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# β-Glucan pretreatment activates lectin pathway to maintain the function of intestinal Th17 cells for infectious enteritis protection

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## ABSTRACT

Enteritis, involving inflammation of the small intestine, is often accompanied by immune cell dysfunction during intestinal infections. Immunomodulatory β-glucans (BGs) have recently been shown to support antibacterial immune responses through the induction of trained immunity. However, little is known about the potential role of BG pretreatment in protecting against infectious enteritis in teleost fish. In this work, by establishing an adult zebrafish enteritis model via infection with the fish pathogen *Edwardsiella piscicida* and pretreating it with BGs, we demonstrated that such pretreatment confers protection against infectious enteritis, accompanied by reduced production of proinflammatory cytokines. Specifically, we found that BG pretreatment could amplify intestinal lectin pathway-associated complement activation to ameliorate the infectious enteritis. Moreover, through comprehensive RNA-seq analysis of immune cell marker genes in zebrafish, we revealed that the lectin pathway amplification by BG pretreatment modulated the responsiveness of intestinal Th17 cells, which was essential for the protection against infectious enteritis. Collectively, these findings identify the intestinal lectin pathway as a potential mediator of the effects of BG pretreatment and reveal its role in maintaining the function of Th17 cells in zebrafish. This suggests that harnessing BG-induced trained immunity might represent a promising therapeutic strategy against infectious enteritis in teleost.

**Keywords:** β-Glucan; Lectin pathway; Th17 cell; *Edwardsiella piscicida*; Infectious enteritis; Zebrafish



## INTRODUCTION

Intestinal inflammation is typically characterized by intestinal barrier disruption and exacerbated inflammatory responses (Neurath, 2024). Central to this pathology is the dysregulation of immune cell populations, including macrophages (Chikina et al., 2020; De Schepper et al., 2018), dendritic cells (Medina Sanchez et al., 2023; Rivera et al., 2022), innate lymphoid cells (Araujo et al., 2024; Lo et al., 2024), as well as Treg and Th17 cells (Bonetti et al., 2024; Gu et al., 2024; Jaeger et al., 2021). Emerging evidence has highlighted the pivotal role of helper T cells in orchestrating intestinal immune homeostasis against infections (Biton et al., 2018; Lin et al., 2023). For example, it has been shown that *Staphylococcus aureus* infection induces a distinct Th17-cell subset producing IL-17 and IL-10 but not secreting IFN-γ, mirroring the non-pathogenic Th17 phenotype observed in colitis models (Zielinski et al., 2012). It has also been reported that cytokines derived from activated Th17 cells could enhance the integrity of intestinal epithelial tight junctions, thereby reinforcing the barrier function (Kumar et al., 2016; Li et al., 2018). Intriguingly, in *Helicobacter hepaticus*-induced colitis, pathobiont-specific inflammatory Th17 cells have been revealed to be major mediators of intestinal inflammation (Xu et al., 2018), while segmented filamentous bacteria-induced intestinal Th17 cells have been revealed to exhibit unique anti-inflammatory phenotypes, characterized by the expression of a transcription factor (c-MAF) and an anti-inflammatory cytokine (IL-10) (Brockmann et al., 2023). Collectively, these findings demonstrate the regulatory capacity of Th17 cells in either maintaining tissue homeostasis or propagating inflammation during infections. This suggests the potential value of precisely characterizing the role of the functional plasticity of Th17 cells in maintaining gut homeostasis in order

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to develop immunotherapies against infectious enteritis.

β-Glucans (BGs), natural polysaccharides formed by D-glucose monomers linked via β-glycosidic bonds, are widespread in the cell walls of yeast, oats, and fungi, where they exhibits immunostimulatory and protective properties (De Marco Castro et al., 2021; Khan et al., 2025). Research on mammalian models revealed that BG pretreatment (also considered as an approach to induce trained immunity) could induce the relatively long-term reprogramming of lymphoid cells through metabolic and epigenetic alterations (Cheng et al., 2014; Netea et al., 2011; Saeed et al., 2014). Mechanistically, this was explained by enhanced inflammatory cytokine production during secondary homologous or heterologous infections (Ding et al., 2023; Kalafati et al., 2020). In addition, in teleost fish, recent studies have characterized the immunomodulatory roles of BGs upon their administration using different methods. For example, in a zebrafish larval model, microinjection of BGs was shown to upregulate *cxcl8* expression and recruit neutrophils to infection sites, thereby conferring protection against subsequent *Salmonella typhimurium* infection (Darroch et al., 2022). Moreover, in seven-band grouper, the intraperitoneal administration of BGs was revealed to enhance the antiviral responses against nervous necrosis virus (NNV; *Betanodavirus*) and increase the rate at which fish survived such infection (Krishnan et al., 2022). Furthermore, in Nile tilapia, the addition of BG to the diet was revealed to exert immunomodulatory effects by augmenting innate immunity and improving resistance to infection by the Gram-negative bacterium *Aeromonas hydrophila* (Koch et al., 2021). Although evidence has revealed that BG pretreatment could alleviate soybean meal-induced intestinal inflammation and maintain structural integrity of the intestine in adult zebrafish (Rehman et al., 2023), whether it can alleviate bacterial infection-induced enteritis in teleost fish remains unclear.

In this study, by establishing an adult zebrafish enteritis model via infection with the fish bacterial pathogen *Edwardsiella piscicida* and pretreating it with BGs, we found that such pretreatment could amplify the lectin pathway-mediated activation of complement. This in turn maintained the responsiveness of intestinal Th17 cells and alleviated the infectious enteritis. Taken together, our results identify the lectin pathway–Th17-cell regulatory axis as a fundamental effector mechanism protecting against infectious enteritis in zebrafish. This axis might provide a target for immunotherapies in aquaculture and other fields.

## MATERIALS AND METHODS

### Zebrafish

Three- to six-month-old AB strain zebrafish were maintained at 27±1°C under a controlled light/dark cycle (14 h light/10 h dark; lights on from 0700h to 2100h). Water quality parameters were strictly monitored and maintained within the following ranges: pH 6.8–7.5, conductivity 500–1500 μS/cm, dissolved oxygen ≥6.0 mg/L, ammonia nitrogen concentration <0.2 mg/L, and nitrite concentration <0.1 mg/L. Healthy zebrafish were randomly selected for the experiment at a sex ratio of approximately 1:1 after 1 week of acclimation.

### Ethics statement

The animal experiments were approved and carried out following the guidelines of the Animal Experiment Committee of the East China University of Science and Technology (Protocol No. 2006272).

### Bacterial culture

*Edwardsiella piscicida* strain EIB202 (CCTCC No. M208068) was used in this study. For bacterial culture, this strain was inoculated in tryptic yeast broth (TYB) medium supplemented with 16.7 μg/mL colistin ($Col^+$). The bacterial solution was cultured in a 30°C incubator for 6 h before being washed and resuspended in PBS to a concentration of $10^9$ CFU/mL ($OD_{600}$=1.0). Gradient dilution was performed for subsequent infection.

### *In vivo* β-glucan (BG) pretreatment and bacterial challenge model

For BG pretreatment, the adult zebrafish (*n*=30) were intraperitoneally (*i.p.*) injected with 10 μL of yeast-derived β-1,3–1,6-(D)-glucan (working concentration of 10 mg/mL resolved in PBS) or the same volume of PBS as a control (Wang et al., 2024a). Five days later, these zebrafish were challenged with *E. piscicida* via rectal injection. Specifically, 5 μL of bacterial solution ($2\times10^6$ colony forming unit (CFU)/mL) was injected into the posterior intestine of the zebrafish, accessed through the anus. At 1, 3, 7, and 14 days post-infection (dpi), zebrafish (samples) were selected at random from the total group and euthanized with MS-222 for each analysis.

### Bulk RNA-sequencing and data analysis

For bulk RNA-sequencing and data analysis, at 7 dpi, the posterior intestines were sampled from 15 zebrafish per group, which were randomly selected from each group and divided into three biological replicates for bulk RNA-sequencing. mRNA was extracted using Trizol (Invitrogen, USA) and resuspended in 20 μL of nuclease-free water, after which it was sequenced on an Illumina Hiseq-PE150. The RNA-seq data were filtered by fastp (v.0.22.0) to remove genes with low read counts. Read counts were then normalized using Trimmed Mean of M-values normalization. The normalized reads were mapped to the reference genome using HISAT2 (v.2.1.0) and HTSeq (v.0.9.1) statistics, and then Fragments Per Kilobase Million was used to standardize the expression. The differentially expressed genes (DEGs), defined as those with an expression difference of $\log_2$(fold change) >1 and *P*-value <0.05, were analyzed by DESeq (v.1.38.3). The DEGs were analyzed using Gene Ontology (GO; gseGO), Kyoto Encyclopedia of Genes and Genomes (KEGG; gseKEGG), and Gene Set Enrichment Analysis (GSEA; fgsea) enrichment, implemented using the R programming language.

To identify the cluster-specific marker genes in bulk RNA-sequencing data, we retrieved the single-cell RNA sequencing data from previously published zebrafish datasets: GSE230044 (Jones et al., 2023), GSE232302 (Matz et al., 2023), and GSE135767 (Gu et al., 2020). To define the immune cell–specific marker gene signatures in zebrafish intestines, the R package Seurat (v.4.0.1) and quality control filtering were performed following a modified version of the standard pipeline: Cells were retained only if they contained >200 and <4 000 detected genes. In addition, data normalization was conducted via the “LogNormalize” method with a scaling factor of 10 000. Principal component analysis (PCA) was applied to genes of which the expression varied markedly among each group. Dimensionality reduction was

then performed using UMAP (RunUMAP function), followed by shared nearest neighbor (SNN) clustering (FindNeighbors function), based on the top 10 principal components. Next, unsupervised clustering was performed with the FindClusters function at a resolution of 0.5. In addition, differential expression analysis was conducted through the FindAllMarkers function with thresholds set at $|\log_2(\text{fold change})| \geq 0.5$ and adjusted *P*-value <0.05. For comparative analyses between conditions, DEGs were determined using the FindMarkers function.

**Enzyme-linked immunosorbent assay (ELISA)**

At 3 and 7 dpi, the zebrafish posterior intestines (*n*=15) sampled from each group with three replicates were weighed and then disrupted with radioimmunoprecipitation assay (RIPA) lysis buffer in a tissue grinder. After centrifugation, the supernatant was collected and stored at −80°C until analysis. The concentration of complement component 3 (C3) in the posterior intestines was measured using a C3 ELISA kit (Fankew, China), in accordance with the manufacturer's protocol.

**Inhibitor treatment**

The inhibitors used in this study were dissolved in DMSO and stored at −80°C. For *in vivo* inhibitor treatment in zebrafish, AMY-101 TFA (inhibitors of C3, 2 mg/mL) or GSK805 (inhibitors of Th17 cells, 2 mg/mL) was intraperitoneally injected into zebrafish at a dose of 20 μg/fish before BG pretreatment and subsequent *E. piscicida* challenge. These concentrations were selected in line with those used on turbot in a previous study (Yang et al., 2024).

**Flow cytometry assays**

At the indicated timepoints, the cell suspensions derived from posterior intestines were obtained as previously described (Zhao et al., 2023) and resuspended in 400 μL of PBS (containing 1% bovine serum albumin) at 4°C for 15 min for blocking to reduce non-specific binding. The cells were resuspended in 200 μL of 4% paraformaldehyde (Sigma, USA) and incubated at room temperature for 15 min to fix them. Then, the fixed cells were washed once with 1×PBS before being resuspended in 200 μL of pre-cooled 90% methanol for permeabilization. These permeabilized cells were then washed again and incubated with rabbit anti-zebrafish IL-17A/F3 antibody (polyclonal, customized, 1:500; AtaGenix, China) at 4°C for 30 min. The control group was administered anti-rabbit IgG as an isotype-matched control. Then, the cells were washed once with 1×PBS and incubated with Alexa Fluor™ 488 goat anti-rabbit IgG (H+L) antibody (A-11034, 1:200; Invitrogen, USA) at 4°C for 30 min. The control group was administered 1×PBS. Subsequently, the cells were washed twice with 1×PBS for subsequent flow cytometry analysis using a BECKMAN COULTER CytoFLEX device, after which FlowJo software (v.10.9.0) was used for data analysis.

**Alcian blue and nuclear fast red staining analysis**

At the indicated timepoints, the posterior intestines of six zebrafish were sampled and rapidly fixed in paraformaldehyde fixative (Servicebio, China) at room temperature for 24–48 h, followed by dehydration using ethanol and vitrification using dimethylbenzene. Next, these samples were embedded in paraffin blocks and sliced to a thickness of 5 μm with a paraffin slicing machine (Leica, Germany). After deparaffinization and rehydration, the sections were stained with Alcian Blue & Nuclear Fast Red Staining Kit (Beyotime, China), in accordance with the manufacturer's protocol. Microscopy and photography of the stained sections were performed after neutral resin sealing and air drying.

**Bacterial enumeration**

At the indicated timepoints, the posterior intestines sampled from three zebrafish were pooled, placed into a sterile 1.5 mL centrifuge tube, and ground with 200 μL of PBS. Tenfold serial dilutions (10-fold to 1000-fold) were prepared with sterile PBS, and 10 μL of them were pipetted into a deoxycholate hydrogen sulfide lactose (DHL) agar plate ($Col^+$) with discrete colonies quantified on plates containing 20–200 colonies. Then, the black colonies were counted (as CFUs in the posterior intestine) after culturing the agar plate at 37°C for 24 h. CFUs=CFUs/posterior intestine.

**mRNA isolation and quantitative PCR (qPCR)**

At the indicated timepoints, the posterior intestines were sampled from three zebrafish, pooled in an RNase-free centrifuge tube, supplemented with 200 mL of RNAstore Reagent (Tiangen, China), and stored at –80°C until analysis. mRNA was extracted using Trizol (Invitrogen, USA) and resuspended in 20 μL of nuclease-free water. cDNA was synthesized using Trans-Script Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen, China), and qPCR was performed using QuantStudio3 (Thermo Fisher, USA) with PerfectStart Green qPCR SuperMix (TransGen), in accordance with the manufacturers' procedures. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method to determine the relative transcription level of genes. The primers used in this study are listed in Supplementary Table S1.

**Statistical analysis**

The statistical analysis was performed using GraphPad Prism software (GraphPad v.8) and ImageJ software (ImageJ v.2.1.0). Statistical significance was calculated using unpaired two-tailed Student's *t* test or two-way ANOVA. Unless indicated otherwise, data are shown as mean±SD from at least three independent experiments. Statistical significance is denoted as follows: $^{*}$: $P<0.05$; $^{**}$: $P<0.01$; $^{***}$: $P<0.001$; $^{****}$: $P<0.0001$; ns: Not significant.

## RESULTS

**β-Glucan (BG) pretreatment alleviates infectious enteritis in zebrafish**

To investigate the effects of BG in preventing infectious enteritis, we employed yeast-derived β-glucan (BGs) to establish an *i.p.* injection-based pretreatment model in adult zebrafish. Five days after the BG pretreatment, the zebrafish were rectally challenged with *E. piscicida* to induce enteritis (Figure 1A). Upon comparison with PBS-pretreated zebrafish, we found that BG pretreatment significantly reduced the mortality induced by *E. piscicida* infection (Figure 1B). Consistent with this, bacterial colonization of the posterior intestine (Figure 1C) and the incidence of bacterial infection-induced inflamed cloaca (Supplementary Figure S1A, B) were significantly reduced in BG-pretreated zebrafish. Moreover, Alcian blue and nuclear fast red staining analysis showed that *E. piscicida* infection resulted in reductions in goblet cells and mucus secretion in zebrafish posterior intestines (Figure 1D, E); however, BG pretreatment significantly ameliorated these phenotypes (Figure 1D, E), suggesting its critical role in

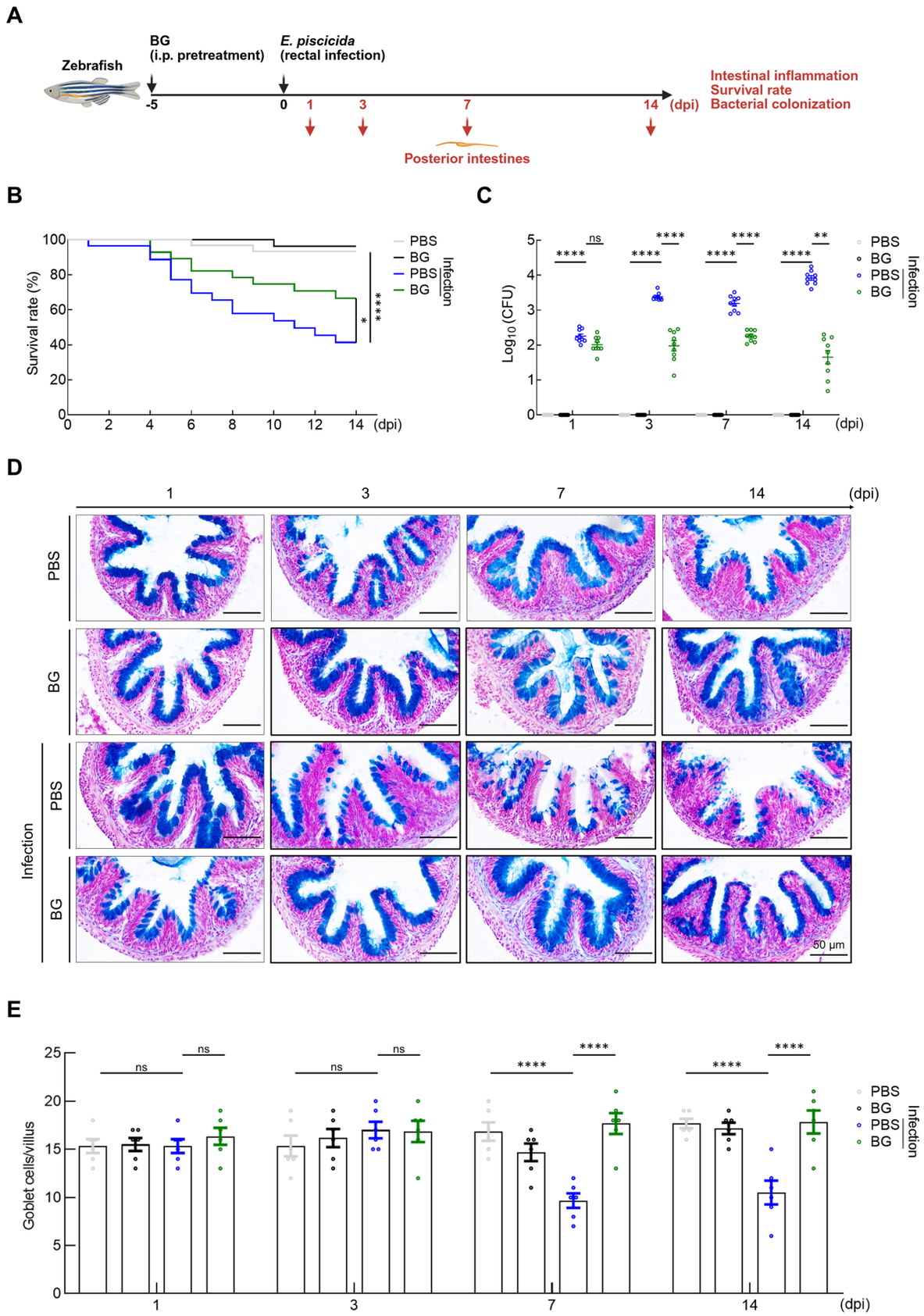


**Figure 1 BG pretreatment alleviates *E. piscicida* infection–induced enteritis in zebrafish**

A: Schematic model of BG pretreatment and *E. piscicida* infection in zebrafish. B: Survival rate (%) analysis over the course of the experiment ($n$=25). C: Bacterial loads in the posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection. D: Histopathological analysis of posterior intestines in PBS- and BG-pretreated zebrafish during *E. piscicida* infection. Scale bar: 50 μm. E: Statistical analysis of the number of goblet cells as indicated in panel D. Data are from three independent replicate experiments and shown as mean±SD, which were analyzed by two-way ANOVA with multiple comparisons. *: $P$<0.05; **: $P$<0.01; ****: $P$<0.0001; ns: Not significant.

alleviating infectious enteritis in zebrafish.

To better understand the mechanism by which BG pretreatment alleviated infectious enteritis, we analyzed the transcriptional expression profile in the posterior intestines of zebrafish using bulk RNA-sequencing (Figure 2A; Supplementary Table S2). By identifying 407 genes that were upregulated in the PBS-pretreated *E. piscicida*-infected group along with 238 genes that were downregulated in the BG-pretreated *E. piscicida*-infected group, we annotated 121 genes that overlapped between these two groups, which were designated as *BG-reversed genes* that act against bacterial infection (Figure 2B, C; Supplementary Table S3). GO analysis revealed that these BG-reversed genes were significantly enriched in pathways associated with 'inflammatory and acute-phase responses' (Figure 2D; Supplementary Table S3). Notably, in the PBS-pretreated zebrafish, the transcriptional expression of proinflammatory cytokines (*il1b*, *il6*, *saa*), chemokines (*cxcl8a*, *cxcl18b*, *ccl34a.4*), and matrix metalloproteinases (*mmp14b*, *mmp9*) was significantly induced after *E. piscicida* infection (Figure 2E) , but these levels were much lower in the BG-pretreated zebrafish (Figure 2E). These results suggest that BG pretreatment could dampen the transcription of inflammatory cytokines, which aligns with the finding of reduced intestinal inflammation in zebrafish (Figure 1).

**BG pretreatment activates intestinal lectin pathway-associated complement cascades against bacterial infection**

To further identify the critical signaling components involved in BG-activates alleviation of infectious enteritis in zebrafish, we analyzed the DEGs whose differential expression was induced by *E. piscicida* infection. We annotated 769 genes (designated as BG-amplified genes) that were upregulated in the BG-pretreated *E. piscicida*-infected group compared with the expression level in the PBS-pretreated *E. piscicida*-infected group (Figure 3A; Supplementary Table S4). Moreover, through KEGG pathway analysis, we demonstrated that 'the complement and coagulation cascades pathway' was significantly upregulated by the BG pretreatment (Figure 3B; Supplementary Table S4). Consistent with this, GSEA to validate the complement-associated genes revealed that they were significantly enriched among the BG-amplified genes (normalized enrichment score [NES]=1.89, $P$=0.00001) (Figure 3C; Supplementary Table S4). Subsequently, by annotation of these complement system-related genes in zebrafish, we analyzed the transcripts of these genes and found that lectin pathway–associated genes (including *mbl2*, *masp1*, *masp2*, *c3a.1*, *c5*, *c7*, *c8*, and *c9*) were preferentially induced during *E. piscicida* infection, and that their expression was relatively elevated in the BG-pretreated *E. piscicida*-infected group (Figure 3D). This suggests that BG pretreatment could amplify the lectin pathway, which might contribute to alleviating infectious enteritis in zebrafish.

**BG-amplified lectin pathway is essential for preventing infectious enteritis**

To further elucidate the role of the BG-amplified lectin pathway in modulating infectious enteritis, we established a pharmacological inhibition assay (Figure 4A). By analyzing the production of complement 3 (C3), a pivotal component of the complement cascade (Kiss et al., 2023; Wu et al., 2024), we confirmed that BG pretreatment significantly elevated the intestinal level of C3 during *E. piscicida* infection, while treatment with the C3 inhibitor AMY-101 mitigated this (Figure 4A). Moreover, upon comparison with the effects of BG pretreatment in alleviating the phenotypes of intestinal inflammation, pretreatment with AMY-101 resulted in reductions in goblet cells and mucus secretion in the zebrafish posterior intestine (Figure 4B; Supplementary Figure S2). Furthermore, the BG-associated downregulation of proinflammatory genes (*il1b*, *il6*) in the posterior intestine during *E. piscicida* infection was also reversed by AMY-101 treatment (Figure 4C, D). Notably, AMY-101 treatment also abolished the survival advantage and bacterial clearance capacity conferred by BG pretreatment (Figure 4E, F), suggesting that the BG pretreatment-associated protection against infectious enteritis is dependent on activation of the lectin pathway in zebrafish.

**BG-amplified lectin pathway is essential for maintaining the function of Th17 cells against infection**

To identify the effector cells associated with the ability of BG pretreatment to mitigate infectious enteritis, we incorporated previously published zebrafish single-cell RNA-seq datasets, namely, GSE230044 (Jones et al., 2023), GSE232302 (Matz et al., 2023), and GSE135767 (Gu et al., 2020), into our study to characterize the marker gene signatures specific to immune cells" or "we performed an integrated analysis of previously published zebrafish single-cell RNA-seq datasets, namely, GSE230044 (Jones et al., 2023), GSE232302 (Matz et al., 2023), and GSE135767 (Gu et al., 2020), to characterize the marker gene signatures specific to immune cells (Figure 5A). We also resolved the dynamics of cell type-specific signatures in our bulk RNA-seq data (Figure 5B; Supplementary Table S5). We found that *E. piscicida* infection induced the transcription of signature genes for macrophages, neutrophils, and dendritic cells, while this was substantially suppressed by BG pretreatment (Figure 5B). Meanwhile, by analyzing the transcription of signature genes for T lymphocytes (e.g., Th17, Treg, Th1, Th2, and NK T cells), we found that *E. piscicida* infection inhibited the expression of these genes, while BG pretreatment counteracted the immunosuppressive phenotype of these signature genes (Figure 5B). Specifically, by performing qPCR analysis to validate the transcription of these genes, we confirmed that the marker gene *il17a/f3* specifically expressed in Th17 cells was significantly downregulated after *E. piscicida* infection, while BG pretreatment significantly reversed this (Figure 5C).

To further validate that the function of Th17 cells is tightly regulated by the BG-amplified lectin pathway during *E. piscicida* challenge, we analyzed the transcription of *il17a/f3* with and without AMY-101 treatment (Figure 5D). Upon comparison with the elevated transcripts of *il17a/f3* in the BG-pretreated *E. piscicida*-infected group, blocking the lectin pathway with AMY-101 significantly inhibited the transcription of *il17a/f3* (Figure 5D). In addition, by performing flow cytometry to validate the efficiency of rabbit anti-zebrafish IL-17A/F3$^+$ antibody (Supplementary Figure S3), we confirmed that *E. piscicida* infection reduced the population of Th17 cells (IL-17A/F3$^+$) in zebrafish posterior intestine (Figure 5E, F); however, BG pretreatment ensured maintenance of the proportion of Th17 cells, while this effect was impaired with AMY-101 treatment (Figure 5E, F). Taken together, these results suggest that the intestinal inflammation induced by *E. piscicida* infection coincided with the impaired proportion of

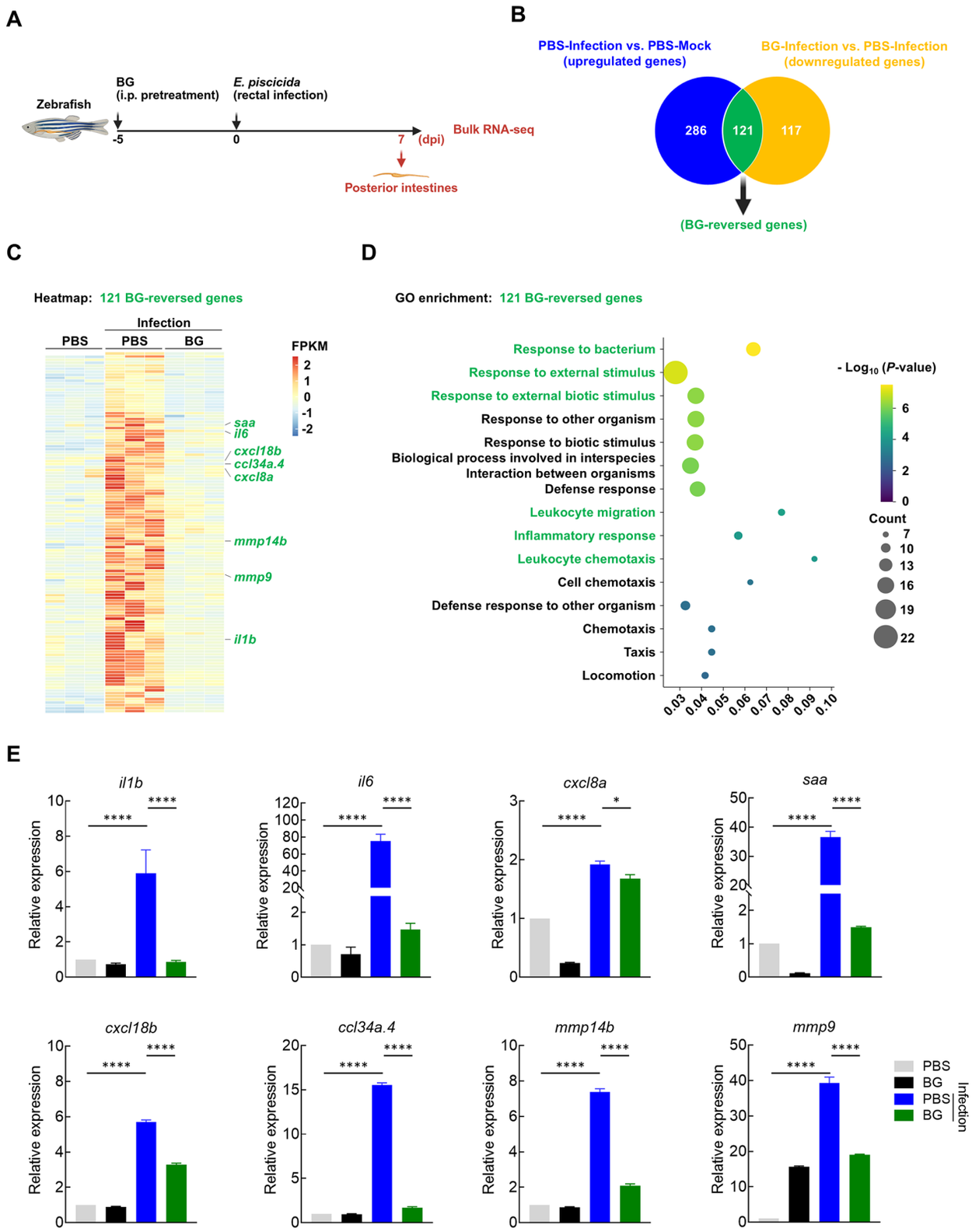


**Figure 2 BG pretreatment reverses the intestinal inflammatory responses during bacterial infection**

A: Workflow of RNA-seq analysis in PBS- and BG-pretreated zebrafish posterior intestine following *E. piscicida* infection. B: Venn diagram of DEGs. C: Heatmap showing 121 BG-reversed genes identified by RNA-seq. D: GO enrichment plot of BG-reversed signaling pathways. E: Representative BG-reversed genes are analyzed by qPCR. Data are from three independent replicate experiments and shown as mean±SD, which were analyzed by two-way ANOVA with multiple comparisons. *: $P<0.05$; ****: $P<0.0001$.

Th17 cells, whereas BG pretreatment could reverse this immunosuppressive effect in a lectin pathway–dependent manner.

**Th17-cell activation is required for preventing infectious enteritis**

To further investigate whether the maintenance of the

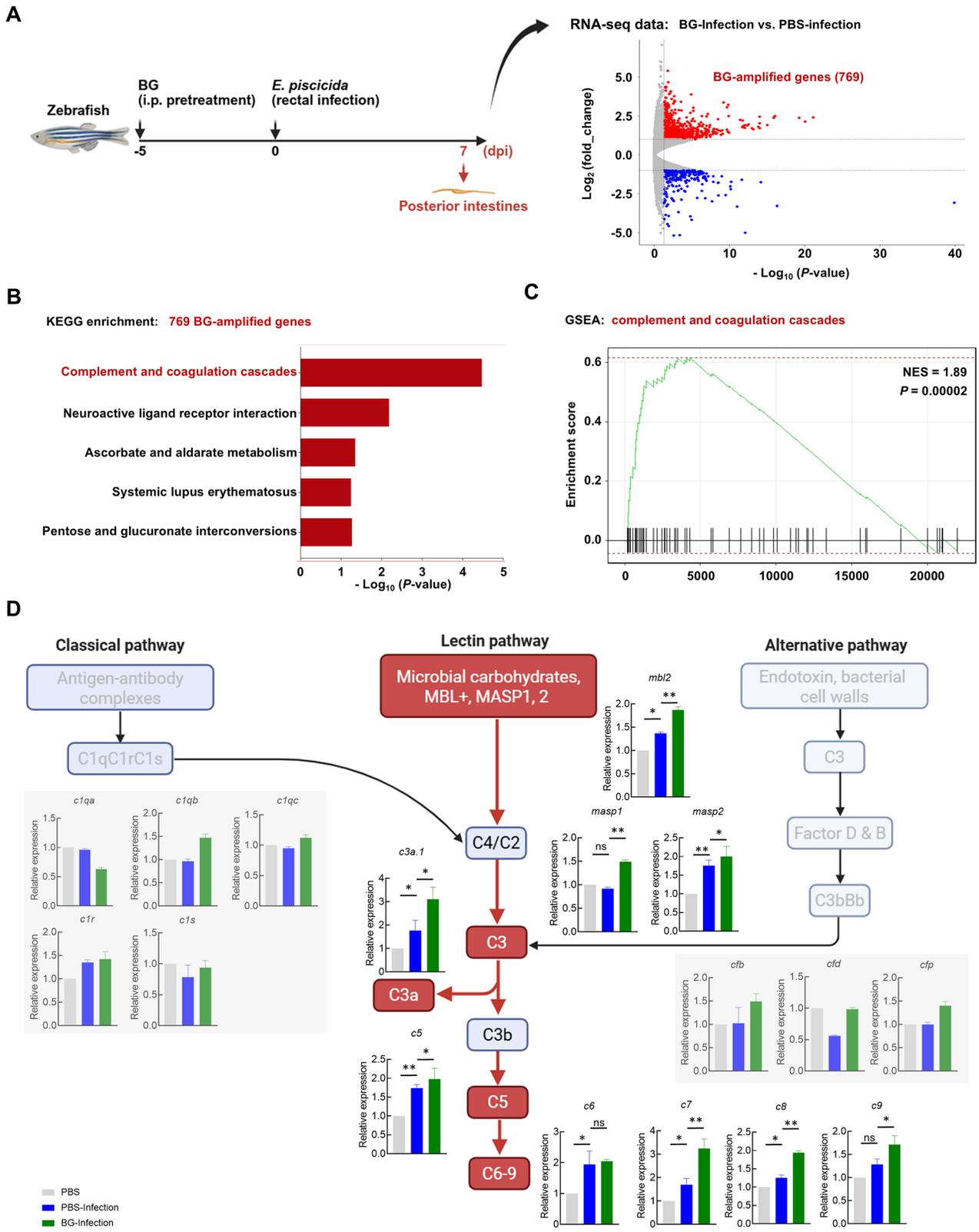


**Figure 3 BG pretreatment amplifies lectin pathway-mediated complement cascade signaling**

A: Workflow of bulk RNA-seq in PBS- and BG-pretreated zebrafish posterior intestines following *E. piscicida* infection; a volcano plot of DEGs is also presented. Red dots indicate upregulated genes, while blue dots indicate downregulated ones. B: KEGG enrichment plot of BG-amplified genes; the plots are ranked by *P*-value. C: GSEA enrichment of "complement and coagulation cascades" in BG-amplified genes. D: Schematic diagram of the complement cascades. The genes encoding components of the BG-amplified complement cascades are analyzed in zebrafish posterior intestines by qPCR. Data are from three independent replicate experiments and shown as mean±SD, which were analyzed by two-way ANOVA with multiple comparisons. *: $P<0.05$; **: $P<0.01$; ns: Not significant.

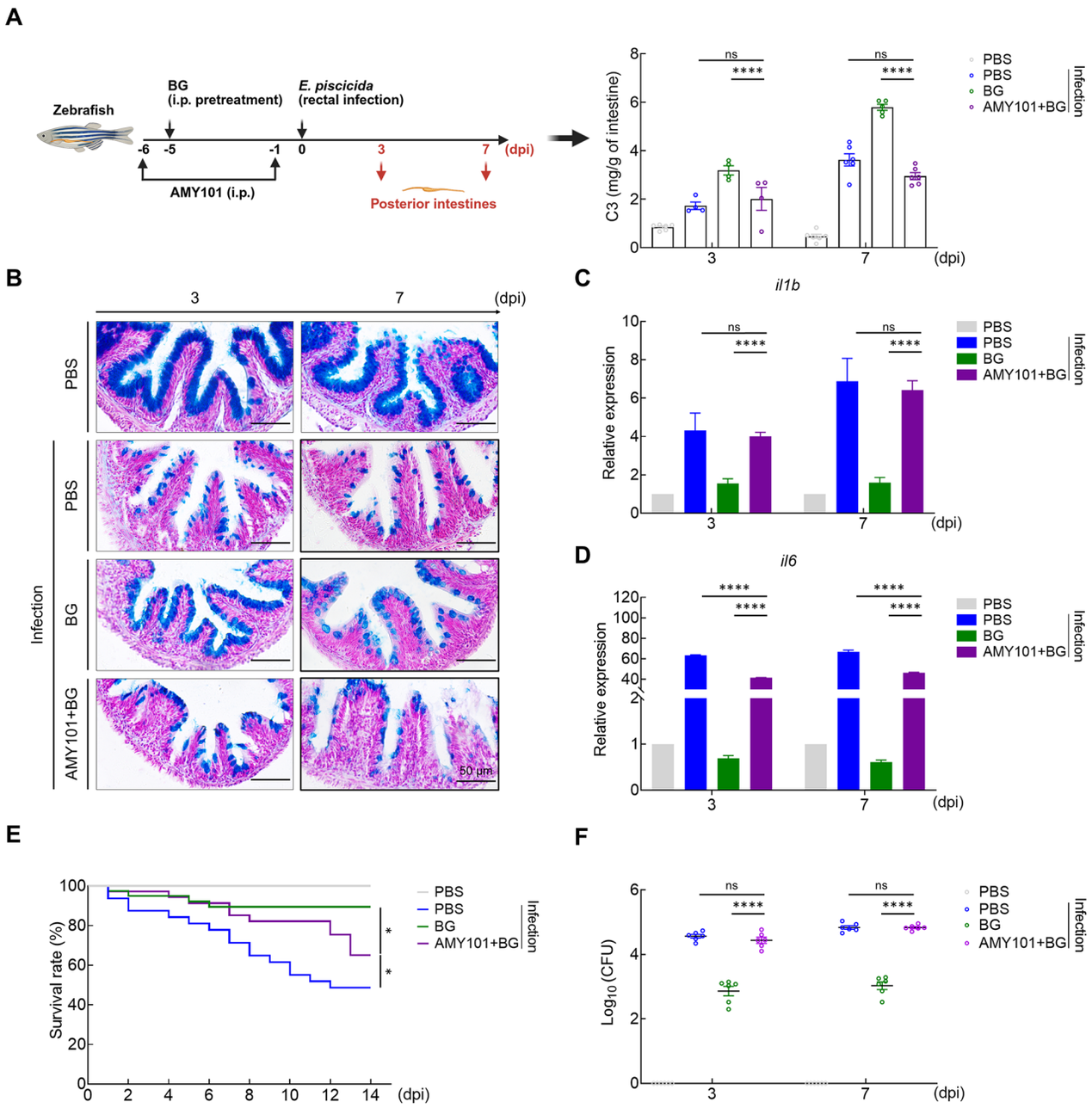


**Figure 4 BG-amplified lectin pathway is essential for preventing infectious enteritis**

A: Schematic model of AMY-101 administration before BG pretreatment and bacterial challenge in zebrafish. The C3 levels in posterior intestines were analyzed by ELISA. B: Histopathological analysis of posterior intestine in PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without AMY-101 administration. Scale bar: 50 μm. C: Transcription of *il1b* in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without AMY-101 administration. D: Transcription of *il6* in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without AMY-101 administration. E: Survival rate (%) analysis over the course of the experiment, with or without AMY-101 administration ($n$=25). F: Bacterial loads in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without AMY-101 administration. Data are from three independent replicate experiments and shown as mean±SD, which were analyzed by two-way ANOVA with multiple comparisons. *: $P$<0.05; **: $P$<0.01; ****: $P$<0.0001; ns: Not significant.

population of Th17 cells achieved by BG pretreatment is essential for alleviating infectious enteritis, we pretreated the zebrafish with GSK805, an inhibitor of Th17-cell differentiation (Nakamoto et al., 2019; Yang et al., 2024; Zhang et al., 2021), and compared its effects with the findings in the BG-pretreated *E. piscicida*-infected group (Figure 6A). Upon comparison with the findings in this latter group, we found that the transcription of *il17a/f3* (Figure 6B) and the proportion of Th17 cells (Figure 6C, D) were significantly attenuated when treating the zebrafish with GSK805 (Figure 6B–D). Meanwhile, treating the zebrafish with GSK805 notably had no effects on *c3a.1* transcription or intestinal C3 production (Supplementary Figure S4A, B), suggesting that the BG-amplified complement pathway might act upstream of Th17 cells. Importantly, we also found that treating the zebrafish with GSK805 abrogated the BG pretreatment-associated protective effects against infectious enteritis, resulting in reductions in goblet cells and mucus secretion in the zebrafish posterior intestine

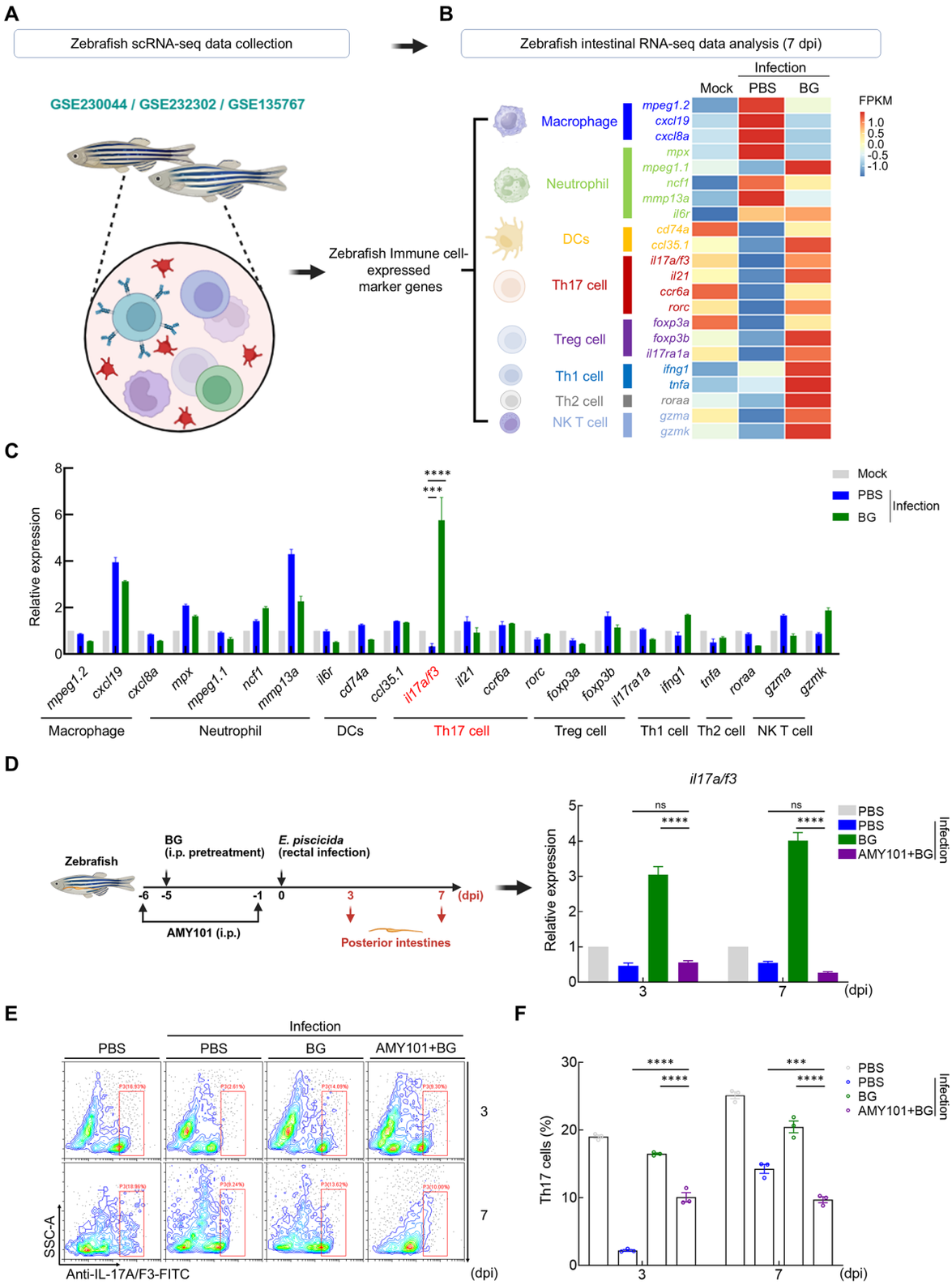


**Figure 5 BG-amplified lectin pathway is essential for maintaining the function of Th17 cells during *E. piscicida* infection**

A: Marker genes expressed by zebrafish immune cells retrieved from scRNA-seq data sets. B: Color-coded heatmap of immune cell marker genes identified in bulk RNA-seq. C: Transcription of the marker genes in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection. D: Gene expression of *il17a/f3* in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without AMY-101 administration. E: Flow cytometry analysis of Th17 cells in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without AMY-101 administration. Red box indicates IL-17A/F3$^+$ Th17 cells. F: Statistical analysis of the proportion of Th17 cells as indicated in panel E. Data are from three independent replicate experiments and shown as mean±SD, which were analyzed by two-way ANOVA with multiple comparisons. ***: $P$<0.001; ****: $P$<0.0001; ns: Not significant.

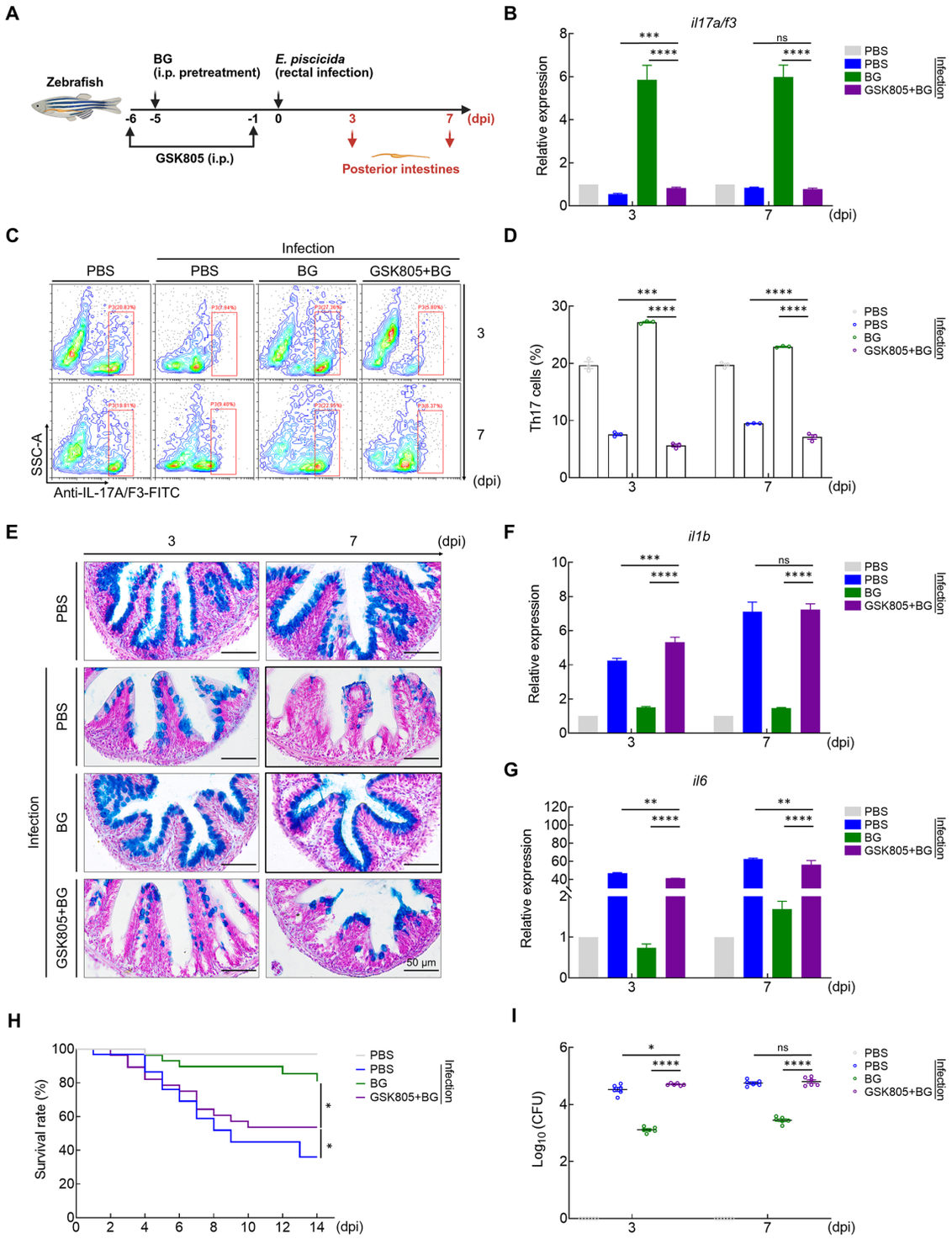


**Figure 6 Activation of Th17 cells is required for BG-associated protection against infectious enteritis**

A: Schematic model of GSK805 administration before BG pretreatment and bacterial challenge in zebrafish. B: Gene expression of *il17a/f3* in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without GSK805 administration. C: Flow cytometry analysis of Th17 cells in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without GSK805 administration. Red box indicates IL-17A/F3$^{+}$ Th17 cells. D: Statistical analysis of the proportion of Th17 cells as indicated in panel C. E: Histopathological analysis of posterior intestine in PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without GSK805 administration. Scale bar: 50 μm. F: Gene expression of *il1b* in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without GSK805 administration. G: Gene expression of *il6* in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without GSK805 administration. H: Survival rate (%) analysis over the course of the experiment, with or without GSK805 administration ($n$=25). I: Bacterial loads in posterior intestine of PBS- and BG-pretreated zebrafish during *E. piscicida* infection, with or without GSK805 administration. Data are from three independent replicate experiments and shown as mean±SD, which were analyzed by two-way ANOVA with multiple comparisons. $^{*}$: $P$<0.05; $^{**}$: $P$<0.01; $^{***}$: $P$<0.001; $^{****}$: $P$<0.0001; ns: Not significant.

(Figure 6E; Supplementary Figure S5). Moreover, the BG-associated downregulation of proinflammatory genes (*il1b*, *il6*) in the posterior intestine during *E. piscicida* infection was also rescued by GSK805 treatment (Figure 6F, G). Notably, treating the zebrafish with GSK805 could also neutralize the survival advantage and bacterial clearance capacity conferred by BG pretreatment (Figure 6H, I). Taken together, these results suggest that BG pretreatment could amplify the lectin pathway to maintain the function of Th17 cells, which play essential roles in protecting against enteritis induced by *E. piscicida* infection in zebrafish (Figure 7).

## DISCUSSION

In this study, by establishing a zebrafish model of infectious enteritis induced by infection with *E. piscicida* and pretreating it with BG, we support the assertion that the intestinal lectin pathway (including but not limited to C3) is activated by BG pretreatment in zebrafish. This suggests that the lectin pathway might be a key mediator of BG-associated trained immunity. Our findings also suggest a central role of the intestinal lectin pathway in maintaining the function of Th17 cells to combat infectious enteritis in zebrafish (Figure 7). In recent years, emerging studies have revealed that BG pretreatment can induce trained immunity (Sadeghi & Divangahi, 2024; Santosa & Sun, 2023). For example, in mammalian models, BG treatment was shown to initiate Dectin-1-dependent metabolic reprogramming, which was characterized by enhanced glycolytic flux, to promote epigenetic changes through histone acetylation/methylation modifications, ultimately potentiating proinflammatory gene expression in innate immune cells during secondary challenges (Cheng et al., 2014; Gomes et al., 2023; Quintin et al., 2012). Consistent with our results, recent reports have also provided evidence that the complement system is involved in mediating localized trained immunity effects. For example, Earhart et al. (2024) revealed a critical mechanism in that C3-C3aR signaling cascades modulate the effects of trained immunity in alveolar macrophages. In addition, Friscic et al. obtained evidence of trained immunity in synovial fibroblasts and revealed that C3 and C3aR are required to

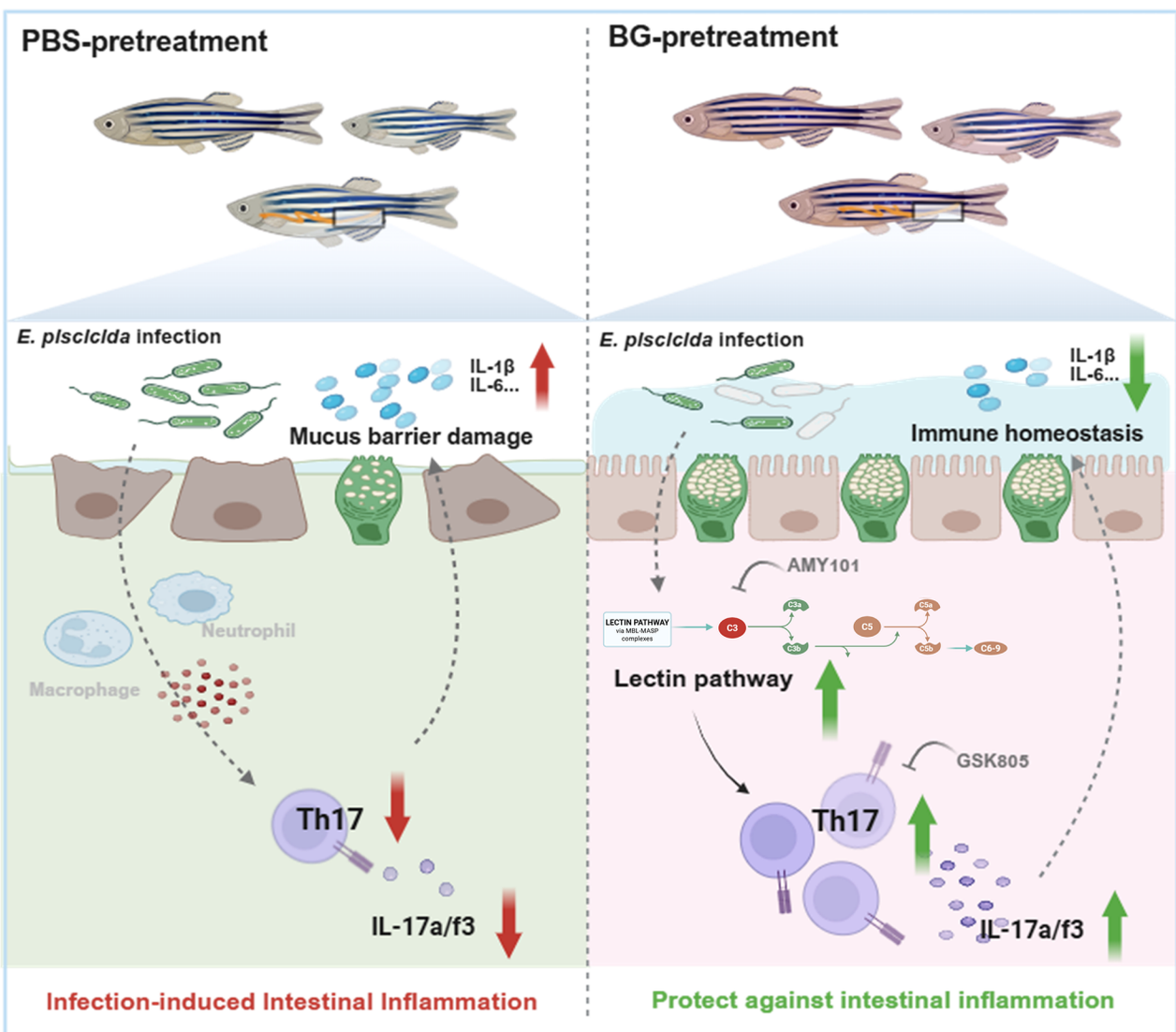


**Figure 7 Proposed mechanism by which the BG-amplified lectin pathway maintains the function of Th17 cells in zebrafish**

BG pretreatment amplifies the lectin pathway-associated complement cascades, contributing to restoration of the cellular function of intestinal Th17 cells, thereby preventing infectious enteritis in the zebrafish model.

trigger the sustained inflammatory response that accompanies damage to articular bone (Friščić et al., 2021). Elsewhere, Wu et al. proposed a novel role of C3 activity in trained immunity, namely, in promoting the site-specific recurrence of inflammation (Wu et al., 2024). These results suggest that the complement pathway might play a critical role in trained immunity. As such, future investigations could focus on delineating the lectin pathway-mediated trained immunity through single-cell epigenomic mapping of complement genes in intestinal immune cells and tissue-resident cells, and on characterizing the metabolic-epigenetic crosstalk that regulates the plasticity of complement expression, which is also a critical issue for maintaining gut homeostasis.

Regarding the cellular signaling that controls intestinal inflammation, our results suggest that *E. piscicida* infection could impair the function of intestinal Th17 cells, indicating its potential function in protecting against infectious enteritis in zebrafish (Figure 7). In mammals, an increasing number of studies have demonstrated that tissue-resident commensal-induced Th17 cells display an anti-inflammatory phenotype capable of regulating effector T-cell responses, which plays essential roles in alleviating colitis (Schnell et al., 2023; Xing et al., 2021). Meanwhile, the dynamic balance between Treg cells and Th17 cells is also critical for maintaining gut homeostasis (Jia et al., 2024; Lin et al., 2023). However, in teleost fish, the functional evidence of Th17 cells was primarily revealed. For example, during *Aeromonas hydrophila* infection in rainbow trout, the proportion of IL-22$^+$ cells was significantly increased in the intestines (Hu et al., 2019), while during its infection in common carp, the expression of genes encoding Th17 cell-related cytokines, including *il-17a/f2*, *il-17a/f3*, and *il-17b*, was induced in the intestines (Sun et al., 2020). Moreover, in a *Mycobacterium marinum*-infected zebrafish model, transcriptional upregulation of *il17a/f2* was also revealed in CD4-1$^+$ lymphocytes (Yoon et al., 2015). Our previous study also found that BG pretreatment could prevent intestinal inflammation induced by *E. piscicida* infection in turbot. That finding, together with our result in zebrafish, suggests that intestinal Th17 cells might be reactivated by BGs (Yang et al., 2024). Against this background, further studies should characterize the function of Th17 cells in teleost fish in pursuit of a deeper understanding of their roles in maintaining gut homeostasis. Our results also indicate that the signature genes for macrophages, neutrophils, and dendritic cells were comparatively upregulated transcriptionally after *E. piscicida* infection, whereas BG pretreatment could effectively mitigate their responses in zebrafish. This might be attributable to the rapid migration of innate immune cells to sites of infection; however, the excessive activation of these cells could also exacerbate inflammation and result in tissue damage (Grayfer et al., 2018; Wang et al., 2024b). Thus, future work should focus on clarifying the molecular mechanisms by which these innate immune cells facilitate BG-induced trained immunity and the roles of BG in maintaining functional Th17 cells, which might offer novel therapeutic strategies against infectious diseases in aquaculture and beyond.

Our study revealed the notable finding that BG pretreatment induced amplification of the lectin pathway, highlighting the critical crosstalk between complement signaling and Th17 cells in maintaining gut homeostasis in zebrafish (Figure 7). However, it is plausible that the microbiota also plays a role in the observed effects of BG pretreatment on Th17-cell function and the prevention of enteritis, since previous studies revealed the mechanism by which the gut microbiota shapes intestinal immune responses, particularly through the induction and regulation of Th17 cells (Alexander et al., 2022; Ang et al., 2020). Moreover, segmented filamentous bacteria have been shown to promote Th17-cell differentiation, which can either protect against pathogens or exacerbate inflammation (Ivanov et al., 2009; Shih et al., 2014). Evidence has also suggested that microbiota can influence trained immunity, with microbial metabolites modulating innate immune memory (Yang et al., 2020). There is thus a need for further exploration of the BG pretreatment–induced changes in microbiota composition and the specific microbial metabolites that play a role in modulating the lectin pathway and Th17-cell responses.

In summary, our study identified the lectin pathway and Th17 cells as key mediators following BG pretreatment and characterized their role in preventing enteritis induced by *E. piscicida* infection in zebrafish. This might provide a novel platform for deepening our understanding of the effectiveness of vaccines, adjuvants, and immune system potentiators (Figure 7). Future work should focus on investigating the epigenetic and metabolic changes induced by BG pretreatment in zebrafish intestinal immune cells or tissue-resident cells and uncovering the participation of the microbiota in infectious enteritis.

## DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request. Bulk RNA-sequencing data have been deposited in GEO under accession code GSE285528 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE285528) and GSA under accession code CRA026239 (https://ngdc.cncb.ac.cn/gsa/browse/CRA026239). The data have also been uploaded to Science Data Bank under DOI: 10.57760/sciencedb.j00139.00253.

## SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

## AUTHORS' CONTRIBUTIONS

Conceptualization, D.Y.; Investigation, Y.Z., X.M., and Z.W.; Formal analysis, Y.Z., J.Y., and D.Y.; Methodology, Y.Z., X.M., and D.Y.; Resources, D.Y., Q.L., Y.Z., and Z.W.; Supervision, D.Y.; Writing–Original Draft, Y.Z. and Z.W.; Writing–Review & Editing, D.Y. All authors read and approved the final version of the manuscript.

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