



Minilaparotomy and full laparotomy, but not laparoscopy, alter hepatic macrophage populations in a rat model

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Abstract

Background: Immune function is better preserved by laparoscopic versus conventional surgery. Numerous mediators of the systemic trauma response are synthesized and/or regulated by the liver. However, it has been stated that the advantages of laparoscopic surgery are no more obvious when conventional operations are performed via mini-laparotomy. We set out to compare the impact of laparoscopy and mini- and full laparotomy on the hepatic macrophage populations.

Methods: Male Lewis rats were subjected to anesthesia alone (control), mini-laparotomy (1 cm), full laparotomy (7 cm), or laparoscopy for 60 min. Endpoints were the total protein in the peritoneal lavage fluid, hepatic ED-1 cells (recruited monocytes), hepatic ED-2 cells (Kupffer cells), the expression of OX-6 in the liver, and C-reactive protein (CRP) in plasma.

Results: Protein in the peritoneal lavage fluid increased significantly after all interventions. Full laparotomy was accompanied by an enhancement in ED-1-positive monocytes in the liver parenchyma compared to all other groups ($p < 0.001$). Mini- and full laparotomy led to an increase in ED-2-positive Kupffer cells ($p < 0.001$). Laparoscopy did not affect the number of monocytes/macrophages. There was no significant alteration of OX-6 expression in either group. No change in the cellular composition in the periportal fields was observed. The CRP plasma levels did not significantly differ between groups.

Conclusions: Laparoscopy completely prevents hepatic macrophage populations from expansion and normal cell disposition is preserved. Laparotomy, irrespective of incision size, increases the number of Kupffer cells. Moreover, full laparotomy, but not mini-laparotomy or laparoscopy, causes an increase in hepatic monocyte recruitment. The regulating pathways after surgery dif-

fer from other immunologic challenges, such as sepsis, in which immunocompetent cells accumulate and are stimulated in the periportal fields.

Key words: Laparoscopy — Laparotomy — Rat — Liver — Macrophage — Monocyte

The response to surgical interventions is initiated by a break in the protective epithelial and endothelial barriers, which results in the activation of numerous pathways of the innate immune system leading to a systemic inflammatory response. Humoral mediators are directly released by the injured tissue or are, to a greater extent, synthesized by the liver [6, 9, 21]. In this regard, Kupffer cells, the resident macrophage population of the liver, play a key role in modeling the local hepatic immune response [5, 14]. Kupffer cells comprise the largest fraction (>50%) of hepatic tissue macrophages. They are predominantly localized in the lumen of hepatic sinusoids and are anchored to the endothelium by long cytoplasmic processes with a pronounced endocytic and phagocytic capacity [3]. They are responsible for the production of potent mediators of the inflammatory response, such as superoxide anions, nitric oxide, interleukin (IL)-6, and tumor-necrosis factor (TNF)- α , and are involved in the regulation of cytokine release by the liver [13].

Numerous studies have demonstrated that laparoscopic surgery leads to less post operative pain and a faster recovery [7, 22]. This was associated with lower serum levels of various cytokines, such as IL-1 β , IL-6, and TNF- α [8, 12, 22, 28], and with lower levels of IL-6 in the peritoneal cavity [28]. The underlying mechanisms have not been clarified, and the advantages of laparoscopic surgery have been attributed to a lower degree of surgical trauma [1], effects of carbon dioxide used for pneumoperitoneum [25, 31], and air contamination that

takes place during conventional surgery [31]. However, McMahon et al. [18] demonstrated that mini-laparotomy, compared to laparoscopy, similarly preserves immune function and leads to similar clinical results. Therefore, it has been a matter of debate whether the advantages of laparoscopic surgery are still obvious when conventional operations are performed via mini-laparotomy [18].

The aim of this study was to examine the impact of laparoscopy versus mini-laparotomy and full laparotomy on the hepatic microenvironment, particularly the liver-associated macrophage subpopulations.

Materials and methods

The Local Animal Care Ethical Committee Hannover (509c-42502-01/405) approved the protocol of the study.

Study design

Male Lewis rats (250–300 g, Central Animal Laboratory, Hannover Medical School) of specific pathogen-free housing were used. They were given 2 days to recover from transport stress and were kept on a 12-h light/dark cycle in a conventional, nonbarrier rodent housing unit in polycarbonate cages. Water and standard rat laboratory diets (V1324-300, SSNIFF, Soest, Germany) were supplied *ad libitum* until 12 h before the experiment. To ensure stress-free anesthetic application, an intravenous cannula was implanted 5 days before the experiment started. The catheter was placed as described previously [19]. Briefly, a catheter (Braun, Melsungen, Germany) was subcutaneously tunneled from the top of the head down to the neck and introduced into the right external jugular vein. The animals were randomized into four groups of six animals: anesthesia alone as the control group, laparoscopy, mini-laparotomy (1 cm), or full laparotomy (7 cm) for 60 min. The animals were killed under general anesthesia after 24 h.

Anesthesia and positioning

Intravenous anesthesia was performed with approximately 2.5 ml of anesthetic solution (0.35 ml ketamine hydrochloride, Ketamin 10%, WDT, Garbsen, Germany, and 0.05 ml medetomidine hydrochloride, Dormitor, Espon, Finland). After induction of anesthesia, the animals were shaved in the abdominal region. They were placed in a supine position on a specially designed small animal operating table with an integrated heated water bath for keeping the continuously measured body temperature constant.

Surgical techniques

All interventions were performed under standard sterile conditions. For laparoscopy, standard reusable equipment was used (Storz, Tuttlingen, Germany). Laparoscopy was performed similarly to the technique described by Gutt et al. [10]. The optic and two working trocars were introduced into the abdominal cavity. The pneumoperitoneum was kept at 5 mmHg and was desufflated after 60 min. The trocar wounds were closed with resorbable sutures.

Midline laparotomy consisted either of mini-laparotomy of 1 cm length or of full laparotomy of 7 cm length. The abdominal cavity was kept open by a spreader without further manipulation for 60 min. The incisions were closed in two layers with resorbable and unresorbable sutures.

Total protein in peritoneal lavage fluids

The amount of protein in the peritoneal lavage fluid was determined using a colorimetric assay (Bio-Rad, Munich, Germany) for protein

concentration following the solution of the detergent. The assay was carried out according to the manufacturer's instructions. The reaction is based on Lowry et al.'s [16] assay. Values were obtained as micrograms of protein per millimeter of peritoneal lavage fluid.

Immunohistochemistry

Liver cryostat sections of 6- μ m thickness were air-dried, wrapped in aluminum foil, and stored at -20°C . To determine the macrophage phenotype, the slides were fixed in acetone for 10 min. After rinsing with TBS-Tween (Tris buffered saline supplemented with Tween-20), the sections were incubated with the primary antibodies (monocytes, ED-1 1:1000; Kupffer cells, ED-2 1:200; or MHC-II, OX-6 1:1000; Serotec, Duesseldorf, Germany) for 30 min in a moist chamber at room temperature. They were rinsed with TBS-Tween, followed by incubation for 30 min with the bridging antibody (100 μ l of DAKO Z 0259, 1:50 in 5% rat serum in phosphate-buffered saline, rabbit anti-mouse antibody). After washing with TBS-Tween, the APAAP complex (100 μ l of DAKO D 0651, 1:50 in TBS-Tween, mouse) was added, and the sections were again incubated for 30 min. The last two steps were repeated for an additional 15 min. To visualize the antibodies, a mixture of APPP substrate (DAKO, Hamburg, Germany) and fast blue (F 3378, Sigma, Darmstadt, Germany) in Tris buffer (pH 8.2) was used for 10 min. Finally, counterstaining was performed with Mayer's hemalaun (Merck, Darmstadt, Germany) and the sections were then mounted in glycerol medium (DAKO). Method specificity was tested by omission of the primary antibody and by corresponding isotype controls. The antibody-labeled cells were stained blue. Each experimental group was composed of six animals. For quantitative analysis, six visual fields/section (0.8 mm²) for each animal were examined blinded to the treatment conditions.

C-reactive protein in plasma

The C-reactive protein (CRP) was determined by a competitive legend-binding assay (CLBA). All CLBAs were done with enzyme-linked immunosorbent assay (ELISA) plates (microlon, 96 well, high binding, Greiner, Frickenhausen, Germany). All plates were incubated at room temperature on a microtiter plate shaker (400 rpm). The washing buffer was Dulbecco phosphate-buffered saline without Ca²⁺ and Mg²⁺ (BBS, pH 7.35) and with 0.1% (v/v) Tween 20 (Sigma-Aldrich, Taufkirchen, Germany, PBST). After the incubation steps, the plates were washed three times with the washing buffer (PBST) using the Nunc Immuno-Washer 12 (Nunc, Wiesbaden, Germany).

Next, the plates were coated with phosphocholine-conjugated bovine serum albumin (PC-BSA) diluted in 0.1 M NaHCO₃ (150 μ l/well, 0.08 μ g/ml PC-BSA and 2 μ g/ml BSA 1 h incubation). After washing, the plates were incubated for 2 h with standard or diluted samples (100 μ l/well) and purified human CRP-conjugated biotin (CRP-biotin, 50 μ l/well, 0.5 μ g/ml). The standard was purified human CRP (Dunn Labortechnik GmbH, Asbach, Germany) and the standard concentration ranged from 2.00 to 0.03 μ g/ml. The samples were diluted 1:100 and 1:1000. The assay buffer for dilution of the standard, the plasma samples, and CRP-biotin was 20 mM Tris/HCl (pH 8.0), 0.1% Tween 20 (v/v), 0.1% BSA (w/v), an 5 mM Ca²⁺. The detection reagent was Avidin conjugated with horseradish peroxidase (Perbio, Bonn, Germany), which was diluted 1:10,000 in assay buffer without Ca²⁺ (150 ml/well, 0.5-h incubation). The horseradish peroxidase was determined on the solid phase with the substrate 3,3', 5,5'-tetramethylbenzidine (TMB, 150 μ l/well). The substrate reaction was stopped with 1 M H₂SO₄ (50 μ l/well). The optical density was measured with microplate ELISA reader at 450 nm.

For the CLBA, the intraassay variation was less than 10% and the interassay variation less than 15%.

Statistical analysis

Results are presented as mean \pm SEM. The Statistical Package for the Social Sciences was used to analyze data. After using analysis of variance on ranks, overall differences between groups were analyzed by the Kruskal-Wallis variance test. In case of a positive result, differences

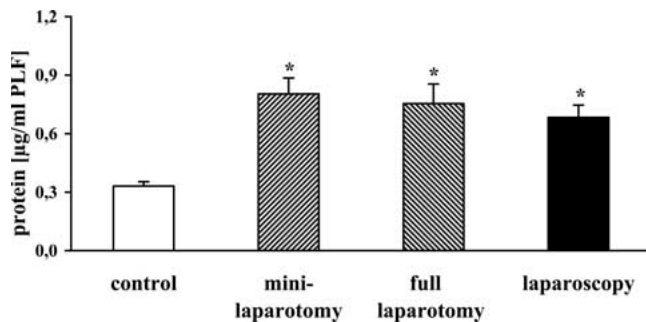


Fig. 1. Protein concentration in the peritoneal lavage fluid obtained 24 h after anesthesia alone (control), mini-laparotomy, foil laparotomy, or laparoscopy. Data are presented as mean \pm SEM ($n = 6$) * $p < 0.005$ versus control.

between two groups were analyzed by the Mann-Whitney U test. $p < 0.05$ was considered significant.

Results

Total protein in peritoneal lavage fluids

The total protein concentration in peritoneal lavage fluids was significantly enhanced after mini-laparotomy ($0.804 \pm 0.082 \mu\text{g/ml}$), full laparotomy ($0.755 \pm 0.100 \mu\text{g/ml}$), and laparoscopy ($0.683 \pm 0.064 \mu\text{g/ml}$) compared to the control ($0.331 \pm 0.022 \mu\text{g/ml}$) ($p < 0.05$) (Fig. 1).

Immunohistochemistry

The number of recruited ED-1-positive monocytes in the liver parenchyma was significantly enhanced by full laparotomy ($93.8 \pm 3.9 \text{ cells/mm}^2$) compared to both control ($58.7 \pm 4.9 \text{ cells/mm}^2$) and laparoscopy ($58.6 \pm 5.4 \text{ cells/mm}^2$) ($p < 0.001$) but not after mini-laparotomy ($76.8 \pm 5.4 \text{ cells/mm}^2$) (Fig. 2). ED-1-positive cells were equally distributed in the liver parenchyma of all animals, and no areas of accumulation were detectable by the increased subpopulation (Fig. 3).

ED-2-positive cells, which represent Kupffer cells, were significantly increased after mini-laparotomy ($94.6 \pm 2.1 \text{ cells/mm}^2$) and full laparotomy ($96.0 \pm 4.2 \text{ cells/mm}^2$) compared to control animals ($57.4 \pm 4.6 \text{ cells/mm}^2$) ($p < 0.001$). There were no significant differences in the number of Kupffer cells in animals after laparoscopy ($73.9 \pm 3.8 \text{ cells/mm}^2$) compared to control animals (Figs. 4 and 5).

The number of OX-6-expressing cells in the liver parenchyma was not significantly different between control animals ($36.6 \pm 8.2 \text{ cells/mm}^2$) and animals after mini-laparotomy ($38.8 \pm 5.8 \text{ cells/mm}^2$), full laparotomy ($42.7 \pm 4.9 \text{ cells/mm}^2$), or laparoscopy ($29.3 \pm 3.7 \text{ cells/mm}^2$). Neither morphological changes nor an increase in OX-6-expressing cells were observed in the areas of the periportal fields (Fig. 6).

C-reactive protein in plasma

The level of CRP in plasma of control animals was within the physiological range ($497 \pm 56 \mu\text{g/ml}$). There

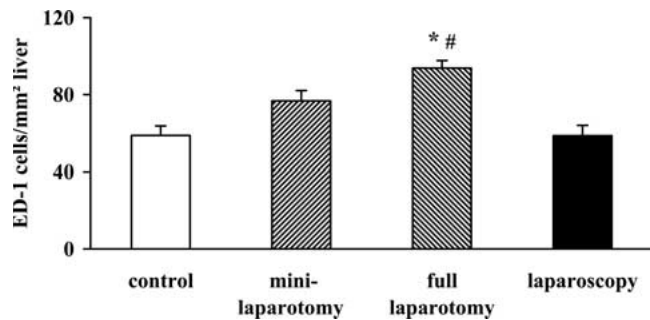


Fig. 2. ED-1-positive recruited monocytes in the liver (mean \pm SEM; $n = 6$) of monocytes after anesthesia alone (control), mini-laparotomy, full laparotomy, or laparoscopy. * $p < 0.001$ versus control. # $p < 0.001$ versus laparoscopy.

were no significant differences in the mean plasma CRP levels 24 h after mini-laparotomy ($561 \pm 16 \mu\text{g/ml}$), full laparotomy ($582 \pm 24 \mu\text{g/ml}$), laparoscopy ($531 \pm 27 \mu\text{g/ml}$), or anesthesia (control) (Fig. 7).

Discussion

Surgical interventions lead to local vascular dilatation and a break in epithelial and endothelial barriers, which is associated with an increase in total protein in the peritoneal cavity. Our experiments showed that the protein concentration in the peritoneal cavity was elevated twofold after any of the surgical procedures investigated. Thus, no difference in the increased permeability was observed between mini- or full laparotomy and laparoscopy.

The production of acute phase proteins and the activation of the cytokine network by the hepatic microenvironment are essential parts of the systemic inflammatory trauma response [26]. Numerous mediators are released by hepatic cells, particularly Kupffer cells, regulating the early phase of the systemic immune reaction [2]. In our experiments, the hepatic cell compartment was histologically unaffected after laparoscopy. No changes in the number of resident ED-2-positive Kupffer cells and ED-1-positive recruited monocytes compared to controls were detected. Mini-laparotomy and full laparotomy induced a significant increase in the number of Kupffer cells. Full laparotomy had an even greater impact on the hepatic macrophage population since the number of resident macrophages and the number of recruited monocytes were increased. It may be concluded that the immunological advantages of laparoscopic surgery become evident when conventional surgery is performed via mini-laparotomy.

An enhanced expression of OX-6 indicates macrophage activation [24]. In the current study, no enhanced expression of OX-6 was detected after any of the investigated surgical interventions. Therefore, it may be assumed that the trauma associated with a laparoscopic or conventional approach to the abdominal cavity without a subsequent operation is not serious enough to activate hepatic macrophages.

However, under physiological conditions, OX-6 is expressed by only 40–60% of Kupffer cells [15]. With no

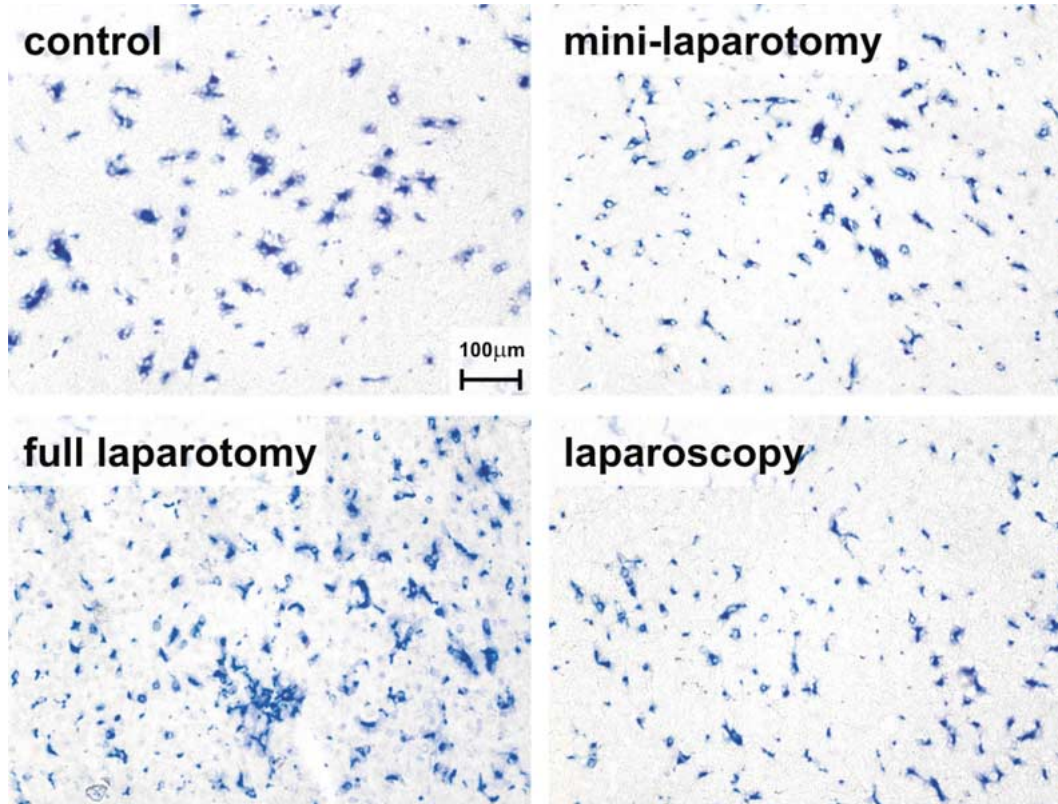


Fig. 3. Immunohistological detection of ED-1-positive monocytes in cryostat sections of the liver. One liver section of each group is shown.

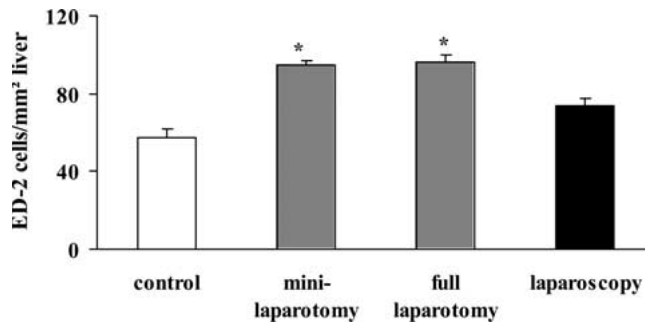


Fig. 4. ED-2-positive resident hepatic macrophages (Kupffer cells) (mean ± SEM, $n = 6$) after anesthesia alone (control), mini-laparotomy, full laparotomy, or laparoscopy. Mini- and full laparotomy caused a statistically significant increase in ED-2-positive macrophages in the liver versus control ($p < 0.001$).

difference in the number of activated Kupffer cells between the experimental groups in this study, but an enlarged macrophage population after laparotomy, it may be speculated that conventional surgery induces a distinct differentiation pathway compared to laparoscopy. Previous data suggest an immunosuppressive effect of laparoscopy. Our data indicate that laparoscopy does not suppress, but preserves, baseline functions of Kupffer cells in the liver. Furthermore, it has been reported that OX-6 also stains endothelial cells of post-capillary venules and other cells scattered throughout the liver parenchyma [4]. We combined OX-6 staining and cell morphology in our study and confirmed the labeling of macrophages in the sections.

A lack of macrophage activation in our study is in accordance with clinical findings of Nguyen et al. [20]. The authors demonstrated that laparoscopic gastric bypass resulted in transient postoperative elevation of hepatic transaminases but did not adversely alter hepatic function to any greater extent than did conventional gastric bypass. Vittimberga et al. [29] confirmed these findings in an experimental setting. No significant differences in the function of isolated Kupffer cells were detected, and hepatic cytokine production after stimulation with lipopolysaccharide was similar after laparotomy, laparoscopy, and in controls. Vittimberga et al. confirmed no alteration of cytokine release by purified Kupffer cells but found differences in the activation of intracellular pathways. The authors demonstrated that the activation of the p38 mitogen-activated protein kinase, an important intracellular messenger, was more pronounced after laparotomy than after laparoscopy. It appears that immunostimulating mediators are different in composition and quantity after laparotomy and laparoscopy. Our study indicates that such mediators may enter circulation and lead to an expansion of the hepatic macrophage population after laparotomy but not after laparoscopy. The levels of CRP in plasma were not elevated in our experiments. This confirms that hepatic acute phase protein release had not been activated.

The periportal fields and OX-6 expression were not affected by any surgical intervention investigated, as would be expected within the scope of other immunological challenges, such as infection [23]. In addition, the enlarged macrophage population after laparotomy was

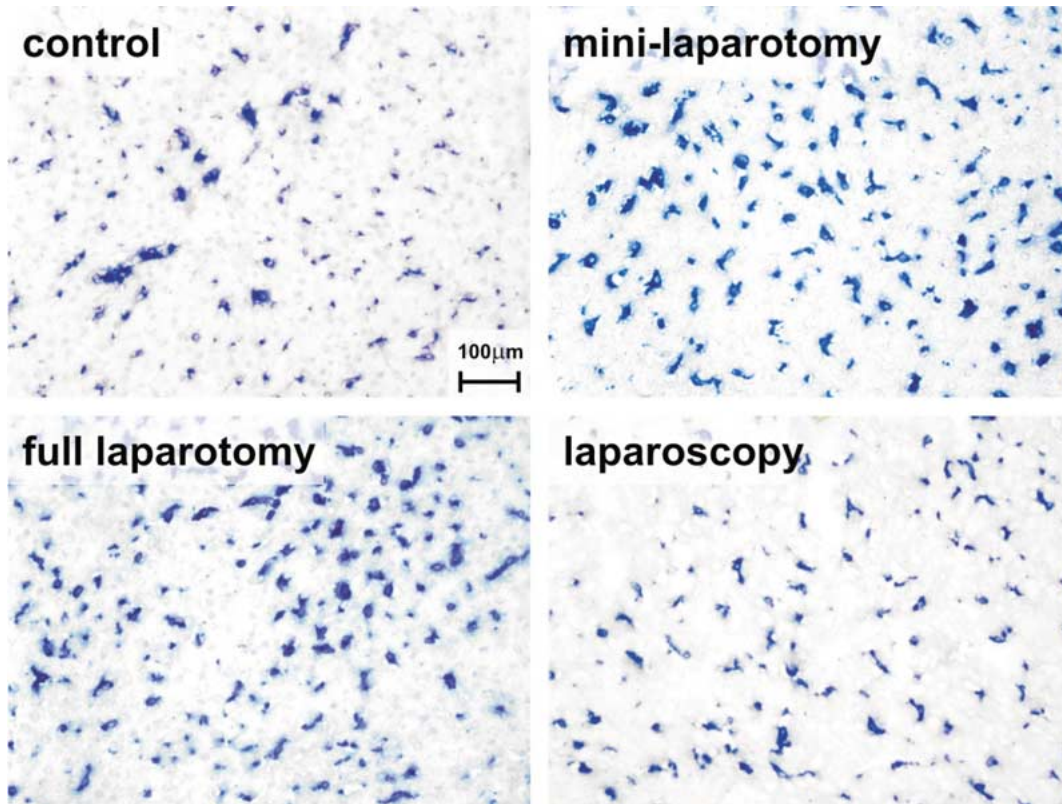


Fig. 5. Immunohistological detection of ED-2-positive Kupffer cells in cryostat sections of the liver. One liver section of each group is shown.

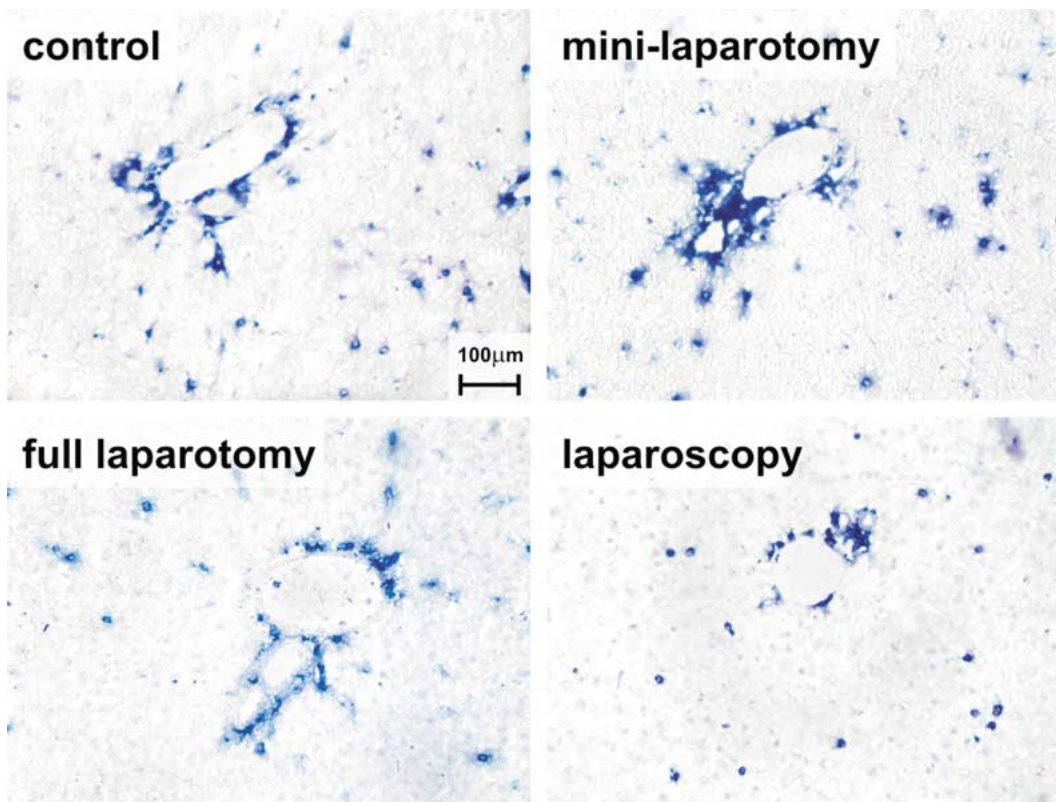


Fig. 6. OX-6 expression in the periportal fields 24 h after anesthesia alone (control), mini-laparotomy, full laparotomy, or laparoscopy. One liver section of each group is shown.

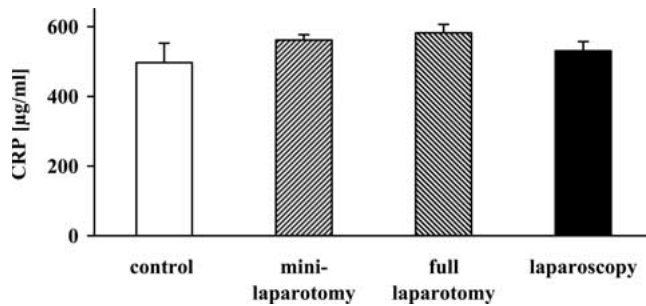


Fig. 7. Concentration of CRP in plasma of Lewis rats 24 h after anesthesia alone (control), mini-laparotomy, full laparotomy, or laparoscopy. Data are presented as mean \pm SEM ($n = 6$).

evenly distributed in the parenchyma without specific areas of accumulation. Many immunologically mediated liver diseases, as well as acute rejection after liver transplantation, are characterized by cell infiltration, especially in the periportal fields. A major increase in OX-6 expression in these areas, as well as an activation of Kupffer cells, was observed [11, 17, 27]. This suggests that the regulating mechanisms of hepatic inflammatory responses are induced differently after infection and surgical trauma.

To our knowledge, this study confirmed for the first time that immunological advantages of laparoscopy are still obvious when compared with mini-laparotomy. Laparoscopy prevents the macrophage population from expanding within the liver and preserves normal cell distribution and baseline function of the hepatic macrophages. Laparotomy, irrespective of the incision size, increases the number of Kupffer cells. Full laparotomy increases hepatic monocyte recruitment. Further studies are required to understand the underlying mechanisms and regulatory pathways that lead to a better preserved immune function after laparoscopy.

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