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SURGICAL ENDOSCOPY
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The role of the spleen in laparoscopy-associated inflammatory response

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Abstract
Background: Carbon dioxide (CO_2) pneumoperitoneum alters the inflammatory response in animal models of sepsis. The spleen is a key organ in inflammation and its removal was predicted to modify this effect.
Methods: The acute phase inflammatory response to lipopolysaccharide (LPS) challenge in male rats was examined for the effects of splenectomy (spx) and the technique of removal (open or laparpscopic). A series of experiments compared LPS-only controls with LPS injection 2 or 9 days following open spx, lap CO_2 spx, open sham, or lap CO_2 sham. The method of splenectomy was studied by randomization to control, open spx, lap CO_2 spx, lap helium (He) spx, or lap air spx with LPS challenge on postoperative day 2. Serum levels of tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ) and, interleutin (IL) 10 were collected at multiple time points, assayed by commercial enzyme-linked immunosorbent assay, analyzed by analysis of variance.
Results: Levels of TNF-α at 1.5 were significantly lower following open sham than following lap sham ($p < 0.05$). Splenectomy drastically reduced INF-γ and TNF-α levels compared to controls ($p < 0.05$) on postoperative day 2. No method of spx preserved TNF-α or INF-γ responses. Recovery of TNF-α response on day 9 was delayed in the spx groups.
Conclusions: Splenectomy dramatically reduces TNF-α and INF-γ responses to LPS challenge, although by different mechanisms. Pneumoperitoneum-mediated modulation of the septic inflammatory response is partially dependent on the spleen.

Key words: Laparoscopy — Pneumoperitoneum — Splenectomy — Inflammatory — response — Lipopolysaccharide — Endotoxemia

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The widespread adoption of laparoscopic surgery in the mid-1990s was driven by improved patient comfort and recovery times. Although these clinical benefits have been documented in numerous outcomes studies, the potential physiologic benefits of laparoscopic surgery are still being elucidated. Interest in this topic has led to previous work by our lab exploring the effect of CO_2 pneumoperitoneum on the immune and inflammatory responses to surgery.

Initial studies demonstrated a preservation of the cell-mediated immune response in mice receiving pneumoperitoneum compared to extraperitoneal incisions or laparotomy [8]. A series of rat studies used bacterial lippolysaccharide (LPS) as a stimulus of the rodent inflammatory cascade and the impact of CO_2 insufflation upon the mediators of inflammation was noted. Production of α_2-macroglobulin was reduced in animals insufflated with CO_2 [1, 9]. This effect was maintained throughout the first 6 h after LPS challenge, regardless of when insufflation was instituted [2]. In an experiment comparing the influence of different pneumoperitoneum gases at the time of LPS challenge, insufflation with CO_2 reduced plasma tumor necrosis factor-α (TNF-α) levels more than He insufflation, and CO_2 increased levels of interteutin (IL)-10, an anti-inflammatory cytokine (unpublished data).

The laboratory models of laparqscppic surgery we previously utilized were either abdominal insufflation alone or laparoscopic cecal ligation and puncture, which can substitute for a number of human conditions, including perforated appendicitis, diverticulitits, or ischemia with perforation. The problems associated with this model include the difficulty of reproducing an identical level of sepsis in each animals and the inability to further manipulate the animal postoperatively.

A colleague examining the response to LPS injection in splenectomized (spx) inbred strains of mice found

Table 1. Design of experiment 1

Group	Intervention	LPS injection POD2		
		1.5 h	3 h	6 h
Control	No operation	$n = 5$	$n = 5$	$n = 5$
Splenectomy	Open	$n = 5$	$n = 5$	$n = 5$
Sham	Open	$n = 5$	$n = 5$	$n = 5$
Splenectomy	CO_2 laparoscopy	$n = 5$	$n = 5$	$n = 5$
Sham	CO_2 laparoscopy	$n = 5$	$n = 5$	$n = 5$

higher LPS-induced IL-10 levels in spx mice compared to nonoperated or sham-operated groups at postoperative day (POD) 9 (A. DeMaio, personal communication). IL-10 is an anti-inflammatory cytokine well-known to downregulate TNF-α. These observations suggest that the spleen plays a role in the regulation of IL-10 cytokines in response to LPS, perhaps dampening the anti-inflammatory response. This led to the idea of using splenectomy to study the rat response to LPS challenge.

We developed a rat model of laparoscopic splenectomy that is technically easy to perform and that is similar to a model previously described [16]. This model is a clean operation, compared to prior laparoscopic cecal ligation and puncture models, and thus allowed the recovery of animals after the procedure for later endotoxin injection. This also allowed us to study the involvement of the spleen in the potential anti-inflammatory effects of pneumoperitoneum. We predicted that laparoscopic splenectomy would lead to preservation of the acute inflammatory response, demonstrating a protective effect compared to open splenectomy.

Materials and methods

Animals

The Johns Hopkins University Animal Care and Use Committee approved the following experimental protocol. Male Sprague–Dawley rats (250–300 g) were caged in central animal facilities with water and chow ad libitum. Animals were fasted overnight prior to the experiment. Animals were initially induced with isoflurane and then placed on an isoflurane flow-by circuit, titrated to keep respirations normal but to provide adequate anesthesia to operative stimuli (usually between 1.75 and 2.0 MAC).

Laparoscopic procedure

After the abdomen was shaved, a 0.5-cm incision was made in the lower midline. The linea alba was sharply incised, and the remaining layers were bluntly spread until the peritoneum was breached. The laparoscope canula was then inserted into the abdomen and retained with a pursestring suture. The abdomen was insufflated with the test gas (insufflator and processor, Olympus) at a flow rate of 0.1 L/min to a-maximum pressure of 4 mmHg. This pressure was chosen as because it provides adequate distention of the abdomen to allow for laparoscopic instrument manipulation but has been shown to reduce portal vein blood flow by only 8% in the rat model [15]. A 0° 2.7-mm laparoscope (pediatric tympanoscope) was then inserted (Karl Storz Endoscopy, Culver City, CA, USA). One-millimeter instruments were then inserted on either side of the camera 1.5 cm away from the camera port.

The spleen was localized in the left upper quadrant and, by gently grasping the mesenteric attachments, was pulled into the middle of the upper abdomen. The spleen was elevated to reveal the vascular inflow along the length of the spleen. A hole was made in the thin, filmy membrane between vascular pedicles approximately two-thirds of the way down the spleen. At this point, manipulation was ceased in the sham animals. For the splenectomy groups, a 5-mm incision was made in the right lower quadrant and a 5-mm clip applier was introduced. Clips were placed around the medial portion of the splenic mesentery and vasculature and then across the remaining lateral mesentery. The spleen was then cut free above the clips. After ensuring hemostasis, the instruments and camera were removed and the spleen was extracted through the camera port. The incisions were closed in two layers with 3–0 vicryl for the fascia and 3–0 nylon for the skin. One stitch was placed in each instrument site. Each animal received a subcutaneous 30 ml/kg bolus of sterile Ringers lactate.

Open procedure

A 1-cm midline incision was made in the upper abdomen. The spleen was located in the left upper quadrant and gently grasped and externalized. The thin, filmy ligaments were ligated. At this point, sham animals had the spleen returned to the abdomen. Splenectomy animals had the splenic mesentery divided with two clips and ligated. Closure was done as discussed previously.

In both operative groups, the manipulation was standardized to 30 min of insufflation or open abdomen, regardless of the actual time of procedure. Buprenorphine 0.1 mg/kg subcutaneous injection was administered for post operative pain control.

Control animals were briefly anesthetized, shaved, and injected with a 30 ml/kg bolus of Ringers lactate.

Animals were then recovered and returned to housing. They were monitored for either 2 or 9 days postoperatively, receiving standard water and feed. The evening prior to injection with LPS, animals were again starved. At 48 h postoperative animals were injected with 1 mg\kg of LPS (purified *Escherichia coil* O26:B6:Σ) in the penile vein. Ninety, 180, or 360 min after injection animals were killed with isoflurance and cardiac puncture was performed. Blood was collected in K2-EDTA tubes and was spun down to separate cells from serum. Serum was then alliquoted and kept frozen at –80° until analysis.

ELISA and Statistics

Commercial enzyme-linked immunosorbent assays (ELISAs) (Biosource International, Camarillo, CA, USA) were used to quantitate serum TNF-α, IL-10, and interferon-γ (INF-γ) levels. Statistics were performed by calculating the average of the results in each identical group of rats. Groups were compared by one-way analysis of variance using the SPSS statistical software package (SPSS, Chicago, IL, USA).

Results

Experiment 1

This experiment compared open splenectomy to laparoscopic splenectomy with CO_2 insufflation (Table 1). To delineate if the physical trauma of surgery was influencing the results, each operative group contained

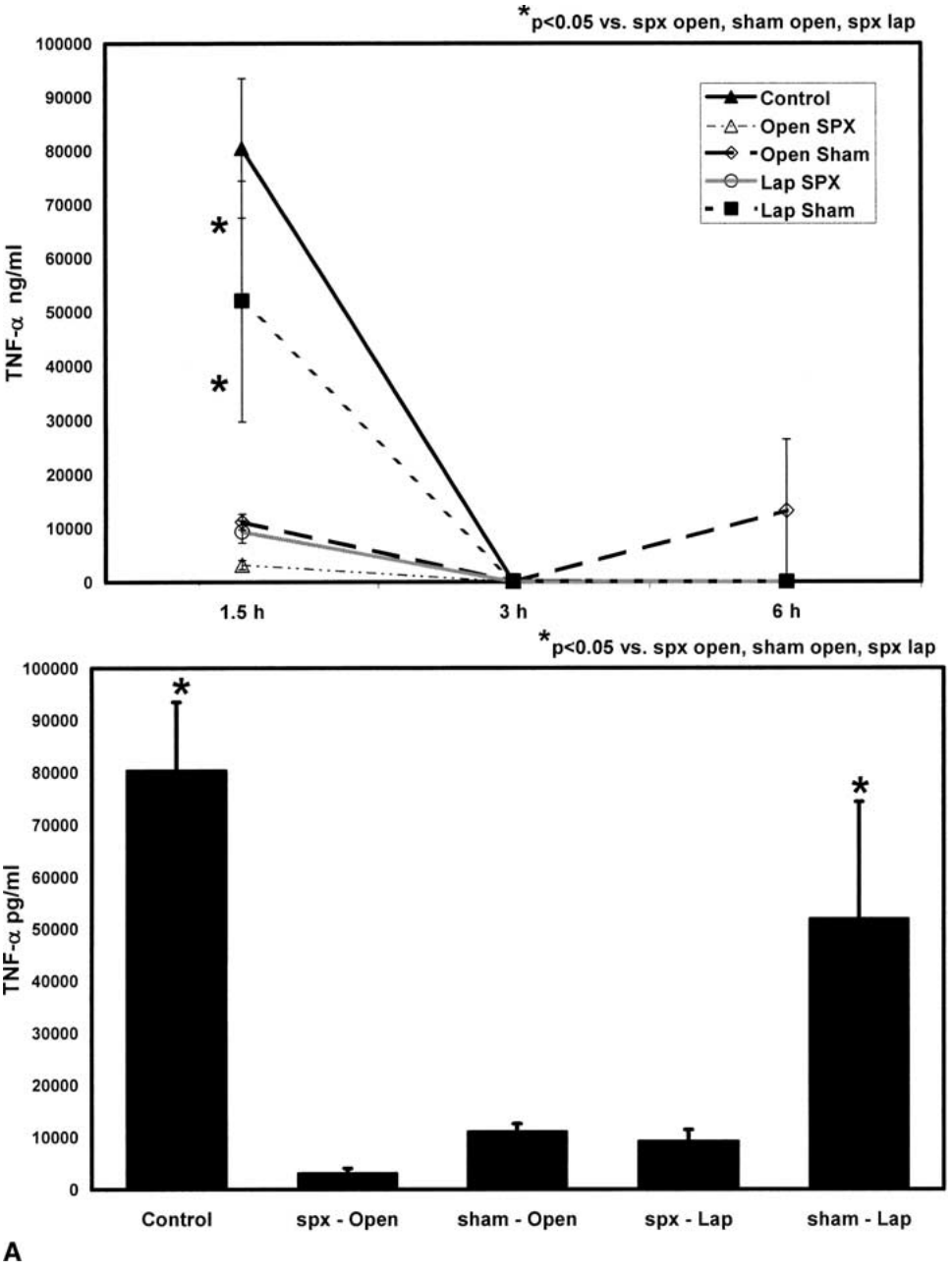

Fig. 1. Plasma cytokine levels as measured by ELISA assay. LPS was injected on POD 2 after sham operation or splenectomy. Cardiac puncture was performed at three time points. All group were compared by ANOVA. **A** Kinetic and 1.5-h time graph of TNF-α levels. The control and sham lap groups are significantly different from the spx open, spx lap, and sham open groups. **B** Kinetic and 1.5 h time point graph of IL-10 levels. The spx open group is significantly elevated in comparison to the control. **C** INF-γ kinetic reveals significantly diminished levels in both spx groups. (Continued on pages 1038–1039.)

an equal number of shams and splenectomized animals. One group received only LPS injection, serving as the, positive control. LPS injection was performed on POD 2 and animals were killed at three time points to obtain a kinetic of cytokine production. ELISAs on the collected plasma were performed for TNF-α, IL-10, and INF-γ.

Analysis of TNF-α revealed a kinetic with the peak production of TNF-α occurring at 90 min (Fig. 1A). At 1.5 h after LPS injection, the control and sham laparoscopy groups were independently significantly elevated compared to spx open, spx lap, and sham open. These three groups had plasma levels reduced by 96, 86, and 88% of the positive control and 94, 79, and 22% of the lap sham, respectively.

Analysis of IL-10 also demonstrated a peak level at 1.5 h. All groups were elevated compared to the control, although only the open spx group had significantly increased plasma level—40% greater than the positive control (Fig. 1B).

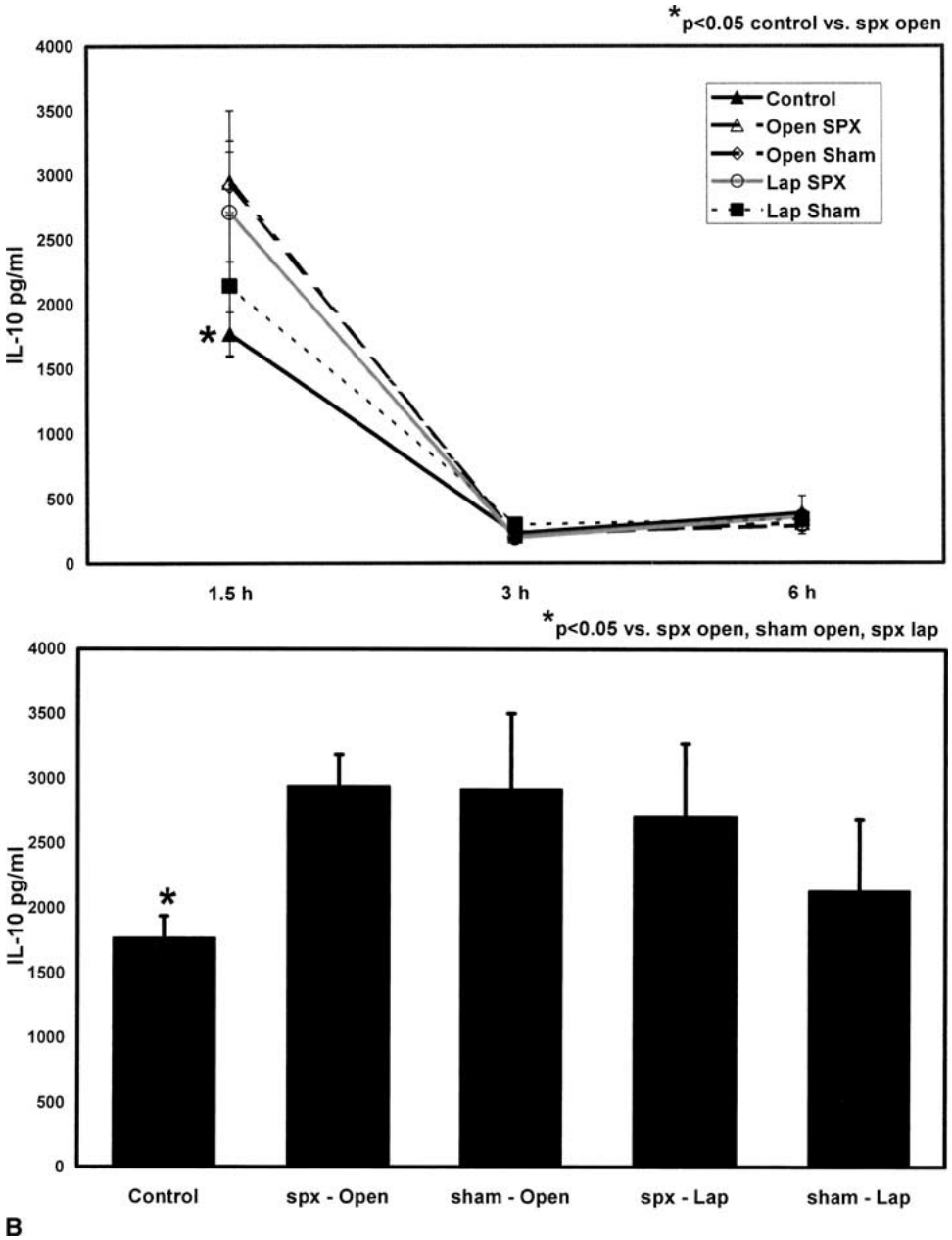

Fig. 1. Continued

INF-γ production was essentially abolished in both splenectomy groups across the 6-h kinetic (Fig. 1C). At 3 h, spx reduced INF-γ levels by 92% compared to the control, whereas at 6 h INF-γ level were reduced by 93% compared to the sham lap group.

Experiment 2

To further investigate the results of TNF-α, and IL-10, the 1.5-h time point was repeated, waiting until POD 9 for LPS injection (Table 2). The plasma TNF-α, in the operative groups did not demonstrate any significant differences from the control at this point, although the two spx group levels were reduced compared to the shams (Fig. 2A). IL-10 levels were also not significantly different from the control (Fig. 2B).

Experiment 3

This experiment studied the effect of lap spx with different gases. Control and open spx groups were compared to lap spx with CO_2, He, and air (Table 3). Animals were injected with LPS on POD 2. Ninety- and 180-min collection times were used as end points.

At 1.5 h TNF-α was significantly elevated in the control group compared to each spx grqup (Fig. 3A). Spx open was reduced by 69%, spx CO_2 by 74%, spx He by 89%, and spx air by 81%. However, there were no

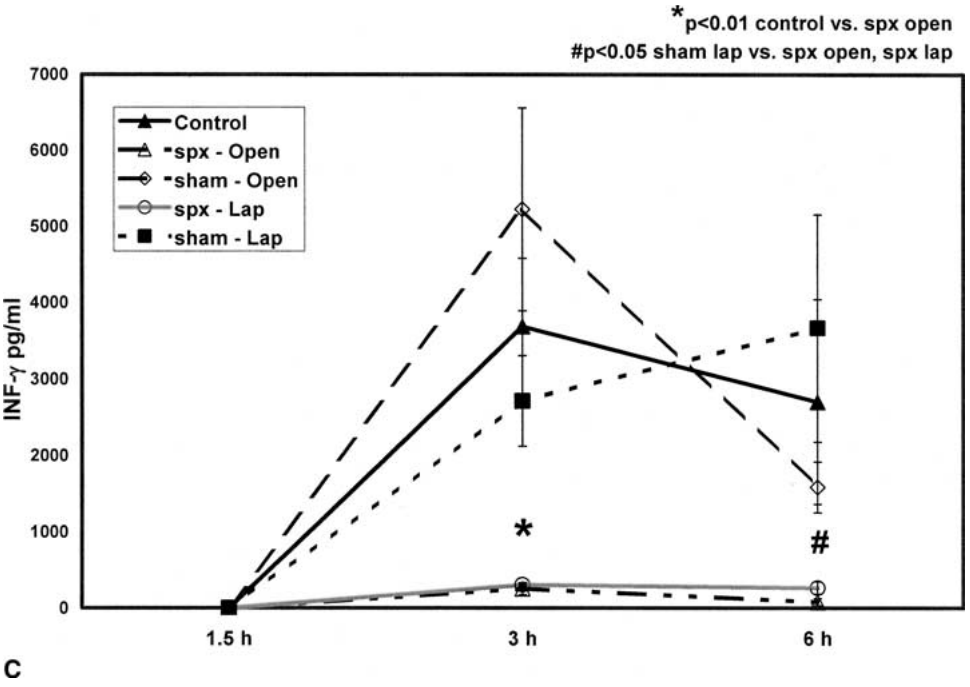

Fig. 1. Continued

Table 2. Design of experiment 2

Group	Intervention	LPS injection POD9 1.5 h
Control	No operation	$n = 10$
Splenectomy	Open	$n = 10$
Sham	Open	$n = 10$
Splenectomy	CO_2 laparoscopy	$n = 10$
Sham	CO_2 laparoscopy	$n = 10$

statistical differences between the types of spx. IL-10 did not demonstrate a significant difference between any groups after 1.5 h (Fig. 3B).

At 3 h INF-γ demonstrated a marked decrease in all spx groups compared to the control, which was significant (Fig. 3C). Spx open was reduced by 74%, spx CO_2 by 93%, spx He by 91 %, and spx air by 89%.

Experiment 4

This experiment was designed to evaluate the sham groups for any alterations in TNF-α, and IL-10, levels between the open and lap sham groups (Table 4). Control and open sham groups were compared to lap sham with CO_2, He, or air. LPS injection was performed on POD2 and blood was collected at 1.5 h. There were no significant decreases in TNF-α plasma levels compared to the positive control in any of the sham-operated animals (Fig. 4A). Likewise, there were no significant increases in IL-10 levels in sham animals compared to the control (Fig. 4B).

Discussion

Elective laparoscopic splenectomy is a commonly performed operation. The rare but life-threatening occurrence of post splenectomy overwhelming bacterial sepsis in both adults and children demonstrates that the spleen plays an important role in the immune/inflammatory cascade. This is due to a variety of functions that the spleen provides, including clearance of bacterial pathogens by filtration, phagocytosis, and opsonization of bacteria. The spleen is also the host to many cells of the reticuloendothelial system, which provide a cellular response to infection. Their removal must influence lymphokine messages throughout the organism [5, 12].

The body's response to an inflammatory challenge, such as injury or infection, is initially local. Activated macrophages at the site of injury release IL-1, TNF-α, and IL-6 [3]. These cytokines activate the innate immune response and go on to act on a multitude of humoral and organ-specific cellular targets. A secondary wave of cytokines is then released. These complex interactions allow one cytokine to enhance the production of some cytokines and provide a negative feedback on others [13].

TNF-α is released from activated macrophages and natural killer (NK) cells. It is best known for its role in producing fever and local inflammation by activating neutrophils and endothelial activation. INF-γ is produced by T1 cells, CD8 cells, and NK cells. It activates macrophages, neutrophils, and NK cells, in addition to increasing MHC expression and promoting differentiation of T1 cells [13].

Our previous work found that CO_2 pneumoperitoneum has, in general, an anti-inflammatory effect in rodent models of sepsis. One possible mechanism for the anti-inflammatory effect of CO_2 pneumoperitoneum is the acidification of peritoneal macrophages. Cultured murine peritoneal macrophages stimulated with LPS produced less TNF-α and IL-1 when exposed to CO_2 versus air or He [17]. One target of the peritoneal

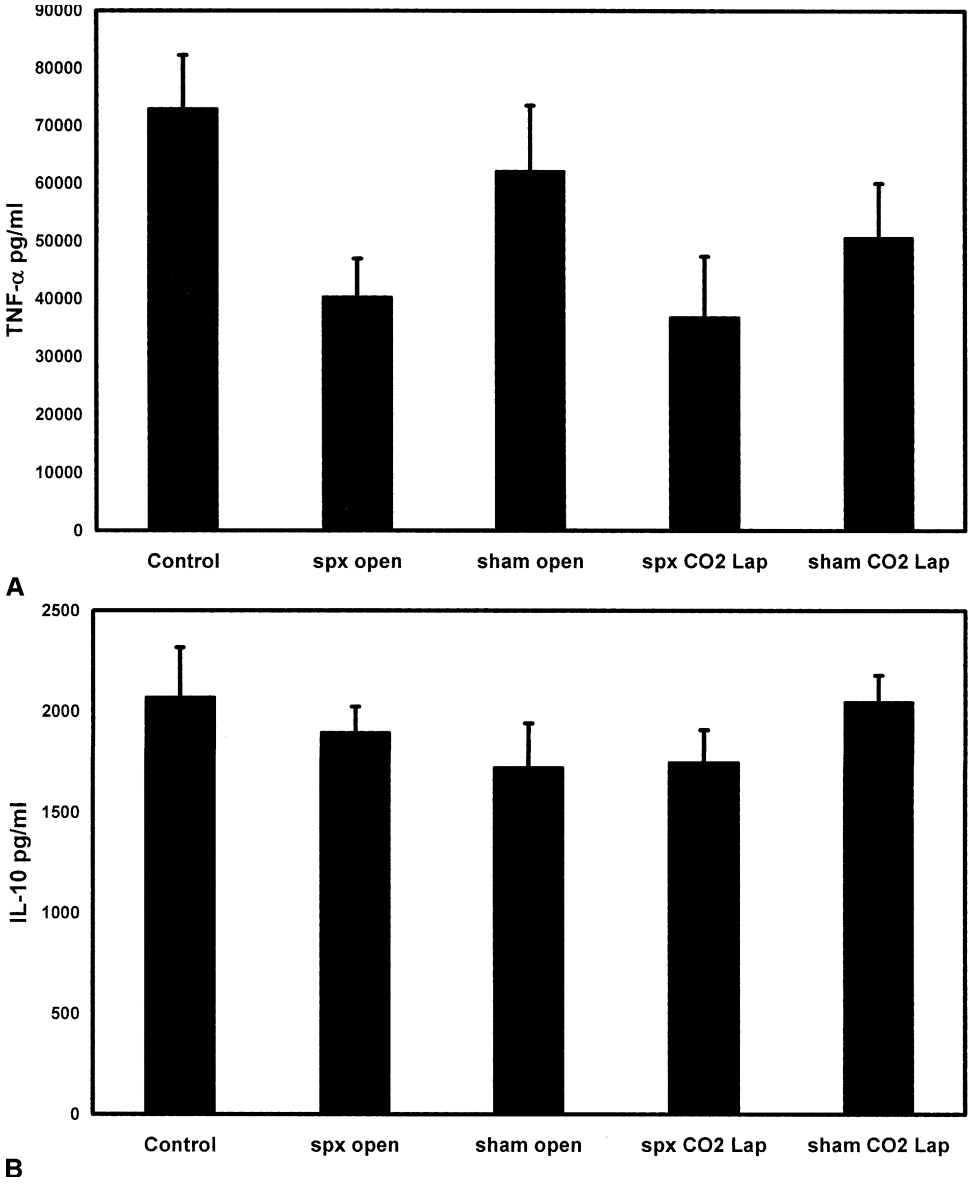

Fig. 2. LPS was injected on POD9 after sham operation or spx. Plasma cytokine levels were measured 1.5 h after LPS injection. **A** TNF-α. **B** IL-10.

macrophages intercellular messaging is the liver hepatocytes and macrophages, which then initiate the hepatic acute phase response with increased-protein production. The interrelated system also responds to input from splenic signaling. INF-γ produced by T cells is known to augment TNF-α expression [13].

Our series of experiments was performed to evaluate laparoscopic versus open splenectomy on the changes to the inflammatory response. At the 1.5-h time point, we found that TNF-α was significantly decreased compared to the positive control in the spx groups and the open sham. The preservation of TNF-α in the lap sham group compared to the open sham demonstrates the suppressive effect of surgical trauma on the postoperative inflammatory response. However, the lap and open spx groups had an equal reduction in TNF-α production after LSP stimulus, suggesting that the reduction of TNF-α in the lap spx group is due to the effect of splenectomy. Alternatively, organ removal may be a traumatic stimulus equal to that of an open incision. This pattern is repeated in experiment 3, in which spx leads to a significant diminishment of TNF-α on POD2, despite the operation technique or the type of gas used for insufflation. The type of gas does not affect preservation of the TNF-α response when comparing only sham-operated animals, as seen in experiment 4. Interestingly, in this experiment, open shams did not demonstrate a decrease in TNF-α.

In experiment 2, injection of LPS was delayed until POD9 to allow time for recovery from the, operative insult. TNF-α production in both spx groups is reduced compared to that in the shams and control, although these differences are not statistically significant. Other investigators have examined the role of splenic involvement in the robustness of the TNF-α response. McCarthy et al. [12] found that TNF-α secretion from harvested peritoneal macrophages was significantly higher following spx compared to controls and shams, but by POD7 the difference was lost. Kohno et al. [11] studied rat plasma TNF-α levels in animals stimulated

Table 3. Design of experiment 3

Group	Intervention	LPS injection POD2	
		1.5 h	3 h
Control	No operation	$n = 10$	$n = 10$
Splenectomy	Open sham	$n = 10$	$n = 10$
Splenectomy	Lap CO_2 sham	$n = 10$	$n = 10$
Splenectomy	Lap He sham	$n = 10$	$n = 10$
Splenectomy	Lap air sham	$n = 10$	$n = 10$

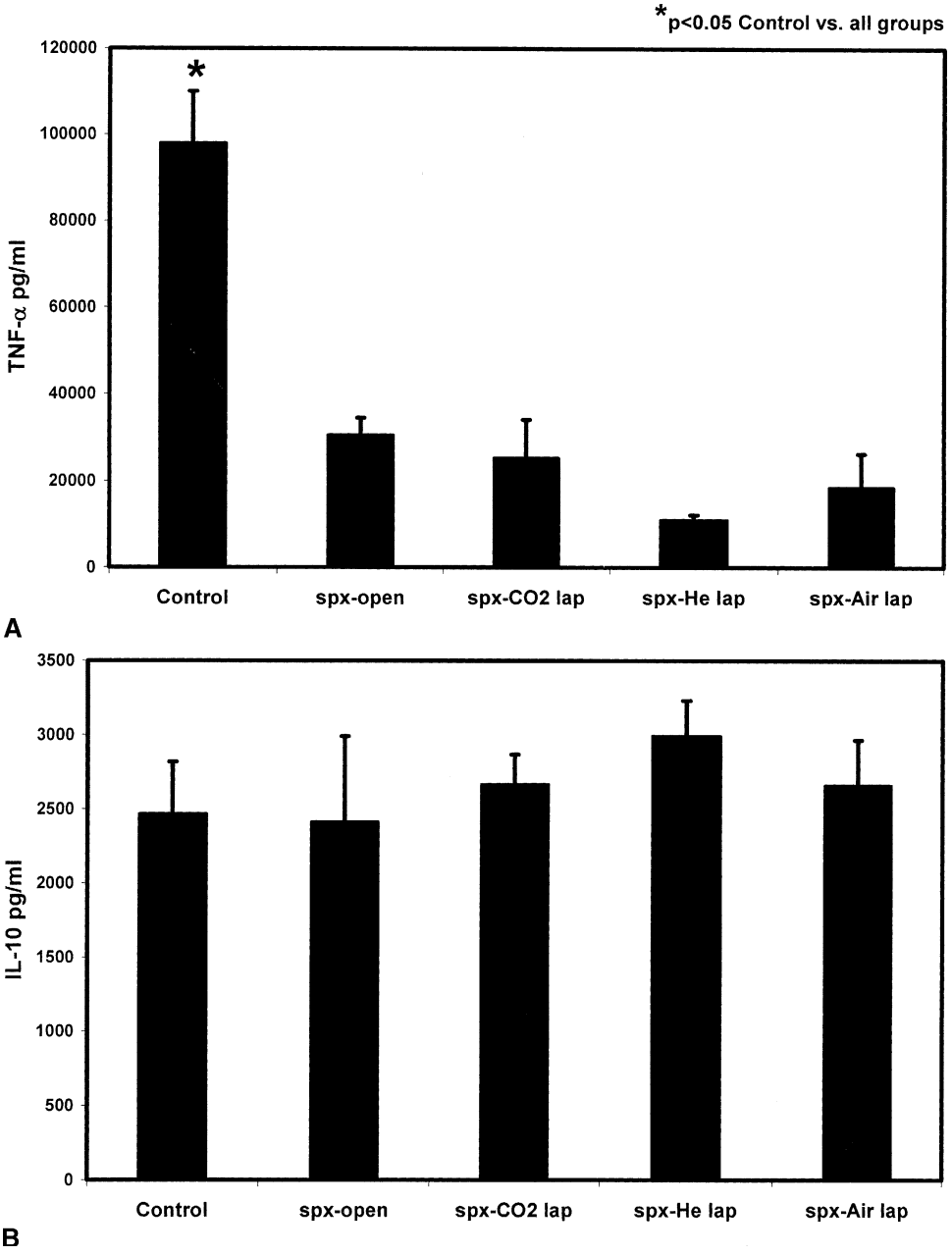

Fig. 3. LPS was injected on POD2 after open or laparoscopic spx. Plasma cytokine levels were measured after LPS injection. **A** TNF-α 1.5 h after LPS injection. The control is significantly elevated compared to each spx group. **B** IL-10 1.5 h after LPS injection. **C** INF-γ 3 h after LPS injection. The control is significantly elevated compared to each spx group. $^{*}p < 0.05$, control vs. all groups. (Continued on page 1042.)

with LPS. There was no significant difference between splenectomized and sham-operated animals, nor did blocking the phagocytic abilites of liver macrbphages decrease the levels. They concluded that splenic macrophages did not affect plasma TNF-α levels [11].

Deriy et al. [5] studied the dose–response curves of TNF-α and INF-γ in splenectomized rats infused with endotoxin. No difference was found in the TNF-α response between spx and sham animals, but INF-γ was significantly reduced in spx but not sham groups. They

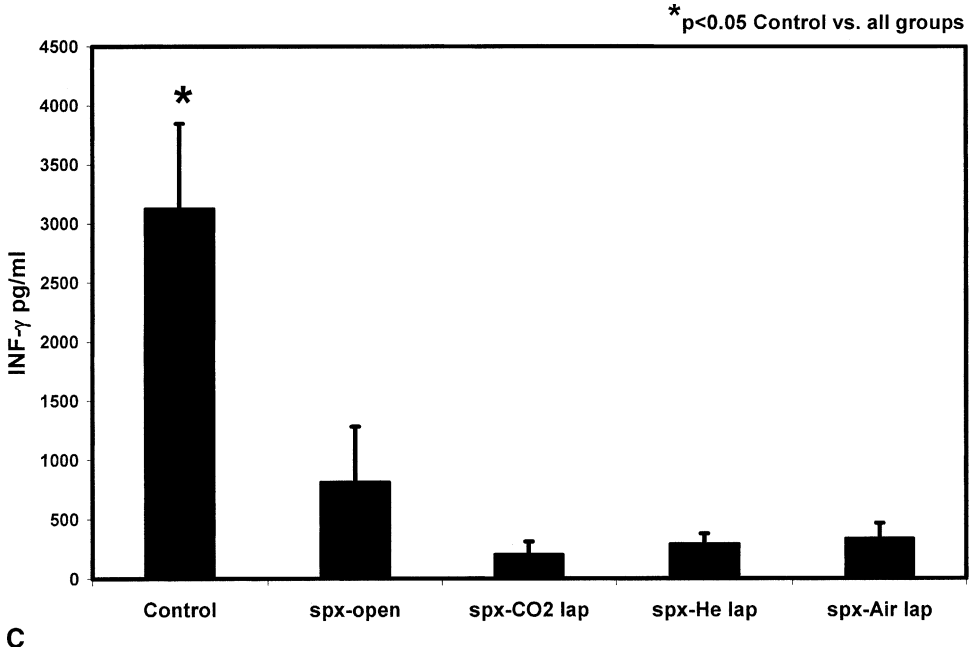

Fig. 3. Continued

Table 4. Design of experiment 4

Group	Intervention	LPS injection POD2 1.5 h
Control	No operation	$n = 10$
Sham	Open Sham	$n = 10$
Sham	Lap CO_2 sham	$n = 10$
Sham	Lap He sham	$n = 10$
Sham	Lap air sham	$n = 10$

also noted that liver damage, as documented by ALT levels, was reduced in spx animals, demonstrating a role for INF-γ in regulating LPS-induced liver injury. Another group studied the response of cultured Kupffer cells to LPS stimulation after spx or sham spx. The group found an initial exaggerated response in hepatocyte protein synthesis in the first 48 h after surgery but a blunted response in the spx group that extended for 60 days. They group theorized that this was due to the loss of priming from splenic lymphokines [4]. Mice treated with anti-INF-γ antibody had significantly reduced mortality from endotoxemia but only minor decreases in plasma TNF-α. Pretreatment with INF-γ caused increased susceptibility to endotoxemia, which was associated with increased serum TNF-α levels [10]. INF-γ is necessary for the toxic and lethal effects of LPS and TNF-α it appears to be an obligatory comediator of TNF-α activity [6].

In our experiments, INF-γ was diminished by any type of splenectomy but not affected by the trauma of surgery alone in the sham groups. Figure 1C demonstrates no significant reduction in INF-γ between the sham lap and open groups. In Fig. 3C, there is a trend towards more production of INF-γ in the open group compared to all three laparscopic groups, but it is not statistically supported. This is consistent with data that demonstrated no significant decrease in INF-γ levels associated with surgical stress, unlike TNF-α, which decreased 92.6% [7].

TNF-α and endotoxin are both potent inductors of IL-10 production, but endogenously produced IL-10 downregulates the production of TNF-α and other proinflammatory cytokines [14]. Our data demonstrate IL-10 levels mirroring the TNF-α results in experiment 1, although the increase in IL-10 levels is only found to be significant between the control and open spx group and is not reflected in the results of experiment 3. Given the very similar values for lap spx and open spx in experiment 1, it is possible that the lack of significance between the control and lap spx groups could be a consequence of this particular experiment being underpowered for detection of differences in IL-10 ($n = 5$). However, a consistent trend is reflected in Fig. 4B, with modest elevations of IL-10 in the open spx and lap CO_2 groups compared to the control, and in experiment 3, in which the three laparoscopic groups are mildly elevated.

In summary, splenectomy dramatically reduces the TNF-α and INF-γ response to LPS challenge, although by different mechanisms. TNF-α is related to the stress of surgery and the local peritoneal response, as well as the loss of INF-γ synergy. INF-γ is obliterated by loss of its locus of production in the spleen, but this effect is not mediated by operative approach. Pneumoperitoneum-mediated modulation of the septic inflammatory response is partially dependent on the spleen. Therefore, the combination of laparoscopic splenectomy and sepsis—as may occur following traumatic splenic injury or following elective laparoscopic splenectomy complicated by infection—may produce a clinically unique immunologic response.

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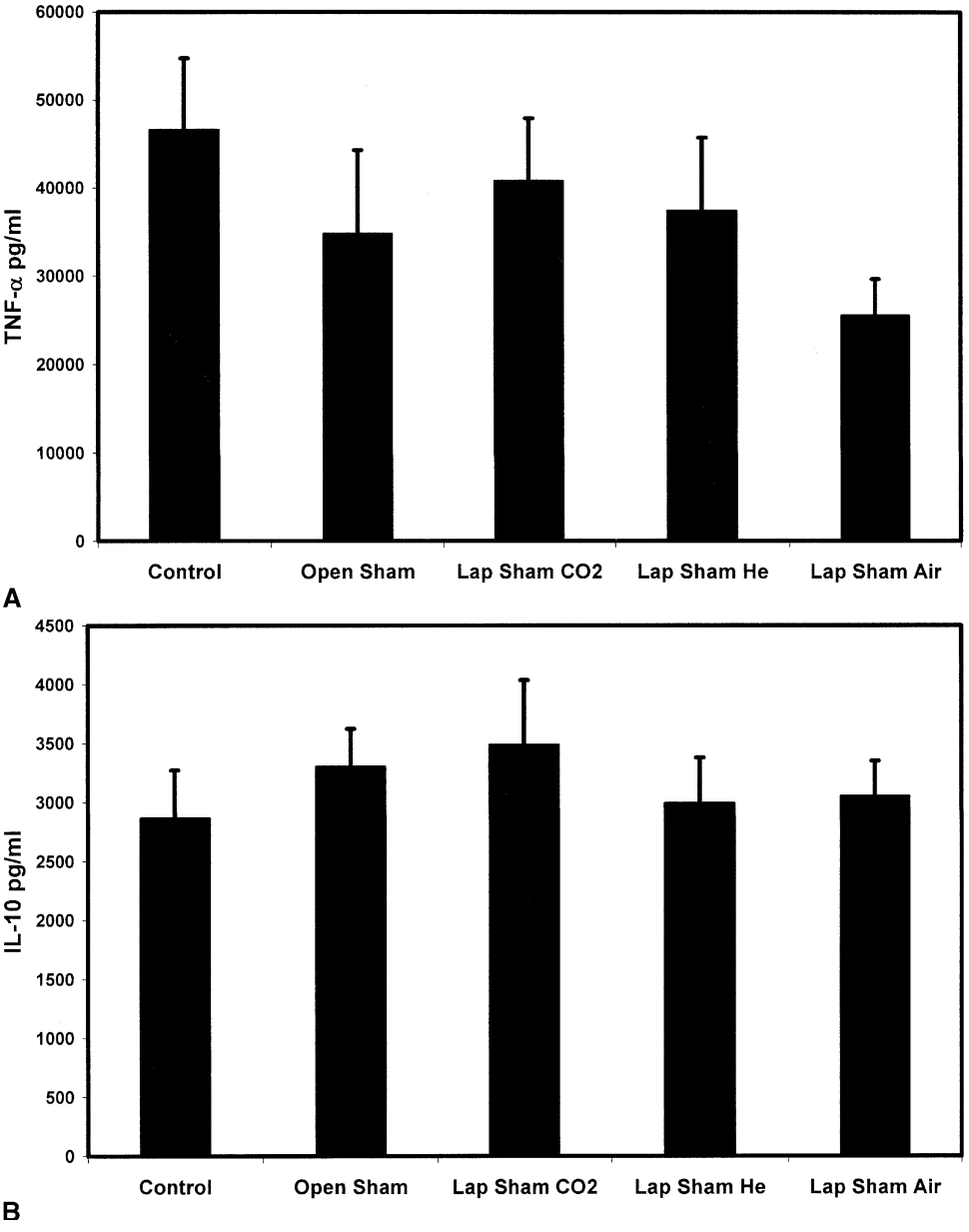

Fig. 4. LPS was injected on POD2 after open or laparpscopic sham manipulation of the spleen. Plasma cytokine levels were measured after LPS injection. **A** TNF-α 1.5 h after LPS injection. **B** IL-10 1.5 h after LPS injection.

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