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Effect of CO_2 Pneumoperitoneum on the Systemic and Peritoneal Cytokine Response in a LPS-Induced Sepsis Model

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Key Words
Pneumoperitoneum · Laparotomy · Cytokines · Sepsis

Abstract
We studied the effect of carbon dioxide (CO_2) pneumoperitoneum on the systemic and peritoneal cytokine response in a rat model of intraperitoneal sepsis. After intraperitoneal injection of bacterial lipopolysaccharide (LPS, 10 mg/kg), rats were divided into 3 groups (n = 49 in each group): control (abdominal puncture); CO_2 pneumoperitoneum, and laparotomy. Blood and peritoneal lavage fluid (PLF) were sampled at 0, 1, 2, 3, 4, 6, and 8 h after LPS challenge. Blood cell counts, plasma endotoxin level, and the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in the plasma and PLF were measured. Blood cell counts did not differ between the 3 groups. Plasma endotoxin levels in the pneumoperitoneum group were significantly increased immediately after the procedure ($p < 0.05$). Although peak plasma TNF-α levels in the pneumoperitoneum group were seen immediately after the procedure, other changes in plasma cytokine levels did not differ significantly between the 3 groups. PLF TNF-α and IL-1β levels in the pneumoperitoneum group were significantly lower than levels in the control and laparotomy groups soon after the procedure ($p < 0.05$). PLF IL-6 levels in the pneumoperitoneum group tended to be lower than those in the laparotomy group. In conclusion, CO_2 pneumoperitoneum might induce different responses between systemic and peritoneal cytokines soon after the procedure in a rat model of intraperitoneal sepsis.

Introduction

Laparoscopic surgery has been widely accepted as a minimally invasive operative treatment because of decreased postoperative pain and length of hospital stay [1–3]. Laparoscopic surgery has been reported to be effective treatment for intra-abdominal inflammatory disease, including acute appendicitis [4–6], diverticular disease [7], and perforated peptic ulcer [8, 9]. Furthermore, these techniques are applied in a variety of surgical conditions complicated by peritonitis resulting in intraperitoneal sepsis [7, 10, 11].

Some immunologic alternations are observed in acute inflammation associated with infection and surgical trauma. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are considered potential mediators of inflammation produced by immunoregulatory cells. The use of a laparoscopic technique has also been associated with decreased production of these bio-

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chemical markers of surgical stress, compared with open procedures [3, 12–14].

However, there is still a theoretical concern that the carbon dioxide (CO_2) pneumoperitoneum used to establish the operative field may enhance endotoxemia and worsen sepsis in conditions complicated by intra-abdominal infection and peritonitis. Only a few controversial experimental data exist with regard to the effect of pneumoperitoneum on bacteremia and physiological changes in sepsis [15–19]. The aim of this study was to evaluate the effect of CO_2 pneumoperitoneum on endotoxemia and the proinflammatory cytokine response in intraperitoneal sepsis.

Materials and Methods

Animals

Animal care and all experiments were approved by the Animal Studies Committee at Oita Medical University according to National Institutes of Health guidelines. Adult male Sprague-Dawley rats (300–380 g) were used for all experiments. They were housed in a controlled environment, exposed to a 12-hour light-dark cycle, and allowed access to food and tap water ad libitum. Lipopolysaccharide (LPS) derived from *Escherichia coli* O111:B4 (Difco Laboratories, Detroit, Mich., USA) was dissolved in 0.9% sterile saline at a concentration of 5 mg/ml, and stored at –30°C until use. Prior to usage the LPS was boiled for 10 min and filtered through a 0.22-μm filter.

Experimental Protocol

All animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (75 mg/kg), which was enough to maintain anesthesia during the following operative procedure. First, the trachea was cannulated with a 7-french multipurpose tube (ATOM, Kyoto, Japan) via tracheotomy to support ventilation. The left femoral artery and vein then were cannulated with a polyethylene tube (PE-50, Clay Adams, Parsippany, N.J., USA) under sterile conditions. The venous catheter was used for continuous infusion of a saline solution at a rate of 4 ml/kg/h until the end of the operative procedure, following which 4 ml/kg of the saline solution was injected subcutaneously every 2 h. The arterial catheter was connected to a transducer (DX-360, Ohmeda, Tokyo, Japan) and the blood pressure and heart rate were displayed on a polygraph system (RM-6000, Nihon Koden, Tokyo, Japan). After stabilization for 30 min, the abdomen was shaved and the skin was cleaned with chlorhexidine in alcohol. An intraperitoneal injection of 10 mg/kg of LPS was administered in the midline with a 27-gauge needle. To avoid variation in the volume of saline solution administered, the LPS dose was suspended in a constant solution volume of 3 ml/kg. Preliminary study showed that the 50% lethal dose of intraperitoneal LPS with a 48-hour observation time was 10 mg/kg.

One hour after intraperitoneal injection of LPS, the animals were randomly divided into the following 3 groups (n = 49 in each group): control = the abdominal cavity was punctured using an 18-gauge needle without gas insufflation for 1 h; pneumoperitoneum = the abdominal cavity was insufflated with CO_2 gas at room temperature, the CO_2 pneumoperitoneum was created for 1 h using an automatically controlled insufflator (Electronic CO_2 Surgiflator 9100, NISCO, Tokyo, Japan) at a constant intra-abdominal pressure of 10 mm Hg, and laparotomy = a 5-cm midline laparotomy was performed, during which the abdominal viscera were exposed for 1 h. The incision was then closed in layers with 4-0 Vicryl.

Samples and Assays

Blood samples were obtained sterilely from the arterial line at 0, 1, 2, 3, 4, 6 and 8 h after LPS intraperitoneal challenge. Arterial blood samples were anticoagulated with EDTA or heparin sodium. The plasma of blood samples was separated by centrifugation at 4°C for 15 min at 3,000 *g*, and then was divided into aliquots and stored at –80°C until assayed.

After arterial blood samples had been obtained, 10 ml of sterile normal saline solution was injected into the abdominal cavity. After gentle massage of the abdomen, 7 ml of the peritoneal lavage fluid (PLF) was taken from each animal. PLF was separated by centrifugation at 4°C for 5 min at 700 *g* and used for measurement of peritoneal cytokines. Each animal subjected to the protocol was sacrificed at this time with an overdose of intraperitoneal pentobarbital sodium so that peritoneal cytokines could be determined.

Arterial blood, anticoagulated with EDTA 2, 4, 6 and 8 h after LPS intraperitoneal challenge, was used for measurement of blood cell counts. The total number of the circulating leukocytes and neutrophils were determined using a Technicon H-1 system (Bayer Diagnostics, Munich, Germany). The plasma of heparinized arterial blood samples at all time points were used for measurement of the endotoxin and cytokine levels. The plasma endotoxin level was measured using a quantitative chromogenic Limulus Amebocyte Lysate assay (Endotoxin Test, LAL-ES, Wako Pure Chemicals Industries, Osaka, Japan) according to the manufacturer's directions. When the LAL-ES was used, a specially designed instrument for turbidimetric kinetic assay, the Toxinometer MT-251 (developed and provided by Wako Pure Chemicals Industries), was employed. All determinations were carried out in duplicate.

The TNF-α concentrations in plasma and PLF were determined using a sandwich enzyme-linked immunosorbent assay with a commercially available kit (Biosource International, Camarillo, Calif., USA) with a threshold level of 4 pg/ml. Briefly, a 96-well microplate was coated with anti-TNF-α antibody. The standard or samples to be tested and a biotin-labeled anti-TNF-α antibody were pipetted into each well and incubated. Streptavidin-horseradish peroxidase complex was then used as an antibody to a second epitope on TNF-α. Optical density was read at 450 nm following the addition of a coloring reagent using an enzyme-linked immunosorbent assay plate reader (Multiskan Multisoft; Labsystems, Helsinki, Finland) for each sample and compared with a standard curve. All determinations were carried out in duplicate.

Similarly, IL-1β and IL-6 concentrations in plasma and PLF were determined using a sandwich enzyme immunoassay with a commercially available kit (Biosource International), each with a threshold level of 3 pg/ml.

Statistical Analysis

All values are presented as the mean ± standard error of the mean (SEM). Overall differences between each of the 3 groups for all time points were analyzed using an analysis of variance (ANOVA). The differences between each of the 3 groups for each time point were analyzed using the Mann-Whitney's U nonparametric test. Differences were considered statistically significant when $p < 0.05$.

Results

All animals survived for the duration of the experiment. During the experiment, the animals in each group were stable and similar with respect to mean arterial blood pressure and heart rate. Leukocyte and neutrophil counts dropped in all 3 groups after the operative procedure. There was no significant difference between the 3 groups (table 1).

Plasma Endotoxin Levels

During the period following the operative procedure, plasma endotoxin levels in the pneumoperitoneum and laparotomy groups were significantly higher than those in the control group ($p < 0.05$). Immediately after the operative procedure, the plasma endotoxin level in the pneumoperitoneum group was significantly higher than that in the laparotomy group (30,003 ± 13,885 versus 1,480 ± 635 pg/ml; $p < 0.05$). The peak level of plasma endotoxin was seen at 4 h in the control group. The plasma endotoxin levels in the pneumoperitoneum and laparotomy groups, however, increased gradually from 2 to 8 h after the procedure (fig. 1).

Plasma Proinflammatory Cytokine Levels

The peak plasma TNF-α level in the pneumoperitoneum group was seen immediately after the operative procedure. Although the peak was earlier than those in the laparotomy and control groups, it was not significantly different with that in the control and laparotomy groups (fig. 2a). Changes in plasma IL-1β levels over time were similar in the laparotomy and pneumoperitoneum groups. Plasma IL-1β levels in the laparotomy and pneumoperitoneum groups were significantly higher at 6 h than those in the control group ($p < 0.05$; fig. 2b). The plasma IL-6 levels were not significantly different between the 3 groups. The peak levels were seen at 6 h in each group (fig. 2c).

PLF Proinflammatory Cytokine Levels

The definite peak PLF TNF-α level in the pneumoperitoneum group was not seen after the operative procedure over the course of the experiment, and was lower than those in the control and laparotomy groups at 3 h ($p < 0.05$; fig. 3a). The PLF IL-1β levels in the pneumoperitoneum group were significantly lower than those in the lap-

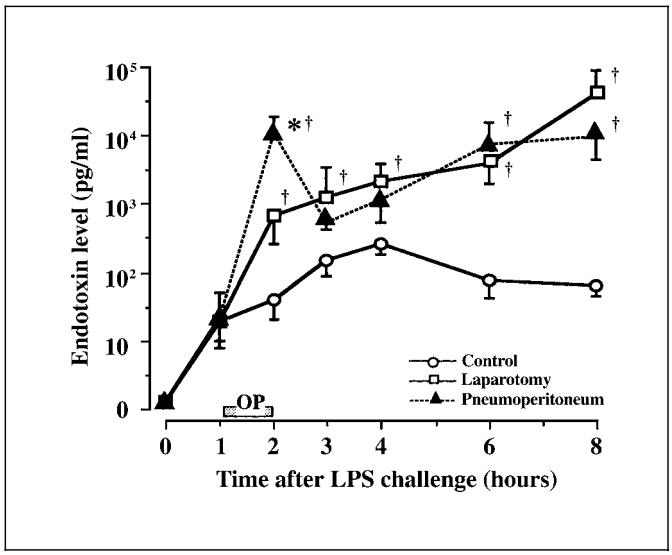

Fig. 1. Plasma endotoxin levels after intraperitoneal injection of lipopolysaccharide (LPS). Values are expressed as mean ± SEM (n = 7 for each point). OP = Operative procedure. * $p < 0.05$ versus laparotomy; † $p < 0.05$ versus control.

Table 1. Blood counts after intraperitoneal injection of lipopolysaccharide (LPS) in rats undergoing an abdominal puncture (control), laparotomy, or pneumoperitoneum (mean ± SEM)

	Time after intraperitoneal injection of LPS, h			
	2 (n = 7)	4 (n = 7)	6 (n = 7)	8 (n = 7)
Leukocytes, 10^6/ml				
Control	5.61 ± 1.20	2.38 ± 0.73	2.13 ± 0.23	1.99 ± 0.25
Laparotomy	3.65 ± 0.50	3.84 ± 0.45	2.12 ± 0.24	2.53 ± 0.38
Pneumoperitoneum	3.99 ± 0.78	3.70 ± 0.38	3.09 ± 1.06	2.89 ± 0.53
Neutrophils, 10^6/ml				
Control	1.13 ± 0.58	0.60 ± 0.31	0.62 ± 0.36	0.20 ± 0.03
Laparotomy	1.19 ± 0.18	0.70 ± 0.34	0.40 ± 0.10	0.16 ± 0.01
Pneumoperitoneum	1.12 ± 0.47	0.96 ± 0.25	0.65 ± 0.19	0.23 ± 0.08

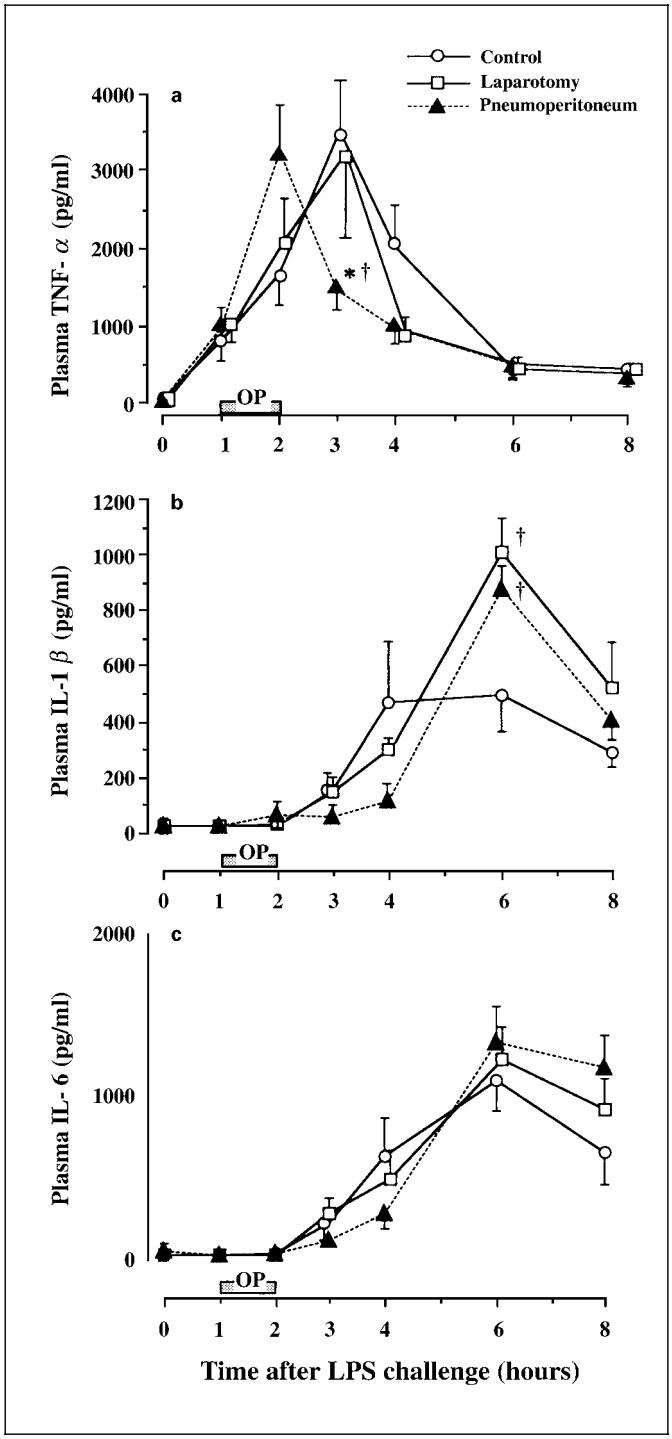

Fig. 2. Changes in plasma proinflammatory cytokine levels after intraperitoneal injection of lipopolysaccharide (LPS). **a** Plasma TNF-α levels. **b** Plasma IL-1β levels. **c** Plasma IL-6 levels. Values are expressed as mean ± SEM (n = 7 for each point). OP = Operative procedure. * $p < 0.05$ versus laparotomy; † $p < 0.05$ versus control.

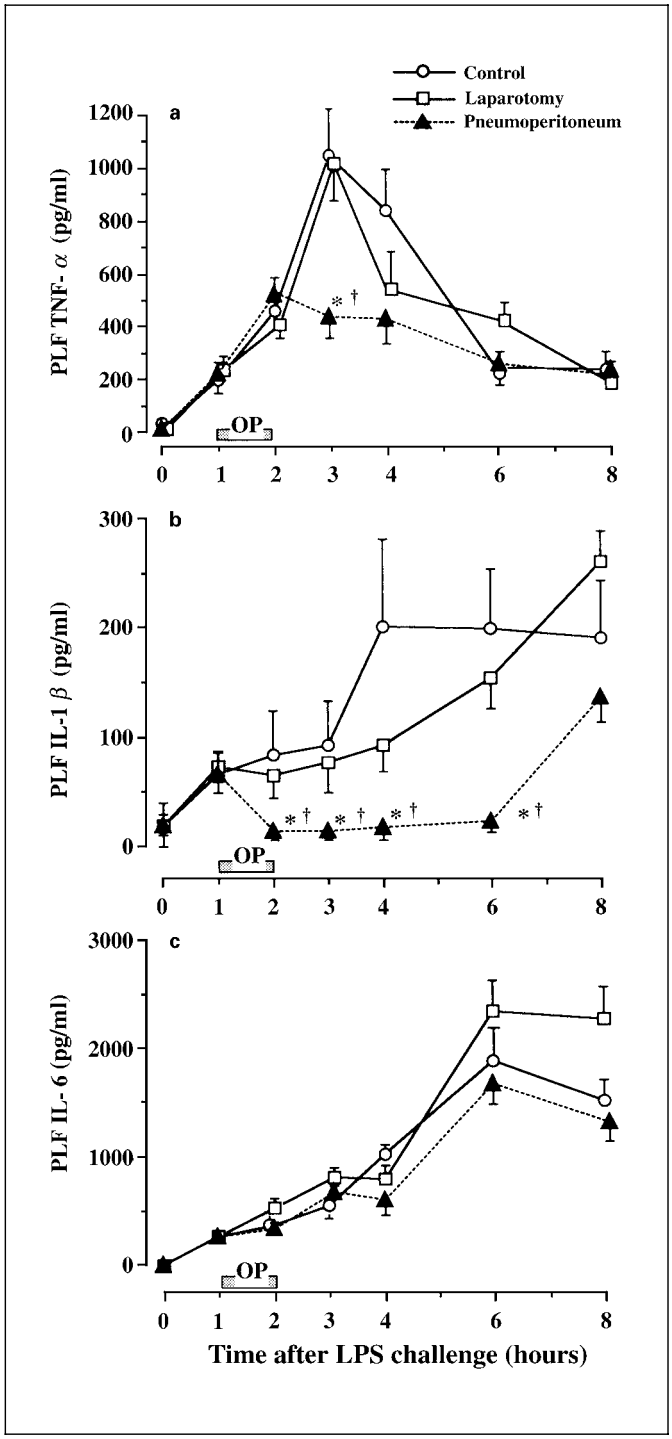

Fig. 3. Changes in peritoneal lavage fluid (PLF) proinflammatory cytokine levels after intraperitoneal injection of lipopolysaccharide (LPS). **a** PLF TNF-α levels. **b** PLF IL-1β levels. **c** PFL IL-6 levels. Values are expressed as mean ± SEM (n = 7 for each point). OP = Operative procedure. * $p < 0.05$ versus laparatomy; † $p < 0.05$ versus control.

arotomy group from 2 to 6 h ($p < 0.05$; fig. 3b).The PLF IL-6 levels had lower tendency than the control and laparotomy groups. The peak levels were seen at 6 h in each group (fig. 3c).

Discussion

The present LPS-induced sepsis animal model was based on a method described by Hadjiminas et al. [20]. The 50% lethal dose (10 mg/kg) of LPS applied in our experiment ensured that the severity of peritonitis would result in intraperitoneal sepsis similar to that requiring surgical therapy in clinical situations. We adopted this intraperitoneal LPS injection method instead of the cecal ligation and puncture method for our animal model of intraperitoneal sepsis because we wanted to exclude the surgical effect of peritoneal damage during this experimental study. It is well known that free-existing LPS following bacterial infection in the peritoneal cavity is a principle mediator of gram-negative septic shock. In the case of intraperitoneal sepsis, LPS may rapidly escape the peritoneal cavity and enter the systemic circulation [21]. In the present study, we investigated changes in the host cytokine response after CO_2 pneumoperitoneum in animals with diffuse peritonitis resulting in intraperitoneal sepsis without shock.

The plasma endotoxin level was significantly higher immediately after CO_2 pneumoperitoneum than after laparotomy. Previous studies have indicated that peritoneal fluid is removed from the peritoneal cavity through large terminal lymphatics and channeled to the thoracic duct. The openings of these lymphatics, called stomata, are located on the peritoneal surface of the diaphragm [22]. Increased abdominal pressure has been shown to increase the patency of the stomata and lead to the transport of particulate materials from the peritoneal cavity to the systemic circulation [23]. It is likely, therefore, that the elevated intra-abdominal pressure used to maintain pneumoperitoneum may increase the plasma endotoxin level. In contrast, some experimental peritonitis studies have reported that positive pressure insufflation of the peritoneal cavity did not increase bacterial translocation from the peritoneal cavity into the bloodstream [15–17]. However, changes in plasma endotoxin levels immediately after the operative procedure were not measured in those studies. Collet et al. [24] reported peritoneal bacterial clearance to be greater in animals undergoing laparoscopic procedures than in those undergoing open procedures. Our data support this observation, and we believe that the transient increase in the endotoxin level may be mediated by increased translymphatic absorption due to the increased intra-abdominal pressure. Furthermore, CO_2 may dilate the peritoneal vessels directly [25] and induce the enhanced absorption of LPS. In this series, we did not observe peritoneal vessels. Further study would be necessary to find out whether CO_2 pneumoperitoneum induces an enhanced absorption of various materials from the intra-abdominal cavity.

In this animal model, the plasma TNF-α level increased immediately after CO_2 pneumoperitoneum was established. Although this level did not differ significantly from that after laparotomy, this increase might represent a response to the increased level of systemic circulating endotoxin. The extent of the rise in plasma IL-1β and IL-6 levels has been correlated with the magnitude of operative trauma. In our study, the changes in plasma IL-1β and IL-6 levels were similar in the laparotomy and pneumoperitoneum groups. Therefore, there may be only a little difference between laparotomy and pneumoperitoneum in terms of the degree of surgical systemic stress in this sepsis model.

Some authors have indicated that systemic cytokine levels do not correlate well with those in the peritoneal cavity [26, 27]. Therefore, we studied the peritoneal proinflammatory cytokine levels. West et al. [28] reported that macrophages cultured in vitro secreted significantly less TNF and IL-1 after LPS stimulation when they were incubated in the presence of CO_2 gas. It is meaningful that the early peritoneal inflammatory cytokine release is relatively low after CO_2 pneumoperitoneum. Excessive margination of activated neutrophils mediated by TNF-α and IL-1β leads to tissue injury [29, 30]. Some evidence, however, indicates that local, not systemic, cytokine levels reflect the severity of a local inflammatory process and that a certain amount of cytokines play an important role in supporting local host defense mechanisms [31, 32]. For example, local action of TNF-α and IL-1β induces local inflammation, recruiting activated neutrophils (IL-8) to the focus and stimulating phagocytosis of bacteria. There are also some reports that laparoscopic appendectomy for perforated appendicitis is associated with a trend toward a higher rate of postoperative intra-abdominal infectious complications than is observed with open appendectomy [33, 34]. The lower peritoneal proinflammatory cytokine release might be associated with postoperative complications. Further examination of the intraperitoneal response after CO_2 pneumoperitoneum is required.

In summary, our report outlines the early response of several proinflammatory cytokines to CO_2 pneumoperi-

toneum. In rats with diffuse peritonitis resulting in intraperitoneal sepsis, CO_2 pneumoperitoneum induces enhanced endotoxemia immediately after the procedure. Systemic proinflammatory cytokine levels after CO_2 pneumoperitoneum were increased to levels similar to those after laparotomy, but peritoneal levels were transiently lower than those in animals undergoing laparotomy. The early inhibited peritoneal proinflammatory cytokine responses might suggest a relatively mild host response after laparoscopic surgery.

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