Role of Gut Microbiota in a Zebrafish Model with Chemically Induced Enterocolitis Involving Toll-Like Receptor Signaling Pathways

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Abstract

Background/Aims: It is believed that inflammatory bowel disease (IBD) involves a breakdown in interactions between the resident commensal microbiota and the host immune response. Recent studies have revealed that gut physiology and pathology relevant to human IBD can be rapidly modeled in zebrafish larvae with a number of advantages compared with murine models. The objective of this study was to evaluate the role of gut microbiota in zebrafish models with IBD-like enterocolitis.

Methods: IBD-like enterocolitis was induced by exposing larval zebrafish to 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). Assays were performed using larval zebrafish collected at 8 and 10 days postfertilization (dpf).

Results: In the absence of gut microbiota, the TNBS-induced enterocolitis was less extensive. The expression of toll-like receptor 3 (TLR3) and the TLRs signaling pathway molecules MyD88 and TRIF, the activation of NF- κ B, and the production of inflammatory cytokine tumor necrosis factor- α were stimulated in TNBS-treated zebrafish but there was no corresponding alteration in germ-free fish. With microbial colonization, all results reverted to a pattern similar to that observed in conventionally reared zebrafish.

Conclusion: We described the key role of gut microbiota in the etiology of a chemically induced larval zebrafish IBD-like model, showing an involvement of TLR signaling pathways.

Introduction

INFLAMMATORY BOWEL DISEASE (IBD), broadly classified into ulcerative colitis (UC) and Crohn's disease (CD), is a chronic inflammatory disease of the gastrointestinal (GI) tract with high morbidity and relapse. Symptoms include abdominal pain, diarrhea, weight loss, ulceration, perforation, and bowel obstruction. Although the precise etiology of IBD remains unclear, perturbed homeostasis between commensal microbiota and mucosal immunity is believed to play a key role in its development and progression in the genetically susceptible individuals.^{1,2} Several investigators have documented that IBD patients contain abnormal compositions of the gut microbiota, characterized by reduced diversity, depletion of specific beneficial commensal species, and overrepresentation of some opportunistic pathogenic bacteria.^{3–5}

There are several mechanisms known for the immune response evoked by pathogenic bacteria, and the host immune system should possess the ability to discriminate between self and nonself rapidly and precisely. Rodent studies have cited that initial recognition of bacteria in the extracellular environment occurs through pattern recognition receptors (PRRs),

which recognize microbial-associated molecular patterns.^{6,7} In mammals, it is revealed that toll-like receptors (TLRs), which comprise a class of transmembrane PRRs, play an essential role in induction of pro-/anti-inflammatory cytokine genes and maintain commensal-mucosal homeostasis. To date, some studies have shown that TLR4, the receptor for lipopolysaccharide, knockout mice did not develop colitic lesions on dextran sodium sulfate (DSS) treatment and TLR4 antagonist antibody-ameliorated inflammation in colitic mice.^{8,9} Moreover, a meta-analysis revealed that genetic variations in TLR4 conferred a statistically significant risk of developing CD and UC.¹⁰ In addition to TLR4, polymorphisms in TLR2, the main receptor for gram-positive bacteria, have been associated with IBD in humans and there is an inflammation-dependent induction of TLR2 expression in intestinal macrophages.^{11,12} On activation of the TLRs, different adaptor proteins, including MyD88, Mal (TIRAP), TRIF (TICAM), TRAM, (TICAM2), or SARM are recruited, and they trigger a signaling pathway, leading to the subsequent downstream activation of signals such as transcription factor NF- κ B (nuclear factor- κ B), which are responsible for induction of pro-inflammatory cytokines and chemokines.^{13,14} Recent findings demonstrated that the TLRs-mediated

MyD88-dependent signaling pathway is critical for spontaneous development of colitis in IL10-deficient mice.⁸ A second pathway is mediated by TRIF and TRAM. Initiation of the MyD88-independent pathway leads to the activation of IFN-regulatory factor 3 (IRF3) and the expression of INF- β and IFN-inducible genes.¹⁴ In zebrafish models, research also shows that TLR signaling pathways play a central role in immune response during infection and inflammation.^{15–17} It is reasonable to speculate that TLR signaling pathway sensing of gut bacteria and provoking inflammatory response is essential in IBD pathogenesis, but it remains to be directly proved how changes in the commensal composition functionally slant TLR signaling in the disease.

Currently, the zebrafish model, as an established developmental biology model, has come to the fore with its specific experimental advantages and characteristics in the study of developmental biology and disease processes. To start with, zebrafish are well suited for investigating host-microbial relationships, as they have innate and adaptive immune systems similar to higher vertebrates.¹⁸ In addition, microbial analysis has demonstrated that the resident commensal microbiota of zebrafish and mammals share most bacterial divisions and serve similar functions in the digestive tract.^{19,20} Moreover, recent research has shown that zebrafish can be raised in a germ-free (GF) environment, providing unique opportunities to study the effects of symbiotic bacteria on intestinal disease development.^{21–23} Furthermore, Fleming and Oehlers *et al.* developed an IBD-like model in zebrafish larvae using 2, 4, 6-trinitrobenzene sulfonic acid (TNBS).^{24,25} An observation of the larvae GI tract after the administration of TNBS reveals region-specific disease changes with biological, pathological, and clinical relevance to the human condition.^{24–27} Finally, the fish environment is relatively easy to manipulate, and embryos can conveniently be produced in large numbers. Recently, zebrafish homologues to mammalian TLRs and adaptor proteins were identified in the zebrafish genome, indicating evolutionary conservation.^{28,29} Some research shows that zebrafish exposed to *Edwardsiella tarda* resulted in an increase in mRNA expression of zTLR3, further demonstrating that zebrafish possess conserved TLR signaling pathways.²⁹

We report here the role of gut microbiota in the pathogenesis of a TNBS-induced larval zebrafish IBD-like model. We find that in the absence of microbiota, TNBS-exposed fish show less extensive pathological changes compared with the control. Overproduction of inflammatory cytokines, such as tumor necrosis factor (TNF)- α , depends on the existence of intestinal microbiota. All traits are later reversed by the colonization of microbes. Furthermore, we present evidence for the involving of TLR signaling pathways, including MyD88-dependent and MyD88-independent pathways, by which the host perceives and responds to its commensal microbiota during enterocolitis development. Together, these studies demonstrate that gut microbiota plays a significant role in the pathogenesis of zebrafish IBD-like models involving TLR signaling pathways. Future studies of the zebrafish TLR signaling pathways will help expand our understanding of how the resident commensal microbiota and the host immunity coordinate and function in both fish and humans.

Materials and Methods

Ethics statement

All experiments with zebrafish were performed in strict accordance with the recommendations in the Guide for the

Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee of Model Animal Research Center, Nanjing University (MARC-AP#: QZ01), in accordance with the Guidelines on the Humane Treatment of Laboratory Animals in China and the Regulations for the Administration of Affairs Concerning Experimental Animals.

Zebrafish husbandry

Adult fish maintenance and embryo collection. Wild-type (AB strain) zebrafish were reared at $28^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ on a 14-h light/10-h dark cycle in a closed flow-through system in charcoal-filtered and fully aerated tap water according to standard procedures. The fish were fed with commercial flakes (TetraMin[®], comprising protein 47%, fat 10%, moisture 6%, fiber 3%, and ash 11%) twice daily.

Zebrafish embryos were collected overnight from spawning adults in groups of about 16 males and 8 females in tanks. Spawning was induced in the morning shortly after the light was turned on. Collected embryos were maintained in embryo medium (13.7 mM NaCl, 0.54 mM KCl, 1.3 mM CaCl_2 , 1.0 mM MgSO_4 , 0.25 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , and 0.42 mM NaHCO_3) at 28.5°C . At 4–5 h postfertilization (hpf), those embryos that had developed normally and reached the blastula stage were selected under a dissecting microscope for subsequent experiments. Zebrafish were raised to 10 dpf without feeding.

Conventionally reared zebrafish. Conventionally reared (CV) control embryos were washed using a cell strainer to get rid of contaminating food residue and reared through 8 days post fertilization (dpf) at a density of approximately one fish per milliliter of embryo medium. A 90% v/v water change was performed each day starting at 3 dpf when larvae hatch from their chorions.

GF zebrafish. GF zebrafish larvae were generated in a Class II, type A2 biological safety cabinet. 6 hpf embryos were soaked in embryo medium containing 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ Streptomycin for 1–3 h. Embryos were then cleaned twice in 0.003% sodium hypochloride (NaOCl) for 15 min in total and rinsed three times with sterile water. After that, embryos were transferred to 20 mL filter-sterilized embryo medium in 50 mL tissue culture flasks at a density of 15–20 embryos per flask. The sterility of GF embryos was assayed by culture under aerobic and anaerobic conditions at 28°C on brain-heart infusion blood agar (BHIBA). Similar rates of hatching and mortality and no developmental defects were observed with these treatments.

Ex-germ-free zebrafish. To generate these larvae, GF flasks were inoculated with 1 mL embryo medium from CV flasks (final density 10^4 CFU/mL, as assayed by aerobic growth on BHIBA at 28°C) at 8 dpf and examined at 10 dpf.

All flasks were maintained at 28.5°C for the duration of the experiment.

TNBS treatment of zebrafish. A stock solution of 5% (w/v) 2, 4, 6-trinitrobenzenesulfonic acid (TNBS; Sigma) in embryo medium was used for the induction of enterocolitis. CV

and GF larvae were immersed in embryo medium and filter-sterilized embryo medium containing 75 $\mu\text{g}/\text{mL}$ TNBS from 3 to 8 dpf, respectively. The concentration was selected based on previously ascertained range-finding studies and information from the available literature.^{24,25} GF zebrafish were monitored routinely for sterility by culturing the aquaculture medium under aerobic and anaerobic conditions at 28°C on BHIBA.

Histology

Larval zebrafish from 8 to 10 dpf were euthanized by immersion in 0.2 mg/mL 3-amino benzoic acid ethylester (MS222; Sigma). For histology, samples were fixed in Bouin's Fixative overnight at 4°C and mounted in SeaPlaque 1% low-melting-point agarose. Then, samples were dehydrated through a standard series of alcohols and Histo-clear and embedded in paraffin. Five-micrometer sections were cut for staining with hematoxylin and eosin. Histological sections were imaged and photographed with an Olympus CX41 system microscope (Olympus USA) and the DS-5M-L1 digital sight camera system (Nikon).

For evaluation of enterocolitis changes caused by TNBS exposure, a simple scoring system was devised (Table 1). Intestinal bulb, mid-intestine, and posterior intestine were assessed separately and total enterocolitis score represented the cumulative values of these separate parameters for all three segments of the intestine. The enterocolitis scores were quantified by an observer who was blinded to the earlier treatment of the fish.

Detection of goblet cells using AB-PAS staining

To study the density of the goblet cell, 5- μm paraffin sections were prepared as described in the methods and stained sequentially with 1% Alcian blue pH 2.5 for 15 min, 1% aqueous periodic acid for 10 min, and Schiff's reagent for 10 min. Goblet cells stain blue using this method. The number of goblet cells was counted manually along the length of the gut from the intestinal bulb to the anus.

Immunofluorescence

Larvae at 8 and 10 dpf were fixed in 4% paraformaldehyde overnight at 4°C. Fixed larvae were soaked in 30% sucrose until they sank, transferred to an embedding chamber filled with OCT Compound (Sakura Finetek USA, Inc.), snap frozen in liquid nitrogen, and stored at -80°C. Five-micrometer frozen sections were cut and blocked with 1% bovine serum albumin before being incubated with monoclonal antibodies

against anti-TNF- α (IN), Z-FishTM, Catalog No. 55383P (1:150, 100 $\mu\text{g}/400 \mu\text{L}$; AnaSpec) overnight at 4°C. Sections were washed in PBS and incubated with Alexa 488-conjugated for 30 min at 4°C, followed by counterstaining with DAPI (1:500). Sections were imaged and photographed with a Leica TCS SP5 confocal scanning microscope (Leica Microsystems, Heidelberg GmbH). The intensity of TNF- α immunofluorescence was quantified for each treatment group, with a minimum of six samples per group, using color threshold and area measurements with Analysis software.

Western blot

Total proteins were extracted from pools of 20 zebrafish larvae with RIPA Lysis Buffer. Protein concentrations were then determined by Bradford assay (Bio-Rad Laboratories). Equal amounts (20 μg) of protein extracts were separated by 12% SDS-PAGE gels on a minigel apparatus (Bio-Rad Laboratories), and then transferred for 2 h at 200 mA to polyvinylidene difluoride membranes (Bio-Rad Laboratories). Membranes were blocked in 3% bovine serum albumin and 0.1% Tween-20 in Tris-buffered saline (TBS) for 1 h at room temperature followed by incubation with primary antibodies anti-GAPDH (CT), Z-Fish, Catalog No. 55339 (1:1000; AnaSpec), anti-TLR3 (CT), Z-Fish, Catalog No. 55356s (1:500; AnaSpec), anti-TICAM-1 (IN), Z-Fish, Catalog No. 55356s (1:500; AnaSpec), anti-MyD88 (CT), and Z-Fish Catalog No. 55449 (1:500; AnaSpec) diluted in 1% bovine serum albumin in TBS (TBST buffer) at 4°C overnight. After five washes with TBST buffer, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:10,000; Molecular probes) for 1 h at room temperature. Finally, after another three washes with TBST buffer, membranes were stained with the ECL system (Amersham Biosciences) and images were acquired using a ChemiDOCTM XRS instrument (Bio-Rad Laboratories). Protein bands were analyzed by Quantity One software (version 4.6; Bio-Rad Laboratories).

Electrophoretic mobility shift assay

To determine the activity of DNA binding NF- κB , electrophoretic mobility shift assays (EMSAs) were performed using a commercial kit (Gel Shift Assay System; Promega). In brief, the extraction of nuclear proteins was carried out as previously described,³⁰ and protein concentrations were determined using a bicinchoninic acid assay kit with bovine serum albumin as the standard (Pierce Biochemicals). For EMSA, the NF- κB oligonucleotide probe (5'-AGTT GAGGGGACTTTCCAGGC-3') was end labeled with

TABLE 1. ENTEROCOLITIS SCORE SYSTEM FOR HISTOLOGY EVALUATION

Numerical score	Intestinal-fold architecture disruption				Goblet cell appearance
	Impaired epithelial integrity	Expanded clefts/ reduced projections	Expanded gut lumen		
0	Normal	Normal	Normal	Normal	
1	Slight disruption	Slight disruption	Slight expansion	Increased in number	
2	Moderate disruption	Moderate disruption	Moderate expansion	Increased in number and different in sizes	
3	Severe disruption	Severe disruption	Severe expansion	Severe morphological changes	

[γ - 32 P] ATP (Free Biotech) and T4-polynucleotide kinase, and nuclear extracts (40 μ g protein) were incubated with the labeled probe for 15 min at room temperature in a volume of 20 μ L containing the binding buffer [10 mM Tris-HCl (pH 7.5), 4% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, and 1% NP-40].³¹ To ensure the specificity of NF- κ B binding to the DNA, a 100-fold excess of unlabeled oligonucleotide was added for a competition reaction. Complexes were then separated by electrophoresis on a 4% native polyacrylamide gel in 0.5 $\times$ TBE. After that, the gel was vacuum dried (80°C, 30 min) and exposed to X-

ray film (Fuji Hyperfilm) at -80°C with an intensifying screen.

Statistical analysis

Biochemical measurements were performed at least in duplicate. Quantitative histological analyses were performed by a blinded scorer. Data are presented as mean $\pm$ standard error of the mean. One-way analysis of variance followed by *post hoc* Bonferroni's multiple-comparison test was used to test the significance between groups. All statistical analyses

FIG. 1. Histological analysis of trinitrobenzene sulfonic acid (TNBS) -induced enterocolitis in zebrafish larvae reared in conventionally reared (CV) conditions or a germ-free (GF) environment and harvested at 8 days post fertilization (dpf), and ex-germ-free (XGF) zebrafish conventionalized at 8 dpf and harvested 2 days later. **(A)** Representative hematoxylin-eosin-stained sagittal sections of the intestine of zebrafish from different groups. *Arrows* indicate goblet cells, and *arrowheads* demonstrate projections and clefts. a, anus; ib, intestinal bulb; Ph, pharynx; s, somite; sb, swim bladder. **(B)** Total enterocolitis score. A total of 30 folds (10 per intestinal segment) were evaluated per intestine, and six intestines were evaluated for each experimental group from three independent experiments. The scores were quantified by a blinded scorer. All error bars are represented as means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Color images available online at www.liebertpub.com/zeb

were performed with Graph-Pad Prism version 5.0 (Graph-Pad Software), and differences were considered significant at $p < 0.05$.

Results

Microbiota are critical in TNBS-Induced enterocolitis development

TNBS treatment induces enterocolitis in CV zebrafish. To investigate the role of gut microbiota in the pathogenesis of TNBS-induced enterocolitis in larval zebrafish, zebrafish were maintained in either a CV or GF environment. For evaluation of enterocolitis changes caused by TNBS treatment, a simple scoring system was devised (Table 1). Intestinal bulb, mid-intestine, and posterior intestine were assessed separately (Fig. 1). Total enterocolitis score, representing the cumulative values of all three segments, enterocolitis score of the intestinal bulb and of mid-intestine are shown in Figure 1B. Consistent with previous reports, TNBS-treated larvae reared in CV conditions and harvested at 8 days post fertilization (dpf) developed enterocolitis. As shown in

Figure 1A, in the intestinal bulb, the epithelium of control samples was characterized by projections and clefts; whereas in TNBS-exposed samples, the lining of the gut appeared smooth and the lumen was expanded. In the mid-intestine region, higher numbers of goblet cells were observed in TNBS-treated larvae compared with controls. Histological analysis did not show any gross epithelial architecture disruption in the posterior intestine of both control and TNBS-exposed fish. In addition, goblet cells were observed in the regions of intestinal bulb and the posterior intestine of fish exposed to TNBS, while the presence of goblet cells remained restricted to the mid-intestine region in controls.

The number of goblet cells was further detected using AB-PAS staining as described earlier. As shown in Figure 2, the number of goblet cells significantly increased with TNBS treatment. Representative pictures of minimum and maximum numbers of goblet cells in all three regions of the intestinal tract are shown in Figure 2A.

GF zebrafish is more resistant to TNBS-induced enterocolitis. To generate GF larvae, our methods described

FIG. 2. Quantification of goblet cells using Alcian blue and periodic acid Schiff reagent (AB-PAS) staining. (A) Representative pictures of maximum and minimum numbers of goblet cells in the intestinal bulb, the mid-intestine, and the posterior intestine. Histochemical staining with AB-PAS demonstrates that goblet cells (blue) synthesize acidic mucins. (B) Goblet cell numbers. All error bars are represented as mean ± SEM. $n = 10$ larvae per group, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Color images available online at www.liebertpub.com/zeb

earlier were similar to other groups.^{32,33} Embryos were cleaned in a series of rinses, at similar concentrations routinely used for fish quarantine without causing developmental defects. No microorganisms were cultured from embryo medium or homogenates of GF fish on BHIBA at 28°C under aerobic or anaerobic conditions.

Zebrafish in control groups reared in GF conditions showed no obvious differences in their gut tissue compared with the controls reared in CV conditions at 8 dpf, as observed in histological sections (Fig. 1A). In the absence of a microbiota, TNBS treatment can still induce enterocolitis, but the enterocolitis score is lower than TNBS-exposed zebrafish reared under CV conditions (Fig. 1B). The total enterocolitis score for TNBS-exposed GF fish was 5.2, which was significantly higher than 2.3 and 1.3 for the control CV group and control GF group, respectively, but lower than TNBS-exposed CV fish, with a total enterocolitis score of 7.6 (Fig. 1B). These results indicate that the resident enteric commensal microbiota play a critical role in the development of TNBS-induced enterocolitis in zebrafish. The aberrant physiologic response (i.e., increase supranuclear vacuoles in intestinal epithelia in the mid-intestine), as we can see in Figure 1A in GF fish, may be caused by a direct toxic effect of TNBS on epithelial cells without involving inflammation or colitis.

To further determine the role of gut microbiota in enterocolitis development, previously, GF fish were transferred to a microbial environment; ex-germ-free (XGF) zebrafish were conventionalized at 8 dpf, harvested 2 days later, and examined. With microbial colonization, these XGF zebrafish with TNBS exposure showed an increase in the enterocolitis score of 6.6, compared with a score of 5.2 in the TNBS-exposed GF fish.

Microbiota influence inflammatory cytokine production

TNF- α expression was assessed using immunofluorescence to measure inflammatory reactions in CV, GF, and XGF zebrafish exposed to TNBS. In our study, TNF- α appeared as red fluorescent light in plasma around the nucleus within the intestinal epithelium (Fig. 3A–F), and the intensity of TNF- α immunofluorescence was quantified for each treatment group, with 6–12 samples per group (Fig. 3G). In the control CV samples, TNF- α staining was absent from the gut (Fig. 3A), whereas TNF- α expression was stimulated significantly with treatment of TNBS (Fig. 3B). However, in GF fish, there was no expression of TNF- α with or without TNBS exposure (Fig. 3C, D). Furthermore, in XGF larvae exposed to TNBS, TNF- α immunofluorescence level increased with microbial colonization (Fig. 3F); meanwhile, there was still no expression in controls (Fig. 3E). These results demonstrate that TNBS exposure primarily evoked an inflammatory response within the intestine depending on the existence of intestinal microbiota.

Microbiota contribute to TNBS-induced activation of TLR signaling pathways

Western blotting was performed to investigate the expression pattern of several components involved in the TLR signaling pathway, including TLR3 and the adapter protein MyD88 and TRIF. As shown in Figure 4, in CV conditions, TLR3 was present only in a small amount in the control group. However, in zebrafish with TNBS-induced enterocolitis,

FIG. 3. Immunofluorescence analysis of tumor necrosis factor (TNF)- α expression in the gut. (A–F) Representative cross-sections of the mid-intestine from different groups. (G) Densitometric analysis of multiple similar experiments. TNF- α staining (red) and DAPI staining (blue) images were visualized by confocal laser scanning microscopy. Cross-sections of the mid-intestine from at least six fish were examined for each condition. Images were shown at 200 $\times$ magnification. Error bars are represented as mean $\pm$ SEM, $n = 13$ –16 sections per group. P values were calculated using a paired t -text, *** $p < 0.001$. Color images available online at www.liebertpub.com/zeb

TLR3 protein level was significantly increased. Interestingly, there was no detectable expression in GF fish with or without TNBS treatment. Moreover, in XGF zebrafish once colonized with resident microbes, TLR3 expression reverted to a pattern similar to that observed in CV fish. TNBS treatment also stimulated proteins of MyD88 and TRIF expression. Although MyD88 and TRIF expression was detected in GF conditions, it showed no obvious stimulation in TNBS-treated groups compared with controls (Fig. 4).

The transcription factor NF- κ B is an important regulator of downstream inflammatory cytokines leading to human IBD

FIG. 4. TNBS exposure increased the expression of TLR3, TRIF, and MyD88 in CV conditions and XGF zebrafish. No detectable expression of TLR3 and no obvious stimulation of MyD88 or TRIF in GF fish. (A) Western blotting with anti-TLR3, anti-TRIF, or anti-MyD88 antibodies. (B) Densitometric analysis of multiple similar experiments. Data are means $\pm$ SEM; $n = 4$ or 5. P values were calculated using a paired t -test, ** $p < 0.01$, and *** $p < 0.001$.

FIG. 5. TNBS treatment induced a significant increase in NF-κB DNA-binding activity in CV and XGF zebrafish, which was strongly decreased and showed no significant difference in GF conditions. The experiment was repeated three times, and the results were consistent.

and is activated by bacterial products by TLR signaling.^{34,35} To analyze the activation of NF-κB, we performed EMSA experiments. As shown in Figure 5, in CV conditions, TNBS treatment induced a significant increase in NF-κB DNA binding activity. By contrast, in GF samples, complex binding to the NF-κB site was strongly decreased and showed no significant difference between the control and TNBS-exposed fish. When XGF zebrafish colonized with microbes, augmentation of NF-κB activity in response to TNBS exposure was again observed.

These results suggest that the presence of commensals was critical in the modulation of mucosal TLRs responsiveness and, subsequently, regulation of inflammatory responses.

Discussion

Host-microbial interactions in the GI tract are complex and incompletely understood, yet previous studies have suggested that they play an essential role in maintaining intestinal homeostasis and inducing intestinal disorders, such as IBD, UC, and CD.^{36,37}

Our studies reveal the critical role of gut microbiota in the pathogenesis of a TNBS-induced larval zebrafish IBD-like model. The present data show that enterocolitis was dependent

on microbiota and TLR signaling, and GF zebrafish are more resistant to TNBS-induced intestinal injury. This is consistent with a previous report that TNBS-induced enterocolitis in zebrafish can be ameliorated by antibiotic treatment.²⁵ Similar data were seen in IL-10-deficient (IL-10^{-/-}) mice, showing that IL-10^{-/-} mice fail to develop spontaneous colitis if reared in GF conditions, strongly suggesting that the microbiota may trigger intestinal inflammation in a susceptible host.⁸ However, DSS-induced colitis in murine models revealed that oral DSS-induced mucosal injury is more extensive in animals with commensal bacterial depletion compared with their conventionalized counterparts.^{38,39} The difference may be caused by a variety of pathogenic mechanisms; however, it further emphasizes the critical role of gut microbiota in the balance between immunosuppression and inflammation in the GI tract.

Inflammatory mediators have long been appreciated for their role in host defense and wound healing.⁴⁰ The overexpression of TLRs in IBD patients suggests that bacteria may stimulate proinflammatory signals in the pathogenesis of enterocolitis. The TLRs are present in intestinal epithelial cells and intestinal lamina propria macrophages.^{12,41,42} Bacteria are recognized TLRs, leading to activation of NF- κ B and inflammatory cytokine production.^{43,44} Our data are consistent with this notion. In our study, TNBS exposure induced TLR3 and TLR signaling pathway molecules' MyD88 and TRIF expression in CV zebrafish. However, there was no detectable expression of TLR3 in both control and TNBS-exposed fish reared in GF conditions, and no stimulation in MyD88 or TRIF. Also consistent with this theory is NF- κ B activation and TNF- α expression. Unsurprisingly, zebrafish with commensal bacterial depletion showing inhibition of TLR signaling pathway showed lower enterocolitis scores. Similar results were reported in previous research. Treatment with the TLR4 antagonist inhibited the development of colitis in MDR 1a-deficient mice, and TLR signal abrogation by MyD88 knockout prevented development of spontaneous colitis in IL-10^{-/-} mice.⁴⁵ Our results showed that the TLR signaling pathway plays a major role in enterocolitis pathogenesis. TLRs may either enhance or suppress intestinal inflammatory and immune response depending on membrane localization, expression pattern, parallel signaling by additional TLRs, cytokine combinations, and interactions with specific intestinal microbiota. This study helps understanding the relationship between microbiota and host immunity and its degradation in inflammatory disease of the intestine, showing the involvement of the TLR signaling pathway, through which the host recognizes gut microbiota and stimulates inflammatory processes.

In our study, zebrafish were raised to 10 dpf without feeding; therefore, the fish might be suffering from starvation, might induce fatty liver, and have other metabolic changes. In addition, immersion of zebrafish in TNBS would be expected to induce injury in regions other than the intestine, such as skin and gill. This may explain the aberrant physiologic response in the intestine, such as an increase in supranuclear vacuoles in intestinal epithelia in the mid-intestine. In order to distinguish goblet cells from fat cells or supranuclear vacuoles, we conducted AB-PAS staining to further examine the goblet cells. The increase in goblet cells observed in TNBS-exposed larvae, along with the up-regulation of TNF- α expression, is more consistent with enterocolitis. Changes in goblet cell number are contrary to a

previous characterization by Oehlers *et al.*, but similar to Fleming's report. Obviously, there were significant differences between the experimental sets. This may be caused by many factors, including genetics, variations in environmental conditions from different geographic locations, the microbiological status of food and water, and experimental methods.

In summary, we represented the critical role of the intestinal microbiota in a chemically induced larval zebrafish IBD-like model. The present study defined a less extensive enterocolitis induced by TNBS treatment in GF zebrafish than CV groups. Furthermore, the present data would also suggest that gut microbiota influence TNBS-induced enterocolitis which may be mediated by TLR signaling pathways, including MyD88-dependent and MyD88-independent pathways. This may possibly provide new clues into determining the etiological mechanisms of IBD and alter these events to prevent or ameliorate the disease.

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Disclosure Statement

No competing financial interests exist.

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