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## CHANGES OF SERUM CYTOKINE ACTIVITIES AND OTHER PARAMETERS IN DOGS WITH EXPERIMENTALLY INDUCED ENDOTOXIC SHOCK

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### ABSTRACT

To study the relationship of changes of cytokines in endotoxic shock, serum tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 like activities, together with physiologic and hemodynamic responses, were examined in dogs before and after intravenous administration of lipopolysaccharide (LPS) purified from *Escherichia coli* in a dose of 500  $\mu\text{g}/\text{kg}$  of body weight. The blood endotoxin concentration increased significantly at 30 min after LPS administration, and maintained high levels for 24 hr. Red blood cell counts, hemoglobin concentration and hematocrit values increased at 30 min, and these high values persisted for 24 hr. The platelet count decreased significantly at 30 min, then showed a tendency to recover, but decreased again at 24 hr. Cardiac output, cardiac index and mean arterial pressure showed transient, significant decreases at 15 min, and then returned to the baseline levels by 24 hr. TNF-like activities increased at 30 min, while IL-1-like activities did so between 30 and 60 min. The former reached the maximal levels at 2 hr and the latter at 1.5 hr. Both activities were then hardly detectable from 6 to 24 hr. IL-6-like activities elevated at 1 hr with the peak at 1.5 hr, and remained high until 24 hr.

Key Words: dog, endotoxic shock, interleukin-1, interleukin-6, tumor necrosis factor.

### INTRODUCTION

Septic shock is defined as peripheral circulatory failure with inadequate tissue perfusion and cell death secondary to an infectious process<sup>10)</sup>. Sepsis is the clinical term for the systemic response to dividing and invading microorganisms of all types; gram-negative and gram-positive bacteria, fungi and viruses<sup>1)</sup>. Endotoxic shock should be equated only with septic shock caused by gram-negative bacteria. In some clinical

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studies in small animals, 60% of the infections were attributable to gram-negative organisms<sup>8,9</sup>). Recently, as a significant factor in sepsis, cytokines, a product of the arachidonic acid cascade and platelet-activating factor (PAF) were described<sup>2,7,26</sup>). Understanding of these variations is important to clarify the mechanism of canine endotoxic shock, and is also useful in prevention and therapy.

The purpose of this study was to investigate the relationship of changes of cytokines in endotoxic shock by measuring the changes of blood endotoxin concentrations and cytokine activities in dogs with experimentally induced endotoxic shock. Furthermore, the hematological and hemodynamic responses were evaluated.

#### MATERIALS AND METHODS

*Experimental animals*: Ten clinically normal adult male beagle dogs, weighing 13–14 kg were used. They were assigned randomly to 2 groups, one for control (group C), and the other for an experimentally induced endotoxic shock (group ES). After induction of the general anesthesia with 0.03 mg/kg atropine sulfate (i.m.), 0.03 mg/kg flunitrazepam (i.v.) and approximately 15 mg/kg thiaminal sodium (i.v.), they were endotracheally intubated, and anesthesia was maintained with halothan. The breathing movements arrested using 0.05 mg/kg vecronium bromide (i.v.). The dogs were then mechanically ventilated using a ventilator (KV-2N, Kimuraikakiki Co. Ltd., Tokyo, Japan). The administration was performed after 30 min, when the hemodynamic state of the dogs had stabilized. After measuring and sampling at 0 min, group ES dogs were given with intravenous injection of lipopolysaccharide (LPS, *E. coli* 055 : B5, Difco, Detroit, Michigan, USA, 500  $\mu$ g/kg) dissolved in 10 ml physiological saline solution, while group C dogs were treated with intravenous injections of 10 ml saline only. In both groups, anesthesia was maintained for 6 hours.

*Blood endotoxin concentration*: Blood was collected from the jugular vein immediately before and 30 min, 3 and 6 hr after the administration of lipopolysaccharide or physiological saline solution. Endotoxin measurement was made with the endotoxin-specific chromogenic test (Endospecy [ET-Test], Seikagakukougyou, Tokyo, Japan)<sup>15</sup>).

*Hemodynamic response and rectal temperature*: The rectal temperature, heart rate and mean arterial pressure were monitored simultaneously throughout the experiment using a polygraph (System 360, Nippondenki Sannei, Tokyo, Japan). For the measurement of MAP and cardiac output, the ordinary catheterization technique was employed. Rectal temperature and heart rate were measured at 24 hr before and 0, 30 min, 1, 2, 3, 4, 5, 6 and 24 hr after LPS administration. Cardiac output, cardiac index and mean arterial pressure were measured at 0, 15, 30 min, 1, 3 and 6 hr after administration. Cardiac output was measured by the thermodilution method using a cardiac output computer (COM-2, Baxter, Santa Ana CA, USA). Cardiac index was calculated using the formula:  $CI = CO / (BSA)^{2/3}$ .

*Hematological examination*: Blood was withdrawn at 24 hr before and 0, 30 min, 1, 2, 3, 4, 5, 6 and 24 hr after administration, and the red blood cell count, hemoglobin concentration, hematocrit value, white blood cell count and platelet count were measured with an automated hematology cell counter (System 9000, Serono-Baker Diagnostics, Inc., Allentown, U.S.A.).

*Coagulative factor*: The prothrombin time, activated partial thromboplastin time and fibrinogen were measured at 24 hr before and 0, 30 min, 3, 6, and 24 hr after administration with a thrombometer (COBAS FIBRO, F. Hoffmann-La Roche & Co. LTD, Basle, Swis).

*Blood serum cytokine activity*: The blood samples collected at 24 hr before and 0, 30 min, 1, 1.5, 2, 3, 4, 5, 6 and 24 hr after administration were placed into a sterile tube and centrifuged at  $2,000 \times g$  for 15 min at room temperature (20–22°C). The serum was collected aseptically and stored at below –80°C until use. Measurements of TNF-like, IL-1-like, and IL-6-like activities were conducted by the methods described by Yamashita *et al.*<sup>23,24</sup>. In the IL-6-like assay, the wells were pulsed with 0.025 ml of 3-(4, 5-dimethylthiazoyl)2, 5 diphenyl tetrazolium bromide (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) for the last 6 hrs of incubation instead of [<sup>3</sup>H]-thymidine used by Yamashita *et al.*<sup>23</sup>. After incubation, 0.1 ml of 50% N, N-dimethylformamide containing 0.7 M sodium dodecyl sulfate (Wako Chem. Ind. LTD., Osaka, Japan) was added to each well and pipetted. Serum samples were diluted to 1:80 to minimize the effects of the uncharacterized factors on MH60<sup>23</sup>. The optical density was read at an excitation wavelength of 540 nm (ImmunoReader NJ-2000, Nippon InterMed, Tokyo, Japan). All determinations were performed in triplicate.

*Statistical analysis*: All values are expressed as mean  $\pm$  SD for 5 dogs. The obtained data were statistically analyzed by Student's *t*-test according to variance equivalence. For nonnormally distributed data, Welch's test was used. A difference was considered statistically significant when the P-value was smaller than 0.05.

## RESULTS

*Blood endotoxin concentration*: The blood endotoxin concentration in group ES increased significantly at 30 min after LPS administration and was maintained at a high level until 24 hr. Group C did not show any significant change. In both groups, significant changes were observed from 30 min to 24 hr (Fig. 1).

*Rectal temperature*: In group C, rectal temperature decreased throughout the anesthetic period, reached the lowest temperature (mean 34.3°C) at 6 hr, and then returned to the baseline levels by 24 hr. In group ES, rectal temperature showed the same tendency as seen in group C, but was always about 0.5°C higher than in group C, and recovered at 24 hr. However, the difference between the two groups was not significant.

*Hemodynamic response*: There was a decrease in heart rate in group C following

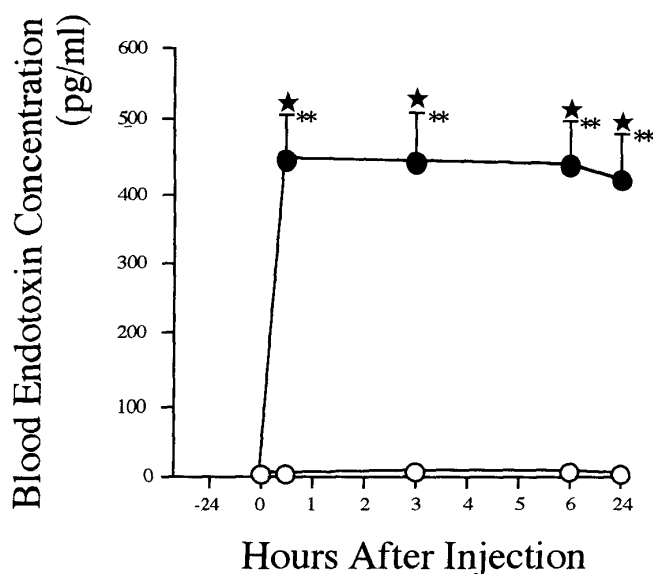


Fig. 1. Changes in blood endotoxin concentration after an injection of lipopolysaccharide.

● : Experimentally induced endotoxic shock group (ES group), n=5;  
 ○ : Control group (C group), n=5. Significantly different  
 (\* :  $P < 0.05$ ; \*\* :  $P < 0.01$ ) compared with the baseline (0min) value,  
 and between groups C and ES (☆ :  $P < 0.05$ ; ★ :  $P < 0.01$ ).

anesthesia. In group ES, heart rate decreased slightly at 30 min after LPS administration, but it returned in a manner similar to that of group C. There were no significant heart rate differences between the two groups.

Group C did not demonstrate significant changes in cardiac output, cardiac index or mean arterial pressure. Group ES had a marked transient decrease in these values at 15 min and maintained low levels until 6 hr, though it recovered slightly. Both groups showed significant changes from 15 min to 3 hr (Fig. 2).

*Hematological findings*: The red blood cell count, hemoglobin concentration and hematocrit value of group C decreased just after anesthesia, then recovered gradually, and finally increased slightly at 24 hr. Group ES showed a similar decrease in these values just after anesthesia, but increased immediately after LPS administration.

There were significant changes in white blood cell count in the 2 groups at 24 hr before the experiment, but the values were within the normal range. Group C showed a tendency to decrease throughout the anesthetic period except for the marked increase at 24 hr, while group ES had a significant decrease at 30 min, maintained low levels until 6 hr, and finally increased significantly at 24 hr.

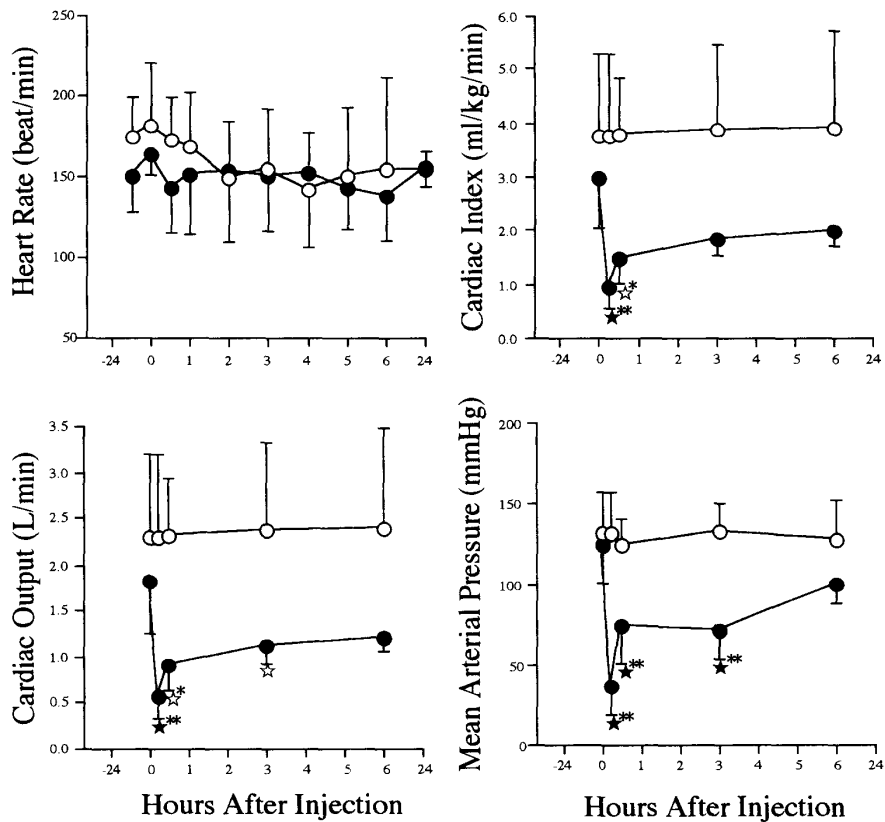


Fig. 2. Changes in heart rate, cardiac output, cardiac index, and mean arterial pressure after an injection of lipopolysaccharide. For key see Fig. 1.

The platelet count of group C decreased immediately after anesthesia and remained low throughout the experiment. In group ES, platelet count decreased significantly at 30 min after treatment, then increased slightly until 5 hr, and finally decreased again significantly at 24 hr (Fig. 3).

*Coagulative factor:* The prothrombin time and activated partial thromboplastin time did not show significant changes in either group. Fibrinogen increased only at 24 hr in both groups (Fig. 4).

*Cytokine activities:* In group C, there were no detectable changes in TNF-, IL-1-, and IL-6-like activities during the experimental period. In group ES, TNF-like activity increased at 30 min after LPS treatment, reached the maximal level at 2 hr, then decreased gradually, finally becoming hardly detectable. IL-1-like activity increased at 30 min after LPS treatment, peaked at 1.5 hr, and subsequently fell to the pretreatment levels. IL-6-like activity increased significantly at 1 hr with the peak at 1.5 hr and maintained a high level until 24 hr (Fig. 5).

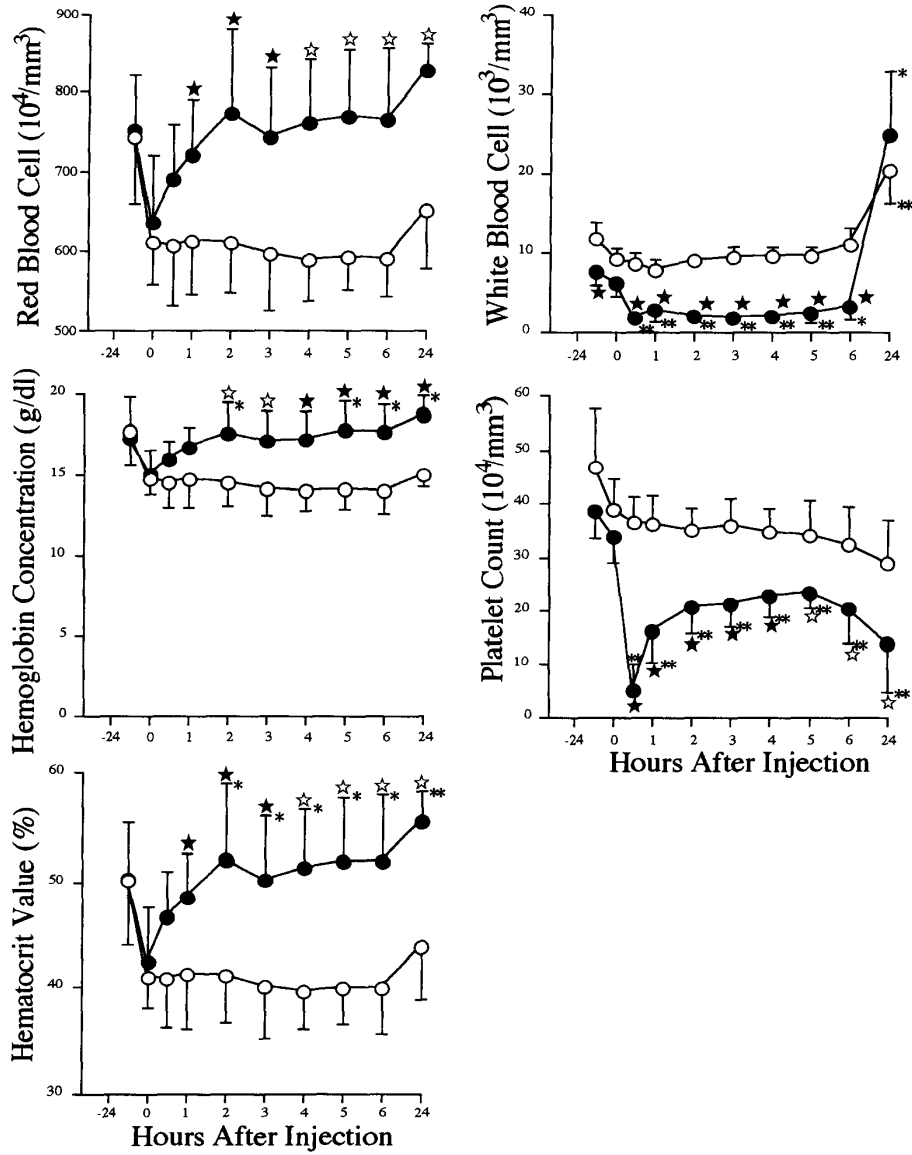


Fig. 3. Changes in red blood cell count, hemoglobin concentration, hematocrit value, white blood cell count, and platelet count after an injection of lipopolysaccharide. For key see Fig. 1.

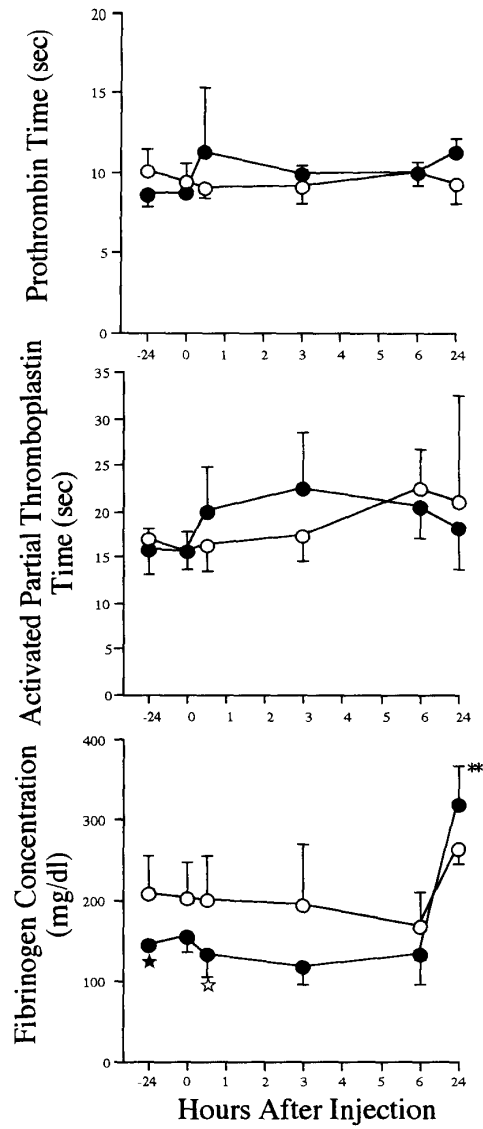


Fig. 4. Changes in prothrombin time, activated partial thromboplastin time, and fibrinogen concentration after an injection of lipopolysaccharide. For key see Fig. 1.



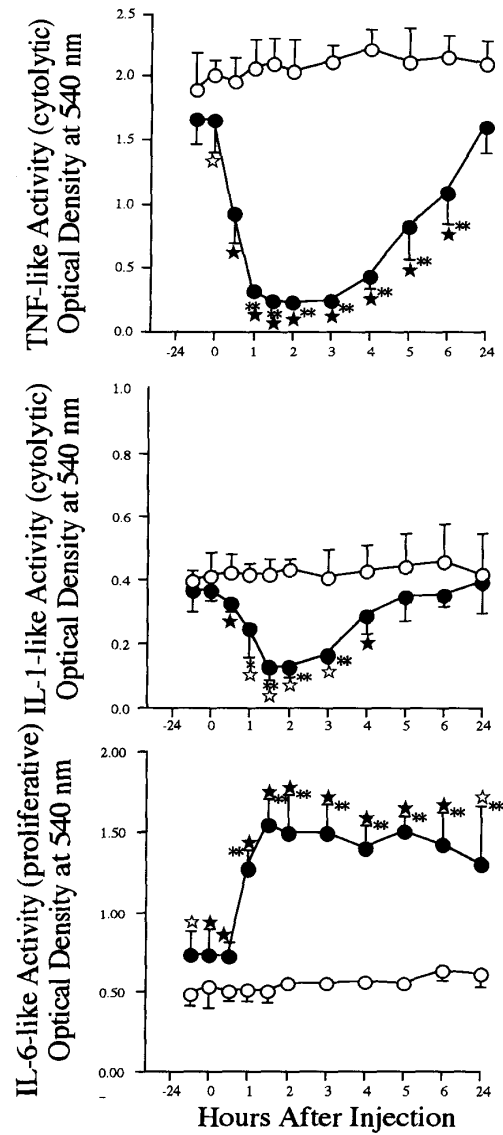


Fig. 5. Changes in TNF-, IL-1-, and IL-6-like activities after an injection of lipopolysaccharide. TNF- and IL-1-like activities were measured with cytolytic assay, and IL-6-like activity was measured with proliferative assay. For key see Fig. 1.

## DISCUSSION

In the canine model of septic shock, a lethal dose of endotoxin (1.5 mg/kg or more) is frequently used<sup>13)</sup>. In this study, 500  $\mu$ g/kg purified endotoxin was administered to study the changes of cytokines in endotoxic shock. Although the dose was not lethal, the blood endotoxin concentration increased markedly just after administration to about 100 times the value before administration, and maintained a high level until 24 hr.

Endogenous pyrogen, which induces fever, is regarded as IL-1 and is an important factor in infectious diseases. In addition, TNF, IL-6 and interferon (IFN) are considered to be co-mediators of fever. LeMay *et al.*<sup>16)</sup> reported that TNF- and IL-6-like activities were related to LPS-induced fever in an all-or-none manner. In the present study, group ES showed a slight but insignificant increase in rectal temperature compared with group C throughout the experimental period (data not shown), suggesting the influence of cytokines as described above. Endotoxin induced fever was probably suppressed by the effect of general anesthesia<sup>11)</sup>.

Tracet *et al.*<sup>21,22)</sup> reported that administration of TNF or IL-1 created clinical symptoms similar to those of endotoxic shock such as hypotension and decrease of cardiac output and cardiac index, and that IL-6 by itself had no acute toxic effect<sup>25)</sup>. On the other hand, Camussi *et al.*<sup>3)</sup> found that TNF alone did not induce endotoxic shock, but did PAF, which was induced by TNF. Our findings of transient hypotension, decreases of cardiac output and cardiac index levels, increases of red blood cell count and hematocrit value, and a change in platelet count resembled those in the experiment where PAF was administered; therefore, supporting the participation of PAF in this process<sup>5,14,20)</sup>. TNF- and IL-1-like activities increased already at 30 min after LPS administration and peaked at about 2 hr, whereas sudden changes in hemodynamic responses occurred just after administration and stabilized again while both cytokine activities were still demonstrated. Therefore, from these findings, it appears that a hemodynamic response after administration of LPS seems to induce the action of PAF, followed by TNF or IL-1 release.

WBC decreased after administration of LPS, which suggested the acceleration of adherence of neutrophils to the endothelium by LPS and LPS-induced TNF<sup>4,17,19)</sup>. In contrast, the significant increase of WBC at 24 hr after treatment in both groups, which was expected, might have resulted from the inflammatory response to the surgical invasion and endotoxin.

Garner and Evensen<sup>6)</sup> reported that a lethal dose of endotoxin induces changes in coagulative factors, but none of the parameters measured changed significantly in the present study. Fibrinogen also increased significantly at 24 hr after treatment, but this value was within the normal range (200–400 mg/dl).

Hesse *et al.*<sup>12)</sup> reported changes of blood TNF, IL-1 and IFN- concentrations

when endotoxin was given to *baboons*. They demonstrated that TNF peaked at 90 min after administration and then disappeared within 6 hr, while IL-1 and IFN- $\gamma$  peaked within 3 and 8 hr, respectively. Our study also showed similar results. These results suggest that the transient release of TNF and IL-1 might be the first response of the host to bacterial infection.

IL-6 is released from various cells in response to stimulation by LPS, TNF and IL-1. Furthermore, IL-6 has a control system of its own for the release of IL-6<sup>18)</sup>. Stimulation by LPS may also induce the release of TNF and IL-1, which subsequently will initiate IL-6 release. IL-6 has the capability to induce various host responses, like hemoconcentration, with stimulation of IL-6 itself. TNF and IL-1 may disappear soon after peaking, while IL-6 may remain at high levels for a long time.

In conclusion, our data showed that cytokines (TNF, IL-1 and IL-6) respond to LPS injection as a model of endotoxin shock. The hemodynamic, complete blood count and coagulative factor response is also shown. However, the exact mechanism of these reactions needs to be further studied.

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