Postoperative ileus: Pathophysiology & treatment strategies
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Chapter 4

Mast cells trigger epithelial barrier dysfunction, bacterial translocation and postoperative ileus in a mouse model


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Abstract

Background: Abdominal surgery involving bowel manipulation commonly results in inflammation of the bowel wall, which leads to impaired intestinal motility and postoperative ileus (POI). Mast cells have shown to play a key role in the pathogenesis of POI in mouse models and human studies. We studied whether mast cells contribute to the pathogenesis of POI by eliciting intestinal barrier dysfunction.

Methods: C57BL/6 mice, and two mast cell deficient mutant mice Kit<sup>W/W-v</sup>, and Kit<sup>W-sh/W-sh</sup> underwent laparotomy (L) or manipulation of the small bowel (IM). Postoperative inflammatory infiltrates and cytokine production were assessed. Epithelial barrier function was determined in Ussing chambers, by measuring transport of luminal particles to the vena mesenterica and by assessing bacterial translocation.

Results: In WT mice IM resulted in pro-inflammatory cytokine and chemokine production, and neutrophil extravasation to the manipulated bowel wall. This response to IM was reduced in mast cell deficient mice. IM caused epithelial barrier dysfunction in WT mice but not in the two mast cell deficient strains. IM resulted in a decrease in mean arterial pressure in both WT as well as mast cell deficient mice, indicating that impaired barrier function was not likely explained by tissue hypoperfusion but involved mast cell mediators.

Conclusions: mast cell activation during abdominal surgery causes epithelial barrier dysfunction and inflammation of the muscularis externa of the bowel. The impairment of the epithelial barrier likely contributes to the pathogenesis of POI. Our data further underscore that mast cells are bona fide cellular targets to ameliorate POI.
Introduction

Postoperative ileus (POI) is characterized by a transient cessation of intestinal motor activity following abdominal surgery, and as a result, patients suffer from complications and prolonged hospital stay \(^1, 2\). The costs related to POI have been estimated to amount 1.47 billion dollars annually in the USA, illustrating its large socio-economical impact \(^1\). Regarding the pathogenesis of POI, it has become evident from animal and human studies that postoperative intestinal hypomotility in POI is the result of an influx of leukocytes into the manipulated muscularis externa \(^3, 4\). Neutrophil infiltrates have been shown to inhibit local contractile activity, i.e. via the release of nitric oxide (NO) \(^5-7\), or general motility via the activation of sympathetic inhibitory neural reflexes \(^8\). The importance of this inflammatory response in POI is underscored for instance by the success of therapeutic strategies aimed at blocking neutrophil recruitment to ameliorate POI \(^1, 3, 9\). However, the pathophysiological mechanisms behind the immune response to bowel manipulation remain to be clarified. In this respect, an important factor could be the reduced epithelial barrier function resulting from bowel handling that was previously observed in rodent models of POI \(^5, 10\). This would be in line with previous observations that bowel wall mechanical stretch \(^11\) and manipulation \(^12\) augments inflammatory responses of bowel wall macrophage populations and local dys-contractility via TLR activation.

We have previously shown that mast cells are crucial players in the intestinal inflammation that mediates POI \(^13\) and that mast cell stabilizers and histamine receptor antagonists are instrumental in reducing POI in animal models \(^13\) and human POI \(^4, 14\). Mast cells are implicated in barrier dysfunction in animal models of chronic stress \(^15, 16\), allergic inflammation \(^17\), parasitic infection \(^18\) and endoxemia \(^19\). Thus, given the implication of mast cells in the pathogenesis of POI, and their potential to regulate intestinal barrier function, we assessed the role of mast cell-induced barrier dysfunction in the occurrence of POI by using two mast cell deficient mouse strains, Kit\(^{W/W-v}\) and Kit\(^{W-sh/W-sh}\). Here, we show that IM during abdominal surgery is associated with intestinal barrier dysfunction and inflammation of the manipulated bowel muscularis externa. Our data indicate that both inflammation and barrier dysfunction are mediated by mast cells and can be considered as factors contributing to POI pathogenesis.

Materials and Methods

Laboratory animals
Mice (C57BL/6, Harlan Nederland, Horst, The Netherlands) were kept under environmentally controlled conditions (light on from 8 AM to 8 PM; water and rodent
nonpurified diet ad libitum; 20°C–22°C, 55% humidity). Mast cell-deficient Kit<sup>W/W-v</sup> (WBB6F1-W/W<sup>v</sup>), the mast cell-sufficient Kit controls (Kit<sup>+/+</sup>) and Kit<sup>W-sh/W-sh</sup> (B6.Cg-KitW-sh/HNihrJaEBsmJ) and their C57BL/6 controls were from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained at the animal facility of the Academic Medical Centre in Amsterdam and were used at 12–20 weeks of age. Animal experiments were performed in accordance with the guidelines of the Ethical Animal Research Committee of the University of Amsterdam.

Surgical procedures: abdominal surgery with intestinal manipulation
Mice were anesthetized by an intraperitoneal (IP) injection of a mixture of fentanyl citrate/fluanisone (Hypnorm; Janssen, Beerse, Belgium) and midazolam (Dormicum; Roche, Mijdrecht, The Netherlands). Surgery was performed under sterile conditions. Mice (8–11 per treatment group) underwent control surgery of only laparotomy (L) or laparotomy followed by intestinal manipulation (IM) as described earlier 13. After 24h mice were anesthetized and killed by cervical dislocation, mesenteric lymph nodes were harvested under aseptic conditions, subsequently the small intestine was removed, flushed in ice-cold saline, divided into several segments and stored for further analysis.

FACS analyses
Mesenteric lymph nodes (MLN) were isolated at indicated time points after surgery and cleared from fat. Tissue was digested for 15 minutes using collagenase IV and cell suspension was obtained after filtering and cells were resuspended, washed, and taken up in RPMI medium/10% FCS and incubated for 3h in BrefeldinA. Cells were then washed and resuspended in FACS buffer, and incubated with the appropriate antibodies. Cells were fixed with 2% PFA; for intracellular FACS, cells were treated with 0.5% saponin in FACS buffer.

Bacterial Translocation
Mesenteric lymph nodes (MLN) were weighed, placed in a tube containing 300 μL of ice-cold Luria-Bertani (LB) broth, homogenized with a sterile grinder, and plated onto blood agar plates under aerobic and anaerobic conditions. After 48 hours of incubation at 37°C, the number of colony forming units (CFU) per milligram lymph node was assessed.

Ussing Chamber Experiments
Segments of tissue of the distal small intestine were opened, cut, and immersed in Modified Meyler’s Buffer (128 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl<sub>2</sub>·6H<sub>2</sub>O, 20.2 mM NaHCO<sub>3</sub>, 0.4 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.33mmol/L Na<sub>2</sub>HPO<sub>4</sub>, and 1.0 MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 mM Hepes, 10 mM glucose at pH 7.4). Ileal segments were removed 45 minutes after surgery and 15
minutes thereafter the tissue was mounted in Ussing chambers (World Precision Instruments, Berlin, Germany). Serosal and mucosal areas were exposed to 2 mL of circulating oxygenated Modified Meyler Buffer maintained at 37°C. After 15 minutes, horseradish peroxidase (HRP, Sigma-Aldrich) was added to the luminal buffer at a final concentration of 10 μM. After 30, 60 and 90 minutes, samples (300 μl) were taken from the serosal side and replaced with fresh buffer. The enzymatic activity of HRP was measured using o-Phenylenediamine dihydrochloride (Sigma-Aldrich) as a substrate. The transepithelial flux of HRP was represented as pMol/h/cm².

In vivo intestinal permeability measurement
Mice (female C57BL/6 and KitW-sh/W-sh) were anaesthetized using mixture of fentanyl citrate/fluanisone (Hypnorm; Janssen) and midazolam (Dormicum; Janssen). Abdominal surgery with intestinal manipulation was performed as described 8. Mice (n = 5-7 per group) were assigned to the following two groups: laparotomy only (sham); laparotomy followed by intestinal manipulation (IM). At 1 hour after surgery, the mice were re-anaesthetised with Isoflurane, 1.2 – 2.5 % vol (Abott Laboratories, Kent, UK) and cannulation of the Superior Mesenteric Vein (SMV) was performed under sterile conditions. After ligating the ileum 5 cm from the ileocecal valve and 5 cm proximal from the initial ligation, 0.5 mL of saline solution containing fluorescent probe (Fitc-Dextran (3-5 kD, Sigma-Aldrich); Rhodamine Dextran (10 kD, Invitrogen, Carlsbad, CA); in saline solution) was gently injected into the lumen. Blood samples (with concomitant fluid replacement) were drawn at intervals of 10 mins until one hour from cannulation using Lithium heparin filled tubes (Greiner bio-one).

Antibiotic treatment
Gut decontamination was performed by administration of polymyxin B (75 mg/kg/d) and neomycin (225 mg/kg/d) as described earlier (turler et al 2007). The antibiotics were instilled into the stomach by a gastric tube for 6 days, once daily. Control animals received vehicle (normal saline) at appropriate time points. Mice underwent surgery with intestinal manipulation as described above on the sixth day of treatment and were killed on day 7.

Measurement of Mean Arterial Pressure
Mean arterial pressure (MAP) was recorded as previously reported 20. In short, after induction of anesthesia, the carotid artery was cannulated and blood pressure and heart rate were recorded using a heparinized saline-filled catheter. The catheter was connected to a pressure transducer (Truwave PX-600F; Baxter, Deerfield, IL, USA) and signals samples and stored using Labview applications (National Instruments, Austin, TX,
USA). Rectal temperature was monitored continuously and remained at 37°C throughout the experiment. Sodium Nitroprusside treatment: after being anesthetized using FFM, mice were injected with 50 µg/kg Sodium nitroprusside (SNP; Sigma-Aldrich) i.p. using an automatic infusion pump. The infusion rate was adjusted whenever necessary in order to maintain the desired MAP drop for the time indicated.

**Gastrointestinal transit**

Gastrointestinal function was determined in vivo by measurement of gastrointestinal transit of liquid FITC-dextran (70,000 Da; Invitrogen, Paisley, UK). Ninety minutes after administration through oral gavage, the animal was killed 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), the cecum, and colon (3 segments of equal length) were collected and assayed in duplicate for the presence of fluorescent label (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 signal along the gastrointestinal tract was determined by calculating the geometric centre (GC): Σ (percent of total fluorescent signal in each segment X the segment number)/100 for quantitative statistical comparison among experimental groups. Individual transit distribution histograms were plotted, and transits were statistically analyzed using the calculated geometric centre (GC).

**Mast cell culture and Reconstitution of KitW-sh/W-sh mice**

KitW-sh/W-sh mice were reconstituted by the injection of bone marrow–derived cultured mast cells into the peritoneal cavity, as described earlier. Sterile, endotoxin-free medium was flushed repeatedly through the bone shaft using a needle and syringe. The suspension of BM cells was centrifuged at 320 g for 10 min and cultured at a concentration of 0.5 × 10^6 nucleated cells/ml in RPMI 1640 with 10% FCS (Sigma-Aldrich) 100 units/ml penicillin, 100 µg/ml streptomycin (Life Technology, Breda, The Netherlands), 10 µg/ml gentamycine, 2 mM L-glutamine, and 0.1 mM nonessential amino acids (referred to as enriched medium) and a combination of IL-3 (5 ng/ml) and SCF (50 ng/ml) for 3 weeks at 37°C in a humidified atmosphere with 5% CO2. Nonadherent cells were transferred to fresh medium at least once a week. After 3–4 weeks when a mast cell purity of ≥ 95% was achieved, as assessed by toluidine blue staining, the cells were harvested for experiments. Three weeks old KitW-sh/W-sh received 10 × 10^5 cells in 100 µL PBS through IP injection. Mice were used 10 weeks after adoptive transfer of mast cells. This procedure reconstitutes the mast cell population without systemic effects.
Immunohistochemistry and visualization of myeloperoxidase positive cells

Immunohistochemical staining for CD11a and CD3 was performed on acetone fixed transverse ileal segments. Endogenous peroxidase activity was eliminated by incubation of segments in methanol containing 0.3% H₂O₂. Nonspecific protein-binding sites were blocked by incubation in PBS, pH 7.4, containing 10% of normal goat serum for 10 min. Sections were incubated overnight with biotinylated hamster anti-mouse CD11a or CD3 antibodies (Pharmingen, San Diego, CA, U.S.A.) (dilution 1 : 1000). Next, sections were incubated with ABComplex/HRP (DAKOCytomation, Glostrup, Denmark) for 30 min. HRP was visualised using SigmaFast DAB (Sigma-Aldrich), incubating 5 min, and counterstained with haematoxilin/eosin. Visualization and quantification of myeloperoxidase (MPO) positive cells in the ileal muscularis externa was performed as described elsewhere ²¹.

Tissue cytokine production

For cytokine measurements, mucosa was separated from the muscle tissue using a glass slide, in icecold modified Meyler’s buffer. Six cm segments were added to 500 µl lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100; pepstatin A, leupeptin, and aprotinin (all 20 ng mL⁻¹; pH 7.4), homogenized, and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 min, and supernatants were stored at -20°C until assays were performed. TNF, IL-6, MCP-1, RANTES, IL-12p70 and KC in supernatants were analyzed by mouse ELISA (R&D systems, Abingdon, United Kingdom) according to manufacturer’s instructions.

Statistical analysis

The data are expressed as mean ± SEM and were analyzed using the nonparametric Mann-Whitney U test. A P value less than 0.05 was considered significant. IM surgery groups were compared to L control groups where indicated.

Results

Intestinal manipulation results in an immune response localized at the muscularis externa

We first established in a mouse model for POI that IM resulted in the activation and infiltration of CD11a-positive leukocytes in the muscularis externa of the small intestine. The intestinal muscularis is known to contain a network of resident macrophage-like cells of which a proportion is F4/80 positive (Fig. 1A) ²². The activation of this cell population has been implicated in the leukocyte recruitment in POI ³, ¹³ leading to an extravasation of CD11a expressing cells that appeared in muscularis

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externa of the small intestine 24h after IM (Fig. 1A and B). The population of CD11a positive cells is negative for CD3 as shown in consecutive sections (Fig. 1B) and comprises extravasated monocytes and neutrophils, as demonstrated earlier. We further assessed the IM induced cytokine production 1h, 3h, 6h and 24h after IM in the muscular layer and mucosa. IL-6 production and signaling is an important characteristic of POI induced inflammation so we choose to measure IL-6 along with chemokines MCP-1 (CCL-2) and KC (the mouse homologue of IL-8) as key parameters of localized inflammation. In 3- to 24 hours after IM we observed a significant increased production of the cytokines CCL-2, IL-6, and KC in the mice that had undergone IM, but not in control L mice (Fig. 1C, left panels); levels of IL-12p70 and TNF were not significantly induced within this time frame. In conjunction with previous reports, the IM procedure did not lead to significant cytokine production in the small intestinal mucosa (Fig. 1C, right panels). Notably, RANTES was constitutively expressed in the mucosa and unaffected by IM (Fig. 1C).

In a recent study intestinal surgery and manipulation was reported to elicit DC derived IL-12 production within 30 minutes after surgery, initiating a Th1 response leading to POI. However in our model, enhanced production of IL-12 at earlier time points after IM in the muscularis tissue was not apparent and IL-12 only increased in muscle tissue after 24 hours. (Fig 1C), i.e. at a time point were an inflammatory infiltrate was already established.

**IM induced inflammation is dependent on the presence of mast cells**

We have previously implicated mast cells in the pathogenesis of POI. Hence, we sought to establish that mast cell activation is involved in the muscularis inflammatory response and POI after intestinal surgery. Upon activation, connective tissue mast cells release chymases such as mouse mast cell protease 1 (mMCP-1). MMCP-1 release in peritoneal lavage fluid was increased one hour after intestinal manipulation compared to L (Fig. 2A). In C57BL/6 mice, mast cells were found in the muscularis externa, Peyer’s Patches and mesentery (Fig 2A). Next, we investigated the role of mast cells in the leukocyte recruitment to the ileum muscularis externa observed after IM. To this end we studied the response to IM in 2 mast cell deficient mice strains that carry different spontaneous mutations in the gene for ckit (White spotting (W) locus) KitW/W-v and KitW-sh/W-sh mice. Reduced c-kit tyrosine kinase-dependent signaling results in mast cell reduction in both mouse strains. In KitW/W-v mice, a reduced inflammatory infiltrate was observed after IM if compared to their mast cell sufficient Kit+/+ cognate controls (Fig
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Figure 1 | IM induced inflammation is localized to the muscularis externa. In animals that underwent laparotomy (L), CD11a-expression, indicating leukocyte activation, is absent from the muscularis externa (A, left panel). Resident macrophages are present in the muscularis externa of unmanipulated mice, as indicated by F4/80 staining (A, right panel). After intestinal manipulation (IM), CD11a expression in the muscularis externa was induced (B, right panel). T-cells (CD3+) do not infiltrate the muscularis externa 24h after IM (B, right panel). IM induced production of cytokines and chemokines in muscularis externa homogenates and in mucosa homogenates is shown at indicated time points after surgery (C). RANTES protein expression was unaltered and constitutively expressed in the small intestinal mucosa (C); * P < 0.05, ** P < 0.01 (compared to the respective L-groups). Bars indicate mean ± SEM. (n = 6 per group).

2B). In addition, the production of cytokines MCP-1 and IL-6 depends on the appropriate development of mast cells as both cytokines are significantly reduced in Kit<sup>W/W<sup>v>v</sup></sup> mice (Fig. 2B). These data were corroborated by a reduced cellular MPO positive infiltrate, and reduced levels of IL-6 and MCP-1 after IM in mice that were treated with mast cell stabilizers ketotifen or doxantrazole (13 and data not shown).

In addition to a mast cell deficiency, in Kit<sup>W/W<sup>v>v</sup></sup> mice haematopoiesis is affected, they are anaemic and infertile26, 27. Therefore we additionally analyzed the alternative c-
kit mutant Kit\(^{W-sh/W-sh}\) and the respective WT strain C57Bl/6, that comprise a more selective mast cell deficient phenotype compared to the Kit\(^{W/W-v}\) strain.\(^{27}\)

In contrast to C57BL/6 controls, IM in the Kit\(^{W-sh/W-sh}\) mice did not lead to a significant increase in MPO positive cells compared to controls (Fig. 2C). The production of IL-6 and CCL-2 in their muscular compartment after IM surgery was significantly reduced compared to their C57BL/6 controls (Fig. 2D). To demonstrate that the observed phenotype was mast cell dependent, we reconstituted Kit\(^{W-sh/W-sh}\) mice with mast cells (MC) obtained from C57BL/6 bone marrow derived mast cell cultures, and measured the cytokine response to IM. Success of reconstitution was demonstrated by presence of mast cells in Kit\(^{W-sh/W-sh}\) ileum (see Fig 3C). From these analyses we concluded that the number, or tissue location, of ileal mast cells found in mast cell reconstituted Kit\(^{W-sh/W-sh}\) mice was not different from those found in C57Bl/6 mice (Fig 3C). In addition, earlier studies have shown that this treatment allows reconstitution to functionally active mast cells.\(^ {13, 27}\) We next assessed whether mast cell reconstitution restores the inflammatory response to IM. In contrast to vehicle treated Kit\(^{W-sh/W-sh}\) mice, in Kit\(^{W-sh/W-sh}\) mice that were reconstituted with C57Bl/6 cultured mast cells the production of CCL-2 after IM was significantly increased, and was comparable to C57BL/6 WT levels. IM-induced IL-6 (Fig. 2D) was unaffected by reconstitution (Fig. 2D), indicating that mast cells do not play a role in the induction of IL-6 in the model.

**IM induced delay in gastrointestinal transit is dependent on the presence of mast cells**

We next examined the role of mast cells in POI by analyzing the functional changes in gastrointestinal transit induced by IM. We choose for Kit\(^{W-sh/W-sh}\) mouse strain to study the effect of mast cell activation on motility because the use of Kit\(^{W-sh/W-sh}\) mice allows the motility parameters to be compared to C57BL/6 mice as these mice are derived on a C57BL/6 background. In the L control group, 24h after surgery, gastrointestinal transit in Kit\(^{W-sh/W-sh}\) mice was delayed compared to C57BL/6 control mice. The majority of the fluorescent marker in Kit\(^{W-sh/W-sh}\) mice is present in segment (sg) 7 and sg8, whereas the marker in the C57BL/6 mice is mainly present in sg9, sg10, and the cecum (Fig. 3A, left panel). This is reflected in the average calculated geometric centre (GC) (Fig. 3B). After IM, in C57BL/6 mice, the gastrointestinal transit is
Figure 2 | Mast cells are crucial in muscularis externa inflammation following bowel handling. Mouse mast cell protease (mMCP-1), determined in peritoneal lavage 1h after surgery, was increased after intestinal manipulation (IM) compared to laparotomy (L) (A). Mast cells (purple) were visualized in C57BL/6 mice with Toluidin Blue staining and were present muscularis externa, serosa, Peyer’s patches and the mesentery (A). The number of myeloperoxidase (MPO) positive cells per mm² and production of proinflammatory cytokines MCP-1 and IL-6 in the muscularis externa was significantly reduced in mast cell deficient Kit<sup>W/W<sub>v</sub></sup> compared to control mice (Kit<sup>+/+</sup>) (B). In Kit<sup>W-sh/W-sh</sup> mice, the number of MPO+ cells was not increased after IM compared to control (C57BL/6). Similarly, in Kit<sup>W-sh/W-sh</sup> reconstituted with C57BL/6 bone marrow derived mast cells, the number of MPO positive cells as unaffected after IM (C). CCL-2, and IL-6 production in Kit<sup>W-sh/W-sh</sup> and C57BL/6 and Kit<sup>W-sh/W-sh</sup> reconstituted with bone marrow derived mast cells (D).

Asterisks indicate significant differences where indicated; ** P < 0.01, * P < 0.05. Bars indicate mean ± SEM, for all groups L: n = 5-7 IM: n = 10 mice per group.
significantly delayed as the fluorescence marker is mainly present in sg4 and sg5 (Fig. 3A, middle panel), also shown by the GC (Fig. 3B). However, the Kit^{W-sh/W-sh} mice display only a minor delay in gastrointestinal transit after IM (Fig. 3A, middle panel), clearly reflected in the GC values shown in Fig. 4B. After Kit^{W-sh/W-sh} mice were reconstituted with WT mast cells, the delay in gastrointestinal transit partially returned, indicated by the more widespread distribution of the fluorescent marker, in sg 2, 3, 5 and also 10 (Fig 3A, right panel). The IM induced delay is also reflected in decreased GC values (Fig 3B).

**Figure 3 |** Role of mast cells in gastrointestinal transit after intestinal manipulation. Twenty four hours after laparotomy (L) or intestinal manipulation (IM), gastrointestinal transit was determined by the % distribution of the fluorescent marker and calculation of the geometric centre (GC) as indicated in materials and methods. St=stomach, cc=cecum. The fluorescent marker (dextran-FITC) in the L groups is located more proximally in the Kit^{W-sh/W-sh} than in the C57BL/6 mice as depicted in (A, left panel). After IM, dextran is localized more proximally in C57BL/6 mice than in Kit^{W-sh/W-sh} mice (A, middle panel). IM induced delay in gastrointestinal transit is partially rescued by reconstitution of Kit^{W-sh/W-sh} with WT mast cells (A, right panel). GC values are shown in (B). * P < 0.05 (compared to the respective L group). Bars indicate mean ± SEM. (L: n = 4, IM: n = 4-7 mice per group). Panel C: Mast cell were visualized using toluidin blue staining in Kit^{W-sh/W-sh} mice (upper panel), or Kit^{W-sh/W-sh} mice that were reconstituted with C57BL/6 cultured mast cells (lower panel). D) the quantification of the number of mast cells recovered in the ileum after reconstitution. N=4 mice per treatment group. ND is not detectable.
A significant decrease in GC value was observed after IM in control C57BL/6 mice, but not in Kit\textsuperscript{W-sh/W-sh}. Although mast cell reconstitution in these mice partially protects from POI as judged by the transit data (Fig. 3A), the GC values were unaffected and were not reduced as seen in C57BL/6 WT mice.

**IM induces mast cell dependent bacterial translocation to MLN**

Given the implication of mast cells in epithelial barrier dysfunction in other models, we next assessed whether mast cells may contribute to the pathogenesis of POI by affecting the epithelial barrier function. To this end we analyzed barrier function after IM in 3 ways: by determining postoperative bacterial translocation to draining mesenteric lymph nodes (MLN), by measuring permeability to HRP in Ussing chambers, and by assessing postoperative particle leakage from the small intestinal lumen into the blood circulation. We first assessed whether IM led to bacterial translocation 24h after surgery by assessing the number of Colony forming units (CFU) in the MLN. In C57BL/6 mice that had undergone IM, the number of bacteria that had translocated to the MLN was significantly increased as compared to the L control group (Fig. 4A). The numbers of CFU cultured from MLN under aerobic and anaerobic culture conditions were comparable and therefore, in subsequent experiments we only cultured bacteria under aerobic conditions. We next assessed whether mast cells play a causal role in the impaired intestinal barrier function after IM. To this end we measured bacterial translocation in mast cell deficient Kit\textsuperscript{W/W-v} mice. Baseline and IM induced bacterial translocation did not differ in Kit\textsuperscript{W/W-v} mice, but this was not a characteristic of the lack of mast cells per se as the number of CFU was also not different in the Kit\textsuperscript{+/+} control mice (Fig. 4B). In the Kit\textsuperscript{W-sh/W-sh} mice the IM induced bacterial translocation was not increased after IM as compared to C57BL/6 control mice, although it should be noted that the actual number of translocating bacteria is generally low in these mice (Fig. 4C). Irrespectively, these data indicate that mast cells are likely to play a role in the bacterial translocation to the MLN after intestinal surgery.

**Intestinal manipulation leads to an impaired barrier function via mast cell activity**

The number of viable bacteria cultured from the MLN is dependent on the integrity of the epithelium, but also largely depends on processes such as uptake, and killing capacity CX3CR1-positive dendritic cells and macrophages. To specify the effect of IM on barrier function we therefore decided to additionally assess epithelial permeability for protein directly after the IM surgery in Ussing chambers. We measured flux of HRP in ileum segments mounted 1h after L or IM. In the IM group, the mucosal to serosal flux of HRP was enhanced as compared to the L group (Fig.5A), reflecting an increase in epithelial permeability after IM. In contrast however, ileal permeability
Figure 4 | Role of mast cells in IM induced bacterial translocation. Bacterial translocation to the mesenteric lymph nodes (MLN) was determined 24h after bowel handling. Following intestinal manipulation (IM), translocation of both aerobic and anaerobic cultured bacteria was significantly increased in C57BL/6 mice as compared to laparotomy (L) controls (L: n = 5, IM: n = 10 mice per group) (A). Bacterial translocation was also increased after IM in Kit<sup>+/+</sup> mice and Kit<sup>W/W-v</sup> mice compared to the L groups but did not differ between the two groups (B). The number of CFU cultured from mesenteric lymph nodes of Kit<sup>W-sh/W-sh</sup> mice was not significantly different between L and IM groups (C). Kit<sup>W/W-v</sup> and Kit<sup>+/+</sup>: L: n = 5, IM: n = 10; Kit<sup>W-sh/W-sh</sup> and C57BL/6: L: n = 3, IM: n = 7 mice per group. Asterisks *P <0.05 (compared to L-group), bars indicate mean ± SEM.

measured in Ussing chambers was not significantly enhanced by IM in mast cell deficient mice Kit<sup>W-sh/W-sh</sup> (Fig 5A), Kit<sup>W/W-v</sup> and Kit<sup>+/+</sup> (Fig 5B). To further substantiate these data we additionally measured intestinal permeability for different sized luminal particles in vivo1h after IM surgery, i.e. at a time point at which no neutrophil recruitment was observed yet. To this end we assessed transport of luminal dextran particles to the draining vena mesenteric vein. We observed a significant increase in the concentration of dextrans of 3-5 kD (Fig 5C, left panel) as well as 10kD (Fig. 5C, right panel) in mesenteric venous blood 1-2hrs after IM, but not after L. As unaffected tight junctions
Figure 5 | Mast cells regulate the transepithelial flux of small particles after intestinal manipulation. Mucosal to serosal transfer of horse radish peroxidase (HRP) was determined in Ussing chambers 1h after surgery in ileal tissue of C57BL/6 and mast cell deficient Kit<sup>W-sh/W-sh</sup> and Kit<sup>W/W-v</sup>. The mucosal to serosal flux of HRP was increased after intestinal manipulation (IM) compared to laparotomy (L) (A) in C57BL/6, but not in the mast cell deficient Kit<sup>W-sh/W-sh</sup> (A) and Kit<sup>W/W-v</sup> (B) (n = 5 mice per group). In C57BL/6, the flux of 3-5 kD (C, left panel) and 10 kD (C, right panel) particles from the small intestinal lumen to mesenteric blood is shown at indicated timepoints 1 hour after IM (open circles) as compared to sham (closed circles). In Kit<sup>W-sh/W-sh</sup>, the IM induced increase in transepithelial flux particles was completely abolished as indicated in D (left panel: 3kD, right panel: 10 kD). Asterisks *P < 0.05 (compared to L-group (A)) ** P < 0.01, ***P <0.001 (compared to sham-group (C)), bars indicate mean ± SEM. (n = 5 mice per group)
only allow transport of particles smaller than about 700D, leakage of particles of a bigger size suggest a disarrangement of tight junction proteins resulting from IM. In conjunction, in vivo measurement of intestinal permeability using luminal 3-5 kD and 10 kD dextran particles demonstrated that in Kit\textsuperscript{W-sh/W-sh} mice were completely protected against IM-induced epithelial permeability changes (Fig 5D). Together, these data indicate that mast cells are required for the pathological barrier dysfunction, associated with inflammatory response to the muscle layer and POI. We next assessed whether bacterial translocation would play a significant role in the pathogenesis of POI. To study this we decontaminated the gut using a 6-day regimen of antibiotic treatment and examined the effect of gut decontamination in our model of POI. As described earlier for colonic ileus\textsuperscript{12} decontamination reduced the number of infiltrating leukocytes significantly (Fig. 6A) and improved post-operative transit after IM (Fig. 6B). These data indicate a role for intestinal bacteria in the pathogenesis of POI and point towards to possibility that enhanced bacterial translocation could contribute to the inflammatory response and POI after IM.

\begin{figure}[h]
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\caption{Antibiotics treatment reduce inflammatory parameters and improve postoperative transit. (A) Antibiotics treatment reduces IM-induced inflammatory infiltrates in the muscularis externa, and improves post-operative transit reflected by an enhanced GC value (B). N=4, data shown are average +/- SEM. Asterisks * P<0.05 (compared to saline treated-group (A) and **P <0.01 (compared to - L group (B).} 
\end{figure}
Intestinal manipulation induced decrease in blood pressure does not account for barrier dysfunction

To assess whether mast cells contribute directly to the decreased barrier function in our POI model, or whether that involves alternative mechanisms, we next explored alternative mechanisms that lead to barrier dysfunction, such as intestinal hypoperfusion\(^{29,30}\). To this end, we next assessed whether IM coincided with a decrease in mean arterial pressure (MAP), by monitoring MAP in arterial cannulated mice during abdominal surgery. As shown in Fig. 7, after opening of the abdomen the MAP was significantly reduced whereas the reduction in the L group was minimal (Fig. 7A). This effect was independent of mast cells as in the Kit\(^W-sh/W-sh\), IM led to a similar decrease in blood pressure if compared to WT (Fig. 7B). For both mouse strains, the decreased MAP sustained until mice recovered from anesthesia after 40 min. (Fig. 7A/B right panels) while no difference in heart rate between L and IM was observed (results not shown). To validate whether the decrease in MAP alone could account for the impaired barrier dysfunction, we administered SNP, a NO donor, to achieve a decrease in MAP comparable that that seen after IM surgery. Intravenous rate-controlled perfusion of SNP allowed dosing SNP to lower MAP to mimic that observed after IM (Fig. 7C). SNP treatment did not trigger an inflammatory reaction 24h after administration (Fig 7D, nor did it lead to bacterial translocation to MLN (Fig 7E), or epithelial barrier dysfunction (Fig.7F). From these data it can be concluded that the reduced MAP during IM does not account for the impaired barrier function, or the inflammatory response after IM, and that mast cells are involved in the pathogenesis of POI via a mechanism that is independent of the reduction in MAP.
Figure 7 | Blood pressure during IM procedure. Mean Arterial Blood pressure (MAP) was recorded during the surgical procedure. Surgery was started when blood pressure was stable (t=0). At t=5’, the % of MAP as compared to MAP at t=0 (basal level) was significantly decreased during intestinal manipulation (IM) but not laparatomy (L) in both C57BL/6 (A, left panel) and mast cell deficient KitW-sh/W-sh mice (B, left panel). Representative MAP recording during surgery is shown in right panels of A (C57BL/6) and B (KitW-sh/W-sh) (L=grey line) and IM (black line). Absolute values are shown at the top of the graphs (A and B) (n = 3 mice per group). Administration of the blood pressure lowering agent Sodium nitroprusside (SNP) gives similar blood pressure reduction as compared to IM (C). SNP administration does not affect MPO count (D), bacterial translocation (E) 24h after IM or epithelial permeability (E) at indicated time points 1 hour after IM. Asterisks *P <0.05 (compared to L-group), bars indicate mean ± SEM. (control: n = 4; SNP: n = 3 mice per group).
Discussion

The prolonged impairment of gastrointestinal motility after intestinal manipulation is a significant confounding factor in postoperative recovery. Rodent models and human studies have demonstrated that surgical inspection and manipulation of the bowel leads to the activation of antigen presenting cells that reside in the intestinal muscularis layer. The general paralysis of the entire GI tract - including the unmanipulated segments - is a commonly seen characteristic of POI. This clinically important aspect of POI involves the activation of an inhibitory neural reflex arch by local inflammatory infiltrates, and was recently also shown to involve the production of IFN-\(\gamma\) by CCR9+T-cells that are activated at the site of manipulation. We have shown previously that the activation of mast cells resulting from local manipulation of the bowel is a pivotal factor in the pathogenesis of POI and the inflammatory response to local manipulation. Hence, we questioned in the current study whether mast cell derived mediators contribute to POI either as local activators of dendritic cells recruited to the gut wall or via epithelial permeability changes.

We show here that intestinal manipulation induces barrier dysfunction via a mechanism that is crucially dependent on mast cells. In patients, barrier dysfunction frequently occurs during abdominal surgery and has been associated with increased postoperative septic morbidity in surgical patients undergoing laparotomy. In addition to this model of POI, as well as in human ileus, mast cell activation has been associated with disturbed intestinal barrier function in ulcerative colitis and several disease entities such as stress-induced hypersensitivity of the bowel and endotoxemia. In these models, the rapid release of serine proteases following triggering of mast cells, possibly via release of Corticotropin Releasing Factor (CRF), is responsible for an increase in epithelial permeability. Likewise, the nature of the mast cell mediators that affect barrier function in our model involves similar rapid mechanisms and mediator release. This mechanism may involve the activation of protease activated receptor-2 (PAR-2) that is expressed on epithelial cells, although most studies have focused on PAR-2 activation by protease activity in the lumen.

Our data are in line with earlier observations in patients and rodent models implicating a disturbed intestinal barrier function after intestinal surgery involving bowel handling. It has been shown that in POI, bacterial products may reach the intestinal muscularis after IM of the small bowel and that antibiotic treatment decreases muscular inflammation after colon manipulation. The implications of this process for the pathogenesis of POI are incompletely understood, but irrespective thereof, the clinical impact of bacterial translocation during surgery is significant. A recent study which included 927 patients over 13 years showed that bacterial translocation was
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associated with increased postoperative septic morbidity in surgical patients undergoing laparotomy. In this study, we assessed barrier integrity by measuring bacterial translocation to the MLN. Most likely this process reflects dendritic cell-mediated uptake of bacteria that are still viable once transported by dendritic cells to the MLN. Thus the bacterial translocation is dependent on a number of immunological processes including phagocytosis, killing and bacterial cultures in the MLN may not reflect merely epithelial integrity. Therefore we performed measurements of barrier function in the small intestine in Ussing chambers, reflecting the para- and transepithelial transport of the 40kD HRP, as well as in vivo measurement of real time changes in epithelial leakage to the vena mesenteria. Using this combination of methods we demonstrated that IM in our model of POI led to a mast cell dependent epithelial barrier dysfunction. This mechanism may explain the important role of mast cells in the pathogenesis of POI and validate mast cells as a bona fide drug target to shorten POI and improve postoperative recovery and barrier function. We performed our experiments in two strains of mast cell deficient mice, Kit<sup>W/W-v</sup>, and Kit<sup>W-sh/W-sh</sup> mice which both carry mutations in the Kit gene (White spotting (W) locus). C-kit is the receptor for Stem Cell Factor (SCF) and involved in regulation of hematopoiesis, proliferation and migration of primordial germ cells and melanoblasts during development. The Kit<sup>W/W-v</sup> mice carry the W mutation, resulting in deletion of the transmembrane domain of the c-kit protein as well as the dominant negative Wv mutation, a point mutation that affects c-kit kinase activity. On the other hand, the mast cell deficient Kit<sup>W-sh/W-sh</sup> mice carry a mutation that reflects an inversion in the kit locus spanning a 2.8 mb segment. Hence, the resulting phenotypes are different: Kit<sup>W-wv</sup> mice have phenotypic abnormalities including sterility, anemia, lack of interstitial cells of Cajal (ICC), and have defects in hematopoiesis that lead to an absence of intra epithelial T-cells, neutropenia, and poor mobilization of blood neutrophils. In contrast, Kit<sup>W-sh/W-sh</sup> mice, bearing the W-sash (W(sh)) inversion mutation, have mast cell deficiency but are not anemic nor sterile. Adult Kit<sup>W-sh/W-sh</sup> mice have been shown to have a profound deficiency in tissue mast cells but normal levels of major classes of other differentiated hematopoietic and lymphoid cells.

As the pathogenesis of POI is mediated by neutrophil influx, we reasoned that the protection after IM in our POI model in these Kit<sup>W/W-v</sup> mice might not be selectively dependent on the lack of mast cells. Therefore, we also carried out our experiments in the Kit<sup>W-sh/W-sh</sup> mice for two reasons: first, in contrast to the Kit<sup>W/W-v</sup>, the Kit<sup>W-sh/W-sh</sup> mice can be tested against C57BL/6 WT control mice. Second, Kit<sup>W-sh/W-sh</sup> have normal neutrophil numbers, are fertile, contain normal number of intra epithelial lymphocytes (IELS), and are not anemic. The latter likely explains why the effects of IM induced
neutrophilic extravasation and inflammatory response were much less pronounced in the KitW-sh/W-sh compared to the KitW/W-v.

Interstitial cells of Cajal (ICC) require intact ckit signaling for proper development and are defective in both ckit mutant mice, although ICC subsets have been shown to develop in KitW-sh/W-sh mice. Because KitW/W-v mice are hemizygous we assessed basic GI motility in KitW-sh/W-sh that are on a C57BL/6 background. Indeed we observed a disturbed motility and delayed transit under normal conditions compared to C57BL/6 mice. But this difference did not reach significance so we decided to include KitW-sh/W-sh in our motility analyses.

The observation that the bacterial translocation in KitW/W-v was almost completely abolished in both affected KitW/W-v as well as its control Kit+/+ may reflect a defective representation of innate immune cells in the lamina propria in these mice, given the purported role of lamina propria APCs in bacterial sampling. In addition, we observed that inflammatory parameters, bacterial translocation, and permeability to HRP differed between the control groups: C57BL/6 and Kit+/+. This is probably due to differences in immune responses that generally exist between mouse strains: for example between Balb/c and C57BL/6.

In this study we found that luminal bacteria were involved in IM induced inflammation and ileus by treating mice with antibiotics, indicating that bacterial translocation contributes to the POI. It has been described that the immune response is induced soon after IM, for example ICAM-1 mRNA is expressed in the muscularis within 15 minutes of manipulation, while luminal products start to appear in the muscularis externa externa 6h after intestinal manipulation, indicating that translocated bacterial antigens may not trigger muscularis immune responses, but may exacerbate immune responses, as shown by our data presented here. It is important to note that lamina propria intestinal macrophages that form the first line of defense generally do not produce high levels of cytokines upon bacterial challenge. Rather, dendritic cells in draining lymph are activated by luminal antigens which may not lead to mucosal inflammation but can contribute to the pathogenesis of POI in unmanipulated areas.

We show in this study that IM is associated with a postoperative decrease in MAP. Hypoperfusion of intestinal tissue following abdominal as well as non-abdominal surgery has been associated with impaired barrier function preceded by hypotension, mesentery hypoperfusion and enterocyte damage. In addition, these studies show that aberrations in actin reorganization, cell proliferation and mitochondrial function are maximal at 60 min. after mechanical bowel manipulation (i.e. the same time point at which we measured intestinal barrier function in the current study) and was partially recovered after 24 hours (at which we measured inflammatory mediators). Although MAP decreases after IM, when MAP was pharmacological lowered, the
intestinal barrier function was not affected and inflammation did not occur to a similar extent. Circulating SNP-derived NO causes smooth muscle relaxation and subsequent microvascular vasodilatation. NO might affect inflammatory processes and intestinal barrier function independent of blood pressure alterations but we show that NO has no effect on these processes. Of note, MAP measured in the carotid artery in our study likely reflects the blood pressure of the internal organs, but we cannot exclude that perfusion in the small intestine is different from the carotid artery.

Concluding, we show that IM elicits a mast cell dependent inflammatory response and intestinal barrier disturbances that may contribute to the pathogenesis of POI. Our study further underscores the potential of mast cell stabilization in ameliorating postoperative recovery, warranting that this treatment strategy should be pursued in clinical setting.
References


