



Original Article

Evaluation of cytokine storms in a disseminated intravascular coagulation monkey model

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(Received February 16, 2020; Accepted March 2, 2020)

ABSTRACT — The purpose of this study was to profile cytokine storms (cytokine release syndrome) in the LPS-induced disseminated intravascular coagulation (DIC)-cynomolgus monkey model by measuring changes in 22 cytokines using Luminex. In this study, increases were noted in 20 cytokines, excluding IL-4 and IL-17A. Specifically, IL-6, IL-8, G-CSF and TNF- α , pro-inflammatory cytokines, and IL-10, an anti-inflammatory cytokine, as well as MCP-1, markedly increased by 10,000 pg/mL or more. In addition to the marked increases in the pro-inflammatory cytokines IL-6 and G-CSF, the concentrations of IL-5, IL-18, IFN- γ , VEGF and IL-15 increased continuously. Also, in addition to the marked increases in the pro-inflammatory cytokine IL-8 as well as in MCP-1, the concentrations of IL-1ra, IL-2, IL-1 β , IL-12/23 (p40), GM-CSF and TGF- α gradually decreased after initially increasing. On the other hand, in addition to the marked increases in the pro-inflammatory cytokine TNF- α and anti-inflammatory cytokine IL-10, MIP-1 β and MIP-1 α transiently increased and then rapidly disappeared from serum. IL-13 increased at 6 hr after administration only. Since the behavior of cytokines in this monkey model was similar to those noted in DIC in humans, this model will be useful for evaluating the efficacy of anti-DIC drugs. In addition, this model will also be useful for assessing the risk of cytokine storm development, which is a serious adverse effect of certain types of antibody drugs and CAR-T cell-based therapies.

Key words: Disseminated intravascular coagulation (DIC), Cytokine storm, Cytokine release syndrome, Cynomolgus monkey

INTRODUCTION

Sepsis is a systemic inflammatory response syndrome (SIRS) triggered by infection, and is thought to be the overproduction of pro-inflammatory cytokines (a cytokine storm) (Ishii, 2014). Disseminated intravascular coagulation (DIC), considered to be highly associated with septicemia (Ishii, 2014), is a serious condition in which marked systemic coagulation activation occurs and microthrombi occur frequently in the microvessels (Asakura, 2014).

In recent years, various DIC animal models have been developed in mice (Yamamoto, 1997; Vizi *et al.*, 2001; Wang *et al.*, 2009; Castellino *et al.*, 2011; Wake *et al.*, 2016) and rats (Okudaira *et al.*, 2001; Asakura *et al.*, 2002) for research into the treatment of DIC. In NHP, Minomo *et al.* (2017) developed the LPS-induced DIC model in cynomolgus monkeys.

It is thought that tissue factors and various cytokines are involved in the onset of DIC. In the DIC mouse model, 32 cytokines were measured after LPS administration and the changes were profiled (Ogawa *et al.*, 2016). How-

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ever, in the cynomolgus monkey DIC model by Minomo *et al.* (2017), increases in TNF- α and IL-1 β , pro-inflammatory cytokines typically increased by LPS, were reported, but changes in other cytokines remained unknown because they were not measured.

Knowledge of changes in cytokines after LPS administration tells us that, compared to the rat, the monkey may provide more useful information to predict therapeutic efficacy in humans. Therefore, the present study evaluated further characterization of cytokine storms and their extrapolation to humans in the LPS-treated cynomolgus monkey DIC model (Minomo *et al.*, 2017), which is expected to offer superior extrapolation to humans.

MATERIALS AND METHODS

Animals

Three male cynomolgus monkeys (4-5 years old) from China or Vietnam were obtained from Trans Genic Inc. (Fukuoka, Japan) and Japan Laboratory Animals, Inc. (Tokyo, Japan) and housed individually in stainless steel cages with high-pressure melamine plated walls (48W \times 85D \times 80H cm, with stainless steel toys for enrichment). The housing conditions were set at 22.0-28.0°C and 40.0-80.0% humidity, and rooms were lit 12 hr/day (from 7:00 to 19:00). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Ina Research Inc., which is fully accredited by AAALAC International.

DIC model induction using LPS

Model animals were prepared according to Minomo *et al.* (2017).

Animals were anesthetized by inhalation of isoflurane, JP (Mylan Seiyaku Ltd., Tokyo, Japan) and administered lipopolysaccharides from *Escherichia coli* K-235 (LPS) via intravenous infusion to the tail vein. At infusion, 0.12 mg/mL of LPS was administered continuously at a rate of 1.2 mg/kg/hr (10 mL/kg/hr) for 2 hr (2.4 mg/kg/2 hr) using a microprocessor single syringe pump (Pump 11 Plus, Harvard Apparatus Inc., Holliston, MA, USA). Successful induction of the DIC model was confirmed by hematological examination of the coagulation/fibrinolysis systems. Approximately 2 mL of whole blood were collected from the femoral vein of unanesthetized animals at pre-dosing and 2 (at completion of administration), 4 and 6 hr post-dosing for analysis of platelets (PLT), the white blood cell count (WBC), prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (Fib) using the ADVIA 120 Hematology System

(Siemens Healthcare Diagnostics K.K., Tokyo, Japan) and Automated Blood Coagulation Analyzer CA-510 (Sysmex Corporation, Hyogo, Japan).

Cytokine measurements

For measurement of cytokines, approximately 3 mL of whole blood were collected from the femoral vein of unanesthetized animals at pre-dosing and 2 (at completion of administration), 4 and 6 hr post-dosing and serum was obtained by centrifugation (approx. 1600 \times g, 10 min, 4°C).

Cytokines were measured using the Bio-Plex 200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA; herein after referred to as "Luminex").

A Non-Human Primate Cytokine/Chemokine Magnetic Panel (Merck Millipore, Darmstadt, Germany) was used to measure the serum samples (undiluted or diluted 100-fold with the Serum Matrix included in the kit) using Luminex. To the wells of a microplate containing 25 μ L of the standard solution, control, serum samples or diluted serum samples, equal volumes of the Serum Matrix or Assay Buffer, followed by antibody-immobilized magnetic beads were added in that order and the plates were incubated at room temperature for 2 hr. Analytes on the surface of the magnetic beads were then combined with a biotin-labeled antibody cocktail and fluorescently labeled with Streptavidin-Phycoerythrin. The fluorescence intensity was measured using Luminex, and IL-1 β , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40 [IL-12/23 (p40)], IL-13, IL-15, IL-17 (IL-17A), IL-18, IFN- γ , G-CSF, GM-CSF, MCP-1, MIP-1 α , MIP-1 β , TNF- α , TGF- α and VEGF were analyzed.

Drug

On the day of the experiment, LPS (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was dissolved in physiological saline, JP (Otsuka Normal Saline, Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) to yield a 0.12 mg/mL formulation.

Data analyses

Means and standard deviations were calculated for each hematological parameter and each cytokine at each measurement time point.

RESULTS

Confirmation of DIC model induction

Hematology showed decreases in PLT, Fib, and WBC, as well as prolongation of PT and APTT, typical changes in a DIC-model animal (Fig. 1).

Cytokine profile in DIC-cynomolgus monkey model

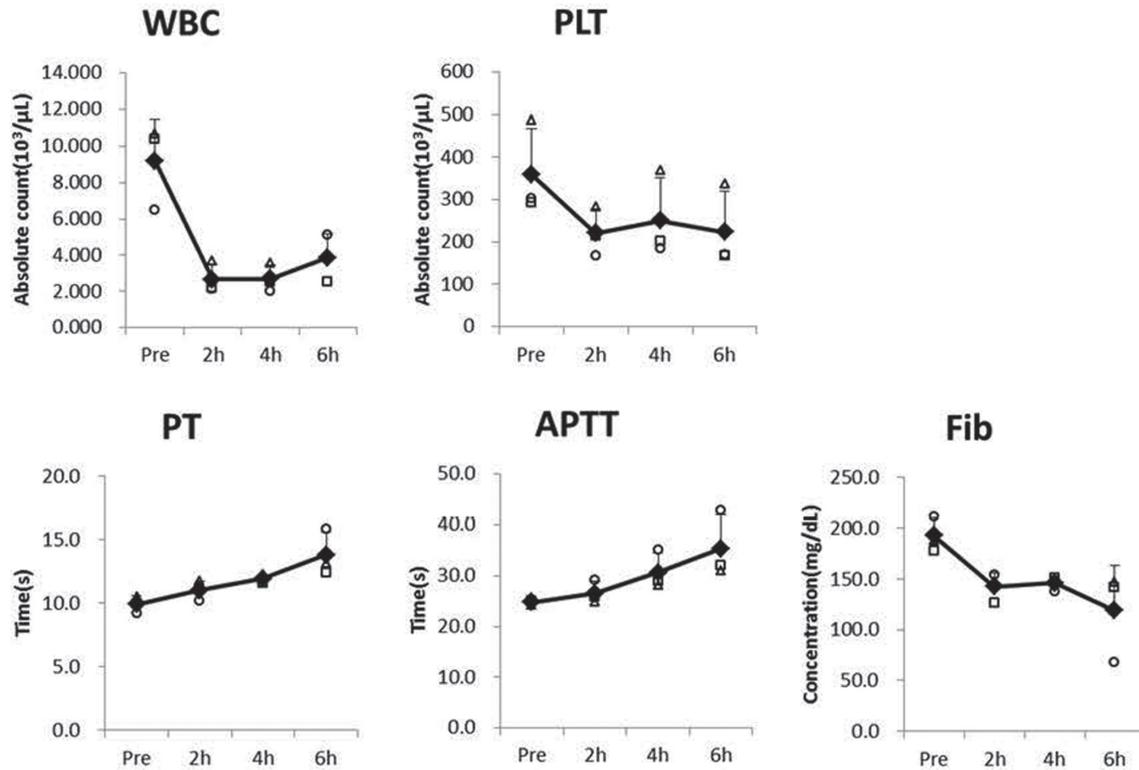


Fig. 1. Hematological confirmation of DIC model induction. Hematology showed decreases in PLT, Fib, and WBC, as well as prolongation of PT and APTT; typical changes in a DIC-model animal.

- ◆: Mean values +SD
- : Individual values (Animal No. 1)
- △: Individual values (Animal No. 2)
- : Individual values (Animal No. 3)

Cytokine measurements

The results of the cytokine assays using Luminex are shown in Figs. 2-1 to 2-3.

Increases of at least 10,000 pg/mL in IL-6, IL-8, IL-10, G-CSF, MCP-1 and TNF- α , and of at least 1000 pg/mL in IL-1Ra and MIP-1 β were noted as compared to pre-dosing (Fig. 2-1). In addition, increases of at least 100 pg/mL in IL-2, IL-5, IL-18, INF- γ , MIP-1 α and VEGF, and of at least 10 pg/mL in IL-1 β , IL-12/23 (p40), IL-13, IL-15 and GM-CSF were noted as compared to pre-dosing (Fig. 2-2). TGF- α increased by at least 1 pg/mL, while no changes were noted in IL-4 or IL-17A as compared to pre-dosing (Fig. 2-3).

Increases were noted in IL-6, IL-18 and VEGF from 2 hr post-dosing (at completion of administration) and in G-CSF, IL-5, INF- γ and IL-15 from 4 hr post-dosing, and these values increased continuously until 6 hr post-dosing. Increases were noted in IL-1ra, IL-2, IL-1 β , GM-CSF and TGF- α until 2 hr post-dosing, and in IL-8, MCP-1

and IL-12/23 (p40) until 4 hr post-dosing, and these values decreased thereafter.

Transient increases were noted in IL-10, TNF- α , MIP-1 β and MIP-1 α at 2 hr post-dosing, and these values rapidly decreased at 4 hr post-dosing. An increase was noted in IL-13 at 6 hr post-dosing only.

DISCUSSION

In this study, decreases in PLT, Fib, and WBC, as well as prolongation of PT and APTT in the hematology, typical changes in a DIC model animal, were noted following administration with LPS, indicating successful induction of the DIC model in the cynomolgus monkeys. In addition, pro-inflammatory cytokines TNF- α and IL-1 β showed peak concentrations at completion of administration. Thereafter, TNF- α rapidly decreased while IL-1 β gradually decreased, changes comparable to those reported by Minomo *et al.* (2017). Furthermore, of the

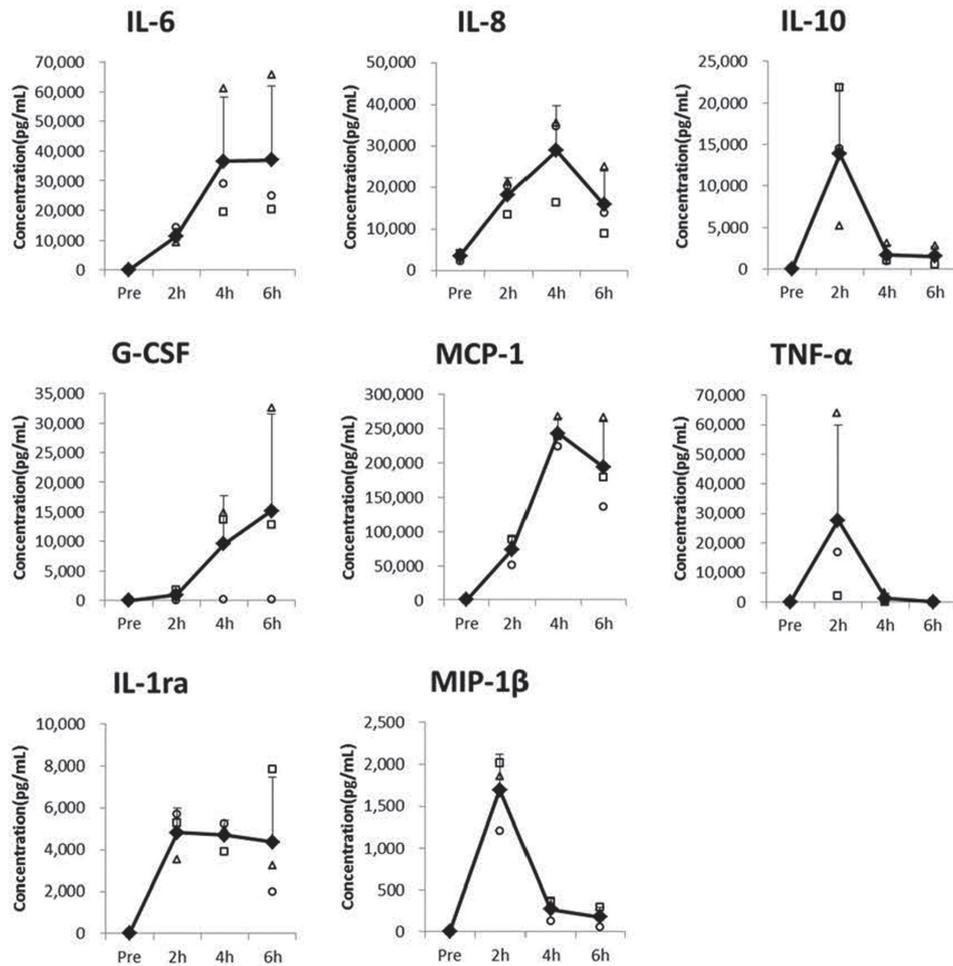


Fig. 2-1. Measurement of cytokines in Luminex. Twenty-two cytokines were determined using Luminex at pre-dosing and 2 (at completion of administration), 4 and 6 hr post-LPS dosing. Increases of at least 10,000 pg/mL in IL-6, IL-8, IL-10, G-CSF, MCP-1 and TNF- α , and of at least 1000 pg/mL in IL-1Ra and MIP-1 β were noted.

◆: Mean values +SD

○: Individual values (Animal No. 1)

△: Individual values (Animal No. 2)

□: Individual values (Animal No. 3)

22 cytokines, including TNF- α and IL-1 β , which were additionally measured in the current study, increases were noted in 20 cytokines following administration with LPS, excluding IL-4 and IL-17A, which did not change.

We compared these changes in cytokines in the cynomolgus monkey DIC model with sepsis patients (Table 1). Our results indicated that IL-6, IL-8, IL-10, G-CSF, MCP-1, IL-1Ra, MIP-1 β , IL-18, MIP-1 α , VEGF, IL-13 and IL-15 increased in both the monkey model and sepsis patients. However, while TNF- α , IL-2, IL-5, IFN- γ , IL-1 β , IL-12/23(p40) and GM-CSF also increased in the monkey model, increases were noted in most, but not

all, sepsis patients. In contrast, IL-4 and IL-17A did not increase in the monkey model, but increased in some sepsis patients. It is possible that these differences were the result of variation in the timing of analyses or the severity of sepsis symptoms. For example, according to Bozza *et al.* (2007), increases in IL-4 only occurred in terminal patients.

When 32 cytokines were measured in the mouse DIC model [18 of which were also evaluated in the current study: IL-1 β , IL-12 (p40), IL-15, IL-6, IL-10, TNF- α , IFN- γ , IL-2, IL-4, IL-5, IL-13, IL-17, MCP-1, MIP-1 α , MIP-1 β , G-CSF, GM-CSF and VEGF; and 14 of which

Cytokine profile in DIC-cynomolgus monkey model

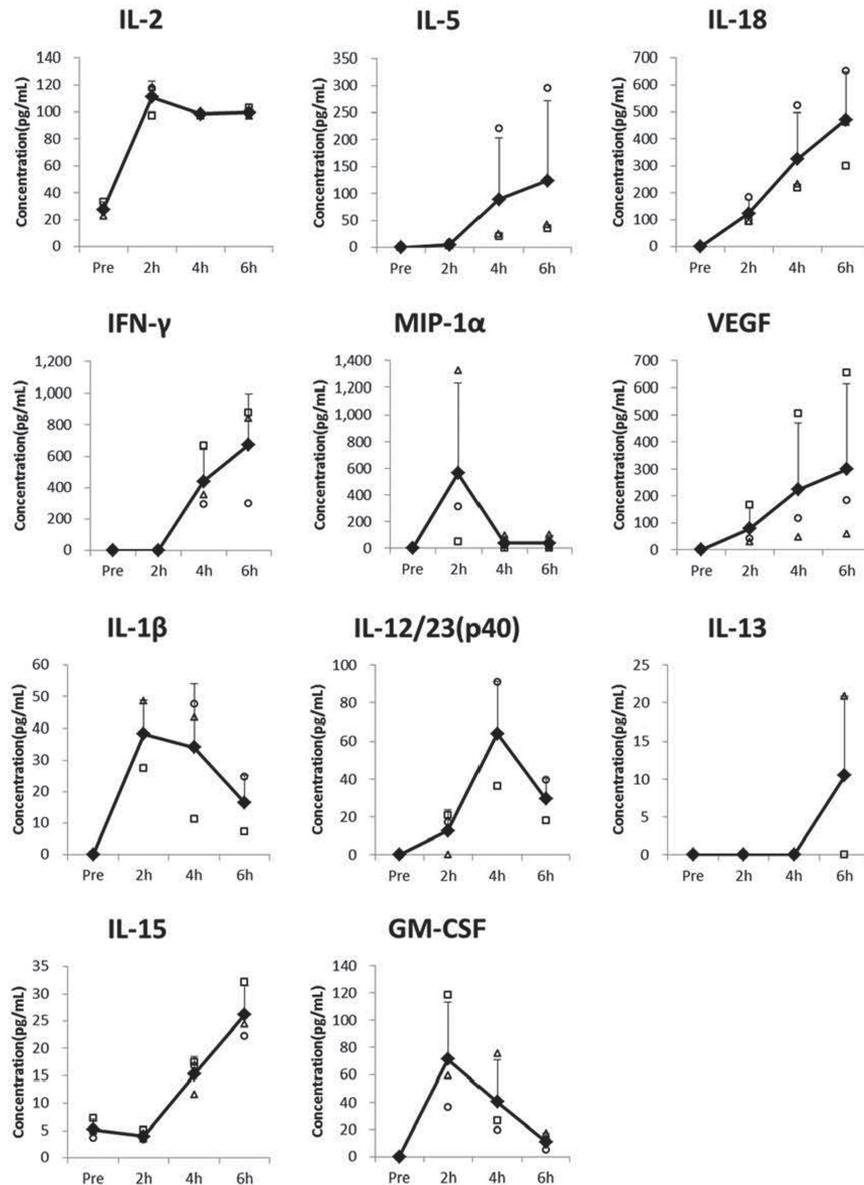


Fig. 2-2. Measurement of cytokines in Luminex. Twenty-two cytokines were determined using Luminex at pre-dosing and 2 (at completion of administration), 4 and 6 hr post-LPS dosing. Increases of at least 100 pg/mL in IL-2, IL-5, IL-18, INF- γ , MIP-1 α and VEGF, and of at least 10 pg/mL in IL-1 β , IL-12/23 (p40), IL-13, IL-15 and GM-CSF were noted.

◆: Mean values +SD

○: Individual values (Animal No. 1)

△: Individual values (Animal No. 2)

□: Individual values (Animal No. 3)

were not included in the current study: IL-1a, IL-12 (p70), IL-3, IL-9, IL-7, RANTES, Eotaxin, KC, MIP-2, MIG, IP-10, LIX, M-CSF and LIF], increases were noted in all at 4 hr post-dosing (Ogawa *et al.*, 2016). In other words, if we exclude the 2 cytokines that did not change (IL-4

and IL-17A), the results obtained in the current study were consistent with those of Ogawa *et al.* (2016). In the mouse DIC model, survival is a key assessment parameter. The survival rate reported by Ogawa *et al.* (2016) at 120 hr post-dosing with the LPS injection was between

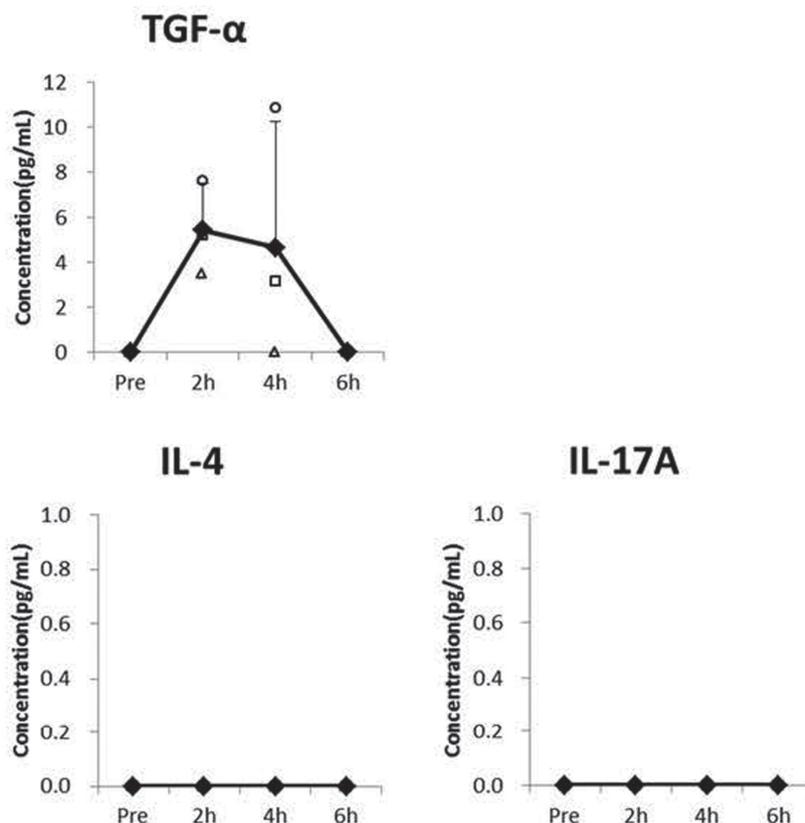


Fig. 2-3. Measurement of cytokines in Luminex. Twenty-two cytokines were determined using Luminex at pre-dosing and 2 (at completion of administration), 4 and 6 hr post-LPS dosing. TGF- α increased by at least 1 pg/mL, while no changes were noted in IL-4 or IL-17A.

- ◆: Mean values +SD
- : Individual values (Animal No. 1)
- △: Individual values (Animal No. 2)
- : Individual values (Animal No. 3)

10 and 30%. In other words, the symptoms inducing acute mortality in the mouse DIC model are of extreme severity, and are thought to mimic severe sepsis cases that lead to death. Therefore, the difference between the mouse and monkey DIC models may not be due to species differences so much as the severity of disease symptoms.

As described above, the cytokine changes in the monkey DIC model used in this study are considered not to be lethal in the acute phase and to be similar to human sepsis. In other words, unlike rodents in which the evaluation endpoint is the acute mortality rate, the fluctuations in cytokine parameters can be observed continuously over longer periods in monkeys, meaning more detailed comparisons are possible. Furthermore, in the presently thriving field of biopharmaceuticals, cases of cross-reactivity have been limited to monkeys, making the monkey model

an indispensable DIC model option.

In a further comparison between rodent and monkey DIC models, according to Minomo *et al.* (2017), “The biomarker changes in the present monkey model resemble the pathophysiologic status in human DIC rather than rat models”. They reasoned that, when LPS was added to peripheral blood mononuclear cells (PBMC), the TNF- α response was comparable between cynomolgus monkeys and humans, while that in rodents was 100-fold weaker than that in humans. Opal *et al.* (1999) and Vaure and Liu (2014) also reported that the TLR4 (receptor for LPS) gene of non-human primates was closer to the human TLR4 gene than rodents.

However, the possibility remains that other animal species may mimic human sepsis more closely than the monkey in the right conditions. Therefore, more detailed

Cytokine profile in DIC-cynomolgus monkey model

Table 1. Comparison of cytokines between monkey DIC model and sepsis patients.

Source	Cytokines																						
	IL-6	IL-8	IL-10	G-CSF	MCP-1 (CCL2)	TNF- α	IL-1Ra	MIP-1 β (CCL4)	IL-2	IL-5	IL-18	IFN- γ	MIP-1 α (CCL3)	VEGF	IL-1 β	IL-12 /23(p40)	IL-13	IL-15	GM-CSF	TGF- α	IL-4	IL-17A	
Monkey DIC	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑	↑	↑	↑	↑	↑	↑	→	→
Matsumoto <i>et al.</i> (2018)	↑↑↑	↑↑	↑	↑	↑↑	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Walborn <i>et al.</i> (2018)	↑↑↑	↑↑↑	↑↑	↑↑	↑↑↑	↑	→	→	→	→	↑↑	↑↑	↑↑	↑↑	↑	→	→	→	→	→	→	→	→
Nomura <i>et al.</i> (2011)	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑	→	→	→	→	→	→	→	→
Ng <i>et al.</i> (2003)	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑	↑	↑	↑	→	→	↑↑	↑↑	↑↑	→	→	→	→	→	→	→	→	→
Costa <i>et al.</i> (2019)	↑↑↑	↑↑	↑	↑	↑↑	↑	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→	→
Remy <i>et al.</i> (2018)	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑	↑↑↑	↑↑	↑↑	↑↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Chen <i>et al.</i> (2018)	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Holub <i>et al.</i> (2018)	↑↑	↑↑↑	↑	↑	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Tamayo <i>et al.</i> (2011)	↑↑	↑↑↑	↑	↑	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	→	→	↑↑	↑↑	↑↑	→	→	→	→	→	→	→	→	→
Jekard <i>et al.</i> (2018)	↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑	↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Sjövall <i>et al.</i> (2014)	↑↑↑	↑↑	↑↑	↑↑	↑	↑	↑	↑	↑	↑	↑	→	→	→	↑	↑	↑	↑	↑	↑	↑	↑	↑
Feng <i>et al.</i> (2016)	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Eidt <i>et al.</i> (2016)	↑↑	↑↑↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Wong <i>et al.</i> (2016)	↑↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Alder <i>et al.</i> (2014)	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Razavi <i>et al.</i> (2019)	↑↑↑	↑↑	↑↑	↑↑	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Bozza <i>et al.</i> (2007)	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
Chung <i>et al.</i> (2015)	↑↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑

Grading scale

1. As the general rule, the first 1/3 of all cytokines that increased the most are indicated with ↑↑↑. The next 1/3 is indicated with ↑↑. The final 1/3 of all cytokines is indicated with ↑.

2. Cytokines with comparable values were allocated with the same grade, regardless of what 1/3 they fell into.

research is still required in this field.

In short, the non-human primate is more useful than the rat as a DIC model because:

- Biomarker changes in primates resemble those in humans;
- Tests in PBMC, showed the TNF- α response is comparable between primates and humans;
- The TLR4 gene in primates is closer to humans than rodents;
- Unlike rodents in which the evaluation endpoint is the acute mortality rate, the fluctuations in cytokine parameters can be observed continuously over longer periods in primates, allowing more detailed comparisons; and
- Due to cross-reactivity, the primate model is useful in evaluating biopharmaceuticals being developed to treat DIC.

Additionally, the cytokine measurement model used in this study has the potential to be useful for detecting other cytokine storms. For example, CAR-T therapy, which is one type of genetically-modified T-cell therapy utilizing CD19-specific chimeric antigen receptors, shows dramatic effects against B-cell tumors. In 2017, the USFDA approved the CD19 CAR-T cell preparations Kymriah™ and Yescarta™ for treatment of refractory/relapsed B-cell acute lymphoblastic leukemia (ALL) and diffuse large B-cell lymphoma (DLBCL). Subsequently, Kymriah™ was approved in Europe in 2018 and in Japan in 2019.

Teachey *et al.* (2016) reported an association between the degree of the cytokine release syndrome and the outcome of the disease by measuring 43 cytokines in 51 patients suffering from refractory/relapsed B-cell acute lymphoblastic leukemia (ALL) treated with CTL019 (tisagenlecleucel, Kymriah™). As a result, in severe cases, G-CSF, GM-CSF, IFN- γ , IFN- α , IL-10, IL-1ra, IL-4, IL-6, IL-8, MCP-1, MIP-1 β , MIP-1 α , TNF- α , VEGF, etc. were significantly increased, and there were no statistically significant differences in IL-13, IL-15, IL-17, IL-1 β , IL-2, IL-5, etc. between severe and non-severe cases of the disease. As a cytokine release mechanism, it is thought that IFN- γ , IL-6, GM-CSF, etc. are released from activated T cells, IL-1ra, IL-10, IL-6, INF- α , MIP-1 α , MIP-1 β and MCP-1 are released from activated monocytes and macrophages, and IL-8, G-CSF, GM-CSF, VEGF, IL-6, etc. are further increased after tissue damage and inflammation.

Apart from IL-4, similar increases in cytokines were noted in the monkey DIC model. Therefore, it is reasonable to surmise that detection of the cytokine release syndrome that occurs during CAR T therapy may be possible in this cytokine measurement model.

Even in the use of TGN1412, predicted to be a super-antibody drug, cytokine storms were induced following symptoms such as initial increases in TNF- α , followed by increases in IFN- γ , and then IL-10, IL-8, IL-6, IL-4, IL-2 and IL-1 β . If you exclude IL-4, the cytokine storms reported in victims receiving TGN-1412 in 2006 were almost the same as those reactions noted in our monkey DIC model. However, Eastwood *et al.* (2010) claimed that due to a lack of CD28 expression on the CD4+ effector memory T-cells in NHPs, detection of the cytokine storms caused by TGN1412 was impossible due to species differences.

Therefore, before the cytokine measurement model used in this study is applied to investigate the effects of unknown substances, such as drugs under development, it is essential to conduct PBMC tests and other studies to confirm cross-reactivity, etc.

As described above, we elucidated the profile of cytokine storms in the LPS-induced DIC cynomolgus monkey model. Since the behavior of cytokines in this monkey model was similar to those noted in DIC in humans, this model will be useful for evaluating the efficacy of anti-DIC drugs. In addition, this model could also be useful for assessing the risk of cytokine storm development, which is a serious adverse effect of certain types of antibody drugs and CAR-T cell-based therapies.

ACKNOWLEDGMENTS

The authors thank Mr. Toshiyasu Hombo, Ms. Penelope Naruta and the technicians at Ina Research Inc. and Sekisui Medical Co., Ltd for providing technical assistance.

Conflict of interest---- The authors declare that there is no conflict of interest.

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Cytokine profile in DIC-cynomolgus monkey model

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