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

PhD thesis

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1. INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed medications worldwide for their analgesic, antipyretic, and anti-inflammatory effects. They inhibit cyclooxygenase (COX) enzymes, blocking the conversion of arachidonic acid to prostaglandins. Non-selective NSAIDs inhibit both COX-1 and COX-2, providing effective relief but causing a 2- to 5-fold increased risk of upper gastrointestinal bleeding and ulceration through depletion of gastroprotective prostaglandins. Selective COX-2 inhibitors reduce gastric toxicity yet carry significant cardiovascular risk, limiting long-term use.

Chronic NSAID exposure also damages the small intestine, a condition termed NSAID-induced enteropathy. Capsule endoscopy studies show that up to 70 % of long-term users develop erosions, ulcers, petechiae, or strictures, often asymptomatic. Unlike upper gastrointestinal injury, enteropathy involves both COX-dependent and COX-independent mechanisms, including enterohepatic recirculation, mucosal barrier disruption, bile-acid toxicity, and gut microbiota alterations. Proton pump inhibitors protect the stomach but offer no benefit to the small bowel, and no approved therapies exist for NSAID enteropathy.

The intestinal microbiota (~3.8×10^{13} microorganisms, dominated by Firmicutes and Bacteroidetes in health) is central to pathogenesis. NSAIDs rapidly induce dysbiosis with loss of Gram-positive commensals

and expansion of Gram-negative bacteria, changes that correlate directly with injury severity. Germ-free or antibiotic-treated animals are markedly protected, and microbiota modulation (antibiotics, probiotics, faecal transplantation) shows preclinical benefit, though clinical use is limited by resistance and inconsistent efficacy.

Bacterial components activate inflammation through Toll-like receptors (TLRs). Among bacterial-sensing TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, TLR9), TLR4 exacerbates enteropathy via lipopolysaccharide-triggered NLRP3 inflammasome and cytokine release; TLR4-deficient mice are protected. In contrast, TLR2 signalling appears protective by limiting leukocyte infiltration and promoting repair. The roles of other TLRs remain largely unexplored.

Particular interest focuses on TLR5, the receptor for bacterial flagellin. TLR5 activation induces antimicrobial peptides (Reg3γ), mucus production, IL-22, and NF-κB-dependent repair, strengthening barrier integrity and restraining Proteobacteria overgrowth—features mirroring NSAID enteropathy. TLR5-deficient mice spontaneously develop colitis and dysbiosis, while flagellin-TLR5 signalling protects against colitis, mucositis, and infection. Despite these parallels, TLR5 expression dynamics and therapeutic activation in NSAID-induced enteropathy have not been studied yet.

Although microbiota dysbiosis and TLR-mediated innate immunity drive NSAID enteropathy, systematic characterisation of TLR expression changes and targeted modulation—particularly of TLR5—remain absent

from the literature. The present thesis therefore aims to identify novel therapeutic strategies targeting Toll-like receptors for the prevention and treatment of NSAID-induced enteropathy.

2. OBJECTIVES

The main objectives of this thesis were:

1. 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. To comprehensively evaluate the correlations between changes in TLR expression and intestinal inflammation and dysbiosis in acute enteropathy.
3. 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.
4. To analyze whether the expression of TLR5 is also downregulated by NSAIDs in mice.
5. To analyze whether TLR5 activation by flagellin prior to IND administration protects against intestinal inflammation and tissue damage in mice.
6. To assess whether TLR5 activation by flagellin after IND administration can mitigate IND enteropathy.

7. To analyze the effect of TLR5 inhibition by TH1020 (TLR5 antagonist) on IND-induced intestinal inflammation.

3. METHODS

Experimental animals: Male Wistar rats (8–10 weeks old, 220–240 g) and male C57BL/6 mice (8–12 weeks old, 23–30 g) were housed under controlled conditions with free access to food and water. All experiments were conducted in compliance with EU regulations and approved by the relevant ethical committee, with efforts made to minimize animal use and suffering.

Study design: The thesis consists of two complementary in vivo studies. The first characterised NSAID-induced changes in small-intestinal Toll-like receptor (TLR) expression, inflammation and gut microbiota in rats. To standardise microbiota, animals were co-housed for one week before randomisation. Acute enteropathy was induced by a single oral dose of indomethacin (IND, 20 mg/kg); rats were sacrificed at 6, 12, 24, 48 or 72 h post-dose (vehicle controls at 72 h only, n = 8 per group). Chronic enteropathy was induced by twice-daily oral IND (2 mg/kg for 14 days or 4 mg/kg for 7 days) or naproxen (10 mg/kg for 14 days or 20 mg/kg for 7 days); vehicle-treated controls were sacrificed after two weeks. At termination, small-intestinal length was recorded, distal jejunal segments were fixed in 10 % formalin for histology, and full-thickness tissue plus luminal contents were snap-frozen at –80 °C. Blood was collected in the chronic study.

The second study evaluated pharmacological modulation of TLR5 signalling in IND-induced enteropathy in mice. Animals (n = 6–7 per group) received a single oral dose of IND (30 or 40 mg/kg) or vehicle. Purified Salmonella typhimurium flagellin (TLR5 agonist, 10 or 30 µg/mouse) or the TLR5 antagonist TH1020 (10 µg/mouse) was administered intraperitoneally at selected time points.

Western blot analysis: Protein expression of COX-2, IL-1β, pentraxin-3 and myeloperoxidase (MPO) was quantified by Western blot using specific primary antibodies, HRP-conjugated secondary antibodies and GAPDH as loading control (Chemidoc XRS+ detection). Luminal flagellin levels were measured by Western blot of boiled and sonicated luminal extracts (anti-flagellin antibody).

qRT-PCR measurements: Total RNA was extracted with QIAzol; reverse transcription was performed with the SensiFAST cDNA kit. Quantitative RT-PCR was run on a LightCycler 480 II using SensiFAST SYBR Green master mix. Target genes (Tlr1, Tlr2, Tlr4, Tlr5, Tlr6, Tlr9, Il1b, Il10, Tnf, Ptgs2, Nos2) were normalised to Rpl13a and expressed as $2^{-\Delta\Delta CT}$ fold changes.

Histology: Histological damage was assessed on haematoxylin-eosin-stained Swiss-roll sections (4 µm) evaluated blindly by two histopathologists. In rats a composite score (0–17) combined villus morphology (0–3), erosions (0–2), ulcers (0–5), mucin depletion (0–3), inflammatory infiltration (0–2) and peritoneal reaction (0–2); severity was graded 0–4. In mice the Chiu/Park scale (0–8) was applied. Digital

slides were scanned with a Pannoramic 1000 scanner.

Hematological analysis: Whole-blood haematological parameters were analysed on a Sysmex XN-1000 analyser.

Gut microbiota analysis: Gut microbiota composition was profiled by 16S rRNA V3–V4 amplicon sequencing (Illumina MiSeq) after DNA extraction with the QIAamp PowerFecal kit. Raw reads were quality-filtered (FastQC, Trimmomatic), classified against the SILVA database (Kraken2/Bracken), CLR-transformed and analysed for alpha diversity (Shannon index), beta diversity (PCA, PERMANOVA) and differential abundance (Wilcoxon rank-sum with Benjamini–Hochberg FDR correction). Absolute bacterial loads of Lachnospiraceae and Enterobacteriaceae were quantified by family-specific qPCR with a standard curve (log CFU/g tissue).

Data analysis: Statistical analysis was performed with GraphPad Prism or R (v4.3.3). Parametric data were analysed by Student's t-test, one- or two-way ANOVA with Fisher's LSD post-hoc; non-parametric data by Kruskal–Wallis with Dunn's test. Outliers were removed by Grubb's test ($p < 0.05$). Spearman rank correlations between TLR expression, inflammatory markers and bacterial abundances were visualised as heatmaps with Benjamini–Hochberg correction for multiple testing.

4. RESULTS

Indomethacin-induced acute enteropathy differentially modulates intestinal TLR expression: In the acute enteropathy model, male Wistar rats received a single high oral dose of indomethacin (20 mg/kg) and were sacrificed at 6, 12, 24, 48, and 72 h (vehicle controls at 72 h). Enteropathy developed rapidly, causing progressive body-weight loss, intestinal shortening, early mucosal erosions, ulcers, and inflammatory infiltration from 6 h onward. This was accompanied by strong upregulation of pro-inflammatory markers (MPO, PTX3, COX-2, IL-1β, TNF-α), peaking at 48 h, together with a gradual decline in the anti-inflammatory cytokine IL-10.

TLR gene expression showed clear differential regulation. TLR1 and TLR2 mRNA increased progressively and were significantly elevated at 24–48 h, paralleling inflammation. TLR6 remained unchanged, while TLR4 and TLR9 exhibited only transient, highly variable increases without statistical significance. In contrast, TLR5 expression was consistently and markedly downregulated throughout the time course, indicating an early and sustained loss of this receptor during acute NSAID-induced small-intestinal injury.

Intestinal dysbiosis and inflammation induced by IND in acute enteropathy are differentially associated with changes in distinct TLRs: TLR expression in acute indomethacin-induced enteropathy correlated differentially with inflammation and dysbiosis. TLR2 (and to

a lesser extent TLR1) showed strong positive associations with pro-inflammatory markers (MPO, PTX3, COX-2, IL-1β, TNF-α), while TLR5 exhibited robust negative correlations. TLR4 associations were weak and marker-specific; TLR6 and TLR9 showed none. 16S rRNA sequencing revealed only minor α-diversity changes but a rapid, highly significant shift in microbiota composition (Permanova $p < 0.001$), with Firmicutes dropping sharply and Gram-negative Proteobacteria/Bacteroidota expanding. Correlations between TLR1, TLR2, TLR5 and bacterial taxa were largely inflammation-driven rather than direct.

Early (6 h) subgroup analysis confirmed that inflammation — not dysbiosis — drove the upregulation of TLR1/TLR2 and downregulation of TLR5. In contrast, TLR4 upregulation was specifically linked to early dysbiosis, particularly Enterobacteriaceae overgrowth. TLR6 and TLR9 remained unaffected. Thus, TLR1/TLR2 upregulation and TLR5 downregulation are tightly coupled to inflammatory severity from the onset of enteropathy, whereas TLR4 changes primarily reflect Gram-negative bacterial expansion.

Severe chronic IND-induced enteropathy exhibits TLR expression patterns similar to severe acute enteropathy: In the chronic enteropathy model, rats received twice-daily indomethacin (IND, 2 or 4 mg/kg) or naproxen (NAP, 10 or 20 mg/kg). High-dose IND (4 mg/kg) produced severe intestinal damage and inflammation comparable to acute high-dose IND, while low-dose

IND (2 mg/kg) induced only mild inflammation (mainly hematological). NAP caused milder, dose-dependent injury.

TLR expression in severe chronic IND enteropathy mirrored the acute pattern: significant upregulation of TLR1 and TLR2, marked downregulation of TLR5, and no significant changes in TLR4, TLR6, or TLR9. Mild chronic IND caused only modest downregulation of TLR1 and TLR5. High-dose NAP showed non-significant trends toward increased TLR2 and TLR4, with significant TLR5 downregulation. Overall, TLR1/TLR2 upregulation occurred selectively in severe disease, TLR5 downregulation was consistent across both mild and severe enteropathy (independent of NSAID type or severity), TLR4 changes remained variable and primarily linked to Gram-negative bacterial abundance, and TLR6/TLR9 expression was unchanged.

NSAID-induced enteropathy downregulates intestinal TLR5 expression in mice while elevates luminal flagellin levels: NSAID-induced enteropathy in mice resulted in a significant reduction in intestinal TLR5 expression and an increase in luminal flagellin levels, mirroring findings previously observed in rats. These results indicate that downregulation of TLR5 is a common feature of NSAID-induced intestinal injury and is associated with elevated bacterial flagellin in the gut.

Systemic flagellin treatment mitigates IND-induced intestinal

inflammation and tissue damage: Systemic flagellin treatment significantly attenuated IND-induced intestinal inflammation and tissue damage in mice, supporting a protective role for TLR5 activation in enteropathy. Flagellin reduced inflammatory markers (IL-1β, COX-2, MPO, and PTX3), limited intestinal shortening, and decreased mucosal injury, although some responses such as Nos2 expression were increased. It also prevented the upregulation of TLR2 and TLR4, reduced total bacterial load and flagellin levels, and was associated with lower levels of key bacterial families. Overall, these findings indicate that flagellin exerts a net anti-inflammatory effect and restricts enteropathy progression at molecular, histological, and morphological levels, although in the absence of inflammation it can induce mild intestinal changes and modulate TLR expression differently.

Flagellin administered after IND also protects against enteropathy: Flagellin administration after IND treatment also provided significant protection against enteropathy, as evidenced by reduced intestinal shortening, decreased inflammatory mediators, and lower TLR4 expression. However, it did not reverse the downregulation of TLR5. These findings demonstrate that TLR5 activation has not only preventive but also therapeutic potential in NSAID-induced enteropathy.

Inhibition of TLR5 by TH1020 aggravates IND-induced intestinal inflammation: Inhibition of TLR5 with the antagonist TH1020 did not

affect intestinal morphology or inflammation in control animals but significantly exacerbated inflammation in IND-induced enteropathy, as shown by increased Il1b, Tlr2, and Tlr4 expression. These findings indicate that endogenous TLR5 signaling plays a protective role, and its blockade worsens intestinal inflammation, supporting its key role in enteropathy pathogenesis.

5. CONCLUSIONS

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.

6. 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.

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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.

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