24 h post-intradermal injection. Firstly, the sample size in MPLA@PLGA-treated group was expanded to repeat the sepsis protection of MPLA@PLGA nanoparticles. It was found that the mice treated with MPLA@PLGA nanoparticles could achieve $\sim$ 83.33% survival, while the mice in PBS group were all death (Fig. 2f). The survival result proved that the activation of TLR4 signaling pathway could promote effective sepsis protection. Notably, the body weight of the survival mice increased to pre-infected level about 5 days after *E. coli*-induced sepsis challenge (Fig. 2g). Furthermore, such sepsis protection induced by MPLA@PLGA nanoparticles was

evaluated by the *E. coli* counts in the organs at 6 h post-lethal infection. It was uncovered that the *E. coli* counts in MPLA@PLGA group were significantly decreased compared to PBS group (Fig. 2h and i), demonstrating enhanced *E. coli* clearance in the MPLA@PLGA-treated mice. Besides, the histology of the major organs in the mice was evaluated by hematoxylin and eosin (H&E) staining at 16 h post-sepsis challenge. As shown in Fig. 2j, the degree of major organ inflammation and damage in MPLA@PLGA-treated mice was lower than PBS-treated mice. All the above results showed that MPLA@PLGA nanoparticles could offer effective sepsis protection.

Thereafter, the *in vivo* mechanisms of MPLA@PLGA nanoparticles in the protection of *E. coli*-induced sepsis were studied in detail (Fig. 3a). Firstly, various immune cells *in vivo*, including natural killer (NK) cells, monocytes, neutrophils and T cells in blood, were analyzed 24 h post-intradermal injection. As shown in Figs. 3b–d and S1A–S1E, the percentages of NK cells, monocytes and CD80⁺F4/80⁺ cells in MPLA@PLGA group were increased, while the percent of CD8⁺ T cells, B cells, CD4⁺ T cells, CD206⁺F4/80⁺ cells and neutrophils was little changed. Furthermore, the concentrations of different cytokines, measured by enzyme linked immunosorbent assay (ELISA), were not significantly changed between the mice treated with PBS or MPLA@PLGA nanoparticles before sepsis challenge (Figs. 3i–n and S2A–S2D), implying the safety of MPLA@PLGA nanoparticles. However, the immune defense in the MPLA@PLGA-treated mice challenged by sepsis was enhanced by up-regulating the percentages of functional immune cells in blood and in abdominal cavity (Figs. 3e–h and S3A–S3E). As exhibited in Figs. 3E–3H&S3A–S3E, the percentages of NK cells, neutrophils and CD80⁺F4/80⁺ cells in the MPLA@PLGA-treated mice were obviously increased after bacterial sepsis challenge, while the percentages of CD8⁺ T cells, B cells, CD4⁺ T cells, monocytes and CD206⁺F4/80⁺ cells were not significantly changed in the two groups. When the mice were challenged by bacterial infection, the functional cytokines were more secreted in MPLA@PLGA group compared to PBS group, implying stronger immune response (Figs. 3i–n and S2A–2D). These results demonstrated that the *in vivo* immune response could be effectively enhanced by MPLA@PLGA nanoparticles for sepsis protection.

Given the role of NK cells, neutrophils and CD80⁺F4/80⁺ cells in the above sepsis protection, we chose RAW 264.7 cells, a kind of F4/80⁺ cell line, to mimic and study the *in vitro* function of MPLA@PLGA nanoparticles. The RAW 264.7 cells were stimulated with PBS or MPLA@PLGA nanoparticles for 6 h. After stimulation, these stimulated cells were cultivated with Cy5.5-labeled and heat-killed *E. coli* (*E. coli*-cy5.5) for 30 min. Thereafter, the polarization of RAW 264.7 cells was evaluated by flow cytometry. It was found that MPLA@PLGA nanoparticles could promote the expression of CD80 and M1 polarization of RAW 264.7 cells (Figure S4A–S4C). Then, the *E. coli* uptake was evaluated by *E. coli*-cy5.5. As exhibited in Figure S5A–S5C, the MPLA@PLGA-treated cells could uptake more *E. coli* compared to the PBS-treated cells, implying that MPLA@PLGA nanoparticles could enhance bacterial endocytosis of RAW 264.7 cells. Furthermore, the cells treated by MPLA@PLGA nanoparticles could more effectively kill bacteria than that treated by PBS (Figure S6A&S6B), demonstrating that MPLA@PLGA nanoparticles could enhance the bactericidal capacity of RAW 264.7 cells. The above results showed that the bactericidal ability of RAW 264.7 cells treated by MPLA@PLGA nanoparticles was enhanced by promoting M1 polarization and increasing bacterial uptake.

The clinical researches showed that more than half of the patients with sepsis would encounter with a period of continuous immunosuppression, in which the patients would easier suffer from secondary infection within a few weeks or months compared to healthy human. Therefore, the immune system in the lived mice was analyzed at 30 days after sepsis challenge (Fig. 4a). It was found that the percentages of CD4⁺ T cells, NK cells, monocytes, B cells and memory CD4⁺ T cells were obviously decreased (Figs. 4b–f and S7A–S7E), implying the destruction of immunity homeostasis in the MPLA@PLGA-treated mice lived from sepsis challenge. However, the percentages of neutrophils, CD8⁺ T cells and memory CD8⁺ T cells in the survived mice were significantly increased, implying the generation of immune memory and enhanced antibacterial capability (Figs. 4g–i and S8A–S8C). Moreover, the secretion of various functional cytokines, particularly including IL-1 β , IL-27, IL-17A, IFN- β and IL-10, was significantly increased in the MPLA@PLGA-treated mice lived from the lethal *E. coli* challenge (Figs. 4j–n and

Figure 3 The in vivo changes of immune phenotypes induced by the MPLA@PLGA nanoparticles. **a** Schematic illustration to evaluate the change of immune phenotypes in the mice with different treatments before or after *E. coli*-induced sepsis challenge. (**b–d**) The proportions of NK cells (NK1.1⁺) in CD45⁺ cells (**b**), monocytes (CD14⁺) in CD45⁺ cells (**c**) and CD80⁺ cells in F4/80⁺ cells (**d**) in blood of the mice pretreated with the MPLA@PLGA nanoparticles before sepsis challenge. (**e–h**) The proportions of NK cells (NK1.1⁺) in CD45⁺ cells (**e**),

neutrophils (Gr-1⁺) in CD11b⁺ cells (**f**), CD80⁺ cells in F4/80⁺ cells (**g**) in blood and NK cells (NK1.1⁺) in CD45⁺ cells (**h**) in peritoneal lavage of the mice from the two groups after sepsis challenge ($n = 4$ per group). (**i–n**) Cytokine levels including TNF-α (**i**), IFN-γ (**j**), IL-6 (**k**), IL-17A (**l**), MCP-1 (**m**) and IL-10 (**n**) in sera from the pretreated mice before or at 6 h after *E. coli* challenge ($n = 4$ per group). Data are presented as means $\pm$ SEM. Statistical significance was calculated by Student's *t*-test.

S9A–S9E), showing the enhanced capacity for secondary *E. coli*-induced sepsis protection. Furthermore, the *E. coli* counts in the organs of the mice, including liver, spleen, lung and kidney, further confirmed the improved capacity to resist *E. coli* infection in the survived mice (Fig. 4o and p).

To evaluate the capacity of MPLA@PLGA-treated mice survived from lethal *E. coli* infection to resist secondary infection, the survived mice and the healthy mice were challenged by various lethal infections

(Fig. 5a). It was amazing that all the MPLA@PLGA-treated mice lived from the first lethal *E. coli* infection, even though in the state of immunity disorder, could resist the second sepsis challenge (*E. coli* 1×10^7 CFU, 200 μ L) (Fig. 5b). The body weight of the mice lived from sepsis challenge could be back to the initial weight about 4 days after the second *E. coli*-induced sepsis (Fig. 5c). However, the MPLA@PLGA-treated mice lived from the first *E. coli* infection could not resist lethal S.a.u infection

Figure 4 In vivo protection for *E. coli*-induced sepsis again in survival mice. **a** Schematic illustration to evaluate the changes of immune cells for repeated *E. coli*-induced sepsis in the survival mice. **(b–i)** The percent of CD4⁺ T cells in CD3⁺ T cells **(b)**, NK cells (NK1.1⁺) in CD45⁺ cells **(c)**, monocytes (CD14⁺) in CD45⁺ cells **(d)**, B cells (CD19⁺) in CD45⁺ cells **(e)**, CD4⁺ T_m cells (CD4⁺CD44⁺) in CD3⁺ cells **(f)**, neutrophils (Gr-1⁺) in CD11b⁺ cells **(g)**, CD8⁺ T cells in CD3⁺ T cells **(h)** and CD8⁺ T_{em} cells (CD44⁺CD62L^{low}) in CD3⁺CD8⁺ cells **(i)** in the blood of normal

healthy mice and the mice survived at 30 days after the first sepsis challenge ($n = 4$ per group). **j–n** Cytokine levels including IL-1β **(j)**, IL-27 **(k)**, IL-17A **(l)**, IFN-β **(m)** and IL-10 **(n)** in sera from the normal mice or the survived mice at 30 days after the first sepsis challenge ($n = 4$ per group). **o** and **p** Representative images **(o)** and the statistic data **(p)** of *E. coli* colonization in the major organs of the mice at 6 h after the second sepsis challenge ($n = 3$ per group). Data are presented as means ± SEM. Statistical significance was calculated by Student's t-test.

Figure 5 In vivo protection for repeated *E. coli*-induced sepsis in the survived mice. **a** Schematic illustration to show the protection effect against for repeated sepsis in the survival mice. **b** and **c** Survival rate (**b**) and individual body weight (**c**) of the mice after repeated *E. coli*-induced sepsis challenge ($n = 10$ per group).

(1.4×10^8 CFU, 200 μ L) to achieve S.a.u.-induced sepsis protection (Fig. 5d and e). Therefore, the MPLA@PLGA nanoparticles as a kind of nano-drug could effectively and specifically protect the mice from repeated *E. coli*-induced sepsis.

Conclusion

In this study, a kind of nano-drug (MPLA@PLGA nanoparticles) was developed by encapsulating MPLA, a hydrophobic TLR4 agonist, with PLGA nanoparticles for sepsis protection. Such nanoparticles could activate in vivo innate immune system by up-regulating the percentages of NK cells and macrophages in the blood and the lesion, increasing the levels of various functional cytokines and accelerating *E. coli* clearance in major organs to achieve sepsis protection after *E. coli*-induced sepsis challenge. Furthermore, the mice pre-treated with the MPLA@PLGA nanoparticles that survived after first sepsis challenge could resist the second sepsis challenge by building acquired immunity for *E. coli* infection. Therefore, this nanoparticle could be served as a simple but effective nano-drug in the prevention and protection of *E. coli*-induced sepsis.

d and **e** Survival rate (**d**) and individual body weight (**e**) of the mice after *S.a.u.*-induced sepsis challenge ($n = 5$ per group). S, survival rate. Data are presented as means $\pm$ SEM. Statistical significance was calculated by log-rank test.

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Declarations

Conflict of interest The authors declare no competing financial interest.

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