Neuro-immunity in intestinal disease: in vivo studies of postoperative ileus and colitis
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Chapter 3

A pathogenic role for IL-1β and MyD88 signalling in postoperative ileus
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Abstract

Background and aims: Postoperative ileus (POI) is a common consequence of intestinal surgery that enhances morbidity and hospitalization. Its pathogenesis involves the activation of intestinal macrophages and dendritic cells (DCs) and a subsequent Th1 response, but it is unclear how these cells are activated. Therefore we investigated whether TLR- and/or IL-1R- signalling is important in the inflammatory cascade after abdominal surgery in a mouse model of POI.

Methods: Experiments were performed in C57Bl/6 (WT) and MyD88-/-, TLR4-/-, TLR2x4-/-, TLR9-/-, NALP3-/- and ASC-/- mice. Mice underwent laparotomy (L) or L followed by a gentle manipulation of the small bowel (IM). Mice were treated i.p. with the IL-1 receptor antagonist (IL-1RA (anakinra); 100 mg/kg), at 1 or 3 h before, 1 h and 6 h after IM. Intestinal inflammation was assessed postoperatively by counting the number of myeloperoxidase (MPO) positive cells per mm² and cytokine production in the muscularis externa of the small intestine. Bacterial translocation was determined at 24 h post surgery by counting the colony forming units (CFU) cultured from the mesenteric lymph node (MLN). Gastrointestinal motility was determined in vivo by measurement of gastrointestinal transit of liquid FITC-dextran 24 h after IM.

Results: Twenty four hours following IM, influx of MPO positive cells and as well as MCP-1 and IL-1β production was dependent on Myd88 signalling. MPO+ cell influx was not dependent on signalling through TLR2, TLR4 or TLR9, while IL-1β and IL-6 production was dependent on TLR9. Peri-operative treatment with the IL-1RA reduced MPO+ cell influx, IL-6 and IL-1β production and significantly improved gastrointestinal transit. In conjunction, IL-1RA reduced IL-12 production by LPS matured bone marrow cells, and counteracted IL-12 production by DCs stimulated by degranulated mast cell conditioned medium. IM induced MPO+ cell influx and cytokine production was not altered in NALP3-/- and ASC-/- mice.

Conclusions: Recognition of bacteria is not a major factor in IM induced POI while signalling through IL-1R and Myd88 is crucial in POI. Treatment with the IL-1R antagonist would be an effective strategy to prevent POI in patients undergoing abdominal surgery.
**Introduction:**
Postoperative ileus (POI) is characterized by a transient period of hypomotility of the gastrointestinal tract, which lasts up to 3–6 days with a significant impact on patient morbidity (1). The pathogenesis of POI involves manipulation of the intestines during abdominal surgery, which initiates an inflammatory response in the muscularis externa that activates inhibiting neural reflex pathways affecting motility of the entire gastrointestinal tract (1). Previous studies have indicated that mast cell activation is a crucial event in the initiation of the inflammatory cascade in the bowel wall after IM (2). In addition, other resident innate immune cells in the muscularis externa are implicated in IM induced POI. In a rodent model of POI, it was shown that muscular inflammation and the subsequent delay in gastrointestinal transit after IM was associated with the activation of resident macrophages in the muscularis externa (3). Most of these cells possess phagocytic properties, express the mouse macrophage marker F4/80, MHC-II, the LPS-binding receptor CD14 and dendritic (DC) markers including CD11c, DEC205 and Langerin (4). In addition, in more recent studies it has been demonstrated that pro-inflammatory cascades are also activated in areas distant from the manipulated site where muscle contractility is inhibited locally; this is referred to as the “field-effect” (5). Recently it was shown that CD103(+) CD11b(+) DCs are activated and stimulate Th1 cells to secrete IFN-γ, thereby activating macrophages (6). Also these CCR9+ Th1 cells migrate to unmanipulated areas and activate local resident muscularis macrophages thereby suppressing motility distant from manipulated areas (6).

How DCs and other phagocytes of the muscularis externa network are activated is not clarified yet. In IM induced POI, we (Snoek SA, submitted) have shown that luminal bacteria pass the epithelial barrier after IM, and may activate resident phagocytes of the muscularis externa. In support of that, it has been shown that orally administered beads appeared in local mesenteric vessels and the muscularis externa after manipulation of the small intestine (7) and the colon (8). Gut decontamination reduced inflammatory markers but not the number of infiltrated leukocytes in the jejunum muscularis, and decreased betanechol induced contractility of jejunal muscle strips after colonic manipulation (8).

In the current study, we further explored how resident innate immune cells including DCs may be activated after IM. We hypothesized that recognition of bacteria through TLRs triggers the immune reaction that underlies POI. We show that the IM induced inflammatory cascade and POI requires MyD88 signalling through the IL-1R rather than through TLRs. Further, our data indicate that mast cells may play a crucial role in POI by activating DCs in an IL-1R dependent fashion.

**Material and methods**

**Animals**
Laboratory animals 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). Twelve to 20 week old (WT) C57BL/6 mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). TLR2−/−, TLR4−/−, TLR2x4−/−, TLR9−/− and MyD88−/− were a kindly gift of the Center for Experimental and Molecular Medicine within the Academic Medical Center and bred in the animal facility of the Academic Medical Center in Amsterdam, The Netherlands. All mouse strains were backcrossed at least 6 times to a C57BL/6 background, except for TLR9−/− mice that were backcrossed 3 times to C57BL/6 background.
TLR4-/- double knockout mice were generated by intercrossing TLR2-/- and TLR4-/- mice. TLR2-/- (9), TLR4-/- (10) and MyD88-/- mice were originally generated as described previously, and the correct phenotype was revealed by genotypic screening. ASC-/- and NALP3-/- mice were generously provided by Dr. R.A. Flavell (Yale University School of Medicine, New Haven, CT). Studies were performed according to the guidelines of the Dutch Central Committee for Animal Experiments and The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments. Surgical procedures and administration of IL-1RA 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 (5–11 per treatment group) underwent control surgery of only laparotomy or laparotomy followed by IM. The surgery was performed as follows: A midline abdominal incision was made, and the peritoneum was opened over the linea alba. The small bowel was carefully externalized, layered on a sterile moist gauze pad, and manipulated from the distal duodenum to the cecum for 5 minutes, using sterile moist cotton applicators. Contact or stretch on stomach or colon was strictly avoided. 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 4 h in a heated (32°C) recovery cage with free access to drinking water but not food. At 4 h postoperatively, mice were completely recovered from anesthesia. After 24 h 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. IL-1RA (Anakinra, Biovitrum, Stockholm, Sweden) (diluted in saline) or saline was injected i.p. (100 mg/kg) at 1h or 3h before, 1h, and 6h after IM.

**Gastrointestinal transit measurements**

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).

**Whole mount preparation**

Ileal segments (3 cm distal from the cecum) were quickly excised, and mesentery was removed. Ileal segments were cut open along the mesentery border, fecal content was washed out in ice-cold PBS, and segments were put into 100 % ethanol. After 30 minutes the segments were transferred to a 70% ethanol solution and pinned flat in a glass-dish. Mucosa was removed, and the remaining full-thickness sheets of muscularis externa were rehydrated by incubation in 50% ethanol and PBS, pH 7.4, for 5 minutes. To visualize myeloperoxidase positive cells, preparations were incubated for 10 minutes with 3-amin-9-ethyl carbazole (Sigma, St. Louis, MO) as a sub-
strate dissolved in sodium acetate buffer (pH 5.0) to which 0.01% H2O2 was added. To quantify the extent of intestinal muscle inflammation, the number of myeloperoxidase-positive cells in 5 randomly chosen 1-mm² fields were counted and expressed as the number of myeloperoxidase-positive cells per mm².

**Bacterial Translocation**

Mesenteric Lymph nodes 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 conditions. After 24 h of incubation at 37°C, the number of colony forming units (CFU) per milligram lymph node was assessed. Culture and stimulation of mouse DCs Bone marrow derived DCs (BMDCs) were obtained by flushing sterile, endotoxin-free medium through the bone shaft using a needle and syringe. The suspension of BMDCs cells was centrifuged at 320 g for 10 min and cultured at a concentration of 0.5 X 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 GM-CSF was added at a 10 ng/mL concentration for 8 days at 37°C in a humidified atmosphere with 5% CO2. Medium was replenished at day 5 and 7 during culture with the addition of fresh GM-CSF. BMDCs were treated with indicated agents and subsequentl matured with 100 ng/mL LPS in overnight incubation.

**Mast cell conditioned medium**

Mast cell conditioned medium (CM) was obtained from mast cells that were cultured for 4-6 weeks at a density of 0.6X10E5/mL medium (RPMI containing 10% fetal calf serum, 1% pen/strep and 6% bone marrow mast cell supplement (containing 20% MEM non-essential amino acids, 1% L-glutamine, 0.22% Sodium Pyruvate, 0.005% β-mercapto-ethanol) and a high concentration of stem cell factor (SCF) (100 ng/ml) allowing only the survival of PMCs. Medium was refreshed every 7 days and mast cell CM was obtained after 7 days in culture. Epithelial resistance measurements CMT93 cells derived from mouse rectum carcinoma were obtained from the ATCC. Cells were cultured in DMEM medium (supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin) and were maintained in a standard cell culture incubator at 37°C and 5% CO2. IL-1β (Peprotech, NJ, USA) and IL-1RA (Biovitrum) were solubilized in culturing media prior to their use. Impedance measurements and Cell Index (CI) determinations were done using a Real Time Cell Analyzer (RTCA) DP Instrument (Roche Applied Sciences, Indiana, USA). Subconfluent cells were trypsinized and plated at a confluent density (50,000 cells/well) into an E-plate 16 (Roche Applied Science) in 150 µl growth media. Cell attachment and growth to confluent phase were monitored using the RTCA DP Instrument. On reaching a confluent state, the cells were treated with murine IL1β (100 or 10 ng/ml) or Kineret (0.5 mg/ml) or both, and real time impedance changes were recorded.

**Cytokine measurements**

For cytokine measurements, 3 cm long small intestinal 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; 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 superna-
tants were stored at -20°C until assays were performed. MCP-1, IL-6, IL-1β and IL-12p70 in supernatants were analyzed by mouse ELISA (R&D Systems, Abingdon, England), 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

Myd88 signalling is crucial in IM induced intestinal inflammation.
We have shown (Snoek SA et al submitted) that IM induces bacterial translocation and therefore, luminal bacteria that pass the epithelial barrier may contribute to IM induced muscular inflammation. Thereupon we firstly analysed the activation of the Myd88 dependent pathway in our model of POI. Twenty four hours after IM, the number of MPO+ cells in the muscularis externa was significantly reduced in Myd88-/- mice as compared to C57Bl/6 (WT) mice (Fig. 1A). In addition, production in of the pro inflammatory cytokines MCP-1 and IL-1β was significantly decreased in Myd88-/- mice compared to WT mice. IL-6 production was also reduced but this was not significant (Fig. 1B). IL-12 production was low and not induced by IM (Fig. 1B). IM-induced bacterial translocation was not altered by Myd88 deficiency as shown in Fig. 1C. These results clearly show a role for Myd88-/- signalling in IM-induced inflammation.
Figure 1: Myd88 signalling is crucial manipulation inflammation. Mice underwent intestinal manipulation (IM) or laparotomy described methods. Twenty four hours after IM, the number of MPO+ cells (A) and production of pro-inflammatory cytokines MCP-1, IL-6 and IL-1β was significantly reduced in compared to WT (C57BL/6) mice. IL-12 levels were low and not enhanced. Translocation of bacteria to the mesenteric lymph node (MLN) (number of colony forming units (CFU)/mg MLN) was not altered in Myd88−/− mice (C). * $P < 0.05$, ** $P < 0.01$ compared to WT IM. Bars indicate mean ± SEM. (L: $n = 4$, IM: $n = 8$ per group) in intestinal induced only (L) in material as and MyD88−/− mice by IM (B).
TLR signalling in IM induced intestinal inflammation.

TLR4 signalling is dependent on the intracellular adaptor molecules TRIF as well as Myd88 (11) and is implicated in IM-induced muscle dysfunction (8). Whether this depends on reduction of intestinal inflammation has not been elucidated so far. As shown in Fig. 2A, leukocyte recruitment is increased in TLR4−/− as compared to WT mice, but this was not significant. In addition, production of MCP-1, IL-1β, IL-6 and was not significantly different between TLR4 deficient and WT mice (Fig. 2B). Also, bacterial translocation was not altered by TLR4 deficiency (Fig. 2C).

Figure 2: No role for TLR4 mediated signalling in intestinal manipulation induced inflammation. Twenty four hours after intestinal manipulation (IM), leukocyte influx, cytokine production and bacterial translocation was determined in TLR4−/−, and WT (C57Bl/6) mice. TLR4 deficiency did not affect the number of MPO positive cells/mm² recruited to the muscularis externa following IM (A) or the production of the proinflammatory cytokines MCP-1, IL-6, IL-1β and (B). The number of colony forming units (CFU) that translocated to the mesenteric lymph node (MLN) was not altered in TLR4 deficient mice (C). Bars indicate mean ± SEM. (L: n = 3, IM: n = 8 per group) enhanced. Translocation of bacteria to the mesenteric lymph node (MLN) (number of colony forming units (CFU)/mg MLN) was not altered in Myd88−/− mice (C). * P < 0.05, ** P < 0.01 compared to WT IM. Bars indicate mean ± SEM. (L: n = 4, IM: n = 8 per group).
To further investigate whether bacterial recognition is involved in IM induced inflammation, we assessed the role of TLR2 and TLR9 in IM induced inflammation. The number of leukocytes that extravasated to the muscularis externa (Fig. 3A), and production of MCP-1 (Fig. 3B left panel) was not significantly different between WT and TLR2-/-, TLR2x4-/-, TLR9-/- mice. However, IL-6 production was significantly reduced in TLR9-/- but not in TLR2-/- and TLR2x4-/- mice (Fig. 3B, middle panel). IL-1β production was significantly reduced in TLR2x4-/-, and TLR9-/- but not in TLR2-/- mice (Fig. 3B, right panel). As is shown in Fig. 3C, the number of CFU cultured after IM is significantly decreased in the TLR2-/- and TLR2x4-/- mice compared to WT mice. These data imply that bacterial recognition is not a major factor in IM induced inflammation.

Figure 3. Role for TLR2 and -9 mediated signalling in intestinal manipulation induced inflammation. Twenty four hours after intestinal manipulation (IM), leukocyte influx, cytokine production and bacterial translocation was determined in TLR4-/-, and WT (C57Bl/6) mice. Leukocyte influx (A) in TLR2-/-, TLR2x4-/- and TLR9-/- and production of MCP-1 (B, left panel) was unaffected in TLR2, 2x4 and 9 deficient mice. IL-6 was significantly decreased in TLR9 KO (B, middle panel) and IL-1β production was dependent on both TLR2 and TLR9 (B, right panel). The number of colony forming units (CFU) cultured from the mesenteric lymph node (MLN) was significantly reduced in TLR2-/- and TLR2x4-/- but not in TLR9-/- mice (C). * P < 0.05 compared to WT IM. Bars indicate mean ± SEM. (L: n = 3, IM: n = 6 per group)
Inflammasome formation in IM induced intestinal inflammation.
Here we show that bacteria are probably not of major importance in IM induced inflammation. Alternatively, during IM, tissue damage may occur during surgery resulting in the release of damage associated molecular patterns (DAMPs) such as reactive oxygen species (ROS) and ATP (12). Activation by DAMPs will induce the assembly of a protein complex called the inflammasome, that is required for IL-1β activation by caspase-1 (12). To assess whether IM induced IL-1β production is dependent on inflammasome activation, IM was performed in NALP3−/− and ASC−/− mice. ASC and NALP3 are crucial proteins in the formation of the inflammasome (12). IL-1β production was significantly increased in NALP3−/− mice and not altered in ASC−/− mice (Fig. 4A). IM induced influx of MPO+ cells (Fig. 4B) and the production of cytokines MCP-1, IL-6 and IL-12 (Fig. 4C) was not dependent on NALP3 or ASC. In addition, distribution of the fluorescent marker in the intestinal segments was not altered in NALP3−/− mice compared to WT mice, indicating that gastrointestinal transit was not affected by NALP3 deficiency (Fig. 4D). These data implicate that inflammasome activation was not crucial in IM induced muscular inflammation.

Figure 4. Intestinal manipulation induced IL-1β production and inflammation is not dependent on inflammasome formation. Intestinal manipulation (IM) was performed in mice deficient in proteins that are crucial in inflammasome formation, NALP3−/− and ASC−/−, and their WT controls (C57Bl/6). Twenty four hours after IM, IL-1β production was significantly higher in NALP3−/− mice, and not affected in ASC−/− mice compared to WT (A). The number of MPO+ cells counted in the muscularis externa (B), and production of MCP-1, IL-6 and IL-12 (C) was not significantly altered in NALP3−/− and ASC−/− mice. In addition the distribution of the fluorescent marker in NALP3−/− mice was not different from WT mice (D). St=stomach, cec=cecum * P < 0.05 compared to vehicle treated mice. Bars indicate mean ± SEM. (n = 4-6 mice per group)
Block of IL-1R signalling reduces IM induced inflammation and improves gastrointestinal transit.

In addition to its crucial role in TLR-signalling, Myd88 is also essential in IL-1R-signalling. Having assessed that IL-1β is produced during IM (see Fig. 1-4), we determined the role for activation of the IL-1R in IM induced intestinal inflammation. IL-1R signalling was blocked by perioperative treatment with human recombinant human IL-1RA, anakinra (13) according to the dosing scheme depicted in Fig. 5A. Mice were treated 1h or 3h before surgery and all mice received IL-1RA 1 h and 6 h after surgery. The number of MPO+ cells that extravasated into the muscularis externa 24 h after IM was significantly reduced after administration of IL-1RA 3 h but not 1 h before IM (Fig. 5B). Production of MCP-1 was unaltered after IL-1RA treatment while IL-6 was significantly reduced only after treatment 3h before IM (Fig. 5C). IL-1β production was significantly reduced only when mice were pretreated 1 h before surgery while IL-12 was low and not significantly different by IL-1RA treatment. We further assessed the effects of IL-1R signalling on gastrointestinal transit (as determined in material and methods). Gastrointestinal motility was disturbed after IM as GC values are around 4.5 (Fig. 5D, left panel). GC values were significantly enhanced by treatment with IL-1RA 3 h but not 1 h before operation (Fig. 5D, left panel). This is further illustrated in Fig. 5D (right panel) showing that the fluorescent marker was distributed more distally in IL-1RA treated mice as compared to vehicle treated mice. These results demonstrate that IM induced inflammation as well as the delay in gastrointestinal transit was dependent on IL-1R signalling.

IL-12 production by LPS matured DCs is dependent on IL-1R activation.

Recently it has been demonstrated that small intestine DCs initiate a Th1 response by secreting IL-12 after IM. We tested whether blocking IL-1R activation by incubation with IL-1RA during LPS induced maturation of mouse bone marrow derived DCs (BMDCs) affected IL-12 production in vitro. IL-1RA significantly reduced IL-10 production only in the highest dose of 5000 μg/mL (Fig. 6A, left panel). IL-12 production was significantly reduced by co-incubation with IL-1RA at 5000, 500 and 50 μg/mL (Fig. 6A, middle panel). LPS induced IL-1β production was not regulated by IL-1R signalling (Fig. 6A, right panel).

Since mast cells may be activated within seconds after manipulation, the release of their mediators will probably initiate the inflammatory cascade after IM, including activation of DCs. Therefore we co-incubated the BMDCs with mast cell conditioned medium (CM) (see material and methods) during maturation with LPS. The addition of 25% and 50 % of mast cell conditioned medium significantly enhanced IL-12 production by LPS matured BMDCs (Fig. 6B, left panel). The increased IL-12 production was significantly reduced in a dose dependent manner by addition of 5, 50 or 5000 μg/mL IL-1RA during maturation (Fig. 6B, left and right panel). These results indicate that mast cell conditioned medium enhances IL-12 production in BMDC through IL-1R signalling. As the IL-1β concentrations in mast cell conditioned medium were generally low (< 17 pg/mL) we aimed to establish that the IL-1R is activated by IL-1β derived from DCs in these experiments. Therefore we assessed the IL-12 production in BMDC deficient in NALP3-/-, a protein that is important the formation of the inflammasome that leads secretion of IL-1β. IL-12 production in LPS matured NALP3-/- BMDC was unaffected by mast cell conditioned medium (Fig. 6C, left panel). As expected, IL-1β production was reduced in NALP3-/- BMDCs and was not affected by addition of mast cell conditioned medium (Fig. 6C, right panel). This indicates that in these experiments mast cell conditioned medium enhances IL-12 production via stimulation of DC derived IL-1β release.
Figure 5. Treatment with IL-1 receptor antagonist IL-1RA reduces IM induced inflammation and ameliorates gastrointestinal transit. IL-1R signalling was blocked during intestinal manipulation (IM) by pretreatment with the IL-1R antagonist IL-1RA (100 mg/kg) at three time points: 1h or 3h before surgery and 1h and 6h after surgery. The dosing scheme is presented in (A). The number of MPO+ cells that extravasated to the muscularis externa was reduced when mice were treated 1h before IM; this reached significance when mice were pretreated 3h before IM (B). Production of MCP-1 was not significantly reduced by IL-RA treatment; production of IL-6 was significantly reduced when mice were pretreated 3h before IM; IL-1β production was significantly reduced when mice were pretreated 1h before IM; IL-12 production was low and not altered by IL-1-RA treatment (C). Twenty four hours after IM, gastrointestinal motility was determined by % distribution of FITC-dextran in intestinal segments 90 minutes after oral gavage. The geometric center (GC) (calculated as indicated in material and methods) was significantly enhanced in the group treated with IL-1RA 3h before IM but not in the group treated 1h before IM (D, left panel). The fluorescent marker in the vehicle group is located more proximally (grey line) than the IL-1RA group (black line) as illustrated in D, right panel. St=stomach, cec=cecum, sg=segment. * P < 0.05 compared to vehicle treated group. Bars indicate mean ± SEM. (n = 6-10 per group)
Figure 6. IL-12 production by mouse DCs is dependent on IL-1R signalling. Immature bone marrow derived mouse DCs (BMDCs) were matured for 16h with LPS (100 ng/mL), with or without preincubation with IL-1RA and/or mast cell conditioned medium for 20 minutes at indicated percentages. IL-10 production by BMDCs was significantly reduced after treatment with 5000 μg/mL IL-1RA but not by lower concentrations (A, left panel); IL-12 production was significantly reduced by 5000, 500 and 50 μg/mL IL-1RA (A, middle panel); IL-1β production was not affected by IL-1RA treatment (A, right panel). LPS matured BMDCs were coincubated with different percentages of mast cell conditioned medium and different doses of IL-1RA. Mast cell conditioned medium alone significantly increased IL-12 production by BMDCs during maturation which is significantly reduced by IL-1RA (B, left panel). IL-12 production is further enhanced by mast cell conditioned medium and this is blocked by cotreatment with different doses of IL-1RA (B, right panel). Mast cell conditioned medium alone significantly increased IL-12 production by BMDCs during maturation which is significantly reduced by IL-1RA (B, left panel). IL-12 production is further enhanced by mast cell conditioned medium and this is blocked by cotreatment with different doses of IL-1RA (B, right panel). Mast cell conditioned medium did not affect IL-12-p70 production in NALP3-/- BMDCs (C, left panel). IL-1β production was significantly reduced in NALP3-/- mice compared to WT; mast cell conditioned medium did not affect IL-1β production in both WT and NALP3-/- BMDCs (C, right panel). * P < 0.05 compared to vehicle treated mice. Bars indicate mean ± SEM. (n = 3)
Activation of IL-1R signalling reduces barrier function in vivo and in vitro.
We (Snoek et al., submitted) have shown that intestinal barrier function is disturbed in POI and we reasoned that blocking of IL-1R signalling may affect epithelial integrity. Indeed, bacterial translocation was significantly reduced when mice were treated with IL-1RA (Fig. 7A) (see protocol Fig. 5A). We further tested the effects of IL-1R signalling in vitro on the epithelial resistance in a confluent CMT93 epithelial cell line (as described in material and methods). IL-1β decreased epithelial resistance during 3 h after addition to CMT93 cell cultures (Fig. 7B). The epithelial resistance returned to start values after 3 h when cells were coincubated with IL-1RA (Fig. 7B). These data indicate that activation of IL-1R signalling in epithelial cells increases permeability and may play a role in barrier disturbances in POI.

**Figure 7. IL-1R signalling reduces barrier function in vivo and in vitro.** Twenty four hours after intestinal manipulation, the number of colony forming units (CFU) cultured from mesenteric lymph nodes (MLNs) was significantly reduced when mice were treated with IL-1RA (A) (n = 10 mice per group). Transepithelial resistance was reduced by 10 ng/mL IL-1β and remained at low levels during 3 h while coincubation with IL-1RA (0.5 mg/mL) returned resistance to control levels within 3 h (B).
Discussion

IM during abdominal surgery induces an inflammatory reaction in the muscularis externa of the small intestine that is initiated by activation of resident mast cells (2) and other innate immune cells including macrophages (3) and DCs (6). In particular, DC mediated activation of memory Th1 cells by IL-12 has recently been implicated in the pathogenesis of POI (6). However, the mechanism of IM-induced activation of these innate immune cells is not clarified yet. In earlier studies we demonstrated that IM induces mast cell dependent translocation of bacteria (Snoek SA et al. submitted (Chapter 1 in this thesis) that may activate muscular phagocytes. In this study, we investigated the possible role of bacterial recognition in IM induced muscular inflammation. We show here that IM induced inflammation was mainly dependent on MyD88 and was only partly affected by TLR 2, 4, or 9-deficiency, indicating a role for IL-1R activation in this process. In conjunction, perioperative treatment with the IL-1R antagonist rhIL-RA (anakinra) prevented IM induced barrier dysfunction, inflammation and subsequent ileus. We also show in that IL-12 production by in vitro LPS matured BMDC is enhanced by mast cell mediators and dependent on IL-1R signalling, further implying a prominent role for IL-1R signalling after bowel handling.

The presence of the TLR adaptor molecule Myd88 is essential in the IM-induced leukocyte influx and cytokine production. However, we show here that bacterial recognition is not the major factor in IM induced inflammation as TLR4 deficiency does not affect leukocyte influx or cytokine production, and TLR2 and TLR9 only play a role in cytokine production. Interestingly, jejunal muscles from colon manipulated TLR4- knockout mice were refractory to muscle dysfunction as compared with manipulated wild-type animals although inflammatory parameters were not assessed and therefore donot oppose our findings (8).

It has been described that orally administered beads appeared in local mesenteric vessels, the muscularis externa and were all ingested by recruited monocytes (7). However, when this pathway was blocked, no beads were present but still a substantial number of neutrophils and monocytes infiltrated the muscularis externa of the small intestine (7). In addition, monocyte loaded beads only started to appear in the muscularis externa 6h after intestinal manipulation (7) while the inflammatory cascade starts earlier after intestinal manipulation (14). For example ICAM-1 mRNA is expressed within 15 minutes of manipulation (14) and subsequent influx of immune cells within 3h after IM (15). Thus, these results indicate that the triggers for the inflammatory cascade after IM are probably not bacteria or their antigens.

Alternatively, we demonstrate here that IL-1R and Myd88-mediated signalling are key events in the inflammatory reaction after IM. The importance of the IL-1R-Myd88 pathway has been implicated in mouse models of sterile, neutrophil dependent inflammation (13;16). In these models, signalling through IL-1R, and Myd88 pathway was crucial in neutrophil influx after bleomycin induced lung damage (13) and intraperitoneal injection of dying cells (16) but less dependent on TLR signalling (13;16) or IL-18R signalling (16). In line, our results indicate a major role for IL-1R-Myd88 signalling in IM induced inflammation, but the effects of defective TLR signalling were less pronounced. This implicates that IM induced inflammation may results from ‘sterile’ inflammation, induced by cell damage during manipulation of the intestine.

It is well possible that handling of the intestine will induce the release of DAMPs, such as ATP and reactive oxygen species (ROS), through mechanical damage of the cells or hypoxic conditions during the operative procedure. DAMPs induce the assembly of the inflammasome, a complex of proteins that is required for caspase-1 dependent cleavage of pro-IL-1β into the active form of IL-1β (12). We performed our experiments in NALP3-/-and ASC-/- mice that are both proteins crucial in inflammasome formation (12). Though, in these animals, production
of IL-1β, other cytokines and MPO+ cell influx into the muscularis externa were not affected. These results suggest that IM induced inflammation may not be initiated by DAMPs and subsequent caspase-1 dependent production of IL-1β. Instead, proteases released from mast cells and neutrophils after bowel handling may cleave pro-IL-1β independently of caspase-1 activation. An example of caspase-1 independent activation of IL-1β in disease has been shown in a model of acute arthritis and involves the neutrophil derived proteinase 3. (17-19).

A recent paper describes the crucial role for CCR9+ Th1 cells that migrate to unmanipulated areas and are responsible for the field effect that characterizes POI (6). These cells are activated by IL-12 producing DCs that accumulate in the manipulated area (6). We show here that IL-12 produced by LPS matured BMDCs is enhanced by mast cells mediators, a process that could be inhibited by IL-1R antagonism. The mast cell conditioned medium itself does not contain IL-1β, implicating that IL-1β is derived from DCs. Proteases such as chymase and cathepsinB secreted from mast cells may cleave pro-IL-1β that is produced by DCs, thereby enhancing IL-1R mediated IL-12 production (18).

Altogether, in IM induced ileus, IL-1R activation is of greater importance than TLR signalling, but neither of the two receptors completely block IM induced inflammation. Therefore we propose that IL-1R signalling may be an early, crucial event that initiates the inflammatory cascade after IM, while bacteria translocate at a later timepoint and contribute to IM induced inflammation via TLR signalling (see Fig. 8). Therefore, perioperative treatment with the rhIL-1RA anakinra during abdominal surgery would be a promising and safe strategy to prevent POI in patients undergoing abdominal surgery.
Figure 8: Hypothetical mechanism of intestinal inflammation induced ileus. Upon intestinal surgery, the release of mast cell mediators activates resident phagocytes and induces barrier dysfunction. IL-1β is released locally and processed by proteinases derived from mast cells and later from incoming neutrophils. The actions of IL-1β in IM induced ileus may include the upregulation of adhesion molecules, and an increase in iNOS and COX-2 expression (20) thereby initiating the inflammatory cascade. Dendritic cells migrate to the manipulated area and activate Th1 cells by IL-12 (6). These CCR9+T-cells will migrate to distant parts of the intestine thereby inducing macrophage activation by IFN-γ locally (6). Also incoming bacteria will further activate local innate immune cells. Together, these events result in a major inflammatory response leading to impaired gastrointestinal motility and postoperative ileus.
Reference List