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Original Article

Inorganic polyphosphate modulates leukocyte accumulation and vascular endothelial cell permeability and ameliorates cecal ligation and puncture-induced lethality

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ABSTRACT — Inorganic polyphosphates with an average degree of polymerization of 150 (polyP$_{150}$) have been shown to improve mortality in a lipopolysaccharide model of sepsis in mice. We aimed to investigate the effects of polyP$_{150}$ in a mouse model of cecal ligation and puncture (CLP) peritonitis, which accurately reflects clinical sepsis, and elucidate its mechanism of action and suitability as a candidate for sepsis treatment. The present study demonstrated that treatment with polyP$_{150}$ significantly improved survival rate in mouse model of CLP peritonitis. polyP$_{150}$ inhibited a CLP-mediated increase in pulmonary vascular permeability as demonstrated by Evans blue dye assay. Pretreatment of polyP$_{150}$ in human vascular endothelial cells, HMEC-1 cells, showed inhibition of tumor necrosis factor-α-induced monocytic THP-1 cell adhesion and intercellular adhesion molecule 1/CD54 gene expression. These results suggest that polyP$_{150}$ ameliorates fatal sepsis by inhibiting expression of the cell adhesion molecule and the accumulation of leukocytes in the vascular endothelium, thereby suppressing the increase in vascular permeability. Our results in this study suggest that polyP$_{150}$ could be a candidate for novel sepsis treatments.

Key words: Sepsis, Inorganic polyphosphates, Cecal ligation and puncture, Vascular permeability

INTRODUCTION

Sepsis is defined as a life-threatening dysfunction of multiple organs caused by a dysregulated host response to infection (Singer *et al*., 2016). The activation of pattern recognition receptors (PRRs) by infection triggers the induction of pro-inflammatory cytokines and tissue damage by inflammation. Endogenous damage-associated molecular patterns released from inflammatory tissues further activate the PRRs, resulting in overproduction of cytokines (hypercytokinemia). In addition, hypercytokinemia results in uncontrolled immune stimulation and suppression, inflammatory responses, and activation of coagulation and fibrinolysis, leading to increased vas-

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cular permeability, disseminated fibrin, decreased blood pressure, hypercoagulation, and circulatory failure. These pathologies in septic shock eventually cause tissue inability to metabolize oxygen, multiple organ damage, and death (Gyawali *et al*., 2019; Hotchkiss *et al*., 2016; McConnell and Coopersmith, 2016).

Currently, it is estimated that one person dies from sepsis every few seconds worldwide (Nishida *et al*., 2018). Various treatments for sepsis have been investigated to date, but no medication has been established. Antimicrobial therapy is the primary treatment for the underlying disease, but has been plagued by the emergence of drug-resistant bacteria and stagnation of antibiotic development (Nishida *et al*., 2018). Other therapies targeting PRRs and cytokines as well as immunoglobulin therapy have been explored, but have not shown clear improvement in prognosis (Alejandria *et al*., 2013; Bernard *et al*., 2014; Opal *et al*., 2013). These situations call for a different approach to the development of therapeutic agents for sepsis.

Inorganic polyphosphate, a compound of phosphoric acid that has undergone linear polymerization, is present ubiquitously in all biological species and is known to have various biological functions depending on its molecular length (Harada *et al*., 2013a, 2013b). Inorganic polyphosphate (polyP) with an average polymerization degree of 150 (polyP$_{150}$) has been shown to improve organ damage and lethality in a mouse model of lipopolysaccharide (LPS)-induced endotoxin shock (Terashima-Hasegawa *et al*., 2019). However, the LPS model is considered to correspond to the end-stage in sepsis, and the basic clinical features of sepsis such as high cardiac output and hypermetabolic state have not been able to be reproduced (Gahhos *et al*., 1981; Wichterman *et al*., 1980). Therefore, studies on agents for sepsis treatment that appeared promising in endotoxin models have not proven efficacious in human clinical trials (Buras *et al*., 2005; Deitch, 1998; Opal *et al*., 2013).

In the present study, we aimed to investigate polyP$_{150}$ in a mouse model of cecal ligation and puncture (CLP) peritonitis, which r displays the typical signs of septic shock, ranging from a high cardiac output state with tachypnea, hyperglycemia, and hyperinsulinemia to a state of reduced cardiac output at the end of life (Li *et al*., 2018; Rittirsch *et al*., 2009; Wichterman *et al*., 1980). We also aimed to elucidate the mechanism of action of polyP$_{150}$ in a mouse model CLP sepsis model and whether polyP$_{150}$ is a suitable candidate for novel sepsis treatments.

MATERIALS AND METHODS

Animals

Seven- to eight-week-old male ddY mice were obtained from Sankyo Labo Service Co. (Tokyo, Japan). They were housed 5 per cage in plastic cages in a temperature-controlled room (22 ± 1°C) with a humidity of 40–60% and kept in a 12 hr light/dark cycle with free access to standard chow diet (F-2, Sankyo Labo Service Co.) and water. All procedures of the animal experiments were conducted according to the National Institutes of Health guidelines for the management and use of laboratory animals and approved by the Animal Care and Use Committee of Showa University (approval #, 28049). Every effort was made to minimize the number of mice used and their suffering. Mice were allowed to acclimate for a week before start of experiments.

Survival rate in CLP mouse model

Standardized protocol was followed to establish a CLP peritonitis mouse model (Murando *et al*., 2019; Rittirsch *et al*., 2009). The cecum was ligated at 50% of its total length and then perforated by a single puncture midway between the ligation and the tip of the cecum with a 21G needle under anesthesia using medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). The experiment was conducted in two sessions: one in which polyP$_{150}$ (0.1 mmol/kg, RegeneTiss, Okaya, Japan) or saline (10 mL/kg) was intraperitoneally administered a day before CLP and one in which either polyP$_{150}$ (0.1 mmol/kg) or saline (10 mL/kg) was intraperitoneally administered a day after CLP. In both sessions, polyP$_{150}$ (0.1 mmol/kg) or saline (10 mL/kg) was administered once daily after CLP until the end of the session. Therefore, the only difference between these two sessions was whether polyP$_{150}$ was administered before CLP or not. Survival of the animals was observed for 14 days after CLP. In the sham animals, the cecum is exteriorized but neither ligated nor punctured.

Vascular permeability in CLP mouse model

Vascular permeability was assessed using the Evans blue dye assay (Radu and Chernoff, 2013; Wick *et al*., 2018). Mice were subjected to CLP and 300 µL of 0.5% Evans blue (Fujifilm, Tokyo, Japan) was administered at the tail vein under isoflurane anesthesia 72 hr later. The chest was opened 30 min after dye injection under anesthesia using medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg), and the lung tissue was removed and weighed. A part of the weighed tissue was dried at 150°C for 24 hr, and the wet/dry weight ratio

was calculated. The other portion of the lung tissue was homogenized with 1.5 mL of formamide and the homogenate was incubated at 55°C for 24 hr. After centrifugation at 15,000 rpm/4°C for 30 min, the optical density of the supernatant was determined using a dual-wavelength spectrophotometer (620/740 nm). The extravasated dye concentration in the lung homogenate was calculated against a standard curve (μg Evans Blues dye/g lung).

Cell culture

Immortalized human dermal microvascular endothelial cells-1 (HMEC-1) and human acute monocytic leukemia THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and Riken Cell Bank (Ibaraki, Japan), respectively. HMEC-1 cells were cultivated in MCDB131 medium (Sigma-Aldrich, St Louis, MO, USA) containing 10 ng/mL recombinant murine epidermal growth factor (rmEGF; Fujifilm), 1 μg/mL hydrocortisone (Fujifilm), 10 mM L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). THP-1 cells were cultivated in RPMI 1640 (Nissui, Tokyo, Japan) containing 10 mM L-glutamine, 20 mM HEPES, 10 units/mL penicillin 100 μg/mL streptomycin, and 10% FBS. Cells were maintained at a logarithmic growth phase in a humidified incubator at 37°C and 5% CO_2.

Monocyte-endothelial cell interactions

HMEC-1 cells were seeded onto 96-well plates (5×10^4/well) and cultured for 48 hr until subconfluence was achieved. The medium was replaced with rmEGF (-) and FBS (-) MCDB131 medium and cells were cultured for another 12 hr. They were pretreated with 10 μM $polyP_{150}$ or phosphate-buffered saline (PBS) as a control for 4 hr and then either treated with 25 ng/mL tumor necrosis factor (TNF)-α (PeproTech, Cranbury NJ, USA) or untreated for 20 hr. The medium of THP-1 cells was replaced with FBS (-) RPMI 1640 the day before the experiment. THP-1 cells were labeled with 5 μM calcein AM (Chemical Dojin, Kumamoto, Japan) for 30 min, washed twice with PBS, and incubated with FBS (-) RPMI 1640 containing antibiotics and 0.5% bovine serum albumin. HMEC-1 cells were co-cultured with labeled THP-1 cells (1×10^5 cells/well) at room temperature in the dark for 30 min (Guo *et al*., 2009; Shu *et al*., 2012). In an experiment where THP-1 cells were treated with TNF-α, THP-1 cells were pretreated with 10 μM $polyP_{150}$ or PBS for 4 hr and then treated with 25 ng/mL TNF-α for 20 hr. The cells were then labeled with calcein AM and co-cultured with HMEC-1. In an experiment where HMEC-1 cells were treated with TNF-α followed by $polyP_{150}$, HMEC-1 cells were either untreated or pretreated with 25 ng/mL TNF-α for 4 hr and then treated with PBS or 10 μM $polyP_{150}$ for 20 hr. The THP-1 cell-attached HMEC-1 cells were washed three times for 5 min with 200 μL of PBS to remove non-adherent cells. The fluorescence intensity was quantified using SpectraMax i3 Multi-Mode Platform (Molecular Devices, San Jose, CA, USA).

RNA isolation and qPCR

HMEC-1 cells were seeded onto six-well plates (2×10^5/well) and cultured for 48 hr. The medium was replaced with rmEGF (-), FBS (-) MCDB131 medium and the cells were cultured for another 12 hr. The cells were pretreated with 10 μM $polyP_{150}$ or PBS as a control for 4 hr and then either treated with 25 ng/mL TNF-α or untreated for 4 hr. RNA was prepared from HMEC-1 cells according to the protocol for the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). cDNAs were prepared using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's protocol. Primers (Thermo Fisher Scientific) used for human intercellular adhesion molecule 1 (ICAM-1) were: forward 5'-CCTACCAGCTCCAGACCTTT-3', reverse 5'-AAGGAGTCGTTGCCATAGGT-3'; those for human vascular cell adhesion molecule 1 (VCAM-1) were: forward 5'- GAAGGTGACGAATGAGGGGA-3' and reverse 5'-CTTGACTGTGTCGGCTTCC-3'; and those for human glyceraldehyde 3-phosphate dehydrogenase were: forward 5'- GCTCACTGGCATGGCCTTCCG-3' and reverse 5'-GTGGGCCATGAGGTCCACCAC-3'. Quantitative PCR (qPCR) was performed on a QuantStudio 3 (Thermo Fisher Scientific) according to the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) protocol. The mRNA levels were measured relative to glyceraldehyde 3-phosphate dehydrogenase mRNA expression in each run. All samples were measured in triplicate and analyzed by the ΔΔCT method.

Statistical analysis

All values are expressed as mean ± standard error of mean. Survival rates were analyzed by the log-rank test and presented as Kaplan-Meier survival curves. Multiple tests were performed using Dunnett's test for comparisons against the respective control group. Statistically significance in all cases was defined at $P < 0.05$.

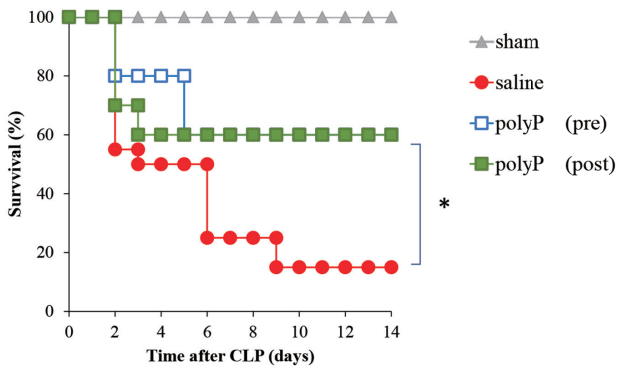

Fig. 1. Effect of $polyP_{150}$ on survival rate in the CLP mouse model. Survival rate of mice administered saline (10 mL/kg, closed circle), $polyP_{150}$ (100 µmol/kg, i.p.) one day before and every day after CLP (pre, open square) or everyday day after CLP (post, closed square). Sham group (closed triangle) received sham operation and saline. Survival curves were compared by log-rank test (*P < 0.05, n = 10 in sham and $polyP_{150}$ groups and n = 20 in saline group).

RESULTS

Effect of $polyP_{150}$ on survival rate in the CLP mouse model

We examined the effects of $polyP_{150}$ on the CLP mouse model in the present study. Based on preliminary experiments, the conditions of CLP were set as a single puncture with a 21G needle. Under these experimental conditions, 85% of animals in the CLP group treated intraperitoneally with saline died after 9 days of treatment in both cases which mice were treated with saline a day before CLP and thereafter as well as with saline after CLP for the observation period. These control groups did not differ significantly, therefore, were combined and are shown as a saline group in Fig. 1. In contrast, daily intraperitoneal $polyP_{150}$ administration at a dose of 0.1 mmol/kg from a day before and every day after CLP significantly improved the mortality rate; 60% of the animals were still alive after 14 days under the experimental conditions (Fig. 1, open square). Similar effect of $polyP_{150}$ was observed when administered after CLP treatment (Fig. 1, closed square).

Effect of $polyP_{150}$ on vascular permeability in CLP mouse model

Increased vascular permeability and disruption of its barrier function are largely responsible for the pathogenesis of CLP-induced septic shock (Jiang *et al.*, 2020; Joffre *et al.*, 2020). Therefore, the effects of $polyP_{150}$ on the increased vascular permeability in the CLP model, which has been known to occur in this animal model, was examined. $polyP_{150}$ was administered a day before and every day for 2 days after CLP, and vascular permeability in lung tissue was examined 72 hr after CLP using the Evans blue assay. Compared with the sham group, CLP increased Evans blue infiltration into lung tissue by approximately two-fold. In contrast, the administration of $polyP_{150}$ almost completely suppressed the CLP-induced accumulation of Evans blue in the lungs (Fig. 2a). Pulmonary edema was examined by determining the ratio of wet to dry weight of lung tissue. Under these experimental conditions, $polyP_{150}$ inhibited the CLP-induced increase in water content of the lungs (Fig. 2b). These results indicate that $polyP_{150}$ suppresses the vascular permeability and pulmonary edema in the CLP model.

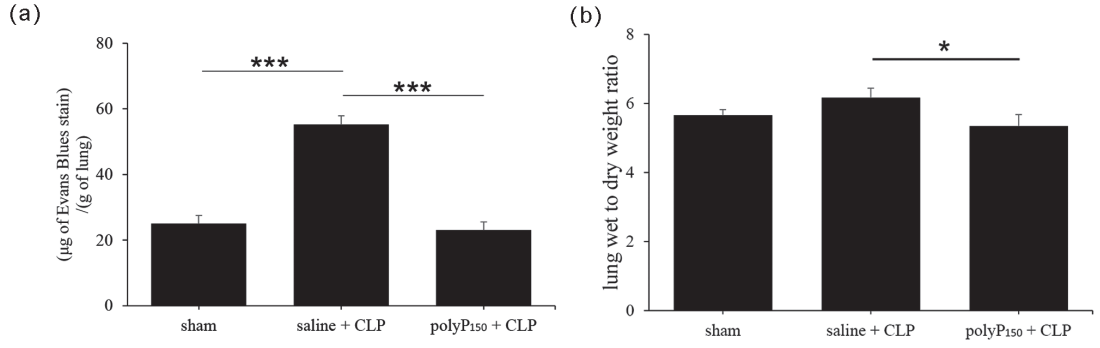

Fig. 2. Effect of $polyP_{150}$ on pulmonary vascular permeability in CLP mouse model. Mice were subjected to CLP and were administered either saline or $polyP_{150}$ (100 µmol/kg), 1 day before and every day after CLP for 2 days. Lung tissues were removed from the animals 3 days after CLP. (a) The amount of Evans blue permeated into the lung tissue is indicated per wet weight of the lung. (b) The wet to dry weight ratio of lung tissue is shown. *P < 0.05, ***P < 0.001 (Dunnett's test compared with CLP group as a control, n = 4).

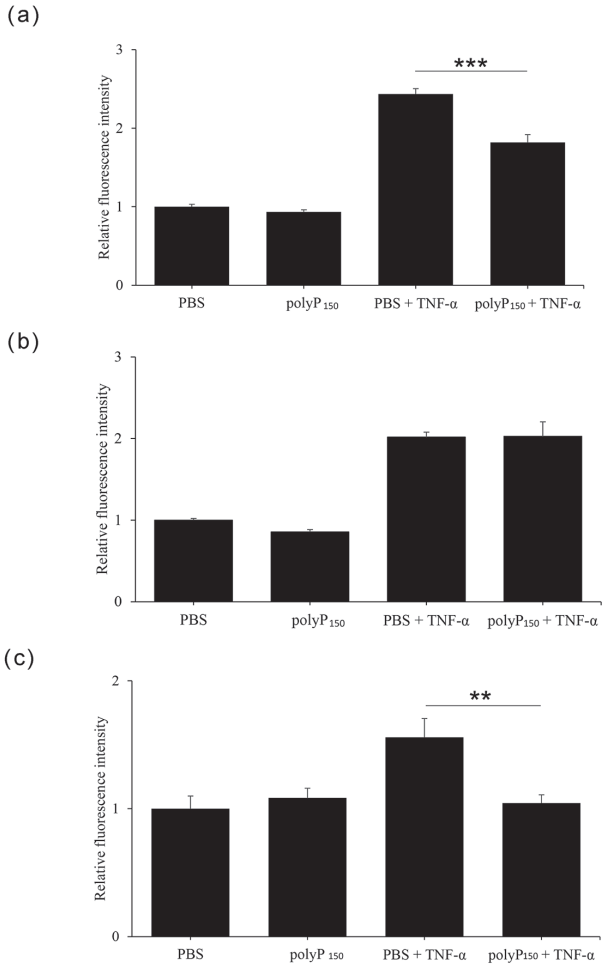

Fig. 3. Effect of $polyP_{150}$ on TNFα mediated monocyte-endothelial cell adhesion. (a) HMEC-1 cells were pretreated with PBS or 10 μM $polyP_{150}$ for 4 hr and either untreated or treated with 25 ng/mL TNF-α for 20 hr. They were co-cultured with fluorescently labeled THP-1 cells. (b) THP-1 cells were pre-treated with PBS or 10 μM $polyP_{150}$ for 4 hr and either untreated or treated with 25 ng/mL TNF-α for 20 hr. They were co-cultured with fluorescently labeled THP-1 cells. (c) HMEC-1 cells were either untreated or pretreated with 25 ng/mL TNF-α for 4 hr and then treated with PBS or 10 μM $polyP_{150}$ for 20 hr. They were co-cultured with fluorescently labeled THP-1 cells. The fluorescence intensity attached to HMEC-1 cells was measured and is shown as relative intensity. $^{**}P < 0.01$, $^{***}P < 0.001$ (Dunnett's test compared with TNF-α group as a control, n = 12).

Effect of $polyP_{150}$ on monocyte-endothelial cell interactions

Inhibition of vascular permeability by $polyP_{150}$ may be one of the causes of improvement in the survival rate of the CLP mouse model. In general, the increased vascular permeability in sepsis is thought to be closely related to the fact that overproduced inflammatory mediators reduce the function of endothelial cell adhesion (Joffre *et al*., 2020). Meanwhile, it has also been shown that adhesion and infiltration of neutrophils to the pulmonary vascular endothelium play an important role in vascular permeability in the acute lung injury secondary to sepsis (Gill *et al*., 2015; Jiang *et al*., 2020; Marcus *et al*., 1997). Therefore, we investigated monocyte-endothelial cell interactions *in vitro* using monocytic cells that are recruited by a mechanism similar to neutrophil adsorption to the vascular endothelium (Ley *et al*., 2007; Nourshargh and Alon, 2014). In this experiment, we observed the adhesion of fluorescence-labeled monocytic THP-1 cells to vascular endothelium-derived HMEC-1 cells.

Compared with the control cells, treatment of HMEC-1 cells with TNF-α increased the relative fluorescence intensity approximately 2.4-fold. In contrast, pretreatment of HMEC-1 cells with 10 μM $polyP_{150}$ significantly attenuated the TNF-α-mediated increase in fluorescence (Fig. 3a). These results indicate that $polyP_{150}$ suppressed the TNF-α-induced monocyte adhesion to vascular endothelial cells. Conversely, when THP-1 cells were treated with $polyP_{150}$ and TNF-α, there was no sign of suppression (Fig. 3b), suggesting that $polyP_{150}$ acts on vascular endothelial cells. In addition, even when HMEC-1 cells were treated with $polyP_{150}$ after TNFα, the adhesion of THP-1 cells to HMEC-1 cells was attenuated (Fig. 3c).

Next, series of experiments were conducted to observe the gene expression of ICAM-1 and VCAM-1 in HMEC-1 cells by qPCR. Compared with the control cells, the expression level of ICAM-1 was increased approximately 144-fold by TNF-α treatment. Pretreatment of the cells with $polyP_{150}$ significantly reduced the effect of TNF-α by about half (Fig. 4a). In contrast, $polyP_{150}$ had little effect on the induction of VCAM-1 by TNF-α treatment (Fig. 4b), indicating that $polyP_{150}$ specifically suppresses ICAM-1 expression by TNF-α in HMEC-1 cells. Thus, our results suggest that $polyP_{150}$-mediated inhibition of the recruitment of monocytes to vascular endothelial cells is at least partly mediated through suppression of ICAM-1 gene expression.

DISCUSSION

This study expands our previous reports with the aim of testing the potential of $polyP_{150}$ as a new therapeutic strategy for sepsis. Although we previously reported that $polyP_{150}$ improves mortality and organ damage in a mouse

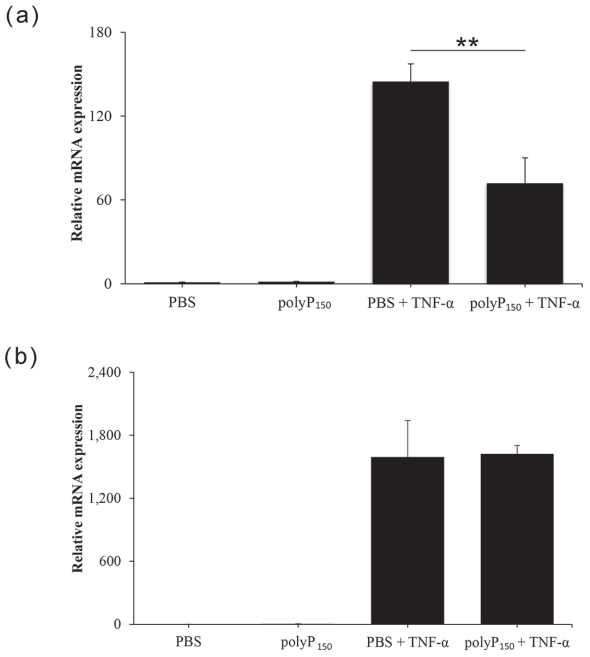

Fig. 4. Effect of $polyP_{150}$ on the gene expression of cell adhesion molecules induced by TNF-α in HMEC-1 cells. HMEC-1 cells were pretreated with PBS or 10 μM $polyP_{150}$ for 4 hr and either untreated or treated with 25 ng/mL TNF-α for 4 hr. The expression levels of ICAM-1(A) and VCAM-1(B) in HMEC-1 cells were determined by qPCR and are shown as the relative gene expression. **P < 0.01 (Dunnett's test compared with TNF-α group as a control, n = 3).

model of LPS sepsis (Terashima-Hasegawa *et al*., 2019), the present study shows that $polyP_{150}$ also has a certain lethality-improving effect in a mouse model of CLP (Murando *et al*., 2019; Rittirsch *et al*., 2009; Wichterman *et al*., 1980), which resembles the clinical picture of sepsis more closely.

Our previous report showed that $polyP_{150}$ protects against LPS-induced death and organ damage by modulating the recruitment of macrophages to tissues such as lung and liver (Terashima-Hasegawa *et al*., 2019). It has been suggested that activated inflammatory cells accumulate in the lungs under septic conditions and release cytokines and reactive oxygen species, disrupting pulmonary vascular endothelial and epithelial cells and increasing pulmonary vascular permeability (Gill *et al*., 2015; Jiang *et al*., 2020; Marcus *et al*., 1997). Excessive leukocyte accumulation may destabilize alveolar barrier function and increase vascular permeability, resulting in further disruption of alveolar barrier function. The major pathologies of acute respiratory distress syndrome secondary to sepsis are hyperpermeable pulmonary edema due to the damage to pulmonary vascular endothelial cells (Matute-Bello *et al*., 2008; Zhou *et al*., 2019). As an initial response, pro-inflammatory cytokines such as IL-1β and TNF-α produced by monocytes/macrophages and activated by endotoxins and other infectious factors lead to an increased expression of adhesion molecules such as ICAM-1 and VCAM-1 on pulmonary vascular endothelial cells (Idriss and Naismith, 2000; De Freitas Caires *et al*., 2018; Lee *et al*., 2011; Niu *et al*., 2020). These adhesion molecules are thought to cause migration and infiltration of leukocytes from the vessels to the interstitium, releasing activated proteases and reactive oxygen species and inducing tissue damage (Amin *et al*., 2006). In the present study, we also observed increased pulmonary vascular permeability in the early stages of CLP treatment, which is thought to be related to CLP-mediated mortality (Aziz *et al*., 2018). Furthermore, increased pulmonary vascular permeability was significantly attenuated by administration of $polyP_{150}$, which reduced it to levels similar to those in the sham group. Thus, it is suggested that $polyP_{150}$ may improve mortality in the CLP sepsis model, at least in part, by suppressing the hyperpermeability of the pulmonary vessels.

According to previous reports, TNF-α released from macrophages is considered to be a major mediator among the diverse inflammatory cytokines that contribute to septic pathology (Brenner *et al*., 2015; Terashima-Hasegawa *et al*., 2019). Furthermore, $polyP_{150}$ inhibits macrophage activation by inhibiting TNF-α-induced phosphorylation of c-Jun N-terminal kinase (JNK)/p38 signal cascade (Terashima-Hasegawa *et al*., 2019). This study showed that ICAM-1 expression is repressed by $polyP_{150}$ in HMEC-1 cells. Thus, it is suggested that $polyP_{150}$ acts not only on autocrine activation of macrophages but also on vascular endothelial cells by TNF-α, suppressing the expression of ICAM-1, which is closely associated with increased vascular permeability. However, the present study also showed that $polyP_{150}$ had virtually no effect on the induction of VCAM-1 expression by TNF-α. The detailed mechanism of this selectivity of $polyP_{150}$ for ICAM-1 and VCAM-1 expression needs to be elucidated in future studies. The effect of $polyP_{150}$ on JNK/p38 signal cascade, an upstream signaling pathway leading to ICAM-1 expression by TNF-α, also needs to be investigated.

The recruitment of neutrophils to the endothelium plays an important role in the disruption of the barrier function of vascular endothelial cells (Gill *et al*., 2015; Jiang *et al*., 2020; Marcus *et al*., 1997). Neutrophils and monocytes are thought to adhere to endothelial cells by

similar mechanisms (Ley *et al*., 2007; Nourshargh and Alon, 2014). Therefore, the present study examined the recruitment of THP-1 cells to HMEC-1 cells as an *in vitro* model (Choi *et al*., 2018; Zhao *et al*., 2017). Treatment of HMEC-1 cells with $polyP_{150}$ before as well as after TNF-α inhibited the adhesion of THP-1 cells to HMEC-1 cells. This inhibition may involve, at least in part, the repression of ICAM-1 induction of HMEC-1 cells. The results obtained here are consistent with the results obtained with *in vivo* experiments where $polyP_{150}$ was effective even when treated after CLP. In the present study, we also tested a system in which THP-1 cells were stimulated with TNF-α, but $polyP_{150}$ had no effect on the induction of cell adhesion to HMEC-1 cells. These results indicate that $polyP_{150}$ acts on the endothelium and has little effect on monocytes with respect to the cell-to-cell adhesion.

Accumulated findings indicate that polyP with 60–100 phosphate residues released from platelets shows pro-inflammatory responses with activation of the coagulation pathway *in vivo* (Bae *et al*., 2012; Dinarvand *et al*., 2014; Müller *et al*., 2009). However, these studies used from 15 to 30 times greater dose of polyP (0.15–0.3 mg/g body weight, which mean approximately 1.5–3 mmol/kg body weight) compared to the current study. In addition, these values were calculated based on the number of moles in monomer, and considering that their studies used a lower molecular weight polyP than ours, the difference in dosage would be much larger. Furthermore, our preliminary study indicates that higher dose of $polyP_{150}$ (1 mmol/kg) was ineffective to LPS-induced lethality (Terashima-Hasegawa *et al*., 2019). It has already been demonstrated that polyP shows biological effects in a chain length-dependent manner (Angelova *et al*., 2016). Indeed, bacterial long-chain polyP has been shown to potentiate pathogenicity by inhibiting phagosome maturation (Roewe *et al*., 2020) and interfering with macrophage function (Rijal *et al*., 2020). Therefore, it is suggested that the response of polyP to inflammation will be different depending on the dose and degree of polymerization.

In summary, the results of the present study demonstrated that $polyP_{150}$ significantly inhibited vascular permeability and increased survival in the CLP animal model. In addition, it was suggested that a part of this mechanism may include the suppression of ICAM-1 expression, which inhibited leukocyte adhesion in pulmonary vascular endothelial cells, and protects the lung tissue from inflammatory response. Although this study focused on pulmonary vascular permeability, there may be other mechanisms involved in $polyP_{150}$-mediated improvement of septic mortality that is yet to be determined. We hope that further elucidation of the mechanism of $polyP_{150}$ will lead to its clinical use in the future.

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Conflict of interest---- The authors T. Shiba and Y. Kawazoe are affiliated with Regenetiss Inc. This does not alter the authors' adherence to the Biomolecules policies on sharing data and materials.

REFERENCES

Alejandria, M.M., Lansang, M.A., Dans, L.F. and Mantaring, J.B. (2013): Intravenous immunoglobulin for treating sepsis, severe sepsis and septic shock. Cochrane Database Syst. Rev., CD001090.

Amin, M.A., Haas, C.S., Zhu, K., Mansfield, P.J., Kim, M.J., Lackowski, N.P. and Koch, A.E. (2006): Migration inhibitory factor up-regulates vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 via Src, PI3 kinase, and NFκB. Blood, **107**, 2252-2261.

Angelova, P.R., Baev, A.Y., Berezhnov, A.V. and Abramov, A.Y. (2016): Role of inorganic polyphosphate in mammalian cells: from signal transduction and mitochondrial metabolism to cell death. Biochem. Soc. Trans., **44**, 40-45.

Aziz, M., Ode, Y., Zhou, M., Ochani, M., Holodick, N.E., Rothstein, T.L. and Wang, P. (2018): B-1a cells protect mice from sepsis-induced acute lung injury. Mol. Med., **24**, 26.

Bae, J.S., Lee, W. and Rezaie, A.R. (2012): Polyphosphate elicits pro-inflammatory responses that are counteracted by activated protein C in both cellular and animal models. J. Thromb. Haemost., **10**, 1145-1151.

Bernard, G.R., Francois, B., Mira, J.P., Vincent, J.L., Dellinger, R.P., *et al*. (2014): Evaluating the efficacy and safety of two doses of the polyclonal anti-tumor necrosis factor-α fragment antibody AZD9773 in adult patients with severe sepsis and/or septic shock: randomized, double-blind, placebo-controlled phase IIb study*. Crit. Care Med., **42**, 504-511.

Brenner, D., Blaser, H. and Mak, T.W. (2015): Regulation of tumour necrosis factor signalling: live or let die. Nat. Rev. Immunol., **15**, 362-374.

Buras, J.A., Holzmann, B. and Sitkovsky, M. (2005): Animal models of sepsis: setting the stage. Nat. Rev. Drug Discov., **4**, 854-865.

Choi, H.J., Kim, N.E., Kim, B.M., Seo, M. and Heo, J.H. (2018): TNF-α-Induced YAP/TAZ Activity Mediates Leukocyte-Endothelial Adhesion by Regulating VCAM1 Expression in Endothelial Cells. Int. J. Mol. Sci., **19**, 3428.

De Freitas Caires, N., Gaudet, A., Portier, L., Tsicopoulos, A., Mathieu, D. and Lassalle, P. (2018): Endocan, sepsis, pneumonia, and acute respiratory distress syndrome. Crit. Care, **22**, 280.

Deitch, E.A. (1998): Animal models of sepsis and shock: a review and lessons learned. Shock, **9**, 1-11.

Dinarvand, P., Hassanian, S.M., Qureshi, S.H., Manithody, C., Eissenberg, J.C., Yang, L. and Rezaie, A.R. (2014): Polyphos-

phate amplifies proinflammatory responses of nuclear proteins through interaction with receptor for advanced glycation end products and P2Y1 purinergic receptor. Blood, **123**, 935-945.

Gahhos, F.N., Chiu, R.C., Bethune, D., Dion, Y., Hinchey, E.J. and Richards, G.K. (1981): Hemodynamic responses to sepsis: hypodynamic versus hyperdynamic states. J. Surg. Res., **31**, 475-481.

Gill, S.E., Rohan, M. and Mehta, S. (2015): Role of pulmonary microvascular endothelial cell apoptosis in murine sepsis-induced lung injury *in vivo.* Respir. Res., **16**, 109.

Guo, Y.L., Bai, R., Chen, C.X., Liu, D.Q., Liu, Y., Zhang, C.Y. and Zen, K. (2009): Role of junctional adhesion molecule-like protein in mediating monocyte transendothelial migration. Arterioscler. Thromb. Vasc. Biol., **29**, 75-83.

Gyawali, B., Ramakrishna, K. and Dhamoon, A.S. (2019): Sepsis: the evolution in definition, pathophysiology, and management. SAGE Open Med., **7**, 2050312119835043.

Harada, K., Itoh, H., Kawazoe, Y., Miyazaki, S., Doi, K., Kubo, T., Akagawa, Y. and Shiba, T. (2013a): Polyphosphate-mediated inhibition of tartrate-resistant acid phosphatase and suppression of bone resorption of osteoclasts. PLoS One, **8**, e78612.

Harada, K., Shiba, T., Doi, K., Morita, K., Kubo, T., Makihara, Y., Piattelli, A. and Akagawa, Y. (2013b): Inorganic polyphosphate suppresses lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) expression in macrophages. PLoS One, **8**, e74650.

Hotchkiss, R.S., Moldawer, L.L., Opal, S.M., Reinhart, K., Turnbull, I.R. and Vincent, J.L. (2016): Sepsis and septic shock. Nat. Rev. Dis. Primers, **2**, 16045.

Idriss, H.T. and Naismith, J.H. (2000): TNF α and the TNF receptor superfamily: structure-function relationship(s). Microsc. Res. Tech., **50**, 184-195.

Jiang, J., Huang, K., Xu, S., Garcia, J.G., Wang, C. and Cai, H. (2020): Targeting NOX4 alleviates sepsis-induced acute lung injury via attenuation of redox-sensitive activation of CaMKII/ERK1/2/MLCK and endothelial cell barrier dysfunction. Redox Biol., **36**, 101638.

Joffre, J., Hellman, J., Ince, C. and Ait-Oufella, H. (2020): Endothelial Responses in Sepsis. Am. J. Respir. Crit. Care Med., **202**, 361-370.

Lee, S.Y., Zaske, A.M., Novellino, T., Danila, D., Ferrari, M., Conyers, J. and Decuzzi, P. (2011): Probing the mechanical properties of TNF-α stimulated endothelial cell with atomic force microscopy. Int. J. Nanomedicine, **6**, 179-195.

Ley, K., Laudanna, C., Cybulsky, M.I. and Nourshargh, S. (2007): Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat. Rev. Immunol., **7**, 678-689.

Li, J.L., Li, G., Jing, X.Z., Li, Y.F., Ye, Q.Y., *et al*. (2018): Assessment of clinical sepsis-associated biomarkers in a septic mouse model. J. Int. Med. Res., **46**, 2410-2422.

Marcus, B.C., Hynes, K.L. and Gewertz, B.L. (1997): Loss of endothelial barrier function requires neutrophil adhesion. Surgery, **122**, 420-426, discussion, 426-427.

Matute-Bello, G., Frevert, C.W. and Martin, T.R. (2008): Animal models of acute lung injury. Am. J. Physiol. Lung Cell. Mol. Physiol., **295**, L379-L399.

McConnell, K.W. and Coopersmith, C.M. (2016): Pathophysiology of septic shock: from bench to bedside. Presse Med., **45**, e93-e98.

Müller, F., Mutch, N.J., Schenk, W.A., Smith, S.A., Esterl, L., Spronk, H.M., Schmidbauer, S., Gahl, W.A., Morrissey, J.H. and Renné, T. (2009): Platelet polyphosphates are proinflammatory and procoagulant mediators *in vivo.* Cell, **139**, 1143-1156.

Murando, F., Peloso, A. and Cobianchi, L. (2019): Experimental Abdominal Sepsis: Sticking to an Awkward but Still Useful Translational Model. Mediators Inflamm., **2019**, 8971036.

Nishida, O., Ogura, H., Egi, M., Fujishima, S., Hayashi, Y., *et al*. (2018): The Japanese Clinical Practice Guidelines for Management of Sepsis and Septic Shock 2016 (J-SSCG 2016). J. Intensive Care, **6**, 7.

Niu, F., Li, H., Xu, X., Sun, L., Gan, N. and Wang, A. (2020): Ursodeoxycholic acid protects against lung injury induced by fat embolism syndrome. J. Cell. Mol. Med.

Nourshargh, S. and Alon, R. (2014): Leukocyte migration into inflamed tissues. Immunity, **41**, 694-707.

Opal, S.M., Laterre, P.F., Francois, B., LaRosa, S.P., Angus, D.C., *et al*. (2013): Effect of eritoran, an antagonist of MD2-TLR4, on mortality in patients with severe sepsis: the ACCESS randomized trial. JAMA, **309**, 1154-1162.

Radu, M. and Chernoff, J. (2013): An *in vivo* assay to test blood vessel permeability. J. Vis. Exp., e50062.

Rijal, R., Cadena, L.A., Smith, M.R., Carr, J.F. and Gomer, R.H. (2020): Polyphosphate is an extracellular signal that can facilitate bacterial survival in eukaryotic cells. Proc. Natl. Acad. Sci. USA, **117**, 31923-31934.

Rittirsch, D., Huber-Lang, M.S., Flierl, M.A. and Ward, P.A. (2009): Immunodesign of experimental sepsis by cecal ligation and puncture. Nat. Protoc., **4**, 31-36.

Roewe, J., Stavrides, G., Strueve, M., Sharma, A., Marini, F., *et al*. (2020): Bacterial polyphosphates interfere with the innate host defense to infection. Nat. Commun., **11**, 4035.

Shu, Q., Amin, M.A., Ruth, J.H., Campbell, P.L. and Koch, A.E. (2012): Suppression of endothelial cell activity by inhibition of TNFα. Arthritis Res. Ther., **14**, R88.

Singer, M., Deutschman, C.S., Seymour, C.W., Shankar-Hari, M., Annane, D., *et al*. (2016): The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA, **315**, 801-810.

Terashima-Hasegawa, M., Ashino, T., Kawazoe, Y., Shiba, T., Manabe, A. and Numazawa, S. (2019): Inorganic polyphosphate protects against lipopolysaccharide-induced lethality and tissue injury through regulation of macrophage recruitment. Biochem. Pharmacol., **159**, 96-105.

Wichterman, K.A., Baue, A.E. and Chaudry, I.H. (1980): Sepsis and septic shock--a review of laboratory models and a proposal. J. Surg. Res., **29**, 189-201.

Wick, M.J., Harral, J.W., Loomis, Z.L. and Dempsey, E.C. (2018): An Optimized Evans Blue Protocol to Assess Vascular Leak in the Mouse. J. Vis. Exp.

Zhao, W., Feng, H., Guo, S., Han, Y. and Chen, X. (2017): Danshenol A inhibits TNF-α-induced expression of intercellular adhesion molecule-1 (ICAM-1) mediated by NOX4 in endothelial cells. Sci. Rep., **7**, 12953.

Zhou, Y., Li, P., Goodwin, A.J., Cook, J.A., Halushka, P.V., Chang, E., Zingarelli, B. and Fan, H. (2019): Exosomes from endothelial progenitor cells improve outcomes of the lipopolysaccharide-induced acute lung injury. Crit. Care, **23**, 44.