Vitamin D Enhances Neutrophil Generation and Function in Zebrafish (*Danio rerio*)

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**Keywords**

Vitamin D\(_3\) · Granulopoiesis · Microbiota · Neutrophil recruitment · Infection

**Abstract**

Vitamin D (VD) is a major regulator of calcium metabolism in many living organisms. In addition, VD plays a key role in regulating innate and adaptive immunity in vertebrates. Neutrophils constitute an important part of the first line of defense against invading microbes; however, the potential effect of VD on neutrophils remains elusive. Thus, in this study zebrafish in different developmental stages were utilized to identify the potential role of VD in the basal homeostasis and functions of neutrophils. Our results showed that addition of exogenous VD\(_3\) promoted granulopoiesis in zebrafish larvae. Reciprocally, neutrophil abundance in the intestine of adult zebrafish with a cyp2r1 mutant, lacking the capacity to 25-hydroxylate VD, was reduced. Moreover, VD-mediated granulopoiesis was still observed in gnotobiotic zebrafish larvae, indicating that VD regulates neutrophil generation independent of the microbiota during early development. In contrast, VD was incapable to influence granulopoiesis in adult zebrafish when the commensal bacteria were depleted by antibiotic treatment, suggesting that VD might modulate neutrophil activity via different mechanisms depending on the developmental stage. In addition, we found that VD\(_3\) augmented the expression of *il-8* and neutrophil recruitment to the site of caudal fin amputation. Finally, VD\(_3\) treatment significantly decreased bacterial counts and mortality in zebrafish infected with *Edwardsiella tarda* (*E. tarda*) in a neutrophil-dependent manner. Combined, these findings demonstrate that VD regulates granulopoiesis and neutrophil function in zebrafish immunity.

**Introduction**

The role of Vitamin D (VD) in the regulation of calcium-phosphate homeostasis and in the control of bone turnover is well known [1]. Vitamin D\(_3\) (VD\(_3\)) is the main form of VD in animals, and it is converted to the hormonal form 1α,25(OH)\(_2\)D\(_3\) by 2 cytochrome P450 (CYP) enzymes, 25-hydroxylase, and 1 alpha-hydroxylase, which are encoded by the *cyp2r1* and *cyp27b1* genes, respectively [2].
Over the past 20 years, numerous studies have shown that VD possesses immunomodulatory functions and plays a critical role in modulating innate and adaptive immunity in mammals [1, 3], and in fish [4]. As one of the first defenders of the innate immunity, neutrophils are rapidly activated upon infection and play an essential role in bacterial clearance [5]. In addition, neutrophils promptly accumulate in large numbers at sites of tissue injury, which limit bacterial translocation, stimulate angiogenesis, and promote tissue restoration [6].

Neutrophil homeostasis is maintained through a careful balance of granulopoiesis, bone marrow (or kidney marrow in fish) storage and release, and migration into vascular compartments and peripheral tissues [7]. Despite extensive evolutionary divergence between teleost fish and mammals, the molecular pathways governing hematopoiesis have been conserved. The process of pluripotent hematopoietic stem cells to myeloid precursors to mature neutrophils is controlled by both extracellular and intracellular factors [8], among which granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3 (CSF3), plays a critical role in directing granulopoiesis and maintaining normal neutrophil numbers [5, 9]. Interestingly, it has also been reported that the microbiota was involved in maintaining steady-state granulopoiesis [10].

In addition, myeloperoxidase (mpx) and matrix metalloproteinase9 (mmp9) are stored in neutrophil cytoplasmic granules, and they play an important role in killing pathogens [5]. Mpx is also a specific marker for neutrophils, which can partly reflect the abundance of neutrophils in homeostasis [11]. In addition, lysozyme C (lysc) can also be used as a granulocyte marker [12].

In this study, we used zebrafish as a model and evaluated the effects of VD on neutrophil generation and confirmed if VD could influence granulopoiesis via gut microbiota. We also analyzed the contribution of VD to neutrophil recruitment to the site of tissue injury and for host resistance to bacterial infection.

**Materials and Methods**

**Zebrafish Maintenance**

Zebrafish were maintained at 28.5°C in a freshwater circulation system with a light: dark cycle of 14 h:10 h. The fish were fed twice daily with newly hatched brine shrimps (*Artemia franciscana*). Husbandry and handling of the fish in the present study were approved by the Experimental Animal Ethics Committee of Ocean University of China.

**Feeding Trial**

Two experimental diets with 0 or 800 IU VD₃/kg diet were designed and formulated in our laboratory. The composition of these experimental diets is shown in Table 1. Wild-type zebrafish at 35 days post fertilization (dpf) around 20–25 mg were randomly assigned into 6 tanks (10 L) with 50 fish/tank, and all fish were fed with 0 IU VD₃/kg diet for 1 week. Afterward, each diet was randomly assigned to triplicate tanks, and the fish were fed twice daily.

For antibiotic treatment, zebrafish (2 months, 70–90 mg) were randomly assigned into 4 tanks (10 L) with 50 fish/tank. Half of them were maintained in an aquaculture system with antibiotics (100 μg/mL ampicillin, 10 μg/mL kanamycin, 0.5 μg/mL amphotericin B, and 50 μg/mL gentamycin) for 1 month. The fish were fed twice daily. At the end of the feeding trial, all fish were euthanized in 0.1% tricaine (MS-222), the intestine of each fish was collected, and saved at −80°C for further analysis.

**Gene Expression Analysis**

Total RNA was extracted from the whole zebrafish larvae treated with control buffer or VD₃ by using the RNAeasy™ Animal RNA isolation kit (Beyotime, Shanghai, China) according to the manufacturers’ instructions. The quantity of total RNA samples was assessed using NanoDrop® One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quality of the extracted RNA was determined by agarose gel electrophoresis. RNA (1 μg) was reversely transcribed to cDNA using the HiScript III RT SuperMix for qPCR with gDNA wiper (Vazyme, Nanjing, China). The *actin2* gene was chosen as the reference gene for sample nor-

**Table 1. Dietary formulation of experimental diet (g/kg)**

| Ingredients                                      | 0 IU-diet | 800 IU-diet |
|--------------------------------------------------|-----------|-------------|
| Casein (vitamin free)                            | 388       | 388         |
| Gelatin                                          | 97        | 97          |
| Fish oil                                         | 115       | 115         |
| Starch                                           | 280       | 280         |
| Cellulose                                        | 55        | 55          |
| Monocalcium phosphate                            | 10        | 10          |
| Choline chloride                                 | 5         | 5           |
| Mineral premix¹                                  | 40        | 40          |
| Vitamin premix (VD₃ free)²                       | 10        | 10          |
| VD₃, IU/kg                                       | 0         | 800         |

¹Mineral premix (mg/g diet): calcium lactate, 327; FeSO₄, 3.125; MgSO₄, 137; NaH₂PO₄, 87.2; NaCl, 43.5; AlCl₃, 0.15; KI, 0.125; KCl, 75; CuCl₂, 0.1; MnSO₄, 0.8; CoCl₂, 1; ZnSO₄, 3; microcrystalline cellulose, 187.2. ²Vitamin premix (mg/g diet): thiamine HCl, 5; riboflavin, 10; calcium pantothenate, 10; D-biotin, 0.6; pyridoxine HCl, 4; folic acid, 1.5; inositol, 200; L-vitamin C-2-magnesium phosphate, 60; niacin, 6.05; α-vitamin E acetate, 50; vitamin K, 4; retinol acetate, 0.11; microcrystalline cellulose, 648.74.

The transgenic *Tg (mpx:eGFP)* zebrafish used in this study was donated by Dr. Eduardo Villablanca, Karolinska Institutet, Sweden. The generation of cyp2r1¹⁻/⁻ zebrafish has been described in a previous report [13].
The Effects of Vitamin D on Neutrophils

malization, and all primer sequences of target genes are listed in Table 2. The qRT-PCR reactions were carried out in a quantitative thermal cycler CFX96™ Real Time System (Bio-Rad, Hercules, CA, USA).

**In vivo Imaging**
Zebrafish were anesthetized in 0.016% Tricaine (MS-222; Sigma-Aldrich, St. Louis, MO, USA), and then mounted in 2% agarose containing 0.016% Tricaine. Imaging was performed with SMZ25 stereo fluorescence microscope (Nikon, Tokyo, Japan).

**Neutrophil Enumeration**
Neutrophil numbers were determined by 2 manners. For direct enumeration, manual counting of neutrophil numbers was assisted by the multipoint function in Image J, which records click to avoid duplicate counting. Alternatively, neutrophil units were computed as previously described and validated [14]. Briefly, total fluorescent area of 1 zebrafish larva was measured by using Image J. In order to acquire the average size of neutrophils in this larva, at least 5 neutrophils located in the tail tip were measured to ensure that the neutrophils do not overlap. Finally, neutrophil units were calculated by dividing the “total fluorescent area” value of each embryo by its average value of “neutrophil size.”

**Gnotobiotic Zebrafish Husbandry**
The generation of gnotobiotic zebrafish in this study followed the established protocols [15]. Briefly, zebrafish embryos were obtained by natural spawning and kept in gnotobiotic zebrafish medium (GZM) at 28.5°C. Next, embryos were incubated in GZM with antibiotics (100 μg/mL ampicillin, 10 μg/mL kanamycin, 0.5μg/mL amphotericin B, 50 μg/mL gentamycin) for 6–8 h. Under sterile conditions, the embryos were washed with 0.1% PVP-I in GZM for 90 s and rinsed twice with GZM. Thereafter, embryos were washed using 0.003% bleach solution in GZM for 20 min. Tryptic soy agar plate, nutrient broth, brain-heart Infusion broth, and Sab-Dex broth were used for checking sterility.

**Caudal Fin Amputation Assay**
Larval zebrafish (5 dpf) was anaesthetized with 0.016% Tricaine (MS-222) and mounted on glass slide. Fish were then observed under a stereomicroscope. Tail amputations were performed posterior to the end of the notochord using a surgical scalpel (Surgical Specialties Sharpont, 72–2,201), and fish were revived into 12-well plates containing GZM. At 0, 4, and 6 h post caudal fin amputation, the fish were euthanized and observed. GFP+ cells at the tail wound margin were enumerated and imaged by using Nikon SMZ25 stereo fluorescence microscope.

**Bacterial Challenge**
*Edwardsiella tarda* (*E. tarda*) was isolated from diseased turbot (*Scophthalmus maximus* L.), and the identity was confirmed by 16S rRNA gene sequencing. Overnight bacterial cultures were washed with GZM and adjusted to 10^8 CFU/mL. Zebrafish larva was infected with *E. tarda* by static immersion at 3 dpf or microinjections at 5 dpf.

**Bacterial Enumeration**
Zebrafish larvae in each group were anesthetized at different time points (24, 48, and 72 h post infection [hpi]) after *E. tarda* immersion. Five larvae were pooled and homogenized in 0.2 mL GZM. Serial dilutions were performed using PBS (10 mM, pH 7.4) of the homogenates. Ten microliters of serial dilutions were plated on deoxycholate hydrogen sulfide lactose agar plates, and black colonies were enumerated after incubation at 28°C for 24 h.

**Generation of Neutrophil-Knockdown Zebrafish Larvae**
Neutrophil-knockdown zebrafish larvae were generated based on a previous method [16]. Briefly, 2 single-guide (sg) RNAs which specifically targeted the zebrafish csf3r gene were generated (listed in Table 3). The mixture of 2 sgRNAs was microinjected into one-cell stage embryos together with Cas9 protein (M0646T; NEB, San...
Diego, CA, USA) at a final concentration of 1 μg/μL. Total RNA was extracted at 6 dpf from the whole zebrafish larvae microinjected with control buffer or sgRNAs, and then reversely transcribed to cDNA by using the HiScript III RT SuperMix. Genotyping primers were designed (listed in Table 2), and qRT-PCR verified the validity of the gene editing.

**Fig. 1.** VD₃ promotes the granulopoiesis in zebrafish larvae. Zebrafish larvae at 2 dpf were exposed to 100 nM VD₃ for 2 days, GZM was replaced at 4 dpf, and 100 nM VD₃ were added simultaneous. a The merged images of Tg(mpx:gfp) zebrafish (6 dpf) showed the abundance and localization of GFP⁺ neutrophils. White dashed line indicates the intestine. b The size of neutrophil population was represented by neutrophil unit as described in Materials and Methods. Graph shows quantification of neutrophil units in whole zebrafish larva at 6 dpf. c Enumeration of intestinal-associated GFP⁺ cells in 6 dpf larvae. d The gene expression by qRT-PCR of csf3a, csf3b, mpx, lysc, and mmp9 from 6 dpf zebrafish larvae treated with 0 or 100 nM VD₃ for 4 days (results are combined from 2 independent experiments, n = 4 replicates/group/experiment, 10–20 larvae/replicate). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. VD₃, vitamin D₃; dpf, days post fertilization; mpx, myeloperoxidase; csf3a, colony-stimulating factor 3a; csf3b, colony-stimulating factor 3b; lysc, lysozyme C; mmp9, matrix metalloproteinase9; GZM, gnotobiotic zebrafish medium.

**Calculations and Statistical Methods**

All data are expressed as mean ± SEM. The software GraphPad Prism 8.0 was used for all statistical evaluations. Differences between the means were evaluated using t test or 2-way ANOVA with Sidak test, and p value <0.05 was regarded as statistical significance.
Results

Vitamin D Increases the Number of Neutrophils in Zebrafish Larvae

To investigate the effects of VD on neutrophil generation in vivo, Tg(mpx: gfp) zebrafish with GFP-labeled neutrophil-specific mpx were utilized. Tg(mpx: gfp) zebrafish larvae at 2 dpf were exposed to 100 nM VD3 for 4 days, and stereomicroscopic evaluation of GFP+ neutrophils in whole 6 dpf zebrafish showed an increase in neutrophil counts in the VD3-treated group compared with the control group (Fig. 1a, b). Notably, the number of neutrophils located in the zebrafish digestive tracts was significantly increased in the VD3-treated group (Fig. 1c). Gene expression analysis with qRT-PCR revealed that VD3 treatment elevated the expression of the granulopoietic cytokine genes colony-stimulating factor 3b (csf3b), whereas no effect on the production of colony-stimulating factor 3a (csf3a) was detected (Fig. 1d). Likewise, the total neutrophil units in the VD3-treated group was also increased (Fig. 1b). In addition, mpx, the granulocyte marker gene (lys), and the neutrophil cytoplasmic granules gene matrix metalloproteinase9 (mmp9) were also increased in the VD3-treated group compared to the control group (Fig. 1d). These data demonstrated that exogenous addition of VD3 upregulated the number of neutrophils in zebrafish larvae.

Vitamin D Regulates Granulopoiesis in Zebrafish Intestines

Next, the effects of VD3 on the neutrophil population in zebrafish intestines at different development stages were examined. Consistent with the results in zebrafish larvae, the genes of csf3b, mpx, lycs, and mmp9, were expressed at higher levels in juvenile zebrafish (35 dpf) fed for 2 weeks with a diet containing 800 IU VD3/kg diets for 2 weeks (n = 12 replicates/group, 2–3 zebrafish/replicate). Transcript levels of csf3a, csf3b, mpx, lycs, and mmp9 in the gut of WT and cyp2r1 mutant zebrafish in 3 months (n = 8 replicates/genotype). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. csf3a, colony-stimulating factor 3a; csf3b, colony-stimulating factor 3b; lycs, lysozyme C; mmp9, matrix metalloproteinase9; dpf, days post fertilization; VD3, vitamin D3; WT, wild-type; mpx, myeloperoxidase.

Microbiota Is Involved in VD-Mediated Regulation of Granulopoiesis

To test whether the microbiota had any role in VD-mediated regulation of granulopoiesis, the expression of csf3b, mpx, and mmp9 was assessed in both conventionalized (CONVED) and germ-free GF zebrafish. Interestingly, VD3 treatment resulted in upregulation of the 3 genes in both CONVED and GF zebrafish (Fig. 3a–c). These findings indicated that VD3-enhanced neutrophil activity was independent of the microbiota in the larvae stage.

Next, the intestines in adult cyp2r1−/− and WT zebrafish were dissected. Consistent with the results of Figure 2b, cyp2r1−/− zebrafish had reduced gene expression of csf3b, mpx, mmp9 compared to WT controls under nor-
Fig. 3. Microbiota impact on granulopoiesis in zebrafish. a–c The gene expression of csf3b (a), mpx (b), and mmp9 (c) in CONVED or GF zebrafish larvae (6 dpf) treated 0 or 100 nM VD₃ for 4 days (results are combined from 2 independent experiments, n = 12 replicates/group, 10–20 larvae/replicate). d–f WT and cyp2r1 mutant zebrafish in 3 months were treated with antibiotics for 1 week, and the gene expression of csf3b (d) mpx (e), and mmp9 (f) transcripts in the gut was analyzed (n = 8 replicates/genotype). Two-way ANOVA with Sidak test was used to test significance. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. csf3b, colony-stimulating factor 3; mmp9, matrix metalloproteinase 9; dpf, days post fertilization; VD₃, vitamin D₃; WT, wild-type; CONVED, conventionalized; GF, germ-free; mpx, myeloperoxidase.
mal conditions. Notably, when the microbiota was depleted with antibiotics, no significant reduction of gene expression was detected in cyp2r1−/− zebrafish compared to WT zebrafish. Thus, there is a significant interaction between VD-mediated effects on intestinal gene expression and the microbiota in adult zebrafish (Fig. 3d–f).

**Vitamin D Promotes Neutrophil Recruitment to the Wound Margin**

To assess the effects of VD on neutrophil migration, we performed caudal fin amputations on Tg(mpx:gfp) zebrafish larvae and quantified the recruitment of neutrophils to the wound margin. We observed that the count of GFP+ neutrophils in the wound margin was higher in the VD3-treated group than the control group at 4 h post wounding (hpw). At 6 hpw, only the group treated with 10 nM VD3 showed increased neutrophil recruitment, while the recruitment of neutrophils to the wound margin in the group treated with a higher VD3 dose (100 nM) was similar to controls (Fig. 4c). The gene expression of neutrophil chemoattractant factor il-8 in whole zebrafish larvae was quantified in homeostasis and after caudal fin amputation. A 2-way ANOVA analysis revealed that both VD3 incubation and caudal fin amputation treatment increased the expression of il-8, while there were no significant interactions between VD3 treatment and caudal fin

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Fig. 4. VD3 enhances neutrophil recruitment to the wound site of amputated fish. a–c The caudal fin of 6 dpf Tg(mpx:gfp) zebrafish. Imaging and quantification of GFP+ neutrophils recruited to tail wound at 4 hpw (n = 4/group) (b). d qRT-PCR analysis of IL-8 transcripts in 6 dpf zebrafish larvae treated 0 nM, 10 nM, or 100 nM VD3 for 4 days, with and without caudal fin amputation (n = 6 replicates/group, 6–15 larvae/replicate). Two-way ANOVA with Sidak test was used to test significance. For c, statistical comparisons were performed within each time point. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. dpf, days post fertilization; VD3, vitamin D3; WT, wild-type; hpw, hours post wounding; mpx, myeloperoxidase.
Fig. 5. VD$_3$ restrains pathogen infection in zebrafish. a–c Transcript levels of mpx (a), mmp9 (b), and csf3b (c) in unstimulated and E. tarda-exposed zebrafish larvae, pretreated with 0 or 100 nM VD$_3$ (n = 4 replicates/group, 10–20 larvae/replicate). Two-way ANOVA with Sidak test was used to test significance. d Zebrafish larvae at 3 dpf were immersed with 1 × 10$^8$ CFU/mL E. tarda. After 24, 48, and 72 h, the bacteria burden in the larvae was counted (n ≥ 24 larvae/group/experiment, 3 independent experiments). e Zebrafish larvae at 3 dpf were pretreated with control buffer or VD$_3$ (50 nM) for 48 h. Afterward, the larvae were microinjected with E. tarda (approximately 200 bacteria/larva). The survival of the larvae in each group was recorded up to 36 hpi (n = 10 larvae/group/experiment, 3 independent experiments). Statistical analysis was conducted by Log-rank test. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. csf3b, colony-stimulating factor 3b; mmp9, matrix metalloproteinase9; dpf, days post fertilization; VD$_3$, vitamin D$_3$; E. tarda, Edwardsiella tarda; hpi, hours post infection; mpx, myeloperoxidase.
amputation. In addition, the group treated with 100 nM VD3 exhibited a moderate increase compared with the group treated with 10 nM, at 6 hpi (Fig. 4d). These results indicate that VD3 enhanced the recruitment of neutrophils to the wound, and it corresponded with increased expression of the il-8 gene.

**Vitamin D Restrains Infection with a Gram-Negative Bacterium in the Zebrafish**

Neutrophils are conditioned by host factors and also respond to microbially derived signals, such as LPS and other pathogen-associated molecular patterns, allowing for proper responses to inflammatory stimuli [17]. To define the effects of VD3 in the process against infection, E. tarda, a Gram-negative bacterium known to cause major damage in aquaculture, was used to challenge 6 dpf zebrafish larvae. The mRNA expression of mpx and mmp9 was increased significantly at the transcriptional level after E. tarda infection (immersion model), and the VD3-treated group had higher mpx and mmp9 expression than the control group. There was a significant interaction between VD3 and E. tarda challenge on mpx and mmp9 expressions (Fig. 5a, b). Additionally, during E. tarda infection, bacterial counts showed a profound increase after 72 h post infection (hpi) in the control group, while the growth of E. tarda was inhibited in the group supplemented with VD3 (Fig. 5d). We also challenged zebrafish larvae by microinjection of E. tarda. Compared with the control group, the VD3 group exhibited significantly greater survival after 36 hpi (Fig. 5e). Collectively, these data demonstrated that supplementation with VD3 enhanced the activity of neutrophils and improved the ability of zebrafish to cope with infection caused by E. tarda in both immersion and injection models.

**Neutrophils Are Required for Vitamin D3-Mediated Control of E. tarda Infection**

Finally, the role of neutrophils in VD3-mediated control of E. tarda infection in zebrafish larvae was investigated. Neutrophil-knockdown zebrafish larvae were generated through the CRISPR/Cas9 system, and F0 mosaic mutant larvae (crispants) were acquired. The efficiency of this gene editing was confirmed by qRT-PCR analysis for csf3r transcription expression (Fig. 6a). When zebrafish larvae were challenged by the microinjection of E. tarda at 5 dpf, csf3r−/− crispants showed lower survival rate than the WT counterparts (Fig. 6b). Notably, all csf3r−/− crispants died at 18 hpi, which was independent of pretreatment with VD3 (Fig. 6b). Consistently, VD3 pretreatment significantly suppressed the bacterial load in WT zebrafish larvae at 48 and 72 hpi (Fig. 6c–e), whereas VD3 did not exhibit any significant effects the bacterial load in csf3r−/− crispants (Fig. 6c–f). Thus, neutrophils are essential for VD3 to control bacterial growth in zebrafish larvae.

**Discussion**

In this study, we have shown that VD is an important regulator of granulopoiesis and neutrophil activities in zebrafish in vivo. Moreover, we provided evidence that the microbiota is involved in VD-regulated neutrophil function depending on the developmental stage of the fish. Finally, VD3 supplementation increased neutrophil recruitment to the wound site in zebrafish and protected zebrafish against bacterial infection.

In recent years, there has been a dramatic resurgence in research interest in VD due to its various biological functions, including the immunomodulation of innate immune cells and inflammatory responses [3, 18]. For example, it was reported that VD can regulate macrophage differentiation [19], the activation of T lymphocytes [20], and B-cell function [21]. Moreover, it has been demonstrated that VD signaling plays an important role in the expansion of hematopoietic stem cells and progenitors [22, 23]. However, little is known about the effects of VD on neutrophil development or functions. As one of the main myeloid cell types, neutrophil development is highly conserved in vertebrates [24], and a high percentage of hematopoiesis is committed to the production of neutrophils [25]. During vertebrate development, successive waves of primitive and definitive hematopoiesis can be found in distinct anatomical sites, and they contribute to both embryonic and adult hematopoiesis [26]. The generation of neutrophils occurs in zebrafish from 2 dpf [27, 28], thus we used this time point and treated zebrafish larvae with exogenous VD3 for 4 days and enumerated neutrophil numbers. Interestingly, our results showed that VD3 promoted the abundance of neutrophils in the early development of zebrafish larvae (Fig. 1a, b). The relative gene expression of the neutrophilic genes mpx, lycs, and mmp9 was increased (Fig. 1d).

It has previously been shown that granulopoiesis is regulated by transcription factors and cytokine signals [29]. In particular, G-CSF/CSF3 is an important cytokine involved in granulopoiesis, and it stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils [9]. In fact, there are 2 csf3 subtypes encoded by the genes csf3a and csf3b in zebrafish [30], and our results showed that only csf3b, but
not csf3a, was upregulated in zebrafish larvae treated with VD3. On account of the different temporal and spatial expressions of csf3a and csf3b during embryogenesis in zebrafish [30], these 2 proteins may have different functions in development. To further confirm the effects of VD on neutrophils, RNA was extracted from the gut of cyp2r1+/+ and cyp2r1−/− zebrafish at the age of 3 mpf. In accordance with the results from zebrafish fed without dietary VD3, the gene expression of csf3b and mpx was decreased in cyp2r1−/− zebrafish (Fig. 2b). Therefore, we believe that VD influences neutrophil granulopoiesis and abundance in the intestine via the regulation of G-CSF/CSF3.

It is noteworthy that zebrafish larvae were treated with VD3 instead of the active metabolite 1α,25(OH)2D3 in our study. So far, there is no evidence to prove that VD3 itself directly stimulates the host immune system. A previous study has demonstrated that the metabolite 25(OH)D3 induces the antimicrobial peptide expression in macrophages when TLRs in macrophages are activated [31]. Further results have shown that TLRs activation upregulates the expression of cyp27b1 in macrophages to metabolize 25(OH)D3 into the active 1α,25(OH)2D3, which is highlighting the importance of the active metabolite 1α,25(OH)2D3 in host immunity. As we analyzed in this study, the gene expression of cyp2r1 and cyp27b1, which encode 2 critical enzymes converting VD3 into the active 1α,25(OH)2D3, was enhanced in exogenous VD3-treated fish larvae (data not shown). Interestingly, it has also been reported that the exogenous estrogen can be absorbed by zebrafish larvae (0–5 dpf) [32]. Accordingly, it is speculated that zebrafish larvae uptake exogenous VD3, which is further metabolized into the active 1α,25(OH)2D3 exerting the immune functions.

The gut can be considered as the largest compartment of the immune system, and it is constantly exposed to multiple foreign antigens [33, 34]. The microbiota plays a fundamental role in the induction, education, and function of the immune system [35]. It has been reported that commensal microbiota may contribute to IL-17 and G-CSF production, thus regulating neutrophil homeostasis [36]. In addition, circulating and bone marrow-derived neutrophils are reduced in antibiotic-exposed neonatal mice [37], and germ-free zebrafish exhibit a severe neutropenia [38]. Thus, there seems to be a close link between microbiota and neutrophil physiology. Interestingly, it has been shown that VD/VDR signaling has a significant impact on the intestinal microbiota [39, 40], and it has been suggested that the effect of VD on health is partially mediated through the microbiome [41]. Therefore, we hypothesized that the communication between VD and microbiota in zebrafish might influence neutrophil behavior. To test our hypothesis, the microbiota in the intestine of juvenile zebrafish was depleted by antibiotic treatment. Notably, we found that the decrease in the gene expression of mpx, csf3b, and mmp9 diminished in the intestine of cyp2r1−/− zebrafish compared to WT fish when the microbiota was depleted (Fig. 3d–f), which confirmed the contribution of microbiota to VD-mediated neutrophil functions. However, the supplementation with VD3 still upregulated the gene expression of csf3b, mpx, and mmp9 in gnotobiotic zebrafish at 6 dpf (Fig. 3a–c), which suggested that VD3-induced granulopoiesis was independent on the microbiota in the early developmental stage of zebrafish. Interestingly, a recent report demonstrated that VD promoted G-CSF production in placental explants [42]. Considering the fact that the whole gastrointestinal tube opens in the zebrafish at 6 dpf, and commensal bacteria colonize the intestine of zebrafish over the course of several weeks [43, 44], we suggest that VD/VDR signaling might directly regulate neutrophil granulopoiesis and the gene expression of csf3 in the early development as a transcription factor.

Neutrophils promptly accumulate in large numbers at sites of tissue injury or infection against the potential microbial invasion. Caudal fin amputation of larval zebrafish is a valuable in vivo model of inflammation and wound repair [45]. Interestingly, the previous studies articulated that neutrophils were the primary cells scavenging apoptotic bodies and small cell debris within 6 h post amputation [46], and IL-8 (also known as CXCL8) is known to be one of the most potent chemoattractant molecules for guiding neutrophils to reach the injured sites.

**Fig. 6.** Neutrophils are required for VD3-mediated control of bacterial growth in zebrafish. a Embryos microinjected with control buffer or sgRNAs targeting csf3r were collected at 6 dpf. Genotyping of csf3r amplicons was analyzed by qRT-PCR, and transcript levels of mpx were also tested. b Zebrafish larvae at 5 dpf were microinjected with E. tarda (approximately 200 bacteria/larva). The survival of the larvae in each group was recorded up to 25 hpi (n = 10 larvae/group/experiment, 2 independent experiments). Statistical analysis was conducted by Log-rank test. c–f Zebrafish larvae were immersed with 1 × 10⁸ CFU/mL E. tarda at 3 dpf. After 24, 48, and 72 h, the bacteria burden in the larvae was counted (n ≥ 14 larvae/group/experiment, 2 independent experiments). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, dpf, days post fertilization; VD3, vitamin D3; E. tarda, Edwardsiella tarda; hpi, hours post infection; mpx, myeloperoxidase; WT, wild-type.
[47]. A previous study revealed that 1α,25(OH)2D3, the active form of VD3, enhanced IL-8 production by neutrophils in vitro [48]. Interestingly, our results confirmed that 1α,25(OH)2D3 augmented gene expression of IL-8 in zebrafish lar-va. However, VD-regulated neutrophil generation independence of the microbiota during early development. VD3 treatment significantly decreased bacterial counts and mortality in zebrafish larvae infected with E. tarda in a neutrophil-dependent manner. VD3 augmented the gene expression of IL-8 and neutrophil recruitment to the site of caudal fin amputation. csf3b, colony-stimulating factor 3b; VD3, vitamin D3; E. tarda, Edwardsiella tarda.

VD deficiency has been associated with a range of bacterial infections [49], and our previous study has identified the importance of VD for pneumococcal killing by human neutrophils [50]. It is well established that neutrophils play a critical role during bacterial infection in zebrafish larvae [16, 51]. In addition, csf3r knockdown zebrafish, in which neutrophil counts are substantially reduced, exhibit a higher mortality and bacterial colonization than WT controls [16]. Interestingly, our results confirmed that VD3 treatment reduced the mortality and bacterial colonization in E. tarda-infected zebrafish larvae (Fig. 5). Consistent with previous reports [16, 51], E. tarda infection resulted in significant upregulation of the neutrophil-specific genes mpx, csf3b, and mmp9. Our results showed that VD3 pretreatment further augmented the expression of mpx and mmp9 in zebrafish larvae following E. tarda infection. In fact, mpx is one of the key components in primary granules of neutrophils, and it is an important part of the innate immune system for host defense against invading microorganisms and neutrophil functions [52]. Besides, mmp9 is also present in the tertiary granules of neutrophils and implicated in neutrophil extravasation, the degradation of extracellular matrix, and activation of IL-1β [5, 53]. Furthermore, we confirmed that neutrophils are essential for VD3-mediated effects on E. tarda bacterial growth in zebrafish, since VD3 was inactive in csf3r−/− crispants (Fig. 6). Combined, our results showed that VD enhanced neutrophil functions during bacterial infection, which is beneficial for bacterial clearance in the host.

**Conclusion**

Our findings have outlined a role for VD3 as an important regulator of neutrophil generation and function. Moreover, VD3 enhances neutrophil recruitment to the wound site and restrains the proliferation of pathogenic bacteria in infected zebrafish. Importantly, neutrophils are required for VD3-mediated control of bacterial infection (Fig. 7).
Statement of Ethics

Husbandry and handling of the fish in the present study were approved by the Experimental Animal Ethics Committee of Ocean University of China, and the procedures were performed strictly according to the Management Rule of Laboratory Animals (Chinese order No. 676 of the State Council, revised on 1 March 2017).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

X.L. designed and performed the experiments, analyzed the data, and wrote the manuscript; Y.L., R.S., and S.L. performed the experiments; Z.Y. generated cyp2r1−/− zebrafish and revised the manuscript, G.H.G. and P.B. supervised the project and revised the manuscript; M.W. acquired the financial supports, supervised the project, designed the experiments, analyzed the data, and wrote the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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