The role of lymphoid tissue in the attenuation of the postoperative ileus

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Koscielny A, Engel D, Maurer J, Wehner S, Kurts C, Kalff JC. The role of lymphoid tissue in the attenuation of the postoperative ileus. Am J Physiol Gastrointest Liver Physiol 304: G401–G412, 2013. First published December 13, 2012; doi:10.1152/ajpgi.00161.2012.—Standardized intestine manipulation (IM) leads to local bowel wall inflammation subsequently spreading over the entire gastrointestinal tract. Previously, we demonstrated that this so-called gastrointestinal field effect (FE) is immune-mediated. The aim of this study was to investigate the role of secondary lymphoid organs [mesenteric lymph nodes (MLN), gut-associated lymphoid tissue (GALT)] in IM-mediated FE by employing mice with deficient secondary lymphoid organs (aly/aly, MLN ex) or by administration of 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol (FTY720), an immunomodulating agent that inhibits emigration of lymphocytes out of lymphoid organs. Small bowel muscularis, and colonic muscularis from wild-type mice as control, from aly/aly mice, FTY720-treated mice (daily dose of 1.0 mg/kg mouse ip starting 3 days before surgical procedure), and wild-type mice that had undergone removal of mesenteric lymph nodes before IM (MLN ex mice) were obtained after selective retrieval of the jejunum or sham operation. FE was analyzed by measuring transit time of orally administered fluorescent dextran in the gastrointestinal tract. Previously, we demonstrated that this so-called gastrointestinal field effect (FE) is immune-mediated. The role of lymphoid tissue in the attenuation of the postoperative ileus; gastrointestinal field effect; aly/aly; 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol

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G401

ABDOMINAL SURGERY OR MAJOR ABDOMINAL TRAUMA RESULT IN POSTOPERATIVE ILEUS WITH VARIABLE GRADUAL MANIFESTATION. ITS IMPLICATIONS INCLUDE POSTOPERATIVE DISCOMFORT, PROLONGED HOSPITALIZATION, AND, SUBSEQUENTLY, HIGHER PATIENT MORBIDITY, ASPIRATION PNEUMONIA, SEPSIS, AND MULTIORGAN FAILURE. THEREFORE, ITS IMPACT ON SOCIOECONOMIC COSTS IS HIGH (28, 39, 46, 58). THE PATHOPHYSIOLOGY OF POSTOPERATIVE ILEUS AND THE POSTOPERATIVE GASTROINTESTINAL DYSMOTILITY HAVE BEEN INVESTIGATED EXTENSIVELY IN RECENT YEARS. POSTOPERATIVE ILEUS IS MARKED BY A NEUROGENIC PHASE AND A CLINICALLY RELEVANT PROLONGED INFLAMMATORY PHASE. NEURONAL MECHANISMS CANNOT EXCLUSIVELY EXPLAIN THE PERPETUATION OF POSTOPERATIVE ILEUS BECAUSE OF EARLY NEURAL RECOVERY SHORTLY AFTER WOUND CLOSURE (5, 6, 15). WE PREVIOUSLY REPORTED A TIME-AND TRAUMA-DEGREE-DEPENDENT INFLAMMATORY CASCADE (29, 30), INFILTRATION OF LEUKOCYTES (34–36, 65, 78), MACROPHAGES (19, 20, 31, 81), IMMUNOLOGICALLY COMPETENT CELLS (18, 29, 30, 34, 60, 62, 65, 76, 78), AND AN UPREGULATION OF PROINFLAMMATORY CYTOKINES WITHIN THE INTESTINAL MUCOSA (30, 32–34, 59, 61, 64, 75–77, 80, 82). PHARMACOLOGICAL OR GENETIC BLOCKAGE OF MACROPHAGES AMELIORATED POSTOPERATIVE ILEUS IN RODENTS (81, 83). IT HAS ALSO BEEN SHOWN THAT INFLAMMATORY INFILTRATES AND HYPOMOTILITY OCCUR IN THE UNMANIPULATED STOMACH AND COLON AFTER STANDARDIZED JEJUNAL MANIPULATION. THIS PROPAGATION OF THE INTESTINAL INFLAMMATION TO UNMANIPULATED PARTS OF THE GASTROINTESTINAL TRACT HAS BEEN TERMED GASTROINTESTINAL FIELD EFFECT (FE) (65, 78). WE ALSO OBSERVED THIS FE IN RODENTS AFTER ORTOPHOTIC SMALL BOWEL TRANSPLANTATION EXCLUDING A SPINAL OR SUPRASPINAL REFLEX (1, 75, 76). WHEN THE LYMPHATIC DUCT WAS CLOSED, FE COULD BE ABRAGATED (34, 75). WE DESCRIBED RECRUITMENT, ACTIVATION, MATURATION, AND MIGRATION OF DENDRITIC CELLS IN THE INTESTINAL MUCOSA. THESE DENDRITIC CELLS INTERACT WITH NAIVE T CELLS IN VITRO (41). WE HAVE ALSO SHOWN THAT INTESTINAL SURGERY ACTIVATES INTESTINAL CD103+CD11b+ DENDRITIC CELLS TO PRODUCE INTERLEUKIN (IL)-12. THIS PROMOTES INTERFERON-γ (IFN-γ) PRODUCTION BY CCR9+ MEMORY T-HELPER TYPE 1 (TH-1) CELLS, WHICH ACTIVATES MACROPHAGES. IL-12 ALSO CAUSED SOME TH-1 CELLS TO MIGRATE FROM SURGICALLY MANIPULATED SITES THROUGH THE BLOODSTREAM TO UNMANIPULATED INTESTINAL AREAS WHERE THEY INDUCED ILEUS. THESE FINDINGS INDICATED THAT POSTOPERATIVE ILEUS IS A TH-1 IMMUNE-MEDIATED DISEASE (18). HOWEVER, WE ONLY DESCRIBED MIGRATING TH-1 CELLS IN THE PORTAL VEIN BLOOD. IT REMAINED UNCLEAR WHETHER THESE CELLS ARE DIRECTLY EMIGRATING FROM THE PERIPHERAL TISSUE TO THE BLOODSTREAM OR WHETHER THEY ARE ALREADY RECIRCULATING AFTER FIRST PASSING THROUGH THE LYMPHATIC SYSTEM AND SECONDARY LYMPHOID ORGANS.

T CELLS ARE USUALLY ACTIVATED IN SECONDARY LYMPHATIC ORGANS. THEREFORE, THE AIM OF OUR STUDY WAS TO ELUCIDATE THE ROLE OF SECONDARY LYMPHOID ORGANS IN GASTROINTESTINAL FE. TO ANSWER THIS QUESTION, WE USED SEVERAL APPROACHES AS FOLLOWS: 1) MICE LACKING SECONDARY LYMPHATIC ORGANS (ALY/ALY) (37, 38, 54, 72), 2) MICE DEVOID OF MESENTERIC LYMPH NODES (MLN EX), AND 3) MICE TREATED WITH 2-AMINO-2-[2-(4-OCTYLPHENYL)ETHYL]-1,3-PROPANEDIOL (FTY720), WHICH ARRESTS ACTIVATED T CELLS IN THE LYMPHOID ORGANS AND INHIBITS THEIR MIGRATION TO AFFECTED SITES AS WELL AS THEIR HOMING BY SPhINGOSINE 1-PHOSPHATE-MEDIATED MECHANISMS (8, 9, 23, 26, 40, 44, 47, 68, 69). HERE, WE REPORT...
that all of these experimental approaches supported a role for secondary lymphatic organs in FE.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice weighing 20–25 g were obtained from Charles River WIGA (Sulzfeld, Germany). Aly/aly knockout mice weighing 20–25 g were obtained from Dr. A. Limmer, Institute of Molecular Medicine and Experimental Immunology (Bonn, Germany). All transplantic models were on a C57Bl/6 background. All experiments were performed in accordance with German legalization regarding the protection of animals. The principles of laboratory animal care (National Institutes of Health Publication No. 85–23, revised 1985) were adhered to. The studies were reviewed and approved by the authority for animal protection rights of the District Council of Cologne/Germany according to the German legalization and the independent ethics committee for animal and human studies of the University of Bonn. The animals were maintained on a 12:12-h light-dark cycle and provided with commercially available chow (Altromin, Lage, Germany) and tap water ad libitum.

Experimental Groups and Studies

For the mice experiments eight groups were defined and studied. Groups 1 and 2 consisted of C57Bl/6 mice, groups 3 and 4 of aly/aly knockout mice, groups 5, 6, and 7 of C57Bl/6 mice that had undergone removal of mesenteric lymph nodes before standardized surgical procedures, and groups 7 and 8 of C57Bl/6 mice treated with a daily dose of 1.0 mg/kg mouse FTY720 intraperitoneally starting 3 days before standardized surgical procedure. In groups 1, 3, 5, and 7, standardized intestinal manipulation (IM) was performed as described previously (3, 34, 65). Surgery was performed under sterile conditions. Animals were lightly anesthetized with isoflurane. After a midline incision into the peritoneal cavity, the manipulation model in groups 1, 3, 5, and 7 was constructed by eventration of the small bowel to the left onto moist gauze with additional light manipulation of the entire small bowel with two moist cotton applicators lasting in total 15 min. Contact with or stretching on the stomach or colon was strictly avoided. Next, the laparotomy was closed with a double-layer running suture. Animals of groups 2, 4, 6, and 8 underwent sham operation. In the sham-operated animals, midline incision into the peritoneal cavity, short dilatation of the abdominal wall by two inserted wall retractors without touching the gastrointestinal tract, and subsequent wound closure by double-layer running suture was performed. In animals of groups 5 and 6, mesenteric lymph nodes were removed shortly after midline laparotomy by eversion of the ileocolonic junctional zone to reach all mesenteric lymph nodes and by subsequent excision of all visible lymph nodes (32-fold magnification of the surgical microscope) under protection of the mesenteric vessels followed by standardized IM or sham operation followed in the same surgical session. None of the sham operation or small bowel manipulation procedures caused mortality. All animals recovered quickly from surgery and began to eat and drink within 12 h. The control animals of each C57Bl/6 group and the aly/aly mouse strain were neither operated nor treated. Controls of groups 7 and 8 were only intraperitoneally injected with FTY720. For this study, animals were killed between 0 and 24 h after manipulation or sham operation. Intestine and colon were used for Taqman-PCR, functional measurements, and histochemistry.

Small Bowel and Colon Preparation

At the determined time points, mice were anesthesitized by isoflurane inhalation, and the abdomen was opened via midline laparotomy. The abdominal aorta was clamped above the superior mesenteric artery and shortly above the aortic bifurcation and flushed with 5 ml of cold (4°C) sodium chloride (0.9%) to remove nonadherent blood cells from the vasculature. Small bowel and colon were removed and placed in iced preoxygenated Krebs-Ringer buffer (KRB). For gene expression analysis, murine jejunal and colonic muscularis was taken 0, 3, 6, and 24 h after surgical procedure and isolated from the mucosa-submucosa by skipping 5-cm-length portions of the intestine or colon over a glass rod and stripping the muscularis from the mucosa, as described previously. The isolated muscularis was snap-frozen in liquid nitrogen and stored at −80°C. Functional studies described below were carried out immediately on bowel specimens taken from the middle jejuntum and colon. Three jejunal and three colonic segments per animal were used for histochemical analysis.

Myeloperoxidase Staining

Specimens for histochemical analysis were prepared 24 h after surgical procedure and performed on whole mounts of the distal jejunum and the colon (n = 5–7 each group) as described before (35). Freshly prepared whole mounts were stained with Hanker-Yates reagent as described previously (82): freshly prepared whole mounts were immered in a mixture of 10 mg Hanker-Yates reagent (Sigma), 10 ml KRB, and 100 μl 3% hydrogen peroxide (Sigma) for 10 min. The reaction was stopped with cold KRB. MPO− cells were counted under a microscope (TE-2000; Nikon, Duesseldorf, Germany) in five randomly chosen areas at a 100-fold magnification in each specimen.

Real-Time Reverse Transcription-Polymerase Chain Reaction

Proinflammatory gene expression was analyzed in control, sham-operated, and manipulated animals of all mouse strains 3, 6, and 24 h after surgical procedure (n = 5–7 each group). Total RNA was analyzed in muscularis externa specimen that was prepared and reverse transcribed to cDNA as described before (81). In brief, total RNA extraction was performed using an RNA extraction kit (Macherey & Nagel, Düren, Germany), and contaminating DNA was eliminated from the RNA preparations using Ambion DNA-free solution (Ambion, Huntington, UK). Aliquoted RNA (200 ng) was processed for cDNA synthesis. Expression of mRNA was quantified in triplicate by a SYBR Green reverse transcription-polymerase chain reaction with gene expression assays for β-actin as housekeeping gene, IL-6, IL-10, tumor necrosis factor-α (TNF-α), and CCL3 [macrophage inflammatory protein-1α (MIP-1α)]. All primer probes were designed by Primer Express Software (Applied Biosystems, Darmstadt, Germany) and purchased from Invitrogen (Karlsruhe, Germany). The designed primer sequences are shown in Table 1. PCR reaction was performed in Universal PCR Mastermix by amplification of 10 ng cDNA for 40 cycles (95°C × 15 s, 60°C × 1 min) on an ABI Prism 7900HT. All PCR reagents were obtained from Applied Biosystems. Dissociation of the PCR products by a melting curve analysis protocol consistently showed specific single melting peaks for all used primer pairs. Data quantification was performed by the ∆∆CT method as described previously by Schmittgen et al. (63).

Functional Studies

Jejunal and colonic smooth muscle activity was measured as previously described (34). In brief, after preparation, mucosa-free
circular muscularis externa strips were equilibrated in KRB-perfused organ chambers at 37°C for 1 h. One end of each strip was tied to a fixed post, and the other was attached to an isometric force transducer (ADL, Heidelberg, Germany) connected to the bridge amplifier and powerlab system (ADI). Dose-response curves of muscle contractions were generated by exposing the muscle strip to increasing concentrations of the muscarinic agonist bethanechol (0.1–300 μmol/l) for 10 min, followed by a 10-min wash period (KRB). The contractile response was analyzed with the ADI Chart, and contractions were calculated as grams per square millimeter per second by conversion of weight and length of the strip (position in the organ bath) to square millimeters of tissue. At the end of each experiment, muscle strips were blotted dry and weighed.

Gastrointestinal transit (GIT) was measured in sham-operated and manipulated animals 25.5 h after surgical procedure (n = 10 each group) as described before (34, 65, 81). Twenty-four hours after surgical procedure, animals were lightly anesthetized and given fluorescent-labeled dextran (70,000 mol wt; Sigma-Aldrich, Taufkirchen, Germany) via gavage into the stomach. Ninety minutes after administration, stomach contents, the entire small bowel (10 equal parts), cecum, and colon (10 equal parts) were divided into 15 pieces to assess gastric and small intestinal transit (29). The intestinal contents were processed according to our previous description, and the fluorescent signal in each bowel segment was read in a multiwell fluorescence plate reader (CytoFluorII; wave length for excitation 485/20 mm and for emission 530/30 mm). Data were expressed as percentage of activity per intestinal segment. A median histogram of the fluorescence was then plotted for analysis of the transit, and geometric center (GC) was calculated from each experiment as \[\frac{1}{n} \sum (\% \text{FITC per segment} \times \text{segment number})/100.\]

Colonic transit (CT) measurement was performed 24 h after operation as described before (10). Briefly, a 2-mm glass ball was peranally inserted 3 cm into the colon. Before insertion, colonic patency was ensured by exclusively inserting the rod 3 cm into the colon. Mice were lightly anesthetized with isoflurane for the whole procedure and woke up within 40 s after glass ball insertion. CT time was calculated as the period between insertion and excretion of the ball.

Drugs and Solutions

A standard KRB solution was used with the following constituents (μmol/l): 137.4 Na⁺, 5.9 K⁺, 2.5 Ca²⁺, 1.2 Mg²⁺, 134 Cl⁻, 15.5 HCO₃⁻, 1.2 HPO₄²⁻, and 11.5 glucose. PBS was purchased from Lonza (Verviers, Belgium). All other chemicals used for this study (if not separately mentioned) were purchased from Sigma-Aldrich. Pure substance of FTY720 was obtained from Novartis Germany (Munich, Germany) and was dissolved in PBS and given intraperitoneally daily starting at day 3 before surgical procedures in a dosage of 1.0 mg/kg mouse.

Data Analysis

Data were compiled as means ± SD. Statistical analysis was performed by the unpaired Student’s t-test or Kruskal-Wallis test. Setup data and PCR data were analyzed by two-way ANOVA using the Bonferroni test comparing all groups with each other. Data were considered statistically significant at P < 0.05; significance levels were P < 0.05, P < 0.01, and P < 0.001.

RESULTS

Polymorphnuclear Cell Infiltration is Significantly Reduced by Loss of Secondary Lymphoid Organs and by Treatment with FTY720

We previously reported that IM initiates the recruitment of polymorphonuclear cells (PMNs) into the manipulated jejunal muscularis over time. A significant PMN recruitment also occurs within the unmanipulated stomach and colon (65).

**Jejunal muscularis.** To analyze infiltration of PMNs into the jejunum, we prepared muscularis whole mounts of the jejunum and counted the number of PMN 24 h after IM. Infiltration of PMNs in aly/aly and FTY720-treated mice did not change significantly (wild-type mice: 221.9 ± 33.4, aly/aly mice: 253.0 ± 19.40, FTY720-treated wild-type mice: 263.8 ± 20.4, MLN ex mice: 177.0 ± 7.7). Control intestinal whole mounts had only minimal PMN counts, with 4.5 ± 1.9 in wild-type mice, 1.3 ± 0.5 in aly/aly mice, 5.3 ± 2.3 in FTY720-treated mice, and 29.0 ± 6.7 in MLN ex mice.

The sham-operated and the manipulated animals of each mouse strain differed significantly (wild-type, P = 0.0030; aly/aly, P < 0.0001; FTY720-treated wild-type, P < 0.0001; MLN ex mice, P < 0.0001). There was no significant difference in manipulated animals of all mouse strains (wild-type vs. aly/aly, P = 0.1396; wild-type vs. FTY720-treated mice, P = 0.8072).

Although there was a significant difference in comparison of sham-operated C57Bl/6 mice vs. sham-operated MLN ex mice (P = 0.0163), this difference between the mouse strains disappeared after IM (C57Bl/6 IM vs. MLN ex IM, P = 0.3455; Figs. 1, A, C, E, G, and I, and 2A).

**Colonic muscularis.** However, PMN infiltrates in the adjacent unmanipulated colonic muscularis of all investigated transgenic, modified, and FTY720-treated animals were significantly reduced (wild-type mice: 126.3 ± 21.7; aly/aly mice: 10.5 ± 4.1, P = 0.0024; FTY720-treated mice: 27.4 ± 2.3, P = 0.0001; MLN ex mice: 46.3 ± 7.5, P = 0.0258), whereas the number of PMNs in the control whole mounts did not vary significantly. The number of PMNs in sham-operated MLN ex animals and manipulated MLN ex mice was not significantly different (46.3 ± 7.5 in sham-operated mice vs. 48.3 ± 6.5 in manipulated mice, P = 0.8435), indicating that removal of MLN without IM also leads to infiltration of PMNs into the colon. Typical PMN infiltrates observed in muscularis whole mounts of the manipulated jejunum and the adjacent unmanipulated colon are shown in Figs. 1, B, D, F, H, and K, and 2B.

**Lack of Either MLN or Secondary Lymphoid Tissue and Treatment with FTY720 Improve Colonic Muscle Function After IM**

The degree of jejunal and colonic muscular dysfunction was analyzed in the following experiments by measurement of in vivo GIT, in vivo CT, and in vitro muscle contractility. All three methods have been described previously using different strains of rodents (15, 34, 65, 81).

**In vivo GIT.** GIT time is one of the most evident parameters for detection and quantification of ileus. We analyzed GIT time in aly/aly mice, FTY720-treated mice, MLN ex mice, and wild-type mice 24 h after IM. To this end, the distribution of orally administered nonabsorbable fluorescent dextran (mol wt 70,000) along the gastrointestinal tract was determined 1.5 h after gavage.

The sham-operated mice of the following mouse strains did not differ significantly from each other: wild-type vs. FTY720-treated mice vs. aly/aly mice (wild type 10.87 ± 0.53 vs. FTY720-treated mice 10.50 ± 0.22, P = 0.6686; wild-type 10.87 ± 0.53 vs. aly/aly mice 12.56 ± 0.32, P = 0.0552).

Manipulated wild-type mice had a significantly delayed GIT (GC: 3.22 ± 0.39) compared with sham-operated wild-type...
mice (GC: 10.87 ± 0.53, P = 0.0001). GIT was significantly better in aly/aly mice and FTY720-treated mice compared with manipulated wild-type mice, but it was still significantly delayed compared with sham-operated mice [in FTY720-treated mice: GC (IM) 7.61 ± 0.51 vs. (sham) 10.50 ± 0.22, P = 0.0032; FTY720 (IM) vs. wild-type (IM), P = 0.0024, FTY720 (IM) vs. wild-type (sham), P = 0.0076; and in aly/aly mice: GC 9.21 ± 0.36 (IM) vs. 12.56 ± 0.32 (sham), P = 0.0012; aly/aly (IM) vs. wild-type (IM), P = 0.0001; aly/aly (IM) vs. wild-type (sham), P = 0.0076].

Removal of MLN before the surgical procedure showed particular results. GIT, which was not significantly different between sham-operated and manipulated mice after MLN removal [GC: 7.77 ± 1.19 (IM) vs. 8.11 ± 0.78 (sham), P = 0.8472] and also not between manipulated MLN ex animals and sham-operated wild-type mice [MLN ex (IM) vs. wild type (sham), P = 0.09], was significantly better than GIT of wild-type mice after IM [MLN ex (IM) vs. wild type (IM), P = 0.0434]. Although GIT in MLN ex mice was improved, we also observed delayed GIT in sham-operated MLN ex mice, indicating that the surgical procedure of MLN removal itself alters GIT.

The improved GIT of FTY720-treated mice (P = 0.0024), MLN ex mice (P = 0.434), and aly/aly mice (P = 0.0001) differed significantly from GIT of wild-type mice after IM. However, a statistically significant difference between FTY720-treated mice and aly/aly mice remained after IM on the one side and the sham-operated wild-type mice on the other side (Fig. 3).

**In vivo CT.** Measurement of GIT revealed a significant improvement of the intestinal motility, which was mainly limited to the stomach and small bowel. To analyze the transit within the colon, we investigated CT time 24 h after IM. As displayed in Fig. 4, IM significantly delayed CT in wild-type mice compared with the sham-operated group [491.8 ± 45.7 vs. 75.00 ± 4.34 s, P < 0.0001], aly/aly mice [178.8 ± 31.9 s, P = 0.0005], FTY720-treated mice [106.8 ± 8.5 s, P < 0.0001], and MLN ex mice [223.2 ± 36.4, P = 0.0018] had a significantly improved CT time after IM compared with wild-type mice. Although differences between aly/aly mice and FTY720-treated mice (P = 0.0606) and between aly/aly mice and MLN ex mice (P = 0.3855) were not statistically significant, this was the case between FTY720-treated mice and MLN ex mice (P = 0.0144). In all investigated transgenic or pretreated mouse groups, CT time did not differ significantly between sham-operated animals and manipulated animals (aly/aly: P = 0.0503; FTY720-treated mice: P = 0.2440; MLN ex mice: P = 0.8619).

**In vitro contractility.** Jejunal and colonic circular smooth muscle specimens were analyzed for spontaneous and bethanechol-stimulated contractions (see Fig. 6). Baseline activity of all groups did not differ significantly. Stimulation of sham-operated muscle strips caused a dose-dependent increase in the generation of large phasic contractions.

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**Jejunal contractility.** After IM, jejunal contractility was significantly (P = 0.0015) decreased in wild-type mice, FTY720-treated mice, and in aly/aly mice (e.g., at a dosage of 100 μmol/l bethanechol, sham-operated animals: 2.84 ± 0.27, 3.38 ± 0.51, and 2.81 ± 0.08 g·mm⁻²·s⁻¹, respectively, vs. manipulated animals: 1.23 ± 0.29, 1.34 ± 0.34, and 1.29 ± 0.45 g·mm⁻²·s⁻¹, respectively). No significant difference in jejunal contractility was observed in wild-type mice, FTY720-treated mice, and aly/aly mice in sham-operated groups. Likewise, there was no significant difference in jejunal contractility in the manipulated animals of all mouse strains (Fig. 5, A and B).

**Colonic contractility.** In contrast, after IM, colonic contractility was significantly improved in FTY720-treated mice and in aly/aly mice, and no significant differences were observed compared with sham-operated mice. However, colonic contractility of wild-type mice remained impaired after IM. Lack of secondary lymphoid organs (aly/aly mice) and arrest of T cells in the secondary lymphoid organs (FTY720) cancelled this contractility suppression, whereas contractile force was at the control (sham-operated) level. For example, colonic control muscle generated 4.65 ± 0.36 g·mm⁻²·s⁻¹, whereas IM caused a significant 50% reduction in colonic contractile force to 2.26 ± 0.48 g·mm⁻²·s⁻¹ in wild-type mice (P = 0.0096) as measured in response to 100 μM bethanechol. Importantly, at this dosage, there was no significant difference between wild-type sham-operated (control) animals (4.65 ± 0.36 g·mm⁻²·s⁻¹) and colonic contractility both in FTY720-treated mice (4.33 ± 1.06 g·mm⁻²·s⁻¹) and in aly/aly mice (4.01 ± 0.28 g·mm⁻²·s⁻¹) after IM (Fig. 5, C and D).

**Lack of Either MLN or Secondary Lymphoid Tissue and Treatment with FTY720 Influence Proinflammatory Gene Expression After IM in Mice**

To further investigate the regulation of proinflammatory cytokines, we analyzed cytokines IL-6, TNF-α, MIP-1α, and IL-10 in sham-operated and manipulated mice. IL-6 is a prototypical pluripotent proinflammatory mediator that has been investigated in the manipulated small intestinal and in the unmanipulated colonic muscularis after IM (29, 30, 32, 34, 65, 75, 82). Furthermore, the inflammatory markers TNF-α, chemokine ligand 3, or MIP-1α and as a representative anti-inflammatory cytokine IL-10, also known as cytokine synthesis inhibitory factor, were analyzed in the colonic muscularis of all mouse strains after sham operation and IM. Gene expression was normalized to intestinal and colonic control tissue and compared with sham-operated and manipulated animals of each mouse group.

Sham-operated animals of all mouse strains showed no significant upregulation of any of the investigated gene mRNA in the study time course and did not differ significantly from each other. In the jejunal muscularis of wild-type mice, IL-6 mRNA upregulation was significantly higher after IM: 170-fold at 3 h,
2,120-fold at 6 h, and 1,660-fold at 24 h after IM. In the colonic muscularis of wild-type mice, the IL-6 mRNA upregulation was also significantly higher after IM: 34-fold at 6 h and 77-fold at 24 h after IM. Additionally, there was a significant upregulation of TNF-α (19-fold), MIP-1α (13-fold), and of the anti-inflammatory cytokine IL-10 (30-fold) in the colonic muscularis 24 h after IM in wild-type mice. In contrast, aly/aly mice, MLN ex mice, and FTY720-treated mice showed no statistically significant upregulation of these mediators in the colonic muscularis 24 h after IM compared with sham-operated animals (Fig. 6).

The IL-6 expression in the jejunal muscularis of all mouse strains was time-dependent and peaked 6 h after IM (Fig. 6A). However, this upregulation of IL-6 could only be seen in the colonic muscularis of wild-type mice where it reached its highest measured level 24 h after IM (Fig. 6B). We also observed a time-dependent upregulation of all other investigated mediators in the colonic muscularis of C57Bl/6 mice (Fig. 6C). PCR analysis of the colonic muscularis of aly/aly mice and FTY720-treated mice could not unveil a time-dependent upregulation of the investigated mediators as shown in Fig. 6. Similar results were obtained in MLN ex mice at 24 h after IM. The differences between the sham-operated mice of all mouse strains and the manipulated aly/aly mice, FTY720-treated wild-type mice, and the MLN ex mice are not statistically significant.

The sham-operated animals of all mouse strains did not differ significantly from each other. Extraordinary roles play both the sham-operated and the manipulated wild-type mice with simultaneous removal of mesenteric lymph nodes, since they show significantly more MPO infiltration in colonic muscularis compared with other sham-operated mice while remaining significantly less than in manipulated wild-type mice. This phenomenon is due to the technique of removal, which requires evertation of the ileocolonic junction, i.e., slight manipulation in itself. Values are expressed as means ± SE counted at a 100-fold magnification; n = 5–7. The field of view was 500 μm².

Fig. 2. Histogram showing counts of MPO⁺ infiltrates in muscularis of the manipulated jejunum (A) and the unmanipulated adjacent colon (B) after IM. Myeloperoxidase (MPO) staining was performed 24 h after sham operation or IM in wild-type mice, aly/aly mice, wild-type mice treated with FTY720 ip, and wild-type mice after removal of mesenteric lymph nodes before IM. IM leads to a significant MPO⁺ infiltration into the jejunal muscularis in all mouse strains (A). This infiltration was significantly less in the colonic muscularis layer of aly/aly mice and pretreated wild-type mouse strains compared with the untreated wild-type mice (B). Colonic MPO⁺ infiltration in aly/aly mice and FTY720-treated mice did not vary significantly from the sham-operated animals (B; ns, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001). The sham-operated animals of all mouse strains did not differ significantly from each other. Extraordinary roles play both the sham-operated and the manipulated wild-type mice with simultaneous removal of mesenteric lymph nodes, since they show significantly more MPO⁺ infiltrates in colonic muscularis compared with other sham-operated mice while remaining significantly less than in manipulated wild-type mice. This phenomenon is due to the technique of removal, which requires evertation of the ileocolonic junction, i.e., slight manipulation in itself. Values are expressed as means ± SE counted at a 100-fold magnification; n = 5–7. The field of view was 500 μm².

Fig. 3. IM delays gastrointestinal transit in wild-type mice, whereas the passage of the nonabsorbable fluorescein isothiocyanate (FITC)-labeled dextran [mol wt (mw) 70,000] in aly/aly mice and wild-type mice treated with FTY720 before IM is significantly improved but not yet as good as in sham-operated animals. Mean geometric center (GC) of FITC distribution in the gastrointestinal tract values of gastrointestinal transit are presented here for all investigated mouse strains. Gastrointestinal transit time was significantly better in manipulated aly/aly mice and FTY720-treated mice but still impaired compared with sham-operated mice. Gastrointestinal transit of animals with simultaneous removal of their mesenteric lymph nodes does not display a significant difference between sham-operated and manipulated animals but is still significantly better than in unmodified manipulated wild-type mice. *P < 0.05, **P < 0.01, and ***P < 0.001.
We have established a model of a locally based pan-intestinal effect of the parasite on the noninfected regions and trauma-degree-dependent inflammatory cascade (29, 30), infiltration of leukocytes (34–36, 65, 78), macrophages (19, 20, 31, 81), and immunologically competent cells (29, 30, 34, 60, 62, 65, 76, 78) and an upregulation of proinflammatory and kinetically active cytokines within the intestinal muscularis with a subsequent hypomotility (30, 32–34, 59, 61, 64, 75–77, 80, 82). Interestingly, this local inflammation is propagated to unmanipulated sections of the gastrointestinal tract, such as the colon and the stomach, with recruitment of inflammatory infiltrates and upregulation of proinflammatory and kinetically active cytokines (65, 65, 79). Anti-inflammatory mediators, such as IL-10, also play a role in the postoperative ileus. During the inflammatory response, they are equally upregulated later and play an obligatory role in postoperative intestinal recovery. We could observe a balanced time-dependent increase of IL-10, which is produced by activated macrophages and T cells, and a decrease in proinflammatory cytokines not earlier than 24 h after IM (67). An age-dependent balance in the release of proinflammatory cytokines and the anti-inflammatory IL-10 has been reported in the postoperative ileus (55).

The pharmacological or genetic blockage of macrophages could ameliorate the postoperative ileus in rodents (81, 83). In recent years, many selective anti-inflammatory strategies have been described for postoperative ileus prevention, mostly focusing on singular proinflammatory mediators such as IL-6 (82), adhesion molecules, and chemokines such as intercellular adhesion molecule-1 (30) and monocyte chemotactic protein-1 (80). Some authors described inhibitory kinetic factors, such as nitric oxide or prostaglandins (33, 36, 64). Most of those strategies only inhibited singular proinflammatory targets with subsequent beneficial effects on postoperative ileus, whereas their clinical implementation has so far failed to display benefits for patients (52). Interventions, such as inhibition of singular mediators of the complex and multifactorially based postoperative ileus, do not seem to be as efficient as the blockage of involved cells, such as macrophages (81, 83) or T cells (18).

Most recently, we have shown that intestinal surgery activates intestinal dendritic cells to produce IL-12. This promotes IFN-γ secretion by CCR9+ memory TH-1 cells, which activate macrophages. IL-12 also caused some TH-1 cells to migrate from surgically manipulated sites through the bloodstream to unmanipulated intestinal areas where they induced ileus. These findings indicated that postoperative ileus is a TH-1 immunemediated disease (18). However, we only described migrating TH-1 cells in the portal vein blood. Attenuation or abolition, respectively, of the gastrointestinal FE through lack of secondary lymphoid tissues or FTY720 induced arrest of T cells within the secondary lymphoid organs or the peripheral tissue (11, 18, 44) suggest that prolonged postoperative ileus is due to an immune interaction mainly localized within the secondary lymphoid organs. Local intestinal inflammation after manipulation was not significantly reduced after removal of mesenteric lymph nodes or by lack of secondary lymphoid organs, but after modulation of T cell activities by FTY720 (18, 81).

Nevertheless, GIT was significantly improved in these mouse strains compared with the wild-type mice, but not quite as good as in sham-operated mice. In contrast, CT was significantly improved and not significantly different from sham-operated animals in aly/aly mice, in wild-type mice after removal of mesenteric lymph nodes, and in wild-type mice...
treated with FTY720. Previously, we have shown that local intestinal inflammation after IM is generated independently from T cell interaction but causes ileus only when a TH-1 memory response is generated. These TH-1 cells play at least two roles: amplification and perpetuation of the local inflammation, and migration via lymphatic organs and blood to the unmanipulated gastrointestinal segments, where they activate resident macrophages resulting in paralysis of the unmanipulated gastrointestinal parts. TH-1 cells and mediators are essential in initiating postoperative ileus (17, 42). Interventions in the immunological interaction between dendritic cells and TH-1 cells as realized by FTY720 or lack of interaction sites (aly/aly and removal of mesenteric lymph nodes) are followed by disturbed activation of TH-1 cells and thus by an improved gastrointestinal and colonic motility. The aly/aly mouse model provides a systemic absence of secondary lymphoid organs, whereas the wild-type mice still have secondary lymphoid organs within the bowel wall after removal of mesenteric lymph nodes. This explains why delay in GIT is longer in MLN ex mice than in aly/aly mice. Measurement of GIT reflects the motility of unmanipulated gastrointestinal segments distant from the selectively manipulated jejunal segment. This possibly explains why GIT is improved while in vitro contractility of the manipulated jejunal segment remains as impaired as in wild-type mice. However, de Jonge et al. described an improved gastric emptying induced by postoperative neural blockade with hexamethonium or guanethidine without affecting small-intestinal transit after IM. However, these authors, unlike us, could not show increased myeloperoxidase-positive infiltrates in the unmanipulated colon (15). In contrast, Mueller et al. could show that vagal inhibition of the intestinal immune response is present at 9 h but not detectable earlier after IM (56). All in all, although the improved gastrointestinal and CT might be explained by an altered immunological activity, our findings do not exclude the contribution of neuronal signals to the postoperative ileus (2, 14, 42).

aly/aly mice, which carry a point mutation in the nuclear factor κB-inducing kinase gene, are characterized by the systemic absence of lymph nodes and Peyer’s patches, disorganized splenic and thymic architectures, and immunodeficiency (21, 66). The organized secondary lymphoid organs play a crucial role in the induction of naïve T and B cells. These structures also provide the basis for cooperative interactions between antigen-presenting cells, T cells, and B cells, which
are, e.g., a prerequisite for recovery from primary virus infections via skin or via blood (25, 37, 38), but do not reject, e.g., skin allografts (54). Lack of secondary lymphoid organs leads to severe immunodeficiency with impaired functions of the dendritic cells and T cells and deficient interaction of these cells (24, 72). Cancellation of the gastrointestinal FE in aly/aly mice implies a central role of organized secondary lymphoid tissue (mesenteric lymph nodes, Peyer’s patches, gut-associated lymphoid tissue of the bowel wall) in the propagation of the pan-enteric inflammatory response to the localized IM as a representative of an intestinal trauma.

However, aly/aly mice generally lack secondary lymphoid organs. Therefore, we sought to specify the role of the mesenteric lymph nodes in the propagation of the FE by removing the mesenteric lymph nodes immediately before IM or sham operation. This procedure revealed no significant differences between sham-operated animals and manipulated animals in GIT, CT, neutrophil infiltrate counts within the colonic muscularis layer, and in upregulation of proinflammatory cytokines on mRNA level, however, compared with the other investigated mouse groups, such as FTY720-treated animals and aly/aly mice, sham-operated and manipulated mice had significantly impaired GIT, CT, and significantly higher neutrophil infiltrate counts and higher upregulation of proinflammatory cytokines on mRNA level, which was, however, still significantly better than in wild-type mice after IM. These alterations in mice after removal of their mesenteric lymph nodes can be explained by the mandatory eventration of the ileocolonic junction when removing the mesenteric lymph nodes, which per se represents a surgical trauma to the ileum and the adjacent colon and induces an inflammatory response of the bowel wall with all its implications as described above. Despite this fact, there was no significant difference between sham-operated animals and animals after IM, suggesting a major role of the mesenteric lymph nodes in the mediation of the gastrointestinal FE. The mesenteric lymph nodes represent a central site of immune interaction between activated dendritic cells and naïve T cells, and they provide the microenvironment for T cell activation and homing (11, 24, 25, 38, 48, 72).

With the data generated by structural loss of secondary lymphoid organs or removal of mesenteric lymph nodes, we next investigated the results of functional alterations in immune reactivity by administration of FTY720. The sphingosine analog of myriocin FTY720 inhibits emigration of lymphocytes from secondary lymphoid organs or mucosal tissues into the blood and sequesters peripheral blood lymphocytes in secondary lymphoid organs without affecting immune reactions (13, 27). FTY720 is converted to FTY720 phosphate. FTY720 phosphate binds on the sphingosine 1-phosphate receptors S1P1, S1P3, S1P4, and S1P5 on the cell surface (9).

Fig. 6. Relative mRNA expression of proinflammatory cytokines, such as interleukin (IL)-6, within jejunal muscularis (A), IL-6 in colonic muscularis (B), and IL-6, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-1α, and anti-inflammatory cytokine IL-10 in colonic muscularis (C) in wild-type mice compared with aly/aly mice and FTY720-treated mice. Values are expressed as means ± SE. Statistically significant differences (***P < 0.001) between displayed samples (n = 4–5). mRNA expression of investigated mediators in the colonic muscularis 24 h after IM of aly/aly mice and FTY720-treated mice did not differ significantly from wild-type mice after sham operation (C). CTL, control.
Most important is the effect of FTY720 phosphate to the S1P1 receptors of T and B lymphocytes, whereby S1P1-dependent emigration of lymphocytes from secondary lymphoid organs to blood is inhibited (8, 50). A single dosage of 1 mg/kg mouse has been described as sufficient to reduce the number of peripheral blood lymphocytes, to block lymphocytes trafficking to peripheral organs (e.g., gut homing) by arresting them within the secondary lymphoid organs, and to inhibit T cell functions without suppressing the proliferative response of T cells (26, 43, 47). Daily administration of FTY720 at the same dosage over 2 wk induced severe systemic lymphopenia and marked suppression of lymphocyte proliferative responses in normal wild-type mice, and it interrupted permanent T cell circulation between gut and secondary lymphoid organs as part of autoimmunity and immune defense actions (47). FTY720 promoted long-term small bowel transplant recipient survival and maintained the architecture of intestinal allografts. FTY720 inhibited intestinal allograft rejection in mice, possibly by enhancing the alloprotective effects of costimulation blockade by prolonged sequestration of lymphocytes in secondary lymphoid organs (40, 53, 69, 84). In our study, FTY720 was also able to attenuate the postoperative inflammatory response to the standardized IM by inducing significantly improved GIT time, CT time, and colonic muscularis contractility and significantly reduced neutrophil infiltrate counts in the colonic muscularis. This is consistent with our previous findings that FTY720 blocks TH-1 memory cell recirculation in the portal vein blood after IM and also prevents IFN-γ production by intestinal TH-1 cells and thereby activation of macrophages (18). The effectiveness of FTY720 in postoperative ileus, which prevents the T cell egress from lymphoid and mucosal tissues (11, 18, 44, 74), is consistent both with a direct FTY720 effect on TH-1 cells (18) and with the sequestration of T cells in the secondary lymphoid organs (11, 12, 44). However, in our studies, this neutrophil infiltrate count in the colonic muscularis of FTY720-treated mice was significantly higher than in sham-operated FTY720-treated mice, although there was no statistically significant difference among the functional data of the colonic muscularis (CT time, in vitro contractility, and proinflammatory cytokine mRNA release) of both groups. This might be explained by an incomplete pharmacological blockade of all T cells, although the selected FTY720 dosages were sufficient for functional improvements and, therefore, for the attenuation and prevention of postoperative ileus.

Alongside these findings, activation of bowel muscle cells by the bioactive sphingolipids ceramide 1-phosphate and sphingosine 1-phosphate in cell culture systems as well as sustained elevated concentration of the two bioactive sphingolipids in bowel muscle tissue with subsequent enhancement of proinflammatory cyclooxygenase-2 expression combined with a release of arachidonic acid, as described previously (16), may also be altered by administration of FTY720 (4).

Although FTY720 shows outstanding immunosuppressive effects (7, 23, 69, 84), it was rejected as immunosuppressant since its safety profile had been less favorable for adverse events than current standard immunosuppression in de novo renal transplant patients (73). Since then, FTY720 has been further evaluated for patients with multiple sclerosis in phase III trials (51, 57). Nevertheless, FTY720 has remained indispensable for the elucidation of underlying immune mechanisms as described above.

Therefore, these results corroborate our previous findings that the described pan-enteric inflammatory reaction after a localized IM is mediated by TH-1 memory cells (18) and the secondary lymphoid tissue of the gastrointestinal tract.

Conclusion

In this report, we were able to show that secondary lymphoid organs are involved in the immunologically mediated postoperative ileus. Obviously, T cells track on two routes to the effector organs: via blood and via lymphatic vessels and secondary lymphoid organs. The lymphatic route appears to be the principal route. Therefore, pan-enteric propagation of intestinal inflammation with subsequent hypomotility as one underlying cascade of the prolonged postoperative ileus depends on T cells and secondary lymphoid organs of the gastrointestinal tract.

Further investigations should analyze the migration route of TH-1 cells activated by IM, e.g., by draining off the thoracic duct at different times after IM and by simultaneous analysis of activated TH-1 cells migrating in the portal vein blood.

Establishing the principal migration route may offer new therapeutic and more specific options to the attenuation of postoperative ileus.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

LYMPHOID TISSUE IN POI


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