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Postoperative Immune Suppression in Visceral Surgery: Characterisation of an Intestinal Mouse Model

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Key Words
Postoperative immune suppression · Mouse model · Surgically induced immune dysfunction · Cytokines · Surgery

Abstract
Background: Postoperatively acquired immune dysfunction is associated with a higher mortality rate in case of septic complications. As details of this severe clinical problem are still unknown, animal models are essential to characterise the mechanisms involved. ***Methods:*** Mice were laparotomised and the small intestine was pressed smoothly in antegrade direction. For extension of trauma, the intestine was manipulated three times consecutively. Following this, the ex vivo cytokine release of splenocytes was determined. The degree of surgical trauma was analysed by detection of HMGB1 and IL-6 in serum and by neutrophil staining in the muscularis mucosae. ***Results:*** We adapted the previously described animal model of intestinal manipulation to provide a model of surgically induced immune dysfunction. Following intestinal manipulation, the mice showed elevated serum levels of HMGB1 and IL-6 and increased infiltration of granulocytes into the muscularis mucosae. Ex vivo cytokine release by splenocytes was suppressed in the postoperative period. The degree of suppression correlated with the extent of surgical trauma. ***Conclusions:*** In this study, we describe a surgically induced immune dysfunction animal model, in which a significant surgical trauma is followed by an immune dysfunction. This model may be ideal for the characterisation of the postoperative immune dysfunction syndrome.

Introduction

Despite considerable progress in intensive care medicine, sepsis remains a severe problem in postoperative patient management [1]. Sepsis is a clinical syndrome defined by the presence of both infection and a systemic inflammatory response. Infection is defined as a pathologic process caused by the invasion of normally sterile tissue, fluid or body cavity by pathogenic or potentially pathogenic microorganisms [2]. Currently, sepsis is the 10th leading cause of death in the United States, with 750,000 cases of severe sepsis per year. The incidence of sepsis is expected to further increase by 1.5% each year,

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resulting in an additional 1 million cases per year by 2020 [3]. For this reason, it is important to investigate the origins of sepsis and, in particular, the contribution of a patient's immune status to the development of sepsis.

It is well known that surgical procedures can induce immune suppression. Following major abdominal surgery, there is a significant reduction of immune competence and this may well contribute to the high mortality associated with postoperative septic complications in situations such as anastomotic insufficiency or pneumonia [4]. In contrast, mortality is significantly lower in cases where sepsis is acquired spontaneously than in cases with perforated diverticulitis [5, 6]. Septic patients can therefore be classified by their immune function status at the time of sepsis development: spontaneously acquired sepsis (type A) develops in the presence of an intact immune system; postoperative sepsis (type B), on the other hand, develops in the context of a partially disabled immune system [7].

Previous studies found that the phenomenon of postoperative immune suppression is associated with a reduction in the expression of HLA-DR on monocytes, caused by a reduced ability of monocytes to respond to challenge with lipopolysaccharide (LPS), with a reduction in proliferation by stimulated T lymphocytes, as well as with a reduction in the secretion of interleukin (IL)-2 and interferon (IFN)-γ [8–10]. Immune dysfunction is already measurable on the first postoperative day. However, depending on the extent and severity of the surgical procedure, normal immune function is re-established within a few days in the case of an uncomplicated postoperative course. In cases in which postoperative sepsis develops, there is a direct correlation between the suppression of monocyte HLA-DR expression and the severity of the septic syndrome [4, 11]. The detailed mechanisms of this clinically important phenomenon are still unknown.

Most studies on the alteration of immune function have been carried out in patients with burn or trauma injury rather than in postoperative patients. Though animal models of immune suppression following trauma or shock have been described, there is as yet no adequate model for the immune suppression phenomenon which follows surgery [12, 13]. To characterise the postoperative immune dysfunction in detail, it will be necessary to develop adequate animal models.

For this purpose, we modified a previously published model used to investigate postoperative ileus [14–17]. Laparotomy followed by manipulation of the small intestine results in surgical trauma, which can be measured by the determination of serum levels of the high-mobility group box 1 protein (HMGB1) and IL-6. We analysed postoperative immune dysfunction, showing a suppressed cytokine release by splenocytes. We then validated these observations in the murine model by comparing them to results obtained from clinical studies on human monocytes. We conclude that repeated intestinal manipulation in mice provides a useful model of postoperative immune dysfunction. We suggest the term SID for 'surgically induced immune dysfunction'.

Methods

Animal Model of SID

We used female C57B/6 mice, aged 8–12 weeks, with an average body weight of 22 g. All animal experiments were designed and carried out according to the guidelines of the German Animal Protection Act. Permission was obtained from the governmental committee on animal welfare (LALLF M-VL/TSD/7221.3-1.1-037/07). As described by Kalff et al. [14], anesthetised mice were laparotomised, and the small intestine was pressed smoothly between two q-tips (intestinal manipulation model, IMM). For extension of surgical trauma, the small intestine was manipulated three times consecutively (SID). In the laparotomy group, the abdomen was laid open and closed after several minutes, without touching the intestine.

Histochemistry: Infiltration of Neutrophil Granulocytes into the Intestinal Muscularis Mucosae

The staining of neutrophil granulocytes in the muscularis mucosae 24 h following SID and IMM was performed as described previously [18, 19]. In brief, whole mounts of mucosa-free muscularis were prepared from the jejunum. Neutrophil granulocytes as myeloperoxidase (MPO)-positive cells were stained with 10 mg Hanker-Yates reagent (p-phenylenediamine/pyrocatechol; Sigma, Taufkirchen, Germany) in 10 ml PBS and 100 μl hydrogen peroxide for 10 min. The MPO-positive cells were detected under a light microscope at 100-fold magnification.

Detection of Serum Cytokine Levels in Mice

Following surgical trauma, mice were anesthetised with 2,2,2-tribromoethanol at four different points in time (at 6, 12, 24 and 48 h). Blood was harvested by retro-orbital puncture. The samples were analysed using a cytometric bead array technique according to the manufacturer's protocol (n = 9 per group; Mouse Inflammation Kit, CBA; BD Pharmingen, Heidelberg, Germany).

Determination of HMGB1 Levels in Murine Serum

Samples were analysed for HMGB1 by an enzyme-linked immunosorbent assay. Levels were quantified using the HMGB1 ELISA Kit II according to the manufacturer's instructions (n = 9 per group; Shino-Test Corporation, Tokyo, Japan).

Ex vivo Cytokine Inducibility of Murine Splenocytes

Spleens were harvested from deeply anesthetised mice. Splenocytes were cultured as described previously [20]. In brief, cells were adjusted to 1×10^7 cells/ml in very-low endotoxin RPMI-

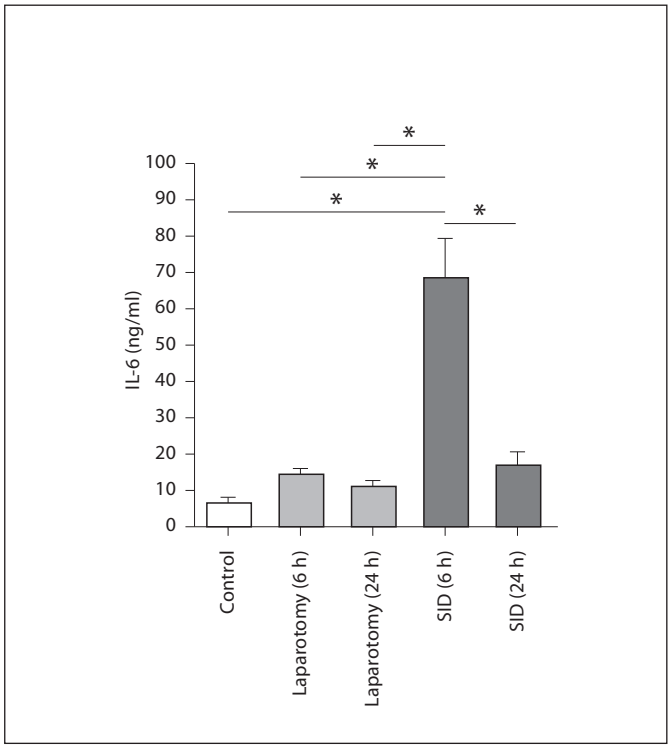

Fig. 1. The SID model induces increased cytokine serum levels. We observed significantly elevated serum levels of IL-6 following SID. Both surgical procedures are followed by an increase in IL-6 levels, whereas IL-6 levels 6 h after SID are significantly increased compared to the control and the laparotomy groups. Twenty-four hours following SID, the IL-6 level equaled the control level again (n = 9 per group, one-way ANOVA and Newman-Keuls, $p < 0.001$). * $p < 0.001$.

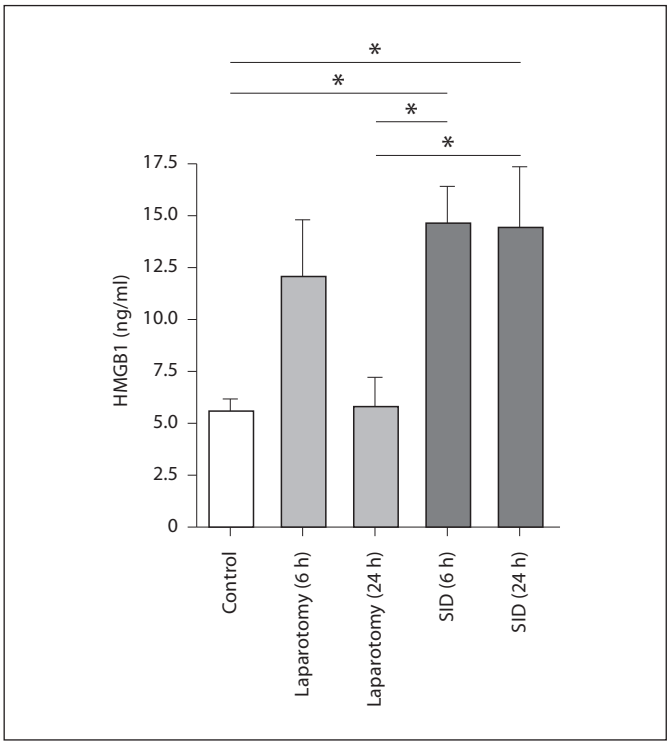

Fig. 2. Elevated HMGB1 levels in serum following surgical procedures as a marker for the severity of the surgical trauma. To describe the extent of surgical trauma, we analysed postoperative serum levels of HMGB1 by ELISA. Following laparotomy, we observed a slight but non-significant increase of HMGB1 in serum at 6 h, whereas the SID group shows significantly increased HMGB1 levels 6 and 24 h postoperatively (n = 9, one-way ANOVA and Newman-Keuls, $p < 0.05$). Mice without surgical procedure served as control group. * $p < 0.05$.

1640 medium (Biochrom, Berlin, Germany) with 2 mM Na-pyruvate (GIBCO, Paisley, UK), 2% D-glucose (Sigma), 4 mM N-acetyl-L-alanyl-L-glutamine (Biochrom), antibiotics [10 mg/ml gentamicin (Sigma), and 10,000 units of penicillin and 10 mg/ml streptomycin (both GIBCO, Paisley, UK)] and 10% fetal calf serum (Biochrom). Cells were stimulated in 500 μl medium with 1 μg/ml lipopolysaccharide from *Salmonella abortus equi* (Sigma). Following incubation for 42 h (at 37°C in a humidified atmosphere with 5% CO_2), the cytokine concentration in the supernatant was analysed by cytometric bead array (n = 7 per group).

Statistical Analyses

Data were analysed using GraphPad Prism Version 3.02 for Windows (GraphPad Software, San Diego, Calif., USA). Differences between two samples at one time point were analysed by unpaired t test and one-way ANOVA (Newman-Keuls post test) for variables with Gaussian distribution. All data are expressed as means ± SEM; $p \leq 0.05$ was considered to be statistically significant.

Results

To examine the characteristics of postoperative immune suppression in mice, female C57Bl/6 mice were laparotomised, and the small intestine was manipulated as described by Kalff et al. [14]. Serum levels of cytokines were assayed at different time points. Six hours after the surgical procedure, we observed a significant increase in IL-6 serum levels, indicating that surgical trauma following intestinal manipulation induces an inflammatory status in mice ($p < 0.001$). Laparotomy did not induce significant elevated IL-6 levels 6 or 24 h following the surgical procedure (fig. 1).

The degree of trauma was assessed by quantifying the levels of HMGB1 in the murine serum. At 6 and 24 h after SID, HMGB1 levels were significantly increased compared to the levels in control mice ($p < 0.05$). Laparotomy

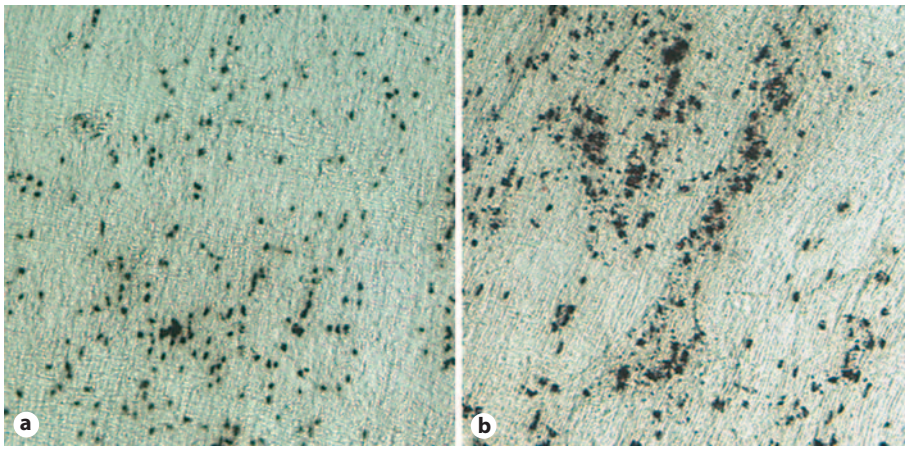

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Fig. 3. The intensification of trauma can be visualised by the elevated number of neutrophils in the intestinal muscularis mucosae. We performed Hanker-Yates MPO staining to visualise the infiltration of neutrophil granulocytes into the muscularis mucosa. Twenty-four hours after a single compression of the small intestine (IMM), several neutrophils infiltrated the muscularis mucosae (**a**). Three-fold compression of the small intestine (SID) results in a markedly increased number of neutrophils in the muscularis mucosae 24 h following the surgical procedure (**b**, 100-fold magnification). Muscularis segments of non-treated mice revealed no neutrophil infiltration (data not shown).

without subsequent intestinal manipulation did not result in significantly elevated HMGB1 release 6 and 24 h following the surgical procedure (fig. 2).

To determine the degree of damage on structural levels, we performed Hanker-Yates MPO staining and visualised the infiltration of neutrophil granulocytes into the muscularis mucosa. Twenty-four hours after SID, the neutrophil invasion into the muscularis was strikingly increased compared to the amount of neutrophils in the single-manipulated intestinal wall (fig. 3).

We observed postoperative immune suppression in two different murine models of intestinal manipulation: the small intestine was surgically manipulated once (IMM) or three times consecutively (SID). At 6 h (fig. 4a, c) or 3 days (fig. 4b, d) after the surgical procedure, splenocytes were isolated and stimulated with LPS. Cytokine release into the supernatant was detected using the cytometric bead array technique. Stimulated splenocytes of non-operated mice served as controls.

We noticed a significant reduction of TNF-α release by murine splenocytes after surgical trauma. Intensification of surgical trauma resulted in increased immune dysfunction, as evidenced by the suppression of cytokine release by splenocytes upon proinflammatory stimulation (fig. 4a). Three days after the surgical procedure, splenocytes of mice with extensive trauma were still suppressed in their response to the proinflammatory stimulus, while splenocytes of laparotomised and IMM-manipulated mice had already reverted to control levels (fig. 4b). Similar effects were seen for the release of IFN-γ by isolated and stimulated splenocytes 6 h (fig. 4c) and 3 days (fig. 4d) after surgery.

Discussion

In 1985, it was reported that surgical procedures induce a reversible depression of cellular immunity [21]. Moreover, irrespective of the nature of the surgical procedure used, all patients suffer from postoperative dysfunction of monocytes [11]. Further studies described a direct correlation between the extent of trauma and the degree of postoperative immune suppression. Decreased immune function induced by extended surgical trauma has been shown by comparing laparoscopic and conventional surgery [22, 23].

A patient's functional immune status plays a decisive role in the induction of postoperative septic complications, and prolonged disarming of the innate immune system is associated with a significantly increased incidence of septic complications [24–26]. Colorectal surgery is associated with a heightened risk of infection, which requires an immediate response from the innate immune system. A limitation of this response evidenced by a blunted release of proinflammatory mediators will considerably increase the risk of septic complications. Since the physiological post-

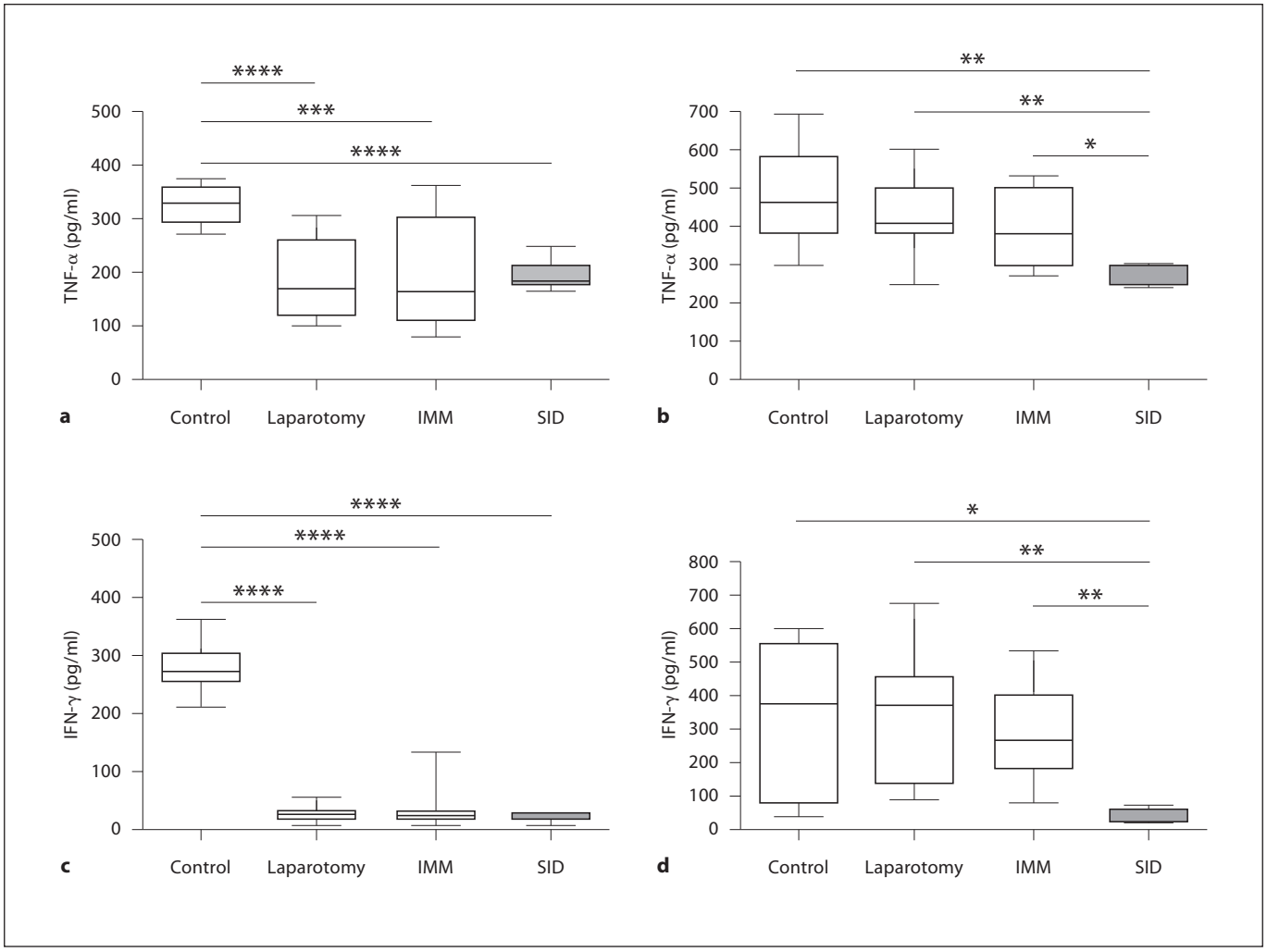

Fig. 4. Increasing surgical trauma causes suppression of ex vivo cytokine inducibility of murine splenocytes: TNF-α and IFN-γ. Six hours after the surgical procedure (**a**, **c**), there is a significant suppression of cytokine release by LPS-stimulated splenocytes ex vivo. The extent of surgical trauma does not influence the degree of suppression, as cytokine release was suppressed following laparotomy, intestinal manipulation (IMM) and SID (**a**, **c**; $p < 0.001$). Cytokine release of laparotomised mice and intestinal manipulated mice reaches normal levels 72 h following the surgical procedure (**b**, **d**). In contrast, splenocytes of the SID group are still suppressed in their answer to the proinflammatory stimulus ex vivo. These differences are significant (**b**, **d**; $p < 0.05$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (n = 7 per group).

operative immune suppression is supposed to be conducive to worsen the outcome in case of surgical complications, especially septic complications like anastomosis leakage, the aim of our study was to first establish an animal model to investigate this phenomenon. As detailed mechanisms of postoperative immune suppression are still unidentified, it is imperative to develop adequate animal models. The presented human data describe the clinic situation in the postoperative period and are the fundament for the development of the mouse model.

Our SID model is based on the procedure of intestinal manipulation first described to study the phenomenon of postoperative ileus [14]. By increasing the degree of intestinal manipulation, we create an immunological situation which mirrors that seen in surgical patients.

In the postoperative period, mice show a significant increase in the serum concentration of the proinflammatory cytokine IL-6, and this mirrors the rise in IL-6 seen in postsurgical patients following conventional colorectal surgery [27].

The degree of surgical trauma can also be assessed by the level of HMGB1 in serum. This protein is a structural component of the nucleus which is released from dead cells and may also be secreted by macrophages [28]. It acts as a proinflammatory mediator, and since in humans its release is elevated early after a mechanical trauma, it is considered an early 'endogenous danger signal', serving as a marker of traumatic injury [29, 30]. Our findings suggest that SID is able to reproduce the situation of surgical trauma in the mouse, as we detected elevated HMGB1 levels in the postoperative period.

Staining of inflammatory cells in the intestinal wall reveals structural changes on cellular levels following surgical trauma induced by SID and IMM. The degree of trauma correlates with the number of neutrophil granulocytes infiltrating the intestinal muscularis mucosae: the increased surgical damage in the model of SID results in an elevated number of neutrophils. These findings suggest that the escalation of local inflammatory reactions could trigger the systemic immunosuppressive response following trauma induced by SID. Further studies evaluating the association between local inflammatory reactions and immune suppression are necessary to analyse the mechanism of the postoperative immune dysfunction.

The local inflammatory response may result in a systemic inflammation not only because of cellular infiltration but also due to bacterial translocation and endotoxin release. As we could not detect any bacterial load in serum and organs following SID (data not shown), we conclude that the immune reaction is triggered by trauma components and not by bacterial invasion. Bacteraemia and high-level release of endotoxins would cause septic processes in the organism as it was described in several septic mouse models [31]. In the postoperative period of SID, there were no signs of septic reactions such as reduced general condition or increased mortality rate. Nevertheless, slightly enhanced levels of endotoxins and inflammatory mediators could be a part of the mechanism that finally leads to the immune suppression induced by SID. The release of prostaglandins and nitric oxide following intestinal manipulation was shown to contribute to the development of postoperative ileus [32]. Therefore, these local inflammatory mechanisms may additionally be the basic origin of the postoperative immune dysfunction.

By analysing isolated murine immune cells, we found evidence for a correlation between the extent of surgical trauma and the degree of postoperative immune dysfunction. Elevated levels of surgical trauma were followed by increased suppression of postoperative immune function. Thus, in this mouse model the degree of trauma can be modified to provide different levels of immune suppression. For quantifying the murine immune status, we chose one early (6 h) and one late point in time (3 days). Our data on human monocytes suggests that on postoperative day 3, there is still immune dysfunction, which correlates well with the results from the mouse model.

Further analysis is now needed to characterise the severe clinical problem of postsurgical immune dysfunction and to develop strategies to control sepsis due to surgically induced immune suppression.

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