Video Article

Murine Distal Colostomy, A Novel Model of Diversion Colitis in C57BL/6 Mice

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Abstract

Diversion colitis (DC) is a frequent clinical condition occurring in patients with bowel segments excluded from the fecal stream as a result of a diverting enterostomy. The etiology of this disease remains ill-defined but appears to differ from that of classical inflammatory bowel diseases such as Crohn's disease and ulcerative colitis. Research aimed to decipher the pathophysiological mechanisms leading to the development of this disease has been severely hampered by the lack of an appropriate murine model. This protocol generates a murine model of DC that facilitates the study of the immune system's role and its interaction with the microbiome in the development of DC. In this model using C57BL/6 animals, distal parts of the colon are excluded from the fecal stream by creating a distal colostomy, triggering the development of mild to moderate inflammation in the excluded bowel segments and reproducing the hallmark lesions of human DC with a moderate systemic inflammatory response. In contrast to the rat model, a large number of genetically-modified murine models on the C57BL/6 background are available. The combination of these animals with our model allows the potential roles of individual cytokines, chemokines, or receptors of bioactive molecules (*e.g.*, interleukin (IL)-17; IL-10, chemokine CXCL13, chemokine receptors CXCR5 and CCR7, and the sphingosine-1-phosphate receptor 4) to be assessed in the pathogenesis of DC. The availability of congenic mouse strains on the C57BL/6 background largely facilitates transfer experiments to establish the roles of distinct cell types involved in the etiology of DC. Finally, the model offers the opportunity to assess the influences of local interventions (*e.g.*, modification of the local microbiome or local anti-inflammatory therapy) on mucosal immunity in affected and non-affected bowel segments and the on systemic immune homeostasis.

Video Link

The video component of this article can be found at https://www.jove.com/video/57616/

Introduction

In recent years, a substantial number of non-infectious colitis entities different from classical inflammatory bowel diseases (IBDs; *i.e.*, Crohn's disease or colitis ulcerosa) have been clinically and histopathologically characterized in humans. The pathophysiological mechanisms leading to the development of these colitis forms are not completely understood partially because appropriate animal models are scarce. Diversion colitis is one of these recently described entities. Although the term was coined in 1980 by Glotzer[1], the initial description of a similar phenotype was given in 1972 by Morson[2]. The disease develops in 50% to 91% of patients with diverting enterostomy, and its clinical intensity varies[3,4]. Given the annual incidence of around 120,000 colostomy patients in the United States of America, this disease entity constitutes an important health problem.

The overall goal of developing this protocol was to provide a murine DC model that relies on a colitis trigger similar to that seen in human DC and that reproduces the primary histopathological features of the human disease. In contrast to other murine colitis models, colitis induction in our model does not require genetically modified animals (*e.g.*, IL-7 transgenic mice, N-cadherin dominant negative mice, or TGFβ$^{-/-}$ mice), the application of chemically irritating substances (*e.g.*, dextran sulfate sodium (DSS)-induced colitis, or trinitrobenzene sulfonic acid (TNBS)-induced colitis), or the transfer of specific cell populations in immune deficient mice (as in the CD45RB^{high} transfer model of colitis) (for a review, see[5]). In contrast to other models, the intact immune system of our DC model allows assessment of the immunological mechanism involved in DC development. The limitation of mucosal inflammation to the excluded bowel segment allows assessment of its repercussion on mucosal immunity in other parts of the gastrointestinal tract, on the immune homeostasis in other immune compartments of the intestinal tract (*e.g.*, Peyer's patches and mesenteriale lymph nodes), and on the immune homeostasis of the entire organism. Finally, our model constitutes an appropriate tool for investigating the mechanisms controlling local inflammatory stimuli originating from changes in the normal local environment for both the local microbiome as well as alimentary antigens.

Protocol

All methods described here have been approved by the veterinary government authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, (LALLF M-V)).

1. Preoperative Care and Preparation of the Animal

1. Upon arrival in the animal facility, divide animals (C57Bl/6) into groups of similar size, cage each group together, and keep groups constant throughout the experiments.
   NOTE: Use animals of the same sex. The results described were obtained with male mice.
   NOTE: If male animals are used, cage groups together starting when they are 7 weeks of age so that a hierarchy can be established, thus minimizing the risk of aggressive behavior during experiments.
2. At least one week before surgery, switch to a high energy (>14 MJ/kg) and high protein (>20%) feed containing all the essential trace elements and vitamins (for details, see list of materials).
   NOTE: Assure all mice weigh at least 25 g when surgery is performed.
3. Induce anaesthesia and analgesia by intraperitoneal injection of ketamine (87 mg/kg i.p.) and xylazine hydrochloride (13 mg/kg i.p.). Wait until the mouse tolerates mechanical stimulation, e.g. toe pitch, without motor response.
4. Secure the narcotized mouse with tapes in a supine position on a heat underlay positioned at the operation desk, guaranteeing stable positioning during operation and avoiding an overwhelming loss of body heat.
   NOTE: The heat underlay should have a surface temperature of 36 °C to 40 °C; the operating room temperature should be 21 °C.

2. Distal Colostomy Operation

1. Shave the abdominal hair. Before starting surgery, disinfect the operation field three times using alcohol 70% and an iodophor. Drape the operation field to guarantee for aseptic conditions.
2. Perform a 15-mm median laparotomy by incising the abdominal muscles and the peritoneum along the linea alba, thus minimizing blood loss.
3. Use two DeBakey atraumatic forceps, carefully pull the cecum, terminal ileum, and ascending and transverse colon from the peritoneal cavity.
   NOTE: Be careful to strictly limit mechanical manipulation of the intestine to prevent injury to mesenteric structures.
4. Identify the cecal pole, the ascending colon, and small intestine (Figures 1a and 1b).
   NOTE: Correct identification of the ascending colon is fundamental for correct placement of the colostomy. In cases where the anatomy of the ileocecal region is ambiguous, the presence of Peyer's patches identifies the small intestine, and the presence of formed stool characterizes the colon.
5. Use a ruler to determine the position of the future colostomy. It should be placed 20 mm distal to the ileocecal valve for a distal colostomy.
6. Make a second 3-mm incision in the abdominal wall in the upper right quadrant. Pull the previously identified colon segment through this incision to form a loop, being careful not to distort the loop.
7. Carefully pass a 22-gauge flexible i.v. cannula through the mesocolon. Take care not to damage mesenteric vascular structures.
8. Return the intestine to the peritoneal cavity.
9. Fix both ends of the flexible tube to the skin using simple stitches and a resorbable suture (*e.g.*, polyglactin 910 or polyfil 4-0 1/2c).
10. Before closing the laparotomy, perform fluid resuscitation using an intraperitoneal injection of 0.5 mL 0.9% saline.
11. Close the peritoneum and the muscle layer with a continuous suture using a resorbable suture (*e.g.,* polyglactin 910 or polyfil 4-0 1/2c). Close the skin with a continuous suture using a resorbable suture (e.g., polyglactin 910 or polyfil 4-0 1/2c).
12. Open the exteriorized colon loop by performing a subtotal transection using a fine scissor. Avoid all injury to the mesentery. Do not transect the colon completely.
13. Fix each colostomy opening using three single full-thickness stitches to the peritoneum and skin using a monofil, absorbable suture (*e.g.*, polydioxanone or monofil 6-0 3/8s). The afferent loop, which is a functional end-colostomy, and the efferent loop, which is a mucous fistula, are clearly separated at this point (**Figure 1c**).
    NOTE: Operation time should be less than 20 minutes to limit fluid and thermal losses.
14. After finishing surgery, disinfect instruments using an aldehyde-free disinfection solution in an ultrasonic bath according to the instructions of the manufacturer.

3. Sham Operation (Colotomy)

1. Perform steps 1.1. through 2.4.
2. Use a ruler to determine the future colotomy position. The colotomy should be positioned the same distance from the ileocecal valve as the colostomy in the experimental group.
3. Open the colon at least two-thirds its circumference using fine scissors.
4. Close the colotomy with a single layer, full-thickness interrupted suture using a monofil, absorbable suture (*e.g.,* polydioxanone, monofil 6-0 3/8s).
   NOTE: Operation time should be less than 20 minutes for an experimented surgeon, limiting fluid and thermal losses.
5. Perform steps 2.10. , 2.11. and 2.14.

4. Postoperative Care

1. Return animals to their cages. Provide a well-tempered atmosphere of 37 °C (*e.g.*, with an infrared lamp) until mice are fully awake. Then, keep mice in a temperature- and humidity-regulated environment (21 °C; 30% ± 10% relative humidity). Allow free access to food and drinking water. To facilitate fluid uptake, provide additional water-soaked animal feed.
2. Start postoperative analgesia by injecting 0.1 mg/kg body weight buprenorphine s.c. when animals show response to mechanical stimulation. Be careful to avoid respiratory depression.
3. Supplement drinking water with 1 mg/mL tramadol for continued analgesia during the first postoperative week.
4. To compensate for decreased fluid intake due to reduced mobility, supply a Solid Drink Pad in the cage during the first postoperative week.
5. Weigh the animals and score animal behavior daily during the first week, every second day during remainder of the first month, and every third day during the second month using the disease severity score described in Kleinwort *et al.*[6].

Representative Results

Surgery is well tolerated both in the experimental (colostomy) and sham (colotomy) groups. Perioperative mortality should not exceed 10% when surgery and perioperative management is correctly performed. In the first postoperative week, significant weight loss is seen in both the experimental and sham groups. Animals of the sham group attain their weight nadir usually toward the fourth postoperative day, but it occurs one day later in the experimental group. Weight loss is more pronounced in the experimental group, reaching 21.7% of initial body weight. In contrast, sham animals lose 10.8% of their initial body weight (**Figure 2**)[6]. After the weight nadir in the first postoperative week, body weight rises continuously in both experimental groups, but with a slower slope in the experimental group. Signs and symptoms of severe intestinal inflammation (*i.e.*, bloody discharge or liquid bowel movements occur neither in the experimental nor the sham group[6]). Total mortality during the first 60 days postoperatively is around 40% in the colostomy group and about 10% in the colotomy group. Most deaths occur during the first postoperative week (61% in the experimental group, 66% in the sham group). Causes of death are shown in **Figure 3**.

Distal colostomy in mice results in the development of predominantly lymphocytic colitis reproducing the hallmark histological features of human DC. These alterations increase with the duration of intestinal exclusion. Crypt length is significantly shortened in excluded bowel segments. This shortening reaches statistical significance after 14 days of fecal diversion (**Figure 3a**). The goblet cell-bearing crypt length is reduced after 30 days in excluded bowel segments (**Figure 3b**). Absolute goblet cell numbers are also significantly reduced in crypts in the excluded bowel segment after 14 postoperative days (**Figure 3c**). The hallmark lesion of DC, the development of lymphoid follicles in the mucosa, requires a longer duration of stool deviation. Although an increased number of the lymphoid follicles can be observed as early as two weeks, differences become significant after two months (**Figures 5a-c**). All histopathological changes described before are more pronounced in distal than in proximal regions of the excluded bowel segments. A typical neutrophilic infiltrate as a sign of acute inflammation is usually not observed (**Figures 5d-e**).

As a sign of a systemic repercussion of local intestinal inflammation, neutrophil counts are significantly increased in colostomy animals as early as 14 days after surgery. This difference is maintained until the end of the observation period (60 days). Platelet counts are slightly increased in animals with colonic diversion after 60 days (**Figure 6**). Hemoglobin level and hematocrit are reduced 14 days after operation in the colostomy group compared to the sham group.

It has been shown that vitamin deficiency in rodents causes reduced mean corpuscular volume (MCH) and mean corpuscular hemoglobin (MCV) values[7]. In our model, we see initial increases in both these parameters after 14 and 30 days. Both MCV and MCH return to normal values after longer follow-up (**Figure 7**). This shows that distal colostomy does not result in clinically significant vitamin deficiency during long-term follow-up.

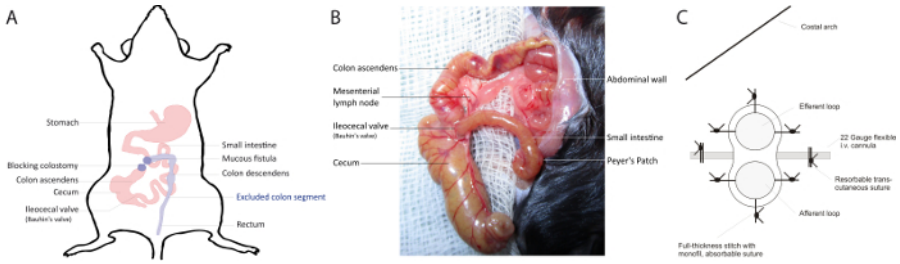

Figure 1: Anatomy of the cecal region and the surgical procedure. (a) Graphical representation of postoperative anatomy. (b) Topography of the cecal pol and anatomical landmarks. (c) Graphical representation of the colostomy openings. **Figure 1a** has been modified from Kleinwort et al.[6]. Please click here to view a larger version of this figure.

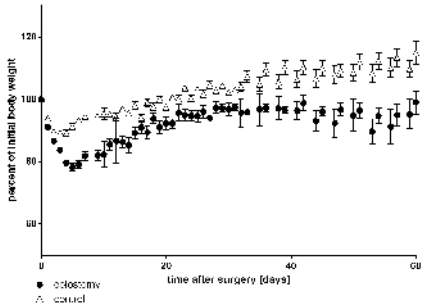

Figure 2: Body weight development. Body weight is shown as percentage of the preoperative body weight. The body weight curves of colostomy and sham (colotomy) animals reveal that the initial postoperative weight loss was significantly higher in the colostomy group compared to the sham group ($p < 0.001$). Values are means ± standard errors of the mean across 21-26 animals per group (21 animals received colostomies; 26 animals were in the sham group). This figure has been modified from Kleinwort et al.[6]. Please click here to view a larger version of this figure.

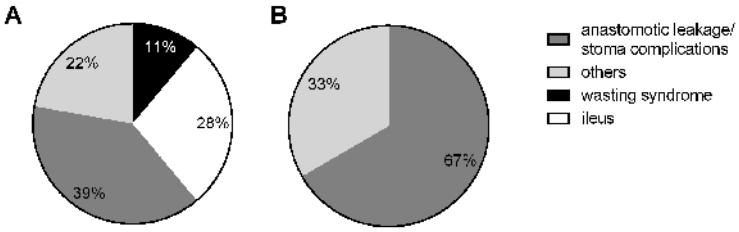

Figure 3: Causes of death. Complications are represented as percentages of all-cause mortality and are illustrated in a pie chart ((a) colostomy group, (b) sham group). Postoperative complications were determined by necropsy. Wasting syndrome was defined as continuous weight loss greater than 33% of the initial body weight and no other findings at necropsy. Ileus and anastomotic leakage were diagnosed at necropsy. Stoma complications were defined as peristomal abscesses and mucocutaneous separations. Other complications comprised wound dehiscence of the laparotomy, ischemia of the cecum, and cases not clear at necropsy. Significant differences in the distribution of postoperative complications were seen between groups ($p = 0.021$, analyzed using the Fisher exact test for two-sided analysis of up to 6 × 6 contingency tables). There were 39 animals in the colostomy group and 29 in the sham group. This figure has been modified from Kleinwort et al.[6]. Please click here to view a larger version of this figure.

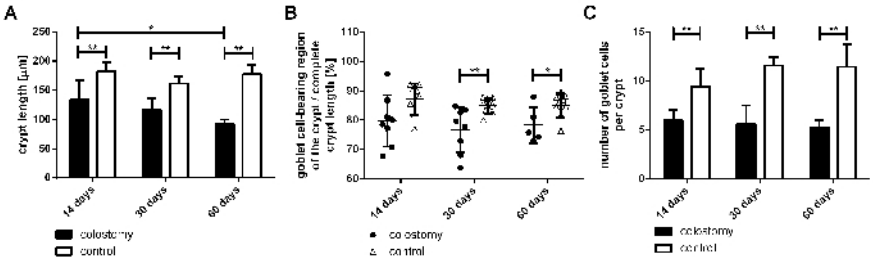

Figure 4: Crypt length and goblet cell population. Crypt length and goblet cell numbers were determined using paraffin sections of the rectum after periodic acid-Schiff staining. (a) Crypt length was significantly decreased in the rectum of animals with diversion colitis (DC). (b) The length of the goblet cell-bearing region of the crypt was measured and set as a ratio to the full crypt length. Thirty and sixty days postoperatively, the percentage of crypt-length-bearing goblet cells was decreased in the DC group. (c) Goblet cell numbers were significantly reduced in DC animals compared to the sham group. Graphs in (a) through (c) show means and standard errors across 5 to 9 animals per group (colostomy 14 and 30 days, sham 14 days: n = 8; colostomy 60 days: n = 5; sham 30 and 60 days: n = 9); #$p < 0.05$; ##$p < 0.01$. This figure has been modified from Kleinwort et al.[6]. Please click here to view a larger version of this figure.

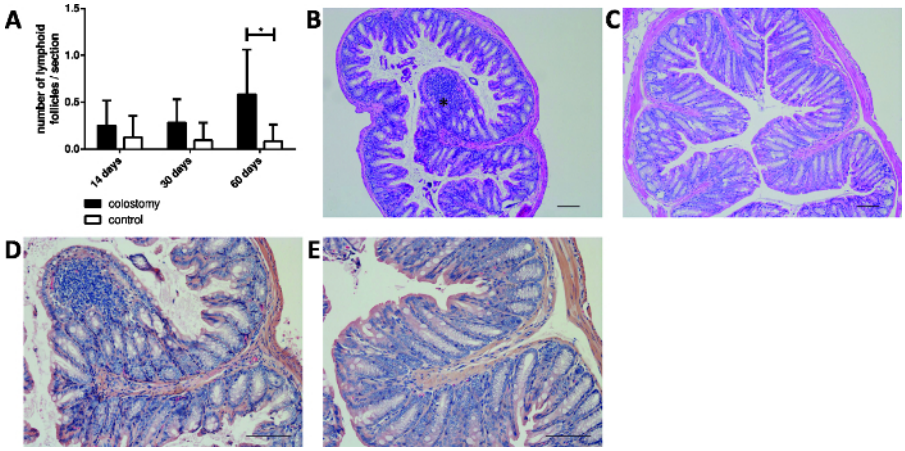

Figure 5: Lymphoid follicles and inflammatory infiltrates. (a) Lymphoid follicles of diverted colons and sham animals were counted on paraffin sections of the rectum stained with hematoxylin and eosin. The graph shows means and standard errors across 5 to 9 animals per group (colostomy 14 and 30 days, sham 14 days: n = 8; colostomy 60 days: n = 5; control 30 and 60 days: n = 9), #p < 0.05; ##p < 0.01. (b) and (c) Representative examples of sections of the rectum of colostomy (b) and sham (c) animals 60 days postoperatively stained with hematoxylin and eosin. A prominent lymphoid follicle (#) was present in the mucosa of a colostomy mouse. Scale bars represent 100 µm. (d) and (e) Staining with chloroacetate esterase reaction on paraffin sections was performed to detect neutrophil granulocytes. On cross-sections of the diverted rectum, an acute inflammatory neutrophilic infiltrate was observed neither in the colostomy group (d) nor in sham animals (e) up to 60 days postoperatively. Scale bars represent 100 µm. This figure has been modified from Kleinwort et al.[6]. Please click here to view a larger version of this figure.

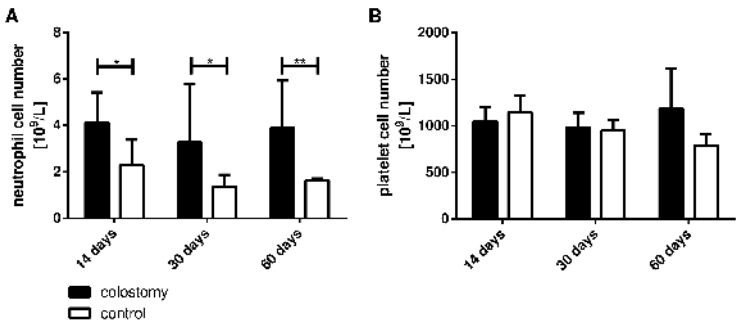

Figure 6: Neutrophil and platelet counts. Blood counts of venous blood samples were determined by a veterinary hematology analyzer. (a) Neutrophil counts were significantly increased in the colostomy group compared to sham animals up to 60 days postoperatively. (b) Platelet counts were slightly elevated in the colostomy group 60 days postoperatively. The graph shows means and standard errors across 5 to 9 animals per group (colostomy 14 and 30 days, sham 14 days: n = 8; colostomy 60 days: n = 5; sham 30 and 60 days: n = 9), #p < 0.05; ##p < 0.01. Please click here to view a larger version of this figure.

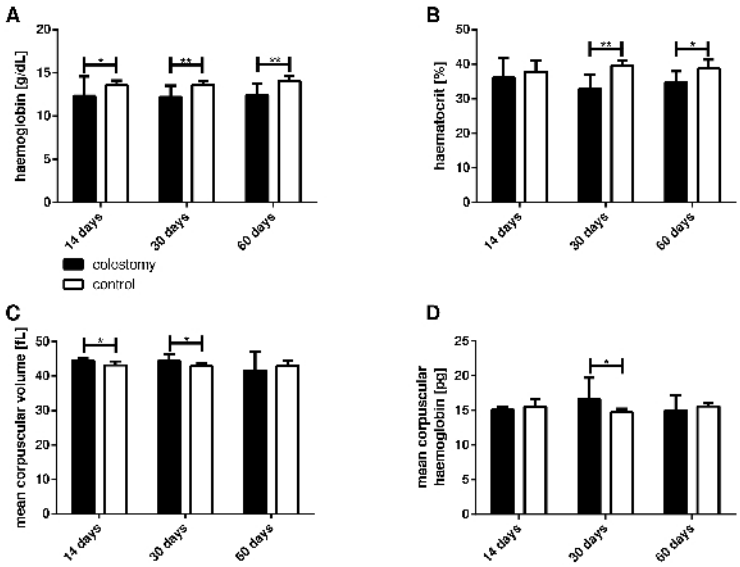

Figure 7: Red blood cell parameters. Blood counts of venous blood samples were determined by a veterinary hematology analyzer. (a) Hemoglobin and (b) hematocrit were significantly decreased in the colostomy group compared to sham group. (c) Mean corpuscular volume (MCH) was significantly elevated 14 and 30 days postoperatively in the colostomy group compared to the sham group but returned to normal after 60 days. (d) Mean corpuscular hemoglobin (MCV) values were elevated after 30 days but no longer after 60 days postoperatively in the colostomy group compared to sham animals. The graph shows means and standard errors across 5 to 9 animals per group (colostomy 14 and 30 days, sham 14 days: n = 8; colostomy 60 days: n = 5; sham 30 and 60 days: n = 9), #p < 0.05; ##p < 0.01. Please click here to view a larger version of this figure.

Discussion

The murine model of DC presented in this protocol reliably reproduces the histopathological features of human DC (*e.g.*, *de novo* development of lymphoid follicles in the submucosa of inflamed bowel segments, crypt shortening, and reductions in goblet cell numbers). Aside from this advantage, this model is induced by a very similar triggering factor and presents with a clinical course of moderate to mild severity as is the case in most affected humans.

To obtain reproducible results and acceptable perioperative lethality, some practice in microsurgical techniques is necessary. However, both colostomy and sham operation (colotomy) can be performed with very low perioperative mortalities (<10%). Some operational steps are of great importance: 1) positively identify the colon and terminal ileum. In cases where the anatomy of the ileocecal region not clear, the colon can be identified by the presence of formed stool and the terminal ileum by the presence of Peyer's patches. 2) When exteriorizing the colostomy loop, assure the mesentery is not contorted, thus avoiding mesenteric ischemia and mechanical ileus. 3) When puncturing the mesentery with the flexible tube, assure a vessel-free sector is chosen, thus preventing bleeding and mesenteric ischemia. 4) When creating the small incision in the abdominal wall, check for sufficient hemostasis. If necessary, use thermo-coagulation to stop bleeding from muscle vessels. 5) Assure the colonic loop is open at least at two-thirds of its circumference and that the posterior wall is sufficiently exteriorized, thus guaranteeing complete blockage of stool passage to the distal bowel segments. 6) Assure that both colostomy ends are well fixed to the skin to avoid an open passage to the peritoneal cavity resulting in higher infection risks or a mechanical ileus because the fecal stream cannot exit. Normally, all surgical procedures can be performed without magnifying glasses or surgical microscopes. In general, utilization of these devices requires further training.

Even when the complexity of the surgical procedures is only moderate, there are many essential operative and perioperative issues to be considered to successfully terminate the experiment with reproducible results.

It is essential for long-term survival with a permanent colostomy that mice have an intact cecum. An experimental group with proximal colostomies adjacent to the ileocecal valve showed a mortality rate of 100% in the first postoperative week because of irreversible weight loss (data not shown). Therefore, it is of utmost importance to choose the colostomy site at least 20 mm distal from the Bauhin`s valve. This observation may be due to the physiology of rodent digestion. Retrograde transport from the proximal colon to the cecum occurs in these species. The cecum is where the synthesis of nutrients by the bacterial microflora occurs8. Normal MCV and MCH values during long-term follow-up indicate that animals with distal colostomies do not suffer from significant vitamin deficiencies. Moreover, coprophagy is an essential part of the nutritional behavior of rodents in their natural environments. When establishing the model, we were highly concerned that perturbing the coprophagy as a consequence of the presence of a permanent colostomy could result in nutritional deficiencies, (*e.g.*, vitamin B). However, while it is a problem under conditions where access to nutrients is limited, it has previously been shown that coprophagy loses its nutritional significance under experimental conditions with free excess to water and nutritionally-balanced food[9]. Under our experimental conditions, it is vital to optimize the conditions of postoperative nutritional intake. We used animal feed that had a high protein content, optimized protein quality, and increased energy density. Animals were acclimatized to this diet at least two weeks before surgery. Postoperatively, water-soaked feed was offered *ad libidum* to faciliate food and fluid intake.

It is crucial to utilize mice of the same sex in both the experimental and sham groups. Sex has been shown to significantly impact the course not only of systemic inflammation but also of intestinal inflammation in various chemically-induced colitis models[10,11,12]. In general, male animals will

develop more pronounced disease with greater inflammation, more crypt damage, less regeneration, higher levels of pro-inflammatory cytokines, and slower recoveries from weight loss[10]. A further advantage of using male animals is better age-matched preoperative body weights, leading to better compensation of the initial postoperative weight loss period. Nevertheless, female mice can be used in the protocol if specific questions (*e.g.*, the influence of sex hormones on the manifestation of DC are to be addressed. Using male mice requires more effort in maintaining stable housing conditions. Male mice are more prone to aggressive interactions leading to injury and death. Male mice live in established hierarchies with one dominant male. Once the hierarchy in the group is established, continued fighting is less common[13,14]. For this reason, we formed the experimental groups upon arrival of the mice in our animal facility at least two weeks before surgery and maintained these groups until experiments ended. In general, experimental groups of 5 to 7 animals were kept in one cage. Maintenance of stable social and housing conditions was of utmost importance not only to minimize aggressive behaviors of experimental animals. Housing conditions have been shown to influence weight gain as well as immunological parameters[15,16,17].

Susceptibility to chemically-induced and genetically-modified colitis models varies considerably between mouse strains[18,19,20]. The present protocol was established in C57Bl/6 mice, a mouse strain with a well-defined genetic background and a well-characterized immune system[21,22,23] that shows intermediate to strong inflammatory response in the dextran sulfate sodium-induced colitis model[20] and is considered a prototypical Th1 strain[23]. The use of other mouse strains can be considered when more severe disease activities or a Th2 environment is required.

In summary, our protocol constitutes a valuable tool for assessing the role of various immune cell types, cytokines, chemokines, and other signalling molecules in the pathogenesis of DC. A large number of genetically-modified mice models are available with the C57BL/6 background and can be combined with our model (*e.g.*, mice deficient for IL-17, IL-10, chemokine CXCL13, chemokine-receptors CXCR5 and CCR7, and sphingosine-1-phosphate receptor 4). The availability of congenic mouse strains on the C57BL/6 background largely facilitate transfer experiments to establish the role of distinct cell types involved in the etiology of DC. Finally, the model offers the opportunity to assess the influence of local interventions (*e.g.*, modification of the local microbiome and local anti-inflammatory therapy) on mucosal immunity in affected and non-affected bowel segments and on systemic immune homeostasis.

Disclosures

All authors declare that they have no competing interests.

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None

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