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Modulating Immune Responses in a Murine Model of Chronic DSS-Induced Colitis Using Tuftsin–Phosphorylcholine

Liu Zhang[1*], Yaping Liu[1], Jian Ming[1], Xiuli Zhuang[1], Alvin Chuen Wei Seah[1]

[1] College of Animal Science and Technology, Shihezi University, Shihezi 832000, China.

Abstract

Helminth-derived compounds can influence the immune system, presenting opportunities for the development of innovative anti-inflammatory therapies. In previous studies, we demonstrated that tuftsin–phosphorylcholine (TPC), a molecule derived from helminths, effectively mitigates inflammation in animal models of autoimmune disorders, including arthritis, colitis, systemic lupus erythematosus, and experimental autoimmune encephalomyelitis. TPC was found to suppress inflammatory activity in peripheral blood lymphocytes and in giant-cell arteritis tissue samples. In the current investigation, we evaluated the therapeutic effects of TPC in a mouse model of chronic colitis. C57BL/6 mice were administered TPC orally after the third cycle of 2% dextran sodium sulfate (DSS)-induced colitis. Treatment with TPC led to notable improvements in colitis symptoms, reflected by a decrease in the disease activity index, an increase in T regulatory cells within mesenteric lymph nodes (analyzed via FACS), a significant reduction in pro-inflammatory cytokine levels (IL-1β, IL-17, IL-6, TNFα), and enhanced expression of the anti-inflammatory cytokine IL-10 (measured by RT-PCR). These results suggest that oral TPC exerts immunomodulatory effects capable of ameliorating chronic colitis, supporting further exploration of this strategy as a potential therapy for inflammatory bowel disease in humans.

Keywords: Tuftsin, Inflammatory bowel disease, Phosphorylcholine, Colitis

Corresponding author: Liu Zhang
E-mail: luizhang@163.com

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Introduction

Inflammatory bowel disease (IBD) encompasses two primary disorders, ulcerative colitis (UC) and Crohn's disease (CD), which share certain symptoms while also presenting distinct clinical features. The underlying causes of UC and CD remain incompletely defined but are generally considered multifactorial. Current understanding emphasizes a complex interplay between genetic predisposition, immune system dysregulation, and environmental influences [1, 2]. Epidemiological studies have linked UC and CD to higher socioeconomic status, possibly reflecting reduced exposure to environmental antigens during early life, consistent with the hygiene hypothesis in the context of intestinal immunity [3]. Joel Weinstock first suggested that the widespread "deworming" practices in industrialized nations could be contributing to the rising incidence of autoimmune disorders [4]. Subsequent research has reinforced this concept, showing a strong association between high helminth prevalence in certain regions and reduced susceptibility to atopic, autoimmune, and auto-inflammatory conditions, including IBD [4–8].

Despite advances in therapy, effective and safe long-term treatments for IBD remain limited. Conventional first-line

interventions include 5-aminosalicylic acids and corticosteroids during acute flares, while maintenance therapy typically relies on aminosalicylates and thiopurines, with corticosteroids being unsuitable for long-term use. Severe or refractory disease may necessitate intravenous corticosteroids, calcineurin inhibitors, or TNF-α antagonists; however, these treatments often have diminishing efficacy over time and carry the risk of significant adverse effects [9–11].

Experimental studies have shown that helminths and their ova can suppress inflammatory responses across multiple immune-mediated conditions [12–15]. This immunoregulatory effect is largely attributed to molecules secreted by the parasites, known collectively as excretory–secretory products, which predominantly contain phosphorylcholine (PC)-bearing glycoproteins [16, 17]. The immunomodulatory properties of these helminth-derived products are primarily linked to PC. Tuftsin–phosphorylcholine (TPC) is a synthetic small molecule inspired by these helminth secretions, combining the tetrapeptide tuftsin with PC. PC is a naturally occurring, non-immunogenic molecule, while tuftsin (Thr–Lys–Pro–Arg) is a physiologically active fragment derived from the enzymatic cleavage of the Fc region of IgG in the spleen [18, 19]. Tuftsin is known to enhance phagocytosis, promote macrophage migration and activation, and stimulate monocyte chemotaxis [18–21]. It has also been applied as a natural adjuvant in influenza vaccine development without reported adverse effects [18,19, 22]. Previous studies have demonstrated TPC's therapeutic potential in various autoimmune models: it delays glomerulonephritis in lupus-prone mice [23], mitigates joint destruction in collagen-induced arthritis [24, 25], prevents colitis in acute DSS-induced murine models [26], and reduces disease severity in experimental autoimmune encephalomyelitis, a model for multiple sclerosis [27]. Ex vivo analyses further show that TPC can suppress inflammatory cytokine production in peripheral blood lymphocytes and in tissue biopsies from patients with giant-cell arteritis [28].

Collectively, these findings highlight the potential for helminth-derived molecules to serve as a novel pharmacological strategy. In this study, we investigated the immunomodulatory effects of TPC in a murine model of chronic colitis to explore its potential as a therapeutic intervention for IBD.

Materials and Methods

Preparation of tuftsin–phosphorylcholine (TPC)

Tuftsin–phosphorylcholine (TPC) was provided by TPCera Ltd. (Jerusalem, Israel). Prior to administration, TPC was reconstituted in commercially available phosphate-buffered saline (PBS) (Biological Industries, Kibbutz Beit-Haemek, Israel) to achieve the desired concentration for experimental use.

Animal model

Forty male C57BL/6 mice, weighing 22–26 g (Envigo, Israel), were maintained under specific pathogen-free (SPF) conditions in ventilated cages at the Sheba Medical Center animal facility. All procedures were conducted in accordance with ethical approvals from the Israeli Ministry of Health (approval numbers 696/11 and 1008/16).

Chronic colitis induction and treatment protocol

Chronic colitis was established in 7-week-old male mice through repeated cycles of 2% dextran sulfate sodium (DSS; MW 36,000–50,000 Da; MP Biomedicals, Germany) administered in drinking water, following a previously published protocol [26]. Each cycle consisted of 5 days of DSS exposure followed by a 5-day recovery period, and the mice underwent three such cycles in total **(Figure 1)**. Disease progression was assessed using a disease activity index (DAI), which incorporated body weight changes, stool consistency, and presence of rectal bleeding.

After the final DSS cycle, mice were treated with daily oral doses of TPC (50 µg in 0.1 ml PBS) administered via a feeding tube for 15 days. On day 45 following DSS initiation, mice were euthanized, and the entire colon was excised and measured for length. A section of the distal colon was fixed in 4% phosphate-buffered formalin for histological analysis. Mesenteric lymph nodes (MLNs) were also collected for downstream isolation of immune cells and extraction of RNA for molecular studies.

Figure 1. Experimental Model of Chronic Colitis and TPC Treatment

Assessment of DSS-induced chronic colitis

Mice were monitored daily for changes in body weight, stool consistency, rectal bleeding, and overall survival. Weight loss was calculated as a percentage relative to baseline weight (measured on the first day of treatment). Occult bleeding was detected using the Hemoccult SENSA test (Beckman Coulter, USA), while visible rectal bleeding indicated overt hemorrhage. Disease severity was quantified using a disease activity index (DAI), which scored three parameters on a 0–4 scale: intestinal bleeding (0 = negative, 2 = positive hemoccult, 4 = gross bleeding), weight loss (0 ≤1%, 1 = 1–5%, 2 = 5–10%, 3 = 10–20%, 4 >20%), and stool consistency (0 = normal, 2 = loose stools, 4 = diarrhea). After 15 days of TPC treatment, mice were euthanized, and the colon was harvested for evaluation of

both macroscopic shortening and histopathological alterations [26, 29].

Histological evaluation

Distal colon segments were excised and fixed in 4% formalin. Tissues were sequentially dehydrated in graded alcohol solutions (70%, 80%, 90%, 100%, 1 hour each), cleared in xylene (three 1-hour incubations), and embedded in paraffin. Sections of 7 μm thickness were cut and stained with hematoxylin–eosin (H&E) for microscopic examination. Two pathologists, blinded to experimental groups, assessed inflammation based on histopathological criteria including epithelial erosion, crypt damage, goblet cell depletion, and infiltration of inflammatory cells.

Flow cytometric analysis of t regulatory cells

Mesenteric lymph node (MLN) cells were labeled with anti-CD4-FITC and anti-CD25-APC antibodies. For intracellular FOXP3 staining, cells were first fixed, washed, permeabilized (Serotec, Oxford, UK), and then incubated with anti-FOXP3 antibodies (eBioscience, San Diego, USA). CD4+CD25+FOXP3+ T regulatory cells (Tregs) were quantified by fluorescence-activated cell sorting (FACS; Becton Dickinson, Franklin Lakes, NJ, USA). Forward and side scatter gates were adjusted to include all cells while excluding debris, with gating focused on the CD4+ population.

Quantitative real-time PCR (qRT-PCR)

Total RNA from MLNs was extracted using the Total RNA Purification Plus Kit (Norgen Biotek, Canada) following the manufacturer's instructions. RNA concentration and purity were determined with a Nanodrop spectrophotometer (Thermo Scientific). One microgram of RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Invitrogen™, Carlsbad, CA, USA). Gene expression analysis was performed using real-time PCR on a StepOnePlus™ system (Applied Biosystems, Foster City, CA, USA), according to the supplier's protocols.

Primer sequences (forward and reverse, respectively) were: IL-1β: 5^J-GGA TGA GGA CAT GAG CAG CAC ATT C-3^J; 3^J- GGA AGA CAG GCT TGT GCT CTG A-5^J; IL-17: 5^JCCT CAA AGC TCA GCG TGT CC-3^J; 3^J-GAG CTC ACT TTT GCG CCA AG-5^J; IL-6: 5^J-ATG CTC CCT GAA TGA TCA CC-3^J; 3^J TTC TTTGCA AAC AG CACA GC-5^J; TNFα: 5^J-AAG CCT GTA GCC CAC GTC GTA-3^J; 3^J-GGC ACC ACT AGT TGG TTG TCT TTG-5^J; β-Actin: 5^J-GAA ATC GTG CGT GAC ATA AAA G-3^J; 3^J-TGT AGT TTC ATG GAT GC CACA G-5^J

The studied genes were normalized by the expression of β-Actin. The results are expressed as relative expression levels for each gene.

Statistical analysis

Data are presented as mean values. To evaluate changes in weight and DAI scores between the control and TPC-treated groups, repeated measures ANOVA followed by Tukey's post hoc test, a single-step multiple comparison approach, was employed. Differences in stool consistency and intestinal bleeding were assessed using the Kruskal–Wallis non-parametric test followed by the Mann–Whitney test. One-way ANOVA with subsequent Student's t-test was used to compare cytokine levels and colon lengths among groups. Statistical significance was defined as $p < 0.05$.

Results

TPC markedly reduces disease severity in a murine chronic colitis model

DSS-induced colitis serves as a widely utilized model for investigating various aspects of IBD, including immune responses, disease pathogenesis, genetic susceptibility, and the influence of gut microbiota. After fifteen days of treatment, mice receiving PBS exhibited significantly higher disease activity (maximum DAI score 2.6) compared to those treated with TPC (maximum DAI score 1.6) (**Figure 2**, $p < 0.001$).

Analysis of intestinal bleeding revealed that PBS-treated mice had considerably more severe bleeding than TPC-treated mice ($p < 0.001$). On Day 15, 13 mice (77%) in the PBS group showed overt rectal bleeding, while 4 mice (23%) tested positive for occult blood. In contrast, TPC-treated mice displayed overt bleeding in 7 mice (35%), occult bleeding in 11 mice (55%), and no bleeding in 2 mice (10%). Mean bleeding scores were 3.6 for PBS-treated mice and 2.5 for TPC-treated mice.

TPC treatment also significantly mitigated diarrhea ($p < 0.001$). At the study's conclusion, 8 PBS-treated mice (47%) experienced diarrhea, and 9 mice (53%) had loose stools, resulting in an average stool consistency score of 3.1. Among TPC-treated mice, 3 exhibited diarrhea, 15 (75%) had loose stools, and 2 (10%) showed normal stool consistency, with an average score of 2.1.

Colon length measurements post-sacrifice indicated that PBS-treated mice had significantly shorter colons (mean 6.1 cm) than TPC-treated mice (mean 7.0 cm; $p < 0.002$) **(Figure 3)**.

Finally, the DSS protocol led to a 15% mortality rate (3/20) in untreated mice, whereas all TPC-treated mice survived.

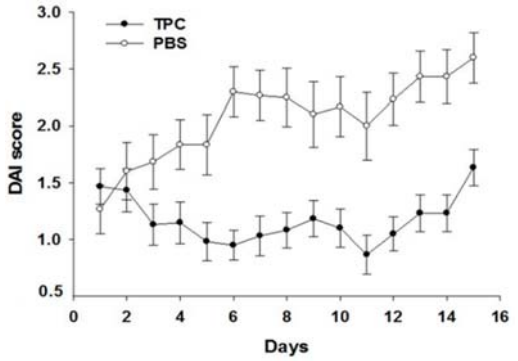

Figure 2. Impact of TPC on Disease Activity in a Chronic Colitis Model. Day 0 on this graph corresponds to Day 30 of chronic colitis. DAI scores are shown as mean ± standard error for each group (n = 20 per group) treated with tuftsin–phosphorylcholine (TPC) or phosphate-buffered saline (PBS). Mice receiving TPC exhibited a significantly lower mean DAI score compared to PBS-treated mice (p = 0.001)

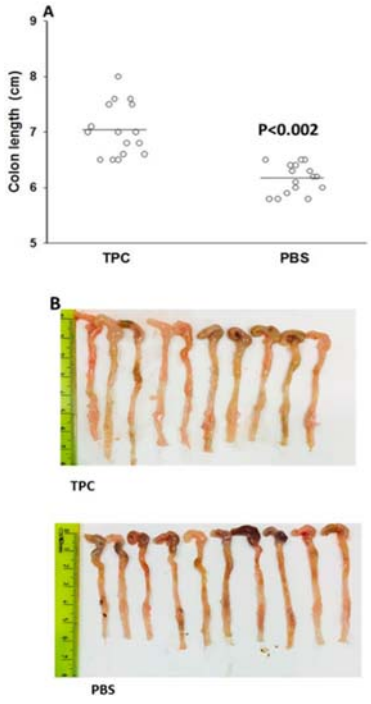

Figure 3. Effect of Tuftsin–Phosphorylcholine (TPC) on Colon Length. Colon lengths were measured in centimeters on Day 45 of the study, following three cycles of 2% DSS administration and 15 days of treatment with either TPC or phosphate-buffered saline (PBS). (A) Mice treated with TPC exhibited significantly less colon shortening compared to PBS-treated controls ($p < 0.002$). (B) Side-by-side comparison of colon lengths between TPC-treated and PBS-treated mice

TPC mitigates inflammation in a murine chronic colitis model

Histopathological evaluation of colonic tissue was performed 45 days after induction of colitis. PBS-treated mice showed pronounced chronic active inflammation affecting the mucosal layer, severe architectural disruption, extensive lymphocyte infiltration, cryptitis, and crypt loss **(Figure 4A)**. In the PBS group, massive lymphocyte infiltration and destruction of normal crypt structures were observed. The histological score for DSS-treated mice was 12 points, comprising: >10% epithelial loss = 3 points, >20% crypt loss = 3 points, severe goblet cell depletion = 3 points, and severe inflammatory cell infiltration = 3 points.

Colon sections from TPC-treated mice displayed notably improved microscopic features. Approximately 30% of these mice still showed severe inflammation comparable to PBS-treated animals. Another 40% exhibited mild to moderate inflammation, characterized by increased lymphocyte presence and mild cryptitis **(Figure 4B)**. The corresponding histological score was 6 points: >10% epithelial loss = 3 points, 10–20% crypt loss = 2 points, moderate goblet cell depletion = 2 points, and mild inflammatory cell infiltration = 1 point.

As shown in **Figure 4C**, the remaining 30% of TPC-treated mice retained largely intact colon architecture with minimal lymphocyte infiltration. No significant cryptitis or immune cell infiltration was noted. The histological score for this group was 0 points, reflecting no epithelial loss, no crypt loss, no goblet cell depletion, and absence of inflammatory cell infiltration.

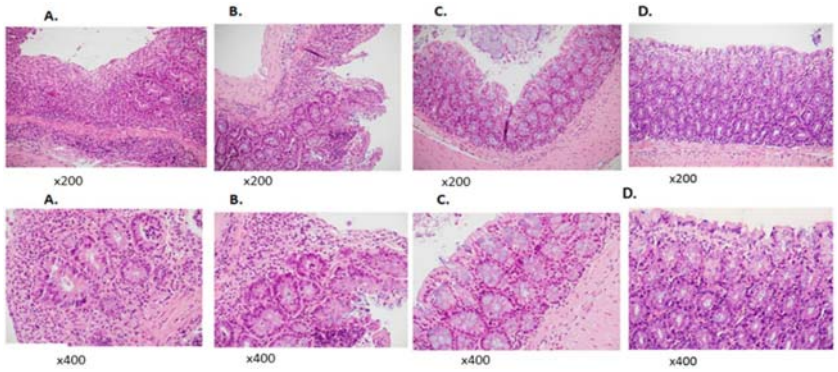

Figure 4. Histopathological Evaluation of Distal Colon Sections in Chronic Colitis Mice. Representative distal colon sections from each experimental group were stained with H&E and examined at ×200 and ×400 magnification. (A) PBS-treated control mice: Displayed extensive lymphocyte infiltration, severe disruption of normal tissue architecture, and pronounced cryptitis. The histological score was 12 points, comprised of >10% epithelial loss = 3 points, >20% crypt loss = 3 points, severe goblet cell depletion = 3 points, and severe inflammatory cell infiltration = 3 points. (B) TPC-treated mice: Showed mild cryptitis with improved tissue integrity. Histological scoring was 6 points: >10% epithelial loss = 3 points, 10–20% crypt loss = 2 points, moderate goblet cell depletion = 2 points, and mild inflammatory infiltration = 1 point. (C) TPC-treated mice: Maintained largely normal tissue architecture, with minimal lymphocyte infiltration and no cryptitis. Histological score was 0 points, reflecting no epithelial loss, crypt loss, goblet cell depletion, or inflammatory infiltration. (D) Healthy control colon: Normal tissue morphology

Expansion of MLN T regulatory cells following TPC treatment in murine chronic colitis

To investigate the impact of TPC on mesenteric lymph node (MLN) T regulatory cells, MLN-derived CD4+ T cells were analyzed for CD4+CD25+FOXP3+ Treg populations via flow cytometry. As shown in **Figure 5A–C**, TPC treatment significantly increased the proportion of Treg cells from 2.58% in PBS-treated mice (n = 20) to 5.6% in TPC-treated mice (n = 20) ($p < 0.002$). All analyses were gated on CD4+ T cells.

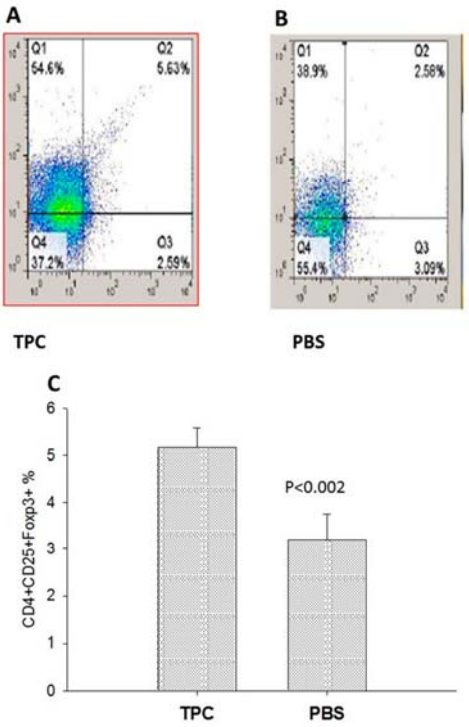

Figure 5. Expansion of T Regulatory Cells in Mesenteric Lymph Nodes of TPC-Treated Chronic Colitis Mice. (A) Percentage of CD4+CD25+FOXP3+ Treg cells in mesenteric lymph nodes of mice treated with TPC or PBS (n = 20). Data are expressed as mean ± SD, with a highly significant difference ($p < 0.0001$). (B) Representative flow cytometry plots showing CD4+CD25+FOXP3+ Tregs in MLNs from TPC- and PBS-treated mice. TPC treatment resulted in 5.63% Tregs, whereas PBS-treated mice had 2.58%. (C) Comparison of Treg percentages between TPC- and PBS-treated groups, illustrating the enhanced Treg expansion following TPC administration

TPC modulates MLN cytokine mRNA expression in murine chronic colitis

At the conclusion of the study, the impact of TPC on mesenteric lymph node (MLN) cytokine mRNA levels was evaluated. **Figure 6** depicts the relative expression of pro-inflammatory cytokines (IL-1β, IL-17, IL-6, and TNFα) compared with the anti-inflammatory cytokine IL-10. TPC treatment significantly reduced the mRNA levels of IL-1β (1.08 ± 0.58, $p < 0.003$), IL-17 (1.32 ± 0.24, $p < 0.001$), IL-6 (1.01 ± 0.19, $p < 0.02$), and TNFα (1.48 ± 0.21, $p < 0.001$). In contrast, IL-10 mRNA expression was elevated in TPC-treated mice (0.57 ± 0.87, $p < 0.009$). These results indicate that TPC effectively suppresses pro-inflammatory cytokine expression while enhancing anti-inflammatory IL-10 transcription in MLNs.

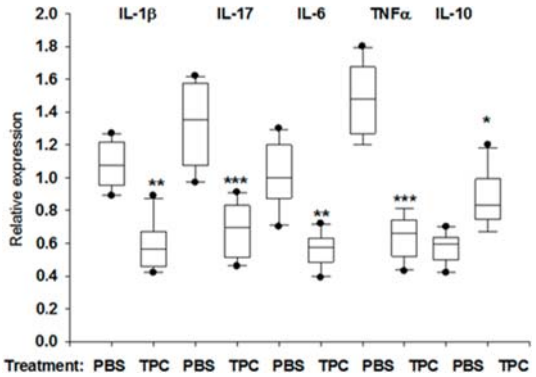

Figure 6. Expression of cytokine genes IL-1β, IL-17, IL-6, TNFα, and IL-10 in mesenteric lymph nodes (MLN) after oral administration of tuftsin–phosphorylcholine (TPC) compared with phosphate-buffered saline (PBS)-treated mice. Data were normalized to β-Actin expression. One asterisk indicates $p < 0.001$, two asterisks $p = 0.02–0.03$, and three asterisks $p < 0.001$

Discussion

This study highlights the immunomodulatory properties of TPC, a small (~1 kDa) helminth-derived molecule, in a

murine model of chronic colitis. Oral administration of TPC, initiated after chronic DSS-induced colitis was established, led to marked improvements in both clinical outcomes and histopathological features. TPC significantly promoted the expansion of CD4+CD25+FOXP3+ regulatory T cells (Tregs) in the MLN. Additionally, TPC reduced mRNA expression of pro-inflammatory cytokines IL-1β, IL-17, IL-6, and TNFα while increasing IL-10 transcription. CD4+CD25+ Tregs are known to prevent and resolve colitis in mice, and their therapeutic effect depends on IL-10 [30, 31]. In ulcerative colitis patients, Tregs also expand in MLN and the lamina propria, but their suppressive function may be impaired or insufficient to control chronic mucosal inflammation [32]. Therefore, our findings emphasize the clinical relevance of increasing MLN Treg numbers and IL-10 expression through oral TPC administration, corresponding with improvements in clinical symptoms and intestinal histology.

These results align with our prior studies demonstrating TPC's immunoregulatory effects, including suppression of pro-inflammatory cytokines and elevation of IL-10, along with enhanced Treg and Breg populations [23–27]. We previously showed that TPC exhibits bifunctional activity in vitro [25]. Specifically, the phosphorylcholine portion binds TLR4 and inhibits NFκB signaling, while tuftsin interacts with macrophages and Tregs via arginine-mediated binding to neuropilin-1. This dual mechanism promotes a shift from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages that secrete IL-10 [25].

Previous work from our group confirmed the critical role of IL-10 in mediating TPC's anti-inflammatory effects [25, 26]. Our findings are further supported by studies showing that parasite-induced amelioration of autoimmune diseases is associated with increased IL-10 production [33–37]. Another potential player in the immune properties of TPC is IL-1β. Mucosal barrier breach induced by IL-1β has been proposed as a trigger of mucositis onset. Studies in mice suggest that epithelial tight junction dysfunction and mucositis significantly improve with antibody neutralization of IL-1β [38, 39].

Previous studies from our group demonstrated that TPC treatment led to a reduction in colonic IL-1β levels [26]. Over the past few decades, research has increasingly explored the therapeutic potential of helminths and their secreted products in disease management [40]. Helminth infections can modulate the host immune system, altering immune responses to antigens, a property with potential applications in treating inflammatory bowel disease (IBD) and other conditions linked to immune dysregulation [41]. Although the use of helminths as a therapeutic strategy is relatively novel, it has already advanced to sophisticated experimental stages in multiple diseases, including through randomized controlled trials (RCTs) [42–49]. Nevertheless, current evidence remains insufficient to draw definitive conclusions regarding the safety and effectiveness of helminth-based therapies in IBD [40].

In summary, TPC, a bifunctional molecule derived from a helminth product, effectively prevented severe colitis in a DSS-induced acute colitis model and demonstrated significant clinical and histological therapeutic benefits in a DSS-induced chronic colitis model. These findings provide experimental proof-of-concept that TPC's therapeutic effects are linked both to the prevention of colitis onset [26] and to the treatment of established chronic colitis. This highlights the potential of TPC as a synthetic small molecule inspired by helminth-derived products for the treatment of colitis in humans. While chemically induced colitis models are well-established and valuable for preclinical therapeutic testing, they do not fully replicate the immune mechanisms of human IBD, underscoring the need for further research.

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