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Identifying Novel Therapeutic Strategies Targeting Toll-Like Receptors for NSAID-Induced Enteropathy

PhD thesis

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List of Abbreviations

ANOVA: Analysis of Variance

CLR: Centered Log-Ratio

COX: Cyclooxygenase

DMSO: Dimethyl Sulfoxide

FDR: False Discovery Rate

GI: Gastrointestinal

IBD: Inflammatory bowel disease

IL1β: Interleukin-1 beta

IL10: Interleukin 10

IND: Indomethacin

I.p.: Intraperitoneal

LPS: Lipopolysaccharide

MPO: Myeloperoxidase

NAP: Naproxen

NOS: Nitric oxide synthase

NSAID: Nonsteroidal anti-inflammatory drug

PBS: phosphate-buffered saline

PG: Prostaglandin

PPI: Proton pump inhibitor

PRR: Pattern recognition receptor

PTX3: Pentraxin 3

qPCR: Quantitative Polymerase Chain Reaction

SEM: Standard Error of the Mean

TLR: Toll-like receptor

VEH: Vehicle

TNFα: Tumor necrosis factor alpha

1. INTRODUCTION

1. 1. Overview of the Characteristics of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent one of the most frequently prescribed classes of medications in all around the world. They are highly valuable for their potential analgesic, antipyretic, and anti-inflammatory effects in managing conditions such as musculoskeletal pain, osteoarthritis, rheumatoid arthritis, and acute inflammatory disorders (1-5). The use of NSAIDs continues to increase because of the global population ages, with chronic pain affecting more than 20% of older adults, and highlighting the essential role of these drugs in therapeutic regimens despite their associated risks (3, 5, 6). NSAIDs exert their beneficial effects mainly by the inhibition of cyclooxygenase (COX) enzymes, which catalyze the conversion of arachidonic acid to prostanoids, including prostaglandins (PGs) and thromboxanes, in this way they can suppress the mediators of inflammation, pain, and fever (2, 3, 7). However, PGs produced by this pathway are also essential for a wide range of physiological functions, such as the maintenance of vascular homeostasis, renal function, ovulation, and parturition, and their inhibition can disrupt these effects, contributing to potential toxicity (2, 4, 8). There are two isoforms of COX enzyme: COX-1, which is constitutively expressed and supports normal physiological functions such as gastric mucosal protection and platelet aggregation, and COX-2, which is induced during inflammatory conditions and promotes the production of pro-inflammatory PGs (2, 9-13). Traditional NSAIDs like ibuprofen, naproxen, and diclofenac, inhibit both COX-1 and COX-2 isoforms in a non-selective manner, thereby reducing inflammatory PGs at the sites of injury while simultaneously depleting gastroprotective PGs which finally result in impairing mucosal integrity and blood flow within the gastrointestinal (GI) tract (2, 3, 14). This dual inhibition explains the "double-edged sword" nature of these drugs which is well-recognized: although they are effective for symptom relief, they also increase the risk of serious adverse effects, particularly GI injury. Case–control studies in NSAID users have estimated a two- to fivefold increase in the relative risk of upper GI complications, with up to 30% of deaths from upper GI bleeding and related complications attributable to NSAID use (1, 2, 15). To reduce NSAID-induced GI toxicity, selective COX-2 inhibitors were developed to inhibit the inducible COX-2 isoform responsible for inflammation (16, 17). Clinical studies have demonstrated that these COX-2–selective inhibitors (such as celecoxib and rofecoxib) significantly reduce upper GI complications compared with non-

selective NSAIDs (18, 19). However, subsequent large-scale trials and meta-analyses revealed that long-term use of these drugs associated with cardiovascular side effects which also limits their use (20-22).

1.2. NSAID-Induced Enteropathy

The widespread use of NSAIDs is associated with significant GI side effects, which, beyond the well-known effects in the stomach and duodenum, also include damage to the small intestine, a condition known as NSAID-induced enteropathy (2, 23, 24). In contrast to upper GI complications, such as peptic ulceration and bleeding, which have long been recognized (2, 25, 26), the prevalence and severity of NSAID-induced small bowel injury have been revealed only due to recent advances in diagnostic technologies. Capsule endoscopy studies showed that around seventy percent of chronic NSAID consumers may have small bowel lesions, such as erosions, ulcers, petechiae or strictures. Importantly, this GI damage often occurs without obvious clinical symptoms (23, 27). These findings suggest that NSAID-induced enteropathy is actually more common than gastric and duodenal lesions, which were previously believed to be the primary sites of NSAID-induced GI toxicity (2, 28). The pathogenesis of NSAID-induced enteropathy is intricate and involves multiple factors, setting it apart from upper GI toxicity (8, 29). While the inhibition of COX enzymes, especially COX-1, is a major contributor to gastric damage, small bowel injury can occur through both COX-dependent and COX-independent mechanisms (2, 8, 29). Several factors can exacerbate intestinal mucosal injury such as enterohepatic recirculation of NSAIDs, along with the disruption of the mucosal barrier and increased intestinal permeability (30). In addition, both bile acids and gut microbiota (particularly Gram-negative bacteria) enhance the pro-inflammatory environment and finally lead to increased tissue damage (31, 32).

Despite current advances, treatment strategies for NSAID–induced enteropathy remain limited (2). Proton pump inhibitors (PPIs), which provide protection against NSAID-induced injury in the upper GI tract, do not offer protection against damage to the small intestine (33). There are no standard treatments available for managing or treating NSAID-induced small intestinal lesions, which is an important gap in clinical practice (34, 35). The incomplete understanding of the pathophysiology of the disease makes it difficult to develop targeted therapies (15, 36).

1. 3. Importance of Gut Microbiota in NSAID enteropathy

The GI tract harbors a complex and diverse microbial community consisting of around $3.8x10^{13}$ microorganisms (37), which is known as gut microbiota. In healthy conditions, most of these bacteria are Gram-positives and belong to the *Firmicutes* phylum (37-39). Besides *Firmicutes*, another dominant phylum is *Bacteroidetes*, while a limited population of *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* are present and contribute to the overall microbial diversity of the healthy gut (40). These microorganisms form symbiotic relationships with their hosts and perform numerous vital activities, from obtaining energy and nutrients from food, synthesizing vitamins, development of the immune system and protection against pathogens (40). Alterations in host-microbiota interactions affect the gut barrier function as well as the local immune system and result in disruption of intestinal homeostasis.

Over the last few decades, multiple studies have highlighted the role of altered microbiota composition (dysbiosis) in the pathogenesis of several diseases. For instance, a reduction in microbial diversity and the specific depletion of the anti-inflammatory commensal Faecalibacterium prausnitzii are recognized as hallmark features of Crohn's disease (41, 42). Similarly, shifts in the Firmicutes/Bacteroidetes ratio have been closely linked to metabolic syndromes, including type 2 diabetes (40). Additionally, dysbiosis affects neurological health through the gut-brain axis, where altered microbial metabolites can influence conditions such as anxiety-like behavior (43, 44). Intestinal dysbiosis is also an important feature of NSAID enteropathy, in which Gram-positive bacteria are gradually replaced by Gram-negative bacteria (27, 45), and these changes exhibit a strong correlation with the severity of intestinal inflammation and damage (46, 47).

Early studies have long recognized the pivotal role of gut bacteria in NSAID-induced enteropathy, demonstrating that germ-free animals (48) or those treated with antibiotics (49) exhibit reduced susceptibility to small intestinal damage caused by NSAIDs. Consistent with this finding, therapeutic modulation of the gut microbiota—through antibiotics, probiotics, or fecal microbiota transplantation—has been shown to confer protection against NSAID-associated intestinal damage (27, 50-52). Nevertheless, the clinical benefit of these strategies is still uncertain (53), and the prolonged use of even „safe" antibiotics, such as rifaximin, can be associated with the development of multidrug-resistant pathogens (54).

1.4. Importance of TLRs in NSAID-Induced Enteropathy

Bacteria can induce intestinal inflammation by activating innate immune responses through several mechanisms, including interplay with pattern-recognition receptors (PRRs) (55, 56). Toll-like receptors (TLRs) are one of the most important PRRs in the innate immune system which can recognize pathogen-associated molecular patterns from different bacteria, viruses, and other microbes (57). They are expressed on intestinal epithelial cells, and immune cells in various tissues (58). Until now, 10 TLRs (TLR1–TLR10) have been identified in humans and 13 in rodents (141). Some of these TLRs are critical for maintaining intestinal homeostasis and responding to pathogenic challenges, including TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9, which are particularly involved in bacterial recognition (57, 58).

Activation of TLRs leads to release of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), which ultimately results in neutrophil accumulation. Although these inflammatory reactions are critical for eliminating pathogens, they can also contribute to tissue damage (55, 56). TLR expression is dynamically regulated by cytokines and the microbiota, and dysregulation of TLR signaling can lead to the perpetuation of chronic inflammation, as observed in inflammatory bowel disease (IBD) and NSAID-induced enteropathy (59-62); therefore, it seems that targeting TLRs, instead of directly modification of the gut microbiota composition, provides a more nuanced strategy for regulation of host–microbe interactions and reducing inflammation in such conditions (58).

However, despite the long-recognized importance of intestinal bacteria in enteropathy, NSAID-induced changes in TLR expression and host-bacterial interactions remain largely unexplored. Of the six TLRs dedicated to bacterial recognition, TLR4 has received the most attention in the context of NSAID-induced enteropathy. TLR4 recognizes lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, and it is believed to play a crucial role in mediating intestinal injury (32). Watanabe et al. (2008) showed that mice lacking TLR4 or its adaptor protein MyD88 were protected against NSAID-induced enteropathy (32). Conversely, LPS administration could aggravate enteropathy in wild-type mice. Subsequent studies have shown that TLR4 activation exacerbated intestinal damage by activation of the NLRP3 inflammasome as a downstream signaling pathway (63, 64). Interestingly, LPS treatment had different effect on enteropathy depending on the time of administration; LPS administration after indomethacin (IND)

aggravated injury by amplifying inflammation, whereas pretreatment with LPS was protective, possibly by inducing tolerance or modulating cytokine responses (32). In addition to TLR4 signaling activity, several studies have investigated changes in TLR4 expression during NSAID-induced enteropathy; however, the results remain controversial (32, 65-67). Thus, although the TLR4/MyD88 pathway appears to be a central contributor to NSAID-induced enteropathy by enhancing inflammatory responses to luminal bacteria following NSAID-related disruption of the mucosal barrier (63), the role of TLR4 may differ at different stages of enteropathy, depending on the expression of TLR4 and the composition of the microbiota.

In contrast, TLR2 which detects Gram-positive bacterial components like peptidoglycans and lipopeptides, is likely to exert a protective effect in NSAID-induced enteropathy. Pretreatment with TLR2 agonists ameliorated IND-induced enteropathy by limiting excessive leukocyte migration to the intestinal mucosa and suppressing pro-inflammatory responses (68). Unlike TLR4, which contributes to disease pathology, TLR2 signaling may support mucosal repair and homeostasis, potentially through IL-10-mediated immunosuppression. Notably, the protective effect of TLR2 agonists in enteropathy was only seen if they were administered prior to development of NSAID-induced injury (68, 69, 70). This suggests that the effect of TLR2 activation in enteropathy depends on its expression and composition of microbiota, like in the case of TLR4. Likewise, it is unclear how the expression of TLR2 changes in enteropathy. It has been reported that TLR2 expression increases during intestinal injury (54, 55). However, in other studies, the expression of TLR2 did not change in NSAIDs induced enteropathy (71, 72).

The roles of other bacterial-sensing TLRs (TLR1, TLR5, TLR6, TLR9) in NSAID enteropathy remain unexplored (63, 71, 73, 74). TLR1 and TLR6 often heterodimerize with TLR2 to recognize bacterial lipopeptides and modulate intestinal inflammation but no specific studies have assessed their changes or functions in NSAID enteropathy (75). Also, direct evidence linking TLR9 to NSAID-induced damage is scarce, though TLR9 agonists show protective effects in other GI injury models, such as radiation syndrome (76).

In terms of TLR5, limited data suggest stable TLR5 expression in mice after transplanted with feces of enteropathic animals (71). Apart from this, little is known about the role of TLR5 in NSAID enteropathy. It is somewhat surprising, given that TLR5 is an important pattern-recognition receptor that detects flagellin of motile Gram-negative β- and γ-*Proteobacteria* (77), whose

intestinal abundance dramatically increases in enteropathy. In addition, several lines of evidence suggest that TLR5 activation protects against various GI injuries. TLR5 prevents dysbiosis-associated inflammation by regulating the gut microbiota composition and localization, thereby plays an essential role in maintaining intestinal homeostasis (78, 79). In intestinal epithelial cells, TLR5 is mainly expressed on the basolateral surface which can recognize translocated flagellin and activates downstream MyD88- and NF-κB–dependent signaling pathways. This activation induces the production of antimicrobial peptides (such as Reg3γ), cytokines (including IL-22), and mucus, which collectively enhance mucosal barrier integrity and limit pathogen overgrowth (80-82). TLR5 deficient mice develop spontaneous colitis, mucus defects, increased intestinal level of *Proteobacteria*, and increased mucosal adherence, features similar to NSAID enteropathy (80, 83). Flagellin-TLR5 signaling protects against colitis, mucositis, and infections by supporting tissue repair and anti-inflammatory responses (81, 84, 85). According to these observations, we hypothesized that TLR5 activation by flagellin can alleviate NSAID-induced enteropathy by reducing inflammatory responses.

Overall, changes in TLRs and their associations with inflammation and dysbiosis in NSAID – induced enteropathy remain largely unexplored. In addition, no studies have been carried out to date to determine whether TLR5 activation provides protection against NSAID enteropathy.

2. OBJECTIVES

2.1. Objectives of the first study

In our first study, we aimed to clarify how NSAID enteropathy affects the expression of TLRs in the small intestine, and we comprehensively evaluated whether changes in TLRs correlate with intestinal inflammation and dysbiosis. To this end, we used both acute and chronic enteropathy models in rats. More specifically, our objectives were as follows.

2.1.1. We aimed to assess the changes in the small intestinal gene expression of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9 at different times during acute enteropathy.

2.1.2. We aimed to comprehensively evaluate the correlations between changes in TLR expression and intestinal inflammation and dysbiosis in acute enteropathy.

2.1.3. We aimed to assess the alterations in the gene expression of TLRs in chronic enteropathy induced by repeated administration of lower doses of NSAIDs, which better reflects the clinical context of chronic NSAID use.

2.2. Objectives of the second study

Because the results of the first study showed that there is a strong inverse correlation between TLR5 expression and IND-induced intestinal inflammation in rats, in our second study we aimed to determine the role of TLR5 in NSAID-induced enteropathy.

2.2.1. We aimed to analyze whether the expression of TLR5 is also downregulated by NSAIDs in mice.

2.2.2. We aimed to analyze whether TLR5 activation by flagellin *prior* to IND administration protects against intestinal inflammation and tissue damage in mice.

2.2.3. We also aimed to assess whether TLR5 activation by flagellin *after* IND administration can mitigate IND enteropathy.

2.2.4. We aimed to analyze the effect of TLR5 inhibition by TH1020 (TLR5 antagonist) on IND-induced intestinal inflammation.

3. MATERIALS AND METHODS

3.1. Experimental animals

The first study was performed using male Wistar rats (8–10-week-old) in a fed state with body weights of 220–240 g, obtained from Toxi-Coop Ltd., Budapest, Hungary. For the second study, we used 8–12-week-old non-fasted male C57BL/6 mice with body weights of 23–30 g, obtained from the National Institute of Oncology. All animals were maintained in a controlled environment with stable temperature (22 ± 2°C) and humidity, following a standard 12-hour light/dark cycle, and had unlimited access to food and water. We took every step possible to ease any animal discomfort and keep the number of animals involved to a minimum. All procedures were performed in accordance with the Directive 2010/63/EU of the European Convention for the Protection of Animals Used for Scientific Purposes. Animal experimentation protocol was approved by the National Scientific Ethical Committee on Animal Experimentation and authorized by the Food Chain Safety and Animal Health Directorate of the Government Office of Pest County (license no. PE/EA/1118-6/2020).

3.2. Study design

This thesis is based on two separate, but complementary studies designed to investigate: (1) the effects of NSAID treatment on small-intestinal TLR expression and its associations with gut inflammation and dysbiosis in rats; and (2) the effect of pharmacological activation and inhibition of TLR5 on IND-induced enteropathy in mice.

In the first study, we first investigated the alterations of intestinal TLRs expression in the context of acute enteropathy. To achieve a uniform gut microbiota in all male Wistar rats, first they were placed in shared cages (4 or 5 rats for each cage) for one week and then randomly assigned to either the vehicle (VEH)-treated group (1% hydroxyethylcellulose) or IND-treated groups. Enteropathy was induced by a single large dose of IND (20 mg/kg; Sigma, St. Louis, MO, USA) administered via gastric gavage (47). Rats were sacrificed at 6, 12, 24, 48, or 72 hours after IND administration, with eight animals for each group. As preliminary experiments demonstrated that VEH-treated rats maintained a stable microbiota composition over time under constant environmental conditions, control animals were sacrificed at 72-hour.

In a second in vivo experiment, we investigated the effects of prolonged NSAID exposure on intestinal TLR expression and inflammation. Rats were randomized into VEH- or NSAID-treated groups. Two groups received IND at doses of 2 mg/kg or 4 mg/kg, which were administered twice daily for 14 and 7 days, respectively. This dosing protocol was selected based on pilot studies indicating that 4 mg/kg/day for 4 weeks resulted in moderately severe enteropathy. However, during the present study, twice-daily administration of 4 mg/kg caused severe enteropathy, necessitating euthanasia of the animals one day earlier than planned due to their poor general condition. Two additional groups were treated with another NSAID, naproxen (NAP, Sigma, St. Louis, MO, USA), at doses of 10 mg/kg or 20 mg/kg (86) twice daily for 14 and 7 days, respectively. Control animals were treated with VEH and were euthanized at a single time point after two weeks.

In both experiments, at the time of sacrifice, the entire small intestine was excised. In the second experiment also blood samples were collected. Intestinal length was recorded as an indicator of inflammation. Full-thickness samples of the distal small intestine and corresponding luminal contents were snap-frozen at –80°C for molecular analyses. Additionally, distal jejunal segments were fixed in 10% formalin for standard histologic analysis.

The second study evaluated the potential protective effects of TLR5 signaling on IND-induced enteropathy in mice, focusing on intestinal inflammation, bacterial load, and mucosal integrity.

To this end, male C57BL/6 mice were randomly assigned to groups of six to seven animals each. Enteropathy was induced by a single high-dose IND administration (30 or 40 mg/kg; Sigma, St. Louis, MO, USA) via gastric gavage, following established protocols (87-89). Control mice received the vehicle (VEH; 1% hydroxyethylcellulose). Purified flagellin isolated from *Salmonella typhimurium* (tlrl-stfla, Invivogen, San Diego, CA, USA) was dissolved in phosphate-buffered saline (PBS) and administered intraperitoneally (i.p.) at dosages of 10 and 30 µg per mouse—consistent with prior research (90, 91)—at the specified time points. The TLR5 antagonist TH1020 (10 µg/mouse) (HY-116961, MedChemExpress, Monmouth Junction, NJ, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted to the required concentration in phosphate-buffered saline (PBS).

3.3. Western blot analysis

Homogenization of tissue samples and Western blotting were conducted according to the procedures detailed in Lázár et al. (2021) (47). The primary antibodies used were anti-COX-2 (D5H5) (12282S, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-IL-1β (ab9722, 1:1000), anti-pentraxin-3 (ab125007, 1:1000) (Abcam, Cambridge, UK), and anti-myeloperoxidase (MPO) (AF3667, 1:1000, R&D Systems, Minneapolis, MN, USA). As secondary antibodies HRP-linked anti-rabbit IgG (7074, 1:10000, Cell Signaling Technology, Danvers, MA, USA) and anti-goat IgG (ab97120, 1:10000, Abcam, Cambridge, UK) were used. Anti-GAPDH (D16H11) (5174S, 1:1000, Cell Signaling Technology, Danvers, MA, USA) was used to control for sample loading and protein transfer and to normalize the content of target protein. Membranes were cut into smaller fragments before antibody incubation if the target protein bands of interest were far apart. The results were replicated at least twice in the experiment. Signals were captured using a chemiluminescence detection kit (Bio-Rad, Hercules, CA, USA) via the Chemidoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA).

Western blot analysis of small intestinal luminal flagellin adhered to methods reported earlier (92, 93). In brief, luminal samples were homogenized in PBS (100 mg/mL), freeze-thawed once, boiled for 10 minutes to lyse the bacteria, sonicated for 45 seconds, and finally centrifuged at 12,000 rpm for 10 minutes at 4 °C. Luminal extracts containing an equal amount of protein (20 μg) were loaded onto a 4-20% precast Tris-glycine SDS polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and separated by electrophoresis. The proteins were then transferred electrophoretically onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) and probed with an anti-flagellin antibody (PA5-144519, 1:1000, Thermo Fisher Scientific, Waltham, MA, USA). The membrane was subsequently incubated with an anti-rabbit HRP-linked secondary antibody (7074, 1:5000, Cell Signaling Technology, Danvers, MA, USA). Signals were detected using a chemiluminescence kit (Bio-Rad, Hercules, CA, USA) with a Chemidoc XRS+ (Bio-Rad, Hercules, CA, USA).

3.4. qRT-PCR measurements

Total RNA was obtained from 10 to 30 mg of small intestine tissue using the QIAzol extraction method (Qiagen, Hilden, Germany). RNA concentration was measured with Nanophotometer (Implen GmbH, Munich, Germany). Reverse transcription was performed from 1 μg of total RNA

with a SensiFAST cDNA synthesis kit (Bioline, London, UK) according to the manufacturer's protocol. Target genes were amplified on a LightCycler® 480 II instrument (Roche, Germany) using the SensiFAST SYBR Green master mix (Bioline, London, UK). Gene expression levels were assessed using the $2^{-\Delta\Delta CT}$ method and Rpl13a was used as reference gene. Primers used are listed in Table 1.

Gene	**Primer sequence (5'-3')**		**Accession number**
Tlr1	forward (rat)	CTG ATC TCT TGC CAC GCA AC	NM_001172120.2
	reverse (rat)	TCT GAG AAC CGC TCA ATC CC	
Tlr1	forward (mouse)	GCA GTT GGT GAA GAA CTC AGG	NM_030682.2
	reverse (mouse)	CAC ATG GGT ATA GGA CGT TTC TG	
Tlr2	forward (rat)	TTG GGT GGA GAA CCT CAT GG	NM_198769.2
	reverse (rat)	GGA ACA AAG TCC CGC TTG TG	
Tlr2	forward (mouse)	AAG ATG CGC TTC CTG AAT TTG	NM_011905.3
	reverse (mouse)	TCC AGC GTC TGA GGA ATG C	
Tlr4	forward (rat)	AAC CTC AAC GAC CTC ACA GG	NM_019178.2
	reverse (rat)	GTT CTC ACT GGG CCT TAG CC	
Tlr4	forward (mouse)	AAT CCC TGC ATA GAG GTA GTT C	NM_021297.3
	reverse (mouse)	TCA GGG ACT TTG CTG AGT TTC	
Tlr5	forward (rat, mouse)	GCC AGA GCC AGA TTG AAG TCT TGA	NM_001145828.1;
	reverse (rat, mouse)	GAG AGG CTG GAG TTC ATC TTC ACA	NM_016928.4
Tlr6	forward (rat, mouse)	CCT CTG GGA TAG CCT CTG CAA C	NM_207604.1;
	reverse (rat, mouse)	CAG AGA ACT GGG TCA TGC TTC C	NM_001384171.2
Tlr9	forward (rat, mouse)	TGC TGG ACC TAA GCG AGA AC	NM_198131.1;
	reverse (rat, mouse)	AAG GAA CTT GCC AGG TGG AG	NM_031178.2
Il1b	forward (rat)	TGG CAA CTG TCC CTG AAC TC	NM_031512.2
	reverse (rat)	GGG CTT GGA AGC AAT CCT TAA TC	
Il1b	forward (mouse)	AAA GCT CTC CAC CTC AAT GGA C	NM_008361.4
	reverse (mouse)	TTG TCG TTG CTT GGT TCT CC	
Il10	forward (rat)	GAA CCA CCC GGC ATC TAC TG	NM_012854.2
	reverse (rat)	AGG AGT TGC TCC CGT TAGC	
Il10	forward (mouse)	TGT GTA TTG AGT CTG CTG GAC	NM_010548.2
	reverse (mouse)	TTC GGA GAG AGG TAC AAA CGA G	
Tnf	forward (rat)	ACT GAA CTT CGG GGT GAT TG	NM_012675.3
	reverse (rat)	GCT TGG TGG TTT GCT ACG AC	
	forward (mouse)	CGG ACT GGA TTC TAT GGT GAA A	NM_011198.5

Ptgs2 (COX2)	reverse (mouse)	CTT GAA GTG GGT CAG GAT GTA G	
Nos2	forward (mouse)	AGG GAC TGA GCT GTT AGA GAC A	NM_010927.4
	reverse (mouse)	GTC ATC TTG TAT TGT TGG GCT GAG	
Rpl13a	forward (mouse, rat)	GGA TCC CTC CAC CCT ATG ACA	NM_173340.2
	reverse (mouse, rat)	CTG GTA CTT CCA CCC GAC CTC	

Table 1. Primer sequences used for assessing the expression of different genes in rats and mice.

3.5. Histology

Distal part of small intestinal samples was excised and prepared using the Swiss-roll technique, then the sample tissue was fixed in 10% formalin, embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin. Histological damage was graded blindly by two histopathologists. In the first study, histological changes were scored in six categories for both acute and chronic NSAID-induced enteropathy in rats: villus morphology (0-3); the presence and extent of erosions (0-2); the presence and extent of ulcers (0-5); the severity of mucin depletion (0-3); the severity of inflammatory cell infiltration (0-2); and the severity of peritoneal reaction (0-2). The sub-scores were then added together to produce a total score out of 17. Based on combined scores, tissue damage was subsequently grouped into 5 grades of severity: grade 0 (0), grade 1 (1–3), grade 2 (4–8), grade 3 (9–13), and grade 4 (14–17).

In the second study, histological injury was graded on an eight-point scale ranging from 0 (normal mucosa) to 8 (transmural infarction) according to the Chiu/Park classification (93, 94), in a blinded manner. In addition, the length of the mucosal area that received a Chiu/Park score of at least 4 was quantified in each histological specimen. The proportion of the total damaged area and the proportion of areas with the highest grade in a given sample were then calculated relative to the total mucosal length. Finally, the severity of neutrophil granulocytic infiltration in the damaged area was graded on a scale of 0-2 (0: normal neutrophil counts; 1: increased number of neutrophils in the submucosa of the affected area; 2: increased number of neutrophils in both the submucosa and the muscularis propria of the affected area). All slides were scanned using a Pannoramic 1000 Digital Slide Scanner (3DHistech, Hungary), and representative images were captured using SlideCenter software (3DHistech, Hungary).

3.6. Hematological analysis

Whole blood samples were collected in Vacuette K-3 EDTA tubes (Greiner Bio-One, Kremsmünster, Austria). Hematological analysis was conducted using a Sysmex XN 1000 hematology analyzer (Sysmex America, Lincolnshire, IL, USA) following standard laboratory protocols. Results were automatically computed via the Sysmex XN 1000 hematology software.

3.7. Gut microbiota analysis

Bacterial DNA was extracted from 80 mg of small intestinal sample using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) and further purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocols. The concentration of genomic DNA was measured using a Qubit 2.0 Fluorometer with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial DNA was amplified with tagged primers (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG and 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C), covering the V3-V4 region of the bacterial 16S rRNA gene. PCR and DNA purifications were performed according to Illumina's demonstrated protocol (Part # 15044223 Rev. B). The PCR product libraries were quantified and qualified by using DNA 1000 Kit on Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Waldbronn, Germany). Equimolar concentrations of libraries were pooled and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v3 (600 cycles PE).

In addition, we assessed the changes in the absolute counts of *Lachnospiraceae* and *Enterobacteriaceae*. To this end, we designed specific primers targeting all bacteria and bacteria belonging to the *Lachnospiraceae* and *Enterobacteriaceae* families. Bacterial 16S rRNA gene sequences were obtained from the European Nucleotide Archive and aligned in UGENE (95) to identify potential specific primers. Final primers were selected based on testing them in SILVA (96) and Ribosomal Database Project (RDP) (97) rRNA gene databases (Table 2). Primers were synthesized commercially (Integrated DNA Technologies, IA, USA). Bacterial DNA was extracted from 50-100 mg luminal samples by ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA).

Then, qPCR was performed on a LightCycler® 480 II instrument (Roche, Germany) using a SensiFAST SYBR Green master mix (Bioline, London, UK). In the first study, bacterial gene expression was determined by the $2^{-\Delta\Delta CT}$ method and normalized for the universal bacterial primer (98). In the second study, a standard curve ranging from 10^5 to 10^9 CFU/ml was produced from amplicons derived from the control strain *E. coli* DH5α (ATCC PTA-1798) to convert cycle threshold (Ct) values into bacterial quantity (log CFU/per gram sample).

Target	**Primer sequence (5'-3')**		**Amplicon size (bp)**	**Applied annealing temperature (°C)**
All bacteria	forward	CCA GCA GCC GCG GTA ATA CG	260	62
	reverse	TGG ACT ACC AGG GTA TCT AAT CCT GTT		
Lachnospiraceae	forward	GGG AAG AWA ATG ACG GTA CCT GAC TAA	140	62
	reverse	RCG CTC CCT TTA CAC CCA GTA AA		
Enterobacteriaceae	forward	TTG ACG TTA CYC GCA GAA GAA GCAC	260	62
	reverse	ACC TGA GCG TCA GTC TTY GTC CAG		

Table 2. *Primer sequences used for bacterial analysis.*

3.8. Data analysis

Bioinformatic analysis was carried out as outlined by Mansour et al. (2020) (99). In brief, raw sequencing reads were initially assessed for quality via FastQC and MultiQC. Low-quality sequences were subsequently filtered and trimmed using Trimmomatic (100), keeping only those sequences with a minimum length of 36 nucleotides and excluding base calls with a Phred score less than 20. Taxonomic classification was performed using the SILVA SSU Ref NR 99 database (release 132), which was preprocessed and indexed via Kraken2 (101) using a k-mer length of 31. The final microbiota composition was estimated using Bracken (102). Following this, a Bayesian-multiplicative zero replacement was applied via the zCompositions package in R (103), after which a Centered Log-Ratio (CLR) transformation was applied using the scikit-bio-Python library (104). Taxa exhibiting a median CLR value under two were omitted from subsequent statistical evaluations. Alpha diversity was measured with the Shannon index, incorporating only taxa supported by a minimum of fifty reads. The overall microbiota composition was visualized by

Principal Component Analysis (PCA), while variations in community structure were evaluated statistically via Permutational Multivariate Analysis of Variance (Permanova). Differential abundance testing was conducted with the Wilcoxon rank-sum test, applying the Benjamini-Hochberg method (105) to control the false discovery rate (FDR).

Statistical analyses of the data were performed with Student's t-test, one-way ANOVA, or two-way ANOVA, followed by Fisher's LSD post hoc test. For non-parametric data, the Kruskal-Wallis test was used with uncorrected Dunn's post hoc test. Outliers identified by Grubb's test were removed from the analyses. A p-value threshold of < 0.05 was considered statistically significant.

Correlations among TLRs, bacterial abundances, and inflammatory markers were examined as reported in earlier studies (47, 106, 107). In brief, two separate correlation analyses were performed across the samples from the first (acute enteropathy induced by a single high-dose IND) and second (enteropathy induced by repeated low-dose IND) *in vivo* experiments, computing Spearman's rank correlation coefficients (Rho values) and associated p-values for parameter pairs (TLRs versus inflammatory markers, TLRs versus bacterial abundances, inflammatory markers versus bacterial abundances). Rho-values were visualized on separate heat maps for each comparison marking correlations considered to be significant according to either the uncorrected p-value or the p-value corrected by the Benjamini-Hochberg procedure (q-value) (105). These correlation analyses were performed using R software version 4.3.3 (108).

4. RESULTS

4.1. Analysis of changes in TLRs and bacterial-host interactions in both acute and chronic NSAID-induced enteropathy.

In the first study, to clarify how NSAID enteropathy modulates intestinal TLR expression and bacterial-host interactions, and to identify prospective therapeutic targets, we used both acute and chronic models of enteropathy in rats.

4.1.1. Indomethacin-induced acute enteropathy differentially modulates intestinal TLR expression.

Our initial objective was to explore the changes in the gene expression of TLR 1, 2, 4, 5, 6, and 9 during severe acute enteropathy. For this purpose, rats received a single high dose of IND (20 mg/kg), and small intestinal samples were collected at five different time points including 6, 12, 24, 48, and 72 h post-dose (Fig. 1A). The severity of IND-induced enteropathy progressively increased over time, resulting in body weight loss (Fig. 1B, left) and intestinal shortening (Fig. 1B, right). Enteropathy was associated with diverse histological alterations including erosions, ulcers, and inflammatory cell infiltrations, observable in certain animals as early as 6 hours after IND treatment (Fig. 1C). Mucosal inflammatory responses were further evidenced by upregulated levels of key inflammatory mediators and cytokines, including MPO, PTX3, COX2, IL1β, and TNFα, which all reached their maximum at 48 hours (Figs. 1D, 1E). Conversely, the gene expression of the anti-inflammatory interleukin 10 (IL10) gradually decreased.

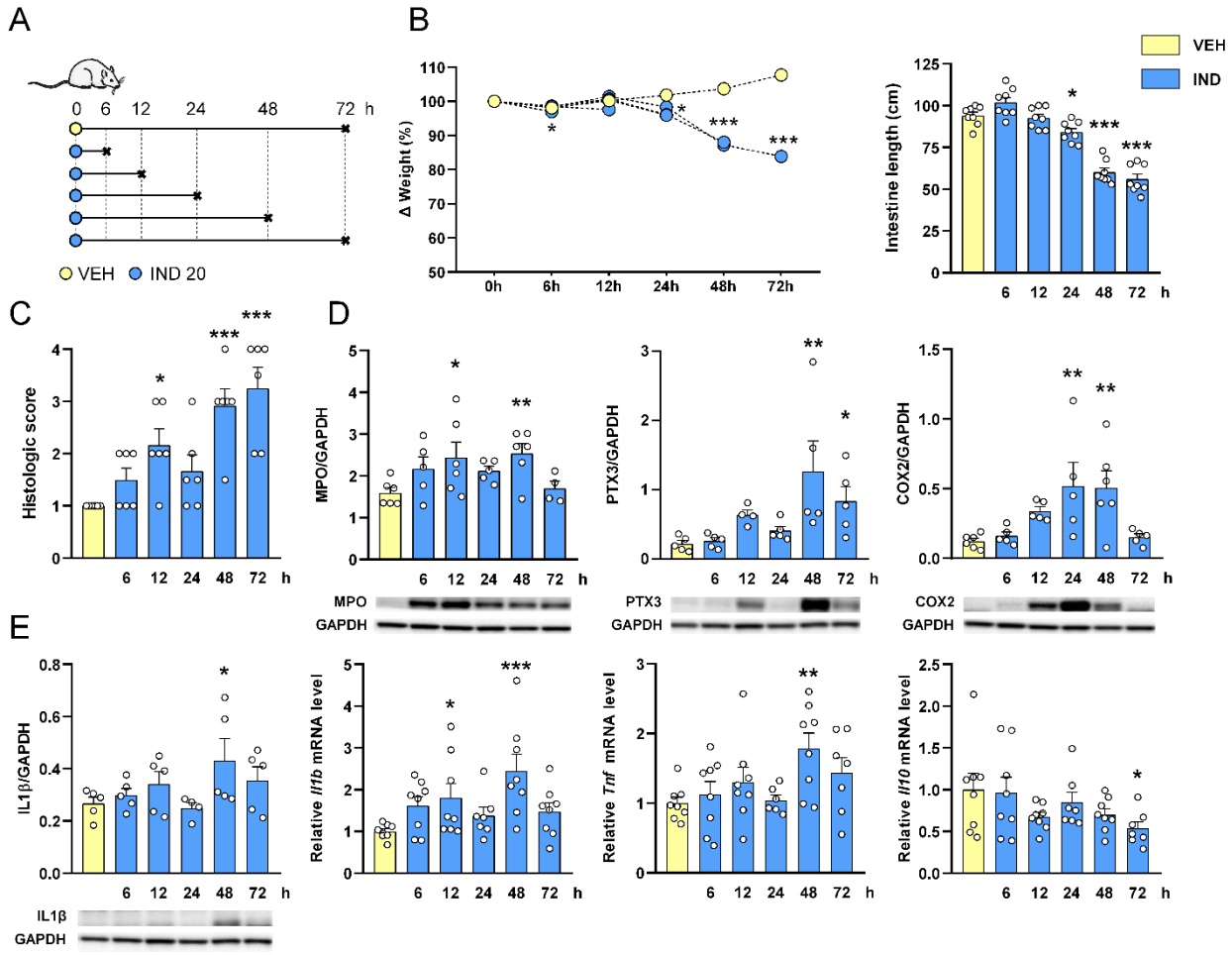


Figure 1. *Acute inflammation and tissue injury in the rat small intestine induced by a single high dose of indomethacin (IND, 20 mg/kg).* ***A)*** *Summary of the study protocol: Rats were treated with vehicle (VEH) or IND and sacrificed at five different time points.* ***B-E)*** *Impact of IND administration on body weight, small intestine length, histological injury, and expression of select inflammatory mediators and cytokines. The results are expressed as the mean + SEM, with individual data points shown as circles. Statistical analyses included two-way repeated measures ANOVA for weight changes, one-way ANOVA for intestinal length and inflammatory gene/protein expression, and Kruskal-Wallis test for histological scoring. These tests were followed by Fisher's LSD and uncorrected Dunn's post hoc analyses, respectively. Sample sizes ranged from 4 to 8 animals per group. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001 compared to the VEH-treated group.*

Next, we measured the gene expression of TLRs in the small intestine of NSAID-treated rats and found that they showed different changes (Fig. 2). TLR2 gene expression was increased with time and was significantly upregulated at 24 and 48 hours as observed for several pro-inflammatory markers. The expression of TLR1, which acts as a co-receptor for TLR2, showed a similar trend

with peak levels at 48 h post IND administration. The level of TLR6, the other heterodimerizing partner of TLR2, did not change. While mRNA levels of TLR4 and TLR9 increased at some time points, they showed high inter-individual variability and were statistically not different from controls. Finally, TLR5 expression was downregulated in enteropathic rats.

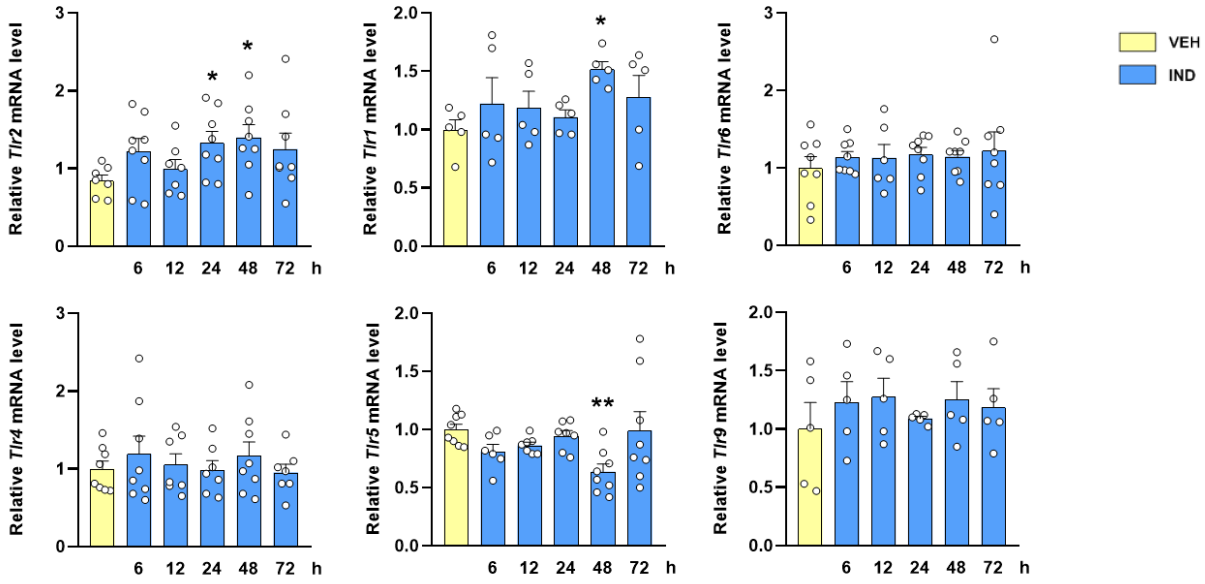


Figure 2. *A high dose of indomethacin (IND, 20 mg/kg) elicits distinctive alterations in intestinal TLR gene expression. Results are shown as mean + SEM, with individual data points illustrated as circles. One-way ANOVA was applied for statistical analysis, followed by Fisher's LSD test. n=5–8 per group. *p < 0.05, **p < 0.01 versus the vehicle (VEH)-treated controls.*

4.1.2. Intestinal dysbiosis and inflammation induced by IND in acute enteropathy are differentially associated with changes in distinct TLRs.

We aimed to determine whether changes in TLRs are associated with the severity of inflammation and/or dysbiosis. With regard to inflammation, we found a strong association between inflammation and TLR2 expression. The association between inflammation and the expression of TLR1 and TLR4 was less pronounced, being significant only in the case of IL1β and COX-2, respectively. In contrast, TLR5 expression exhibited a strong negative correlation with intestinal inflammation in IND-induced acute enteropathy, while the expression of TLR6 and TLR9 did not correlate with any of the tested inflammatory markers (Fig. 3).

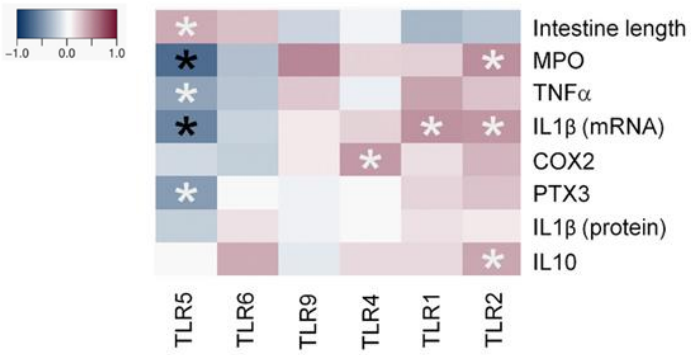


Figure 3. *Heat map of Spearman's correlation coefficients between TLRs and tissue injury markers in animals treated with VEH or IND 20. p-values were corrected by the Benjamini–Hochberg procedure yielding q-values. White asterisk: uncorrected $p < 0.05$, black asterisk: $q < 0.05$.*

Since TLRs and gut microbiota are closely interrelated, we analyzed whether IND-induced changes in small intestinal TLRs are associated with specific microbiota signatures. To this end, we determined the small intestinal microbiota composition by 16S rRNA gene sequencing IND had only minor effect on the Shannon index, an indicator of α-diversity (Fig. 4A) which is consistent with literature data (45). However, IND induced significant changes in the overall microbiota composition. As illustrated in the PCA score plot (Fig. 4B), IND-induced dysbiosis was already detectable at 6 h (Fig. 4B) and was characterized by a dramatic shift from Gram-positive to Gram-negative bacteria (Figs. 4C-4E) (Permanova $p < 0.001$ between VEH and IND-treated groups). More specifically, the relative abundance of Gram-positives like *Firmicutes* decreased from 96.7% to 57.1% within 72 hours. This reduction was mainly seen in *Lactobacillus*, *Limosilactobacillus*, *Ligilactobacillus*, *Romboutsia*, and *Turicibacter* genera. At the same time, several genera from the Gram-negative *Proteobacteria* and *Bacteroidota* phyla, such as *Escherichia–Shigella* and *Bacteroides*, showed a significant increase in enteropathy. Several Gram-negatives, particularly those belonging to the *Gammaproteobacteria*, showed a strong positive association with inflammation, whereas several Gram-positives correlated inversely with that (not shown.)

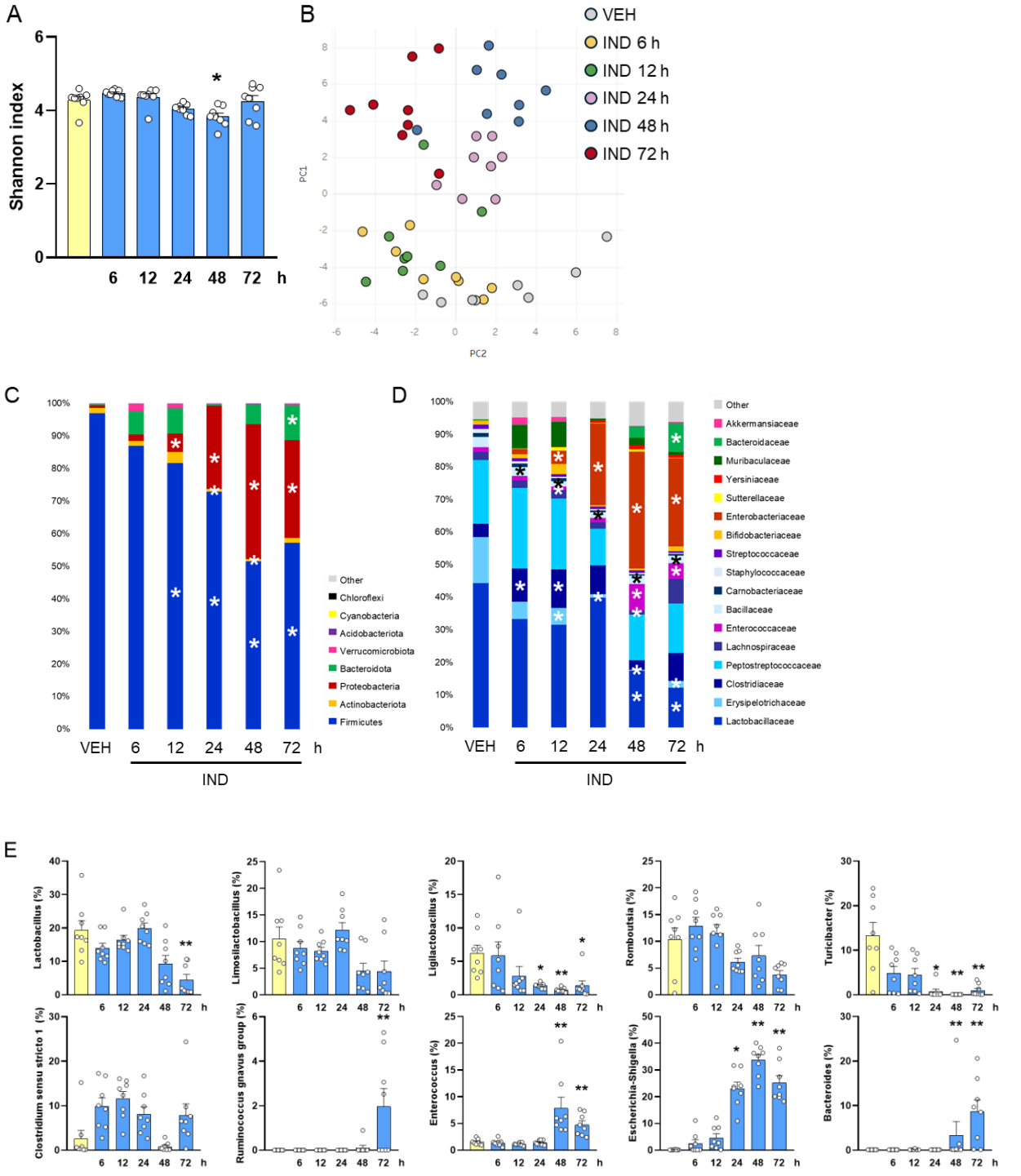


Figure 4. *A high dose of indomethacin (IND, 20 mg/kg) causes marked changes in the small intestinal microbiota composition.* ***A)*** *Impact of IND on bacterial diversity in the small intestine, measured by the Shannon index (Kruskal-Wallis test, followed by uncorrected Dunn's tests, n = 8/group).* ***B)*** *Principal component analysis (PCA) score plot illustrating the small intestinal microbiota composition of rats received vehicle (VEH) or IND.* ***C-E)*** *IND-induced changes in the relative abundance of bacterial phyla*

*and major families and genera. The differential abundance tests were performed using ANCOM with Holm-Bonferroni correction for multiple comparison, n = 8/group. *q < 0.05, **q < 0.01 compared to VEH-treated group.*

Next, we comprehensively evaluated the correlation between TLR expression levels and the relative abundances of all detected bacterial families and genera in control and enteropathic rats. Changes in TLR1, TLR2, and TLR5 expression correlated with the luminal levels of multiple bacterial families (Fig. 5) and genera (not shown), although only the positive correlation between TLR2 and the genus *Vibrio* remained significant when individual p-values were corrected for multiple testing. However, the observed associations between TLRs and intestinal bacteria were likely driven mainly by inflammation rather than by direct TLR–bacterial interactions. Specifically, inflammation-associated bacteria, such as members of *Gammaproteobacteria* and *Enterococcus,* showed positive correlations with TLR1 and TLR2 and negative correlations with TLR5, whereas several Gram-positive bacteria (e.g., *Lactobacillaceae*, *Erysipelotrichaceae*), which decreased in inflammation, showed the opposite correlation with these TLRs. In contrast, TLR4, TLR6, and TLR9, whose expression was minimally or not influenced by acute intestinal inflammation, did not show significant correlations with bacteria. Notably, TLR6 and TLR9 showed significant negative correlations only with *Pectobacteriaceae* and *Bifidobacteriaceae*, respectively.

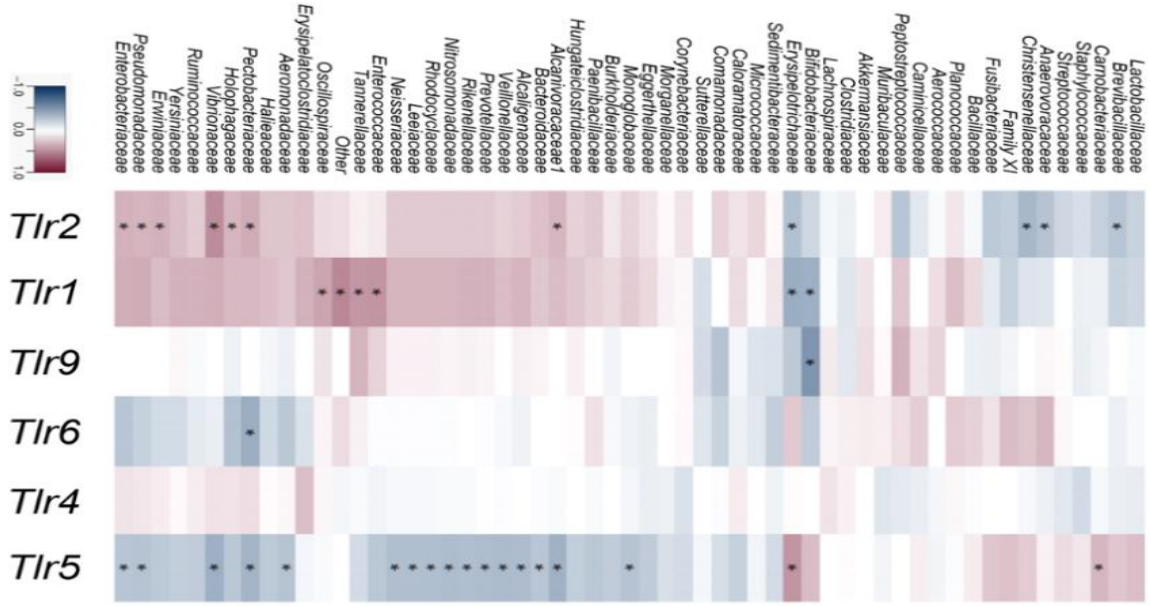
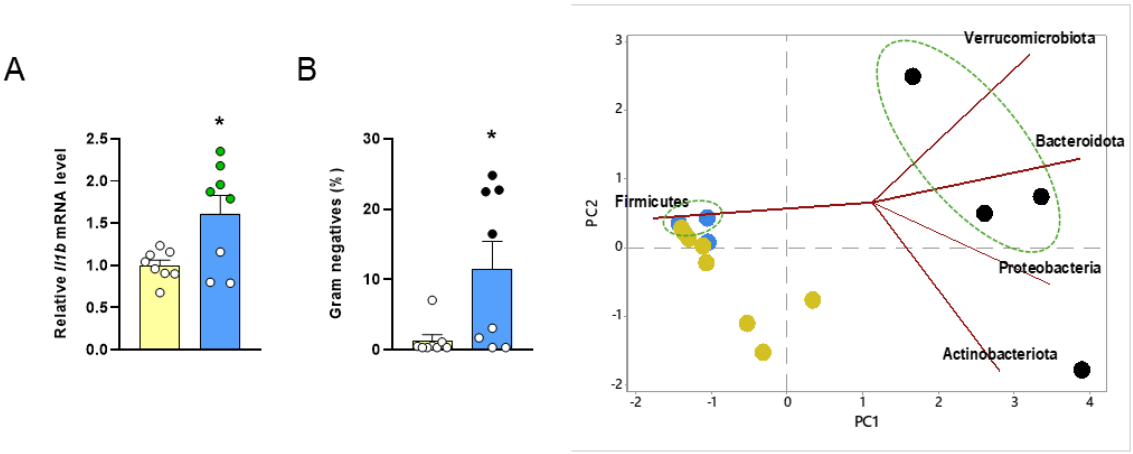
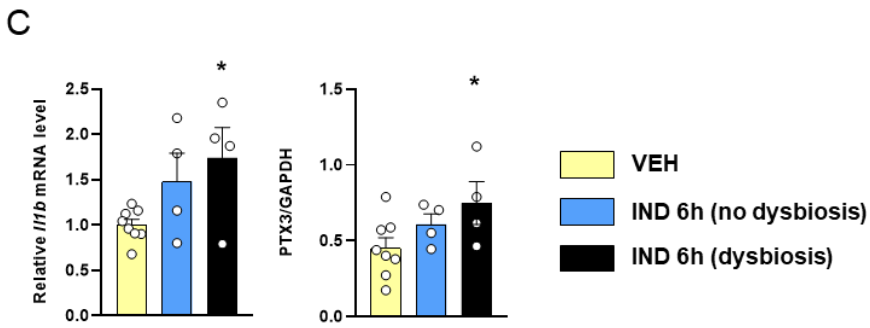


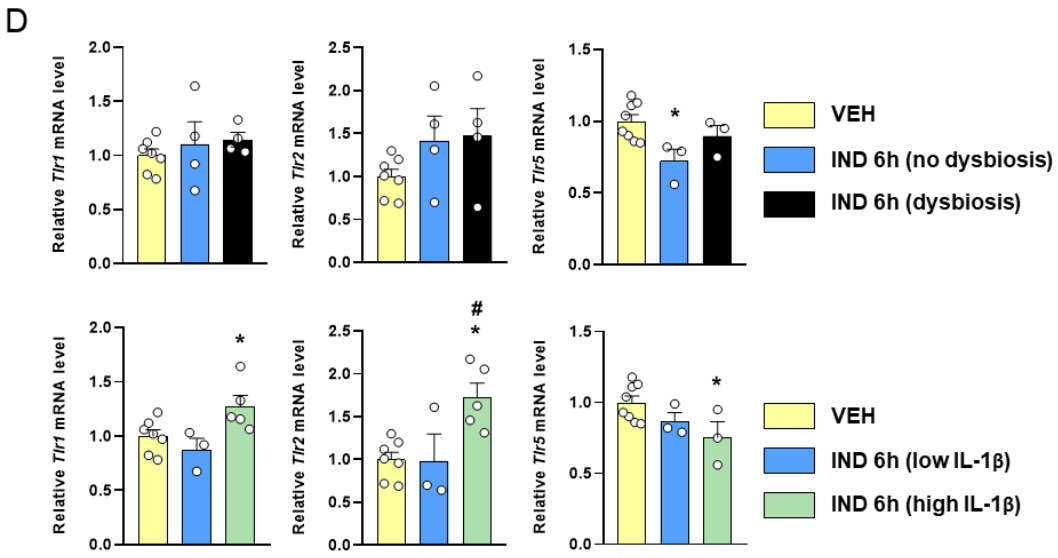
Figure 5. *High-dose indomethacin (IND)-induced TLR alterations display distinct associations with intestinal bacterial populations. Heat map of Spearman's correlation coefficients between TLRs and the small intestinal luminal levels of detected bacterial families in rats treated with VEH or 20 mg/kg IND. * uncorrected $p < 0.05$.*

To uncover direct relationships between TLRs and bacteria independent of the confounding effects of inflammation, we conducted a subgroup analysis on rats euthanized at the earliest time point, 6 hours following 20 mg/kg IND administration. This allowed us to differentiate animals with uninflamed intestinal mucosa from those having already mild inflammation, as indicated by higher tissue levels of inflammatory markers such as IL-1β (Fig. 6A). In addition, some animals that only partially matched those with detectable inflammation, showed small intestinal dysbiosis with a shift towards Gram-negative bacteria (*Verrucomicrobiota*, *Bacteroidota*, *Proteobacteria*) and *Actinobacteriota* (Fig. 6B). These changes were attributed to changes in the *Akkermansiaceae*, *Muribaculaceae*, *Enterobacteriaceae* and *Bifidobacteriaceae* families, respectively. When we compared IND-treated animals with and without dysbiosis, we found mild inflammation in non-dysbiotic animals, but more severe inflammation in animals with dysbiosis (Fig. 6C), implying that microbiota alterations aggravated inflammation but did not initiate it. When we compared TLR expression in animals with and without inflammation, and also in animals with and without dysbiosis (Fig. 6D, E), we found that the changes in TLR1, TLR2 and TLR5 expression did not depend on the presence of dysbiosis but showed a strong association with inflammation (Fig. 6D). In contrast, the expression of TLR4 showed a strong association with dysbiosis in early enteropathy, in particular with the expansion of the *Enterobacteriaceae* family (Fig. 6E). There was no difference in TLR6 and TLR9 expression between animals with and without dysbiosis or inflammation (not shown).

Overall, our results showed that TLR1 and TLR2 are upregulated and TLR5 is downregulated in severe enteropathy. The association between inflammation and the expression of these TLRs can be observed in the early stages of enteropathy. In contrast, changes in TLR4 expression are more strongly associated with the overgrowth of Gram-negative bacteria than with the severity of inflammation.


A
Relative Il1b mRNA level
B
Gram negatives (%)
Verrucomicrobiota
Bacteroidota
Firmicutes
Proteobacteria
Actinobacteriota
PC1
PC2


C
Relative Il1b mRNA level
PTX3/GAPDH
VEH
IND 6h (no dysbiosis)
IND 6h (dysbiosis)


D
Relative Tlr1 mRNA level
Relative Tlr2 mRNA level
Relative Tlr5 mRNA level
VEH
IND 6h (no dysbiosis)
IND 6h (dysbiosis)
IND 6h (low IL-1β)
IND 6h (high IL-1β)

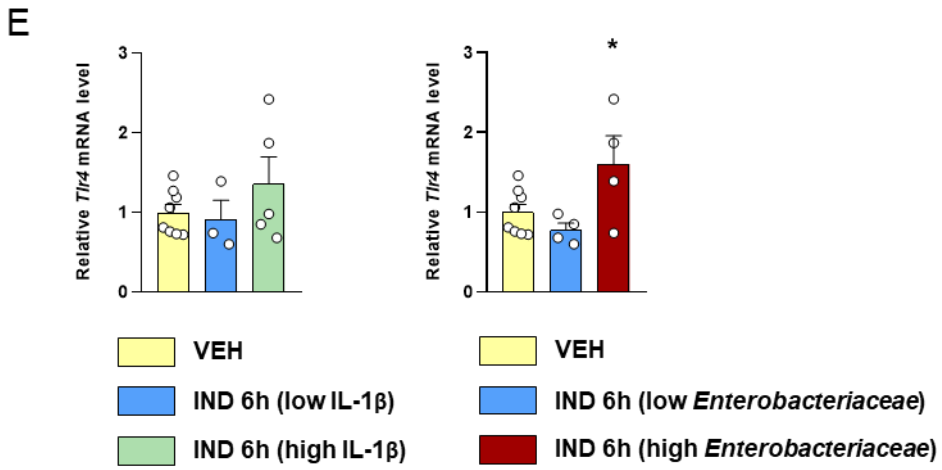


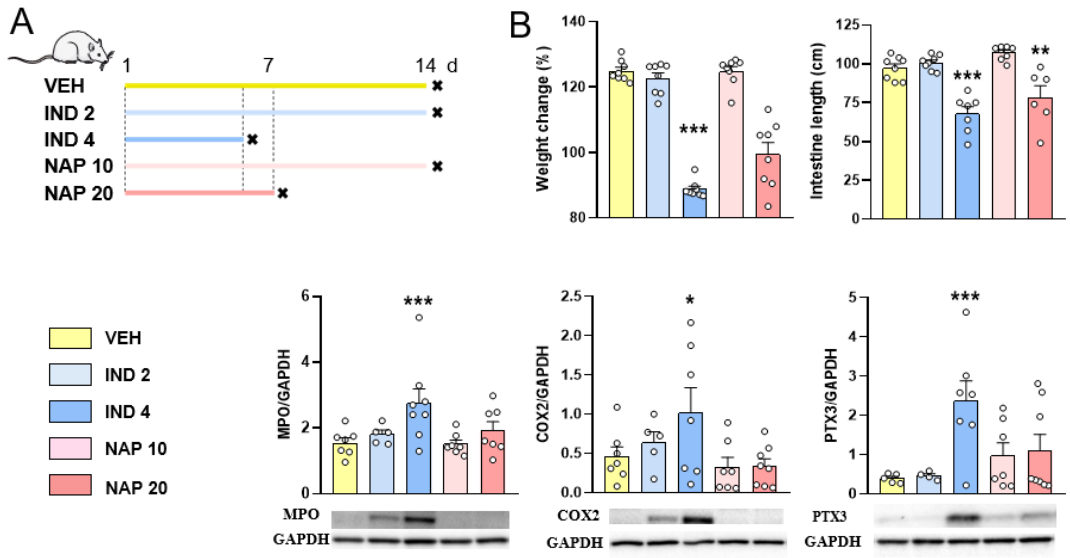
Figure 6. *The expression of TLRs is differently associated with inflammation and dysbiosis in the early stage of acute enteropathy.* ***A-B)*** *6 h after treatment with indomethacin (IND, 20 mg/kg) a part of the animals had mild inflammation (marked with green individual data points in panel A and green dotted circles in the PCA score plot in panel B) and/or significant small intestinal dysbiosis (black data points).* ***C-E)*** *The expression of inflammatory markers, TLRs in VEH-treated and different sub-groups of IND-treated animals. The results are expressed as the mean + SEM, circles represent individual data points. For statistical analysis one-way ANOVA was used, followed by Fisher's LSD test. n = 3–8/group. *p < 0.05, compared to VEH-treated group, # p < 0.05 compared to IND-treated animals with low IL-1β levels.*

4.1.3. Severe chronic IND-induced enteropathy exhibits TLR expression patterns similar to severe acute enteropathy

To investigate whether the observed changes in TLRs could be recapitulated by treating animals repeatedly with lower doses of NSAIDs over a longer period of time, rats were administered either IND (2 or 4 mg/kg) or naproxen (NAP) (10 and 20 mg/kg) twice daily for different time periods (Fig. 7A). 4 mg/kg IND given repeatedly caused severe intestinal damage and inflammation similar to 20 mg/kg IND given once in the first study, whereas repeated administration of 2 mg/kg IND caused only mild inflammation (Fig. 7B), and enteropathy was mainly reflected by changes in hematological parameters, such as reduced hemoglobin levels and increased neutrophil to lymphocyte ratio (Fig. 7C). NAP was less damaging than IND, causing only mild-to-moderate enteropathy at the higher dose (Fig. 7A, 7B).

Severe enteropathy induced by repeated administration of 4 mg/kg IND caused changes in TLR expression similar to 20 mg/kg IND given once. TLR1 and TLR2 genes were upregulated and TLR5 was downregulated, whereas the expressions of TLR4, TLR6 and TLR9 were not significantly different between control and enteropathic animals (Fig. 7D). In contrast to severe enteropathy, chronic mild enteropathy caused by IND had only a mild and rather inhibitory effect on TLR expressions, in particular on those of TLR1 and TLR5 (Fig. 7D). Given that NAP at the dose of 10 mg/kg did not cause any appreciable intestinal pathology, we measured the expression of TLRs only in rats treated with the higher dose of NAP (Fig. 7E). In these animals, we found that TLR2 and TLR4 tended to increase in enteropathy, although neither increase was statistically significant. In contrast, TLR5 expression decreased significantly.

Overall, our results showed that TLR1 and TLR2 are significantly upregulated in severe enteropathy, whereas TLR5 is downregulated in both mild and severe disease. Changes in TLR4 expression are highly variable and do not show a strong association with the severity of inflammation, instead, likely depend stronger on the abundance of Gram-negative bacteria, at least in the early stages of the disease. In addition, TLR6 and TLR9 expression do not change significantly in enteropathy.



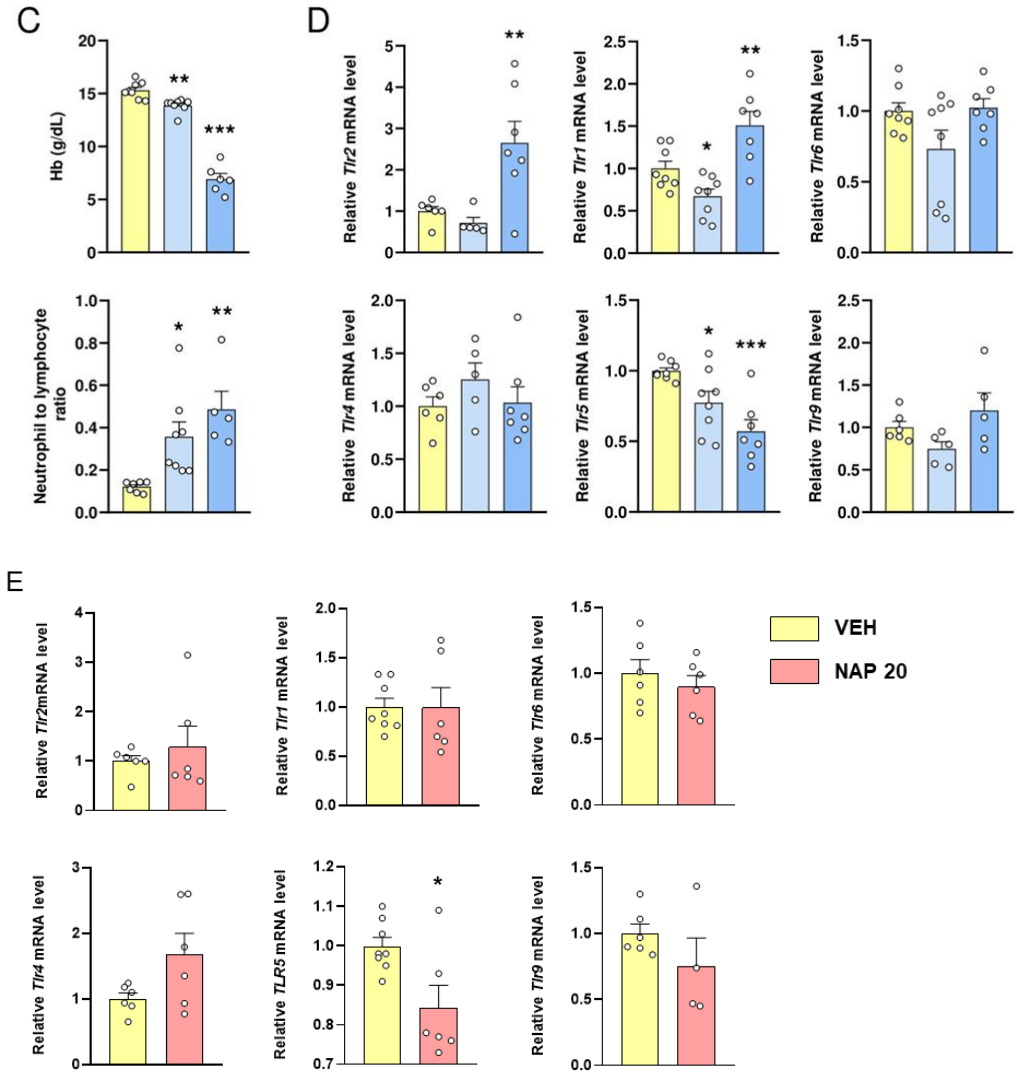


Figure 7. *The repeated administration of indomethacin (IND) and naproxen (NAP) at lower doses induces enteropathy of varying severity and causes distinct alterations in TLR expression.* ***A)*** *Study design; higher-dose groups (IND 4 mg/kg and NAP 20 mg/kg) were euthanized on days 6 or 7, respectively, due to the rapid onset of severe enteropathy, while lower-dose groups were treated for 14 days. Impacts of prolonged IND or NAP exposure on* ***B)*** *animal weight, small intestinal length, levels of select inflammatory mediators and cytokines,* ***C)*** *blood parameters,* ***D-E)*** *TLR expression. Data are presented as mean + SEM, with individual values shown as circles. One-way ANOVA (followed by Fisher's LSD test) or Student's t-test was employed for statistical evaluation. n=5–8 per group. *p < 0.05, **p < 0.01, ***p < 0.001 versus the vehicle (VEH)-treated controls.*

4.2. Analysis of the Role of TLR5 Activation and Inhibition in NSAID-Induced Enteropathy in Mice

4.2.1. NSAID-induced enteropathy downregulates intestinal TLR5 expression in mice while elevates luminal flagellin levels

The first study has identified TLR5 as a particularly sensitive responder to NSAID exposure in rats, being downregulated in both mild and severe enteropathy. To assess whether the expression of the TLR5 gene (*Tlr5)* is similarly reduced in mouse enteropathy, we treated mice with a 30 mg/kg dose of IND via gavage and collected the intestines 24 h later. The presence of inflammation was indicated by significant shortening of the intestine and increased ileal gene expression of IL-1β (*Il1b*). Similar to rats, IND enteropathy was associated with a significant decrease in *Tlr5* expression in mice (Fig. 8A), indicating that it is a common feature of NSAID enteropathy.

Furthermore, we aimed to quantify the levels of the TLR5 ligand flagellin protein in the small intestines of control and IND treated mice using Western blotting. Consistent with earlier studies (92, 109), the antibody targeting *Salmonella*-derived flagellin detected various flagellin monomers and/or dimers across multiple bacterial species, leading to several bands on the immunoblot (Fig. 8B). While ileal flagellin concentrations displayed considerable interindividual variation, IND-induced enteropathy was generally associated with a higher intestinal flagellin load compared to control animals.

Overall, these findings confirmed that NSAID enteropathy is associated with downregulation of TLR5, and demonstrated that it also results in increased intestinal flagellin levels.

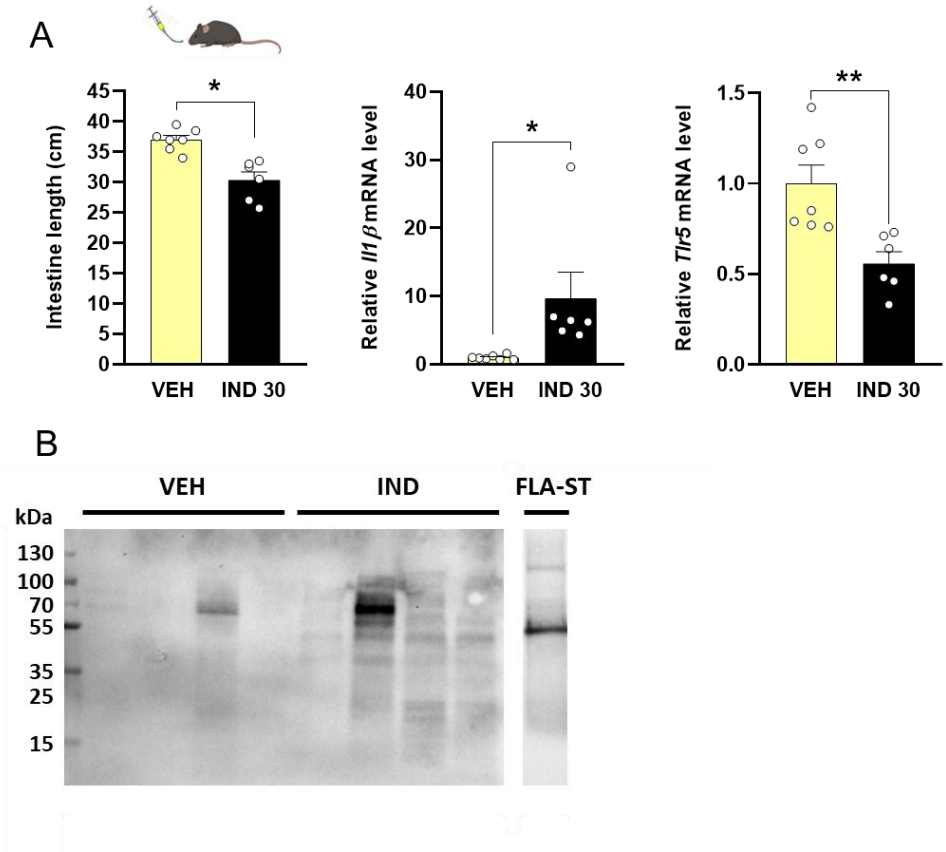


Figure 8. *Small intestinal Tlr5 expression is reduced and luminal flagellin level is increased in NSAID enteropathy.* ***A)*** *The effect of a single 30 mg/kg dose of indomethacin (IND) on ileal TLR5 mRNA levels and inflammatory markers. Circles represent individual data points, while bars indicate mean + SEM. *p < 0.05, **p < 0.01 (Student's t-test, n=6–8/group).* ***B)*** *Western blot of small intestinal samples from vehicle- (VEH) and IND-treated mice was performed by an anti-flagellin primary antibody. Purified flagellin from Salmonella typhimurium (FLA-ST, 0.4 µg) considered as the positive control. Because of the strong band intensity resulting from the amount of FLA-ST loaded, images of the luminal samples and positive control were captured after different exposure times.*

4.2.2. Systemic flagellin treatment mitigates IND-induced intestinal inflammation and tissue damage

The strong inverse correlation between intestinal inflammation and TLR5 expression led us to hypothesise that TLR5 plays an as yet unidentified protective role in enteropathy. To test whether flagellin treatment can ameliorate NSAID enteropathy, we administered flagellin at two different doses (10 or 30 µg/mouse) twice - 2 h prior to and 4 h after IND - to IND-treated mice (Fig. 9A). IND induced small intestinal inflammation within 24 h, characterized by shortening of the small intestine (Fig. 9B) and increased levels of IL-1β, COX-2, and MPO (Fig. 9C). It also elevated

PTX3, which is released by a variety of cell types in response to proinflammatory cytokines and other inflammatory signals. In addition, the anti-inflammatory cytokine IL-10 was elevated, likely as part of a counterregulatory mechanism. These inflammatory responses were largely prevented by flagellin treatment. Interestingly, the expression of *Nos2*, the gene encoding the inducible form of nitric oxide synthase (NOS), increased in flagellin-treated enteropathic animals, suggesting that some aspects of the complex inflammatory response in enteropathy are not influenced - or can even be promoted - by flagellin.

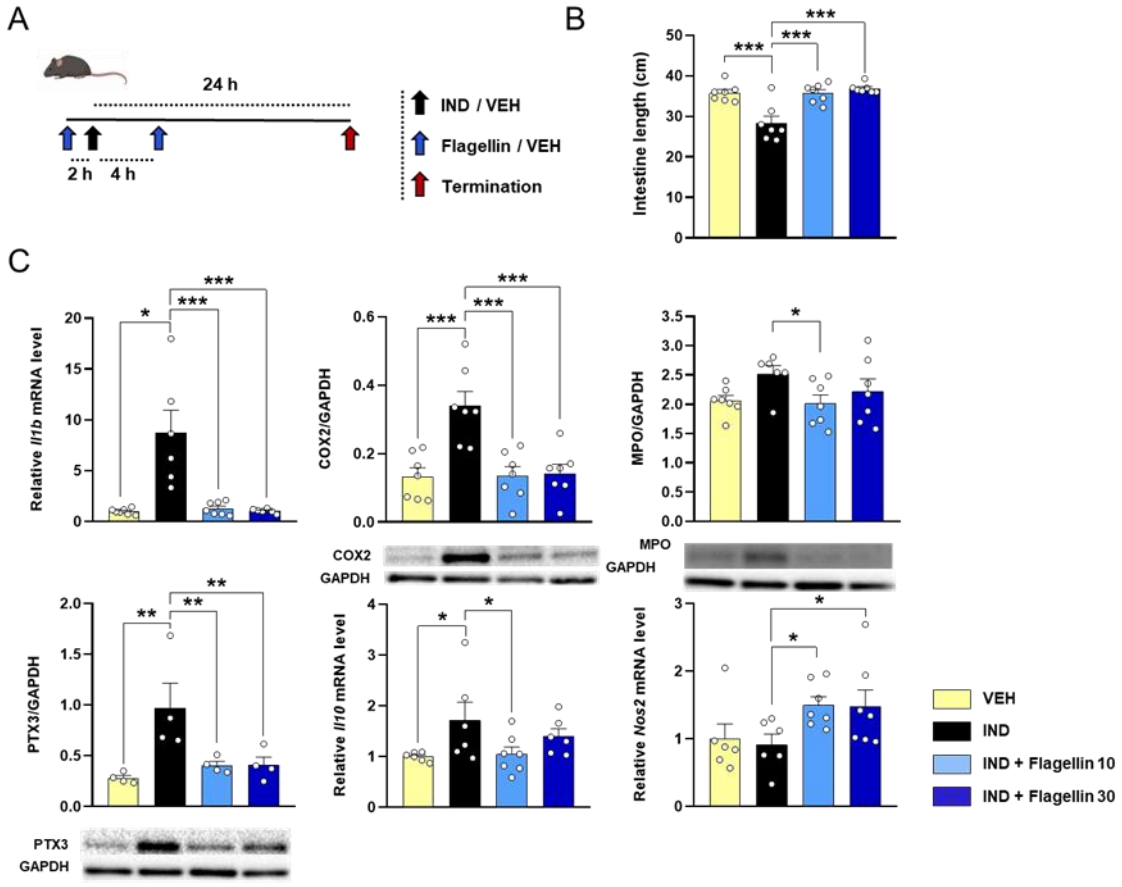


Figure 9. *Systemic flagellin administration mitigates small intestinal inflammation in IND-induced enteropathy.* ***A)*** *Experimental protocol.* ***B)*** *Impact of flagellin treatment on IND-associated intestinal shortening.* ***C)*** *The effect of flagellin on select inflammatory markers in IND-treated mice. Dots represent individual data points, while bars indicate the mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA with Fisher's LSD test, n=4–7/group).*

Nevertheless, the net effect of flagellin on enteropathy was clearly anti-inflammatory, as reflected by the reduced mucosal injury, which would otherwise be significantly exacerbated by

inflammation and neutrophil accumulation (32, 108). The small intestinal mucosa of enteropathic mice exhibited various pathologies, ranging from mild epithelial damage to patchy transmural lesions (Fig. 10). To evaluate the extent of mucosal injury, we initially employed the Chiu/Park scale. Mice treated with IND alone or with IND and the lower dose of flagellin had higher histopathology scores than VEH-treated controls. However, mice receiving the higher dose of flagellin showed less severe histological injury, suggesting a protective effect of flagellin. Quantifying the proportion of damaged mucosal area relative to the total mucosal length in each histological specimen revealed that flagellin treatment reduced the proportion of both the total affected area and the area exhibiting the most severe damage. Nevertheless, neutrophil granulocytic infiltration at the sites of injury was similar in all groups, as indicated by the neutrophil scores. This suggests that, while flagellin treatment protected the epithelium against IND, it did not significantly affect neutrophil infiltration following epithelial damage.

Collectively, these results indicate that targeted administration of a TLR5 agonist restricts enteropathy progression at morphological, histological, and molecular levels.

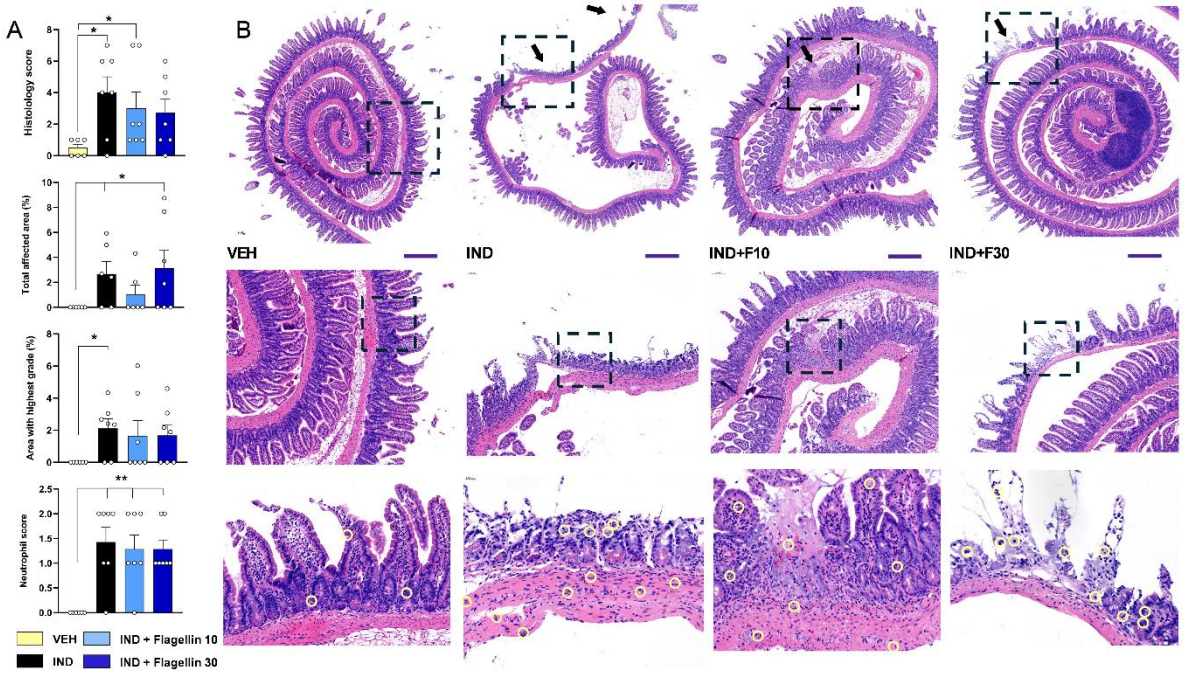


Figure 10. *Systemic flagellin administration reduced the histological injury in the small intestine during enteropathy.* ***A)*** *The effect of flagellin treatment on the overall histology score, on the proportions of the total damaged area and the proportion of areas with the highest grade in a given sample, and on neutrophil*

*infiltration associated with mucosal damage. Dots indicate data for individual mice, bars indicate the mean+SEM. *p<0.05 (Kruskal-Wallis, uncorrected Dunn's test, n=6-7/group).* ***B)*** *Representative histological images (haematoxylin and eosin staining, scale bar: 0.5 mm) of the small intestines. Arrows denote mucosal injury; yellow circles denote neutrophil granulocytes.*

In previous studies, pretreatment with TLR2 or TLR4 ligands mitigated IND enteropathy due to downregulation of TLR4 on intestinal macrophages and their hyporesponsiveness to subsequent NSAID exposure (32, 68). We therefore measured the gene expression of *Tlr2* and *Tlr4* in control and enteropathic animals and found that flagellin treatment prevented the IND-induced upregulation of both TLRs (Fig. 11A). In contrast, flagellin had no effect on the downregulation of TLR5 in enteropathy (Fig. 11A).

Next, we assessed the effect of flagellin treatment on the small intestinal bacterial count and also on the levels of the *Enterobacteriaceae* and *Lachnospiraceae* families, which are major contributors to the flagellin content in the inflamed and non-inflamed gut, respectively (110, 111). The amount of total ileal bacteria was increased in enteropathy compared to control animals, but it was significantly reduced by flagellin (Fig. 11B). In addition, in line with previous studies (46, 47), the level of *Enterobacteriaceae* was higher, whereas that of *Lachnospiraceae* was lower in enteropathic animals compared to controls, although the differences were not significant. Except in two mice, which exhibited exceptionally high ileal levels of *Enterobacteriaceae*, flagellin-treated mice had lower levels of both selected bacterial families than mice receiving only IND. Notably, the level of *Enterobacteriaceae* correlated with the levels of MPO (not shown), as well as with those of TLR2 and TLR4 (Fig. 11C), but not with TLR5. The lower levels of total bacteria, *Enterobacteriaceae*, and *Lachnospiraceae* in flagellin-treated mice suggested that the intestinal flagellin protein load is reduced in these animals, which was confirmed by immunoblotting (not shown). Collectively, these results suggest that reduced inflammation in flagellin-treated enteropathic mice was associated with lower intestinal bacterial and flagellin levels and reduced expression of TLR2 and TLR4.

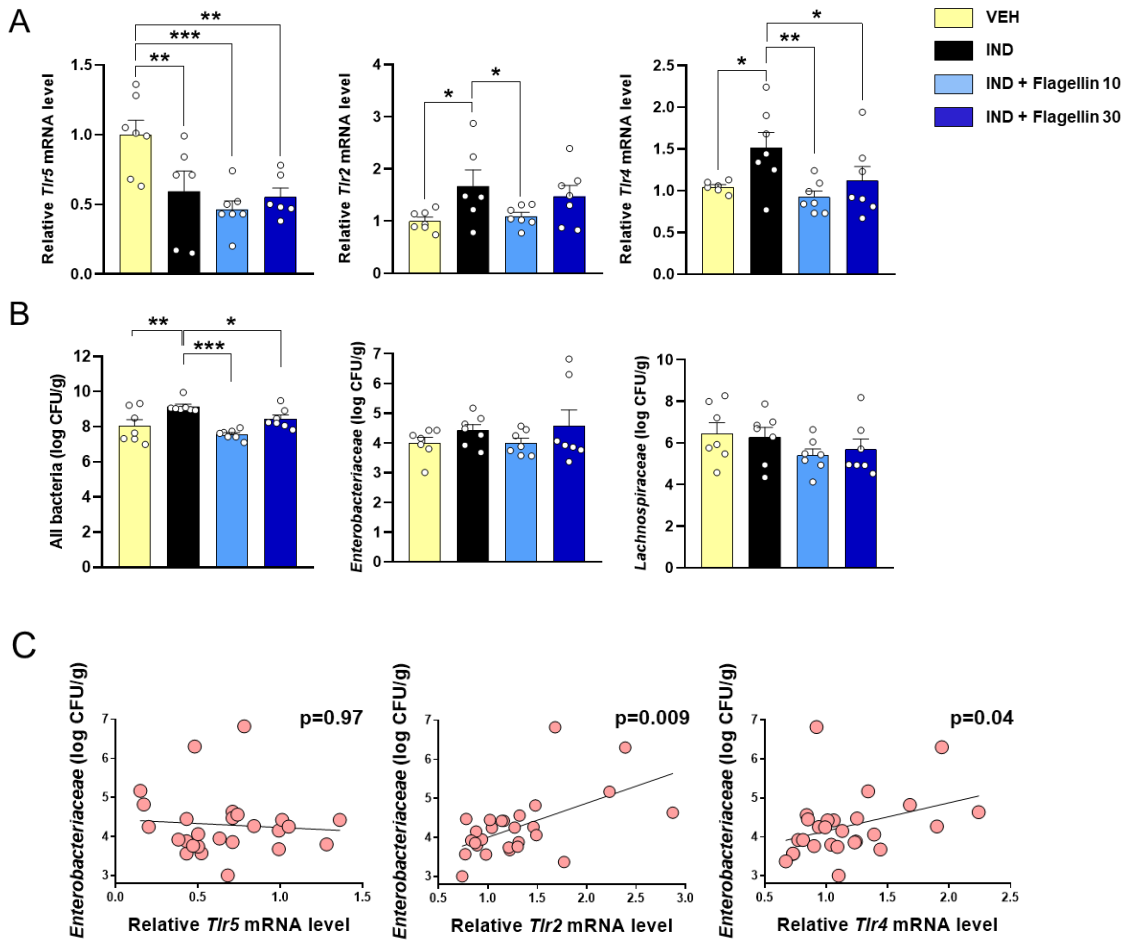


Figure 11. *Systemic flagellin administration reduces Tlr2 and Tlr4 expression along with overall bacterial numbers in the ileum of mice with enteropathy.* ***A)*** *Influence of flagellin treatment on TLR gene expression.* ***B)*** *Effects of flagellin on total bacterial levels, as well as on the abundances of Enterobacteriaceae and Lachnospiraceae. Dots represent individual data points, while bars represent mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA with Fisher's LSD test, n=5–7/group). C) Associations between Tlr expression levels and the abundance of Enterobacteriaceae, determined by Spearman's test.*

We wondered whether flagellin treatment downregulates TLR2 and TLR4 in the non-inflamed gut as well. Mice were treated with flagellin and euthanized as in the previous study but received only hydroxyethylcellulose instead of IND (Fig. 12A). Flagellin treatment caused no visible changes in the general condition or behavior of the animals, nor inflammatory shortening of the small intestine (not shown), supporting the previous notion that flagellin at moderate doses does not induce severe adverse events (91). Nevertheless, flagellin-treated mice exhibited mild ileitis, indicated by higher levels of the inducible form of cyclooxygenase (COX-2) and *Nos2* (Fig. 12B). This inflammation was associated with downregulation of TLR5 and upregulation of TLR2 (Fig. 12C). In addition,

the expression of TLR4 tended to increase in response to flagellin (p=0.15) (Fig. 12C). These results suggest that flagellin treatment induces mild intestinal inflammation and upregulates - rather than downregulates - TLR2 and TLR4 in the absence of enteropathy.

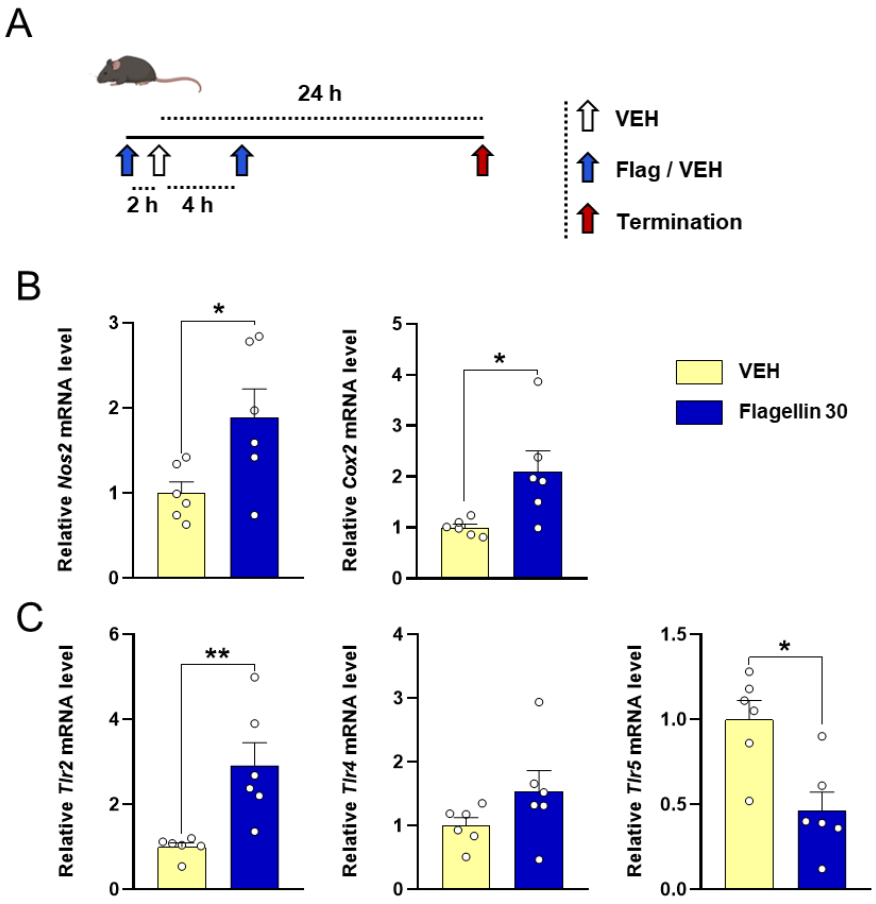


Figure 12. *Systemic flagellin administration induces mild inflammation and elevates Tlr2 and Tlr4 expression in mice without enteropathy.* ***A)*** *Experimental design.* ***B-C)*** *Impact of flagellin treatment on inflammatory markers and Tlr expression. Dots represent individual data points, while bars indicate the mean + SEM. *p < 0.05, **p < 0.01 (Student's t-test, n=6/group).*

4.2.3. Flagellin administered after IND also protects against enteropathy

Given that TLR2 and TLR4 ligands aggravated enteropathy when administered after IND - in contrast to the protection caused by pretreatment with them (32, 68) - we investigated whether a

single flagellin injection 4 h after IND treatment could also mitigate enteropathy (Fig. 13A). We found that flagellin induced robust protection in this experimental setting as well, as indicated by reduced inflammatory shortening of the intestine (Fig. 13B) and reduced levels of inflammatory mediators and TLR4 expression (Fig. 13C, 13D). However, flagellin treatment did not affect the IND-induced downregulation of TLR5. This data indicates that pharmacological activation of TLR5 is not only preventive but also shows therapeutic capacity.

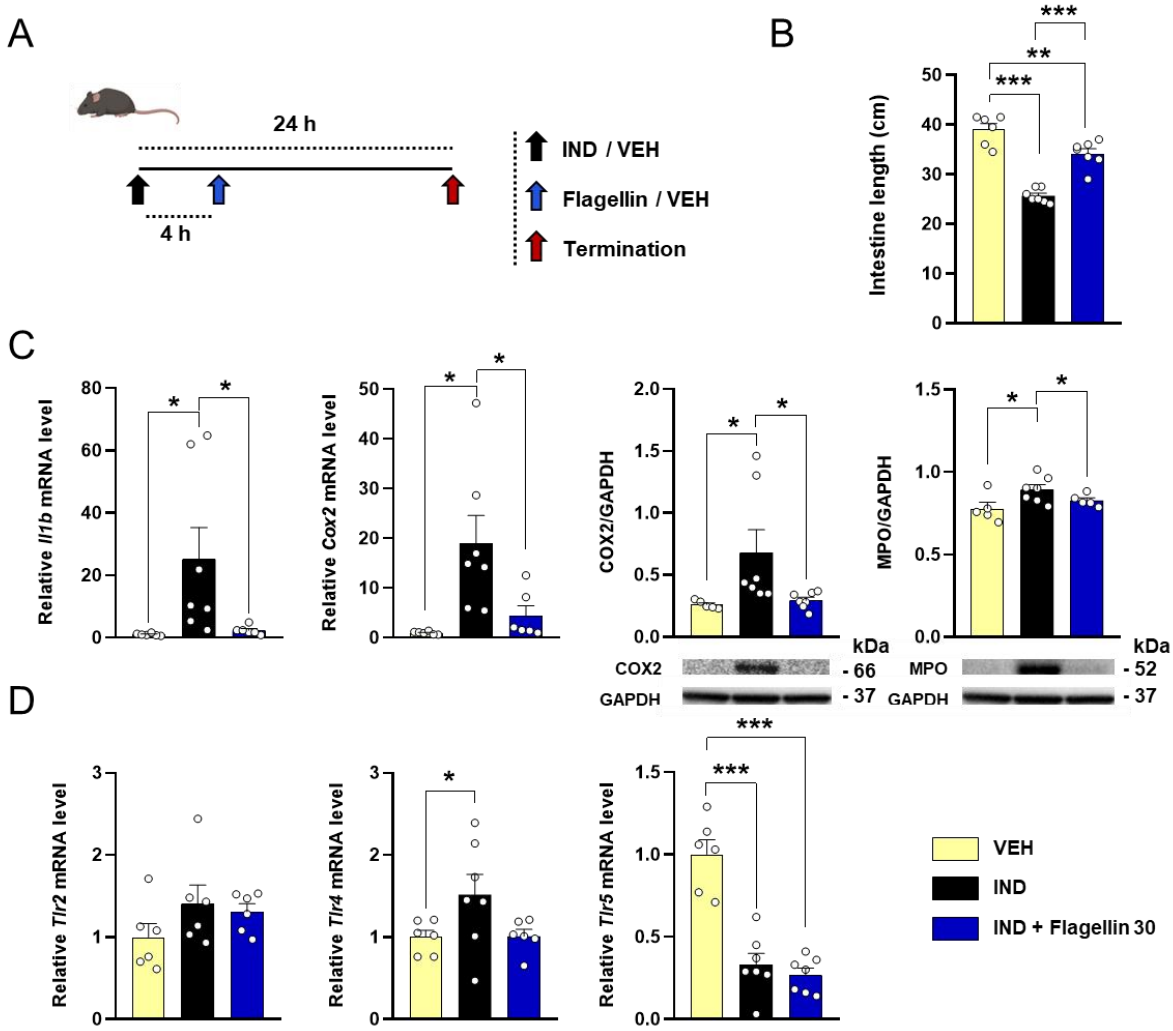


Figure 13. *Flagellin administration mitigates small intestinal inflammation when delivered only after IND administration.* ***A)*** *Experimental design.* ***B)*** *Impact of flagellin on IND-induced intestinal shortening.* ***C)*** *Influence of flagellin on select inflammatory markers in IND-treated mice.* ***D)*** *Effect of flagellin on Tlrs expression. Dots represent individual data points, while bars indicate the mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA with Fisher's LSD post hoc test, n=5–7/group).*

4.2.4. Inhibition of TLR5 by TH1020 aggravates IND-induced intestinal inflammation

Finally, we treated VEH and IND-treated animals with the potent and selective TLR5 antagonist TH1020. TH1020 treatment alone did not cause any visible morphological damage to the intestine or any change in the measured inflammatory parameters. However, it significantly increased the expression of *Il1b*, *Tlr2*, and *Tlr4* in enteropathic mice (Fig. 14), suggesting that blockade of endogenous TLR5 signaling pathways promotes inflammation in enteropathy. These results are in line with the previous results, confirming the pivotal role of TLR5 in enteropathy pathogenesis.

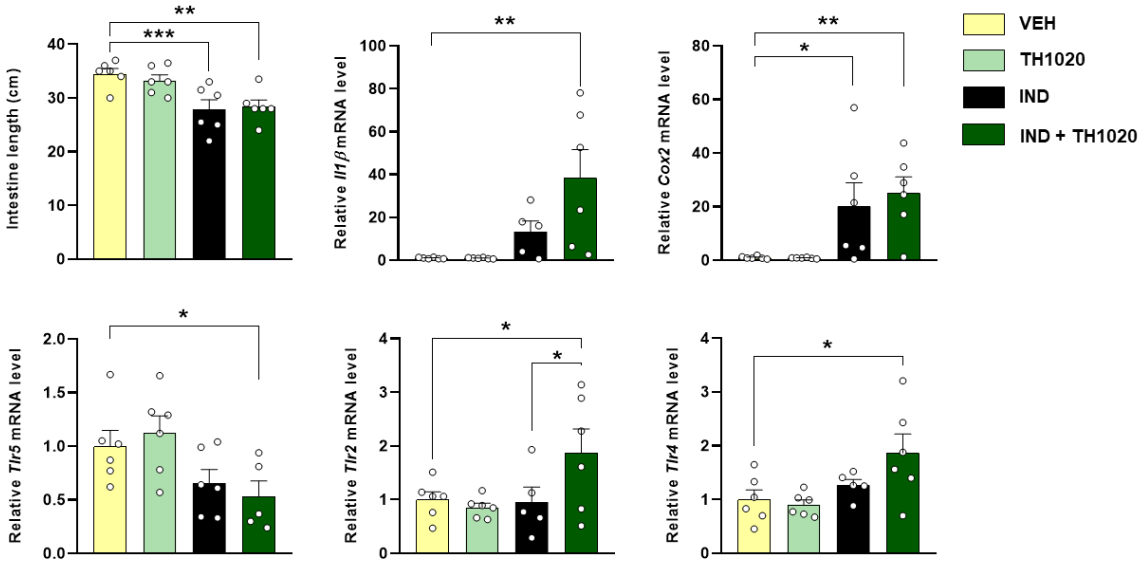


*Figure 14. Blocking TLR5 pharmacologically exacerbates indomethacin (IND)-induced small intestinal inflammation. Impact of the TLR5 antagonist TH1020 (10 µg, delivered intraperitoneally four hours post-IND) on intestinal shortening, inflammatory markers, and expression of Tlrs. Dots represent individual data points, while bars indicate the mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA with Fisher's LSD post hoc test, n=5–6/group).*

5. DISCUSSION

Our results demonstrated that NSAID-induced enteropathy has differential effects on the expression of six bacterial-sensing TLRs. Specifically, TLR2 and TLR1 showed elevated expression in the severe inflamed intestine, while TLR6 and TLR9 did not show significant changes in enteropathy. The association between TLR4 expression and the severity of tissue inflammation was less clear, but TLR4 expression showed a strong association with the abundance of the *Enterobacteriaceae* family. Importantly, our study identified TLR5 as a particularly sensitive responder to NSAID exposure, being downregulated in both mice and rats independent of the severity of enteropathy, and we provided clear evidence of its protective effect against enteropathy.

The essential function of TLR-bacterial interactions in preserving intestinal homeostasis is well recognized (69, 112), and disruptions in TLR signaling have been connected to inflammatory gut disorders such as IBD (113). However, existing data on alterations in TLR expression during NSAID enteropathy remained limited and inconclusive. Thus, the main objective of the first study was to evaluate the gene expression pattern of all six primary bacterial-detecting TLRs in both acute and chronic IND-induced enteropathy, and to investigate whether these changes correlate with inflammation and dysbiosis.

Our results indicated that severe intestinal inflammation, induced by either a single high dose of IND or repeatedly administered low doses, was associated with a moderate upregulation of ileal TLR2 gene expression. A comparable 1.5-fold rise in ileal TLR2 in IND-treated rats was previously reported by Teran-Ventura et al. (66), though not observed by others (71, 72). Such inconsistencies are likely due to variations in experimental protocols resulting in different intestinal inflammation, on which TLR2 upregulation is strongly dependent. In acute enteropathy, TLR2 increased mainly when inflammation peaked, whereas in chronic mild enteropathy, it did not increase but rather tended to decrease, similar to that in mild inflammation caused by other triggers (61).

Nonetheless, the overexpression of TLR2 in severe IND induced enteropathy aligns with reports of increased TLR2 in diclofenac-treated rats (114), colitic mice (115), and IBD patients (116-118) as well as those with diverticulitis (116). The results of our second study also confirmed the upregulation of TLR2 in enteropathy, as discussed below. Overall, these findings suggest that increased TLR2 signaling plays a role in promoting intestinal inflammation induced by diverse triggers, including NSAIDs.

A key feature of TLR2 is its ability to heterodimerize with either TLR1 or TLR6, influencing its ligand preferences (75, 115). Our observation that TLR1 increased in parallel with TLR2, while TLR6 remained largely unchanged, indicates that IND-induced inflammation is associated with overexpression of the TLR2/TLR1 complex rather than the TLR2/TLR6 receptor complex. This aligns with previous evidence associating the TLR2/TLR1 dimer— but not TLR2/TLR6—with pro-inflammatory effects in the gut mucosa (119). It also matches the ligand profile of TLR2/TLR1, which preferentially recognizes triacyl lipopeptides from Gram-negative bacteria (120). Although we detected several significant correlations between TLR2/TLR1 expression and the luminal abundance of Gram-negatives in enteropathic animals, these do not necessarily reflect direct interactions between TLR2/TLR1 and bacteria, considering the strong association of both with inflammation. Indeed, our finding showing similar TLR2/TLR1 elevations in animals with and without dysbiosis in the early phase of enteropathy, yet significantly higher levels of TLR2/TLR1 in those with elevated IL-1β regardless of microbiota gut composition, suggests that inflammation was the primary driver of TLR2/TLR1 upregulation, rather than an increase in Gram-negative bacteria.

Based on existing research, both LPS - a key component of the outer membrane of Gram-negatives - and its receptor TLR4 play a central role in the pathogenesis of enteropathy. NSAID-induced small intestinal damage is markedly attenuated in TLR4-mutant or TLR4-knockout mice (32, 71), while LPS exposure increases injury in antibiotic-treated animals (121). Interestingly, whether enteropathy involves changes in TLR4 expression is less clear. Some reports suggest an overexpression of TLR4 in NSAID enteropathy (65, 71), but others found no changes in small intestinal TLR4 mRNA or protein levels in response to IND treatment (32, 72), or only mild changes compared to the colon (66). In the first study, TLR4 expression showed only trends towards elevation in enteropathic rats, although there was clear elevation of TLR4 in a subset of IND-treated animals. Notably, we found a clear association between TLR4 expression and the luminal levels of the *Enterobacteriaceae* family in the early stage of enteropathy. This association was later confirmed by our second study on enteropathic mice, although in that study TLR4 also showed a significant increase in enteropathy, as will be discussed later. Overall, these observations suggest that changes in TLR4 expression are less dependent on the severity of inflammation, and are stronger dependent on the presence of Gram-negative bacteria, particularly *Enterobacteriaceae*, expressing the TLR4 ligand LPS.

A hallmark of NSAID-associated intestinal dysbiosis is the marked expansion of *Gammaproteobacteria* (45), and several members of this class are flagellated and detected by TLR5 (77). However, little is known about the involvement of TLR5 in enteropathy. A key finding of our first study is that TLR5 expression was significantly decreased in enteropathic animals. This pattern was found consistently in all IND-treated groups including animals with both severe and mild enteropathy, resulting in a strong negative correlation between TLR5 expression and inflammation. In addition, we found a similar reduction in TLR5 expression in NAP-treated rats. A similar TLR5 reduction has been observed in the colonic mucosa of colitic mice (122) and IBD patients (123), though there is also some data indicating no change or even elevation of TLR5 in IBD (116, 117). Given that both intestinal microbes (61) and cytokines (122, 124) regulate TLR5 expression, and *Gammaproteobacteria* increased in parallel with inflammation, dissecting each factor's role in TLR5 downregulation is challenging. However, the consistent decrease in TLR5 expression in early enteropathy among both dysbiotic and non-dysbiotic animals suggests the dominant influence of inflammation on TLR5 expression. Moreover, considering that TLR5 deficiency has been linked to dysbiosis and reduced ability to manage *Proteobacteria* (83, 91), our observation that TLR5 reduction preceded the overgrowth of *Proteobacteria* in some animals suggests that it was a cause rather than a consequence of dysbiosis. From these results, we hypothesized that an early decrease in TLR5 signaling contributes to NSAID enteropathy and dysbiosis, and that early administration of a TLR5 ligand my provide protection against it, similar to gut injury of other origins (91).

Building on this, the aim of our second study was to assess the effect of TLR5 activation and inhibition on NSAID enteropathy. Our results confirmed our hypothesis, as we showed that activation of TLR5 either before or shortly after NSAID administration markedly reduces tissue inflammation and mucosal injury, whereas inhibition of TLR5 exacerbates the severity of inflammation. These findings suggest that the downregulation of TLR5, an endogenous protective receptor, may represent a key event in the development of NSAID-induced enteropathy, and therapeutic activation of TLR5 may offer an effective strategy for its treatment.

In our second study we confirmed that enteropathy is associated with reduced TLR5 expression. However, the mechanisms responsible for this change remain to be identified. Cell culture studies have shown that flagellin can suppress TLR5 expression (125). As described here, IND enteropathy is accompanied by increased flagellin levels, consistent with earlier reports describing

inflammation-associated alterations in microbial gene expression (110, 126, 127). However, we also detected decreased TLR5 expression in animals treated with flagellin, despite these mice being largely protected from IND and displaying bacterial loads and flagellin levels similar to control animals. This indicates that luminal bacteria and flagellin are unlikely to be primary regulators of TLR5 expression, at least in acute enteropathy. This interpretation is consistent with the basolateral expression of TLR5 in the intestinal epithelium (128) and with previous studies showing that faecal microbiota transplantation from enteropathic mice does not change TLR5 expression in recipient animals (129). Moreover, it is improbable that exogenously administered flagellin directly caused the reduction, as TLR5 was assessed 20 hours after administration, and circulating flagellin levels decline rapidly (130).

The precise role of TLR5 in intestinal injury remains controversial, with both protective (84, 91, 131-133) and detrimental (134-137) effects reported. The impact of TLR5 is likely context-dependent, varying with underlying disease mechanisms and the intensity of inflammation. Our findings show that in acute NSAID-induced enteropathy, systemic administration of flagellin provides strong protection and partially ameliorates mucosal injury.

We initially hypothesized that TLR5-mediated protection might operate through the downregulation of TLR4 and the induction of hyporesponsiveness in intestinal macrophages, as previously reported in mice pretreated with the TLR4 ligand LPS (32) or the TLR2 ligands LAM and LTA (68). While reduced expression of both TLR4 and TLR2 was observed in flagellin-pretreated mice, this change is more likely to reflect a downstream consequence of protection rather than its primary cause.

In enteropathic mice, TLR4 and TLR2 expression correlated with the degree of inflammation and *Enterobacteriaceae* abundance, and both were decreased in flagellin-treated mice. In contrast, TLR2 and TLR4 expression was increased in flagellin-treated control (non-enteropathic) animals with mild intestinal inflammation. Moreover, flagellin induced significant protection and reduced TLR4 expression even when administered four hours after IND exposure. This contrasts with the effects of TLR4 and TLR2 ligands, which exacerbated enteropathy under comparable experimental conditions (68). These observations indicate that the protective action of flagellin is unlikely to result from “endotoxin tolerance” and instead causes by other mechanisms.

Regarding potential therapeutic implications, our findings suggest that short-term administration of flagellin, or of a less immunogenic and pharmacologically optimized TLR5 agonist such as entolimod (CBLB502) (84), may enhance the resilience of the intestinal mucosa in patients who require long-term NSAID therapy. At the same time, our results also draw attention to possible gastrointestinal risks associated with TLR5 inhibition. This concern is particularly relevant given the growing recognition of TLR5 as a contributor to inflammatory joint diseases such as rheumatoid arthritis (138, 139). Consequently, although therapeutic strategies targeting TLR5 in rheumatoid arthritis may provide benefit to joint pathology, they could also increase susceptibility to NSAID-induced gastrointestinal complications, for which these patients are already at elevated risk (140).

In summary, our results showed that IND-induced enteropathy is associated with distinct changes of intestinal TLR expression. TLR1 and TLR2 are upregulated, TLR5 is downregulated, TLR4 exhibits only modest and highly variable alterations, and TLR6 and TLR9 remain largely unchanged in enteropathic rats. Although these expression changes are associated with both tissue inflammation and intestinal dysbiosis—two closely interconnected processes—analysis in animals with early-stage enteropathy indicates that individual TLRs are differentially influenced by these factors (Fig. 9). Specifically, alterations in TLR1, TLR2, and TLR5 expression were strongly associated with inflammation, whereas changes in TLR4 expression were more closely related to gut dysbiosis during early disease. In addition, our study provides evidence for the first time that TLR5 plays a protective role in NSAID-induced enteropathy. Systemic administration of the TLR5 ligand flagellin markedly attenuates the development of severe intestinal inflammation following exposure to high-dose IND. In contrast, inhibition of TLR5 enhances the inflammatory response characteristic of enteropathy. Taken together, these findings emphasize the role of continuous TLR5 signaling as an endogenous protective mechanism against NSAID-induced intestinal damage, with early reduction of TLR5 expression representing a key step in the development of enteropathy. Notably, our pharmacological results show that modulation of TLR5 can both prevent disease initiation and provide therapeutic benefit, underscoring its potential as both a preventive and interventional strategy.

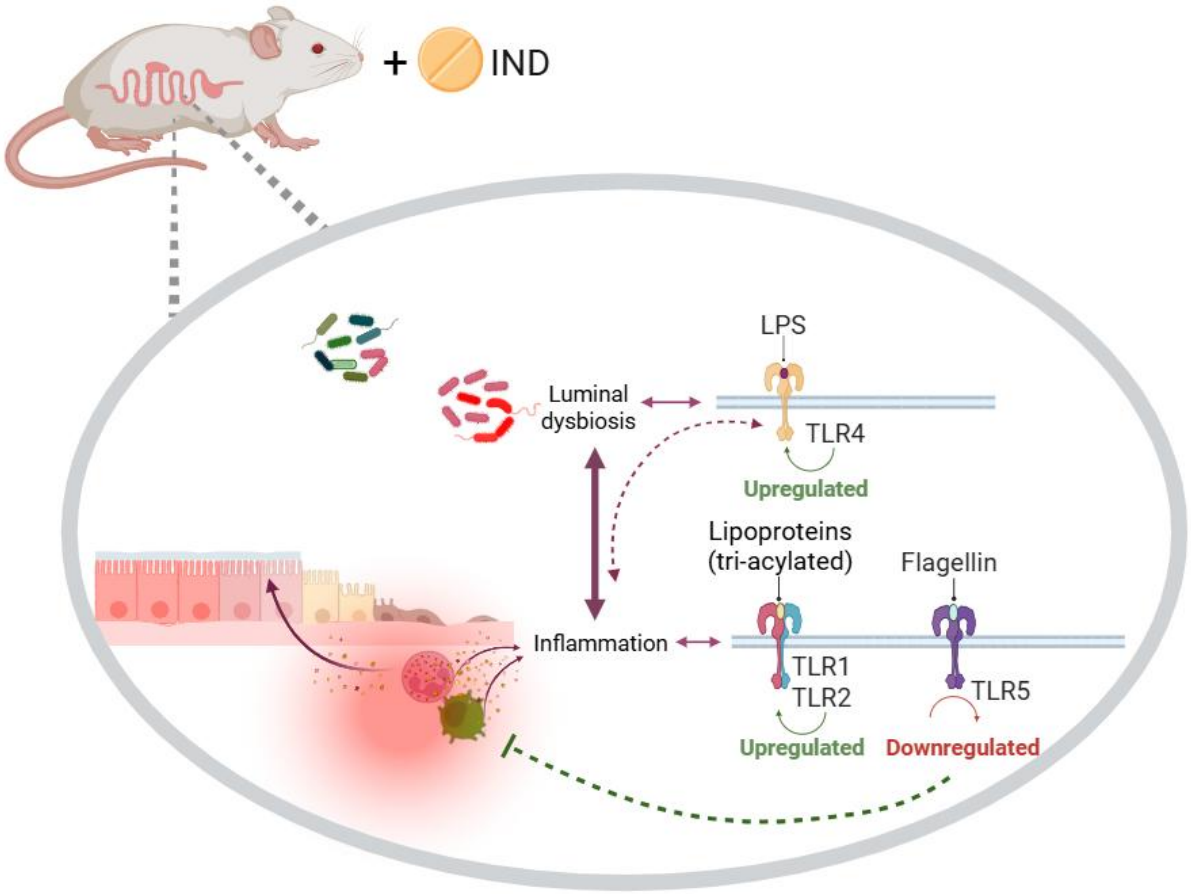


Fig. 15. Associations between toll-like receptors (TLRs), inflammation, and intestinal microbiota in enteropathy. Alterations in TLR1, TLR2, and TLR5 expression were primarily associated with inflammatory changes, whereas TLR4 expression change was largely dependent on intestinal dysbiosis. TLR5 activation ameliorated NSAID-induced inflammation and mucosal injury.

6. CONCLUSION

This thesis provides comprehensive evidence that intestinal expression of TLRs is differentially regulated during NSAID-induced enteropathy and differentially associated with intestinal inflammation and dysbiosis. Among the TLRs studied, TLR5 emerged as a key endogenous protective factor against NSAID-induced small intestinal injury.

The major conclusions of our studies are:

1. **NSAID-induced enteropathy is characterized by distinct TLR expression patterns**, with upregulation of TLR1 and TLR2 associated with inflammation, relatively stable TLR6 and TLR9 expression, and variable TLR4 changes linked more strongly to dysbiosis than inflammation.
2. **TLR5 is consistently and markedly downregulated in NSAID enteropathy**, independent of NSAID type, disease severity, or experimental species. This downregulation occurs early in disease development and may precede significant dysbiosis.
3. **TLR5 signaling plays a protective role in intestinal homeostasis**, as pharmacological activation of TLR5 with flagellin significantly reduces NSAID-induced inflammation and tissue injury, while inhibition of TLR5 exacerbates enteropathy.
4. **The protective effects of TLR5 activation are not mediated by TLR4 suppression**, indicating that TLR5 regulates intestinal inflammation through distinct mechanisms.

Collectively, these findings identify TLR5 as a critical regulator of host–microbiota interactions in NSAID-induced enteropathy and highlight its potential as a novel therapeutic target. Pharmacological activation of TLR5 may offer a promising preventive and therapeutic strategy to protect the small intestine in patients requiring long-term NSAID therapy. At the same time, the results emphasize caution regarding therapeutic strategies that inhibit TLR5, as such approaches may increase gastrointestinal vulnerability in NSAID-treated individuals.

7. SUMMARY

Chronic use of NSAIDs is frequently associated with intestinal damage and dysbiosis. Several studies highlight the important role of gut microbiota–host interactions and innate immune signaling, particularly through TLRs, in the development and progression of NSAID-induced enteropathy.

In the present thesis we investigated how NSAID-induced enteropathy alters intestinal TLR expression during acute and chronic enteropathy in rodents, and how these changes correlate with inflammation and changes in gut microbiota composition. We induced acute enteropathy by a single high dose of IND, while chronic enteropathy was induced by repeated administration of low-dose IND or NAP.

We found that NSAID enteropathy is associated with distinct changes of the expression of intestinal TLRs. TLR1 and TLR2 were consistently upregulated in parallel with inflammation severity, whereas TLR6 and TLR9 remained largely unchanged. TLR4 showed modest and variable changes that were more closely associated with intestinal dysbiosis, particularly the expansion of *Enterobacteriaceae*, during early disease stages. In contrast, TLR5 expression was significantly and consistently downregulated in both acute and chronic enteropathy, regardless of NSAID type, species, or inflammation severity.

Importantly, our studies in mice demonstrated a protective role for TLR5 signaling in enteropathy. Systemic administration of the TLR5 ligand flagellin, either before or after NSAID exposure, significantly attenuated intestinal inflammation, reduced tissue injury, and limited bacterial overgrowth. Conversely, pharmacological inhibition of TLR5 exacerbated NSAID-induced intestinal inflammation. These protective effects were not primarily mediated through suppression of TLR4, indicating a distinct and independent role for TLR5 in maintaining intestinal homeostasis.

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9. PUBLICATIONS

Publications related to the thesis:

1. **Haghighi A**, Tóth AS, Demeter ZO, Hutka B, Zsidai A, Lengyel L, Haghighi S, Pannier M, Le Cosquer G, Meunier ES, Ágg B, Makra N, Ostorházi E, Ligeti B, Kovács K, Kelemen Á, Jakab A, Wachtl G, Kökény G, Szabó D, Zádori ZS. *Oral indomethacin modifies small intestine biofilms and host-microbe interaction mediators*. Life Sciences. 2025; 384:124114. Impact factor, journal quartile – 5.1, D1.

2. Haghighi S, **Haghighi A**, Zádori ZS, Kovács K, Manzéger A, Kökény G. *Celecoxib and naproxen disrupt autophagy and activate EGR1 in kidney tubules.* Experimental and Molecular Pathology. 2025; 144:105000. Impact factor, journal quartile – 3.7, Q1.

3. **Haghighi A**, Demeter ZO, Zsidai A, Lengyel L, Haghighi S, Ostorházi E, Jakab A, Kökény G, Görbe A, Szabó D, Magierowski M. *Toll-like receptor 5 protects against nonsteroidal anti-inflammatory drug-induced enteropathy in mice*. Life Sciences. 2026 Jun 11:124532. Impact factor, journal quartile – 5.1, D1.

Publications not related to the thesis:

1. László SB, Hutka B, Tóth AS, Hegyes T, Demeter ZO, **Haghighi A**, Wachtl G, Kelemen Á, Jakab A, Gyires K, Zádori ZS. *Celecoxib and rofecoxib have different effects on small intestinal ischemia/reperfusion injury in rats.* Frontiers in Pharmacology. 2024;15: 1468579. Impact factor, journal quartile – 4.4, Q1.

2.Haghighi Bardineh SA, Balou HA, Sedigh Ebrahim-Saraie H, Mobayen M, Esmailzadeh M, Haghighi S, **Haghighi A**, Sadeghi M. *Predictive value of serum albumin and calcium levels in burn patients with Pseudomonas aeruginosa infection: A comprehensive analysis of clinical outcomes.* International Wound Journal. 2024;21(3):14786. Impact factor, journal quartile – 2.8, Q1.

3. Salehi Z, **Haghighi A**, Haghighi S, Aminian K, Asl SF, Mashayekhi F. *Mitochondrial DNA Deletion Δ4977 in Peptic Ulcer Disease.* Molecular Biology. 2017;51(1):30-33. Impact factor, journal quartile – 1.0, Q3.

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