Neuromodulation of intestinal inflammation
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Chapter 6

Systemic inflammation with enhanced brain activation contributes to more severe delay in postoperative ileus

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Abstract

Background: The severity of postoperative ileus (POI) has been reported to result from decreased contractility of the muscularis inversely related to the number of infiltrating leukocytes. However, we previously observed that the severity of POI is independent of the number of infiltrating leukocytes, indicating that different mechanisms must be involved. Here, we hypothesize that the degree of tissue damage in response to intestinal handling determines the upregulation of local cytokine production and correlates with the severity of POI. Methods: Intestinal transit, the inflammatory response, I-FABP (marker for tissue damage) levels and brain activation were determined after different intensities of intestinal handling. Key results: Intense handling induced a more pronounced ileus compared with gentle intestinal manipulation (IM). No difference in leukocytic infiltrates in the handled and non-handled parts of the gut was observed between the two intensities of intestinal handling. However, intense handling resulted in significantly more tissue damage and was accompanied by a systemic inflammation with increased plasma levels of pro-inflammatory cytokines. In addition, intense but not gentle handling triggered enhanced c-Fos expression in the nucleus tractus solitarius (NTS) and area postrema (AP). In patients, plasma levels of I-FABP and inflammatory cytokines were significantly higher after open compared with laparoscopic surgery, and were associated with more severe POI. Conclusions and inferences: Not the influx of leukocytes, rather the manipulation-induced damage and subsequent inflammatory response determine the severity of POI. The release of tissue damage mediators and pro-inflammatory cytokines into the systemic circulation most likely contribute to the impaired motility of non-manipulated intestine.
Introduction

Postoperative ileus (POI) is characterized by a transient inhibition of gastrointestinal (GI) motility following surgery. Patients experience significant discomfort, such as abdominal distention, nausea, and inability to pass stool or tolerate food. Especially prolonged ileus leads to an increased risk for wound dehiscence, pulmonary, and thromboembolic complications and a prolonged hospital stay and is associated with an enormous economic burden [1]. During the last decade, evidence has accumulated that intestinal inflammation evoked by handling of the intestine is a key mechanism underlying impaired GI motility following surgery, both in humans and in animal models. These studies demonstrated that infiltrating leukocytes inhibit the contractile activity of the manipulated intestine by local release of pro-inflammatory mediators such as nitric oxide and prostaglandins [2,3]. It is becoming increasingly clear that POI mainly results from intestinal handling of the intestine during surgery [4]. In rodents, Kalff et al. elegantly showed that manipulation of the intestine triggered the influx of leukocytes in the muscularis, starting from 3h onwards and further increasing up to 24h after surgery. Of note, the number of infiltrating leukocytes increased with the severity of intestinal manipulation (IM) with compression of the intestine yielding more influx than running along the intestine with cotton swabs [5]. These infiltrating leukocytes, mainly monocytes, subsequently release inflammatory mediators such as prostaglandins and nitric oxide impairing the contractility of smooth muscle strips of the intestine [6]. The latter has been proposed to underlie the delay in intestinal transit observed 24h after the abdominal surgical procedure. Recently, however, we observed that eventration of the small intestine and graded manipulation of the intestine up to 3.5 g did not delay GI transit, but was associated with influx of leukocytes to the same level as more intense manipulation that did lead to POI [7]. These data would imply that other mechanisms must be involved. Based on the above, we reasoned that the degree of tissue damage evoked by intestinal handling may be an important determinant of the severity of POI. Several clinical studies indeed have reported an increased postoperative inflammatory response related to increased operative trauma with systemic release of cytokines and systemic spread of the inflammatory response [5,8,9]. Tissue damage can trigger an innate immune response via the local release of damage associated molecular pattern molecules [10], evoking an inflammatory response involving macrophages and/or mast cells. The resulting enhanced local inflammation may result in a more systemic inflammatory response with increased serum levels of pro-inflammatory cytokines.
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The latter will consequently affect distant regions of the gut and contribute to the generalized aspect of POI. In this study, therefore, we investigated the mechanism behind severe POI by studying the local and systemic inflammatory response, including brainstem activation after different intensities of intestinal handling.

Material and methods

Animals

Laboratory animals were kept under environmentally controlled conditions (light on from 8:00 AM to 8:00 PM with water and food *ad libitum*; 20–22 °C, 55% humidity). Ten to twelve weeks old C57NL/BL6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). Mice were maintained at the animal facility of the Academic Medical Centre in Amsterdam and were used at 12–14 weeks of age. Studies were performed according to the guidelines of the Dutch Central Committee for Animal Experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands).

Patients

Patients undergoing elective segmental colectomy for colonic cancer were invited to participate. The protocol was approved by the Medical Ethics Review Board of the Academic Medical Center in Amsterdam (The Netherlands) and was conducted in accordance with the principles of the Declaration of Helsinki and good clinical practice guidelines.

Surgical procedures

Anesthesia was performed by an intraperitoneal (i.p.) injection of a mixture of Ketamine (Ketalar 100 mg.kg⁻¹) and Xylazine (Rompun 10 mg.kg⁻¹). Mice (five to eight per group) underwent a laparotomy (L) alone, or a L followed by small IM [11]. Surgery was performed as follows: a midline abdominal incision was made and the peritoneum was opened over the linea alba and the small bowel was carefully layered on a sterile moist gauze pad. The small intestine was manipulated from the distal duodenum to the cecum and back for a total of three times. Contact with or stretch on stomach or colon was strictly avoided. Gentle standardized bowel manipulation (gentle IM) was constructed
using a sterile moist cotton applicator attached to a device enabling the application of a constant pressure of 9 g to the intestine. The more intense manipulation (intense IM) was performed by compression of the small bowel using moist cotton applicators such that the luminal content was moved aborally as previously described [12]. After the surgical procedure, the abdomen was closed by a continuous 2-layer suture (Mersilene, 6–0 silk). After closure, mice were allowed to recover for 3h in a heated (32 °C) recovery cage.

Gastrointestinal transit measurements

Gastrointestinal function 24h postoperatively was determined in vivo by measurement of GI transit of liquid non-absorbable fluorescein isothiocyanate–dextran (FITC-dextran; 70 000 Da; Invitrogen, Paisley, UK). Ten microlitres of FITC-dextran dissolved in 0.9% saline (6.25 mg.mL$^{-1}$) was administered via oral gavage. Ninety minutes later, animals were sacrificed and the entire bowel from stomach to distal colon was collected. The contents of the stomach, small bowel (divided into 10 segments of equal length), cecum, and colon (three segments of equal length) were collected and assayed in duplicate (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA; excitation wavelength: 485 nm, emission wavelength: 528 nm) for the quantification of fluorescent signal in each bowel segment. The distribution of the fluorescent label along the GI tract was determined by calculating the geometric center (GC): Σ (% of total fluorescent signal in each segment x the segment number)/100 for quantitative statistical comparison among experimental groups [13].

Colonic transit

Colon function was determined in vivo by the measurement of colon transit of a glass ball. One and a half hour before sacrifice, mice were briefly anesthetized with isoflurane (Abbott). Patency of the colon was carefully checked by inserting a polished metal rod 3 cm into the colon. The rod was pulled out and a 2.2 mm plastic ball was transanally inserted with blunt surgical forceps and pushed forward for 3 cm into the colon with a polished metal rod. The time from insertion until excretion of the plastic ball was considered as colonic transit time [14].
c-Fos expression in the brain

Twenty-four hours after surgery mice were sacrificed by transcardiac perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA; pH 7.4). Brains were collected, postfixed for 4h (4 °C) and cryo-protected by immersion in 30% sucrose in 0.2 mol.L\(^{-1}\) PBS (pH 7.4) at 4 °C overnight. Coronal sections of 30 µm of the brainstem were collected. After rinsing in 0.05 mol.L\(^{-1}\) Tris-buffered saline TBS (pH 7.4), sections were incubated overnight at 4°C with goat anti-Fos (1 : 1500; SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) primary antibodies. However, sections were incubated 1h with biotinylated secondary antibody and after with avidin-biotin complex (ABC; Vector, Burlingame, CA, USA) for 1h. The reaction product was visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide H\(_2\)O\(_2\) for 5 min to count the number of c-Fos immunoreactive neurons, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 color camera (Sony Corp., Tokyo, Japan). A minimum of seven sections was used for c-Fos counting in the NTS (from Bregma -7.20 to -7.76 mm) and Area Postrema (AP; Bregma -7.32 to -7.76 mm), and 9–11 sections for PVN (Bregma -0.58 to -1.22 mm).

Immunohistochemistry staining for leukocytic infiltration of the small intestinal muscularis.

To quantify the degree of inflammation in whole mounts of the intestinal muscularis, ileal segments were cut open and rinsed in ice-cold modified Krebs solution. The segments were fixed with 100% ethanol for 10 min, transferred to ice cold modified Krebs solution, and pinned flat in a glass-dish. Mucosa and submucosa were removed, and the remaining full-thickness sheets of muscularis externa were stained for polymorphonuclear neutrophils with Hanker Yates reagent (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 10 min. To quantify the extent of intestinal muscle inflammation, the number of myeloperoxidase (MPO) positive cells in 10 randomly chosen representative high-power fields (HPF; 668.4 x 891.2 µm) was counted and the average was calculated. Tissue sections were coded so that the observer was unaware of the surgical treatment of the specimens.
Blood analysis: tissue damage and plasma levels of inflammatory cytokines

*Mice*

Tissue damage was assessed by determining plasma levels of intestinal fatty acid-binding protein (I-FABP) [15]. Levels of I-FABP in the plasma were determined using standard enzyme-linked immunosorbent assay (ELISA) for mouse I-FABP (Hycult Biotechnology (Hbt), Uden, The Netherlands). Interleukin (IL)-6, the murine IL-8 homologue KC, Monocyte Chemoattractant Protein-1 (MCP-1), Tumor Necrosis Factor (TNF-α) and IL-1β plasma levels of venous blood retrieved by cardiac puncture 1, 6 and 24h after surgery were determined using cytometric bead array kits (CBA) according to the manufacturer’s instructions (BD Biosciences, Erembodegem, Belgium). Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Biosciences). Cytometric bead assay results were analyzed using the FCAP ArrayTM software (BD Biosciences).

*Patients*

IL-6, IL-8, MCP-1, TNF-α and IL-1β plasma levels of venous blood retrieved 2h after surgery were determined using CBA kits for human IL-6, IL-8, MCP-1, TNF-α (for human TNF-α and IL-1β the enhanced sensitivity flex set kits were used) according to the manufacturer’s instructions (BD Biosciences). Flow cytometric analysis was performed using a FACSArray flow cytometer (BD Biosciences). Cytometric bead assay results were analyzed using the FCAP ArrayTM software (BD Biosciences). Before determination of I-FABP in the human samples, blood was centrifuged two times and the obtained plasma was concentrated with the use of Vivaspin 23 000 MW sample concentrators (Bio-Sciences AB, Uppsala, Sweden) by centrifuging at 3506 g at 4 °C for 2h and further processed according to the manufacturer’s instructions. Levels of I-FABP in the concentrated plasma were determined using ELISA human I-FABP (Hbt).

**RNA extraction and inflammatory gene expression**

Total RNA was extracted from the intestinal muscularis externa of the distal stomach, jejunum, and distal colon at 6 and 24 after start surgery. The muscularis was microscopically dissected from the submucosa and immediately snap frozen in Tripure (Roche diagnostics, Mannheim, Germany) and stored at -80 °C. Tissue was homogenized by a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France).
RNA extraction was performed using RNeasy Mini Kit (Qiagen # 74104; Qiagen Benelux BV, Venlo, the Netherlands) according to manufacturer’s instructions. Total of RNA were transcribed into complementary cDNA by qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) according to manufacturer’s instructions. Quantitative real-time transcription polymerase chain reactions (RT-PCR) were performed with the LightCycler 480 SYBR Green I Master (Roche) on the Light Cycler 480, Roche (Roche). Results were quantified using the $2^{\Delta\Delta C_t}$ method (PMID:11328886). The expression levels of the genes of interest were normalized to the expression levels of the reference gene (RPL32). PCR experiments were performed in triplicate, and standard deviations calculated and displayed as error bars. Primer sequences used are listed in Table S1.

**Statistical analysis**

The data on human plasma cytokine levels were not normally distributed. The Kruskal–Wallis test was performed to assess whether the cohort of data was statistically different. When variance of medians was statistically significant, the Mann–Whitney U test was used to identify the statistical differences within the cohort. For comparison of the time to recovery of GI function and plasma I-FABP levels between open and laparoscopy-treated patients, the Mann–Whitney test was used and results were shown as median with interquartile ranges (IQR). All other data were statistically analyzed by one-way ANOVA followed by Tukey’s Multiple Comparison analysis and are presented as mean±SEM. A probability level of $P < 0.05$ was considered statistically significant. Graph Pad Prism version 5.01 software was used to perform statistical analysis and create graphs.

**Results**

**GI transit and colonic transit**

Twenty four hours after surgery, the intestinal transit was significantly delayed by IM compared with L (GC: 8.9±0.7). Notably, intense IM (GC: 3.8±0.2) induced a more severe delay in intestinal transit compared with gentle IM (GC: 6.3±0.8; Fig. 1A). Colonic transit did not differ significantly between L and gentle IM, but was significantly delayed after intense IM compared with L (Fig. 1B).
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Figure 1. Intense manipulation leads to a more pronounced delay of intestinal transit compared to Gentle manipulation with similar leukocytic infiltrate in the manipulated small intestine. Mean geometrical center (GC) of orally administered FITC-dextran (A), or colonic transit time of a 2-mm plastic ball (B) after intestinal manipulation (IM) or sham operation (laparotomy) at 24 hours after surgery. Results are representative of three independent experiments in groups of 7-8 mice and data are mean±SEM. * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis

Intense handling leads to enhanced inflammation in the manipulated part of the intestinal tract

In the small intestine, IM but not L resulted in an influx of MPO-positive inflammatory cells in the muscle layer of the small intestine with similar leukocytic counts in the gentle and intense IM groups 24h after surgery (Fig. 2). In addition, IM resulted in the upregulation of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α at 6 and 24h compared with L. Importantly, intense manipulation induced significantly more upregulation of IL-6 and TNF-α as compared with gentle manipulation (Fig. 3). In the non-handled colon, leukocyte infiltration was significantly increased after IM compared with L. However, no difference in leukocyte infiltration in the colon was observed after intense and gentle IM (Fig. 3). In line, IL-6, IL-1β and TNF-α mRNA levels were not significantly different after IM compared with L, indicating that there was no detectable inflammatory response (Fig. 3). However, in the stomach, manipulation of the intestine did not result in an influx of MPO positive cells in the muscularis (data not shown), or an increase in mRNA levels of IL-6, IL-1β and TNF-α (Fig. 3).
Figure 2. Gentle and intense manipulation lead to a similar leukocytic infiltrate in the manipulated and non-handled parts of the intestinal tract. Leukocyte recruitment reflected as the number of myeloperoxidase (MPO) positive cells per high power field (HPF) in the muscularis externa in the different parts of the intestinal tract at 24 hours after surgery. However, the influx did not differ significantly (NS) between gentle IM (grey bars) and intense IM mice (black bars) in the small intestine or colon. Results are representative of three independent experiments in groups of 6-8 mice and data are mean±SEM. * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.

Figure 3. Expression of inflammatory cytokines in different parts of the gastrointestinal tract. Quantitative PCR for IL-6 (A), IL-1β (B) and TNF-α (C) in muscularis of the stomach, small intestine and colon at 6 and 24 hours after surgery. Results are representative of three independent experiments in groups of 4-7 mice and data are mean±SEM. * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.
**Intense IM results in tissue damage with release of inflammatory cytokines into the circulation**

Next, we investigated whether increased levels of tissue damage could be associated with a more pronounced upregulation of pro-inflammatory cytokines reflected in increased cytokine plasma levels. One hour after surgery, plasma levels of I-FABP, a marker of intestinal tissue damage, were significantly elevated in mice subjected to more intense IM (Fig. 4A). After 24h, I-FABP was no longer detectable. To study whether intense IM results in a systemic inflammatory response, circulating pro-inflammatory cytokine levels were determined at 1, 6 and 24h following surgery. As shown in Figure 4, plasma levels of KC, MCP-1 and IL-6 were significantly increased following intense IM, but not after gentle IM or L, whereas manipulation of the intestine did not result in enhanced plasma levels of IL-1β and TNF-α (data not shown for TNF-α).

![Figure 4. Tissue damage and plasma levels of inflammatory cytokines after different intensities of surgical manipulation.](image)

*Results are representative of three independent experiments in groups of 6-8 mice and data are mean±SEM; * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.*

**Intense IM-induced tissue damage is associated with AP activation**

Previous studies have reported activation of brain areas following abdominal surgery, a mechanism that was proposed to contribute to the development of POI [16]. To investigate whether brain activation contributes to the severity of POI or is associated
with increased plasma levels of cytokines, we investigated the expression of c-Fos to determine the amount of neuronal activation in the brainstem 24h after surgery. The neurons of the AP, which is exposed to systemic circulation, relay their signal to the nucleus tractus solitarius (NTS) and can thereby result in an enhanced activation of the NTS [17,18]. The number of c-Fos positive neurons in the AP and NTS was significantly higher after intense compared with gentle IM (Fig. 5). c-Fos expression was also significantly higher after intense IM at higher levels of the neurocircuitry, namely in the hypothalamic paraventricular nucleus (PVN; data not shown). In line, we observed a positive correlation of c-Fos expression in the AP with plasma I-FABP levels [Spearman’s ρ correlation coefficient 0.65 (95% CI: 0.30–0.85; P = 0.0013)], suggesting that the degree of tissue damage is associated with activation of the AP (Fig. 5).

Figure 5. Intensity of IM and tissue damage are associated with enhanced brainstem activation. Representative images of IM-induced c-Fos expression in brainstem nuclei 24 hours after surgery (A). Panel B correspond to c-Fos expression in the nucleus tractus solitarius (NTS). Panel C shows c-Fos expression in the area postrema (AP). Data are expressed as mean±SEM for 6-8 mice per group. Activation of the AP is associated with tissue damage (plasma I-FABP levels 1 hour after surgery); Spearman’s ρ correlation coefficient 0.65 (95% CI: 0.30 - 0.85; P = 0.0013 (D). * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.
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Recovery of GI function, tissue damage, and systemic inflammatory cytokines in humans after different intensities of surgical handling

In patients undergoing elective intestinal surgery baseline characteristics (age, sex, type of surgery, body mass index, and American Society of Anesthesiologists grade) did not differ significantly between open and laparoscopic surgery, except duration of surgery (median time: 139 min after open surgery vs 186 min after laparoscopy, P < 0.001). Plasma levels of I-FABP and the inflammatory cytokines IL-6, IL-1β, MCP-1, and IL-8 were significantly higher after open compared with laparoscopic intestinal surgery (Fig. 6B-C). This was associated with a longer duration of POI (median time until tolerance of solid food and passing defecation: 96h after open colonic surgery vs 72h after laparoscopic; Fig. 6A), confirming that more intense manipulation of the intestine leads to an increase in plasma levels of pro-inflammatory cytokines and more severe POI.

![Graphs and images](image)

Figure 6. Tissue damage, systemic inflammation and duration of POI in patients after different intensities of surgical manipulation. (A) Recovery of gastrointestinal (GI) function: time until passing stool and tolerance of solid food after laparoscopic (white bars; n=26) and open (i.e., requiring more intense IM) colonic surgery (black bars; n=20). (B) Plasma levels of I-FABP (pg/mg protein) in concentrated plasma samples 2h after surgery (open (n=16); laparoscopy (n=19). (C) Plasma levels IL-1β and TNF-α (fg/ml), IL-6, MCP-1, IL-8 (pg/ml) 2h after surgery (open (n=15); laparoscopy (n=19). * P < 0.05; Median±IQR, Mann-Whitney U test.)
Discussion

Inflammation of the intestinal muscularis is abundantly demonstrated to underlie POI. Here, we demonstrated that not the number of infiltrating leukocytes, but that rather tissue damage and the release of inflammatory cytokines into the circulation are important factors determining the severity of POI. Concomitantly, we found in humans that open abdominal surgery leads to more tissue damage and increased levels of circulating cytokines compared with minimally invasive laparoscopic surgery. Finally, increased tissue damage and plasma levels of cytokines lead to activation of the AP and PVN, possible contributing to the development of more severe POI. Taken together, our findings indicate that more severe upregulation of pro-inflammatory cytokines, in response to increased tissue damage, with 'leakage' of pro-inflammatory cytokines into the systemic circulation significantly contribute to the severity of POI.

The pathophysiology of POI involves recruitment of leukocytes into the intestine impairing smooth muscle contractility [19]. Incremental degrees of manipulation of the small intestine cause a progressive increase in leukocyte infiltration [5]. These infiltrating leukocytes subsequently release inflammatory mediators such as prostaglandins and nitric oxide impairing the contractility of smooth muscle strips of the muscularis and have been proposed to underlie the delay in intestinal transit [20]. Up to date, there is still a scarcity of information on the influence of the severity and extent of surgery on the duration of POI [1]. Graber et al. subjected six monkeys to three operations varying in extent and site of dissection. In this cross-over study, the duration of postoperative dysmotility was independent of the extent, and site of the operative procedure [21]. However, years later Uemura et al. showed in rats that the magnitude of the abdominal incision does affect the duration of POI [22]. We previously demonstrated that only externalization of the intestine outside the abdominal cavity already induced a significant influx of leukocytes without resulting in POI [7]. However, in this study, no significant difference in leukocyte recruitment was observed in the small intestinal muscularis following intense IM compared with gentle IM. As we failed to demonstrate that increased influx of leukocytes is associated with prolonged POI, other mechanisms seem to determine the severity of POI.

It is reasonable to speculate that more severe handling of the intestine will result in more tissue damage. Veenhof et al. recently demonstrated a significant increase in
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IL-6 in serum of patients undergoing open rectal procedures compared with patients undergoing a laparoscopic procedure [8]. In line, several studies have reported an increased postoperative inflammatory response related to increased operative trauma [6,8,9]. Damaged tissue releases pro-inflammatory mediators (also called pro-inflammatory damage-associated molecular patterns, or DAMPs), such as heat shock proteins, uric acid, HMGB-1, SAP130, DNA, and S100 proteins that are normally intracellular. Mast cells and macrophages, two cell types known to be involved in the pathogenesis of POI, may be activated by interaction with these DAMPs [10]. We reasoned that with increasing intensity of manipulation, the contribution of tissue damage in the pathogenesis of POI will increase, not only leading to more intense local inflammation reducing small intestinal motility but also leading to release of cytokines in the systemic circulation. The latter will affect neuromuscular function of unmanipulated segments of intestine, that is, the colon in our model. In this study, we indeed recorded higher levels of I-FABP both in mice and patients undergoing more severe intestinal handling. I-FABP is part of a family of nine different FABP types, each named after the tissue of its first detection, and is involved in the intracellular buffering and transport of long-chain fatty acids. I-FABP is currently used as a marker of intestinal tissue injury in experimental rodents and clinical studies [15,23,24]. Elevated plasma levels of I-FABP are detected in patients suffering from intestinal diseases. Hence, I-FABP shows to be a useful plasma marker for the detection of intestinal injury, especially in patients undergoing intestinal surgery [25]. In addition, we found a significant correlation between I-FABP plasma levels 1h after surgery and intestinal IL-6 production 24h postoperatively (Pearson correlation coefficient 0.7; P = 0.0006). Moreover, intense IM was associated with more pronounced upregulation of pro-inflammatory cytokines, associated with detection of these cytokines in the systemic circulation. Clearly, this increased inflammatory response in the handled intestine will impair smooth muscle function. A possible additional factor contributing to more severe ileus may result from the increased levels of plasma cytokines activating the hypothalamic-pituitary-adrenal axis. During tissue trauma, immune cells release the pro-inflammatory cytokines IL-1, IL-6, and TNF-α into the general circulation. These cytokines result in enhanced activation of the hypothalamus triggering hypothalamic-pituitary-adrenal activity [16,26-29]. This results in an enhanced sympathetic inhibition of intestinal motility through stimulation of α2-adrenergic receptors on monocytes leading to an increased release of nitric oxide [30]. Indeed, our preliminary brain histology data (unpublished) indicated enhanced activation of the hypothalamic PVN in the intense IM mice that still
had detectable systemic IL-6 levels 24h after surgery. Finally, the more enhanced delay in transit might result from direct activation of residential macrophages by circulating cytokines, DAMPs, and other tissue damage products, or even bacterial products. These muscularis-resident macrophages can induce nitric oxide synthase thereby further contributing to the postoperative impairment of GI motility [6,31].

As POI is characterized by impaired motility of the entire GI tract, including areas that have not been manipulated, other factors than local inflammation should be involved. Previously, evidence has been reported that the local inflammation, mainly via prostaglandins, activates afferent nerves triggering inhibitory neural pathways affecting motility of distant non inflamed areas [2,6,31-33]. More recently, Engel et al. showed that IM evokes local IL-12 production and thereby triggers TH1 memory cells to egress into the systemic circulation and migrate to non-manipulated areas of the intestine. There, these TH1 memory cells stimulate macrophages in the muscularis externa leading to dissemination of the inflammatory response [14]. In previous experiments, however, we were unable to demonstrate increased levels of IL-12 [34]. Moreover, we showed that RAG1−/− mice, devoid of T cells, developed POI to the same extent as wild type mice [35] suggesting that other mechanisms must be involved. In this study, we observed that IM of the small intestine resulted in an influx of leukocytes into the colon, but the degree of influx was not related to impaired motility. Notably, colonic transit was only delayed after intense IM but not following gentle manipulation although the influx of leukocytes was comparable. Similarly, the upregulation of inflammatory cytokine levels after intestinal handling, both after intense and gentle IM, did not differ from L mice (data not shown), indicating that reduction in colonic motility does not result from disseminated inflammation. Based on the observation that delayed colonic transit is rather associated with increased systemic levels of pro-inflammatory cytokines, we speculate that impaired colonic motility rather results from the known inhibitory effects of pro-inflammatory cytokines on smooth muscle function. Pro-inflammatory cytokines such as TNF-α, IL-1β, KC, and MCP-1 may affect directly enteric neural coordination of motility or intestinal muscle contractility [15-19]. In addition, we showed that increased plasma levels of inflammatory cytokines, only observed following intense IM, activate the AP and NTS [17,18]. This activation may subsequently trigger enteric inhibitory pathways, and thereby further contribute to more severe POI [2,6,31-33][36].

In conclusion, our findings indicate that more severe upregulation of pro-inflammatory
cytokines, in response to increased tissue damage, with ‘leakage’ of proinflammatory cytokines into the systemic circulation significantly contribute to the severity of POI. Our observations may aid in the development of strategies to selectively block this response and reduce the severity of ileus. In addition, more insight into how tissue damage triggers the release of systemic cytokines may also lead to therapeutics to prevent this response.

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### Supplementary table 1. Primer sequences for qRT-PCR.

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</tbody>
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