Neuro-immunity in intestinal disease: in vivo studies of postoperative ileus and colitis
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Chapter 2

Intestinal manipulation causes mast cell-mediated epithelial barrier dysfunction in a mouse model of postoperative ileus
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Intestinal manipulation causes mast cell-mediated epithelial barrier dysfunction in a mouse model of postoperative ileus

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Abstract

**Background:** Abdominal surgery involving bowel manipulation commonly results 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 can contribute to the pathogenesis of POI by eliciting a disturbance of intestinal barrier function.

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

**Results:** IM resulted in pro-inflammatory cytokine and chemokine production, and neutrophil extravasation to the manipulated bowel of WT, but not in mast cell deficient mouse strains. IM led to impaired barrier function 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.

**Conclusions:** IM during abdominal surgery leads to a mast cell dependent epithelial barrier dysfunction and inflammation of the muscularis externa. The impaired barrier function may contribute to the pathogenesis of POI. Our data reveal mast cells as a bona fide drug target to ameliorate POI following bowel surgery.
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-economic impact (2). 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), or general motility via the activation of sympathetic inhibitory neural reflexes (6). 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 (2;4;7). 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;8). This would be in line with previous observations that bowel wall mechanical stretch (9) and manipulation (10) 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 (11) and mast cells are implicated in barrier dysfunction in animal models of chronic stress (12;13), allergic inflammation (14), parasitic infection (15) and endoxemia (16). 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, KitW/W-v and KitW-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. Therefore, targeting mast cells could be instrumental in the treatment of POI.

Materials and Methods:

Laboratory animals
Mice (C57BL/6, Harlan Nederland, Horst, The Netherlands) were kept under environmentally controlled conditions (light on from 8:00 to 8:00; water and rodent nonpurified diet ad libitum; 20°C–22°C, 55% humidity). Mast cell-deficient KitW/W-v (WBB6F1-W/Wv), the mast cell-sufficient Kit controls (Kit+/+) and KitW-sh/W-sh (B6.CgKitW-sh/HNihrJaeBsmJ) and their C57/BL6 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, Beese, 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 (11). 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 CaCl₂·6H₂O, 20.2 mM NaHCO₃, 0.4 mM NaH₂PO₄·H₂O, 0.33mmol/L Na₂HPO₄, and 1.0 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 C57/Bl6 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 (6;11). Mice (n = 5 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-anesthetized 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 FD4 (Sigma-Aldrich); Rhodamine Dextran (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).
Measurement of Mean Arterial Pressure

Mean arterial pressure (MAP) was recorded as previously reported (17). 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 sampled 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, 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 center (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 center (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 (11). 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 × 10⁶ 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 gentamycin, 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 achieved, as assessed by toluidine blue staining, the cells were harvested for experiments. Three weeks old KitW-sh/W-sh received 10 × 10⁶ 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 anti-
bodies (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 visualized using SigmaFast DAB (Sigma-Aldrich), incubating 5 min, and contra-stained with haematoxilin/eosin. Visualization and quantification of myeloperoxidase (MPO) positive cells in the ileal muscularis externa was performed as described elsewhere (18).

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.
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) (19). The activation of this cell population has been implicated in the leukocyte recruitment in POI (4;6) leading to an extravasation of CD11a expressing cells that appeared in muscularis externa of the small intestine 24h after IM (Fig.1A). 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 (2). We further assessed the IM induced cytokine production 1h, 3h, 6h and 24h after IM in the muscular layer and mucosa. In 3 to 24 h after IM we observed an increased production of the cytokines MCP-1, IL-6, and KC, IL-12p70 and TNF in the mice that had undergone IM, but not in control L mice (Fig. 1C, left panels). IM did not induce significant cytokine release in the small intestinal mucosa (Fig. 1C). Notably, RANTES was constitutively expressed in the mucosa (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 (20). 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 h. (Fig 1C). In addition, no apparent changes in CD11c+CD103+MHCII+ DC populations were noted in mesenteric lymph nodes at 1, 3, or 24 hr after IM (Fig 2A), and no changes in IL-12 production by these subsets were noted at these time points after surgery (Fig 2B). These results seem to indicate that in our model IM elicits a local activation of antigen presenting cells in the intestinal muscularis rather than a systemic T cell response.
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 MCP-1, IL-6 and KC was significantly increased in muscularis externa homogenates (C, left panels) but not in mucosa homogenates (C, right panels) at indicated time points after surgery. IL-12 and TNF production was low and increased only after 24h (C). RANTES protein expression was unaltered (C left panels) and constitutively expressed in the small intestinal mucosa (C, right panels) * P < 0.05, ** P < 0.01. Bars indicate mean ± SEM. (n = 6 per group)
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. 3A). In C57BL/6 mice, mast cells were found in the muscularis externa, Peyer’s Patches and mesentery (Fig. 3A). 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 mast cell deficient mice. A reduced inflammatory infiltrate in mast cell deficient Kit<sup>W/-v</sup> was observed after IM compared to Kit<sup>+/+</sup> (controls) (Fig. 3B). In addition, the production of cytokines MCP-1 and IL-6 depends on the presence of mast cells as both cytokines are significantly reduced in Kit<sup>W/-v</sup> mice (Fig. 3B). We also analyzed the alternative c-kit mutant Kit<sup>W-sh/-sh</sup> and C57BL/6 controls, that are proposed to serve as a more selective mast cell deficient phenotype compared to the Kit<sup>W/-v</sup> strain (21). Similar to Kit<sup>W/-v</sup> mice, the Kit<sup>W-sh/-sh</sup> mice displayed a reduction of the IM induced influx of MPO positive cells (Fig. 3C) and production of MCP-1 IL-1β (Fig. 3D), though this was not significant. IL-6 production was significantly reduced in Kit<sup>W-sh/-sh</sup> (Fig. 3D). To demonstrate that the observed phenotype was mast cell dependent, we reconstituted Kit<sup>W-sh/-sh</sup> mice with mast cells (MC) obtained from C57/BL6 bone marrow derived mast cell cultures, and measured the cytokine response to IM. In mast cell reconstituted Kit<sup>W-sh/-sh</sup> mice the levels of MCP-1, but not IL-6 or IL-1β, were recovered to WT levels.

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Figure 3. 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 were visualized in C57BL/6 mice with Giemsa 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 KitW/W-v compared to control mice (Kit+/+) (B). In KitW/v/W-sh mice, the number of MPO+ cells was reduced compared to control (C57BL/6), but not sh/W-sh significantly (C). In KitW/v/W-sh reconstituted with C57BL/6 bone marrow derived mast cells, the MPO count did not differ from C57BL/6 and KitW/v/W-sh (C). MCP-1 production was reduced in KitW/v/W-sh compared to C57BL/6 (D, left panel), and KitW/v/W-sh reconstituted with bone marrow derived mast cell cultures show restoration of MCP-1 production (D, left panel). IL-6 production and IL-1β was reduced in KitW/v/W-sh mice but not restored after reconstitution with WT mast cells (D, middle and right panel). ** P < 0.01, * P < 0.05. Bars indicate mean ± SEM. (B,: L: n = 5, IM: n = 10 mice per group; A, C,D: L: n = 3, IM: n = 4-7 mice per group)
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 KitW-sh/W-sh mouse strain to study the effect of mast cell activation on motility because the use of KitW-sh/W-sh mice allows the motility parameters to be compared to C57/BL6 mice as these mice are derived on a C57BL/6 background. In the L control group, 24h after surgery, gastrointestinal transit in KitW-sh/W-sh mice was delayed compared to C57BL/6 control mice. The majority of the fluorescent marker in KitW-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. 4A, left panel). This is reflected in the average calculated geometric center (GC) (Fig. 4B). After IM, in C57BL/6 mice, the gastrointestinal transit is significantly delayed as the fluorescence marker is mainly present in sg4 and sg5 (Fig. 4A, middle panel), also shown by the GC (Fig. 4B). However, in the KitW-sh/W-sh mice, the fluorescent marker is primarily distributed in sg8 and sg9, indicating only a minor delay in gastrointestinal transit (Fig. 4A, middle panel), clearly reflected in the GC values shown in Fig. 4B (right panel). After KitW-sh/W-sh mice were reconstituted with WT mast cells, the delay in gastrointestinal transit partially returned, indicated by the distribution of the fluorescent marker, in sg 2, 3, 5 and also 10 (Fig. 4A, right panel). The IM induced delay is also reflected in decreased GC values (Fig 4B). A significant decrease in GC value was observed after IM in control C57/Bl6 mice, but not in KitW-sh/W-sh. Although mast cell reconstitution in these mice partially protects from POI as judged by the transit data (Fig. 4A), the mast cell reconstitution did not significantly reduce the GC values back to WT levels.

Figure 4. Role of mast cells in gastrointestinal transit after intestinal manipulation. Twenty four hours after L or IM, gastrointestinal transit was determined by the % distribution of the fluorescent marker and calculation of the geometric center (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 KitW-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 KitW-sh/W-sh mice (A, middle panel). IM induced delay in gastrointestinal transit is partially rescued by reconstitution of KitW-sh/W-sh with WT mast cells (A, right panel). GC values are shown in (B). * P < 0.05. Bars indicate mean ± SEM. (L: n = 3, IM: n = 4-7 mice per group)
Intestinal manipulation 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 via enhanced barrier dysfunction. To this end we analyzed barrier function after IM in 3 ways: by determining postoperative bacterial translocation to draining lymph nodes, 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 bacterial translocation 24h after IM. In C57/BL6 mice that had undergone IM, the number of bacteria cultured from MLN was significantly increased as compared to the L control group (Fig. 5A). 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 KitW/Wv mice. Baseline and IM induced bacterial translocation did not differ in KitW/Wv 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+/+ control mice (Fig. 5B). In the KitW-sh/W-sh mice the IM induced bacterial translocation was not increased after IM as compared to C57/Bl6 control mice (Fig. 5C) pointing towards a crucial role for mast cells in this process.

Figure 5. Role of mast cells in IM induced bacterial translocation. To assess barrier function, bacterial translocation to the mesenteric lymph nodes (MLN) was determined 24h after bowel handling. Following intestinal manipulation (IM), translocation of both aerobic an 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+/+ mice and KitW/Wv 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 KitW-sh/W-sh mice was not significantly different between L and IM groups (C). (KitW/Wv and Kit+/+: L: n = 5, IM: n = 10; KitW-sh/W-sh and C57BL/6: L: n = 3, IM: n = 7 mice per group). *P <0.05, bars indicate mean ± SEM.
Figure 6. Mast cells regulate the transepithelial flux of small particles after intestinal manipulation. In addition to measurement of bacterial translocation after IM, mucosal to serosal transfer of horse radish peroxidase (HRP) was determined 1h after surgery in ileal tissue of C57BL/6 and mast cell deficient KitW-sh/W-sh and KitW/W-v. The mucosal to serosal flux of HRP was increased by intestinal manipulation (A) in C57BL/6. The intestinal manipulation induced increase was not significant in the mast cell deficient KitW-sh/W-sh (A) and KitW/W-v (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 was significantly increased at indicated timepoints 1 hour after intestinal manipulation (open circles) as compared to sham (closed circles). In KitW-sh/W-sh, the IM induced increase in transepithelial flux particles was completely abolished as indicated in D (left panel: 3kD, right panel: 10 kD). *P <0.05, **P < 0.01, ***P <0.001, bars indicate mean ± SEM. (n = 5 mice per group)
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, killing and migration of CX3CR1-positive dendritic cells and macrophages (22).

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. 6A), reflecting an increase in epithelial permeability after IM. In contrast however, ileal permeability measured in Ussing chambers was not significantly enhanced by IM in mast cell deficient mice Kit\(^{W^{-/-}}\) (Fig. 6A) Kit\(^{W^{-/-}}\) (Fig. 6A) Kit\(^{+/+}\) (Fig. 6B). To further substantiate these data we additionally measured intestinal permeability for different sized luminal particles in vivo 1h 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. 6C, left panel) as well as 10kD (Fig. 6C, right panel) in mesenteric venous blood 1-2hrs after IM, but not after L. As unaffected tight junctions only allow transport of particles smaller than about 700D, these data demonstrate disarrangement of tight junction and leading to barrier dysfunction 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\(^{W^{-/-}}\) mice were completely protected against IM-induced epithelial permeability changes (Fig. 6D). Together, these data indicate that mast cell activation leads to a pathological barrier dysfunction, associated with inflammatory response to the muscle layer and POI.

Intestinal manipulation is associated with a decrease in blood pressure.

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 (23). 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\(^{W^{-/-}}\), 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’ (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 mimick 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 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 drops during intestinal manipulation. (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 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. *P <0.05, 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 (2;20). 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 arc by local inflammatory infiltrates (6), and was recently also shown to involve the production of IFN-γ by CCR9+T-cells that are activated at the site of manipulation (20).

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 (3;11). Hence, mast cells are likely to act as local activators of dendritic cells residing in the gut wall. 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 (24-26). In addition to a key role for mast cells in IM induced inflammation in this model of POI (11), as well as in humans (3), mast cell activation has been associated with disturbed intestinal barrier function in several disease entities such as stress-induced hypersensitivity of the bowel (12) and endotoxemia (16). In these models, the rapid release of serine proteases, including tryptase, following triggering of mast cells is responsible for an increase in epithelial permeability, possibly via the activation of protease activated receptor-2 (PAR-2) that is expressed on epithelial cells (27). We do not know the exact nature of the mast cell mediators that affects barrier function in our model but this likely involves similar rapid mechanisms and mediator release.

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 (26;28). It has been shown that in POI, bacterial products may reach the intestinal muscularis after IM of the small bowel (28) and that antibiotic treatment decreases muscular inflammation after colon manipulation (29). 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 associated with increased postoperative septic morbidity in surgical patients undergoing laparotomy (25).

In this study, we assessed barrier integrity by measuring bacterial translocation to the MLN. Most likely this process reflects dendritic cell (30) (or CX3CR1 expressing macrophages (31))- mediated uptake of bacteria that are still viable in 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 mesenterica. 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, KitW/W−/− and the KitW-sh/W-sh which both carry mutations in the Kit gene. Kit expression is abolished in mast
cells and mutant mice have a mast cell deficit (32;33). The KitW-sh/W-sh mice display a tissue-specific phenotype; c-kit mRNA was shown to be lacking in KitW-sh/W-sh mast cell cultures, but the c-kit mRNA was normally detectable in the cerebellum, testis and spleen of KitW-sh/W-sh mice. In contrast to KitW-sh/W-sh mutants, KitW/W-v are neutropenic and mobilize neutrophils poorly (34). As the pathogenesis of POI is mediated by neutrophil influx, we reasoned that the protection after IM in our POI model in these KitW/W-v mice might not be selectively dependent on the lack of mast cells. This is likely to be the reason why the effects of IM induced MPO influx were much less pronounced in the KitW-sh/W-sh. 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 KitW/+ is indicative of 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 (30). Furthermore, in contrast to the KitW/W-v, the KitW-sh/W-sh mice are fertile, contain normal number of intraepithelial lymphocytes (IELs), and are not anemic (35), and can be tested against C57/Bl6 WT control mice. In KitW/W-v heterozygotes, interstitial cells of Cajal (ICC) failed to develop in various regions of the GI tract, although some ICC subpopulations have been observed (36). The loss of ICC may affect basic motility in Kit mutants. However, in contrast to KitW/W-v we observed that gastrointestinal transit under healthy conditions in KitW-sh/W-sh was only mildly–and not significantly delayed. Together, we conclude that the widely used KitW/W-v mutants display mast-cell independent aberrancies that make these mice less useful for our studies specifically aimed at the role of mast cells in orchestrating innate immune responses compared to the KitW-sh/W-sh mutant.

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 (37;38). 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 (38;39) (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 (40;41) 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.
Reference list

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