Essential title page

Title

Establishment of Disseminated Intravascular Coagulation (DIC) Model by a Single iv Administration of *E. coli*-derived Lipopolysaccharide (LPS) to Cynomolgus Monkeys and Evaluation of its Pathophysiological Status

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Running title

DIC Model by LPS in Cynomolgus Monkeys

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Word count

3997

Abstract

We prepared a DIC model by administrating LPS to cynomolgus monkeys, and investigated its potential for evaluations of new medicines for DIC therapy. Peripheral blood mononuclear cells (PBMC) collected from cynomolgus monkeys were incubated with LPS (8 types), and TNF-α levels in the media were measured. LPS from *E. coli* (K-235) was most appropriate in terms of larger increases and smaller variation in TNF-α levels. PBMC from rats, cynomolgus monkeys or humans were incubated with LPS (K-235), and the TNF-α response to LPS was investigated. The response was comparable between cynomolgus monkeys and humans but small in rats. In an *in vivo* experiment, LPS (K-235) was administered once intravenously to cynomolgus monkeys with or without recombinant human thrombomodulin (rhTM) to investigate any changes in coagulation and fibrinolysis biomarkers and the suppressive effect of rhTM. The liver, kidney, and lung were examined histopathologically. Almost all of the changes resembled the pathophysiological status of human DIC and were suppressed by co-administration of rhTM. The DIC model resembling human DIC was established by LPS (K-235) treatment in cynomolgus monkeys, and therapeutic effect of rhTM was noted, suggesting that this model is useful in evaluations of the efficacy of new medicines for DIC therapy.

Key words: Thrombomodulin, LPS, DIC

1. Introduction

Disseminated intravascular coagulation (DIC), a severe clinical condition caused by an underlying disease, involves a marked, continuous, and widespread activation of coagulation in circulating blood and the formation of many microvascular thrombi. When the clinical condition progresses, a consumptive coagulopathy occurs. The typical underlying diseases in DIC are sepsis, solid cancer, aortic aneurysm, and acute promyelocytic leukemia, and the activation of coagulation and fibrinolysis differ depending on the underlying disease (1). Sepsis is the most common underlying disease for DIC, and many studies have been conducted with sepsis-induced DIC models (2, 3, 4).

Most of these reports are for rat models induced by lipopolysaccharide (LPS) (5, 6). No report was found regarding DIC model induced by LPS in NHPs. Many therapeutic agents for thrombosis are protein formulations, and if homology of the reactive site between animals and humans is low, the therapeutic agents for humans do not react to animals. Therefore, the cross reactivity between animals and humans is an important issue in the evaluation of the therapeutic efficacy of agents in animal models. rhTM has been widely used for DIC therapy in humans, and Mohri *et al*. (7) reported the cross reactivity of rhTM in several animal species. Cross reactivity of rhTM in rodents is very low, and the therapeutic efficacy of rhTM observed in both humans and cynomolgus monkeys was not observed in rats (7). In light of the issue of cross reactivity, the development of an LPS-induced DIC model in NHPs is important for the development of new medicines.

In the present study, we investigated appropriate types of LPS and animal species by *in vitro* experiments and produced a DIC model by a single intravenous administration of LPS in cynomolgus monkeys which were confirmed by several biomarkers noted in the diagnostic criteria for human DIC. Furthermore, the effects of rhTM were investigated by co-administration with LPS using the DIC model in cynomolgus monkey.

2. Materials and Methods

2.1. Animals

Male cynomolgus monkeys aged 4 to 7 years, purpose-bred in China and maintained at Shin Nippon Biomedical Laboratories, Ltd. (SNBL, Tokyo, Japan), were used. The animals were housed individually in temperature and humidity controlled room on a 12-h light/dark cycle. Approximately 108 g (approximately 12 g × 9 pieces) of pellet food (HF Primate J 12G 5K9J, Purina Mills, LLC., Missouri, USA) were provided to each animal daily, and water was available *ad libitum*. Male Sprague-Dawley rats aged 9 to 10 weeks, purpose-bred in Charles River Laboratories Japan, Inc. and maintained at SNBL, were used. The animals were housed individually in temperature and humidity controlled room on a 12-h light/dark cycle. Solid food (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were available *ad libitum*. These monkey and rat studies were approved by the Institutional Animal Care and Use Committee of SNBL, and were conducted in accordance with the bylaws of the committee.

2.2. Humans

Six healthy mongoloid men aged from 20 to 40 years were involved. This study using human PBMC was approved by the Human Ethics Committee of SNBL, and conducted in accordance with the bylaws of the committee.

2.3. Blood sampling

Cynomolgus monkey blood was drawn from the femoral vein. Rat blood was drawn from the abdominal aorta under anesthesia by an intraperitoneal injection of sodium pentobarbital (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) solution (6.48 mg/mL, 5 mL/kg). Human blood was drawn from the vein of the upper arm.

2.4. Preparation of PBMC

Equal volume of Hank's Balanced Salt Solution (Life Technologies, MA, USA) was mixed with heparinized blood from cynomolgus monkeys, rats or humans. The mixture was slowly added to a tube already containing Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The tube was centrifuged and PBMC was obtained. The PBMC and Hank's Balanced Salt Solution were mixed and centrifuged. The supernatant after centrifugation was discarded, and the remaining was mixed with Hank's Balanced Salt Solution again. The mixture was centrifuged and the supernatant was discarded. The pellet obtained was mixed with RPMI (Life Technologies, MA, USA) containing 10% fetal bovine serum (Equitech-Bio Inc., TX, USA) and 1% penicillin/streptomycin (Invitrogen Ltd., Paisley, UK), and prepared to 3×10^6 cell/mL of PBMC.

2.5. Experiment I (Selection of appropriate LPS)

LPS purified from *Escherichia coli* (*E. coli*, 0111:B4, 055:B5, 026:B6, 0127:B8, K-235, and 0128:B12), *Pseudomonas aeruginosa* 10 (*P. aeruginosa*), and *Salmonella enterica* serotype enteritidis (*S. enterica*) were purchased from Sigma-Aldrich Co. LLC (Missouri, USA). Each LPS was dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan), and diluted with medium to prepare a 30 pg/mL solution. The 8 types of LPS solutions were mixed with PBMC from 6 cynomolgus monkeys in 1:2 (v/v, LPS final concentration: 10 pg/mL). The medium without LPS was mixed with PBMC as a control. These mixtures were incubated for 24 hrs at 37°C under 5%CO_2. They were centrifuged after incubation, and the TNF-α levels in the supernatant were measured with a kit for NHP (ProcartaPlex NHP Basic Kit and NHP TNF-α Simplex, Affymetrix, CA, USA). In addition, inter-lot variation was investigated by 2 lots (Lot A and Lot B) of LPS from *each E. coli* (K-235) and *S. enterica* in the same manner.

2.6. Experiment II (Selection of appropriate animal species)

LPS from *E. coli* (K-235) was dissolved in physiological saline, and diluted with medium to prepare 0.3, 3, 30, 300 pg/mL, 3, 30, 300 ng/mL, 3, and 30 μg/mL solutions. These 9 concentrations of LPS solutions were mixed with PBMC from each of the 6 cynomolgus monkeys, rats, or humans in 1:2 ratio (v/v, LPS final concentrations: 0.1, 1, 10, 100 pg/mL, 1, 10, 100 ng/mL, 1, and 10 μg/mL). As a control, medium without LPS was mixed with PBMC. These mixtures were incubated for 24 hrs at 37°C under 5% CO_2. These mixtures were centrifuged after incubation. The TNF-α levels in the supernatant were measured with respective kits for each animal species (NHP TNF-α Simplex, Rat TNF-α Simplex and Human TNF-α Simplex, Affymetrix, CA, USA).

2.7. Experiment III (Changes in biomarkers and histopathology after LPS treatment)

LPS from *E. coli* (K-235) was dissolved in physiological saline. Fifteen naïve cynomolgus monkeys were divided into 3 groups (control, low LPS dose, and high LPS dose groups) with 6, 3, and 3 animals, respectively. LPS dose levels were 0.6 and 2.4 mg/kg in the low and high LPS groups, respectively. LPS was administered into the cephalic vein of the forearm at a rate of 20 mL/kg/2 hrs using a syringe pump under anesthesia by nitrous oxide and isoflurane. For the control group, physiological saline was administered once into the cephalic vein of the forearm in the same manner used for LPS. Blood samples (approximately 5.5 mL/point) were collected before dosing, and at 2, 4, 6, and 8 hrs after initiation of LPS dosing. Special examination on the following parameters was conducted with whole blood treated with the anticoagulant EDTA-2K: general hematology test, citrated plasma: Prothrombin time (PT, Dade Innovin, Sysmex Corporation, Hyogo, Japan), Activated partial thromboplastin time (APTT, Dade Actin, Sysmex Corporation, Hyogo, Japan),

Fibrinogen (Fng, Dade Thrombin Reagent, Sysmex Corporation, Hyogo, Japan), Thrombin-antithrombin complex (TAT, Enzygnost TAT micro, Siemens Healthcare K.K., Eschborn, Germany), D-dimer (DD, RIAS AUTO D-dimer NEO, Sysmex Corporation, Hyogo, Japan), Plasmin-α_2-plasmin inhibitor complex (PIC, RIAS AUTO PIC, Sysmex Corporation, Hyogo, Japan), Soluble fibrin monomer (SF, IATRO SF II, LSI Medience Corporation, Tokyo, Japan) and Plasminogen activator inhibitor 1 (PAI-1, AssayMax Human Plasminogen Activator Inhibitor (PAI-1) ELISA Kit, Assaypro LLC., MO, USA) and serum: TNF-α (NHP TNF-α Simplex, Affymetrix, CA, USA), IL-1β (NHP IL-1β Simplex, Affymetrix, CA, USA) and general blood chemistry test. These reagents for human were validated using plasma or serum samples from cynomolgus monkeys at SNBL, and their specificity, accuracy, precision, recovery and stability were confirmed. After blood sampling at 8 hrs after the initiation of LPS dosing, the animals were anesthetized by an intravenous injection of sodium pentobarbital (26 mg/kg) and euthanized by exsanguination. The liver, kidney, and lung were fixed in 10% neutral buffered formalin. They were embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) and phosphotungstic acid hematoxylin (PTAH). Slide specimens were examined microscopically.

2.8. Experiment IV (Suppressive effect of rhTM on DIC induction by LPS)

rhTM (640 U/kg, Asahi Kasei Pharma Corporation, Tokyo, Japan) was administered concomitantly with 2.4 mg/kg LPS from *E. coli* (K-235) to 3 cynomolgus monkeys. rhTM was injected at 1 mL/kg/2 hrs into the cephalic vein of the forearm which was opposite the side used in LPS administration with a syringe pump. The LPS administration and examination were performed in the same manner as in experiment III.

2.9. Statistical analysis

In Experiments I and II, the TNF-α levels were analyzed by the Tukey-Kramer HSD (Honestly Significant Difference) test with pairwise comparisons of least squares means. In Experiments III and IV, the data for PT, APTT, Fng, TAT, DD, PIC, SF, PAI-1, TNF-α, IL-1β, and hematology and blood chemistry parameters were statistically analyzed by Dunnett's test comparing the values of the low and high LPS dose groups with the control group. Pairwise comparisons of the high LPS dose group and LPS + rhTM dose group in Experiment IV were performed by the t-test based on a one-way ANOVA model for each sampling. A value of $p<0.05$ was considered statistically significant. These statistical analyses were performed using JMP (version 12.2, SAS Institute, NC, USA).

3. Results

3.1. Experiment I (Selection of appropriate LPS)

For the selection of an appropriate type of LPS, PBMC from cynomolgus monkeys was

incubated with LPS purified from *E. coli*, *P. aeruginosa*, or *S. enterica* for 24 hrs at 37℃, and the *in vitro* production of TNF-α was measured and compared against each other. The TNF-α levels were statistically significantly increased by 5 types of LPS from *E. coli* (055:B5, 026:B6, 0127:B8, and K-235) and *S. enterica* (Figure 1, A). In particular, the TNF-α levels in 3 types of LPS from *E. coli* (0127:B8 and K-235) and *S. enterica* were increased 7 to 9-fold the control. In addition, the coefficient of variation (CV) of TNF-α levels in 3 types of LPS from *E. coli* (055: B5 and K-235) and *S. enterica* were smaller than the others and the CV for *E. coli* (K-235) was smallest. In the additional experiment, LPS from *E. coli* (K-235) showed a smaller inter-lot variation in TNF-α production than that from *S. enterica* (Figure 1, B). From these results, we selected the LPS (K-235) for a preparation of an LPS-induced DIC model in cynomolgus monkey in the next Experiments II, III and IV.

3.2. Experiment II (Selection of appropriate animal species)

Species-differences in the TNF-α producing potency of PBMC from 6 animal species by LPS (*E. coli,* K-235) were investigated by 24–hr incubation at 37°C. In rats, cynomolgus monkeys, and humans, the TNF-α levels were increased to 100, 2000, and 5000 pg/mL, respectively, at up to 10^7 pg/mL of LPS (Figure 2), with statistical significances being comparable with the control, while the TNF-α levels in rats was very low at up to 10^7 pg/mL. The TNF-α levels in cynomolgus monkeys were comparable to that in humans at the LPS concentration of 10^2 to 10^4 pg/mL.

3.3. Experiment III (Changes in biomarkers and histopathology after LPS treatment)

In an *in vivo* experiment, 0.6 or 2.4 mg/kg of LPS (*E. coli,* K-235) was administered once intravenously to cynomolgus monkeys. Heart rate increased during LPS dosing at 0.6 and 2.4 mg/kg. Also, slight decrease in spontaneous activity was observed from 4 to 8 hrs after dosing at 2.4 mg/kg. The biomarkers were measured at 2-hr intervals up to 8 hrs after initiation of LPS dosing (Figure 3). TNF-α levels increased dose-dependently, reached a peak (80-fold increase) at 2 hrs, and then decreased promptly. IL-1β levels also showed a dose-dependent sharp increase with a peak at 2 or 4 hrs, and there was statistical significance from 2 to 8 hrs after dosing at 2.4 mg/kg. Neutrophil counts were decreased at 2 hrs, and then increased with statistical significance at 4 to 6 hrs when compared with the time-matched control. Monocyte counts were decreased at up to 8 hrs. Platelet counts were continuously lower than the control up to 8 hrs. Fng levels decreased dose-dependently up to 8 hrs at 2.4 mg/kg. PT and APTT showed prolongation up to 8 hrs at 2.4 mg/kg. TAT levels increased dose-dependently with a peak at 4 hrs, and very high levels continued up to 8 hrs. SF levels increased dose-dependently up to 8 hrs. DD and PIC levels were increased at 2 hrs and thereafter. PAI-1 levels increased, and very high levels were maintained up to 8 hrs. Almost all of these changes described above showed statistical

significance when compared with time-matched control values as shown in Figure 3. In the histopathology of the liver, kidney, and lung, slight deposition of brown pigment (HE stain) and livid material (PTAH stain) were observed in sinusoid of the liver (Table 1 and Figure 4); no noticeable findings were noted in the kidney or lung.

3.4. Experiment IV (Suppressive effect of rhTM on DIC induction by LPS)

In the investigation of the efficacy of DIC therapeutic medicine, rhTM (640 U/kg) was co-administered with 2.4 mg/kg LPS (*E. coli*, K-235) and biomarkers were measured in the same manner as in experiment III (Figure 3). The co-administration of rhTM showed a suppressive effect on the increases in TNF-α, TAT, SF, DD, and PIC levels, prolongation of PT and APTT, and decreases in Fng levels. No suppressive effect of rhTM was noted in neutrophil, monocyte, platelet, PAI-1 or hepatic histopathology.

4. Discussion

Administered LPS binds to LPS binding protein, and is recognized by TLR4 expressed on the surface of monocytes and macrophages. The stimulation of TLR4 regulates the production of inflammatory cytokines such as TNF-α and IL-1β. Monocytes and macrophages secrete these cytokines and tissue factor (8). The secreted tissue factor activates the coagulation cascade, and promotes the production of fibrin. Microthrombi are induced by these activators in the model of sepsis-associated DIC.

TNF-α was reported to be a triggering factor for an induction of DIC (1). Therefore, in Experiment I, TNF-α production by PBMC from cynomolgus monkeys was examined to select an appropriate type of LPS among 8 types for an induction of DIC in cynomolgus monkeys. LPS from *E. coli* (055: B5) is often used for the preparation of sepsis-associated DIC rat models (1, 5, 6) and TNF-α level after incubation with it increased at 10 pg/mL statistically significantly to approximately 4-fold that of the control in the present study. However, LPS from *E. coli* (0127:B8 and K-235) and *S. enterica* showed an approximately 7-fold increase (Figure 1, A). In a comparison of the interindividual differences of the TNF-α levels, the variation in the LPS from *E. coli* (055:B5 and K-235) and *S. enterica* was smaller than the others. The difference in *in vitro* TNF-α production by the 2 lots (Lots A and B) of LPS from *E. coli* (K-235) was smaller than LPS from *S. enterica* (Figure 1, B). From these results, the LPS from *E. coli* (K-235) was considered to be the most appropriate inducer for DIC in cynomolgus monkeys rather than that from *E. coli* (055:B5) and *S. enterica*.

In *in vitro* Experiment II using PBMC from cynomolgus monkeys, rats, or humans with 10^{-1} to 10^{7} pg/mL of LPS from *E. coli* (K-235), human PBMC showed dose-dependent increases in TNF-α level with a statistical significance at the LPS concentrations of 10^{2} to 10^{7} pg/mL. In cynomolgus monkey PBMC, the TNF-α level showed a statistically significant increase between 1 and 10^{7}pg/mL, and it reached a plateau at 10 pg/mL of LPS. Between 10^{2} to

10^4 pg/mL of LPS, the TNF-α response was comparable between cynomolgus monkeys and humans, while that in rats it was 100-fold lower than that in humans (Figure 2). Opal et al. (9) reported that serum LPS levels in patients with sepsis were 110 to 726 pg/mL, suggesting a similar response in cynomolgus monkeys to humans at pathophysiological range of serum LPS. It was considered to be appropriate to prepare a human DIC model in cynomolgus monkeys rather than rats. Vaure and Liu (10) reported that TLR4 of non-human primates (NHP) was closer to humans in TLR4 gene, its protein sequence, and its regulation than to other animals and that NHP showed the best response to the TLR4 agonist as in humans.

In *in vivo* Experiment III, conducted to evaluate the pathophysiological status of the LPS-induced DIC model in cynomolgus monkeys, LPS from *E. coli* (K-235) was administered once intravenously at 0.6 and 2.4 mg/kg to cynomolgus monkeys, and biomarkers for both coagulation and fibrinolysis were measured, and histopathological examination was conducted on the liver, kidney, and lung. At 2.4 mg/kg of LPS, prolonged PT and APTT, decreased Fng, and increased TAT and SF were observed with statistical significance as the coagulation biomarkers. As the biomarkers for fibrinolysis, increased DD, PIC, and PAI-1 were observed with statistical significance. Increased PAI-1 which characterizes sepsis-associated DIC, was also observed (Figure 3). At 0.6 mg/kg of LPS, similar change patterns were noted, to a lesser extent, in these coagulation and fibrinolysis biomarkers, but the changes were not statistically significant in Fng, PT, APTT or DD (Figure 3). From these results, a DIC model with low interindividual differences can be prepared by an administration of 2.4 mg/kg of LPS (K-235) to cynomolgus monkeys. Changes in biomarkers were summarized up to 8 hrs after LPS dosing based on the present results (Figure 5). In contrast to the sharp increases in inflammatory cytokines (TNF-α and IL-1β), the decreases in neutrophils and monocytes reached a trough 2 hrs after LPS dosing in this model. These decreases in monocytes and neutrophils in the present *in vivo* study were speculated to be due to a formation of complexes of neutrophils and monocytes with platelets because Stahl *et al*. (11) reported that LPS caused increases in platelet-monocyte complexes and platelet-neutrophil complexes in human blood in *in vitro* experiment. TNF-α promote the induction of tissue factor, resulting in the activation of coagulation. From the sharp increases in TAT and SF, the activation of coagulation reached a peak at almost 4 hrs which was 2 hrs later than the peak of the inflammatory cytokines (TNF-α and IL-1β), and the production of fibrin was promoted. The slightly delayed increase in DD, PIC, and PAI-1 after the rises in TAT and SF probably show an induction of fibrinolysis slightly after the activation of coagulation, and fibrinolysis continued up to at least 8 hrs which was the time of necropsy. Also, due to the inhibiting effect of PAI-1 on fibrinolysis, the fibrinolysis was not fully achieved. In the histopathological examinations, slight brown pigment deposition (HE stain) and livid material deposition (PTAH stain) were observed in the sinusoid of the liver, and those were considered to be deposition of fibrin complexes. Also, slight focal angiectasis was observed

in the liver, and it was considered to be liver microvascular endothelial dysfunction (Table 1 and Figure 4) (12). Almost all the results in biomarkers suggest that the present status in cynomolgus monkeys is considered to resemble the coagulation and fibrinolysis changes in human DIC (13).

In Experiment IV for an evaluation of the therapeutic efficacy of DIC medicine (rhTM) by measuring biomarkers, 640 U/kg (twice the clinical dose) of rhTM was administered concomitantly with LPS (K-235) to cynomolgus monkeys, and the suppressive effect of rhTM on the biomarker level induced by LPS in Experiment III was evaluated up to 8 hrs. The prolongation of PT and APTT, decrease of Fng and increase of TAT, SF, DD, and PIC were statistically significantly suppressed by co-administration of rhTM (Table 2). The statistically significantly suppression of these changes is considered to be induced by activation of the protein C. rhTM activate the protein C. The activated protein C cleave the coagulation cofactor Va and VIIIa, and suppress the coagulation (7). However, no suppressive effect of rhTM was noted in neutrophils, monocytes, platelets, PAI or hepatic histopathology. These changes in neutrophils, monocytes, and platelets were speculated to be due to a formation of complexes of neutrophils, monocytes, and platelets with LPS. Also, PAI-1 was released from vascular endothelial cell by a stimulation of TNF-α and IL-1β. The effect of rhTM was not associated with these mechanisms. These are probable reasons why changes were not suppressed by co-administration of rhTM. It may be possible to show a suppressive fibrinolytic effect of rhTM in histopathology if the necropsy is conducted earlier than 8 hrs after dosing.

In conclusion, a sepsis-associated DIC model can be prepared in cynomolgus monkeys by a single intravenous administration of 2.4 mg/kg LPS of *E. coli* (K-235). The biomarker changes in the present monkey model resemble the pathophysiologic status in human DIC rather than rat models. In addition, this cynomolgus monkey model could be applicable for evaluation of the therapeutic efficacy of new medicinal candidates because the therapeutic efficacy of rhTM was quantitatively evaluated by measuring the biomarker levels in blood, plasma, or serum at the close concentration of LPS in serum of DIC patients. Furthermore, a greater volume of peripheral blood can be collected more frequently in cynomolgus monkeys than rats, indicating many kinds of biomarkers for DIC can be measured sequentially in individual animals. Further analysis of the biomarkers using this cynomolgus monkey model will contribute to the recognition of the pathophysiology in human sepsis-associated DIC.

Conflicts of interest

The authors declare that they have no conflict of interests.

Acknowledgments

The authors are grateful to Dr. Fumio Chatani and Dr. Kazumasa Yamashita (Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories, Japan) for their help in the preparation of this manuscript.

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Figure Captions

Figure 1. In vitro production of TNF-α after LPS exposure in PBMC of cynomolgus monkeys.
A: PBMC of cynomolgus monkeys was incubated with either 8 types of LPS (10 pg/mL) for 24 hrs at 37˚C, and TNF-α levels in the incubation media were measured. The mean ± SE is represented as bar graph for each LPS (n=6). The average control level is shown as 100%. The CV is represented as line graph (n=6). Pairwise comparisons of least squares means were performed using the Tukey-Kramer HSD test. Statistical significance is denoted with * for $p < 0.05$ and ** for $p < 0.01$.
B: PBMC of cynomolgus monkeys was incubated with either 2 lots of LPS (10 pg/mL) from *E. coli* (K-235) or *S. enterica* for 24 hrs at 37˚C, and TNF-α levels in the incubation media were measured. The mean ± SE is represented (n=6). Lot A of LPS was used for in vitro study in Figure 1 A. The TNF-α level for each Lot A is shown as 100%.

Figure 2. *In vitro* production of TNF-α after LPS exposure in PBMC of humans, cynomolgus monkeys, or rats.
PBMC of humans, cynomolgus monkeys, or rats was incubated with LPS (10 pg/mL) from *E. coli* (K-235) for 24 hrs at 37˚C, and TNF-α levels in the incubation media were measured. The mean ± SE is represented (n=6). Pairwise comparisons of least squares means were performed using the Tukey-Kramer HSD test. Statistical significance is denoted with ** for $p < 0.01$.

Figure 3. Biomarker levels in DIC cynomolgus monkeys receiving LPS.
LPS from *E. coli* (K-235) was administered once intravenously to 3 or 6 cynomolgus monkeys at 0.6 or 2.4 mg/kg to produce DIC model. In addition, rhTM (640 U/kg) was administered concomitantly with 2.4 mg/kg LPS (n=3) to investigate any suppressive effect of rhTM on the DIC induction by LPS. TNF-α, IL-1β, neutrophil, monocyte, platelet, Fng, PT, APTT, TAT, SF, DD, PIC, and PAI-1 levels in serum, blood or plasma were measured before dosing, and up to 8 hrs after the initiation of LPS dosing. The mean ± SE is represented (n=3 or 6). Dunnett's test was applied to compare the LPS groups (0.6 or 2.4 mg/kg) with the control group. Statistical significance is denoted with * for $p < 0.05$ and ** for $p < 0.01$. Pairwise comparisons of the LPS 2.4 mg/kg group and the LPS 2.4 mg/kg + rhTM 640 U/kg group were

performed using the t-test based on a one-way ANOVA model. Statistical significance is denoted with # for $p < 0.05$.

Figure 4. Microscopic pictures of the liver in cynomolgus monkey treated with LPS.
After 8 hrs after initiation of LPS dosing, liver, kidney, and lung were collected from each animals (n=3 or 6). Slide specimens stained with HE and PTAH were examined microscopically. Brown pigment deposition (HE) and livid material deposition (PTAH) were observed in sinusoid of the liver (arrows).

Figure 5. Schematic profile for the changes of biomarkers in DIC model in cynomolgus monkeys.
Changes in biomarkers for inflammation, coagulation, and fibrinolysis were summarized up to 8 hrs after LPS dosing based on the results of the present study.

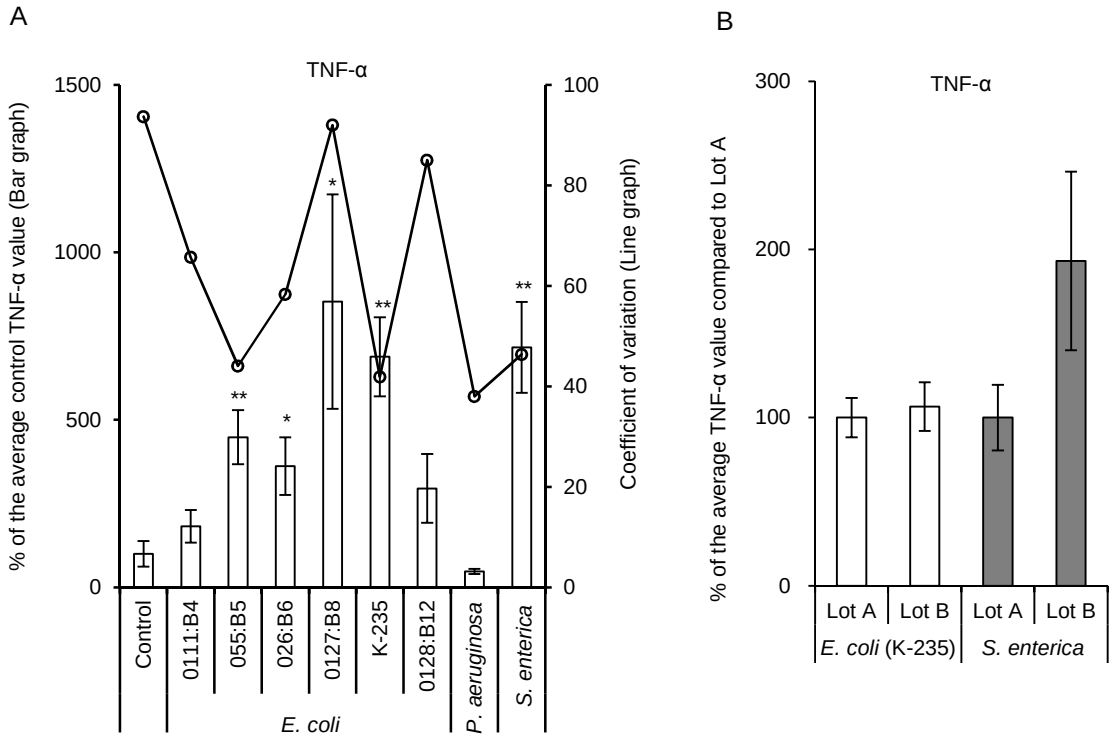
A
TNF-α
% of the average control TNF-α value (Bar graph)
Coefficient of variation (Line graph)
1500
1000
500
0
100
80
60
40
20
0
**
*
*
**
**
Control
0111:B4
055:B5
026:B6
0127:B8
K-235
0128:B12
P. aeruginosa
S. enterica
E. coli
B
TNF-α
% of the average TNF-α value compared to Lot A
300
200
100
0
Lot A
Lot B
Lot A
Lot B
E. coli (K-235)
S. enterica

Figure 1

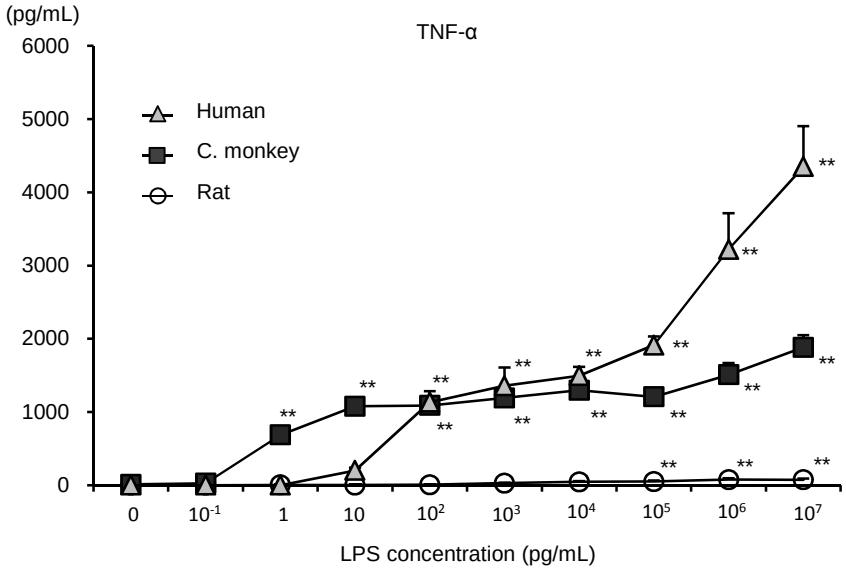
(pg/mL)
TNF-α
6000
5000
4000
3000
2000
1000
0
Human
C. monkey
Rat
**
0
10-1
1
10
102
103
104
105
106
107
LPS concentration (pg/mL)

Figure 2.

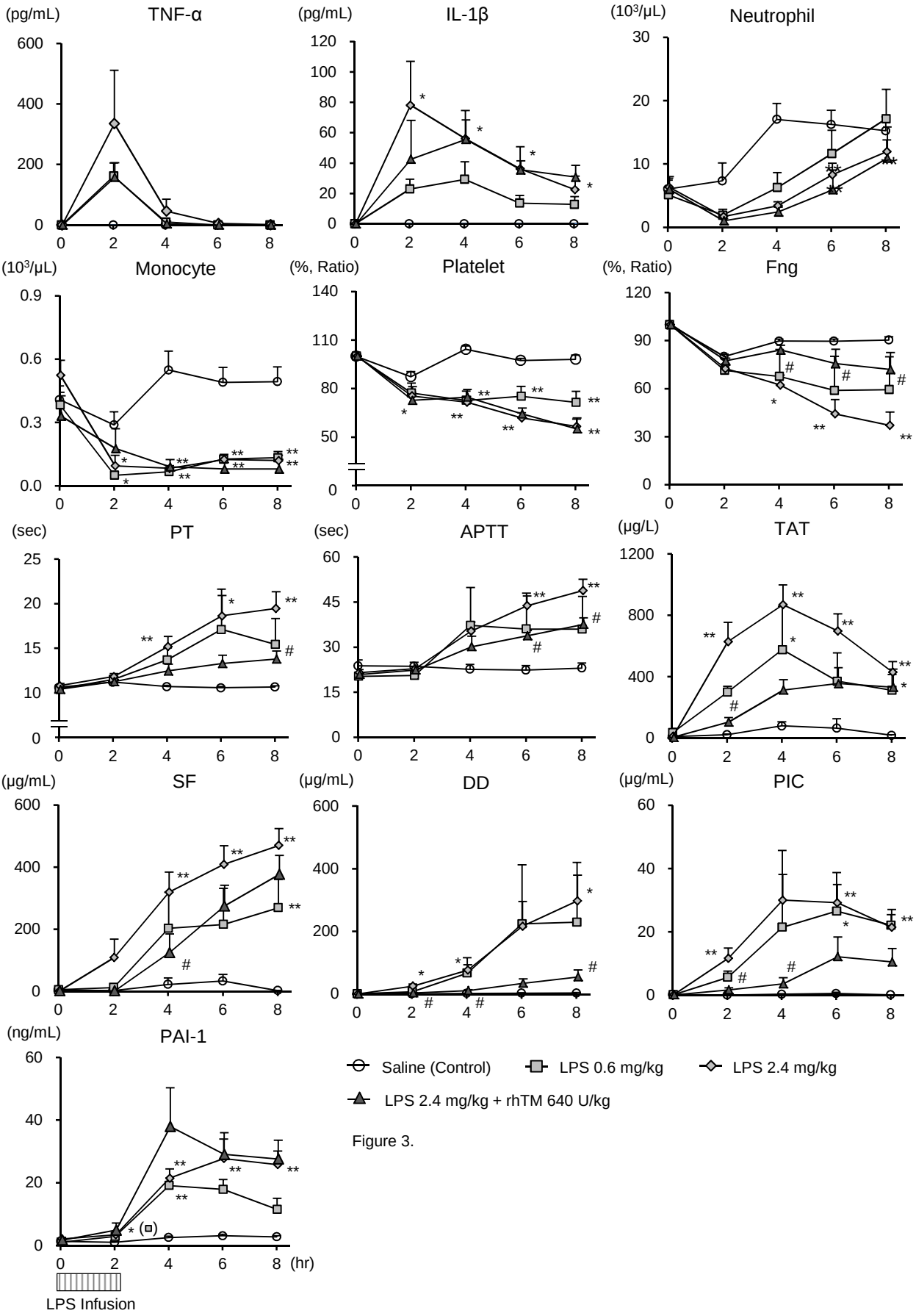
(pg/mL)
TNF-α
(pg/mL)
IL-1β
(10³/μL)
Neutrophil
(10³/μL)
Monocyte
(%, Ratio)
Platelet
(%, Ratio)
Fng
(sec)
PT
(sec)
APTT
(μg/L)
TAT
(μg/mL)
SF
(μg/mL)
DD
(μg/mL)
PIC
(ng/mL)
PAI-1
(hr)
LPS Infusion
Saline (Control)
LPS 0.6 mg/kg
LPS 2.4 mg/kg
LPS 2.4 mg/kg + rhTM 640 U/kg

Figure 3.

HE PTAH

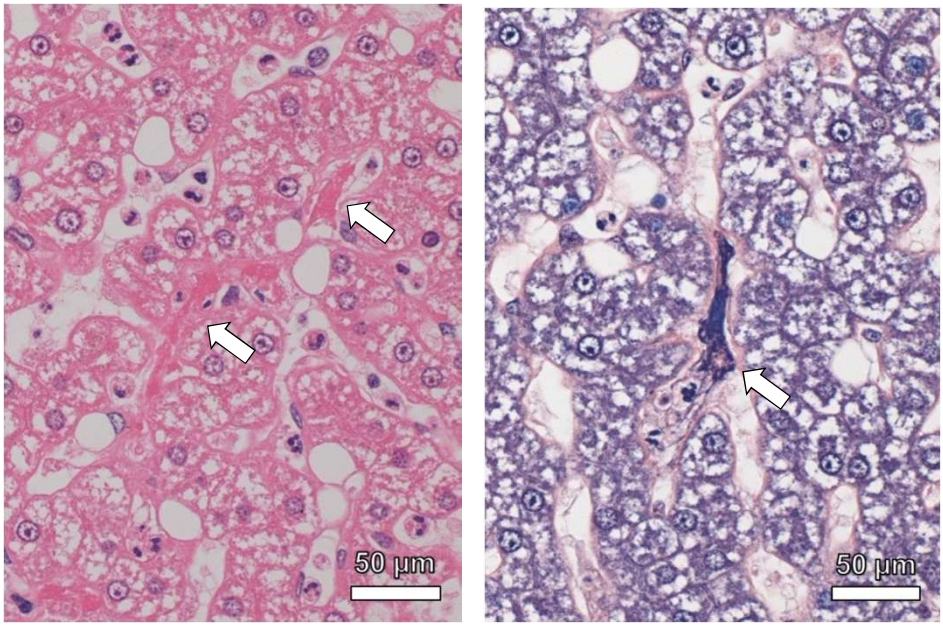

Figure 4.

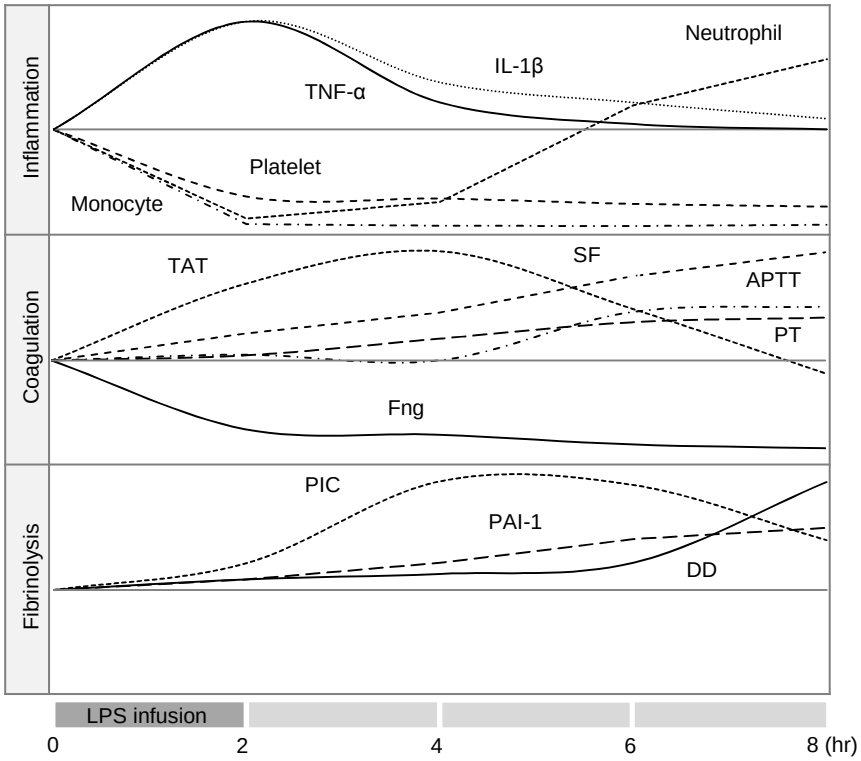
Inflammation
Neutrophil
IL-1β
TNF-α
Platelet
Monocyte
Coagulation
TAT
SF
APTT
PT
Fng
Fibrinolysis
PIC
PAI-1
DD
LPS infusion
0
2
4
6
8 (hr)

Figure 5.

Tables

Table 1. Numbers of animals with histopathological findings in the liver.

Staining	Findings	Groups (mg/kg)			
		Saline	LPS (0.6)	LPS (2.4)	LPS (2.4) + rhTM
HE	Brown pigment deposition in sinusoid	0/6	2/3	1/6	1/3
	Focal angiectasis	0/6	1/3	1/6	0/3
PTAH	Livid material deposition in sinusoid	0/6	1/3	2/6	1/3

The 4 groups were treated with physiological saline, LPS (0.6), LPS (2.4), or LPS (2.4) + rhTM. The liver, kidney, and lung were examined histopathologically after staining with HE and PTAH.

Table 2. Summary of changes in biomarkers in the LPS treatment group when compared with the saline control or 2.4 mg/kg groups.

Type	Biomarkers[*1]	LPS (0.6)	LPS (2.4)	LPS (2.4) + rhTM
Diagnostic biomarkers	Platelet decrease	○	○	×
	FDP (or D-dimer) increase	△	○	●
	Fibrinogen decrease	△	○	●
	PT prolongation	△	○	●
	ATIII decrease	–	–	–
	TAT increase	○	○	●
	SF increase	○	○	●
	F1+2 increase	–	–	–
Reference biomarkers[*2]	PIC increase	○	○	●
	α_2 plasmin inhibitor decrease	–	–	–
	Protein C decrease	–	–	–
	PAI-1 increase	○	○	×
	HMGB-1 increase	–	–	–
	e-XDP decrease or severe increase	–	–	–

*1: Biomarkers noted in the Provisional Diagnostic Criteria for DIC released on October by the Japanese Society of Thrombosis and Hemostasis (14)

*2: Biomarkers used for classification of disease type, an evaluation of pathophysiological condition in human DIC (14).

–: Not examined, △: Tendency without statistical significance (vs. control group), ○: Statistically significant change (vs. control group), ●: Statistically significant suppression (vs. LPS 2.4 mg/kg alone group), ×: No significant suppression (vs. LPS 2.4 mg/kg alone group)