Postoperative ileus: Pathophysiology & treatment strategies
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Chapter 3

Systemic inflammation and enhanced brain activation contribute to more severe delay in postoperative ileus

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Submitted
Abstract

Objective: To investigate the mechanism behind severe postoperative ileus (POI) by studying the local and systemic inflammatory response, including brain stem activation after different intensities of intestinal handling.

Summary Background Data: The severity of 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: Mice were subjected to gentle manipulation of the small intestine (gentle IM), more intense manipulation (intense IM), or only laparotomy. Postoperative intestinal transit, local and systemic inflammatory response, I-FABP (a marker for tissue damage) levels and brain activation were determined. Finally, in humans the duration of POI, plasma levels of I-FABP and inflammatory cytokines after open and laparoscopic segmental colectomy were measured.

Results: Intense IM induced a more pronounced ileus compared to gentle IM ($P=0.0001$). No difference in leukocytic infiltrates in the handled and non-handled parts of the gut was observed between the two IM procedures. However, intense IM resulted in significantly more severe tissue damage and was accompanied by a systemic inflammation with increased plasma levels of pro-inflammatory cytokines. In addition, intense but not gentle IM triggered enhanced c-Fos expression in the nucleus of the solitary tract and area postrema ($P=0.0014$). In patients, plasma levels of I-FABP and inflammatory cytokines were significantly higher after open compared to laparoscopic surgery, and were associated with more severe POI.

Conclusions: Not the influx of leukocytes, but rather the manipulation-induced tissue 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 3 hours onwards and further increasing up to 24 hours after surgery. Of note, the number of infiltrating leukocytes increased with the severity of intestinal manipulation with compression of the intestine yielding more influx than running along the intestine with cotton swaps.\(^5\) 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 24 hours 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 grams 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. The latter will consequently affect distant regions of the gut and contribute to the generalized aspect of POI.

In the present study, therefore, we investigated the mechanism behind severe POI by studying the local and systemic inflammatory response, including brain stem activation after different intensities of intestinal handling.

**Materials 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°C–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**
Anaesthesia was performed by an intraperitoneal (i.p.) injection of a mixture of Ketamine (Ketalar 100 mg/kg) and Xylazine (Rompun 10 mg/kg). Mice (5–8 per group) underwent a laparotomy alone, or a laparotomy followed by small intestinal manipulation (IM). 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 grams 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 abroad as previously described. 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 3 hours in a heated (32 °C) recovery cage.

**Gastrointestinal transit measurements**

Gastrointestinal function 24 hours postoperatively was determined in vivo by measurement of gastrointestinal transit of liquid non-absorbable fluorescein isothiocyanate–dextran (FITC-dextran) (70,000 Da; Invitrogen, Paisley, UK). 10µl of FITC-dextran dissolved in 0.9% saline (6.25 mg/mL) 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 (3 segments of equal length) were collected and assayed in duplicate (Synergy HT, BioTek Instruments Inc., VT, USA; excitation wavelength: 485 nm, emission wavelength: 528 nm) for quantification of the fluorescent signal in each bowel segment. The distribution of the fluorescent label along the gastrointestinal tract was determined by calculating the geometric center (GC): \( \Sigma (\% \text{ of total fluorescent signal in each segment} \times \text{the segment number})/100 \) for quantitative statistical comparison among experimental groups.13

**Colonic transit**

Colon function was determined in vivo by 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, post-fixed for 4 h (4 °C) and cryo-protected by immersion in 30% sucrose in 0.2M PBS (pH 7.4) at 4 °C overnight. Coronal sections of 30 μm of the brainstem were collected. After rinsing in 0.05 M Tris-buffered saline TBS (pH 7.4), sections were incubated overnight at 4°C with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies. Then, sections were incubated 1 hour with biotinylated secondary antibody and after with avidin-biotin complex (ABC, Vector, Burlingame, USA) for 1 hour. The reaction product was
visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulphate and 0.01% hydrogen peroxide H₂O₂ for 5min. 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 colour camera (Sony Corp., Tokyo, Japan). A minimum of 7 sections was used for c-Fos counting in the NTS (from Bregma -7.20 mm to -7.76 mm) and Area Postrema (Bregma -7.32 mm to -7.76 mm), and 9-11 sections for PVN (Bregma -0.58 mm to -1.22 mm).

**Immunohistochemistry**

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 minutes, 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 minutes. 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 µm x 891.2 µm) was counted and the average was calculated.

**Blood analysis: tissue damage & plasma levels of inflammatory cytokines**

Mice: tissue damage was assessed by determining plasma levels of Intestinal fatty acid-binding protein (I-FABP). 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, Interleukin (IL)-8, Monocyte Chemoattractant Protein-1 (MCP-1), Tumour Necrosis Factor (TNF-α ) and Interleukin (IL)-1β plasma levels of venous blood retrieved 1, 6 and 24 hours 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, Erembodegem, Belgium). Cytometric bead assay results were analyzed using the FCAP Array™ software (BD Biosciences, Erembodegem, Belgium).

Patients: IL-6, IL-8, MCP-1, TNF-α and IL-1β plasma levels of venous blood retrieved 2 hours 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, Erembodegem, Belgium). Flow cytometric analysis was performed using a FACSArray flow
cytometer (BD Biosciences, Erembodegem, Belgium). Cytometric bead assay results were analyzed using the FCAP Array™ software (BD Biosciences, Erembodegem, Belgium). 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 23000 MW sample concentrators (GE Healthcare) by centrifuging at 3506 X g at 4 °C for 2 hours and further processed according to the manufacturer’s instructions. Levels of I-FABP in the concentrated plasma were determined using ELISA human I-FABP (Hycult Biotechnology (Hbt), Uden, the Netherlands).

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). RNA extraction was performed using RNeasy Mini Kit (Qiagen # 74104) according to manufacturer’s instructions. Total of RNA were transcribed into complementary cDNA by qScript cDNA SuperMix (Quanta Biosciences) according to manufacturer’s instructions. Quantitative real-time transcription polymerase chain reaction (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 supplementary table 1.

Statistical Analyses
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 analysis of variance (ANOVA) followed by Tukey’s Multiple Comparison analysis and are presented as means ± s.e.m. A probability level of P less than 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 intestinal manipulation (IM) compared to laparotomy (GC: 8.9 ± 0.7). Notably, intense IM (GC: 3.8 ± 0.2) induced a more severe delay in intestinal transit compared to gentle IM (GC: 6.3 ± 0.8) (Fig. 1A). Colonic transit did not differ significantly between laparotomy and gentle IM, but was significantly delayed after intense IM compared to laparotomy (Fig. 1B).

![Figure 1](image)

**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 (Panel A), or colonic transit time of a 2-mm plastic ball (Panel B) after intestinal manipulation (IM) or sham operation (laparotomy) at 24 hours after surgery. Results are representative of three independent experiment in groups of 7-8 mice and data are means ± s.e.m.. * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.

**Intense handling leads to enhanced inflammation in the manipulated intestine**

In the small intestine, IM but not laparotomy 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 24 hours after surgery (Fig. 2). In addition, IM resulted in the upregulation of the pro-inflammatory cytokines IL-6, IL-1β and TNFα at 6 and 24 hours compared to laparotomy. Importantly, intense manipulation induced significantly more upregulation of IL-6 and TNFα as compared to gentle manipulation (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 means ± s.e.m. * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.

In the non-handled colon leukocyte infiltration was significantly increased after IM compared to laparotomy. 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 TNFα mRNA levels were not significantly different after IM compared to laparotomy, indicating that there was no detectable inflammatory response (Fig. 3). Also 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 TNFα (Fig. 3).
Figure 3 | Expression of inflammatory cytokines in different parts of the gastrointestinal tract.
Quantitative PCR for IL-6 (Panel A), IL1β (Panel B) and TNFα (Panel 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 means ± s.e.m; * P < 0.05, one-way ANOVA followed by Tukey’s Multiple Comparison analysis.

Intense IM results in tissue damage with release of 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 24 hours, 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 24 hours 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 laparotomy, whereas manipulation of the intestine did not result in enhanced plasma levels of IL-1β and TNFα (data not shown for TNFα).
Figure 4 | Tissue damage and plasma levels of inflammatory cytokines after different intensities of surgical manipulation.

Plasma I-FABP (marker for tissue damage) levels 1 hour after surgery (Panel A). Plasma levels of IL-6 (Panel B), KC (CXCL-1) (Panel C), IL-1β (Panel D) and MCP -1 (Panel E) were determined in blood retrieved at 1, 6 and 24 hours after surgery. Results are representative of three independent experiments in groups of 6-8 mice and data are means ± s.e.m; * P < 0.05, one-way ANOVA followed by Tukey's Multiple Comparison analysis.

Intense IM induced tissue damage is associated with area postrema activation

Previous studies have reported activation of brain areas following abdominal surgery, a mechanism that was proposed to contribute to the development of POI.15 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 24 hours after surgery. The neurons of the area postrema, which is exposed to systemic circulation, relay their signal to the nucleus of the solitary tract (NTS) and can thereby result in an enhanced activation of the NTS.16, 17 The number of c-Fos positive neurons in the area postrema and NTS was significantly higher after intense compared to 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 area postrema with plasma I-FABP levels (Spearman's \( \rho \) 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 area postrema (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 (Panel A). Panel B correspond to c-Fos expression in the Nucleus of the solitary tract (NTS). Panel C shows c-Fos expression in the area postrema (AP). Data are expressed as mean ± s.e.m. 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 \( \rho \) correlation coefficient 0.65 (95% CI: 0.30 - 0.85; \( P = 0.0013 \) (Panel D).

\* \( P < 0.05 \), one-way ANOVA followed by Tukey's Multiple Comparison analysis.

**Recovery of GI function, tissue damage and systemic inflammatory cytokines in humans after different intensities of surgical handling**

In patients undergoing elective intestinal surgery, plasma levels of I-FABP and the inflammatory cytokines IL-6, IL-1\( \beta \), MCP-1 and IL-8 were significantly higher after open compared to 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: 72 hours after laparoscopic vs 96 hours after open colonic surgery (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.
Discussion

Inflammation of the intestinal muscularis is abundantly demonstrated to underlie POI. Here we demonstrated that not the number of infiltrating leukocytes, but 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 area postrema 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. Incremental degrees of manipulation of the small intestine cause a progressive increase in leukocyte infiltration. 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. 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. Graber et al. subjected 6 monkeys to 3 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. However, years later Uemura et al. showed in rats that the magnitude of the abdominal incision does affect the duration of POI. We previously demonstrated that only externalization of the intestine outside the abdominal cavity already induced a significant influx of leukocytes without resulting in POI. Also in the present study, no significant difference in leukocyte recruitment was observed in the small intestinal muscularis following intense IM compared to 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 IL-6 in serum of patients undergoing open rectal procedures compared to patients undergoing a laparoscopic procedure. In line, several studies have reported an increased postoperative inflammatory response related to increased operative trauma. 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. In the present study, we indeed recorded higher levels of I-FABP, a marker for tissue damage, both in mice and patients undergoing more severe intestinal handling. 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 TNFα into the general circulation. These cytokines result in enhanced activation of the hypothalamus triggering hypothalamic-pituitary-adrenal activity. 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. 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 24 hours 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.

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. 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. In previous experiments, however, we were unable to demonstrate increased levels of IL-12. Moreover, we showed that RAG1-/- mice, devoid of T cells, developed POI to the same extent as wild type mice, suggesting that other mechanisms must be involved. In the present 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 laparotomy 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 TNFα, IL-1β, KC and MCP-1 may affect directly enteric neural coordination of motility or intestinal muscle contractility. In addition, we showed that increased plasma levels of inflammatory cytokines, only observed following intense IM, activate the area
postrema and NTS.\textsuperscript{16, 17} This activation may subsequently trigger inhibitory neural motor pathways affecting distant regions of the gut with enhanced sympathetic inhibition of intestinal motility, and thereby further contribute to more severe POI.\textsuperscript{6, 26-28, 31}

In conclusion, 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. Our observations indicate that identification of the host cell(s) that initiate release of inflammatory cytokines into the circulation 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 Material**

**Supplementary table 1 |** Primer sequences for qRT-PCR.

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