Anti-TNF-α therapy alters the gut microbiota in proteoglycan-induced ankylosing spondylitis in mice

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Abstract
Ankylosing spondylitis is a chronic, progressive disease, and its treatment is relevant to the gut microbiota. Anti-tumor necrosis factor-alpha (anti-TNF-α) therapy alters the gut microbiota in many diseases, including inflammatory bowel disease. However, little is known about the effect of TNF-α blocker treatment on the gut microbiota in ankylosing spondylitis. Herein, the effect of a TNF-α blocker on the gut microbiota in proteoglycan-induced arthritis was investigated. Proteoglycan-induced mice were treated with an rhTNFR:Fc solution of etanercept (5 µg/g) for 4 weeks. rhTNFR:Fc treatment attenuated the arthritis incidence and severity of arthritis in the proteoglycan-induced mice and decreased inflammation in the ankle joints and ameliorated ileal tissue destruction. Moreover, high gut permeability occurred, and zonula occludens-1 and occludin protein levels were reduced in proteoglycan-induced mice. These levels were significantly restored by the administration of rhTNFR:Fc. The serum TNF-α and IL-17 levels were also decreased. In addition, flora analysis via 16S rDNA high-throughput sequencing revealed that rhTNFR:Fc treatment restored the gut microbiota composition to a composition similar to that in control mice. In conclusion, anti-TNF-α therapy attenuated proteoglycan-induced arthritis progression and modulated the gut microbiota and intestinal barrier function. These results provide new insights for anti-TNF-α therapy strategies via regulating the gut microbiota in ankylosing spondylitis.

Keywords
16S rDNA high-throughput sequencing, ankylosing spondylitis, anti-TNF-alpha therapy, gut microbiota, proteoglycan-induced mice
1 | INTRODUCTION

Ankylosing spondylitis (AS) is a chronic autoimmune disease that mainly affects the axial spine and sacroiliac joints. Osteophyte formation and ankyloses often develop later in this disease, which may lead to a typical spine imaging manifestation known as “bamboo spine” (Fattahi et al., 2018). Human leukocyte antigen (HLA)-B27 presents in over 90% of patients with AS (Reveille et al., 2018). Recent studies have shown that the gut microbiota may be a vital factor relevant to AS (Ranganathan, Gracey, Brown, Inman, & Haroon, 2017). Investigations into the fecal microbiota of AS patients support the association between AS and the gut microbiota (Ranganathan et al., 2017). Notably, subclinical gut inflammation was observed in more than 60% of patients with AS (VanPraet, Jacques, Van den Bosch, & Elewaut, 2012), indicating that intestinal disorders are related to AS pathogenesis.

Ankylosing spondylitis treatment mainly targets the remission of disease progression. Tumor necrosis factor-alpha (TNF-α) blockers have been shown to improve inflammatory conditions in the spine and sacroiliac joints (Taurog, Chhabra, & Colbert, 2017). The mechanism underlying the therapeutic effects of TNF-α blockers on AS remains uncertain. Further studies have proposed the gut microbiota as an important factor that may influence anti-TNF-α treatment outcomes. A study involving 18 patients with AS found that the host’s gut microbial community can predict the clinical response related to anti-TNF-α therapy and that the order Burkholderiales is considered a biomarker determining the response to anti-TNF-α treatment (Bazin et al., 2018). In addition, imbalanced gut microbial communities in patients with inflammatory bowel disease (IBD) showed normal microorganism colonization after anti-TNF-α therapy (Busquets et al., 2015; Magnusson et al., 2016). Moreover, age-related alterations in the gut microbiota were reversed in TNF−/− mice. These mice exhibited decreased intestinal permeability and reduced systemic inflammation compared with those in mice of the same age (Thevarajan et al., 2017).

The gastrointestinal tract of mammalian hosts is colonized by many commensal microbes, herein referred to as the gut microbiota (Clavel, Gomes-Neto, Lagkov vardos, & Ramer-Tait, 2017), and disorder of the gut microbiota is regarded as important to the pathogenesis of many autoimmune diseases, including IBD, allergies, diabetes, and axial spondyloarthritis (Jiménez et al., 2017; Leone, Cham, & Chang, 2014; Tilg & Moschen, 2014; Wen et al., 2017). Although gut microbiota alterations in anti-TNF-α-treated AS have been reported (Bazin et al., 2018), the interactions between anti-TNF-α therapy and the gut microbiota remain unknown. In this study, we hypothesized that the treatment effect of TNF-α blockers on AS benefited mucosal barrier protection and regulated an imbalanced intestinal microbiota and that IL-17A mediated by Th17 cells in the host’s intestines was implicated in the treatment responses. Hence, in this study, we constructed an animal model of ankylosing spondylitis by injecting proteoglycan and dimethylglycine (DDA) into BALB/c mice (Gnant, Mikecz, Arzoumanian, & Poole, 1987; Tseng et al., 2017). The composition and function of the fecal microbiota were analyzed via 16S rDNA high-throughput sequencing.

2 | MATERIALS AND METHODS

2.1 | Mice and construction of the proteoglycan (PG)-induced AS model

Thirty female BALB/c retired breeder mice (8 months old) weighing 26 ± 2.4 g were purchased from Beijing HFK Bioscience Co., Ltd. Mice were housed in the Central Animal Laboratory of Southern Medical University under standard conditions with uninterrupted access to food and water. All mice were adapted for 2 weeks before the experiments commenced.

As previously described (Boldizsar, Tarjanyi, Nemeth, Mikecz, & Gant, 2009; Ishikawa, Colavite, Rosa, et al., 2014), 100 μg of cartilage proteoglycan emulsifier (Sigma) plus 2 mg of DDA adjuvant (Sigma) were mixed in 200 μl of normal saline and injected into the abdominal cavities of the mice on days 0, 21, and 42.

2.2 | Treatment protocols and arthritis assessment

The thirty mice were randomly allocated to three groups (n = 10 per group): the control, PG, and rhTNFR:Fc groups. The control group contained 10 healthy mice. The mice in the PG group were intra-peritoneally injected with normal saline, and the PG-induced mice in the rhTNFR:Fc group were treated with an rhTNFR:Fc solution of etanercept (5 μg/g) diluted in normal saline via intraperitoneal injection three times per week. This dosage was based on previous publications showing that rhTNFR:Fc efficiently inhibited murine TNF-α (Fei et al., 2011; Fries et al., 2008). rhTNFR:Fc treatment was initiated 4 weeks after the third PG induction to modify the clinical effect of the TNF-α inhibitor on AS.

The arthritis score and body weight were evaluated weekly from the third immunization until euthanasia (week 14) and blindly assessed by two investigators. Arthritis severity was assessed using a standard visual scoring system (Ishikawa, Colavite, da Rosa, et al., 2014).

The mice were euthanized with avertin at 14 weeks; the ankle joints were then removed, and hematoxylin and eosin (HE) staining was performed as previously described (Gnant, 1982).

3 | ELISA

The serum TNF-α and IL-17A concentrations were determined using commercial ELISA kits (ABclonal Technology) in accordance with the manufacturer’s instructions. Each cytokine was measured three times independently, and the final concentration was calculated using a standard curve.

3.1 | Pathological and immunohistochemical assessment of ileal tissue

Ileal tissues were obtained immediately after the mice were sacrificed. After irrigation with normal saline, ileal tissues were fixed
in 4% phosphate-buffered paraformaldehyde. The operation was conducted later as previously described (Xue et al., 2018). In addition, immunohistochemistry was conducted in accordance with standard procedures (Ruan et al., 2014). The primary antibodies used were rabbit anti-ZO-1 (diluted 1:150; Thermo Fisher Scientific) and anti-occludin (diluted 1:200; Thermo Fisher Scientific). Samples were incubated with primary antibody overnight at 4°C, warmed at 37°C for 45 min, and then incubated with secondary antibody. The final products were imaged and analyzed using a Zeiss microscope (Carl Zeiss).

### 3.2 Gut permeability in vivo

Mice were fasted with water supplementation for 6 hr and were then administered fluorescein isothiocyanate-conjugated dextran (4 kDa; DX-4000-FITC, Sigma-Aldrich) by gavage. The reagent dose was calculated as 60 mg per 100 g of body weight (Cani et al., 2008). Venous blood (300 µl) was obtained from behind the eye sockets after 1 hr and centrifuged at 12,000 g for 3 min. The supernatant was diluted with an equal volume of phosphate-buffered saline (PBS). Fluorescence measurements were subsequently conducted with excitation at 485 nm and emission at 535 nm.

### 3.3 Western blot analysis

Ileal tissues were obtained and stored in liquid nitrogen. Samples were incubated in radioimmunoprecipitation assay (RIPA) buffer (Beyotime) for the extraction of total protein. Protein sample concentrations were measured by the bicinchoninic acid (BCA) method (Beyotime). The prepared proteins were separated by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were incubated with the primary antibodies (1:500; Thermo Fisher Scientific), anti-occludin (1:500; Thermo Fisher Scientific) and anti-β-actin (1:5,000; Bioss) at 4°C overnight and were then incubated with secondary antibody (goat anti-rabbit) for 1 hr. An electrochemiluminescence (ECL) Western blotting kit (Beyotime) was used to detect the target bands on the membranes. The target bands were visualized, and the protein intensity was analyzed using the Gene Gnome Bio Imaging System (Syngene Bio-imaging).

### 3.4 16S rDNA high-throughput sequencing analysis

The cecal contents were collected from the mice in sterile, tightly closed containers, and immediately preserved at −80°C. DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen Ltd.) in accordance with the manufacturer’s protocol. The fecal microbiota in 15 fecal samples was determined via 16S rDNA gene sequencing at the Realbio Genomics Institute (Shanghai, China). The F341F forward primer (5’-ACTCCTACGGGRSGGCAGCAG-3’) and the R806R reverse primer (5’-GGACTACVGGGTATCTAATC-3’) were used to amplify the V3-V4 hypervariable regions of the bacterial 16S rDNA. Files were clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold for the reads. The data from all samples were then normalized for analysis of alpha and beta diversity. Briefly, the number of sequences in the sample with the fewest sequences (21,246 reads/sample) was selected to set the threshold value, and the same number of sequences from each of the samples was randomly extracted for subsequent analysis (Huang et al., 2018). Principal coordinates analysis (PCoA) was conducted on UniFrac distance matrices using Quantitative Insights Into Microbial Ecology (QIIME) software based on community differences, and the distance distribution was determined by heatmap analysis. The relative species abundances were calculated using the annotation results in R software. Metagenome function was predicted by PICRUSt (Qi et al., 2018).

### 3.5 Statistical analysis

SPSS 22.0 software was used for statistical analyses. All statistical analyses were conducted at least three times. Statistical differences among the three groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s test. The Kruskal–Wallis rank sum test was used to analyze the relative species proportions. Statistical significance was defined as p < .05.

## 4 RESULTS

### 4.1 RhTNFR:Fc attenuated body weight loss and severity in PG-induced mice

Figure 1a shows the experimental program schedules. Figure 1b,c shows the increased arthritis incidence and scores in PG-induced mice. Arthritis incidence was delayed after rhTNFR:Fc inoculation, and the arthritis scores in the rhTNFR:Fc group were significantly decreased between 12 and 14 weeks. Figure 1d shows the dynamic variations in body weight. Histological features of the hind limbs of PG-induced mice showed inflammatory cell infiltration, synovial hyperplasia, cartilage destruction, and bone erosion, which were not present in control mice. rhTNFR:Fc-treated mice exhibited mild inflammatory cell infiltration and joint space narrowing. However, synovial hyperplasia, cartilage destruction, and bone erosion were not observed in rhTNFR:Fc mice (Figure 1e).

### 4.2 RhTNFR:Fc improved the mucosal morphology of the ileal tissue and decreased gut permeability in PG-induced mice

In control group mice, the intestinal villi were smooth and showed normal glands, while the intestinal villi of the PG-induced mice were disorganized, structurally disrupted, and often separated...
from the basement membrane. After rhTNFR:Fc treatment, the intestinal damage showed mild villus shortening (Figure 2a). Gut permeability was significantly increased in the PG group compared with that in the control group. After rhTNFR:Fc supplementation, gut permeability in the mice markedly declined (Figure 2b).
4.3 | RhTNFR:Fc increased the expression of tight junction (TJ) proteins in PG-induced mice

Figure 2c and 2 shows the ZO-1 and occludin protein expression levels, which were lower in PG mice than in control group mice. However, the ZO-1 and occludin expression levels were significantly increased after rhTNFR:Fc treatment. These results were consistent with the immunohistochemistry results, in which PG-induced mice showed lighter brown staining than did control mice, and the intensity of brown staining in
rhTNFR:Fc mice was stronger than that in PG-induced mice (Figure 2g).

4.4 | RhTNFR:Fc decreased the serum TNF-α and IL-17A levels

The TNF-α and IL-17A levels in PG-induced mice were significantly increased compared with those in control mice, and rhTNFR:Fc treatment distinctly decreased the PG-induced upregulation of serum TNF-α. In addition, the decline in IL-17A expression was more subtle than that in TNF-α expression (Figure 2e,f).

4.5 | RhTNFR:Fc modulated the composition of the microbiota in PG-induced mice

Four hundred forty-seven OTUs were obtained. Venn diagrams indicated 17, 9, and 14 unique OTUs in the control, PG, and treatment groups, respectively (Figure 3a). The α-diversity of the gut microbial communities in the three groups was evaluated, and the richness estimators, including the Chao1 index (Figure 3b, p = .33) and observed species (Figure 3c, p = .05), were not significantly different among the groups. However, the PD diversity estimator was significantly lower in the PG group than in the control and rhTNFR:Fc groups (Figure 3d, p = .018).

Analysis of similarities (ANOSIM; Figure 3e,f) indicated that the gut microbial communities differed among the three groups (p < .05). Principal coordinates analysis (PCoA) was conducted based on compositional similarities of the gut microbial communities. Figure 3g,H shows that the PG samples were separated from the control samples, whereas the rhTNFR:Fc group was a short distance from the control group (the control and rhTNFR:Fc groups did not statistically differ) and was distant from the PG group (p < .05).

The gut microbiota profiles were dominated by Bacteroidetes and Firmicutes at the phylum level (Figure 3i). PG-induced mice

![Figure 3](https://example.com/figure3.png)
showed an altered gut flora with a decreased abundance of Bacteroidetes (38.4%) and an increased abundance of Firmicutes (54.8%) and Proteobacteria (5%) compared with the abundances of Bacteroidetes (61.5%), Firmicutes (34.2%), and Proteobacteria (2.6%) in control mice. However, the relative abundances of Bacteroidetes (62%), Firmicutes (34.6%), and Proteobacteria (2.3%) in the gut flora of mice in the rhTNFR:Fc treatment group did not significantly differ, and the ratio of Bacteroidetes to Firmicutes was similar to that of the control group.

Figure 3j shows the changes in relative abundance at the genus level. PG-induced mice exhibited notably increased relative abundances of Alistipes, Odoribacter, and Clostridium XIVa and markedly decreased relative abundances of Alloprevotella, Barnesiella, and Bacteroides. Helicobacter, Flavonifractor, and Akkermansia showed little variation after PG induction. At the genus level, the relative abundances of Alloprevotella, Barnesiella, Odoribacter, and Clostridium XIVa were significantly restored by rhTNFR:Fc treatment, and the relative abundance of Lactobacillus was prominently increased compared with that in the PG and control groups.

Figure 4a shows the hierarchical clustering heatmap analysis of Bacteroidetes at the phylum and order levels. The abundance of the class Bacteroidia was significantly decreased in PG mice compared with that in control and rhTNFR:Fc mice. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways predicted for the intestinal flora differed significantly among the three groups (Figure 4b). The enrichment of the KEGG pathways involving “cell motility,” “membrane transport,” and “signal transduction” was significantly increased in the PG group. After rhTNFR:Fc treatment, the genes were highly enriched in “nucleotide metabolism,” “signaling molecules and interaction,” and “digestive system.”

Gut bacteria interact with each other rather than act in isolation. Next, we performed a co-abundance network analysis using Cytoscape software to study how different intestinal microbes interact with each other. As shown in Figure 4c, the rhTNFR:Fc group contained high abundances of Alloprevotella, Barnesiella, and Lactobacillus and showed a higher number of positive connections than the PG and control groups. Furthermore, positive connections generally occurred within groups, while negative connections occurred among different groups.

5 | DISCUSSION

Ankylosing spondylitis is a chronic inflammatory disease that is thought to be relevant to the gut microbiota (Ranganathan et al., 2017). Recent research has revealed that anti-TNF-α therapy improves the gut microbial community, which is relevant to its therapeutic effect (Bárdos et al., 2005; Thevaranjan et al., 2017). However, the underlying mechanism is unclear. In our study, we established a model of PG-induced AS (Glant et al., 1987). rhTNFR:Fc treatment effectively suppressed arthritis onset, increased the clinical scores,
and decreased body weight loss. 16S DNA sequencing revealed that rhTNFR:Fc treatment reversed the gut microbial changes due to PG induction, indicating that the therapeutic effect of anti-TNF-α on AS may be associated with regulating the gut microbiota and improving gut barrier function.

IL-17 is produced by the Th17 subset of helper T cells and is considered to play key genetic and therapeutic roles and to exhibit elevated serum concentrations in AS (Gracey et al., 2016; Smith & Colbert, 2014). In our study, the concentration of IL-17A was significantly decreased after rhTNFR:Fc treatment, indicating that the effect of anti-TNF-α therapy may be related to the inhibition of serum IL-17A.

The gut microbiota may be responsible for gut barrier function, and high gut permeability reduces the gap between the external environment and the circulatory system. Some microbial antigens are structurally similar to self-antigens, and immune cells activated by microbial antigens also react with self-antigens, consequently leading to autoimmune attacks. This effect is called “molecular mimicry” and is proposed as the pathogenic mechanism of AS (Cusick, Libbey, & Fujinami, 2012). An investigation of patients with AS determined the general existence of high gut permeability in AS (Martínez-González et al.,). In addition, the gut permeability of older mice with TNF knockdown was similar to that of either young or old germ-free mice, indicating that TNF can regulate gut barrier function (Thevaranjan et al., 2017). Noth R et al. found that the increased intestinal permeability in patients with Crohn’s disease was significantly decreased after 7 days of infliximab treatment (Noth et al., 2012). Consistent with these results, PG-induced mice had high gut permeabilities compared with those of healthy control mice; however, this trend was reversed by rhTNFR:Fc treatment. Moreover, the levels of the TJ proteins ZO-1 and occludin were decreased in PG-induced mice, but rhTNFR:Fc restored the barrier function by upregulating ZO-1 and occludin protein expression. Although inhibiting TNF-α contributed to the improved gut permeability, whether this effect was caused by rhTNFR:Fc directly or by indirect regulation of the gut microbiota is unclear.

Decreased gut microbiota diversity occurs in many diseases, including IBD and AS (Costello et al., 2015; Leone et al., 2014). A decreased Bacteroidetes/Firmicutes abundance ratio indicates an imbalanced gut microbiota (Murphy et al., 2010). rhTNFR:Fc treatment significantly increased the Bacteroidetes/Firmicutes ratio to 1.79, which was approximately equal to that in healthy mice. These results are consistent with those from SpA patients (Breban et al., 2017). In addition, an increase in the relative proteobacterial abundance in healthy individuals is relevant to the emergence of diseases, including metabolic disorders and inflammatory diseases (Shin, Whon, & Bae, 2015). In this study, the relative proteobacterial abundances decreased after rhTNFR:Fc treatment, suggesting that rhTNFR:Fc exerted a protective effect on the recovery of the gut microbiota composition.

Next, we predicted the KEGG pathways with PICRUSt. A previous gut microbial study on AS revealed that the genes belonging to “membrane transport” and “cell motility” were notably enriched (Wen et al., 2017). The present study yielded similar results, indicating that dysbiosis in PG-induced mice leads to functional changes in the gut microbiota that are possibly related to upregulation of membrane transport and cell motility genes. However, rhTNFR:Fc treatment altered this state by enriching genes related to nucleotide metabolism, signaling molecules, and interactions. Curtis et al. (2015) reported that nucleotide metabolism, especially adenosine signaling, shows an important metabolic signature of promoting inflammation resolution (Wen et al., 2017). In addition, Tang et al. revealed that inflamed tissues from patients with ulcerative colitis exhibited mucosal communities that lacked the genes related to nucleotide and carbohydrate metabolism (Tang et al., 2015). The anti-inflammatory effect of rhTNFR:Fc on mice might be related to nucleotide metabolism, which is regulated by the gut microbiota. However, the effects of rhTNFR:Fc on the composition and function of the gut microbiota remain to be clarified.

6 | CONCLUSION
In conclusion, rhTNFR:Fc treatment modulated the gut microbiota, decreased the levels of inflammatory cytokines, and recovered intestinal barrier function in PG-induced mice. This study provides new insights for anti-TNF-α therapeutic strategies via regulating the gut microbiota in mice with ankylosing spondylitis.

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CONFLICTS OF INTERESTS
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
Hai L and Bin L: conceptualized the study. Lianjun Y and Zhifei C: involved in data curation. Bin L and Zhifei C: performed formal analysis. Hai L and Bin L: involved funding acquisition. Bin L, Lianjun Y, Zhihai S, Min W, Weicong Zh, Jinshi L, and Tingxuan W: investigated the manuscript. Jincheng H and Qinghao Zh: involved the methodology. Hai L and Qingchu L: involved in project administration. Junchi Zh and Jincheng H: Software. Hai L: supervised the study. Zhifei C and Junchi Zh: validated the manuscript. Bin L: wrote the manuscript and prepared the original draft. Lianjun Y: wrote, reviewed, and edited the manuscript.

ETHICS STATEMENT
All animal experiments were approved by the ethics committee of Southern Medical University and were conducted in accordance with the institutional guidelines.
DATA AVAILABILITY STATEMENT
The raw data were uploaded to the National Center for Biotechnology Information SRA database under the accession number SRP216934

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