Alas1 is essential for neutrophil maturation in zebrafish

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ABSTRACT

Neutrophils play essential roles in innate immunity and are the first responders to kill foreign micro-organisms, a function that partially depends on their granule content. The complicated regulatory network of neutrophil development and maturation remains largely unknown. Here we utilized neutrophil-deficient zebrafish to identify a novel role of Alas1, a heme biosynthesis pathway enzyme, in neutrophil development. We showed that Alas1-deficient zebrafish exhibited proper neutrophil initiation, but further neutrophil maturation was blocked due to heme deficiency, with lipid storage and granule formation deficiencies, and loss of heme-dependent granule protein activities. Consequently, Alas1-deficient zebrafish showed impaired bactericidal ability and augmented inflammatory responses when challenged with *Escherichia coli*. These findings demonstrate the important role of Alas1 in regulating neutrophil maturation and physiological function through the heme. Our study provides an *in vivo* model of Alas1 deficiency and may be useful to evaluate the progression of heme-related disorders in order to facilitate the development of drugs and treatment strategies for these diseases.

Introduction

Neutrophils are the most abundant leukocytes in the circulation and the first responders to sites of infection, where they attack pathogens by phagocytosis, degranulation, and by generating neutrophil extracellular traps.1,2 Neutrophil development is highly conserved in vertebrates, making zebrafish a suitable model for investigation. Neutrophils are derived from granulocyte-monocyte progenitors, and undergo determination and differentiation from myeloblasts to mature neutrophils.1,3 During neutrophil differentiation and maturation, neutrophil granules are formed and assembled.1 Anti-microbial proteins are thought to be the major constituents of neutrophil granules, and they play important roles in neutrophil diapedesis, chemotaxis, and the phagocytosis of micro-organisms.1,4

Several transcription factors have been reported to be involved in neutrophil development and physiological function in mammals, including SPI.1/PU1 and C/EBP-ε.3 A recent study of embryonic myelopoiesis revealed that the Pu.1-Runx1 regulatory loop controlled embryonic myeloid cell fate in zebrafish.5,6 We previously demonstrated that c-Myb and Cebp1 co-operatively acted in parallel to govern neutrophil maturation.7 In addition to these transcription factors, zebrafish deficient for the neutrophil granule protein myeloperoxidase (Mpx) or the neutrophil-specific marker nephrosin (Npsn) have altered neutrophil maturation and inflammatory responses to fungal and bacterial infection, respectively.8,9 Nevertheless, the complicated regulatory network of neutrophil maturation, as well as the impact on physiological function remain poorly understood.

Heme (iron protoporphyrin IX) functions as a prosthetic group on various proteins, so-called hemoproteins, such as hemoglobin, myoglobin, cytochromes, catalases, and peroxidases.10 Hemoproteins are involved in diverse biological functions, including oxygen transport, energy metabolism, and drug biotransformation.11 Moreover, heme also plays important roles in the regulation of transcription,12,14

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The 5-aminolevulinate synthase 1 (ALAS1) is a mitochondrial enzyme that catalyzes the condensation of glycine and succinyl-CoA, forming 5-aminolevulinic acid. ALAS1 is the first and rate-limiting enzyme of the heme biosynthetic pathway, which is conserved from lower to higher organisms. ALAS1 (also called hepatic ALAS or non-specific ALAS) is ubiquitously expressed throughout the body, whereas another isoform, ALAS2 (also called ALAS-E), is predominantly expressed in erythroid cells, to meet the need of the large amounts of heme required for hemoglobin synthesis. It has been reported that human ALAS2 mutations cause X-linked sideroblastic anemia; ALAS2-deficient mice and zebrafish also display severe anemia, revealing the major contribution of ALAS2 to erythroid heme biosynthesis, and hence how it is essential to erythroid differentiation. In contrast to ALAS2, there are no reported human diseases directly caused by mutations in ALAS1. In mice, Alas1-null embryos are lethal by embryonic day 8.5 (E8.5), thus, the in vivo physiological role of ALAS1 is unclear. Using GFP knock-in mice (Alas1fl/fl), ALAS1 was found to be highly expressed in the liver, exocrine, endocrine glands, and myeloid cells, where large amounts of heme are required to meet the needs of tissue-specific hemoproteins, such as MPO, NADPH oxidase, and CYP450. Notably, Alas1 is also expressed higher in neutrophils than macrophages, suggesting cell-specific roles in neutrophils. However, the function of ALAS1 in neutrophils is still unknown.

Taking advantage of their transparent body, we can observe neutrophil morphology to trace neutrophil behaviors in live zebrafish. Zebrafish is an ideal model for studying neutrophil biology. Here we report a novel role of Alas1 in regulating neutrophil development using a neutrophil lineage-deficient mutant zebrafish line (previously representing neutrophil peroxidase activity was absent in mutants). Therefore, we speculated that the loss of the SB signal was due to defects in neutrophil maturation. To test this possibility, we detected the transcript and protein activity of the myeloid-specific peroxidase (Mpx), which is an abundant granule protein in neutrophils. Whole-mount in situ hybridization (WISH) showed that mpx mRNA expression was intact (Figure 1C), suggesting the presence of neutrophils in smu350 mutants. To further examine Mpx enzyme activity, diaminobenzidine (DAB, a peroxidase substrate) staining was performed. The results showed that while the signal in the yolk sac (representing hemoglobin peroxidase activity) was present, signals representing neutrophil peroxidase activity were absent in smu350 mutants (Figure 1D), suggesting that Mpx lost its catalytic activity.

**Methods**

**Fish maintenance**

Zebrafish were maintained under standard conditions. Embryos were maintained in egg water containing 0.2 mM N-phenylthiourea (Sigma-Aldrich, St. Louis, MO, USA) to prevent pigment formation. All work involving zebrafish was approved by the Institutional Animal Ethics Committee of the Southern Medical University. The following strains were used: AB, Tg(lyz:DsRed), Tg(gata1-DsRed), Tg(vitrino) and alas1+/-.

**Treatment with succinylacetone**

Succinylacetone (SA) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in egg water. Zebrafish embryos were placed in culture dishes containing 1 mM SA at 5 hours (h) post fertilization (hpf) until the desired stage.

**Bacterial infection**

The eGFP-labeled E. coli strain XL10 was cultured in LB broth with 50 μg mL⁻¹ ampicillin at 37°C until reaching an optical density at 600 nm between 0.5-0.8. Bacteria were washed with sterile phosphate buffered saline (PBS) three times, harvested by centrifugation at 5000 x g for 5 minutes (min) and resuspended in sterile PBS. The working concentration of E. coli was 2x10⁶ mL⁻¹, and approximately 0.5 mL of bacterial suspension was subcutaneously injected over a somite into 3-day-post-fertilization (dpf) embryos with 0.02% tricaine using a PLI-100A Pico-Injector (Warner Instruments, Hamden, CT, USA) as previously described. For bacterial colony forming assays, every injected embryo was washed with sterile PBS three times, and then homogenized in 200 μL of sterile PBS at the desired time points. Then, 10 μL of homogenate was plated on LB medium with ampicillin and cultured at 37°C overnight. The results are the average of two separate experiments.

**Statistical analysis**

Data were recorded and analyzed using GraphPad Prism 7 and IBM SPSS v.23. Two-tailed Student t-test and Mann-Whitney U test were used for comparisons between parametric and non-parametric data, respectively. One-way analysis of variance (with Bonferroni or Dunnett T3 post-test adjustment) was used for parametric data to make multi-comparisons. Differences were considered significant at P<0.05. Data are expressed as the mean±Standard Deviation (SD).

**Results**

**Neutrophil deficiency in smu350 mutant zebrafish**

To identify new regulators of neutrophil development, we conducted a genetic screen for neutrophil-deficient zebrafish mutants using Sudan black B (SB) staining. From this screen, we isolated the neutrophil-deficient smu350 mutant, which lacked the SB signal as early as 36 hpf (Figure 1A). The early loss of the SB signal in smu350 mutants suggested defects in embryonic neutrophils, as SB cells represent embryonic neutrophils that are initiating from embryonic myelopoietic tissue. Because myeloid progenitors that are derived from rostral blood islands will progress to neutrophils during embryonic myelopoiesis, we first determined if there were defects in the formation of myeloid progenitors. The results showed that mpx expression at 22 hpf was normal (Figure 1B), suggesting the presence of myeloid progenitors in smu350 mutants. Therefore, we speculated that the loss of the SB signal was due to defects in neutrophil maturation. To test this possibility, we detected the transcript and protein activity of the myeloid-specific peroxidase (Mpx), which is an abundant granule protein in neutrophils. Whole-mount in situ hybridization (WISH) showed that mpx mRNA expression was intact (Figure 1C), suggesting the presence of neutrophils in smu350 mutants. To further examine Mpx enzyme activity, diaminobenzidine (DAB, a peroxidase substrate) staining was performed. The results showed that while the signal in the yolk sac (representing hemoglobin peroxidase activity) was present, signals representing neutrophil peroxidase activity were absent in smu350 mutants (Figure 1D), suggesting that Mpx lost its catalytic activity. As neutrophil granules are abundant with Mpx, we directly monitored neutrophil granule morphology via video-enhanced differential interference contrast (VE-DIC) analyses of live embryos. The results showed that neutrophils from siblings (alas1+/- and alas1+/- embryos from a heterozygous alas1+/+ in-cross) had abundant visible and highly mobilized granules, while neutrophils from smu350 mutants lacked such granules.
These results suggest that neutrophil maturation is defective in smu350 mutants.

The alas1 mutation was responsible for neutrophil defects of smu350 mutant

Positional cloning was then performed to identify the causative gene in smu350 mutants. Initial mapping with bulk segregation analysis located the mutated site to linkage group 11 (data not shown), then fine mapping placed the mutated gene within a region between two simple sequence length polymorphism markers, CU638745-M and CU929297-M, from the Massachusetts General Hospital panel (Figure 2A). The mutation was then mapped to a 100-kb region partly covered by two bacterial artificial chromosomes (BACs) (Figure 2A). There were 9 predicted genes in this region (Figure 2A). By sequencing, we found a T-to-A mutation in alas1 intron 7 next to the...
Figure 2. The *alas1* gene was mutated in *smu350* mutants. (A) The mutated gene in *smu350* mutants mapped to a 100-kb region between two simple sequence length polymorphism markers, CU633745-M (two recombinants in 6160 *smu350* mutant embryos) and CU929297-M (three recombinants in 6160 *smu350* mutant embryos), on linkage group 11. The 100-kb region, partly covered by two bacterial artificial chromosomes (BACs) (CU633745 and CU929297), contains 9 predicted genes. (B) The structure of the zebrafish *alas1* gene. The red asterisk indicates a T-to-A mutation in intron 7 of *alas1* in *smu350* mutants. The black arrow indicates the position of the CRISPR/Cas9 target in *alas1*. Numbers of constitutive exons are indicated. (C) Agarose gel electrophoresis of *alas1* RT-qPCR amplification products from 3-day post fertilization (dpf) wild-type zebrafish, siblings, and *smu350* mutants. Four major products (indicated by black arrows) were identified in *smu350* mutants compared with wild-type transcripts (461 bp). The *actb2* was used as an internal control. (D) The mutated *alas1* transcripts and their predicted translation products in *smu350* mutants. The blue arrow indicates the position of the transcriptional start site. Black arrows indicate the RT-qPCR primers used in (C), and black boxes indicate the wild-type peptides. Red boxes indicate the incorrect peptides generated by the altered splicing. Pink boxes indicate the pre-sequence domain (pfam09029). Green boxes indicate the aspartate aminotransferase superfamily domain (fold type I) of pyridoxal phosphate-dependent enzymes (cl18945). Boxes with zigzag edges indicate truncated regions. Blue boxes with white numbers indicate exons. Black and red numbers denote distances to the start codon in wild-type and mutants, respectively. (E) *alas1* expression was up-regulated in *smu350* mutants. Relative expression of *alas1* transcripts assessed by RT-qPCR in *smu350* mutants (gray column) and wild-type (black column) at 2, 3, 5, and 7 dpf [mean±Standard Deviation (SD); n=10 in each group, performed in triplicate]. Statistical significance was determined using Student t-test, ***, P<0.001. (F) Alas1 protein was absent in *smu350* mutants. Alas1 protein expression in the whole fish body assessed by western blotting at 5 dpf. GAPDH was used as the loading control.
exon-intron boundary in smu350 mutants (Figure 2B), which is likely to be a splicing mutation. By amplifying alas1 cDNA from smu350 mutants, we found at least four unexpected alas1 transcripts (Figure 2C), which were confirmed by sequencing analysis following TA cloning. These unexpected transcripts were predicted to produce truncated Alas1 proteins or in-frame-insertion Alas1 proteins, all of which would interrupt the enzyme activity domain of Alas1 (Figure 2D). Expression analyses by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) showed elevated alas1 mRNA expression in smu350 mutants compared with siblings throughout development (Figure 2E). However, we found that neither the wild-type form nor the abnormal variants of Alas1 protein were present in smu350 mutants, as determined by western blotting (Figure 2F). These data strongly suggest that this alas1 mutation is responsible for the smu350 mutant (hereafter named alas1smu350/smu350) phenotype and that the alas1smu350/smu350 mutant is a loss-of-function mutant.

To confirm that the alas1smu350/smu350 mutant phenotype was indeed caused by the alas1 mutation, we used CRISPR/Cas9 to create alas1-knock-out mutants. A homozygous alas1 mutant (alas1Δ2/Δ2) with a 2-bp deletion within exon 7 of alas1 was obtained, and the mutation resulted in a frameshift of the alas1 product, causing a loss of Alas1 protein in the alas1Δ2/Δ2 mutant (Figure 3A and B). Similar to the alas1smu350/smu350 mutant, the alas1Δ2/Δ2 homozygous mutant and the alas1Δ2/Δ2 bi-allelic mutant also showed loss of SB staining (Figure 3C and D), indicating that alas1 is indeed the causative gene for the altered neutrophil development phenotype.

The alas1 mutation caused heme deficiency

ALAS1 is the first and rate-limiting enzyme for heme biosynthesis, and heme negatively regulates ALAS1 expression through a feedback mechanism. As the Alas1 protein was undetectable (Figure 2F), we postulated that the heme levels of alas1smu350/smu350 mutants might be decreased. To test this hypothesis, we measured heme levels in alas1smu350/smu350 mutants using a fluorescence heme assay. Surprisingly, total heme levels of alas1smu350/smu350 mutants were abnormally elevated (Figure 4A).

As erythroid tissue is the major site of heme production in the body and depends on the isozyme Alas2,33 we then checked if this elevated heme was derived from erythrocytes. We first compared the erythrocyte numbers between 4-dpf alas1smu350/smu350 mutants and their siblings and

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Figure 3. The alas1 was the causative gene of the smu350 mutant. (A) Sequencing analysis revealed a 2-bp deletion within exon 7 of alas1 in CRISPR/Cas9 generated alas1Δ2/Δ2 mutants. Uppercase sequences highlighted blue indicate exons; sequences in lowercase indicate introns. Sequences underlined in red indicate the CRISPR/Cas9 target in alas1. The red asterisk indicates the smu350 mutation site. Black boxes indicate the wild-type peptides, and the red box indicates the incorrect peptide generated by the altered splicing. The pink box indicates the pre-sequence domain (pfam09029). The green box indicates the aspartate aminotransferase superfamily (fold type I) domain of pyridoxal phosphate-dependent enzymes (cl18945). The box with zigzag edges indicates the truncated region. Black and red numbers denote distances to the start codon in wild type and mutants, respectively. (B) Alas1 protein was absent in alas1Δ2/Δ2 mutants. Examination of Alas1 protein expression in the whole fish body by western blotting at 5 days post fertilization (dpf). GAPDH was used as the loading control. (C) The Sudan black B (SB) signal was absent in alas1Δ2/Δ2 mutants. SB staining in siblings (upper) and alas1Δ2/Δ2 mutants (lower) at 3 dpf. Boxed regions are magnified in the lower right-hand corner. Scale bars: 200 μm. (D) SB signal was absent in alas1smu350/smu350 mutants. SB staining in siblings (upper) and alas1smu350/smu350 mutants (lower) at 3 dpf. Boxed regions are magnified in the lower right-hand corner. Scale bars: 200 μm.
Figure 4. The alas1 mutation caused heme deficiency. (A) Whole fish heme levels of alas1<sup>smu350/smu350</sup> mutants were significantly higher than those of siblings. Relative whole fish heme levels in siblings (circles) and alas1<sup>smu350/smu350</sup> mutants (squares) at 4 days post fertilization (dpf). Lines show means±Standard Deviation (SD), 6 individual data points in each group, each data point was based on 3 measurements for 5 embryos; Student t-test, ***P<0.001. The relative heme level was normalized to per-fish level. (B) No significant differences in erythrocyte numbers between siblings and alas1<sup>smu350/smu350</sup> mutants. gata1:DsRed<sup>+</sup> erythrocyte numbers were measured by flow cytometric analysis using 4-dpf self-progeny of Tg(gata1:DsRed)<sup>+</sup>;alas1<sup>+</sup>;gata1<sup>+</sup> transgenic line. Numbers were normalized to per-fish level in siblings (black column) and alas1<sup>smu350/smu350</sup> mutants (gray column). Means±SD; performed in triplicate; Student t-test, ns: not significant. (C) Cellular heme levels of alas1<sup>smu350/smu350</sup> mutant neutrophils were decreased compared with those of siblings. Relative cellular heme levels in sorted neutrophils (lyz:DsRed<sup>+</sup> cells) of siblings (black column) and alas1<sup>smu350/smu350</sup> mutants (gray column) at 4 dpf. Means±SD; performed at least in triplicate; Student t-test, *P<0.05. The relative heme level was normalized to per-cell level. (D) Cellular heme levels of alas1<sup>smu350/smu350</sup> mutant erythrocytes were increased compared with those of siblings. Relative cellular heme levels in sorted erythrocytes (gata1:DsRed<sup>+</sup> cells) of siblings (black column) and alas1<sup>smu350/smu350</sup> mutants (gray column) at 4 dpf. Means±SD; performed in triplicate; Student t-test, *P<0.05. The relative heme level was normalized to per-cell level. (E) Aberrant whole fish heme increment was dampened in alas1<sup>smu350/smu350</sup> mutants without erythrocytes. Relative whole fish heme levels in 4-dpf alas1;gata1a siblings, alas1 single mutants, gata1a single mutants, and alas1;gata1a double mutants from alas1<sup>smu350/+ ;gata1am651/+</sup> in-cross. Lines show means±SD, 4 individual data points in each group, each data point was based on 3 measurements for 5 embryos; one-way ANOVA followed by Dunnett T3 post test, *P<0.05, ns: not significant; nd: not detectable. The relative heme level was normalized to per-fish level. (F) Relative expressions of alas2 and genes related to heme degradation and transport. The assay was performed by RT-qPCR in 4-dpf siblings (black column) and alas1<sup>smu350/smu350</sup> mutants (gray column). Means±SD; n=8 in each group, performed in triplicate. Statistical significance was determined using Student t-test; ****P<0.0001, **P<0.01, *P<0.05. ns: not significant.
found no significant differences (Figure 4B), indicating the elevated whole heme in mutants was not due to increased erythrocyte numbers. We next isolated neutrophils and erythrocytes of alas1smu350/smu350 mutants and their siblings by fluorescence-activated cell sorting (FACS) using 4-dpf self-progeny of Tg(lyz:DsRed); alas1smu350/+ and Tg(gata1:DsRed); alas1smu350/+ transgenic lines, respectively, to directly measure heme levels in the two cell types. By comparing relative heme levels in neutrophils or erythrocytes between alas1smu350/smu350 mutants and their siblings, we found that heme was less abundant in neutrophils of alas1smu350/smu350 mutants than that of siblings (Figure 4C), while in erythrocytes, heme was more accumulated in erythrocytes of mutants (Figure 4D). These data indicate that alas1 mutation results in heme insufficiency in neutrophils but abnormal accumulation in erythrocytes. To further confirm that the elevated heme of the whole body was derived from erythrocytes in alas1smu350/smu350 mutants, we introduced vlt-651,34 (a gata1a mutant with a ‘bloodless’ phenotype having no erythrocytes but intact white blood cells) into the smu350 mutant background to eliminate the effect of erythrocytes. As expected, whole fish heme levels of alas1smu350/smu350 mutants were almost undetectable compared with those of siblings in the gata1a mutant background (Figure 4E). These data indicate that the aberrant heme accumulation of the whole body is indeed derived from erythrocytes in alas1smu350/smu350 mutants.

Alas2, the other isozyme of Alas1, is essential for the heme biosynthesis in erythrocytes and predominantly expressed in erythrocytes.39 To test whether the erythrocyte heme increment resulted from the elevated alas2 expression, we checked alas2 expression in alas1smu350/smu350 mutants. The data showed that alas2 was not altered compared with that in siblings (Figure 4F), suggesting that the erythroid heme accumulation was not due to the compensatory of alas2, at least at the transcription level. Since heme content is tightly controlled by the homeostasis of heme biosynthesis, degradation, and transport pathways,38 we then detected the expression of heme oxygenase enzymes (hmox1a and hmox2a),10,36 which encode the rate-limiting enzymes for heme degradation. The results showed that both gene expressions were down-regulated in alas1smu350/smu350 mutants (Figure 4F), suggesting the impaired heme degradation in the absence of alas1. The results suggest that the dysregulation of heme biosynthesis affects the heme degradation in alas1smu350/smu350 mutants, and the elevated heme might be attributed to the reduced heme degradation. The elevated heme in erythrocytes could not be utilized by heme-deficient neutrophils in alas1 mutants, which is likely due to the fact that the synthesized heme in erythrocytes could not be transported to neutrophils. To test this hypothesis, we further detected the expressions of genes encoding heme transporters. Flvcr1a is reported to export heme out of the cell as a plasma membrane heme exporter.39 Hpx, a high-affinity heme-binding protein, is reported to interact with FLVCR in heme transfer.35 HRG-1 is reported to deliver heme to the cytosol,34 which is encoded by slc48a1a (heme transporter hrgr1-B and slc48a1b (heme transporter hrgr1-A) in zebrafish. MRPS/ABC5 is reported to reside on the plasma membrane and endosomal compartments and regulate the export of cytosolic heme.39 RT-qPCR showed that the

Figure 5. Heme was essential for neutrophil maturation. (A) Whole fish heme levels of succinylacetone (SA)-treated embryos were significantly decreased than those of untreated control. Relative whole fish heme levels in untreated control (circles) and SA-treated embryos (triangles) at 2 days post treatment (dpt). Lines show Means±Standard Deviation (SD), 6 individual data points in each group, each data point was based on 3 measurements for 5 embryos; Student t-test; ***P<0.001. (B) The o-Dianisidine signal was totally absent in SA-treated embryos. o-Dianisidine staining in untreated wild-type (left, 11 of 11 embryos) and SA-treated (right, 10 of 10 embryos) embryos at 2 dpt. (C) The Sudan black B (SB) signal was totally absent in SA-treated embryos. SB staining in untreated wild-type (left, 20 of 20 embryos) and SA-treated (right, 17 of 17 embryos) embryos at 2 dpt. (D) The DAB signal was totally absent in SA-treated embryos. DAB staining in untreated wild-type (left, 18 of 18 embryos) and SA-treated (right, 14 of 14 embryos) embryos at 2 dpt. (E) The DMB signal was totally absent in SA-treated embryos. DMB staining in untreated wild-type (left, 18 of 18 embryos) and SA-treated (right, 14 of 14 embryos) embryos at 2 dpt. (F) The mpox signal was totally absent in SA-treated embryos. mpox staining in untreated wild-type (left, 23 of 23 embryos) and SA-treated (right, 15 of 15 embryos) embryos at 2 dpt. Scale bars: 200 μm (B-F).
Figure 6. alas1 deficiency caused impaired host immunity against E. coli infection. (A) Arrowhead indicates the site of bacteria injection. The imaged region is boxed. (B–E) The in vivo behavior of neutrophils against E. coli was monitored by confocal microscopy. eGFP+ E. coli were subcutaneously injected over one somite into 3-day post fertilization (dpf) sibling (upper panels) and alas1smu350/smu350 mutant (lower panels) larva of the Tg(lyz:DsRed) background. Neutrophil behavior was analyzed through live imaging at 0.5 hours post injection (hpi) (B), 3 hpi (C), 5 hpi (D), and 24 hpi (E). All images are maximum-intensity projections from 25 steps x 2 μm. Scale bars: 50 μm. (F) Bacterial burden of embryos injected with E. coli. Significantly more bacterial cells were detected in alas1smu350/smu350 mutants (gray column) compared with siblings (black column) at 5 and 24 hpi. Mean±Standard Deviation (SD); n>10 in each group, Mann-Whitney U test: ***P<0.0001, **P<0.01, ns: not significant. (G) Quantification of recruited DsRed+ neutrophils at infection sites in live embryos injected with E. coli. Significantly more neutrophils were observed at the infection sites of alas1smu350/smu350 mutants (gray column) compared with siblings (black column) at 24 hpi. Mean±SD; n>19 in each group; Student t-test: ****P<0.0001. nd: not detectable, ns: not significant. (H) Relative il1b expression assessed by RT-qPCR. il1b expression was up-regulated in alas1smu350/smu350 mutants (gray column) compared with siblings (black column) at 3, 5, and 24 hpi. Mean±SD; n=4 in each group, performed in triplicate. Expression levels were adjusted for trauma (phosphate buffered saline (PBS) injection only). Statistical significance was determined using Student t-test: **P<0.01, *P<0.05, ns: not significant. (I) Relative cxcl8a expression assessed by RT-qPCR. cxcl8a expression was up-regulated in alas1smu350/smu350 mutants (gray column) compared with siblings (black column) at 3 and 5 hpi. Mean±SD; n=4 in each group, performed in triplicate. Expression levels were adjusted for trauma (PBS injection only). Statistical significance was determined using Student t-test: **P<0.01, ns: not significant.
expressions of these heme transporter genes were decreased in \textit{alas1}^{smu350/smu350} mutants compared with their siblings (Figure 4F), probably due to the feedback regulation of aberrant heme contents in \textit{alas1}^{smu350/smu350} mutants. Thus, the heme transport deficiency might be one of the reasons that the elevated erythroid heme could not be utilized by neutrophils in \textit{alas1}^{smu350/smu350} mutants.

**Heme was essential for neutrophil maturation**

To confirm whether the neutrophil maturation defects were caused by inadequate heme levels, we next treated wild-type zebrafish embryos with SA, an inhibitor of δ-aminolevulinic acid dehydratase, which catalyzes the second step in heme biosynthesis pathway, to inhibit the endogenous heme levels. Total heme levels of SA-treated embryos were significantly decreased compared with untreated control embryos (Figure 5A). As reported, the o-Dianisidine staining signal was decreased as hemoglobin synthesis is inhibited without heme (Figure 5B). When we monitored the neutrophil phenotypes, we found that SA-treated embryos showed loss of SE and DAB staining but intact lyz and myx expression (Figure 5C-F), which mimics the neutrophil maturation defects in Alas1-deficient mutants. These data suggest that the neutrophil defects in \textit{alas1}^{smu350/smu350} mutant are indeed caused by inadequate effective heme in neutrophils.

**Neutrophil bactericidal defects in \textit{alas1}^{smu350/smu350} mutants**

Neutrophils play key roles in various functions, including action against certain infections, largely depending on granule proteins. The neutrophil granule defects suggest that the anti-infection ability of \textit{alas1}^{smu350/smu350} mutant neutrophils may be attenuated. To detect whether \textit{alas1} deficiency affected neutrophil bactericidal function, \textit{alas1}^{smu350/smu350} mutants were challenged with a bacterial infection. We subcutaneously injected eGFP labeled \textit{E. coli} over one somite in \textit{Tg(lyz:DsRed);alas1}^{smu350/smu350} intercrossed embryos, in which DsRed was expressed specifically in neutrophils (Figure 6A). Neutrophil behavior and immune responses were then monitored. We first monitored in vivo bacterial growth and detected the kinetic curves of the bacterial burden of infected embryos. In sibling embryos, bacteria growth was inhibited effectively in the host, as green fluorescent bacteria decreased rapidly in the infection site (Figure 6B-E). By further plating the homogenized embryos/larvae on LB medium for quantification, we found that bacterial colonies were gradually decreased from 5 h post injection (hpi) and eventually became almost absent at 24 hpi (Figure 6F), suggesting the inhibition of bacterial growth in the host. In mutant embryos, the eGFP bacterial load was similar to siblings within the first 3 hpi (Figure 6B and C), but fluorescent bacteria were still accumulating at 5 hpi and persisted at 24 hpi, when clearance had been completed in the siblings (Figure 6D-E). Quantification data consistently showed that the plated colony numbers from mutants were similar to those of siblings within the first 3 hpi, but the numbers were significantly higher at 5 and 24 hpi than those of the siblings (Figure 6F), suggesting antimicrobial activity was impaired in \textit{alas1}^{smu350/smu350} mutants. We further counted the number of neutrophils recruited to the infection site. Within the first 5 hpi, \textit{alas1}^{smu350/smu350} embryos showed similar neutrophil recruitment to sibling embryos (Figure 6B-D, G). However, neutrophils were still accumulating at the infection site in \textit{alas1}^{smu350/smu350} larvae at 24 hpi, when recruited neutrophils had almost completely disappeared in the siblings (Figure 6E and G), confirming the defect in neutrophil-specific antibacterial response in \textit{alas1}^{smu350/smu350} mutants.

To gain further insight into the infection-induced inflammatory alterations, we detected the expression of inflammatory factors \textit{il1b} and \textit{cxcl8a}, which induce neutrophil chemotaxis and promote immune responses. Expression analyses showed that both genes were significantly up-regulated in infected \textit{alas1}^{smu350/smu350} mutants compared with siblings. The \textit{il1b} and \textit{cxcl8a} expressions peaked in the mutants at 5 hpi, at which point their expressions had already been down-regulated in the siblings (Figure 6H and I), suggesting a more dramatic inflammatory response in \textit{alas1}^{smu350/smu350} mutants. Taken together, these data demonstrate that \textit{alas1} deficiency causes impaired immune responses to bacterial infection.

**Discussion**

In this study, we showed a role for the heme biosynthesis pathway enzyme Alas1 in regulating neutrophil maturation and function. Neutrophils in Alas1-deficient zebrafish had heme deficiency, which led to the loss of heme-related granule protein activities, defective granule formation, and altered immune responses against pathogenic bacteria.

Here, we found that the heme dysregulation caused by the \textit{alas1} mutation led to neutrophil defects in zebrafish. Given the important role of neutrophils in immunity, it was expected that Alas1-deficient zebrafish would show impaired bactericidal ability. Inflammatory factors, such as \textit{il1b} and \textit{cxcl8a}, mediate neutrophil recruitment and promote immune responses. The over-elevated expression of inflammatory factors in infected \textit{alas1}^{smu350/smu350} mutants might be explained by the ineffectiveness of killing bacteria, thereby leading to greater pathogen growth and stronger immune responses in the host. We also noticed that the inflammatory responses eventually subsided while the bacterial burden and recruited neutrophils were still present at 24 hpi in Alas1-deficient zebrafish, so it was likely that the inflammatory factors had been excessively depleted.

As far as we know, the regulatory genes and pathways of heme biosynthesis, degradation and transport are largely conserved between mammals and zebrafish in general. Vertebrates contain two ALAS isozymes encoded by 2 distinct genes located on different chromosomes; \textit{ALAS2} is expressed in erythrocytes, whereas \textit{ALAS1} is ubiquitously expressed. By searching the integrated RNA-seq database on BloodSpot, we found that, in humans and mice, \textit{ALAS1} is highly expressed in myeloid cells, which is consistent with our zebrafish data and partially explains the importance of \textit{alas1} for neutrophils. In mice, \textit{ALAS1} is also highly expressed in the liver, exocrine, and endocrine glands, suggesting specific roles in those tissues. Accordingly, even though \textit{alas1} is ubiquitously expressed, we suspect that \textit{alas1} may play specific roles in certain tissues to meet the need of hemoproteins. It is reported that \textit{alas1}-null mice died in utero until E8.5, with a severely retarded morphology, indicating that \textit{alas1} is essential for the early development of mouse embryos.
It is likely that because of the lethality resulting from ALAS1 deficiency, no reported human diseases directly caused by mutations in ALAS1 have been reported so far. Although the Alas1-deficient zebrafish were indistinguishable on morphology from wild type at embryonic stages, the Alas1-deficient zebrafish are not viable past 8 dpf and showed some morphological defects from 4 dpf onwards, such as delayed disappearance of the yolk sac, abnormal liver, and failed swim bladder formation (data not shown). Thus, the specific functions of ALAS1 in different tissues and organs remain to be clarified.

Alas1-deficient zebrafish showed impaired heme levels in neutrophils but elevated heme levels in erythrocytes. It is known that in addition to mitochondrial heme synthesis, degradation, and trafficking. In this study, we found that the expression of alas2, encoding the other rate-limiting enzyme of heme synthesis, was not changed, while the expressions of heme degradation and transporter genes were down-regulated in Alas1-deficient mutants. In Alas1-deficient zebrafish, cellular heme levels were unexpectedly elevated in erythrocytes, partly due to the decrease in heme degradation. The excessive heme could not be effectively used by neutrophils, probably because the heme transport was impaired. Similar to mice, the over-produced heme by alas2 in erythrocytes could not compensate for the function of alas1 in zebrafish, indicating the essential roles of alas1. The feedback mechanisms for heme homeostasis remain unclear, so future studies will be needed to elucidate the molecular mechanisms of heme metabolism and trafficking.

Impaired heme biosynthesis or heme deficiency leads to heme-related disorders, such as anemia, acute porphyrias, and leukemia. Acute intermittent porphyria is characterized by the accumulation and/or excretion of excess heme precursors. As Alas1 is the key enzyme in heme biosynthesis, repressing Alas1 activity by RNAi is now being used to prevent acute porphyria attacks. Thus, Alas1-deficient zebrafish may serve as an in vivo animal model for evaluating the risks of therapeutic strategies, since Alas1 deficiency causes neutrophil defects, as well as other potential defects in Alas1-abundant tissues. This study may also contribute to the development of new drugs or treatment strategies for heme-related diseases.

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