Reports

Novel mouse model of colitis characterized by hapten-protein visualization

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Trinitrobenzene sulfonic acid (TNBS) and oxazolone are used to induce colitis for the investigation of inflammatory reactions in the colon. Although these chemicals are presumed to bind proteins in the colonic mucosa and then induce colitis as haptens, hapten-protein formation has not yet been confirmed in the colonic mucosa. We developed a mouse model of colitis characterized by hapten-protein visualization, using 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl), which emits fluorescence after binding to proteins. The enema of 1 mg/mL NBD-Cl induced severe diarrhea, rectal bleeding, and body weight reductions in BALB/c mice. Mucosal signs indicative of colitis, such as redness and swelling observed under stereomicroscopy or inflammatory cell infiltration and crypt-epithelium destruction under microscopy, were manifested around NBD-proteins visualized with fluorescence. Fluorescence microscopy showed the infiltration of F4/80⁺ cells around areas of NBD-proteins, and flow cytometry indicated the uptake of NBD-proteins by CD11b⁺ cells. We also found critical roles for T cells and interleukin-6 in colitis induction with NBD-proteins. NBD-Cl-induced colitis presents a unique model to study the relevance between hapten-protein formation and inflammatory reactions and offers a method to assess experimental interventions on colitis induction in the mucosa, where hapten-protein formation is confirmed.

Ulcerative colitis and Crohn's disease are chronic inflammatory disorders of the gastrointestinal tract and are broadly categorized as inflammatory bowel disease (IBD) (1). The precise pathogenesis of IBD still remains unclear, and a cure for IBD is not yet available. Although glucocorticoids, 5-aminosalicylic acid, immunosuppressive agents, anti-tumor necrosis factor- α antibodies, and leukocyte/granulocyte apheresis are useful to induce remission and prevent disease recurrence, IBD can be a refractory disease (2,3). Therefore, there is a need for the development of novel anti-inflammatory treatments for IBD patients.

Experimental colitis in mice is a useful model for the investigation of inflammatory reactions in the colon and also for the evaluation of novel anti-inflammatory treatments. Intrarectal administration of trinitrobenzene sulfonic acid (TNBS) or oxazolone is a well-known method to induce colitis in mice (4–7). TNBS and oxazolone are considered to be haptens, because these chemicals are presumed to bind to endogenous proteins

in the colonic mucosa and induce a local immunologic response through macrophage and T cell activation. However, we cannot observe hapten-protein formation in either TNBS- or oxazolone-induced colitis, because TNBS- and oxazolone-protein conjugates are not distinguishable from TNBS or oxazolone alone, respectively, in the colonic mucosa. Hapten-protein visualization allows us to investigate the relevance between hapten-protein formation and inflammatory reactions. Furthermore, the visualization of hapten-protein conjugates can enable the assessment of experimental interventions on colitis induction in the mucosa, where hapten-protein formation is confirmed.

4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) is a chemical that fluoresces upon binding to proteins through the substitution reaction of 4-Cl with amino or thiol groups of amino acid residues (8). The fluorescence of NBD-proteins (excitation 460 nm, emission 535 nm) is similar to those of fluorescein and green fluorescence proteins, so NBD-proteins can be detected

using experimental settings for the observation of fluorescein and green fluorescence proteins. NBD-Cl resembles dinitrochlorobenzene in chemical structure and reaction patterns, thereby inducing allergic contact dermatitis in a manner similar to dinitrochlorobenzene (9). Thus, we expected that the intrarectal administration of NBD-Cl might induce colitis in mice through the formation of NBD-proteins and that these could be visualized in the colonic mucosa under fluorescence observation.

In the present study, we examined colitis induction by NBD-Cl in mice and the fluorescence observation of NBD-proteins in the colonic mucosa.

Materials and methods

Reagents

We obtained NBD-Cl from Tokyo Chemical Industry (Tokyo, Japan or TCI-America, Portland, OR, USA). NBD-Cl was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 200

Figure 1. Body weight following NBD-Cl enema and observation of NBD-Cl-induced colitis. (A) Mice were intrarectally administered 100 μ L 40% ethanol containing 0, 0.5, 1, or 2 mg/mL NBD-Cl ($n = 6$ in each group). Body weight of living mice was measured at days 0–5. $*P < 0.05$ (paired two-group t -test, compared with body weight at day 0). (B) Mice were treated with 0- or 1-mg/mL NBD-Cl enemas ($n = 6$). Two days later, the length of the dissected colon was measured. $*P < 0.05$ (Student's t -test). An arrow indicates severe redness and swelling of the colon at the portion 2–4 cm from the anus. (C) Under stereomicroscopic observation, intense fluorescence was detected exclusively in the colonic mucosa that exhibited severe redness and swelling. Scale bar, 200 μ m. (D) After the stereomicroscopic observation, the colon was fixed to prepare paraffin sections. After removal from paraffin, the sections were stained with hematoxylin and eosin, and adjacent ones were not stained for fluorescence microscopic observation. Intense fluorescence was detected exclusively in the colonic mucosa that exhibited inflammatory cell infiltration and crypt-epithelium destruction. Scale bar, 50 μ m. Similar results were obtained in all six mice.

mg/mL for the stock solution, which was stored at -80°C . NBD-Cl stock solution was diluted with ethanol (Wako Chemicals, Osaka, Japan) and then with distilled water to prepare the enema solution. Rat anti-mouse interleukin-6 receptor (IL6R) monoclonal IgG antibody MR16-1 (10,11) was kindly provided by Chugai Pharmaceutical Company (Shizuoka, Japan). Control rat IgG was purchased from Sigma-Aldrich. Diethyl ether, 4% paraformal-

dehyde solution, ammonium chloride, acetone, and olive oil were purchased from Wako Chemicals. RPMI1640, HEPES, gentamicin, penicillin-G, streptomycin, amphotericin B, fetal bovine serum, and phosphate-buffered saline were purchased from Invitrogen (Carlsbad, CA, USA).

Colitis induction

Eight-week-old female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and kept in a 12-h light/dark cycle with controlled humidity (60–80%) and temperature ($22^{\circ} \pm 1^{\circ}\text{C}$) under specific pathogen-free conditions. Food and water were freely available. All animal experiments were performed according to the guidelines of the Institute for Laboratory Animal Research and with the approval of the ethics committee of the Nagoya University School of Medicine.

To induce colitis, NBD-Cl was intrarectally administered to mice in a similar manner to TNBS and oxazolone (7,12–14). Briefly, we lightly anesthetized mice with diethyl ether and inserted a rubber catheter (2-mm outer diameter) fitted onto a 1-mL syringe via the anus. The tip was positioned 2 cm proximal to the anus, and then 100 μ L 40% ethanol containing 0–2 mg/mL NBD-Cl (0 mg/mL NBD-Cl enemas contained only

40% ethanol) was slowly administered to the mice through the catheter. Mice were kept in a head-down position for 30 s and then returned to their cages. Ethanol (40%) is used to help haptens go through the intestinal epithelial barrier (7,12–14).

Macroscopic, stereomicroscopic, and microscopic observation of the colon

For macroscopic observation, the colon was dissected from mice 2 days following administration of NBD-Cl enemas. After the macroscopic observation, the colon was opened longitudinally, washed with physiological saline to remove stools, and pinned down on plates with the mucosal side facing up for observation with the SZX-RFL2 fluorescence stereomicroscopy system (Olympus, Tokyo, Japan). After stereomicroscopic observation, the colon was fixed overnight in 4% paraformaldehyde solution and embedded in paraffin to prepare sections (6 μ m). After removal from paraffin, sections were observed with the BZ-8000 fluorescence microscopy system (Keyence, Osaka, Japan) using Aqua-Poly/Mount coverslipping medium (Polysciences, Warrington, PA, USA). To detect macrophages, sections were incubated with anti-Fc γ III/II receptor antibody (BD Biosciences, San Diego, CA, USA) and

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Figure 2. NBD-proteins and macrophages. (A) To prepare paraffin sections for fluorescence microscopic observation using anti-F4/80 antibody-biotin and streptavidin–Alexa Fluor 647, the colon was dissected from mice 1 day following 1-mg/mL NBD-Cl enema ($n = 3$). Similar results were observed in all three mice. Scale bar, 50 μ m. (B) To isolate mononuclear cells for flow cytometry, the colon was dissected 1 day following 1-mg/mL NBD-Cl enema ($n = 3$). A total of 10,000 cells were analyzed in each experiment. The cells in the boxed region were defined as FL1⁺. (C) Isolated mononuclear cells were exposed to PerCP-Cy5.5-conjugated anti-CD11b antibody and PE-conjugated anti-CD11c antibody before flow cytometry. Percentages of CD11b⁺ and/or CD11⁺ cells were assessed within the FL1⁺ cell pool. A total of 100,000–200,000 cells were analyzed in each experiment.

Table 1. Colitis symptoms within 2 days following NBD-Cl enema.

NBD-Cl	<i>n</i>	Diarrhea ^a		Rectal bleeding
		Mild	Severe	
BALB/c wild 0 mg/mL	6	0	0	0
0.25 mg/mL	6	0	0	0
0.5 mg/mL	6	3	0	0
1 mg/mL	6	3	3	2
2 mg/mL	6	0	6	4
Wild 1 mg/mL	6	3	3	3
Nude 1 mg/mL	6	3	0	0
Wild + IgG 1 mg/mL	6	2	3	1
Wild + anti-IL-6R 1 mg/mL	6	3	0	0

^aApparent discharges of loose stool were defined as severe diarrhea, and traces of loose stool around the anus were defined as mild diarrhea. One mouse died at day 3 after the 1-mg/mL NBD-Cl enema, while three mice died at days 2, 3, and 4 after the 2-mg/mL NBD-Cl enema. They all exhibited severe diarrhea and rectal bleeding, and their data is included here.

then with biotinylated anti-F4/80 antibody (Acris Antibodies GmbH, Hiddenhausen, Germany), followed by incubation with streptavidin–Alexa Fluor 647 (Molecular Probes, Eugene, OR, USA). L.A.B. solution (Polysciences, Warrington, PA, USA) was used for antigen liberation according to the manufacturer’s instructions.

Histological evaluation of colitis was performed as previously described (15). Colitis scores were determined by the following histological criteria: (i) low level of mononuclear cell infiltration with infiltration seen in <50% high-power field (hpf; $\times 200$) and no structural changes observed; (ii) low level of mononuclear cell infiltration, crypt distortion, and no destruction of epithelia; (iii) high level of mononuclear cell infiltration with infiltration seen in $\geq 50\%$ hpf and no destruction of epithelia; (iv) focal destruction of epithelia covering <1 hpf; (v) destruction of epithelia covering ≥ 1 hpf; and (vi) extensive destruction of epithelia covering ≥ 2 hpf. All sides were blinded for scoring.

Flow cytometry

Mononuclear cells were isolated from the portion of the colon 2–4 cm from the anus 1 day following administration of NBD-Cl enemas, as described previously (16), and analyzed with a flow cytometer (Beckman, Fullerton, CA, USA). Alternatively, isolated mononuclear cells were incubated with anti-Fc γ III/II receptor antibody and then with PerCP-Cy5.5-conjugated anti-CD11b antibody and phycoerythrin (PE)–conjugated anti-CD11c antibody (BD Biosciences) before flow cytometry analysis.

Fluorescence observation of NBD-protein formation in the serum

NBD-Cl stock solution was diluted with dimethyl sulfoxide to a concentration of 20 mg/mL and then with fetal bovine serum or phosphate-buffered saline to 50 μ g/mL. The fluorescence emission of the samples was determined with a fluorescence plate reader (excitation 485 nm, emission 535 nm; Twinkle

LB970; Berthold, Bad Wildbad, Germany) 2, 6, and 18 h following incubation at 37°C.

Assay for macrophage endocytosis of NBD-proteins and the effect of NBD-proteins on T cell activation

Cells were obtained from the spleen of BALB/c mice, and mononuclear cells were isolated via lysis of red blood cells in 0.75% NH₄Cl solution buffered with 17 mM Tris-HCl, pH 7.6. Isolated splenic mononuclear cells (6×10^5) were resuspended in 5-mL polystyrene round-bottom tubes (BD Biosciences) containing 300 μ L RPMI1640 with 10 mM HEPES, 10 μ g/mL gentamicin, 100 U/mL penicillin-G, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B, and 5% FBS pretreated with 0 or 50 μ g/mL NBD-Cl for 2 h at 37°C, and incubated for 24 h at 37°C in 5% CO₂. The depletion of CD11b⁺ cells in isolated splenic mononuclear cells was performed before the incubation using CD11b MicroBeads (Miltenyi Biotec, Gladbach, Germany). Following treatment with anti-Fc γ III/II receptor antibody, the cells were stained for flow cytometry analysis with PE-conjugated anti-F4/80 antibody (eBioscience, San Diego, CA, USA) to identify macrophages or with PE-conjugated anti-CD4 antibody and PerCP-Cy5.5-conjugated anti-CD69 antibody (BD Biosciences) to identify activated T cells.

Skin test with NBD-Cl

We performed a skin test with NBD-Cl on the ear of mice as previously reported (15,17). Briefly, 4 days following administration of NBD-Cl enemas, both sides of the ear were treated with 20 μ L 1 mg/mL NBD-Cl in 4:1 acetone:olive oil. Ear

Figure 3. Comparison between wild-type and nude mice. BALB/c wild-type and nude mice were intrarectally given 100 μ L 40% ethanol containing 1 mg/mL NBD-Cl ($n = 6$). Two days later, body weight reduction was assessed (A), and the colon was dissected for histological analysis (B and C). * $P < 0.05$; (A) Student's t -test, (B) Mann-Whitney U test. Scale bar, 50 μ m.

thickness, E , was measured with a digital caliper (Niigata Seiki, Niigata, Japan) before and 24 h after the treatment. Ear swelling (%) was calculated as $[(E_{\text{post}} - E_{\text{basal}}) \div E_{\text{basal}}] \times 100$.

Organ culture to determine cytokine production in the colon

One or two days following administration of NBD-Cl enemas, a 3–4-cm portion of the colon from the anus was dissected, longitudinally opened, washed with PBS, and weighed. Organ culture of the samples was performed as previously reported (18). Briefly, the samples were placed on culture insert filters (0.4- μ m pore size) in 6-well plates (BD Falcon, Franklin Lakes, NJ, USA) containing RPMI1640 with 10 mM HEPES, 10 μ g/mL gentamicin, 100 U/mL penicillin-G, 100 μ g/mL strepto-

mycin, 0.25 μ g/mL amphotericin B, and 5% FBS (600 μ L in the upper chamber and 1400 μ L in the lower chamber). The culture was incubated in 5% CO_2 at 37°C for 24 h, after which the culture medium in the lower chamber was collected and stored at -80°C until being assayed for cytokine concentrations with a Multi-Analyte Profiler ELISArray kit for mouse Th1/Th2/Th17 cytokines (SABiosciences, Frederick, MD, USA) and a Mouse IL-1 β ELISA kit (Pierce, Rockford, IL, USA).

Statistical analysis

Data are presented as means $\pm$ SD. We used a paired two group t -test, Student's t -test, or Mann-Whitney U test as indicated in the figure legends. A P value of <0.05 was considered statistically significant.

Results and discussion

Colitis induction with NBD-Cl enema

BALB/c mice were given 100 μ L 40% ethanol containing 0–2 mg/mL NBD-Cl via intrarectal administration. Severe diarrhea and rectal bleeding were observed within 2 days following 1- and 2-mg/mL NBD-Cl enemas (Table 1). The intrarectal administration of 1 and 2 mg/mL NBD-Cl also induced body weight reduction (Figure 1A). One mouse out of 6 died at day 3 after the 1-mg/mL NBD-Cl enema, while 3 mice died at days 2, 3, and 4 after the 2-mg/mL NBD-Cl enema. We decided to use NBD-Cl enemas at a concentration of 1 mg/mL (0.1-mg dosage per mouse) to efficiently induce colitis with low mortality.

Fluorescence observation of NBD-proteins in the colonic mucosa

For macroscopic observation, the colon was dissected 2 days after administration of a 1-mg/mL NBD-Cl enema, at which time body weight reductions peaked (Figure 1A). Macroscopic observation showed significant shrinkage of the colon (Figure 1B). Severe redness and swelling were also observed in the distal colon, especially at the portion 2–4 cm from the anus (Figure 1B, arrow). Under fluorescence observation with stereomicroscopy, an intense fluorescence signal was detected exclusively in the colonic mucosa that exhibited severe redness and swelling (Figure 1C). For microscopic observation, the dissected colon was fixed, embedded in paraffin, and sectioned. After removal from paraffin, an intense fluorescence signal was detected exclusively in the colonic mucosa showing inflammatory cell infiltration and crypt-epithelium destruction (Figure 1D). These findings indicate that 1-mg/mL NBD-Cl enema leads to NBD-protein formation in the colonic mucosa and that these conjugates can induce colitis.

The involvement of macrophages, T cells, and interleukin-6 in colitis induction

Usage of dyes with fluorescence emissions that differed from NBD-proteins allowed us to investigate events following NBD-protein formation. Fluorescence immunohistochemical staining showed the infiltration of F4/80 $^{+}$ cells in the vicinity of NBD-proteins in the colon dissected from mice 1 day following 1-mg/mL NBD-Cl enema (Figure 2A). Fluorescence (FL1, 530 nm) corresponding to the emission from NBD-proteins was detected in a very small fraction of isolated colonic mononuclear cells using flow cytometry analysis (Figure 2B). Flow cytometry also demonstrated that the majority of the FL1 $^{+}$ cells expressed CD11b (Figure 2C). Both F4/80 and CD11b are used

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Figure 4. Cytokine involvement in colitis induction. (A) Mice were treated with 0- or 1-mg/mL NBD-Cl enemas. One day (open bars) or two days (closed bars) later, colon samples were taken for organ culture ($n = 4$). Cytokine concentrations in culture media were divided by the weight of the colon samples. (B–D) Mice were given 100 μ L 40% ethanol containing 1 mg/mL NBD-Cl intrarectally, and then 1 mg anti-IL6R antibody or control IgG was subcutaneously administered ($n = 6$). Two days later, body weight reduction was assessed (B), and the colon was dissected for histological analysis (C and D). * $P < 0.05$; (B) Student's t -test, (C) Mann-Whitney U test. Scale bar, 50 μ m.

as a macrophage marker (19). These findings suggest that NBD-proteins are endocytosed by macrophages. To further assess the endocytosis of NBD-proteins by macrophages, we performed in vitro assay using isolated splenic mononuclear cells. NBD-Cl–proteins were formed in the serum ≤ 2 h at 37°C judging from fluorescence emission (Supplementary Figure S1A). Isolated splenic mononuclear cells were incubated for 24 h in the culture media containing 5% serum, which was pretreated with 50 μ g/mL NBD-Cl for 2 h, and then used for flow cytometry analysis. FL1 fluorescence was detected in $9.05\% \pm 3.77\%$ ($n = 4$) of isolated splenic mononuclear cells. Flow cytometry also demonstrated that F4/80 was expressed by $71.76\% \pm 1.76\%$ of the FL1⁺ cells. Furthermore, the percentage of cells expressing CD69, a marker for T cell activation (20), was increased within the CD4⁺ T cell pool in the presence of the serum pretreated with 50 μ g/mL NBD-Cl (Supplementary Figure S1B). The increase was inhibited with the depletion of CD11b⁺ cells in isolated splenic mononuclear cells, which reduced the population of F4/80⁺ cells from

$8.33\% \pm 1.77\%$ to $0.98\% \pm 0.21\%$, before the incubation (Supplementary Figure S1B). These findings suggest that NBD-proteins are endocytosed by macrophages and presented to T cells, leading to T cell activation.

The visualization of hapten-protein conjugates can enable the assessment of experimental interventions on colitis induction in the mucosa, where hapten-protein formation is confirmed. To assess the involvement of T cells, we compared colitis induction with NBD-Cl enemas between BALB/c wild-type and thymus-deficient nude mice. Neither severe diarrhea nor rectal bleeding was observed in the nude mice (Table 1). Body weight reduction was much less in nude mice than in wild-type mice (Figure 3A). Histological analysis also showed that inflammatory cell infiltration and crypt-epithelium destruction were not remarkable in nude mice, although the presence of NBD-proteins was detected in the colonic mucosa of these mice via fluorescence microscopy (Figure 3, B and C). To assess T cell sensitization, we performed a skin test with NBD-Cl on the ear 4 days following

administration of NBD-Cl enemas. Ear swelling, which is used as an indicator of dermatitis (15,17), was more severe in wild-type mice pretreated with 1-mg/mL NBD-Cl enemas ($24.0\% \pm 5.0\%$, $n = 4$) than in ones pretreated with 0-mg/mL NBD-Cl enemas ($11.9\% \pm 4.9\%$, $P < 0.05$, Student's t -test). However, in nude mice, there was no statistically significant difference in ear swelling between pretreatments with 1- and 0-mg/mL NBD-Cl enemas ($5.9\% \pm 2.4\%$ and $5.1\% \pm 1.5\%$, respectively). These findings suggest that administration of 1-mg/mL NBD-Cl enemas induces T cell sensitization.

Colitis symptoms appeared within 2 days (Table 1), and body weight reduction peaked at day 2 (Figure 1A) following 1-mg/mL NBD-Cl enema. Thus, we collected colon samples 1 and 2 days following the NBD-Cl enema and performed organ culture of the samples for 24 h to determine cytokine production in colitis induction. The concentration of interleukin-6 was markedly greater in the culture media than that of other cytokines (Figure 4A). Interleukin-6 was also detected in the culture media of colon samples taken from nude mice 1 day following 1-mg/mL NBD-Cl enema, although the concentration of interleukin-6 (66 ± 18 pg/mL/mg colon sample, $n = 4$) was less than in the culture media of colon samples taken from wild-type mice (111 ± 20 pg/mL/mg colon sample, $P < 0.05$, Student's t -test), indicating the contribution of both T and non-T cells to interleukin-6 production. Previous studies have shown that interleukin-6 is expressed in a wide variety of cell types, including T cells and macrophages (21–24). To assess the involvement of interleukin-6 in colitis induction, anti-IL6R antibody was subcutaneously administered following 1-mg/mL NBD-Cl enema. Neither severe diarrhea nor rectal bleeding was observed in mice treated with anti-IL6R antibody (Table 1). The administration of anti-IL6R antibody also attenuated body weight reduction and histological colitis scores (Figure 4, B and C). Inflammatory cell infiltration and crypt-epithelium destruction were suppressed with anti-IL6 antibody administration, while NBD-protein formation was confirmed in the colonic mucosa under fluorescence observation (Figure 4D). Previous studies have indicated the involvement of interleukin-6 in the pathogenesis of inflammatory bowel diseases (25–27). Interleukin-6 signal blockade with anti-IL6R antibody attenuates experimental colitis (10,28) and is described as a novel strategy for the treatment of Crohn's disease (29). We also confirmed this critical contribution of interleukin-6 to

NBD-Cl-induced colitis. Interleukin-6 is a pleiotropic cytokine, and it has important regulatory functions in the immune system including Th17 cell development (21–24). The concentration of interleukin-17A was increased in the culture media of colon samples taken 1 day following 1-mg/mL NBD-Cl enema (Figure 4A). The concentration of interleukin-4 was also elevated, while that of interferon γ was not (Figure 4A). These findings suggest that Th17 and Th2 cells may be involved in colitis induction with the NBD-Cl enema rather than Th1 cells.

As with TNBS and oxazolone (7,12), NBD-Cl induces colitis, in which inflammatory cell infiltration and crypt-epithelium destruction are observed microscopically, via intrarectal administration. In BALB/c mice, body weight reduction peaks at day 2 following TNBS enema (dosage 1 mg per mouse) (12) and NBD-Cl enema (100 μ g per mouse) (Figure 1A), while at day 1 following oxazolone enema (1 mg per mouse) (7). In either the TNBS- or oxazolone-induced colitis model, the specific visualization of hapten-proteins is not available, and therefore, we cannot confirm hapten-protein formation in the colonic mucosa. In contrast, NBD-Cl-induced colitis presents the fluorescence visualization of NBD-proteins in the colonic mucosa, which helps us to study the relevance between hapten-proteins and inflammatory reactions, and allows for the confirmation of hapten-protein formation in the colonic mucosa when we assess the effects of experimental interventions on colitis induction. In conclusions, NBD-Cl-induced colitis model can facilitate the investigation of inflammatory reactions to hapten-proteins and will aid in the evaluation of novel anti-inflammatory treatments.

Acknowledgments

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Competing interests

The authors declare no competing interests.

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