


ARTICLE



Gut microbiota involved in leptospiral infections

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Leptospirosis is a re-emerging zoonotic disease worldwide. Intestinal bleeding is a common but neglected symptom in severe leptospirosis. The regulatory mechanism of the gut microbiota on leptospirosis is still unclear. In this study, we found that *Leptospira interrogans* infection changed the composition of the gut microbiota in mice. Weight loss and an increased leptospiral load in organs were observed in the gut microbiota-depleted mice compared with those in the control mice. Moreover, fecal microbiota transplantation (FMT) to the microbiota-depleted mice reversed these effects. The phagocytosis response and inflammatory response in bone marrow-derived macrophages and thioglycolate-induced peritoneal macrophages were diminished in the microbiota-depleted mice after infection. However, the phagocytosis response and inflammatory response in resident peritoneal macrophage were not affected in the microbiota-depleted mice after infection. The diminished macrophage disappearance reaction (bacterial entry into the peritoneum acutely induced macrophage adherence to form local clots and out of the fluid phase) led to an increased leptospiral load in the peritoneal cavity in the microbiota-depleted mice. In addition, the impaired capacity of macrophages to clear leptospires increased leptospiral dissemination in *Leptospira*-infected microbiota-depleted mice. Our study identified the microbiota as an endogenous defense against *L. interrogans* infection. Modulating the structure and function of the gut microbiota may provide new individualized preventative strategies for the control of leptospirosis and related spirochetal infections.

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INTRODUCTION

Leptospirosis is a worldwide zoonotic disease caused by *Leptospira* spp. [1]. Because of global warming, this disease is re-emerging and affects one million people per year, causing ~58,900 deaths [2]. Humans and animals can be infected by pathogenic *L. interrogans* through contacting mucous membranes or abraded skin with *Leptospira*-contaminated water or moist soil [3, 4]. The clinical manifestations of human leptospirosis range from mild illness to multiorgan failure, which are characterized by jaundice, kidney and liver failure, and pulmonary hemorrhage [3]. The causes of differences in sensitivity to the infection between individuals are still unknown. Because of the ill-defined pathogenesis of leptospirosis, antibiotic therapy is still the preferred treatment. However, due to the difficulty of early diagnosis of leptospirosis, some *L. interrogans*-infected patients often develop multiorgan dysfunction [5]. The Jarisch–Herxheimer reaction is also a risk factor that should be considered in antibiotic therapy [6]. Thus, it is imperative to exploit precision medicine strategies for developing leptospirosis treatment.

Although there is no literature on *L. interrogans* as an intestinal pathogen, intestinal bleeding is a common but neglected symptom in severe leptospirosis [7, 8]. The intestine harbors a densely populated resident microbial community, which consists of bacteria, viruses, and fungi, called microbiota [9]. This system is

important for many physiological processes, including development of the immune system, improvement of the intestinal epithelial barrier and nutrient acquisition [9]. In addition to defending against enteropathogenic infection, the gut microbiota also plays a positive role in nonenteropathogenic infections [10–12]. However, the interaction between *L. interrogans* and the gut microbiota remains elusive.

The intraperitoneal challenge route is the most common route of leptospiral infection in experimental models [13]. Resident macrophages are nonadherent in peritoneal fluid during homeostasis. However, bacterial entry into the peritoneum has been shown to induce macrophage adherence to form local clots that effectively bring macrophages and bacteria in proximity and out of the fluid phase, which helps control infection. This response is called the “macrophage disappearance reaction” [14]. In this study, we show that *L. interrogans* infection changes the composition of the gut microbiota in mice. Microbiota dysbiosis increases leptospiral colonization and weight loss. We show that the gut microbiota influence different compartment-derived macrophages during infection. In addition, our study demonstrates that the gut microbiota influence the macrophage disappearance reaction that helps to control leptospiral burden. Our work highlights the essential role of the gut microbiota in leptospirosis and offers a new strategy for the control of leptospirosis and related spirochetal infections.

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MATERIALS AND METHODS

Ethics statement

A previous study reported that sex influenced host susceptibility to leptospirosis [15] and that sex may affect the composition of microbiota [16]. To exclude the influence of sex on the results, all experiments were conducted in male C57BL/6J mice. Specific pathogen-free male C57BL/6J mice were maintained on standard rodent chow with water supplied ad libitum and with a 12-h light/12-h dark cycle during the experimental period. All animal experiments were conducted according to the regulations of the Administration of Affairs Concerning Experimental Animals in China. The protocol was approved by the Institutional Animal Care and Use Committee of Jilin University (20170318).

Bacterial strains and animals

Pathogenic *L. interrogans* serovar Lai strain Lai (56601) was cultured in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium at 29 °C. *L. interrogans* were counted in a Petroff-Hauser chamber and diluted in phosphate buffer solution (PBS) before use. Male C57BL/6J mice were provided by Liaoning Changsheng Biotechnology Co. Ltd.

Experimental infections

Eight-week-old male C57BL/6J mice were injected intraperitoneally with 10^8 leptospires. Fecal pellets were collected aseptically at 0, 2, and 7 days post infection (p.i.). Mice were euthanized at 0, 2, 4, and 7 days p.i.. The small intestine and colon were collected aseptically. The intestinal contents were washed out from the intestine with PBS, and the tissues were stored at -80°C .

To explore the role of the gut microbiota in male C57BL/6J mice during *L. interrogans* infection, we depleted the gut microbiota with broad-spectrum antibiotics (Abx) as previously described [10]. Eight-week-old male C57BL/6J mice were treated with Abx (1 g/L ampicillin, 1 g/L metronidazole, 1 g/L neomycin sulfate (purchased from Sigma), and 0.5 g/L vancomycin (BiochemPartner, China)) in drinking water for 3 weeks. The antibiotic-treated water was then stopped 2 days prior to infecting animals intraperitoneally with 10^8 leptospires [10, 17]. Mice were monitored daily for variations in body weight and clinical signs of leptospirosis. Mice were euthanized at 2, 4, and 7 days p.i.. The kidneys, livers, and lungs were collected aseptically. For the fecal microbiota transplantation (FMT) experiment, eight-week-old male C57BL/6J mice were treated with Abx in drinking water for 3 weeks. Fresh fecal pellets were collected from the untreated mice and then resuspended in sterile normal PBS (1 fecal pellet/1 mL of PBS). The pellets were immediately homogenized. The homogenate was centrifuged (100 rpm, 5 min, 4 °C), and 200 μL of the supernatant was given to the Abx-treated mice by oral gavage for 4 consecutive days after antibiotic treatment was stopped. The control mice were given an equal volume of PBS [10]. The mice were intraperitoneally infected with 10^8 leptospires and euthanized at 4 days p.i.. The kidneys, livers, and lungs were removed aseptically and stored at -80°C .

To explore the role of the gut microbiota on the macrophage disappearance reaction after infection, eight-week-old male C57BL/6J mice were treated with antibiotics as described above. After a 2-day washout period, the control mice and the Abx-treated mice were intraperitoneally injected with PBS or 150 U heparin/mouse (an inhibitor of macrophage disappearance reaction) immediately after infection with 10^8 leptospires [14].

Peritoneal lavage

For peritoneal macrophage proportion and number detection, male C57BL/6J mice were euthanized at 2, 6, and 24 h p.i.. Peritoneal cavities were lavaged twice with 5 mL of sterile PBS, and 4 mL of fluid was recovered. The peritoneal lavage was centrifuged at 2000 rpm for 10 min, and the pellets were analyzed by flow cytometry.

To detect the leptospiral load in the peritoneal cavity, male C57BL/6J mice were euthanized at 2, 6, and 24 h p.i.. The peritoneal cavities were lavaged with 5 mL of sterile PBS, and 2 mL of fluid was recovered. The fluid was centrifuged at 12,000 rpm for 5 min. Then, the DNA of the pellets was extracted, and the leptospiral burden was detected by qPCR.

For the peritoneal cytokine detection, male C57BL/6J mice were euthanized at 24 h p.i.. The peritoneal cavities were lavaged with 5 mL of sterile PBS, and 2 mL of fluid was recovered. The peritoneal lavage was centrifuged at 2000 rpm for 10 min, and the supernatants were examined for cytokine production. For analysis of ex vivo cytokine production, a total

of 1×10^6 cells were cultured in 1 mL of RPMI supplemented with 10% FCS for 24 h, and the supernatants were collected for cytokine examination [18].

In vivo barrier permeability

The permeability of the intestine was determined as previously described [19, 20]. Mice were starved for 6 h before gavage with FITC-dextran (4 kDa, 600 mg/kg body weight/mouse). After 2 h, blood was collected and centrifuged at 3000 rpm and 4 °C for 10 min. The samples were collected at 0, 2, 4, and 7 days p.i. and analyzed with a fluorescence spectrophotometer (Synergy II plate reader with Gen5 software; BioTek Instruments, Winooski, VT).

Cell culture and infection

Bone marrow-derived macrophages (BMDMs) and thioglycolate-induced macrophages were isolated as previously described [18, 21]. In brief, cells were isolated from bone marrow and cultured in RPMI 1640 supplemented with 10% FCS, 20% L929 supernatant, and 1% penicillin and streptomycin for 7 days. Eight-week-old male C57BL/6J mice were injected intraperitoneally with 2 mL of 3% thioglycolate. Mice were euthanized after 4 days, and macrophages were enriched by plating the lavage cells on tissue culture plates for 2 h at 37 °C. Then, the plate was washed 3 times with sterile PBS to remove nonadherent cells. Resident peritoneal macrophages were isolated from the mice as described above without a thioglycolate treatment. A total of 2×10^6 cells were infected with leptospires at a multiplicity of infection (MOI) of 100.

For the phagocytosis experiment, cells were incubated with *L. interrogans* for 1 h [22]. After trypsinization and three washes with RPMI, the cells were centrifuged at 1200 rpm for 5 min, and the pellets were stored at -80°C for DNA extraction.

For the gentamicin protection assay, cells were washed with RPMI to remove extracellular bacteria after 1 h of infection and then incubated for 1 h in medium containing gentamicin ($100 \mu\text{g mL}^{-1}$) to kill the remaining extracellular bacteria. Then, the cells were incubated in a medium containing gentamicin ($25 \mu\text{g mL}^{-1}$) for another 4 h. The cells were lysed in 1 mL of distilled water, and 100 μL aliquots were used to inoculate 2 mL of EMJH broth. The number of bacteria was determined using a Petroff-Hauser chamber after 6–8 days.

For cytokine detection, 2×10^6 cells were infected with *L. interrogans* at an MOI of 100 for 24 h. The supernatant was collected and stored at -80°C .

Cell depletion and adoptive transfer

For macrophage depletion experiments, liposomes containing PBS or 5 mg/mL clodronate (Liposoma) were injected into the peritoneal cavity in a volume of 200 μL 3 days before infection according to the instructions. Thioglycolate-induced macrophages (1×10^6) were injected intraperitoneally into mice 1 day before infection.

Quantitative real-time PCR

For detecting the leptospiral load in the tissues or cells, the specimens (0.09–0.15 g) of tissues were homogenized with PBS (w/v, 1/10). The homogenate was centrifuged at 2000 rpm and 4 °C for 5 min. The supernatant was transferred into a new tube. Then, it was centrifuged at 12,000 rpm and 4 °C for 5 min. The pellets of tissues and cells were extracted using the TIANamp Bacteria DNA kit (Tiangen, China) according to the manufacturer's instructions [23]. The primers specific for *LipL32* was used to detect leptospires (Forward primer, 5'-TCGCTGAAATRGGWGTTCTGT-3'; Reverse primer, 5'-CGCCTGGYTCMCCGATT-3') [24]. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15 s and 60 °C for 60 s [25]. qPCR was conducted by an Applied Bioscience 7500 thermocycler and FastStart Universal SYBR Green Master Mix (Roche Applied Science, Germany). The genomic DNA of a counted number of *L. interrogans* was used as a calibrator. The leptospiral load was presented as the number of genome equivalents per μg of tissue DNA [26].

Flow cytometry

Peritoneal lavage was performed as described above, and the cells were preincubated with anti-CD16/CD32 mAb on ice for 15 min. Then, the cells were stained with a cocktail of antibodies (PECy7-labeled anti-CD45, FITC-labeled anti-F4/80 and APC-labeled anti-CD11b) for 30 min. Antibodies

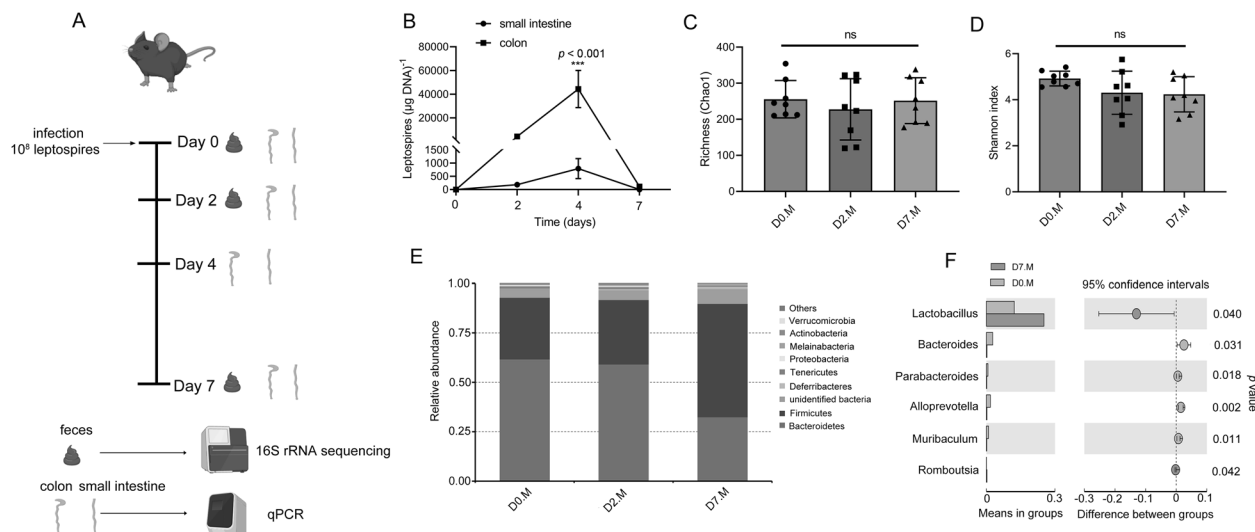


Fig. 1 *L. interrogans* infection altered the composition of the gut microbiota. **A** Flow diagram of the experiment. Eight-week-old male C57BL/6J mice were injected intraperitoneally with 10^8 leptospores. Mice were euthanized at 0, 2, 4, and 7 days p.i., and the small intestines and colons were collected for leptospiral load detection. Fresh fecal pellets were collected aseptically for 16S rRNA gene sequencing at 0, 2, and 7 days p.i. **B** Leptospiral burden in the small intestines and colons of *Leptospira*-infected mice was examined by qPCR ($n = 4$). **C** Chao1 α -diversity in the feces of mice. **D** Shannon diversity in the feces of mice. **E** Relative abundance of the top 10 phyla in the feces of mice. **F** The difference in the gut microbiota at the genus level was examined between D0.M and D7.M groups. **C–F** D0.M, mice 0 day post infection, $n = 8$; D2.M, mice 2 days post infection, $n = 8$; D7.M, mice 7 days post infection, $n = 8$. Each experiment was repeated twice. Data are shown as the mean \pm SEM. Statistical significance was determined using the Wilcoxon rank-sum test. *** $p < 0.001$. ns not significant.

were purchased from Invitrogen and BD Pharmingen. Cells were analyzed on a BD FACSCalibur, and data were analyzed with FlowJo software (Treestar software, USA).

Enzyme-linked immunosorbent assay (ELISA)

Secreted cytokines from cells and peritoneal lavage were performed following the instructions of the manufacturer (BioLegend, CA, USA) for cytokine detection.

Microbiota assay

Fresh fecal pellets were collected under sterile conditions, immediately frozen in liquid nitrogen and then stored at -80°C . Total genomic DNA from the samples was extracted using the CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. Then, 16S rRNA genes of distinct regions were amplified using specific primers with barcodes (27F, 5'-AGAGTTTGATCMTGGCTCAG-3'; 1492R, 5'-ACCTGTTCAGACTT-3'). All PCRs were carried out with TransStart FastPfu DNA Polymerase (TransGen Biotech). PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using the SMRTbellITM Template Prep Kit (PacBio) following the manufacturer's recommendations. The library quality was assessed on the Qubit 2.0 Fluorometer (Thermo Scientific) and FEMTO Pulse system. Finally, the library was sequenced on the PacBio Sequel platform. Raw sequences were initially processed through the PacBio SMRT portal. Sequences were filtered for a minimum of 3 passes, and a minimum predicted accuracy of 90% (minfullpass = 3, minPredictedAccuracy = 0.9). The predicted accuracy was 90%, which was defined as the threshold below which CCS was considered noise. The files generated by the PacBio platform were then used for amplicon size trimming to remove sequences outside the expected amplicon size (minLength 1340 bp, maxLength 1640 bp). The reads were assigned to samples based on their unique barcode and trimmed by removing the barcode and primer sequence. The reads were compared with the reference database using the UCHIME algorithm to detect chimeric sequences, and then the chimeric sequences were removed to obtain clean reads. Alpha diversity (Chao1 and Shannon) was calculated with QIIME (Version 1.9.1) and displayed with R software (Version 2.15.3). Principal coordinate analysis (PCoA) was performed to obtain principal coordinates and visualize complex, multidimensional data. A distance matrix of weighted Unifrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum

variation factor was demonstrated by the first principal coordinate, and the second maximum variation factor was demonstrated by the second principal coordinate. PCoA analysis was displayed by the WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3).

Statistical analysis

All values are expressed as the mean \pm SEM. Differences between mean values of normally distributed data were analyzed using the Wilcoxon rank-sum test. The results were considered statistically significant at $p < 0.05$.

RESULTS

L. interrogans infection changed the composition of the gut microbiota in mice

We first examined the leptospiral load in the intestine. The results showed that the leptospiral load reached a peak at 4 days p.i., and it was cleared at 7 days p.i.. There were almost 100X more leptospores in the colon (44406 ± 15699) than in the small intestine (793.4 ± 379.5) at 4 days p.i. (Fig. 1B). Then, we examined whether *L. interrogans* infection could influence the gut microbiota in mice. We analyzed the fecal pellets from mice by 16S rRNA gene sequencing. Although the bacterial richness and diversity of the gut microbiota in mice were not significantly altered between the two groups (Fig. 1C, D), the relative abundance of Firmicutes and Bacteroidetes between uninfected mice (D0.M) and infected mice 7 days post infection (p.i.) (D7.M) were significantly different at the phylum level and genus level (Fig. 1E and Supplementary Figs. S1A and 1B). The relative abundance of *Lactobacillus* was significantly higher on the D7.M group compared with the D0.M group (Fig. 1F). Then, we analyzed the ratio of Firmicutes to Bacteroidetes, which has been used as a marker of disease state in infection [27] and obesity [28]. This value was significantly increased in the D7.M group compared with the D0.M group (Supplementary Fig. S1C). We pooled all groups together to perform principal coordinate analysis (PCoA). The results showed that the composition of the gut microbiota in the D7.M group was different from that in the D0.M group (Supplementary Fig. S1D).

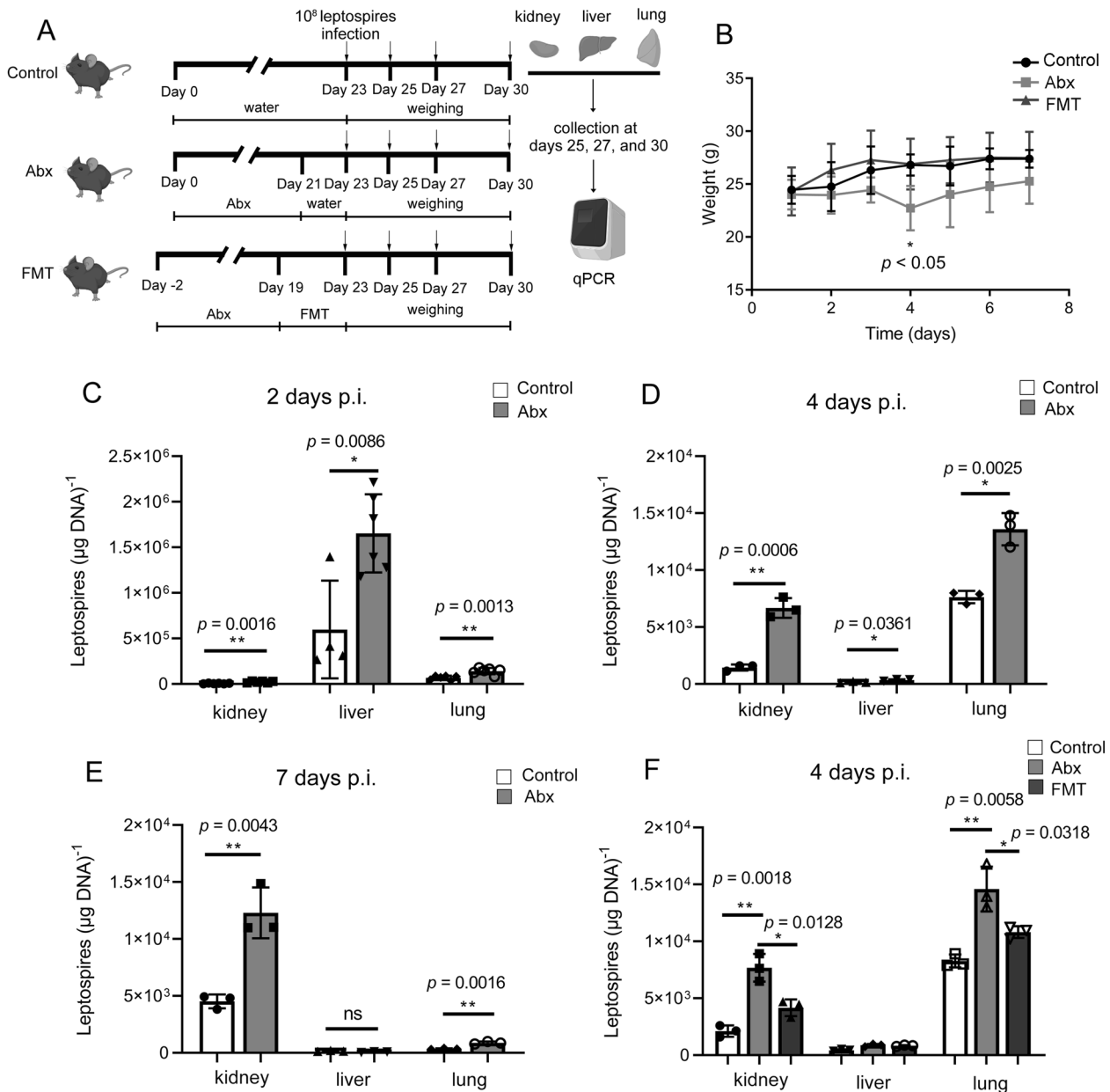


Fig. 2 Depletion of microbiota increased leptospiral burden in the tissues after infection. **A** Flow diagram of the experiment. Antibiotic treatment and FMT to Abx-treated mice are described in the Methods. Then, mice were intraperitoneally infected with 10⁸ leptospire. The kidneys, livers, and lungs were collected at 2, 4, and 7 days p.i.. Mice were weighed daily after infection. **B** Body weight curve of the control mice ($n = 12$), the Abx-treated mice ($n = 12$) and FMT to gut microbiota-dysbiosis mice ($n = 12$). Statistical significance was determined using the Wilcoxon rank-sum test. $*p < 0.05$, the Abx group versus control group. Leptospiral burdens of the control mice ($n = 3-4$) and the Abx-treated mice ($n = 3-6$) in the kidneys, livers, and lungs were detected by qPCR at 2 (**C**), 4 (**D**), and 7 days p.i. (**E**). **F** Leptospiral burdens of the kidneys, livers, and lungs of control mice ($n = 3$), Abx-treated mice ($n = 3$) and FMT to gut microbiota-dysbiosis mice ($n = 3$) were detected by qPCR at 4 days p.i. Each experiment was repeated three times. Data are shown as the mean \pm SEM. Statistical significance was determined using the Wilcoxon rank-sum test. $*p < 0.05$, $**p < 0.01$. ns not significant.

The intestinal barrier is essential for maintaining intestinal homeostasis [29]. Our results showed that the intestinal permeability of mice did not change significantly (Supplementary Fig. S1E). These results demonstrated that *L. interrogans* infection could change the composition of the gut microbiota in mice.

Microbiota depletion increased leptospiral burden in the organs after *L. interrogans* infection

To explore the role of the gut microbiota in mice during infection, we depleted microbiota with broad-spectrum antibiotics in mice (Fig. 2A). During the first week of antibiotic treatment, the mice

first lost weight and then gained weight. At the end of the antibiotic treatment, there was no difference in the weight between the two groups (Supplementary Fig. S2). However, there was a significant weight loss in the Abx-treated mice compared with the control mice at 4 days p.i. (Fig. 2B). The results showed that the leptospiral load in the kidneys, livers, and lungs of the Abx-treated mice was increased at 2, 4, and 7 days p.i. (Fig. 2C-E). However, fecal microbiota transplantation (FMT) to Abx-treated mice partially reversed these changes at 4 days p.i. (Fig. 2B, F). These data indicated that the microbiota helped enhance the antileptospiral capacity.

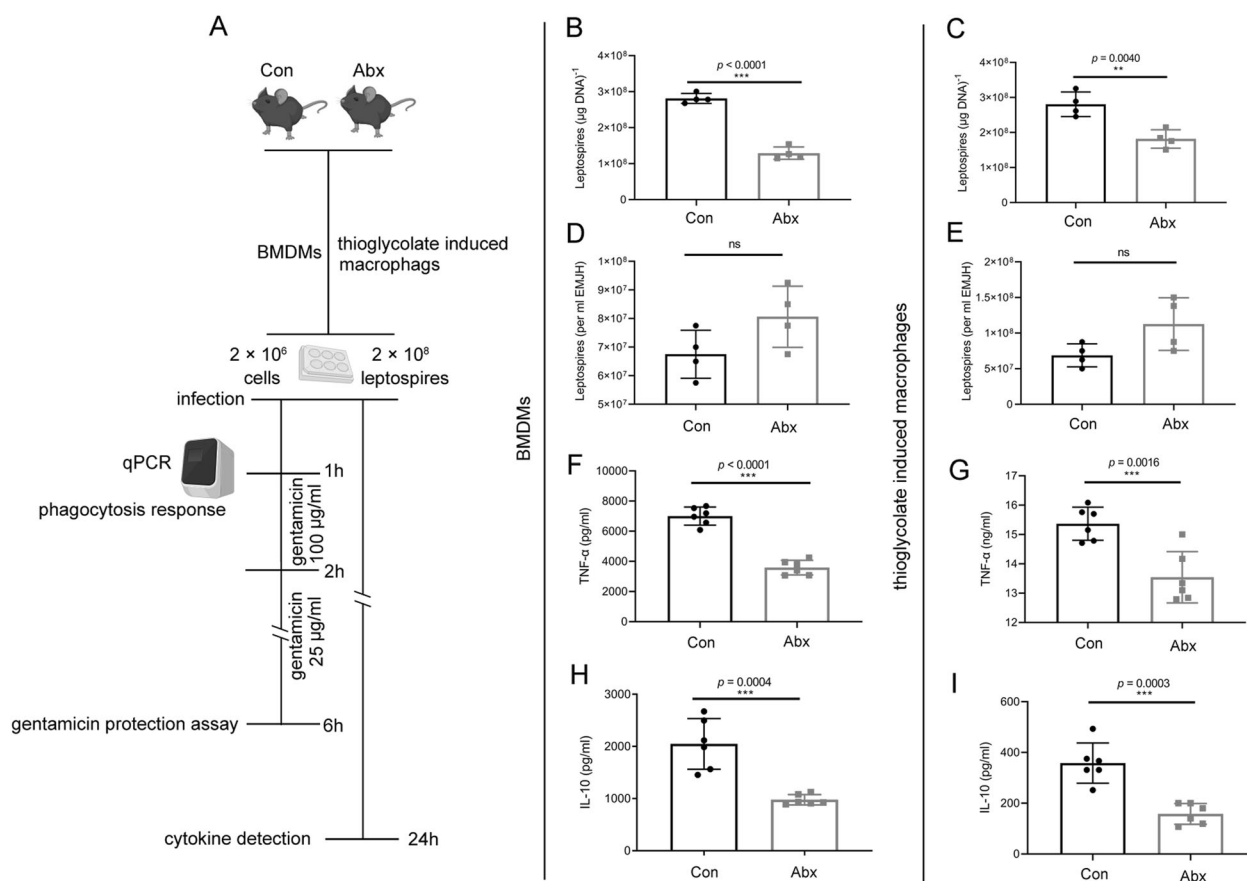


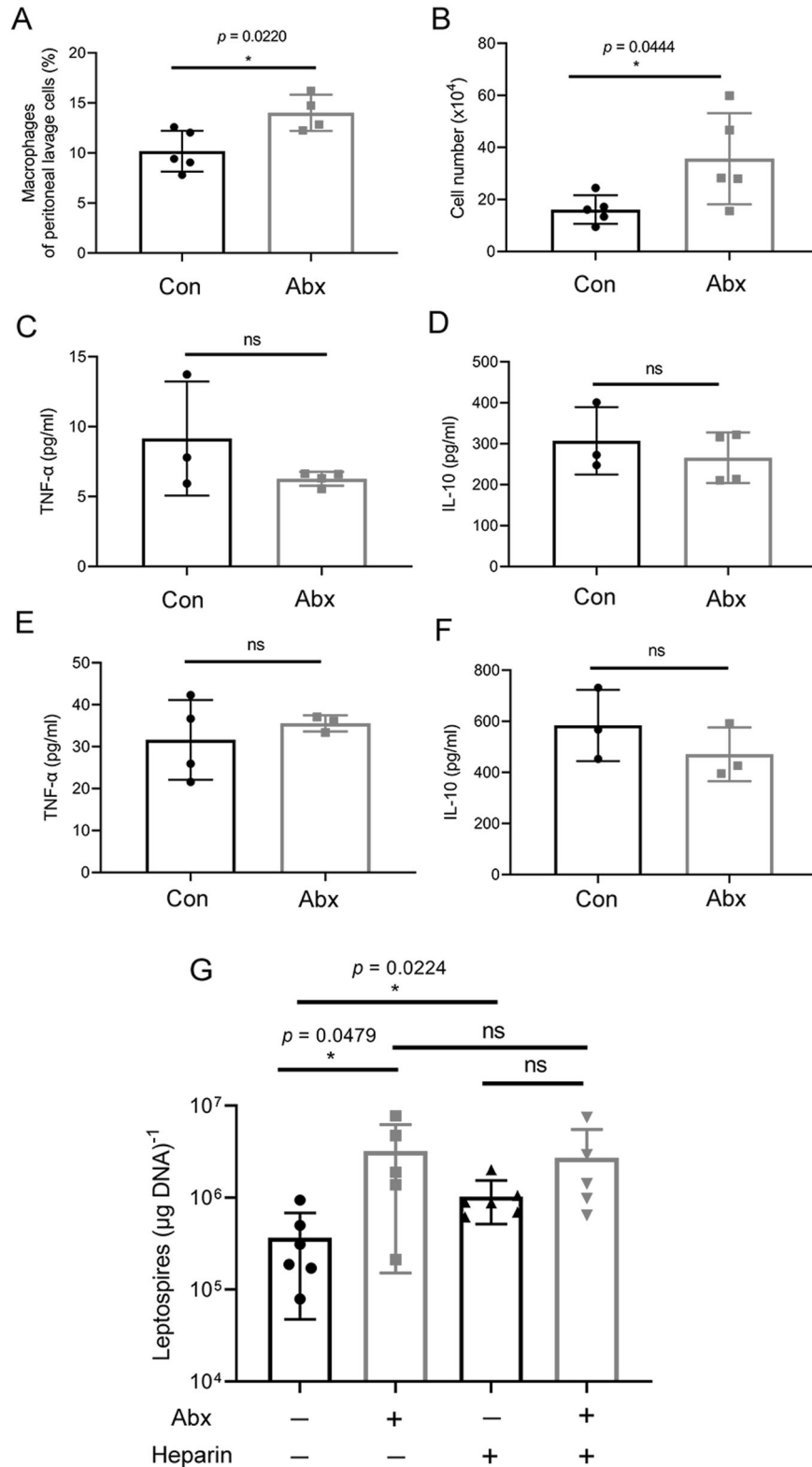
Fig. 3 Diminished antibacterial ability and inflammatory response of BMDMs and thioglycolate-induced macrophages in Abx-treated mice. **A** Flow diagram of the experiment. BMDMs and thioglycolate-induced macrophages were isolated from the control mice and the Abx-treated mice. A total of 2×10^6 cells were infected with leptospire at a multiplicity of infection (MOI) of 100. Then, the cells were treated as described in the Methods. The phagocytosis response of BMDMs (**B**) and thioglycolate-induced macrophages (**C**) in the control mice ($n = 4$) and the Abx-treated mice ($n = 4$) was analyzed by qPCR after 1 h of infection. Gentamycin protection assay of BMDMs (**D**) and thioglycolate-induced macrophages (**E**) in the control mice ($n = 4$) and the Abx-treated mice ($n = 4$). BMDMs (**F, H**) and thioglycolate-induced macrophages (**G, I**) were infected with leptospire for 24 h, and the protein levels of TNF- α and IL-10 were examined by ELISA in the control mice ($n = 6$) and the Abx-treated mice ($n = 6$). Each experiment was repeated three times. Data are shown as the mean \pm SEM. Statistical significance was determined using the Wilcoxon rank-sum test. ** $p < 0.01$, *** $p < 0.001$. ns not significant.

The gut microbiota induced different functions in various compartment-derived macrophages during *L. interrogans* infection

Previous studies reported that macrophages play an important role during *L. interrogans* infection [30, 31]. Thus, we examined the effect of the gut microbiota on various compartment-derived macrophages responding to *L. interrogans* infection. The results showed that the phagocytosis and bactericidal ability were diminished in BMDMs and thioglycolate-induced macrophages of the Abx-treated mice compared with those of the control mice (Fig. 3B–E). However, the phagocytosis of resident peritoneal macrophages from the Abx-treated mice was equal to that of the control mice (Supplementary Fig. S3A). The production of TNF- α and IL-10 was also attenuated in BMDMs and thioglycolate-induced macrophages of Abx-treated mice after *L. interrogans* infection (Fig. 3F–I). However, the protein levels of TNF- α and IL-10 in resident peritoneal macrophages were not different between the Abx-treated mice and the control mice after infection (Supplementary Figs. S3B and 3C). These data indicated that the effects of the gut microbiota on macrophages derived from different sources were diverse during infection.

Diminished macrophage disappearance reaction led to impaired leptospire elimination in the peritoneal cavity of *Leptospira*-infected mice after microbiota depletion

Early control of *Leptospira* was shown to predict the future extent of organ colonization [32]. Thus, we examined the immune response in the peritoneal cavity at the early stages of infection. The proportion and number of macrophages in the peritoneal cavity were comparable between the control mice and the Abx-treated mice at 2 h and 6 h p.i. (data not shown). However, the proportion and number of macrophages were significantly higher in the Abx-treated mice compared with that of the control mice at 24 h p.i. through peritoneal lavage (Fig. 4A, B). The protein levels of TNF- α and IL-10 were not changed between the control mice and the Abx-treated mice at 24 h p.i. (Fig. 4C, D). We wondered whether this phenomenon was due to the various numbers of cells in the peritoneal cavity between these two groups. Thus, we examined cytokine production from the same number of peritoneal cells of the control mice and the Abx-treated mice *ex vivo*. There were no differences in the protein levels of TNF- α and IL-10 with the same number of cells (Fig. 4E, F). In Fig. 4A, B, we detected more macrophages in the Abx-treated mice than in the control mice at 24 h p.i., which was indicative of macrophage



disappearance reaction. Examination of the leptospiral load in the peritoneal cavity showed that the leptospiral load in the peritoneal cavity was comparable between the control mice and the Abx-treated mice at 2 h p.i. and 6 h p.i. (Supplementary Figs. S4B and S4C). However, the leptospiral load was higher in the Abx-treated mice than in the control mice at 24 h p.i. (Fig. 4G). Heparin (an

inhibitor of macrophage disappearance reaction) administration significantly repressed the bactericidal ability in the peritoneal cavity of the control mice, while it resulted in no difference in the leptospiral load of the Abx-treated mice (Fig. 4G). Collectively, the macrophage disappearance reaction contributed to the control of *L. interrogans* infection, which was dependent on the gut microbiota.

Fig. 4 The diminished macrophage disappearance reaction increased the leptospiral load in the peritoneal cavity after microbiota depletion. Eight-week-old male C57BL/6J mice were treated with antibiotics or water for 3 weeks. After a 2-day washout period, the mice were intraperitoneally infected with 10^8 *L. interrogans*. **A, B** The proportion and numbers of macrophages in the peritoneal cavity after 24 h infection in the control mice ($n = 5$) and the Abx-treated mice ($n = 4-5$) were analyzed by flow cytometry. **C, D** The protein levels of TNF- α and IL-10 in the peritoneal cavity were examined by ELISA after 24 h of infection in the control mice ($n = 3$) and the Abx-treated mice ($n = 4$). **E, F** Peritoneal cells were lavaged from the peritoneal cavity of the control mice ($n = 3-4$) and the Abx-treated mice ($n = 3$) after 24 h p.i. and were cultured ex vivo. The supernatant of TNF- α and IL-10 from peritoneal cells (1×10^6) after 24 h incubation was examined by ELISA. **G** The control mice and the Abx-treated mice were intraperitoneally injected with or without 150 U heparin/mouse immediately after infection, and peritoneal lavage of leptospiral load was examined by qPCR at 24 h p.i. $n = 5-6$ animals per group. Each experiment was repeated three times. Data are shown as the mean \pm SEM. Statistical significance was determined using the Wilcoxon rank-sum test. * $p < 0.05$. ns not significant.

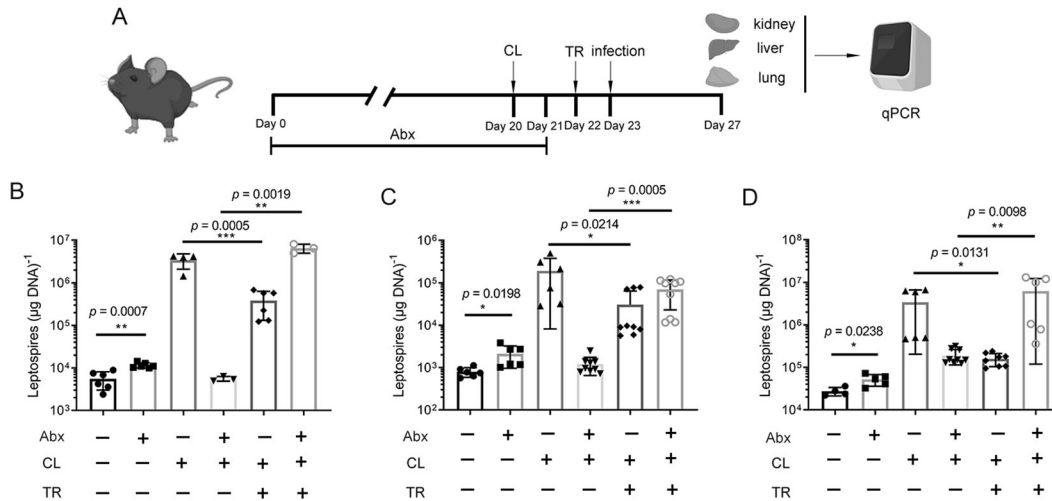


Fig. 5 The impaired antileptospiral capacity of macrophages increased leptospiral dissemination after microbiota depletion. A Flow diagram of the experiment. Eight-week-old male C57BL/6J mice were treated with antibiotics or water for 3 weeks. After a 2-day washout period, the mice were intraperitoneally infected with 10^8 *L. interrogans*. Liposomes containing PBS or 5 mg/mL clodronate were intraperitoneally injected into the peritoneal cavity 3 days before infection. A total of 10^6 thioglycolate-induced macrophages from the control mice were transferred into clodronate-treated mice 1 day before infection. The mice were euthanized at 4 days p.i. Leptospiral burdens in the kidneys (B), livers (C), and lungs (D) of mice was determined by qPCR at 4 days p.i. $n = 3-9$ animals per group. CL Clodronate, TR Transfer. Each experiment was repeated twice. Data are shown as the mean \pm SEM. Statistical significance was determined using the Wilcoxon rank-sum test. * $p < 0.05$; ** $p < 0.01$. *** $p < 0.001$.

The impaired antileptospiral capacity of macrophages increased leptospiral dissemination after microbiota depletion

To further explore the role of macrophages during *L. interrogans* infection under microbiota dysbiosis, we depleted macrophages with clodronate-loaded liposomes in the control mice and the Abx-treated mice prior to infection (Fig. 5A) [11, 32]. Clodronate pretreatment increased susceptibility to *L. interrogans* infection in the control mice as previously reported [32]. Interestingly, clodronate pretreatment did not significantly promote the leptospiral load in Abx-treated mice. Instead, the bacterial load in the kidney and liver decreased slightly in Abx-treated mice after macrophage depletion. Adoptive transfer of thioglycolate-induced peritoneal macrophages reversed the defect in clodronate-pretreated control mice. However, these transferred cells significantly enhanced susceptibility to *L. interrogans* infection in clodronate-pretreated mice upon microbiota dysbiosis at 4 days p.i. (Fig. 5B–D).

DISCUSSION

To the best of our knowledge, this is the first study to explore the role and underlying mechanism of the gut microbiota in leptospirosis. A previous study reported that the kidneys, lungs, and livers are the major organs affected by severe leptospirosis [33]. Intestinal bleeding is a common but neglected symptom in severe leptospirosis [7]. A healthy gut environment, shaped by the presence of a healthy functional microbial ecosystem, is

fundamental to guide the immune system towards homeostasis [34, 35]. A previous study reported that host susceptibility to disease is frequently heterogeneous and is increasingly attributed to the gut microbiota [36]. Alavi et al. also demonstrated that interpersonal gut microbiome variation determined an individual's susceptibility and resistance to cholera infection [37]. Differences in the composition of the gut microbiota may explain a series of clinical symptoms in leptospirosis patients. Our study proved that although *L. interrogans* infection did not alter the abundance or diversity of the gut microbiota, it did change the composition of the specific gut microbiota in hosts. Infections with some nonenteropathogens, such as *Mycobacterium tuberculosis* [38], *Influenza* [36], and *Burkholderia pseudomallei* [12], have also been shown to alter the composition of the microbiota. Jacobson et al. demonstrated that microbiota community composition, rather than species richness, drives microbiota-mediated colonization resistance against pathogen infection [39]. Changes in the gut microbiota might be the result of the activation of intestinal immunity caused by *L. interrogans* infection. A previous study demonstrated that host immune activation induced rapid transcriptional and metabolic adaptation of intestinal microbes [40]. The disturbance of the intestinal environment caused by infection will lead to the disruption of the homeostasis between intestinal immunity and the microbiota and eventually lead to the expansion or decrease of certain bacteria [34].

The alterations in the gut microbiota caused by infection have recently been linked to a concept called "microbiome memory", which is different from trained immunity. Stacy et al. showed that

host-initiated metabolite pathways that functionally alter the microbiota after primary infection augment the colonization resistance to subsequent infection [41]. Macrophages and monocytes possess memory properties involving fungal cell-wall constituents, such as β -glucan [42]. Trained immunity can be induced in Abx-treated mice with *Candida albicans* [43]. These studies show that microbiota or their components and metabolites can regulate host immunity against secondary infection in different ways. However, Sencio et al. demonstrated that gut dysbiosis caused by *Influenza* infection contributed to pulmonary pneumococcal superinfection [44]. Thus, not all pathogen infections increase resistance to subsequent infections. It will be interesting to explore whether *L. interrogans* infection can also train the microbiota to enhance resistance to pathogens. 16S rRNA gene sequencing data showed that the relative abundance of *Lactobacillus* was significantly increased after infection. Potula et al. reported that oral administration of *L. plantarum* to C3H/HeJ mice alleviates acute leptospirosis [45]. Zhang et al. reported that *Influenza* infection elicited an expansion of endogenous *Bifidobacterium animalis*, which protected mice against infection [36]. Although Potula et al. did not discuss the relation between the protective effect of *L. plantarum* and the gut microbiota, we proposed that probiotic therapy, especially endogenous lactobacillus isolate treatment, was an effective way to prevent leptospirosis. Our study also demonstrates that gut microbiota depletion increases the leptospiral load in organs accompanied by weight loss. A decrease in body weight was the earliest clinical sign of leptospirosis, which was usually accompanied by anorexia [46]. Recently, anorexia was presumably linked to microbiota dysbiosis [47]. Our study indicates that disturbance of the gut microbiota, such as antibiotic treatment, could increase susceptibility to *L. interrogans* infection.

Previous studies suggested an important role for macrophages during *L. interrogans* infection [30–32]. In this study, we further explored the effect of the gut microbiota on various compartment-derived macrophages during *L. interrogans* infection. Our data revealed that the antibacterial ability and inflammatory response to infection of BMDMs and thioglycolate-induced macrophages, which are of hematopoietic origin, but not of resident peritoneal macrophages, which are largely derived from embryonic yolk sac progenitors, were blunted after microbiota depletion [11]. Schuijt et al. also reported that Abx treatment impaired the phagocytosis response of whole-blood neutrophils but not of peritoneal macrophages to *Streptococcus pneumoniae* infection [10]. Consistent with the results of the resident peritoneal macrophages in vitro, there was no difference in the inflammatory condition of the peritoneal cavity between the control mice and the Abx-treated mice at 24 h p.i.. Interestingly, the proportion and number of peritoneal macrophages (nonadherent) were higher in the Abx-treated mice than in the control mice at 24 h p.i. through peritoneal lavage. A previous study reported that bacterial entry into the peritoneum acutely induced macrophage adherence to form local clots that effectively brought macrophages and bacteria in proximity and out of the fluid phase, which is called the “macrophage disappearance reaction”. This process was proven to promote *Escherichia coli* clearance in the peritoneal cavity [14]. The diminished macrophage disappearance reaction resulted in more nonadherent macrophages in the fluid phase. Thus, more macrophages were detected in the Abx-treated mice through peritoneal lavage after infection. Our data demonstrated that the macrophage disappearance reaction contributed to *L. interrogans* clearance, which was dependent on the gut microbiota. Our study identified a gut-peritoneal cavity axis that might provide the basis for other abdominal-related diseases. Although the intraperitoneal route of infection is widely used to experimentally inoculate animals, this challenge route does not represent a natural route of infection [46]. Many groups have explored the effect of different

routes on *Leptospira* infection [13, 46, 48]. *Leptospira* dissemination exhibit significant variation depending on the inoculation route used. The number of leptospires that breach the tissue and disseminate is contingent on the immune defense capability at each port of entry [13]. In addition, signals from the gut microbiota to distant organs could regulate local immunity [49]. Further work should focus on the role of the gut microbiota on the natural route of infection.

The different immune responses of BMDMs and resident peritoneal macrophages to *L. interrogans* infection might be related to the dynamic changes in the microbiota from birth to adulthood. The gut microbiota in early life might imprint specific immune instruction into resident macrophages, which could potentially lead to a differential immune response to infection compared with that of BMDMs in microbiota-depleted adult mice. Recently, Archita et al. demonstrated that the selective presence of live microbes in fetal organs had broader implications for the establishment of immune competency and priming before birth [50]. Events in early life that can alter the gut microbiota composition (e.g., infection/inflammation, antibiotic exposure, and caesarean section) can pathologically imprint the immune system and increase the risk of diseases [51]. However, the interaction between the gut microbiota and the immune system is also dynamic [52]. Germ-free mice inoculated by oral gavage with the Oligo-Mouse-Microbiota bacterial consortia exhibited more resistance to *Salmonella* infection [53]. Further investigation of the immunomodulatory effect of specific intestinal bacteria on macrophages in early life will help us understand the heterogeneity of immune responses of different macrophages to infection. Our results showed that the bacterial load in the kidneys and livers of the Abx-treated mice was comparable after macrophage depletion. This could be explained by the impaired antileptospiral capacity of peritoneal macrophages in Abx-treated mice. We transferred thioglycolate-induced peritoneal macrophages from the control mice into the control mice and the microbiota-depleted mice that were pretreated with clodronate before infection. Adoptive cell transfer reduced the bacterial load in the kidneys, livers, and lungs of the control mice pretreated with clodronate. However, adoptive cell transfer increased the bacterial load in the kidneys, livers, and lungs of the microbiota-depleted mice pretreated with clodronate. These results indicate that impaired antileptospiral capacity in the peritoneal cavity could lead to leptospiral transmission after microbiota depletion.

In summary, effective cross-protective vaccines are not available, and at present, antibiotic treatment is only effective if used early in the course of *L. interrogans* infection [45]. Our study shows that the gut microbiota may serve as an endogenous barrier against *L. interrogans* infection. Modulating the structure and function of the gut microbiota may provide new targets for individualized preventative strategies for leptospirosis and related spirochetal infections.

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AUTHOR CONTRIBUTIONS

XX and JL share co-first authorship. JL and YC conceived ideas, and XX and WZ designed experiments. XX, JL, RT, XW and XC performed the mouse experiments. JL, SZ and JC conducted the bacteriology experiments. XX and JL analyzed data. WZ and

YC performed bioinformatics analysis. XX and JL wrote the original draft. WZ and YC supervised the study and reviewed the final version of the text.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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