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ORIGINAL RESEARCH

Tnfrsf1b Protects Zebrafish Against *Klebsiella pneumoniae* Infection

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Objective: *Klebsiella (K). pneumoniae* is an opportunistic pathogen that causes severe infections in the lungs, urinary tract, and liver, yet its pathogenesis remains unclear. *Tnfrsf1b*, a member of the tumor necrosis factor receptor superfamily, is associated with inflammation and tissue damage. The objective of this study was to investigate the functional role of *tnfrsf1b* in zebrafish during *K. pneumoniae* infection and *tnfrsf1b* should be considered a candidate target for further investigation.

Methods: A *tnfrsf1b*-knockout zebrafish line was generated using CRISPR/Cas9 technology. Both wild-type and mutant zebrafish were infected with the hypervirulent *K. pneumoniae* strain NTUH-K2044. Phenotypic changes, immune cell recruitment, and cytokine production were assessed using Alcian blue staining, Sudan Black B staining, neutral red staining, qRT-PCR, and ELISA. Rescue experiments were performed by injecting capped *tnfrsf1b* mRNA into mutants and wild-type zebrafish embryos. Pharmacological inhibition was tested using the TNFR inhibitor R7050.

Results: Compared with wild-type zebrafish, *tnfrsf1b*-knockout mutants exhibited significantly higher malformation and mortality rates, increased recruitment of macrophages and neutrophils, and elevated levels of pro-inflammatory cytokines (TNF-α, IL-1β). Similar phenotypes were observed in R7050-treated zebrafish. *K. pneumoniae* infection further exacerbated these immune dysfunctions in *tnfrsf1b* mutants. Injection of capped *tnfrsf1b* mRNA into mutants partially rescued developmental and immune defects, while overexpression in wild-type zebrafish conferred protection against *K. pneumoniae*-induced damage.

Conclusion: *tnfrsf1b* plays a critical role in regulating host immune responses and protecting zebrafish from *K. pneumoniae* infection. Targeting the *tnfrsf1b* signaling pathway may represent a promising therapeutic approach for managing infections caused by hypervirulent *K. pneumoniae*.

Keywords: *tnfrsf1b*, *Klebsiella pneumoniae*, zebrafish, inflammation, infection susceptibility

Introduction

Tumor necrosis factor-alpha (TNF-α), a member of the TNF superfamily, can be produced by many immune cells under pathologically related conditions and plays different roles.[1–4] For example, TNF-α is involved in many biological processes, including autoimmunity,[4] late tumor development,[5] and arthritis.[6] TNFR1 and *tnfrsf1b* are TNF-α signal receptors. TNFR1 is expressed in most cell types; however, *tnfrsf1b* is restricted primarily to endothelial, epithelial, and subsets of immune cells.[7] The TNFR1 signaling pathway is involved in inflammation and apoptosis pathways, whereas the *tnfrsf1b* signaling pathway is involved in the anti-inflammatory response and initiation of apoptosis.[8] Inhibiting TNFR1 alters autoimmune-related homeostasis; however, *tnfrsf1b* initiates repair after injury[9] and participates in

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developmental processes, such as heart development.[10] Recently, *tnfrsf1b* was reported to play a vital role in treating many diseases as a new drug candidate,[11] but the functional mechanism of *tnfrsf1b* remains unclear.

Klebsiella (K). pneumoniae, an opportunistic pathogen, is widely distributed in nature and in almost all hospitals.[12] Under normal circumstances, *K. pneumoniae* does not result in pathological infections;[13] however, when the body's immunity is low, *K. pneumoniae* infection can cause pneumonia, urinary tract infection, liver abscess, bacteremia, and other symptoms.[13] *K. pneumoniae* infection can become serious and even life-threatening, especially for patients in the intensive care unit or older people.[14] Unfortunately, there are still no effective drugs or methods to treat this infection. Previous research has focused on the virulence of pathogenic bacteria and the response of the host immune system.[15] Pro-inflammatory responses have been found to play important roles in the elimination of *K. pneumoniae*.[16] Hu and Cai reported that immunodeficient mice can be easily infected by *K. pneumoniae*.[17] The clearance of *K. pneumoniae* is significantly reduced and the susceptibility to infection is increased in mice deficient in TNFR1 and CXCL15.[18,19]

In recent years, the clinical significance of *K. pneumoniae* has dramatically increased due to the emergence of hypervirulent strains, such as NTUH-K2044, which are associated with liver abscess, endophthalmitis, and disseminated infections even in otherwise healthy individuals. These strains often exhibit enhanced capsule production, increased invasiveness, and resistance to phagocytosis, leading to poor clinical outcomes. At the same time, the global rise of antimicrobial resistance in *K. pneumoniae*, including carbapenem-resistant and extended-spectrum β-lactamase (ESBL)-producing isolates, has created serious challenges in treatment. The dual threat of hypervirulence and multidrug resistance makes *K. pneumoniae* a pathogen of urgent clinical concern, underscoring the need to better understand host–pathogen interactions and identify new candidate targets such as *tnfrsf1b*.

The zebrafish is a model organism[20] that has been widely used to study the pathogenicity, virulence, and drug resistance of bacteria, as well as host reactions to bacterial infection.[21–24] Our previous study demonstrated that *K. pneumoniae* infection leads to intestinal inflammation and microbial dysbiosis in adult zebrafish, using clinically isolated strains with varying virulence levels.[25] However, the host genetic determinants contributing to susceptibility or resistance to *K. pneumoniae* infection remained largely unexplored. The current study is fundamentally distinct from the previous work in the following aspects: (1) Mechanistic Focus: While the earlier study centered on comparative pathophysiological outcomes caused by different *K. pneumoniae* strains, the current research explores the functional role of *tnfrsf1b* signaling in host-pathogen interactions. (2) Genetic Manipulation: We employed a CRISPR/Cas9-generated *tnfrsf1b*-knockout zebrafish line, which allows us to dissect the immunological role of *tnfrsf1b* in infection susceptibility and inflammatory regulation. (3) Therapeutic Relevance: This study demonstrates the protective effects of *tnfrsf1b*-capped mRNA and identifies *tnfrsf1b* as a potential therapeutic target against hypervirulent *K. pneumoniae*. (4) Functional Rescue and Pharmacological Inhibition: We further validate our findings by both genetic rescue (mRNA injection) and pharmacological inhibition (R7050), revealing mechanistic depth that was not addressed in the previous publication. The TNF-α receptors TNFR1 and *tnfrsf1b* both exist in zebrafish, but their function is still not completely understood. In this study, the *tnfrsf1b* gene in zebrafish was knocked-out using CRISPR/Cas9 technology, and then the wild-type (WT) and *tnfrsf1b*$^{-/-}$ zebrafish were infected with *K. pneumoniae* strain NTUH-K2044 to study the function of *tnfrsf1b* in the interaction of *K. pneumoniae* and host. The immune factors and immune cells and partial gene expression patterns were investigated. This work may provide a theoretical basis for the clinical treatment of *K. pneumoniae* infection.

Materials and Methods

Fish Maintenance

All zebrafish embryo experiments were performed in compliance with national and institutional animal welfare regulations. Adult AB-strain zebrafish were maintained in a recirculating water system under a controlled 14-hour light and 10-hour dark cycle at 28 °C, and fed three times daily. For embryo collection, male and female fish were placed together in mating tanks the night before, and fertilization occurred within an hour of light onset the following morning. Collected embryos were incubated in 10 cm Petri dishes containing egg water supplemented with 0.3% methylene blue and maintained in a 28 °C incubator under the same 14/10 h light/dark schedule. Embryos were sampled at 4–6 days post-fertilization (dpf) for whole-mount Alcian blue staining. At 5 dpf, additional samples were harvested for behavioral testing and qRT-PCR analysis.

Generation of tnfrsf1b Mutant Zebrafish

The *tnfrsf1b* knockout zebrafish line was established using CRISPR-Cas9 genome editing. A target sequence within the first exon was selected, and the corresponding guide RNA (gRNA) was synthesized based on established protocols. To induce mutations, zebrafish embryos at the one-cell stage were co-injected with 100 pg of gRNA and 300 pg of Cas9-capped mRNA. Following injection, embryos were cultured at 28.5 °C in E3 medium (composed of 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$). At 2 days post-fertilization (dpf), genomic DNA was extracted from three pools of 10 embryos each. A 194 bp DNA fragment encompassing the target region was amplified by PCR (primer sequences provided in Table S1). To assess mutation efficiency, 7 μL of PCR product was digested with T7 Endonuclease I (New England Biolabs) at 37 °C for 3 hours, and cleavage efficiency was quantified using NIH ImageJ software. PCR amplicons were cloned into the pMD-19T vector (Takara) for Sanger sequencing. To confirm germline transmission, founder (F0) zebrafish were raised to adulthood and crossed with wild-type fish. Genomic DNA from 10 F1 embryos per cross was analyzed by enzymatic digestion (data not shown). Sibling F1 carriers of heritable mutations were grown to maturity, and individual mutants were re-verified via PCR and sequencing of fin-derived DNA. Homozygous *tnfrsf1b* mutants were obtained by mating male and female F1 fish carrying the same deletion. A stable mutant line harboring a 5 bp deletion in *tnfrsf1b* was successfully established.

Bacterial Immersion Experiments

Bacterial cultures were grown to the logarithmic phase, and the concentration of the suspension was standardized by measuring OD600. A conversion curve between OD600 and colony-forming units (CFU) was established in preliminary experiments, and the immersion infection dose was adjusted to 5×10^6 CFU/mL accordingly. To further assess infection progression, bacterial loads in zebrafish larvae were determined at designated time points post-infection by homogenizing groups of 10 larvae, plating serial dilutions on LB agar, and counting colonies after overnight incubation at 37 °C.

The *K. pneumoniae* immersion infection model was modified from previously established protocols. Bacteria were cultured to the logarithmic growth phase (OD600 = 0.4–0.6), then collected by centrifugation, washed, and resuspended in sterile E3 medium. At 3 days post-fertilization (dpf), both wild-type and *tnfrsf1b* mutant zebrafish larvae were rinsed with fresh E3 medium and allocated into sterile 6-well plates, with 10 larvae placed in each well. Each well was filled with 8 mL of bacterial suspension at a concentration of 5 × 10□ CFU/mL. Larvae were maintained in this suspension at 28 °C for 72 hours. After incubation, the larvae were collected and processed for subsequent experimental procedures. Each immersion assay was independently repeated six times, with a total of 75 larvae assigned to each experimental group.

Staining of Neutrophils and Macrophages in Zebrafish

Zebrafish larvae were initially fixed in 2% paraformaldehyde and gently agitated for 2 hours, followed by overnight staining with Sudan Black B (Solarbio, USA), a lipid-based dye that specifically labels neutrophil granules. After an 8-hour co-incubation with bacteria, 10 larvae from each group were fixed in 4% paraformaldehyde, rinsed with phosphate-buffered saline containing Tween, and again stained with Sudan Black B to visualize neutrophils. Following a 70% ethanol wash, neutrophil recruitment was examined microscopically. The preparation of the staining solution followed a previously published protocol.

For assessing macrophage phagocytic activity, groups of 10 zebrafish larvae were placed in 12-well plates containing 2.5 mg/L neutral red dye solution (Solarbio, China). This dye accumulates in macrophages as red puncta due to active endocytosis. After 8 hours of bacterial exposu*re, the larvae were anesthetized, and macrophage phagocytosis of neutral red was observed under a microscope.*

RNA Isolation, cDNA Synthesis, and qRT-PCR

Total RNA was isolated from pools of over 25 zebrafish larvae using TRIzol reagent (Invitrogen), followed by reverse transcription into complementary DNA (cDNA) using Superscript III Reverse Transcriptase (Invitrogen, USA). Quantitative real-time PCR (qRT-PCR) was conducted on an ABI Step-One Plus system employing the SYBR Green

detection method (TaKaRa, Dalian, China). The thermal cycling conditions included 40 amplification cycles consisting of denaturation at 95 °C for 10 seconds and annealing/extension at 58 °C for 30 seconds. Each reaction was run in triplicate using independently collected biological replicates. Gene expression levels were normalized to the reference gene β-actin, and relative quantification was determined using the $2^{-\Delta\Delta CT}$ method. Primers used for qRT-PCR (listed in Table S1) were designed to span exon-exon junctions using Primer Express 5.0 software and were evaluated for secondary structures, such as self-dimers and hairpins, using Oligo Analyzer.

Inflammatory Factor Analysis by Enzyme-Linked Immunosorbent Assay (ELISA)

A total of 50 zebrafish larvae were homogenized in 0.5 mL of 0.9% sodium chloride solution using a tissue homogenizer. The resulting homogenate was centrifuged at 3000 rpm for 10 minutes at 4 °C, and the supernatant was collected for subsequent cytokine analysis, including interleukin-1α (IL-1α), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α). Enzyme-linked immunosorbent assay (ELISA) was conducted following the manufacturer's protocols (Jianchengbio, Nanjing, China). Each sample and standard was analyzed in triplicate to ensure accuracy.

Statistical Analyses

All data are expressed as mean ± standard deviation (SD). Statistical comparisons between two groups were performed using Student's *t*-test, while multiple group comparisons were analyzed by one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Each experiment was independently repeated a minimum of three times. A p value less than 0.05 was regarded as statistically significant.

Results

Bioinformatics of Zebrafish tnfrsf1b and Construction of Mutation

We first analyzed the protein sequences of zebrafish *tnfrsf1b*, comparing with the homologous protein from human and mice. Although zebrafish *tnfrsf1b* shared only 24.15% and 21.8% amino acid sequence identity with the homologues of human and mice, respectively (Figure 1A), the functional domains of the three proteins are conserved, composed of four TNFR domains and a transmembrane region (Figure 1B).

The zebrafish *tnfrsf1b* gene contains 9 exons (Figure 1C). To construct a mutant strain with complete gene inactivation, we designed the gRNA site in the first exon region of *tnfrsf1b* gene and obtained a homozygous mutant with 5 bases depletion (Figure 1C).

Phenotype Analysis of Zebrafish tnfrsf1b Mutant

We first investigated the impacts of *tnfrsf1b* knockout on development by observing egg production, fertilization and hatchling. The homozygous zebrafish *tnfrs1b* mutant reduced egg production of female zebrafish (Figure 2A). Fertilization rate of the eggs also showed a ~20% decrease (Figure 2B). Hatchling rate, a key step for development, was also affected by absence of *tnfrsf1b*, while introduction of *tnfrsf1b* mRNA also partially reversed the impacts by mutation (Figure 2C–H).

In addition, we observed the impacts of *tnfrsf1b* knockout on death and malformation rate of larvae fish. *tnfrsf1b* mutation caused significant increase of death rate at 96 hpf, which could be partly reversed by introduction of *tnfrsf1b* mRNA (Figure 2D and G). The *tnfrs1b* mutant exhibited a malformation rate is as high as 34% at 72hpf. The impacts of mutation could also be reversed by introduction of *tnfrsf1b* mRNA from 48–96 hpf (Figure 2E–I). The results indicate that the *tnfrs1b* mutant has obvious reproductive and developmental defected phenotype.

Zebrafish tnfrsf1b Mutant Has an Evident Immune Dysfunction Phenotype

Neutral red staining showed that the number of macrophages in the *tnfrsf1b* mutant significantly increased compared with that in the WT (Figure 3A and B). Sudan black staining showed that the number of neutrophils in the *tnfrsf1b* mutant also significantly increased (Figure 3C and D). Using qRT-PCR, we found that the mRNA level of *tnf-α, il-1β*, and *il-6* was significantly upregulated, while the mRNA level of *tnf-β, ccl2, ccl3, cxcl2*, and *ifng1* did not change significantly (Figure 3E).

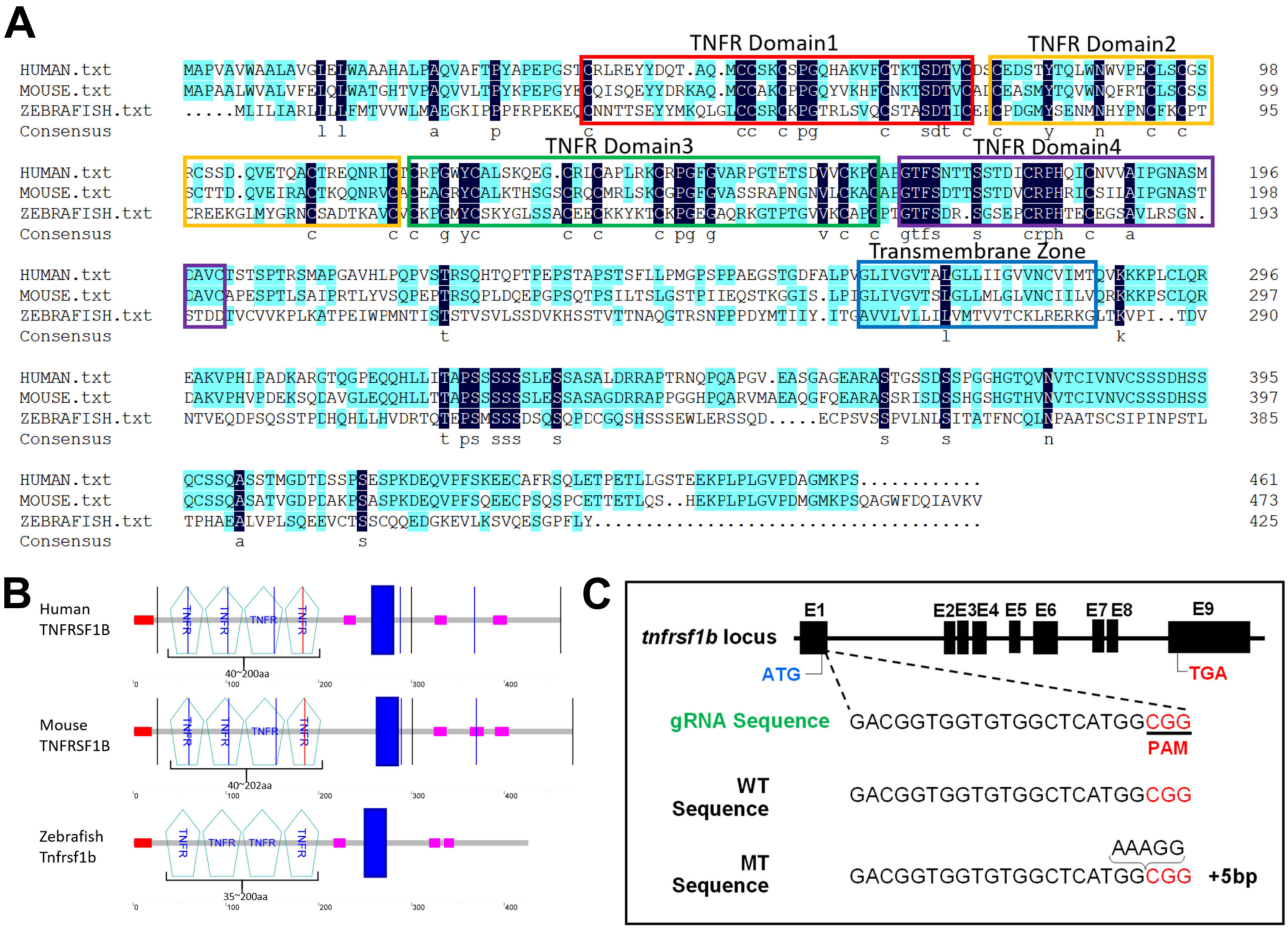

Figure 1 Evolutionary conservation and mutant of zebrafish tnfrs1b. (**A**) Conservative comparison of human, mouse, and zebrafish amino acid sequences. (**B**) Conservation type comparison of the functional domain of human, mouse, and zebrafish tnfrs1b. (**C**) The structure of zebrafish tnfrs1b gene and the acquisition of mutants. Red box indicates TNFR Domain 1, yellow box indicates TNFR Domain 2, green box indicates TNFR Domain 3, the purple box indicates TNFR Domain 4, and blue box represents the Transmembrane Zone.

ELISA results showed that the protein expression of TNF-α and IL-1β was significantly increased in the *tnfrsf1b* mutant (Figure 3F). These results indicate that there is an evident immune dysfunction phenotype in the *tnfrsf1b* mutant.

Treatment of WT Zebrafish with the TNFR Inhibitor R7050 Leads to Evident Immune Dysfunction

R7050 is an inhibitor of TNFR, which can inhibit the activities of TNFR1 and *tnfrsf1b*. After treating WT zebrafish with R7050, the malformation rate of zebrafish was significantly increased in the 72 hpf (Figure 4A). ELISA showed that *tnfrsf1b* protein expression was inhibited, as expected. Furthermore, the number of macrophages (Figure 4B and C) and neutrophils (Figure 4D and E) was also significantly increased. qRT-PCR revealed that tnfa, tnb, il1b, il6, ccl2, ccl3, and cxcl2 were significantly upregulated (Figure 4F). In addition, the protein expression of TNF-α and IL-1β increased significantly after R7050 treatment (Figure 4G). These results indicate that the treatment of WT zebrafish with the TNFR inhibitor R7050 leads to significant immune dysfunction.

Infection of Zebrafish with K. pneumoniae Strain NTUH-K2044 Enhances Immune Dysfunction in tnfrsf1b Mutant

WT and *tnfrsf1b* mutants were infected with *K. pneumoniae* NTUH-K2044. There was no significant difference in the proportion of deformities in the *tnfrsf1b* mutants in the presence and absence of *K. pneumoniae* (Figure 5A). After

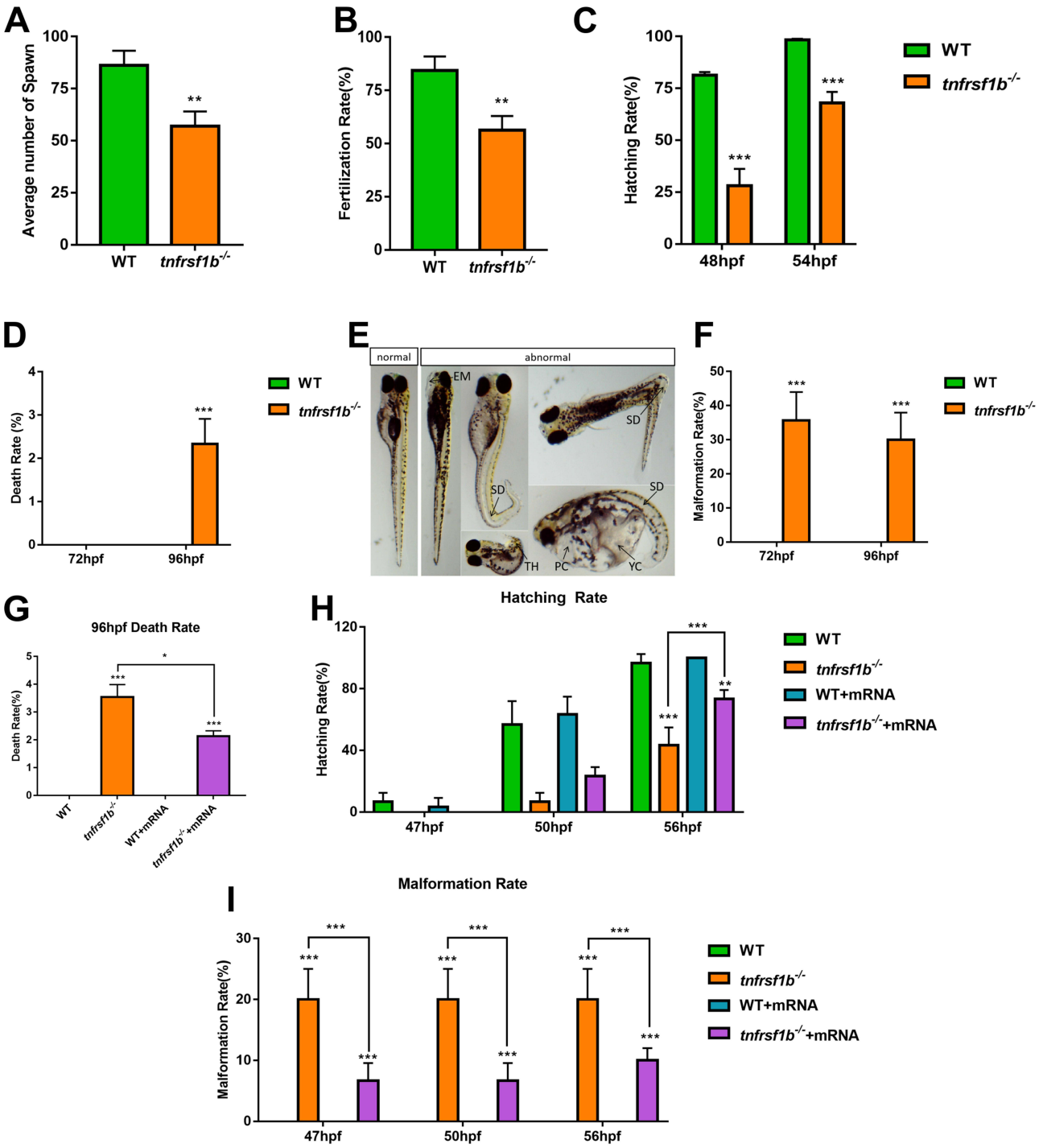

Figure 2 Phenotype analysis of zebrafish tnfrs1b mutant. (**A**–**C**) Egg production, fertilization rate, and hatching rate of zebrafish tnfrs1b mutant. (**D**) Mortality rate of zebrafish tnfrs1b mutant at 72 and 96hpf; (**E** and **F**) Deformed appearance and ratio of zebrafish tnfrs1b mutant. (**G**–**I**) Mortality, hatching rate, and deformity rate of rescue zebrafish tnfrs1b mutant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

treating the WT with the same concentration of *K. pneumoniae*, there was no significant increase in mortality at 72 and 96 hpf compared with the control group; however, compared with that of *tnfrsf1b* mutants not treated with *K. pneumoniae*, the mortality rate of *tnfrsf1b* mutants treated with *K. pneumoniae* increased significantly at the same time point (Figure 5B).

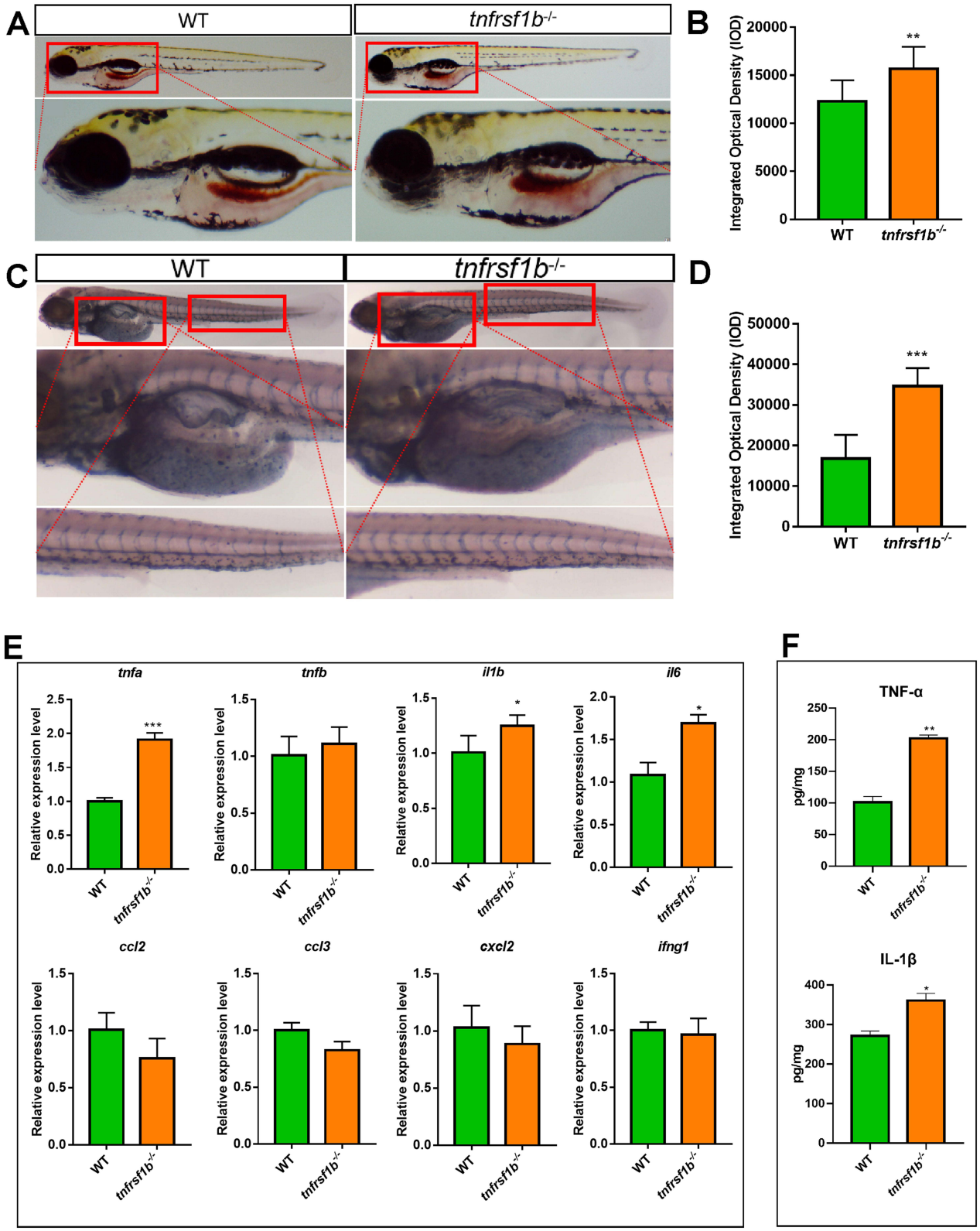

Figure 3 Deletion of zebrafish *tnfrsf1b* leads to dysfunction. (**A**) Zebrafish *tnfrsf1b* mutant macrophage staining. (**B**) Statistics of optical intensity of macrophages. (**C**) Zebrafish *tnfrsf1b* mutant neutrophil staining. (**D**) Optical density statistics of neutrophils. (**E**) Expression of immune-related genes in the zebrafish *tnfrsf1b* mutant. (**F**) ELISA to detect the protein expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in *tnfrsf1b* mutants. Data are expressed as mean ± standard deviation (SD),* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the WT group.
Abbreviation: WT, wild type.

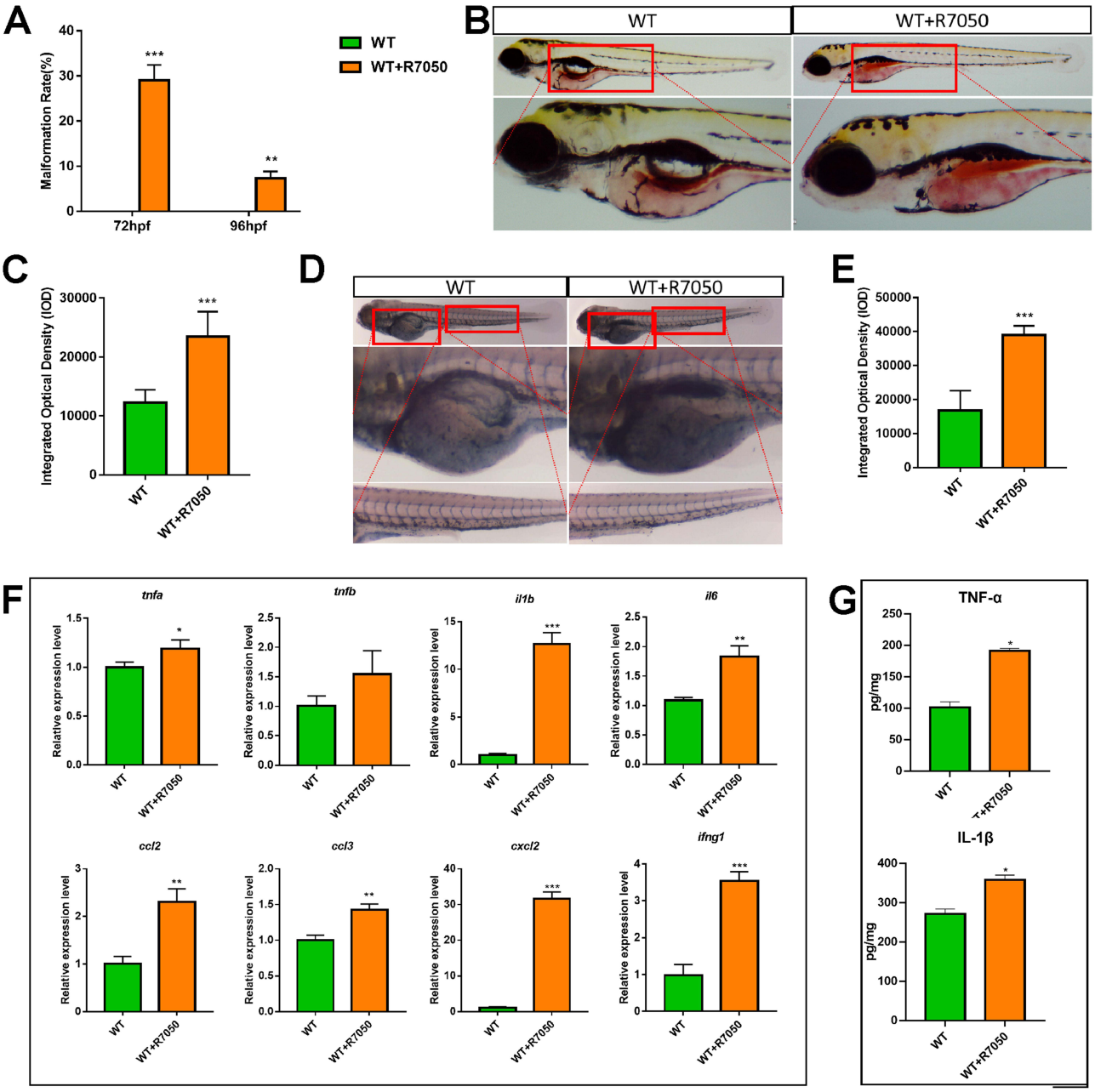

Figure 4 Treatment of wild-type (WT) zebrafish with TNFR inhibitor R7050 results in evident immunodeficiency. (**A**) Treatment of WT zebrafish with TNFR inhibitor R7050 resulted in evident malformations at 72 and 96 hours post-fertilization (hpf). (**B**) Changes in macrophages in WT zebrafish treated with R7050. (**C**) Statistical analysis of macrophages. (**D**) Changes in neutrophils in WT zebrafish treated with R7050. (**E**) Statistical analysis of neutrophils. (**F**) Changes in immune-related genes in WT zebrafish treated with R7050. (**G**) ELISA was used to detect the changes in tumor necrosis factor (TNF)-α and interleukin (IL)-1β protein concentrations in WT zebrafish treated with R7050. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the WT group.

As shown in Figure 5C–F, *K. pneumoniae* treatment increased the number of neutrophils and macrophages, with a more severe phenotype observed in the *tnfrsf1b* mutant both in WT and mutant zebrafish. However, the increase in the *tnfrsf1b* mutant was more considerable. qRT-PCR results showed that *K. pneumoniae* treatment of the *tnfrsf1b* mutants significantly increased the mRNA level of inflammation-related genes, such as *il-1β, il-8, ccl2, ccl3*, and *cxcl2* (Figure 5G). Furthermore, ELISA showed that the protein concentrations of TNF-α and IL-1β significantly increased in *K. pneumoniae*-treated WT and *tnfrsf1b* mutant zebrafish (Figure 5H).

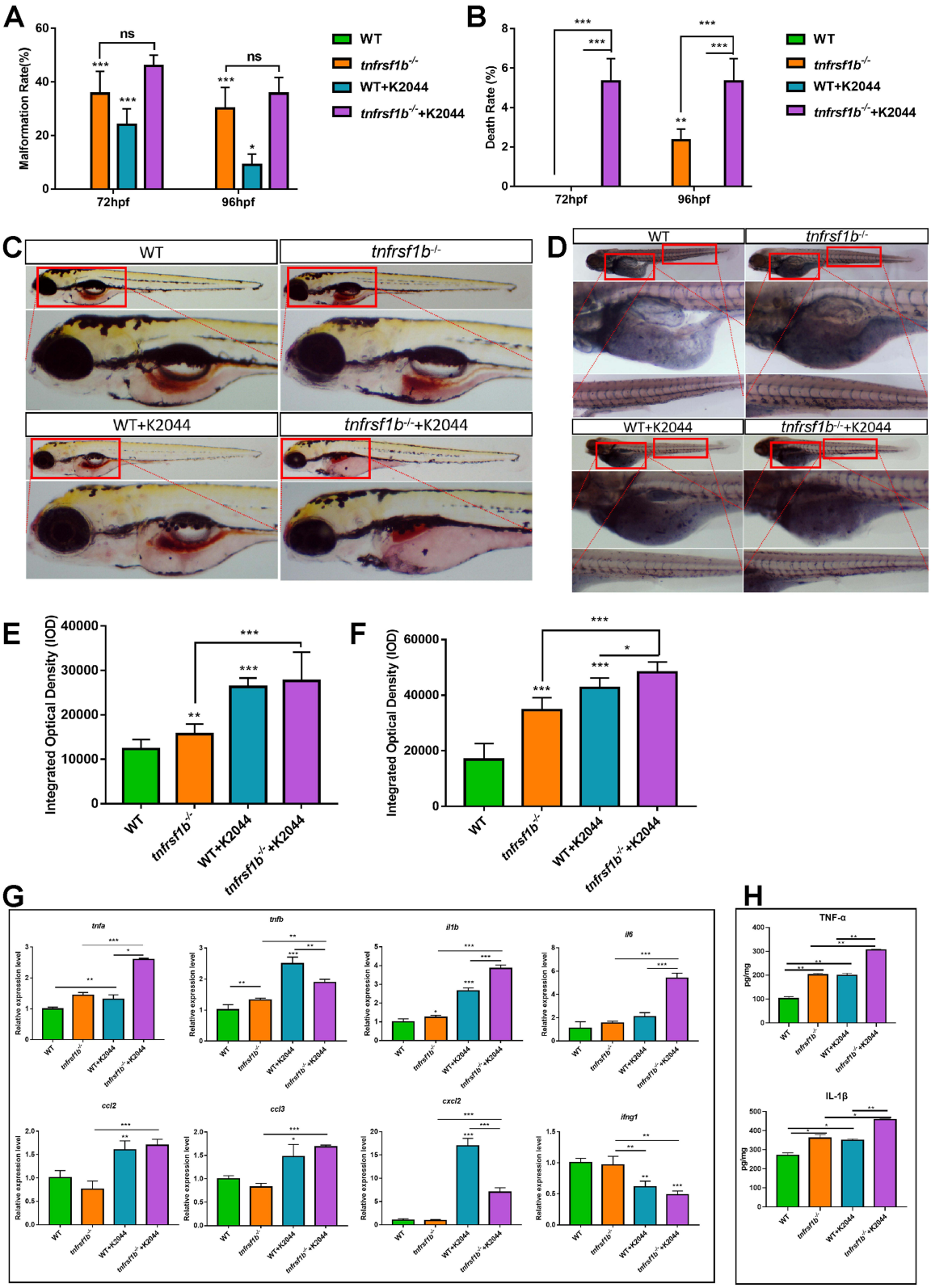

Figure 5 Treatment of *tnfrsf1b* mutants with *K. pneumoniae* strain K2044 enhances immune damage caused by *tnfrsf1b* deficiency. Changes in the deformity rate (**A**) and mortality (**B**) of *K. pneumoniae* strain K2044-treated *tnfrsf1b* mutants. (**C**) Staining of macrophages after treatment with *K. pneumoniae* strain K2044 in *tnfrsf1b* mutants. (**D**) Staining of neutrophils after treatment with *K. pneumoniae* strain K2044 in *tnfrsf1b* mutants. (**E**) Statistics of macrophages. (**F**) Statistics of neutrophils. (**G**) *K. pneumoniae* strain K2044 treatment of *tnfrsf1b* mutants resulted in immune-related gene expression changes. (**H**) ELISA was used to detect the changes of tumor necrosis factor (TNF)-α and interleukin (IL)-1β protein concentrations after treatment of *tnfrsf1b* mutants with *K. pneumoniae* strain K2044. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Abbreviations: WT, wild type; hpf, hours post-fertilization; ns, not significant.

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R7050-Treated WT Zebrafish Subjected to K. pneumoniae NTUH-K2044 Treatment Show a Phenotype Similar to That of tnfrsf1b Mutants Treated with K. pneumoniae

WT zebrafish pre-treated with R7050 were infected with *K. pneumoniae*. The malformation rate (Figure 6A) and the number of macrophages and neutrophils (Figure 6B–E) increased significantly compared with those in WT zebrafish not treated with R7050. Furthermore, the mRNA level of *il-1β, il-8, ccl2, ccl3, cxcl2*, and *ifng1* was significantly increased after the addition of R7050. *K. pneumoniae* added to R7050-pre-treated WT zebrafish resulted in a significant down-regulation in the expression of *cxcl2* and *ifng1*, but no significant differences in other detected genes (Figure 6F), compared to that in zebrafish treated with R7050 alone. Furthermore, ELISA analysis revealed that the protein concentrations of TNF-α and IL-1β were significantly increased in WT zebrafish treated with R7050 following *K. pneumoniae* infection, compared to controls (Figure 6G). These results further confirm that combined pharmacological inhibition of TNFR signaling and bacterial challenge exacerbates immune dysfunction.

tnfrsf1b-Capped mRNA Can Rescue the Phenotype of tnfrsf1b Mutants and Protect Zebrafish Against Damage Caused by K. pneumoniae

tnfrsf1b-capped mRNA was injected into single-celled WT and *tnfrsf1b* mutants. Compared with that in WT zebrafish, *tnfrsf1b*-capped mRNA significantly inhibited the increase in macrophages and neutrophils in *tnfrsf1b* mutants (Figure 7A–D).

To verify whether *tnfrsf1b*-capped mRNA had a protective effect during *K. pneumoniae* infection, *tnfrsf1b*-capped mRNA was then injected into WT and *tnfrsf1b* mutants; when the embryos reached 3 dpf, NTUH-K2044 was added. The results showed that *tnfrsf1b* -capped mRNA significantly rescued the malformation rate and death rate caused by NTUH-K2044 in the *tnfrsf1b* mutants compared with the WT (Figure 7E and F). In addition, *tnfrsf1b*-capped mRNA significantly reduced the number of neutrophils and macrophages following NTUH-K2044 infection in the *tnfrsf1b* mutants (Figure 7G–J).

Discussion

In this study, we first successfully obtained a zebrafish *tnfrsf1b* mutant with a 5-bp deletion using CRISPR/Cas9 technology. The *tnfrsf1b* mutant showed a clear immunodeficiency phenotype, manifested as an increase in the number of macrophages and neutrophils and an increase in levels of some inflammatory factors. Infecting the *tnfrsf1b* mutant with *K. pneumoniae* increased the malformation rate, mortality rate, and immune responses. *tnfrsf1b*-capped mRNA could rescue this phenotype and protected zebrafish against *K. pneumoniae* infection. The results indicated that *tnfrsf1b* plays a role in the development of zebrafish. Smith et al found that TNF-α/*tnfrsf1b* signaling is required for glial ensheathment at the dorsal root entry zone and that *tnfrsf1b* is also involved in the regulation of bone by the proteasome. In this study, there was a significant increase in the levels of immune cells and cytokines in the *tnfrsf1b* mutant. Macrophages are mainly fixed cells or free cells that phagocytose cell fragments and pathogens (that is, phagocytosis and digestion) and activate lymphocytes or other immune cells to respond to pathogens.[26] Neutrophils exert chemotactic, phagocytic, and bactericidal effects.[27] Under normal conditions, the increase in these two types of cells indicates that *tnfrsf1b* reduction may be caused by the susceptibility to some microorganisms in the environment. Therefore, the establishment of the *tnfrsf1b* mutant can be used as an immune disorder zebrafish model to study its related immune homeostasis.

The *tnfrsf1b* mutant causes an increased susceptibility to *K. pneumoniae*. Studies in mice have found that the deletion of many immune genes can increase the susceptibility to *K. pneumoniae* infection.[18,28] Since *tnfrsf1b* can initiate pro-inflammatory signaling pathways, the host's pro-inflammatory signaling can limit *K. pneumoniae* infection. Our findings support the idea that zebrafish *tnfrsf1b* functions in a manner comparable to its mammalian counterparts, despite sequence divergence. Previous studies in mice have demonstrated that *tnfrsf1b* signaling promotes regulatory T cell expansion, macrophage polarization, and endothelial repair by activating NF-κB and MAPK signaling cascades. These pathways are also implicated in balancing pro-inflammatory and anti-inflammatory responses during bacterial infections. In zebrafish, the observed increase in macrophages, neutrophils, and inflammatory cytokines in *tnfrsf1b* mutants may reflect the loss of *tnfrsf1b*-mediated regulatory signaling, thereby leading to excessive inflammatory responses. Taken together, these results

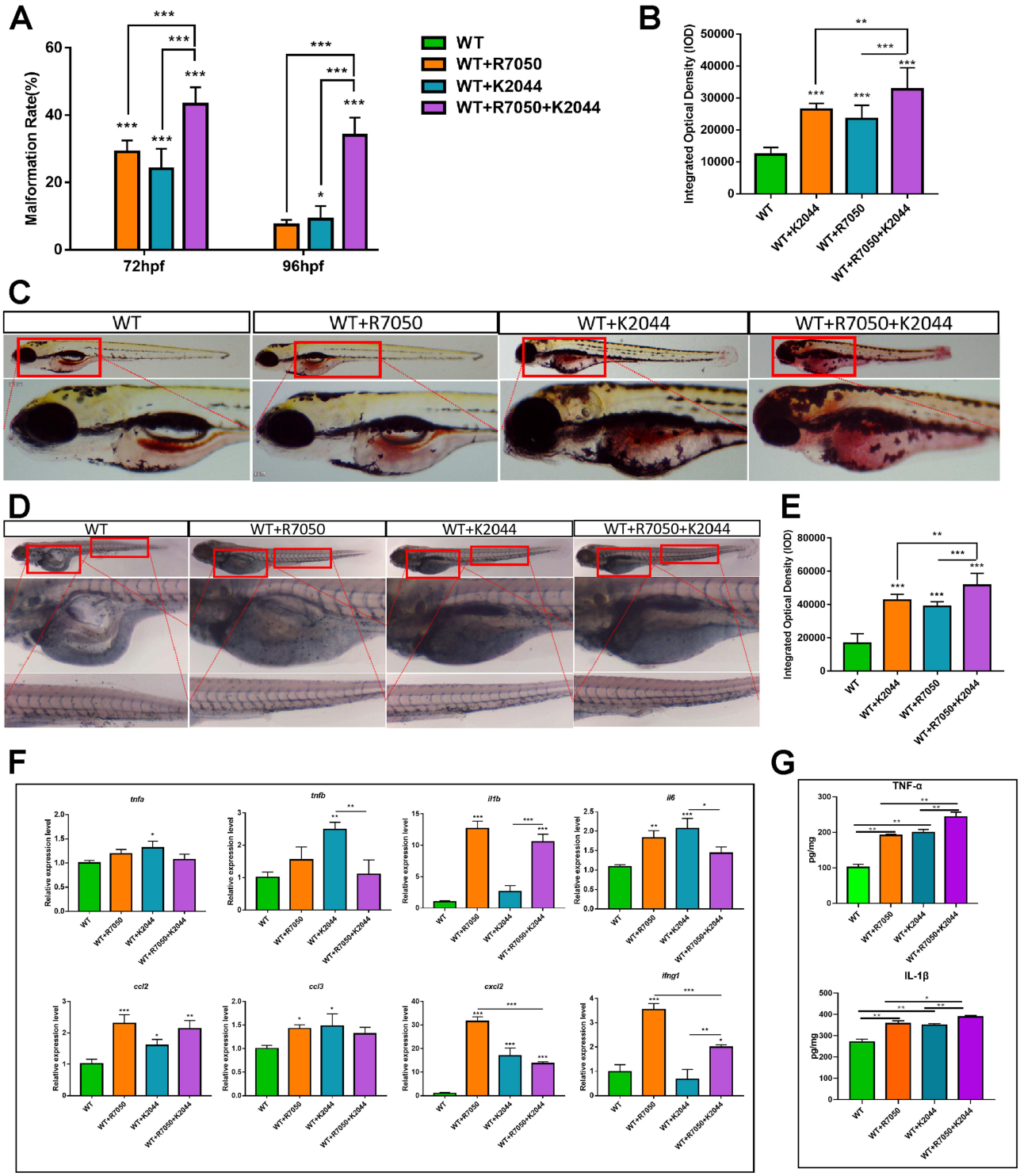

Figure 6 Addition of *Klebsiella pneumoniae* strain K2044 after treatment of wild-type (WT) zebrafish with R7050 resulted in a more severe phenotype. (**A**) Changes in the malformation rate of *K. pneumoniae* strain K2044 after treatment of WT zebrafish with R7050. (**B** and **C**) Changes in macrophages after the addition of *K. pneumoniae* strain K2044 following treatment with R7050 in WT zebrafish. (**D** and **E**) Changes in neutrophil granulocytes in WT zebrafish treated with R7050 after adding *K. pneumoniae* strain K2044. (**F**) Changes in immune-related genes after adding *K. pneumoniae* strain K2044 to WT zebrafish treated with R7050. (**G**) ELISA was used to detect the changes of tumor necrosis factor (TNF)-α and interleukin (IL)-1β protein concentrations after adding *K. pneumoniae* strain K2044 to WT zebrafish treated with R7050. Hpf, hours post fertilization. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

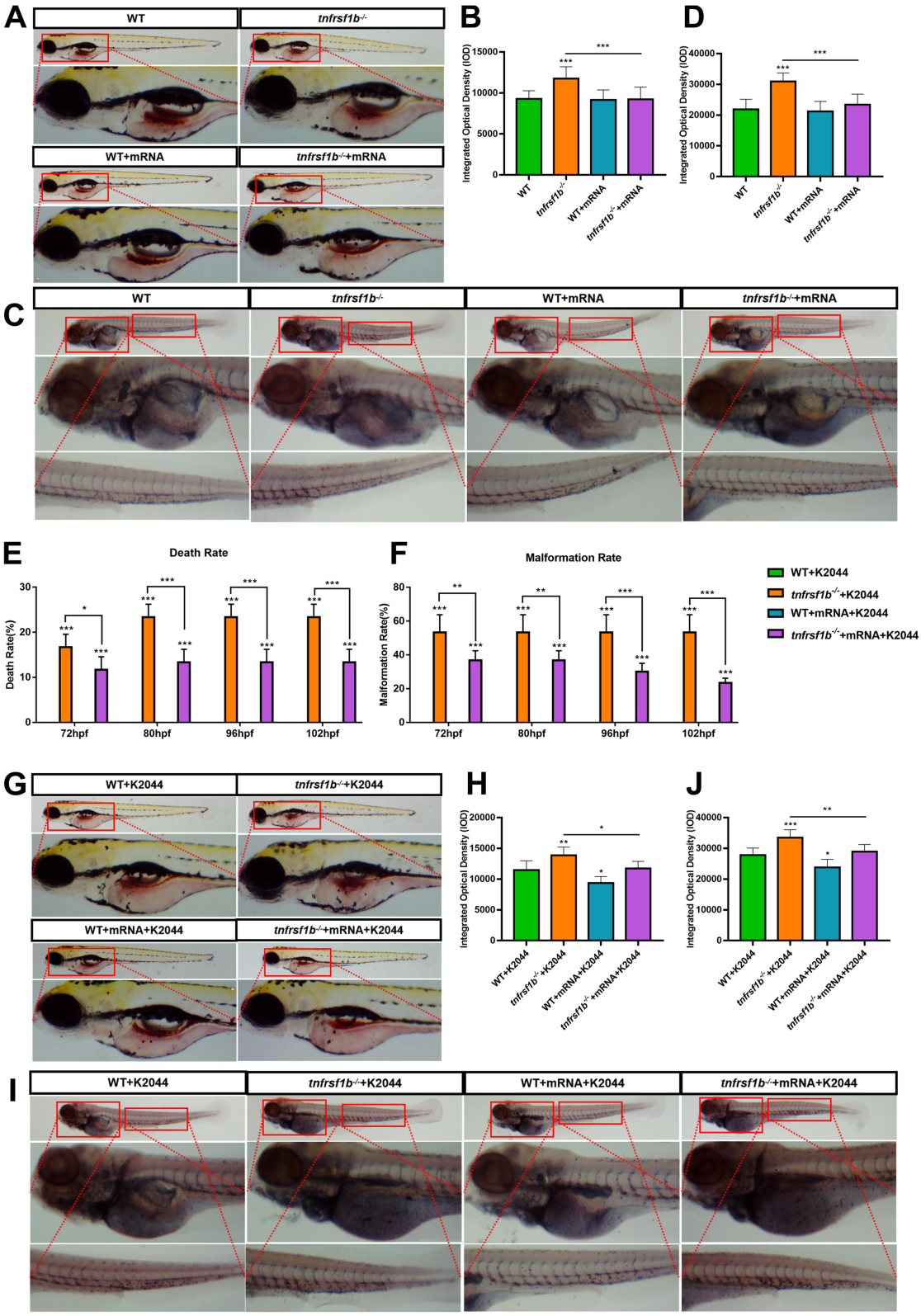

Figure 7 *Tnfrsf1b*-capped mRNA can rescue immunodeficiency caused by *tnfrsf1b* deletion. Changes in macrophages (**A** and **B**), neutrophils (**C** and **D**), mortality (**E**), and malformation rate (**F**) after injection of *tnfrsf1b*-capped mRNA into *tnfrsf1b* mutants. Changes in macrophages (**G** and **H**) and neutrophils (**I** and **J**) after injection of *tnfrsf1b* -mRNA into wild-type (WT) and *tnfrsf1b* mutant zebrafish. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Abbreviation: Hpf, hours post-fertilization.

suggest that the protective role of *tnfrsf1b* against *K. pneumoniae* infection in zebrafish is at least partly mediated through conserved downstream pathways such as NF-κB and MAPK, which parallels findings from mammalian systems.

One of the advantages of zebrafish as a research model organism is that they can facilitate pharmacological operations because we can dissolve drugs into the culture water, and zebrafish larvae can absorb the drugs through the skin.[29] Inhibitors of TNFR can act on both of the receptor proteins.[30] In this study, an inhibitor of TNFR was tested to mimic the phenotype observed in the *tnfrsf1b* knockout. The inhibitor plus *K. pneumoniae* infection of larvae zebrafish led to a significant increase in teratogenicity and mortality and an increase in immune cell numbers; however, the result is inconsistent in the quantification of immune genes. The main reason may be that TNFR inhibitors not only act on *tnfrsf1b* but also act on the activity of TNFR1. Research has shown that TNFR1 and *tnfrsf1b* participate in different immune processes.[31] TNFR1 can activate the transcription factor NF-κB, mediate apoptosis, and function as a regulator of inflammation.[32] *tnfrsf1b* and TNFR1 form a heterocomplex that mediates the recruitment of two anti-apoptotic proteins, c-IAP1 and c-IAP2, which possess E3 ubiquitin ligase activity.[33] *tnfrsf1b* has no intracellular death domain; thus, this protein protects neurons from apoptosis by stimulating antioxidative pathways.[34] Whether there are other targets for the pharmacological mechanism of action needs further study.

tnfrsf1b can be used as a candidate target to treat *K. pneumoniae* infection. Injecting the *tnfrsf1b*-capped mRNA into the *tnfrsf1b* mutant rescued the phenotype caused by *K. pneumoniae* and also improved the phenotype of *K. pneumoniae* infection in WT zebrafish. It is noteworthy that in Figure 5A the deformity rate of *tnfrsf1b* mutants showed an increasing trend after *K. pneumoniae* infection, although the difference did not reach statistical significance. In contrast, mortality significantly increased (Figure 5B). This discrepancy may be explained by the fact that larvae with more severe developmental deformities were less tolerant to infectious stress, thereby contributing to the elevated mortality. These findings suggest that *tnfrsf1b* deficiency may synergize developmental abnormalities and immune dysfunction to exacerbate susceptibility to bacterial infection. Previous research has mainly focused on the development of antibiotics to kill or resist pathogens; however, whether *K. pneumoniae* can cause disease through internal changes in the body and the role of bacterial elimination is a direction worth studying in the future. Previous studies on *tnfrsf1b* have found that it can play a role in neurodevelopment and anti-oxidation.[34]

Recent studies have further elucidated the immunomodulatory role of *tnfrsf1b* (TNFR2) in bacterial infection contexts. For instance, Liang et al (2022) demonstrated that while TNFR1 signaling promotes inflammatory responses and effector T cell expansion, TNFR2 signaling is distinctly associated with anti-inflammatory activity and tissue homeostasis, underscoring the balancing role of *tnfrsf1b* in host defense.[35] Moreover, Zhang et al (2024) reported that GRN can upregulate TNFR2 expression and promote M2 macrophage polarization, further supporting a role for *tnfrsf1b* in orchestrating macrophage phenotype toward tissue repair and resolution of inflammation.[36] Additionally, in a bacteremia model, Xu et al (2023) found that TNFR2+ regulatory T cells are essential for protecting against pulmonary injury, indicating that *tnfrsf1b* contributes to immune tolerance mechanisms during severe infection.[37] Collectively, these findings align with our observations in zebrafish where *tnfrsf1b* deficiency leads to exaggerated inflammation and immune cell infiltration, suggesting that macrophage polarization and regulatory T cell pathways may be key mechanisms through which *tnfrsf1b* modulates host responses to *K. pneumoniae* infection.

Our study demonstrated that *tnfrsf1b*-deficient zebrafish exhibit exaggerated inflammatory responses and increased mortality upon *K. pneumoniae* infection, suggesting that *tnfrsf1b* plays a protective role. However, whether *tnfrsf1b* is strictly essential for resistance to *K. pneumoniae* remains an open question. It is possible that *tnfrsf1b* functions as a modulatory rather than indispensable factor, fine-tuning the balance between pro- and anti-inflammatory signals during infection. Moreover, the interaction between host genetic deficiency and bacterial virulence is complex, and the exacerbated phenotypes observed may reflect a synergistic effect rather than independent contributions. Future work employing double manipulations (eg, *tnfrsf1b* mutants combined with infection by different virulence strains, or selective pathway inhibition) will be important to dissect whether the effects of *tnfrsf1b* deficiency and *K. pneumoniae* infection are additive or synergistic.

It should be noted that R7050 is a pan-TNFR inhibitor that blocks the activity of both TNFR1 and *tnfrsf1b*. Therefore, the phenotypes observed in R7050-treated zebrafish may not exclusively reflect the inhibition of *tnfrsf1b*. TNFR1 is known to activate pro-inflammatory signaling pathways such as NF-κB and apoptosis in mammalian systems, and its inhibition could potentially alter the balance between inflammatory activation and resolution. Nevertheless, the fact that *tnfrsf1b* mutants and R7050-treated zebrafish showed comparable immune

dysfunction supports the conclusion that *tnfrsf1b* plays a major role in the observed phenotypes. We have revised the Discussion to clarify this point and to highlight that future work using more selective inhibitors or genetic approaches will help to dissect the relative contributions of TNFR1 and *tnfrsf1b*.

While our findings highlight *tnfrsf1b* as a potential therapeutic target for mitigating immune dysfunction during *K. pneumoniae* infection, caution must be exercised when considering its translational application. In mammalian models, excessive activation of *tnfrsf1b* has been linked to immunosuppression and impaired pathogen clearance. Therefore, therapeutic strategies aimed at enhancing *tnfrsf1b* signaling may reduce damaging inflammation but could simultaneously increase susceptibility to secondary infections. This dual effect underscores the need for carefully balanced approaches that restore immune homeostasis without tipping toward excessive suppression. Future studies should evaluate the safety and long-term consequences of modulating *tnfrsf1b* activity in the context of bacterial infections.

One limitation of this study is the relatively low amino acid sequence identity between zebrafish *tnfrsf1b* and its mammalian counterparts (21.8–24.15%). This raises concerns regarding the direct extrapolation of our findings to humans. Although structural analysis suggests that key signaling domains are conserved, the differences in immune system architecture between zebrafish and mammals may result in species-specific variations in *tnfrsf1b* function. Therefore, while zebrafish provide a powerful vertebrate model for dissecting host–pathogen interactions in vivo, caution is warranted in directly translating these results to human *K. pneumoniae* infections. Future studies in mammalian models will be important to validate the mechanistic insights obtained from zebrafish.

In conclusion, our study demonstrates that *tnfrsf1b* deficiency in zebrafish leads to increased susceptibility to *K. pneumoniae* infection, whereas restoration of *tnfrsf1b* signaling confers protection. These findings suggest that *tnfrsf1b* may represent a candidate pathway for further investigation as a potential therapeutic target against hypervirulent *K. pneumoniae* infection, although validation in mammalian models and clinical settings will be required.

Ethical Approval

The Committee for the Management and Use of Laboratory Animals of Hangzhou Huante Bio-technology Co., Ltd. approved this animal experiment. (IACUC-2024-8957-01).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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