

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

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Keywords

Vitamin D₃ · 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 VD₃ 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 VD₃ augmented the expression of *il-8* and neutrophil recruitment to the site of caudal fin amputation. Finally, VD₃ 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.

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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 D₃ (VD₃) is the main form of VD in animals, and it is converted to the hormonal form 1 α ,25(OH)₂D₃ by 2 cytochrome P450 (CYP) enzymes, 25-hydroxylase, and 1 α -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.

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 premix ¹	40	40
Vitamin premix (VD ₃ free) ²	10	10
VD ₃ , IU/kg	0	800

VD₃, vitamin D₃. ¹Mineral premix (mg/g diet): calcium lactate, 327; FeSO₄, 3.125; MgSO₄, 137; NaH₂PO₄, 87.2; NaCl, 43.5; AlCl₃, 0.15; KIO₃, 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 *cyp2r1^{-/-}* zebrafish has been described in a previous report [13].

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 RNeasy™ 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 2. The sequences of the primers (5'–3') used in this study

Test	Symbol	Forward	Reverse
Genotyping	<i>cyp2r1</i>	CCCCAAGTTTGCATCTAAGA	GATGCATTACACTGCTATGC [13]
	<i>csf3r</i>	CTTGTGTGTGTTCCAGGAGGA	GGGTGAGTTTCAGAGGATCAG [16]
qRT-PCR	<i>Mpx</i>	TCCAAGCTATGTGGGATGTGA	GTCGTCGGCAAACTGAA [38]
	<i>lysc</i>	TCGTGTGAAAGCAAGACACTGGGA	ACTCGTGGGTCTTAAACCTGCTT [12]
	<i>csf3a</i>	AACTACATCTGAACCTCCTG	GACTGCTCTTCTGATGTCTG [30]
	<i>csf3b</i>	AGAGAACCTACTGAACGACCT	CTTGAACCTGGCTGAGTGGAG [16]
	<i>il-8</i>	GTCGCTGCATTGAAACAGAA	CTTAACCCATGGAGCAGAGG
	<i>mmp9</i>	CATCCGCAACTACAAGACATTC	GGTCCAGTATTATCATCGTCATCA [16]
	<i>actin2</i>	GATGATGAAATTGCCGCACTG	ACCAACCATGACACCCTGATGT [13]

csf3b, colony-stimulating factor 3b; *mmp9*, matrix metalloproteinase 9; *mpx*, myeloperoxidase; *lysc*, lysozyme C.

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, 0.5 µg/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 pos-

Table 3. *csf3r* sgRNA targets used in this study

sgRNA	Sequence (5'–3')
Target #1	TGCCAGCCTCCGGTGTGAC [16]
Target #2	CGTAGAAGTGCTGCCCGGGT [16]
sg, single-guide.	

terior to the end of the notochord using a surgical scalpel (Surgical Specialties Sharp, 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 10⁸ 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

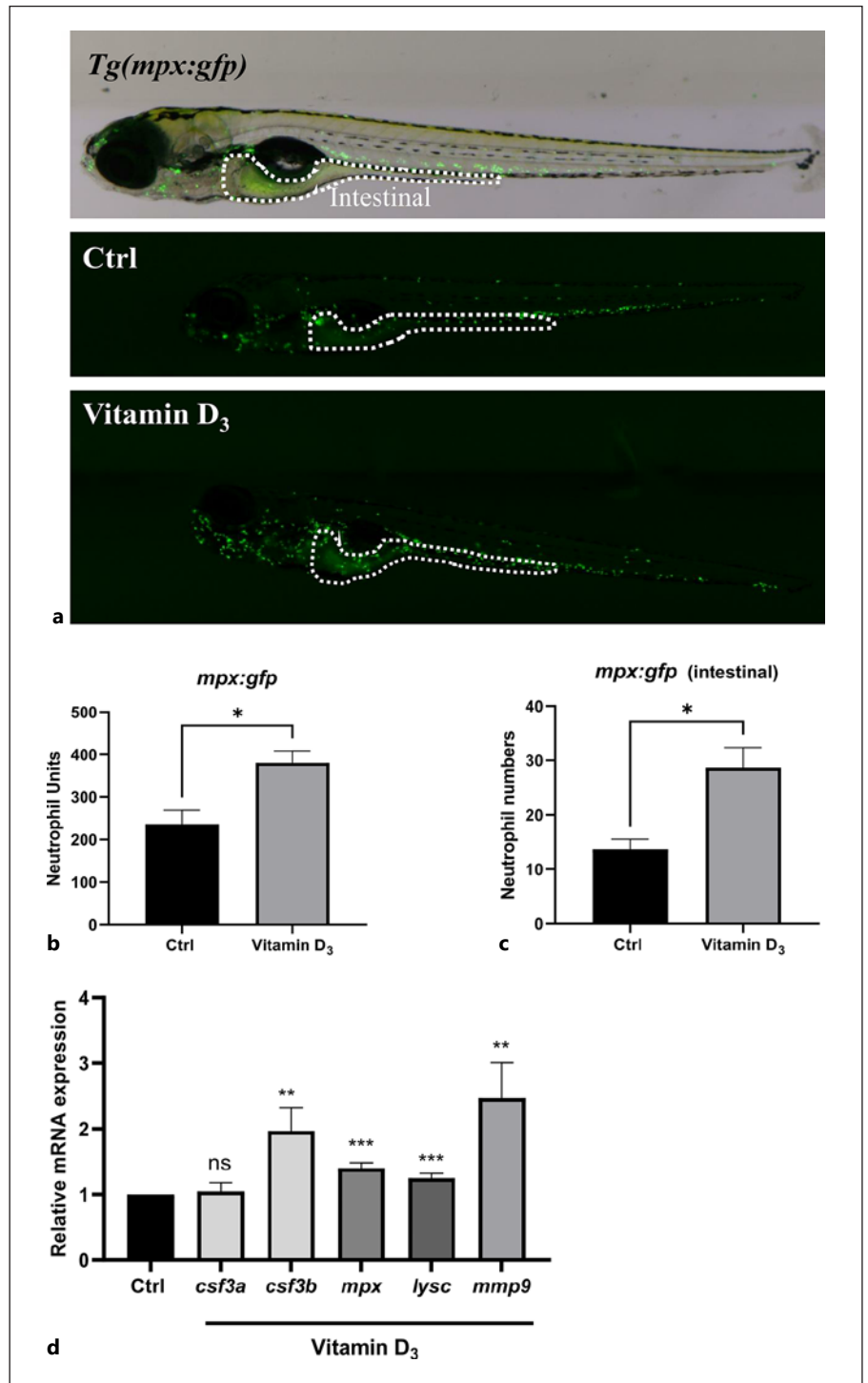


Fig. 1. VD₃ promotes the granulopoiesis in zebrafish larvae. Zebrafish larvae at 2 dpf were exposed to 100 nM VD₃ for 2 days, GZM was replaced at 4 dpf, and 100 nM VD₃ were added simultaneous. **a** The merged images of *Tg(mpx:gfp)* zebrafish (6 dpf) showed the abundance and localization of GFP⁺ neutrophils. 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; lysozyme C; mmp9, matrix metalloproteinase9; GZM, gnotobiotic zebrafish medium.

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.

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*, *lys*, and *mmp9*, were expressed at higher levels in juvenile zebrafish (35 dpf) fed for 2 weeks with a diet containing 800 IU VD₃ compared to the 0 IU group (Fig. 2a). Moreover, the gene expression of *csf3a*, *csf3b*, *mpx*, *mmp9* in *cyp2r1*^{-/-} zebrafish was significantly decreased compared to wild-type (WT) control zebrafish at adult stages (Fig. 2b). These transcriptional differences suggested that VD₃ contributed to the number and distribution of the neutrophil population in zebrafish intestines.

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. Interest-

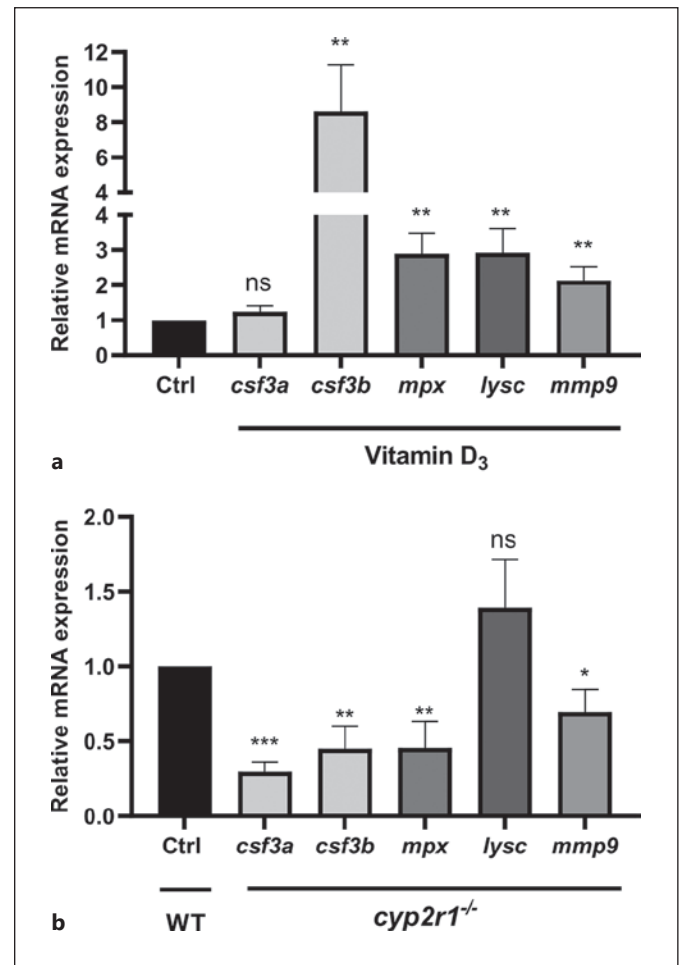


Fig. 2. VD₃ contributes to the granulopoiesis in zebrafish intestine. **a** The gene expression of *csf3a*, *csf3b*, *mpx*, *lysc*, and *mmp9* in the gut of zebrafish (35 dpf) fed 0 or 800 IU VD₃/kg diets for 2 weeks ($n = 12$ replicates/group, 2–3 zebrafish/replicate). **b** Transcript levels of *csf3a*, *csf3b*, *mpx*, *lysc*, and *mmp9* in the gut of WT and *cyp2r1* mutant zebrafish in 3 months ($n = 8$ replicates/genotype). Data are represented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *csf3a*, colony-stimulating factor 3a; *csf3b*, colony-stimulating factor 3b; *lysc*, lysozyme C; *mmp9*, matrix metalloproteinase9; dpf, days post fertilization; VD₃, vitamin D₃; WT, wild-type; *mpx*, myeloperoxidase.

ingly, 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-

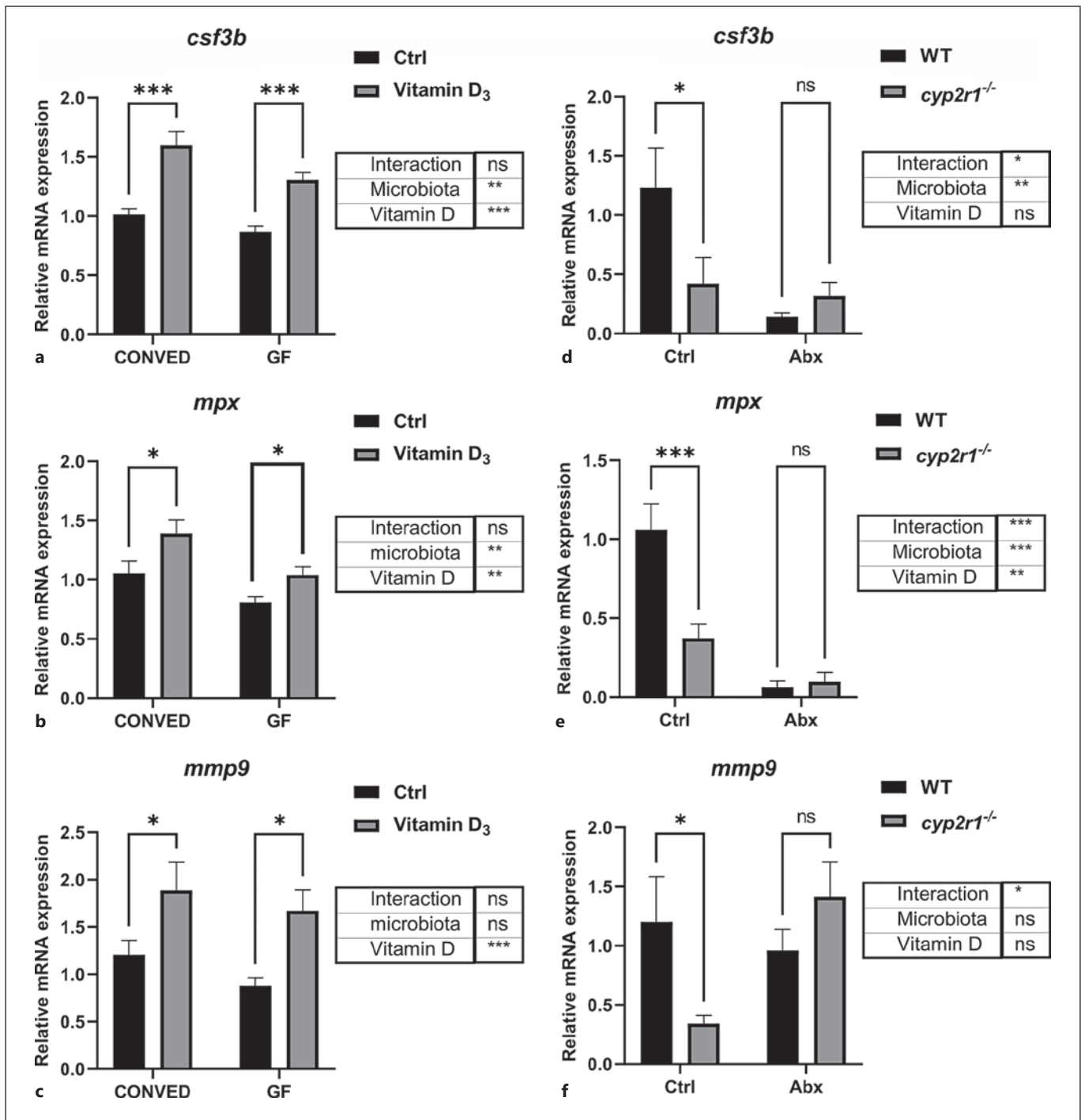


Fig. 3. Microbiota impact on granulopoiesis in zebrafish. **a–c** The gene expression of *csf3b* (**a**), *mpx* (**b**), and *mmp9* (**c**) in CONVED or GF zebrafish larvae (6 dpf) treated 0 or 100 nM VD₃ for 4 days (results are combined from 2 independent experiments, $n = 12$ replicates/group, 10–20 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**) tran-

scripts 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 \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *csf3b*, colony-stimulating factor 3; *mmp9*, matrix metalloproteinase9; dpf, days post fertilization; VD₃, vitamin D3; WT, wild-type; CONVED, conventionalized; GF, germ-free; *mpx*, myeloperoxidase.

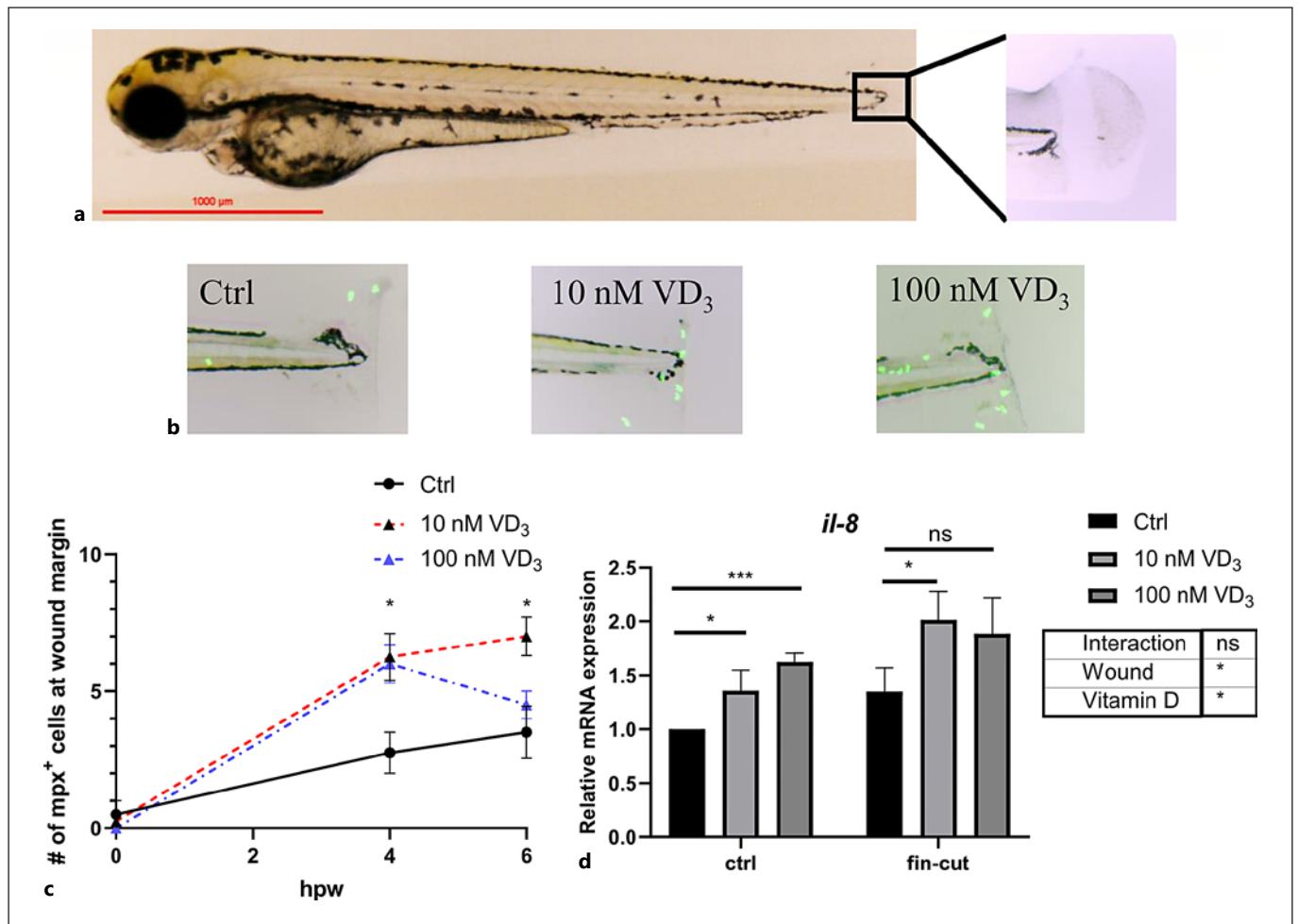


Fig. 4. VD₃ enhances neutrophil recruitment to the wound site of amputated fish. **a–c** The caudal fin of 6 dpf *Tg(mpx:gfp)* zebrafish. Imaging and quantification of GFP⁺ neutrophils recruited to tail wound at 4 hpw ($n = 4$ /group) (**b**). **d** qRT-PCR analysis of IL-8 transcripts in 6 dpf zebrafish larvae treated 0 nM, 10 nM, or 100 nM VD₃ for 4 days, with and without caudal fin amputation ($n = 6$ rep-

licates/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 \pm 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.

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

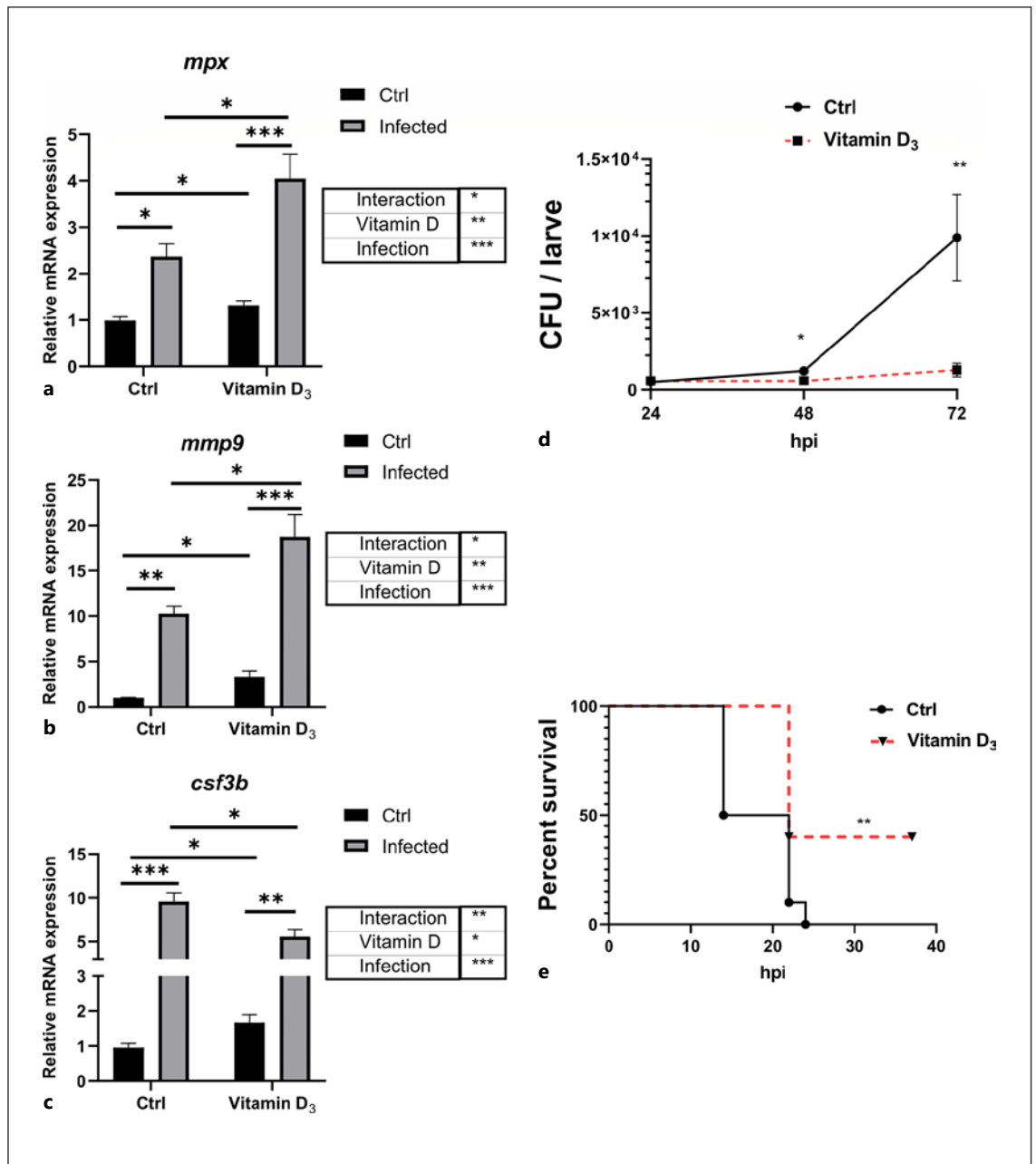


Fig. 5. VD₃ restrains pathogen infection in zebrafish. **a–c** Transcript levels of *mpx* (**a**), *mmp9* (**b**), and *csf3b* (**c**) in unstimulated and *E. tarda*-exposed zebrafish larvae, pretreated with 0 or 100 nM VD₃ ($n = 4$ replicates/group, 10–20 larvae/replicate). Two-way ANOVA with *Sidak* 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 ($n \geq 24$ larvae/group/experiment, 3 independent experiments). **e** Zebrafish larvae at 3 dpf were pretreated with control buffer or

VD₃ (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 \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *csf3b*, colony-stimulating factor 3b; *mmp9*, matrix metalloproteinase9; dpf, days post fertilization; VD₃, vitamin D₃; *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 hpw (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 zebra-

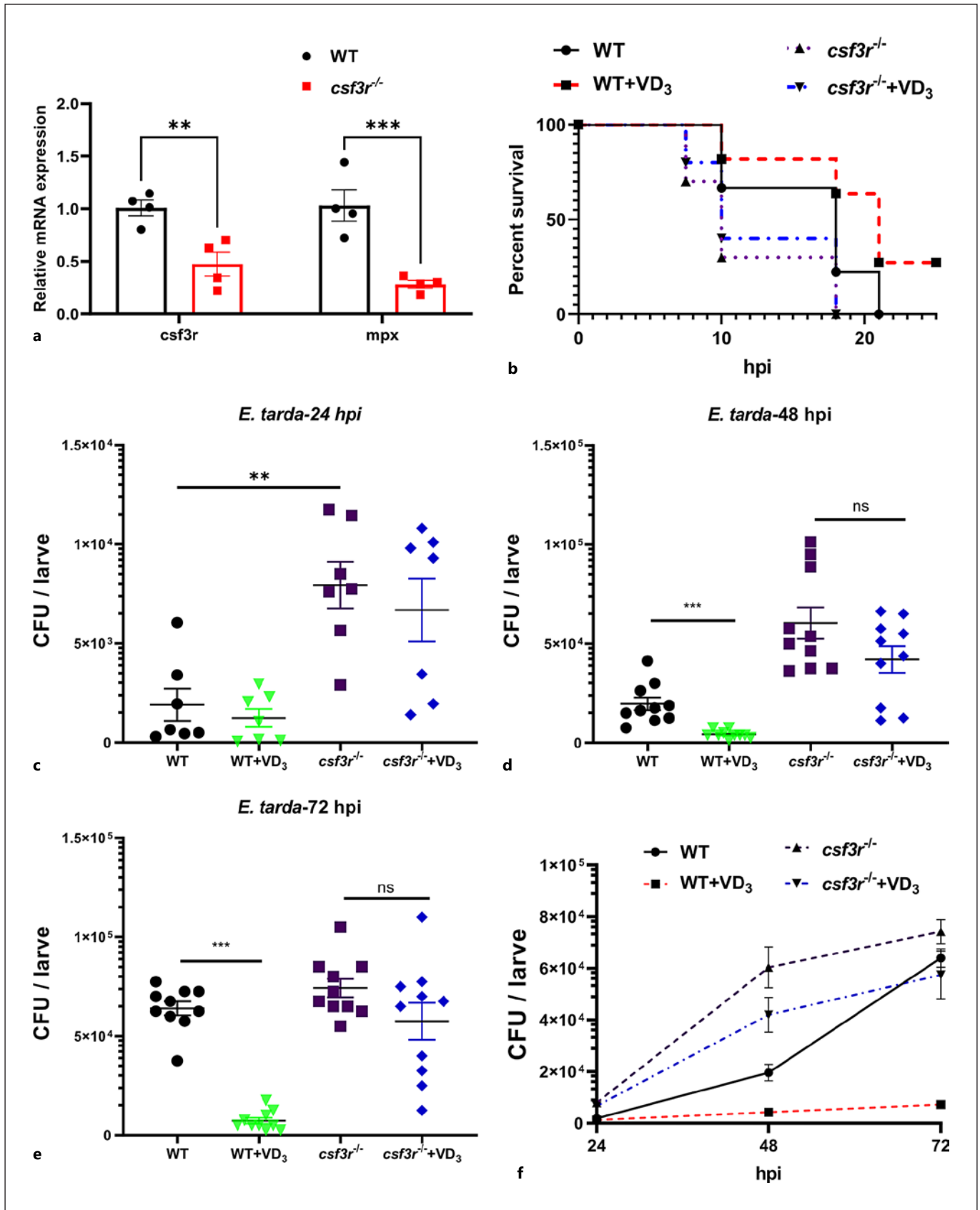
fish larvae at 48 and 72 hpi (Fig. 6c–e), whereas VD₃ did not exhibit any significant effects on the bacterial load in *csf3r*^{-/-} crispants (Fig. 6c–f). Thus, neutrophils are essential for VD₃ to control bacterial growth in zebrafish larvae.

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*, *lysc*, 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



(For legend see next page.)

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 1 α ,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 1 α ,25(OH)₂D₃ [31], which is highlighting the importance of the active metabolite 1 α ,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 1 α ,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 1 α ,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

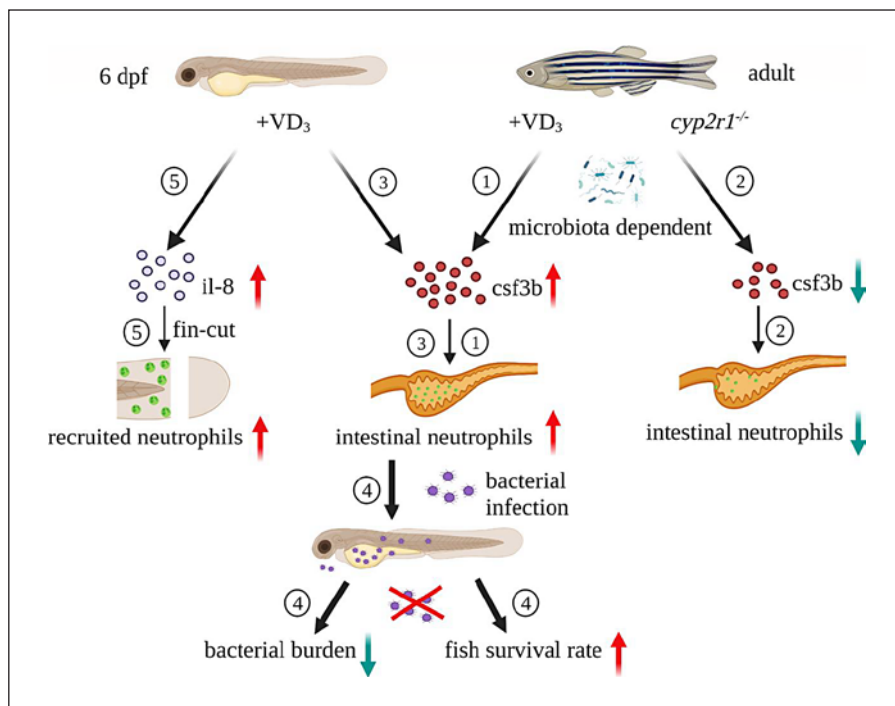
neutrophils are reduced in antibiotic-exposed neonatal mice [37], and germ-free zebrafish exhibit a severe neutropenia [38]. Thus, there seems to be a close link between microbiota and neutrophil physiology. Interestingly, it has been shown that VD/VDR signaling has a significant impact on the intestinal 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). Statisti-

cal analysis was conducted by Log-rank test. **c–f** Zebrafish larvae were immersed with 1×10^8 CFU/mL *E. tarda* at 3 dpf. After 24, 48, and 72 h, the bacteria burden in the larvae was counted ($n \geq 14$ larvae/group/experiment, 2 independent experiments). Data are represented as mean \pm 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.

Fig. 7. Schematic model of the regulation of granulopoiesis and neutrophil functions by VD₃ in zebrafish. ① In adult zebrafish, the gene expression of *csf3b* and the neutrophil markers were enhanced in the intestine of the fish fed with VD₃-containing diet, compared to those fed with non-VD₃ diet. ② The gene expression of *csf3b* and neutrophil abundance in the intestine of adult zebrafish with a *cyp2r1* mutant were reduced. Interestingly, microbiota is involved in VD-mediated granulopoiesis in adult zebrafish. ③ Addition of exogenous VD₃ promoted granulopoiesis in zebrafish larvae. However, VD-regulated neutrophil generation independent of the microbiota during early development. ④ VD₃ treatment significantly decreased bacterial counts and mortality in zebrafish larvae infected with *E. tarda* in a neutrophil-dependent manner. ⑤ 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*.



[47]. A previous study revealed that 1 α ,25(OH)₂D₃, the active form of VD₃, enhanced IL-8 production by neutrophils in vitro [48]. Interestingly, our results confirmed that 1 α ,25(OH)₂D₃ augmented gene expression of IL-8 in zebrafish larva, and further improved neutrophil recruitment to the wound site (Fig. 4). These results demonstrated that VD could regulate neutrophil behaviors and promote neutrophil recruitment to the injury sites in an IL-8 dependent manner, which potentially contributes to injury protection and subsequent wound healing.

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 fol-

lowing *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-1 β [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.

References

- 1 Sassi F, Tamone C, D'Amelio P. Vitamin D: nutrient, hormone, and immunomodulator. *Nutrients*. 2018 Nov;10(11):1656.
- 2 Jones G, Prosser DE, Kaufmann M. Cytochrome P450-mediated metabolism of vitamin D. *J Lipid Res*. 2014 Jan;55(1):13–31.
- 3 Cantorna MT, Rogers CJ, Arora J. Aligning the paradoxical role of vitamin D in gastrointestinal immunity. *Trends Endocrinol Metab*. 2019 Jul;30(7):459–66.
- 4 Lock E-J, Waagbø R, Wendelaar Bonga S, Flik G. The significance of vitamin D for fish: a review. *Aquac Nutr*. 2010 Sep;16(1):100–16.
- 5 Liew PX, Kubes P. The neutrophil's role during health and disease. *Physiol Rev*. 2019 Apr;99(2):1223–48.
- 6 Phillipson M, Kubes P. The healing power of neutrophils. *Trends Immunol*. 2019 Jul;40(7):635–47.
- 7 Lawrence SM, Corriden R, Nizet V. The ontogeny of a neutrophil: mechanisms of granulopoiesis and homeostasis. *Microbiol Mol Biol Rev*. 2018 Feb;82(1):e00057–17.
- 8 Havixbeck JJ, Barreda DR. Neutrophil development, migration, and function in teleost fish. *Biology*. 2015 Nov;4(4):715–34.
- 9 Pazhakh V, Clark S, Keightley MC, Lieschke GJ. A GCSFR/CSF3R zebrafish mutant models the persistent basal neutrophil deficiency of severe congenital neutropenia. *Sci Rep*. 2017 Mar;7:44455.
- 10 Salva S, Alvarez S. The role of microbiota and immunobiotics in granulopoiesis of immunocompromised hosts. *Front Immunol*. 2017 May;8:507.
- 11 Renshaw SA, Loynes CA, Trushell DM, Elworthy S, Ingham PW, Whyte MK. A transgenic zebrafish model of neutrophilic inflammation. *Blood*. 2006 Dec;108(13):3976–8.
- 12 Murdoch CC, Espenschied ST, Matty MA, Mueller O, Tobin DM, Rawls JF. Intestinal Serum amyloid A suppresses systemic neutrophil activation and bactericidal activity in response to microbiota colonization. *PLoS Pathog*. 2019 Mar;15(3):e1007381.
- 13 Peng X, Shang G, Wang W, Chen X, Lou Q, Zhai G, et al. Fatty acid oxidation in zebrafish adipose tissue is promoted by 1 α ,25(OH) $_2$ D $_3$. *Cell Rep*. 2017 May;19(7):1444–55.
- 14 Ellett F, Lieschke GJ. Computational quantification of fluorescent leukocyte numbers in zebrafish embryos. *Methods Enzymol*. 2012 Feb;506:425–35.
- 15 Pham LN, Kanther M, Semova I, Rawls JF. Methods for generating and colonizing gnotobiotic zebrafish. *Nat Protoc*. 2008 Dec;3(12):1862–75.
- 16 Wang Z, Lin L, Chen W, Zheng X, Zhang Y, Liu Q, et al. Neutrophil plays critical role during *Edwardsiella piscicida* immersion infection in zebrafish larvae. *Fish Shellfish Immunol*. 2019 Apr;87:565–72.
- 17 El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie JC, Gougerot-Pocidalo MA, Dang PM. Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Immunol Rev*. 2016 Sep;273(1):180–93.
- 18 Martens PJ, Gysemans C, Verstuyf A, Mathieu AC. Vitamin D's effect on immune function. *Nutrients*. 2020 Apr;12(5):1248.
- 19 Wasnik S, Rundle CH, Baylink DJ, Yazdi MS, Carreon EE, Xu Y, et al. 1,25-Dihydroxyvitamin D suppresses M1 macrophages and promotes M2 differentiation at bone injury sites. *JCI Insight*. 2018 Sep;3(17):e98773.
- 20 Von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Ødum N, Geisler C. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. *Nat Immunol*. 2010 Apr;11(4):344–9.
- 21 Rolf L, Muris AH, Hupperts R, Damoiseaux J. Vitamin D effects on B cell function in autoimmunity. *Ann N Y Acad Sci*. 2014 May;1317(1):84–91.
- 22 Cortes M, Chen MJ, Stachura DL, Liu SY, Kwan W, Wright F, et al. Developmental vitamin D availability impacts hematopoietic stem cell production. *Cell Rep*. 2016 Oct;17(2):458–68.
- 23 Medrano M, Carrillo-Cruz E, Montero I, Perez-Simon JA. Vitamin D: effect on haematopoiesis and immune system and clinical applications. *Int J Mol Sci*. 2018 Sep;19(9):2663.
- 24 Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. *Immunol Rev*. 2016 Sep;273(1):11–28.
- 25 Kennedy AD, DeLeo FR. Neutrophil apoptosis and the resolution of infection. *Immunol Res*. 2009 Dec;43(1–3):25–61.
- 26 Ottersbach K, Smith A, Wood A, Göttgens B. Ontogeny of haematopoiesis: recent advances and open questions. *Br J Haematol*. 2010 Feb;148(3):343–55.

- 27 Le Guyader D, Redd MJ, Colucci-Guyon E, Murayama E, Kissa K, Briolat V, et al. Origins and unconventional behavior of neutrophils in developing zebrafish. *Blood*. 2008 Jan 1; 111(1):132–41.
- 28 Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, et al. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity*. 2006 Dec;25(6):963–75.
- 29 Ward AC, Loeb DM, Soede-Bobok AA, Touw IP, Friedman AD. Regulation of granulopoiesis by transcription factors and cytokine signals. *Leukemia*. 2000 Jun;14(6):973–90.
- 30 Stachura DL, Svoboda O, Campbell CA, Espín-Palazón R, Lau RP, Zon LI, et al. The zebrafish granulocyte colony-stimulating factors (Gcsfs): 2 paralogous cytokines and their roles in hematopoietic development and maintenance. *Blood*. 2013 Dec;122(24):3918–28.
- 31 Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*. 2006 Mar;311(5768):1770–3.
- 32 Souder JP, Gorelick DA. Quantification of estradiol uptake in zebrafish embryos and larvae. *Toxicol Sci*. 2017 Aug;158(2):465–74.
- 33 Mowat AM. To respond or not to respond: a personal perspective of intestinal tolerance. *Nat Rev Immunol*. 2018 Jun;18(6):405–15.
- 34 Martins RR, Ellis PS, MacDonald RB, Richardson RJ, Henriques CM. Resident immunity in tissue repair and maintenance: the zebrafish model coming of age. *Front Cell Dev Biol*. 2019 Feb;7:12.
- 35 Belkaid Y, Harrison OJ. Homeostatic immunity and the microbiota. *Immunity*. 2017 Apr; 46(4):562–76.
- 36 Mei J, Liu Y, Dai N, Hoffmann C, Hudock KM, Zhang P, et al. Cxcr2 and Cxcl5 regulate the IL-17/G-CSF axis and neutrophil homeostasis in mice. *J Clin Invest*. 2012 Mar;122(3): 974–86.
- 37 Deshmukh HS, Liu Y, Menkiti OR, Mei J, Dai N, O’Leary CE, et al. The microbiota regulates neutrophil homeostasis and host resistance to *Escherichia coli* K1 sepsis in neonatal mice. *Nat Med*. 2014 May;20(5):524–30.
- 38 Kanther M, Tomkovich S, Xiaolun S, Grosser MR, Koo J, Flynn EJ, et al. Commensal microbiota stimulate systemic neutrophil migration through induction of serum amyloid A. *Cell Microbiol*. 2014 Jul;16(7):1053–67.
- 39 Wang J, Thingholm LB, Skiecevičienė J, Rausch P, Kummel M, Hov JR, et al. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. *Nat Genet*. 2016 Nov;48(11):1396–406.
- 40 Thomas RL, Jiang L, Adams JS, Xu ZZ, Shen J, Janssen S, et al. Vitamin D metabolites and the gut microbiome in older men. *Nat Commun*. 2020 Nov;11(1):5997.
- 41 Waterhouse M, Hope B, Krause L, Morrison M, Protani MM, Zakrzewski M, et al. Vitamin D and the gut microbiome: a systematic review of in vivo studies. *Eur J Nutr*. 2019 Oct; 58(7):2895–910.
- 42 Zhang JY, Wu P, Chen D, Ning F, Lu Q, Qiu X, et al. Vitamin D promotes trophoblast cell induced separation of vascular smooth muscle cells in vascular remodeling via induction of G-CSF. *Front Cell Dev Biol*. 2020 Dec;8: 601043.
- 43 Brugman S. The zebrafish as a model to study intestinal inflammation. *Dev Comp Immunol*. 2016 Nov;64:82–92.
- 44 Wallace KN, Pack M. Unique and conserved aspects of gut development in zebrafish. *Dev Biol*. 2003 Mar;255(1):12–29.
- 45 Miskolci V, Squirrell J, Rindy J, Vincent W, Sauer JD, Gibson A, et al. Distinct inflammatory and wound healing responses to complex caudal fin injuries of larval zebrafish. *Elife*. 2019 Jul;8:e45976.
- 46 Li L, Yan B, Shi YQ, Zhang WQ, Wen ZL. Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration. *J Biol Chem*. 2012 Jul; 287(30):25353–60.
- 47 De Oliveira S, Reyes-Aldasoro CC, Candel S, Renshaw SA, Mulero V, Calado A. Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response. *J Immunol*. 2013 Apr;190(8):4349–59.
- 48 Chen L, Eapen MS, Zosky GR. Vitamin D both facilitates and attenuates the cellular response to lipopolysaccharide. *Sci Rep*. 2017 Mar;7:45172.
- 49 Derbyshire EJ, Calder PC. Respiratory tract infections and antibiotic resistance: a protective role for vitamin D? *Front Nutr*. 2021 Mar; 8:652469.
- 50 Subramanian K, Bergman P, Henriques-Normark B. Vitamin D promotes pneumococcal killing and modulates inflammatory responses in primary human neutrophils. *J Innate Immun*. 2017 Feb;9(4):375–86.
- 51 Liu X, Chang X, Wu H, Xiao J, Gao Y, Zhang Y. Role of intestinal inflammation in predisposition of *Edwardsiella tarda* infection in zebrafish (*Danio rerio*). *Fish Shellfish Immunol*. 2014 Dec;41(2):271–8.
- 52 Aratani Y. Myeloperoxidase: its role for host defense, inflammation, and neutrophil function. *Arch Biochem Biophys*. 2018 Feb;640: 47–52.
- 53 Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood*. 2000 Oct;96(8):2673–81.