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Myeloperoxidase Inactivation Affects Neutrophil Recruitment in Zebrafish Injury-Induced Model

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Abstract

Uncontrolled migration and excess recruitment of neutrophils can lead to persistent inflammation, tissue damage and disease. Myeloperoxidase is a remarkable target for further understanding the immune cell migration. This study tests the hypothesis that myeloperoxidase may regulate the immune cell activity and provides a new perspective on the treatment of immune diseases by exploring the mechanism of neutrophil migration. Studies of leukocyte migration *in vivo* in the zebrafish model, which set a collection of advantages both mammalian and cell lines, have gained widespread attention. In this study, we used tg (*coro1a*: eGFP; *lyz*: dsred2) and tg (*lyz*: eGFP) lines labelling both macrophages and neutrophils to study the effect of mpx on neutrophil chemo taxis to wounds. We found that myeloperoxidase was required for neutrophil migration to the wound site in injury-induced inflammation, but not required for neutrophil migration to the infection site in the infection-induced inflammation. Further, the regulation of myeloperoxidase was specific to neutrophil migration to wound inflammation, but was not necessary for macrophage migration. Thus, myeloperoxidase activity shows therapeutic potential for inflammatory disease related to neutrophil migration.

Keywords: Zebrafish; Leukocyte; Migration; Inflammation; *In vivo*

Introduction

Immune cell migration is a key aspect of inflammatory response. In recent years, it has been the focus to explore the pathogenesis of inflammation and find appropriate treatment methods among a great deal of research in the life sciences. In the early stages of autoimmune disease, the infiltration of innate immune cells is very important. Neutrophils exert an immediate effect via antimicrobial products and reactive oxygen, and through the secretion of cytokines with more long-lasting effect, influencing other immune cells [1]. Neutrophils can also regulate the activity of antigen-presenting B and T cells, thereby regulating adaptive immune responses [2-4]. All of these effects depend on neutrophils arriving at sites of inflammation through a specific mechanism. However, neutrophils cannot precisely distinguish between host and foreign antigens; this nonspecific reaction is the main mechanism leading to normal tissue damage. So targeting the migration function of neutrophils is one of important therapeutic strategies to treat immune disease.

In recent years, studies of leukocyte migration *in vivo* in the zebrafish model, which set a collection of advantages both mammalian and cell lines, have gained widespread attention [5-8]. The use of *in vivo* imaging of transgenic zebrafish whose neutrophils express green fluorescence protein (GFP), as well as increased real-time tracking of immune cell migration, provides a new perspective on how chemicals and genetic interference operate on neutrophils' chemotactic response. After injury, wound epithelial cells release H_2O_2, which forms a decreasing concentration gradient. This gradient is required for rapid recruitment of leukocytes to the wound [8,9]. The inhibition of hydrogen peroxide production only inhibits the first wave of neutrophil wound infiltration. The factors that mediate the later sustained phase of neutrophil recruitment are still unknown. When inflammation occurred, immune cells swiftly migrated to the inflammation site in response to signalling by cytokines, chemokine, and ROS. Many experiments in vitro have demonstrated that IL-8 induced neutrophil migration [10,11]. There is IL-8 homologue in zebrafish, cxcl8, play an important role in acute inflammation, and is the major regulator of neutrophil chemotactic response [12]. The zebrafish inflammation model will be instrumental in elucidating mechanisms that regulate leukocyte migration *in vivo* and factors that affect neutrophil migration, and will continue to provide new insight into the onset and resolution of inflammation.

Myeloperoxidase (mpo), and 146 kDa heme peroxidase, is mainly derived from neutrophils. Increased mpo activity is a potential sign of inflammation [13], and mpo regulates inflammation through positive or negative feedback [14,15]. In sum, due to its effect on cascade amplification of inflammatory signals, mpo plays an important role in inflammatory processes. It is dependent or independent of activity characteristics that have functional effects on regulating immune cells and are involved in inflammatory disease. However, some hypotheses have been based on in vitro experiments, and they need further validation based on exploration of the regulatory effect of mpo on innate immune cells.

Zebrafish has the conserved ortholog of mammalian mpo, myeloperoxidase (mpx), is expressed specially in neutrophils in the developmental stage [16]. Using a new neutrophil-replete but myeloperoxidase-deficient mutant (durif), Pase et al. [17] showed that myeloperoxidase-deficient zebrafish had abnormally sustained high concentrations of H_2O_2 in wounds despite similar numbers of arriving neutrophils. Another study reported that inhibiting myeloperoxidase activity resulted in decreased inflammatory cell recruitment in a

mouse model of multiple sclerosis [18]. Thus in this study, we aim to model mpx inactivity in zebrafish using a pharmacologically irreversible mpx activity inhibitor, 4-amino-benzoyl (acid) hydrazine (abah), to explore innate immune cell migration and test a neutrophil-targeted drug *in vivo*, and to try to further understand how mpx regulates neutrophil recruitment behavior. This can provide insight into the mechanism of neutrophil migration.

Materials and Methods

Drugs

An mpx inhibitor, 4-amino-benzoyl (acid) hydrazine (abah, Sigma, Cat #A41909), was ordered from Sigma-Aldrich. The concentration for immersion was 10 mm, and the concentration for microinjection was 50 mm Recombinant Human IL-8 (77aa) cytokine was purchased from PEPROTECH (USA).

Experimental Animal

Four days post fertilization (dpf) AB/wild type zebrafish strains and tg(*lyz*:GFP), tg(*coro1a*:eGFP; *lyz*:DsRed2) transgenic lines were used in this study [19].

All the treatment of experimental animals was approved by the Committee on the Ethics of Animal Experiments of the USTC (License Number: USTCACUC1103013), and was in accordance with the guidance and provisions of the Committee on Laboratory Animal Resource Centre and the Animal Care and Use Guidelines of the University of Science and Technology of China.

Experimental bacterial strains

GFP-expressing strains of *Staphylococcus aureus* (*S. aureus*, NCTC8325) were used and diluted to a uniform concentration of 1,000 colony-forming units (cfu)nl-1.

Mechanical damage model

Tail fin and ventral fin injury were used to induce chemotactic response. To make a tailfin amputation, a sterile scalpel was used to cut an incision along the spinal cord ends as straight as possible [20,21]. To make the ventral fin acupuncture injury, a sterile pin was used at in the ventral fin area, 4-5 somites from the cloaca.

Muscle injection

Four dpf larvae were pre-incubated with abah, then performed an injection in the muscle at the 4th-6th somite from the cloaca with 20 nl PBS, 13 nl IL-8 recombinant protein at 5.6 μM, or 13 nl Staphylococcus aureus at 1,000 cfu nl-1, respectively. The control group was not pre-incubated with abah. Images were taken at 2 hours post amputation (hpa).

Zebrafish *in vivo* imaging

For *in vivo* observation of macrophages and neutrophils behavior, we used 4 dpf tg (*coro1a*:dGFP;lyz:DsRed2) hkz04t;nz50, tg (*lyz*:eGFP) transgenic lines and tg (*flk*:GFP;*lyz*:DsRed) hybrid lines. All larvae prior to imaging were treated in 0.01% MS222 (3-amino benzoic acid ethyl ester). In order to prevent damage to the living body samples, the exposure time was as short as possible when imaging. Imaris software (Bitplane, Switzerland), ImageJ software (NIH, USA) and Adobe Photoshop CS2 (USA) were used for image processing.

Long-term continuous imaging: The leukocyte in the whole body or damaged area was observed using OLYMPUS FV-1000 (BX61WI, Japan) confocal microscopy with 10x OLYMPUS Plan Fluor objective (NA 0.30) and 60x OLYMPUS Plan water-dipping objective (NA 0.9). For observation of the dynamic migration of neutrophils to the wound area, we continuously photographed from 15 minutes post amputation (minpa) to 4 hpa, 1 min/z-stack, then made the photographs into a real-time video.

For analysis of neutrophil trajectory, we used data obtained from Imaris software (Bitplane, Switzerland) with an OLYMPUS-FV1000 (BX61WI) continuous imaging confocal microscope, 1 min every one z-stack, from 0.75 hpa-1.25 hpa or 1.5 hpa-2 hpa, to analyze the motion characteristics of neutrophils. Only the neutrophils that speed was greater than the 0.015 μm s-1, and migration along the X-axis direction of the wound were included in the statistics.

Fluorescence images obtained: An OLYMPUS (BX60WI) fluorescence microscope with 10x Plan Fluor objective (NA 0.40) was used to obtain fluorescence images for counting the number of neutrophils and macrophages recruited to the wound site.

Gene expression analysis

At the time points indicated, using a sterile scalpel, the body portion between the cloaca and the wounded tail tip was excised from 100 larvae at each point. Tail tissue samples were then pooled and frozen in RNAiso Plus (TAKARA, China). RNA was extracted according to standard procedures. RT-PCR was performed by one-step RT-PCR kit (TAKARA, China).

Real-time PCR was performed with a BioRadicuclermyIQ2 instrument using a two-step real-time quantitative PCRSYBR Green Supermix kit (Bio-Rad, USA). The primers used are shown in Table 1. In all cases, PCR was performed with triplicate samples and repeated at least twice.

Statistical analysis

Data were analysed in Graph Pad Prism 5.0 (Prism, USA) using one-way ANOVA and t-test. The results were shown as mean ± SEM (performed as three independent experiments). $P<0.05$ was considered statistically significant. *, **, and *** represent $P<0.05$, $P<0.01$, and $P<0.001$, respectively.

Results

Inhibition of mpx activity reduces the number of neutrophils recruitment to the wound site in zebrafish injury-induced inflammation model

Inflammation is divided into two types: sterile and infection inflammation. To study the role of mpx in both types of inflammation, we pharmacologically inhibited mpx activity with abah using 4 dpf tg(*lyz*:eGFP) transgenic lines. Tail amputation (Figure 1A) and bacterial injection were then performed in both the abah-treated and untreated groups. At 3 hpa of the tail fin, about 17 ± 2 neutrophils were found in the region of 200 μm away from the wound site of 4 dpf normal zebrafish. However, a significantly lower number of neutrophils were observed in the abah treatment group (Figure 1B and

1C, p<0.0001). The results showed that inhibition of mpx activity reduces the number of neutrophils recruitment to the wound site.

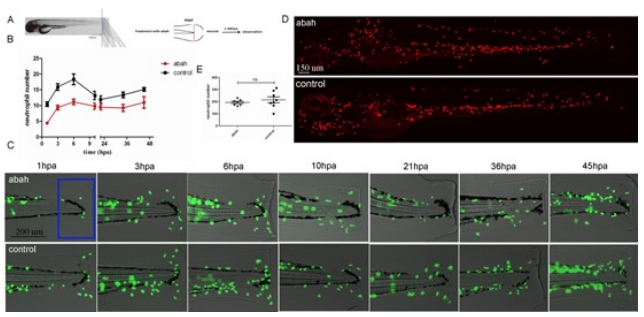

Figure 1: Inhibitory effect of abah on neutrophil migration toward the wound site. Neutrophil migration assay was carried out using tg (*lyz*:GFP) larvae. (A) A schematic view of zebrafish tail fin amputation and operation process. (B) Quantification of neutrophils recruited to the region of 200 μm away from injury site in abah-treated and control group. N=30-50. (C) A schematic of zebrafish tail fin at the indicated time points; blue boxes indicate the statistical area of neutrophil numbers. (D) The distribution of neutrophils in the whole zebrafish is shown in the abah treatment and control larvae groups, respectively. (E) Quantification of (D). N=8.

To confirm that abah acts to block the recruitment rather than the ontogeny of neutrophils, whole body neutrophil counts were performed in the presence of the abah and the vehicle-only control. No differences in morphology, number or distribution of neutrophils were observed after abah treatment (Figure 1D and 1E), and the average total number of neutrophils was 193 ± 2 and 200 ± 2 in the abah treatment and control groups (p=0.0973), respectively. In addition, the number of neutrophils in the hematopoietic tissue was also counted; the result was consistent with above (data not shown). In contrast, when we performed a microinjection of *S. aureus* into the muscles of the fifth somite rostral from the cloaca, the number of neutrophils accumulated at the bacterial injection site at 2 hours post infection (hpi) was similar: 25.7 ± 2 and 25 ± 2 in the abah treatment and the control group (p=0.8740), respectively (Figure 2A and 2B, in the middle of the row). In addition, sterile PBS injection induced a local sterile inflammation, causing minor accumulation of inflammatory cells at the site of injection. Similar to the tail fin injury model, in the abah treatment group the number of recruitment neutrophils was a little lower than those in the control group (p=0.0438), respectively (Figure 2A and 2B, in the right of row). These results illustrated that mpx was necessary to neutrophil migration to wounds in a mechanic injury-induced inflammatory response, whereas it was dispensable in a bacterial infection inflammation response.

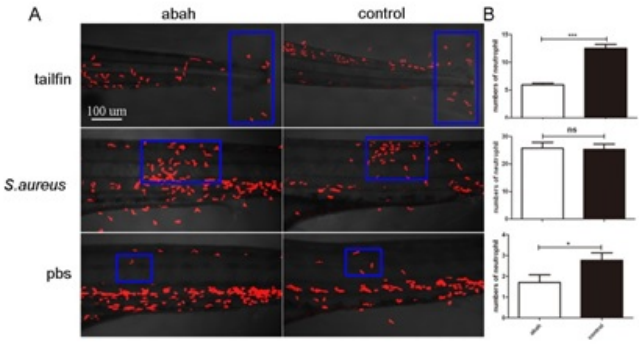

Figure 2: Inhibition of mpx activity plays a different role in injury and infection inflammation. (A) Confocal fluorescence views of the distribution of neutrophils after being subjected to tail amputation, muscle microinjection of PBS, and S. aureus, respectively. Tg (*lyz*:DsRed) transgenic fish were used. Images were taken at 2 h after injection. (B) Quantification analysis shows the neutrophil number in the wound area or *S. aureus* injection site. N=30-50.

Three hours after tail fin injury, abah treatment reduced the number of neutrophils that migrated to the wound area. To find out what happened to the neutrophil number later, whether it exist a delayed migration effect, we observed the number of neutrophils that migrated to the wound at different time points after tail fin injury. As shown in Figure 1B and 1C, from 0-6 hpa, neutrophils gradually migrated to the caudal damage area, reaching a peak at 6 hpa. After this, the number of neutrophils reduced gradually. However, at 48 hpa, a significant short-term rebound phenomenon appeared. This proved again that inhibition of mpx activity reduced the number of neutrophils recruited to the wound site. This trend of neutrophil numbers was very consistent, without any delay effect.

To verify the specific effect of mpx on neutrophil migration, tg (*coro1a*:eGFP; *lyz*:DsRed2) hkz04t; nz50 transgenic zebrafish lines were used in which macrophages and neutrophils were labeled with green and yellow fluorescent colors, respectively. As shown in Figure 3C, the number of macrophages migrating to the wound area at 3 hpa was 11 ± 2 and 10 ± 2 in the abah-treated group and the control group, respectively. This showed no significant difference in the number of macrophages (p=0.3062), whereas there were 8 ± 2 neutrophils in the abah-treated group and 17 ± 2 neutrophils in the control group (p<0.0001). This result demonstrated that mpx played a special role in neutrophil migration, but not in macrophage migration. In addition, acupuncture injury at the 4th-5th somite from the cloaca was performed for *in vivo* observation of the dynamic macrophage and neutrophil migration process (Figure 3A and 3B). Our results showed that only macrophages migrated into the wound site at 3 hpa in the abah-treated group, while almost no neutrophil came in. Interestingly, we observed a phenomenon of neutrophils coming across the vessel and maintaining a position in the lower side of the vessel (Figure 3B, top row, white arrows), but these neutrophils were not recruited to the wound site. However in the control group, consistent with the previous results, around 8 ± 2 neutrophils migrated into the wound area. The number of green macrophages recruited was 4 ± 2 (Figure 3B, in the bottom row). This result also revealed that inhibition of mpx activity specifically influenced neutrophil migration to the wound site. All of

the above results demonstrated that mpx specifically regulated neutrophil migration to the wound site in injury-induced inflammation, but exerted no effect on macrophages, and was dispensable for neutrophil migration in the bacteria-infection inflammation response.

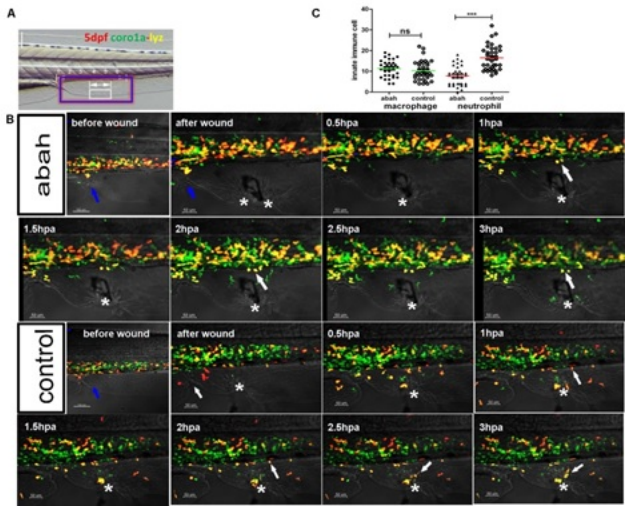

Figure 3: Inhibition of mpx activity specifically influences neutrophil migration to the wound area, but not macrophage in tg (*coro1a*:eGFP; *lyz*:DsRed2)hkz04t; nz50 transgenic zebrafish. (A) Schematic view of pelvic fin injury, about 4-5 somites away from cloaca. (B) The distribution of neutrophils and macrophages from 0-3 h after zebrafish pelvic injury in abah-treated and control groups. The white star and red arrow show the wound areas, the blue arrow indicates the position of cloaca, and the white arrows indicate neutrophil migration out of the blood vessel. (C) Quantification of neutrophils and macrophages respectively recruited to the region of 200 μm away from injury site in abah-treated and control group at 3 hpa. N=30-50.

Mpx plays different roles in basal random movement and injury-induced directional migration of neutrophils

To further characterize the effect of mpx on neutrophil responses, time-lapse analysis was performed to assess different aspects of neutrophil migration and movement behavior. Under normal conditions, the neutrophils residing in the tissues or blood vessels were constantly moving within a certain area as basal random movement, presumably patrolling the surrounding tissues to check for damaged cells and apoptosis (Figure 4A). This suggests that abah treatment had no effect on the motion behavior of the neutrophil itself. Upon tissue injury with extracellular inflammation, neutrophils migrated swiftly to the injury site, taking a relatively straight migration trajectory as a directional movement, with a change of migration velocity, speed, and forward migration distance (Figure 4B). We next assessed the migratory behavior of neutrophils in abah treatment before and after tail fin amputation. We found that in both the abah-treated and control groups, neutrophils displayed a basal migratory pattern under injury-free conditions (sham). Upon tail fin injury during 0.75 hpa-0.25 hpa or 1.5 hpa-2 hpa, neutrophils rapidly migrated toward the wound site in the control group, whereas neutrophils in abah treatment moved randomly and failed to reach the wound site, resulting in a reduction of the number of neutrophils in the wound area. Track length, velocity, and speed of neutrophil migration to the wound site were similar to those in the control group. These data indicated that the mpx was necessary for injury-induced directional migration of neutrophils, but was dispensable for the basal random movement of neutrophils. It could be said that mpx was required for a sufficient number of neutrophils to migrate into the wound site, but not for some other neutrophil migration, which showed the same behavior as those in the control group after tail fin injury.

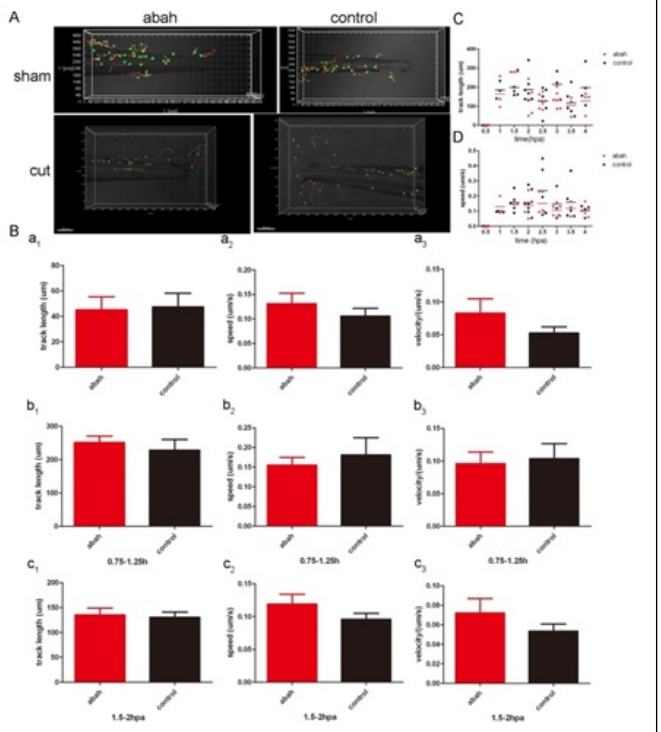

Figure 4: The injury-induced migration tracks of the neutrophils. (A) A schematic diagram of neutrophil migration tracks (B) Statistical data on the injury-induced migration tracks, speed, and velocity of neutrophils to wound area in 4 dpf tg(lyz: Dsred) were analyzed before and after tail fin injury in the abah-treated and control groups. Each panel consists of ≥ 20 neutrophils from 7 larvae. In (a), the basal random movement of neutrophils without injury is presented. The directional movement of neutrophils from 0.75 h to 1.25 h after tail fin injury is shown in (b). The directional movement of neutrophils from 1.5 h to 2 h after tail fin injury is shown in (c). A continuous real-time analysis of trends in neutrophil migration track (C) and speed (D) for each neutrophil every half an hour in the abah-treated and control groups. The sample consists of 8 cells from the same larvae.

Real-time analysis of trajectory length and speed for a single neutrophil was performed. We found that the neutrophil rapidly migrated into the wound site at 1 hpa. Later, it migrated into the wound site with the similar speed (Figure 4D). At different time points, the change in migration speed and trajectory length of neutrophil recruitment was consistent both in the abah treatment and in the control group (Figure 4C and 4D).

In order to verify whether neutrophil behavior across the vascular wall was affected by inhibition of mpx activity, we performed an acupuncture pelvic injury in 4 dpf tg (*flk*:GFP;*lyz*:DsRed) transgenic zebrafish, in which neutrophils were labelled by red fluorescent protein and blood vessels were labeled by green fluorescent protein. The result showed that neutrophils were capable of crossing the blood vessels in both the abah treatment and the control group, although they soon moved back to the blood vessels (Figure 5A, blue arrows). While in the control group, the neutrophils that migrated across the vascular wall into the wound site continued to stay in the wound area, then came into an active state, as morphological changes indicated (Figure 5A, blue arrow).

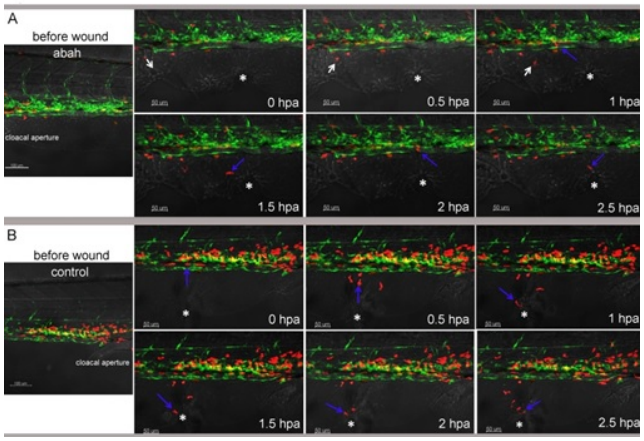

Figure 5: Abah does not affect the behavior of neutrophils across vessels in 5 dpf tg(*flk*:GFP;*lyz*:DsRed) zebrafish. The diagram of neutrophils recruited to the wound site at indicated time points before and after acupuncture injury of pelvic fin, distance from cloaca about third-fourth somite in the abah-treated group (A) and the control group (B). White asterisk indicates the wound area; scale bar 50 μm. White arrows indicate random neutrophil migration back and forth between the wound site and the vessel. Blue arrows indicate neutrophils moving normally out across the blood vessels.

Cxcl8 is involved in the regulation effect of mpx on recruitment of neutrophils to the injured site

Inhibition of mpx activity by pharmacological inhibitor (abah) decreased the migration of neutrophil to the wound site in injury-induced inflammation. Thus whether the inhibition of mpx activity affect the chemokine signaling upon inflammation needs to be verified.

After inducing inflammation in zebrafish by tail fin amputation, we first investigated genes that were considered likely to be involved in the process of inflammation. We found that cxcl8 mRNA expression at the wound sites was lower in abah treatment than those in the no treatment group at 2 h post amputation (Figure 6A and 6B), which partly explained why the abah treatment reduced the number of neutrophils recruited to the wound area. Similarly, ELISA assay showed that inhibition of mpx activity decreased the expression level of cxcl8 (Figure 6C). In addition, we also investigated it with an adult tail fin injury model by Q-RT-PCR tests (Figure 6D).

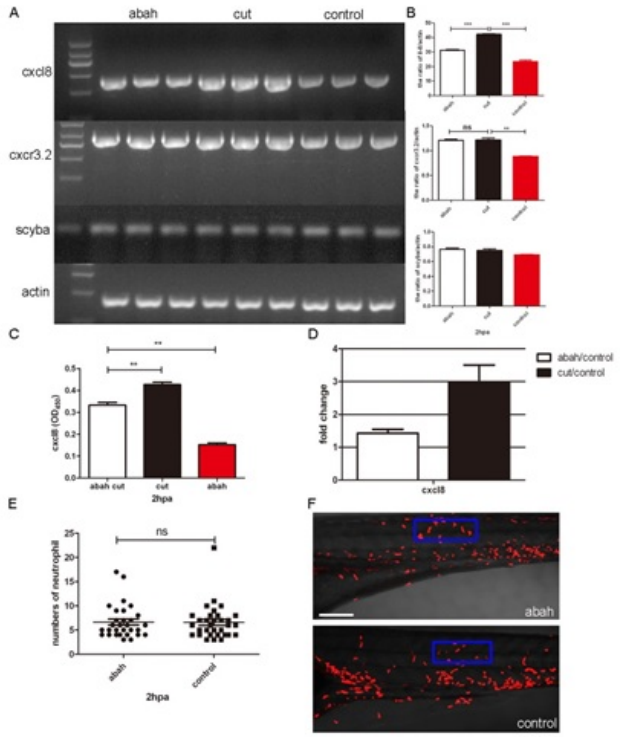

Figure 6: Mpx regulates injury-induced neutrophil directional migration via regulating cxcl8 expression. (A) The tail fins from 4 dpf zebrafish larvae were wounded and mRNA levels of indicated genes (cxcl8, macrophage-specific chemokine scyba and receptor gene cxcr3.2) were detected by RT-PCR (A), ELISA (C) at 2 hpa (80-100 tail fins per group). Quantitative RT - PCR (D) of cxcl8 in adult wound model. Control group is from tail fin tissue of unwounded zebrafish larvae. (B) Statistical data for band brightness in (A). Gene expression is normalized against actin or ef1a. (E) Counts of fluorescent neutrophils recruiting to the muscle injection area were taken at 2h after injection. Data indicate means ± SEM. N=30-50. (F) Representative wide-field fluorescence microscope micrographs of 4 dpf tg (*lyz*:DsRed) being pretreated with abah followed by muscle injection of human recombinant IL-8 into the fifth somite from the cloaca. Blue boxes indicate neutrophil number for statistical area.

It has been reported that the chemokine receptor cxcr3.2 is specifically expressed in zebrafish embryo macrophage, regulating macrophage migration to the inflammation site [22]. Scyba, a homologue of human cxcl14 in zebrafish due to its chemotactic properties, could activate macrophages and dendritic precursor cells [23]. Therefore, we needed to detect whether mpx also regulated the expression of macrophage-specific chemokine. By semi-quantitative reverse transcription we found that the mRNA expression of cxcr3.2 and scyba, after inhibition of mpx activity, was similar to those in the control group (Figure 6A and 6B). These results illustrated that abah treatment specifically reduced the expression of cxcl8 chemokine, which regulated the neutrophil migration to the wound area, but did

not affect the expression of cxcr3.2 and scyba expression, which activated macrophage migration behavior.

Mpx activity–cxcl8 axis plays an essential role in injury-induced neutrophil recruitment response

To address in this study whether the mpx signal for inducing neutrophil recruitment in zebrafish was also through cxcl8, recombinant human IL-8 protein was used to detect the role of mpx on IL-8 chemotaxis in 4 dpf zebrafish.

The microinjection of IL-8 protein significantly increased the number of neutrophils recruitment in the 4 dpf tg (*lyz*:eGEP) zebrafish line (Figure 6F). Two hours post-injection, it was observed that 7 ± 2 neutrophils were recruited to IL-8 injection site whether in the presence or absence of abah treatment (Figure 6E and 6F); this was higher than those in the PBS injection group (Figure 2). This result again *in vivo* proved the role of IL-8 in neutrophil chemotactic function and also suggested that IL-8 acts downstream of mpx in the regulation of injury-induced migration of neutrophils, therefore explaining that the inhibition of mpx activity did not affect the migration of neutrophils to IL-8.

Discussion

Although many previous reports have suggested that myeloperoxidase can regulate the functional effects of immune cells involved in the inflammatory disease of tissue injury using vitro models [24-26], further evidence is required to validate the effect of mpx regulation on innate immune cells.

When applied to zebrafish, abah, identified in the zebrafish screen model, showed a dose-dependent inhibitory effect on neutrophil migration. The results showed that mpx was required for neutrophil migration to the wound area in injury-induced inflammation, but not for neutrophil migration to the infection site in infection-induced inflammation. Here, we only tested the number of neutrophil at 2 hpi, the inflammatory response at different time points after infection did not given observation. We thought our result was not contrary to previous findings in myeloperoxidase-deficient zebrafish against *C. albicans* infection showing an equal number of neutrophil at 12 hpi, but significantly higher from 24 hpi in mutant embryos than in control embryos [27]. In fact, the infection reachs to a peak at 12hpi; then the neutrophils decrease gradually later because of the resolution of inflammation or the death of immune cells. Whether treatment zebrafish with abah also affect neutrophil recruitment to *S. aureus* infection site at a more comprehensive infection time need further to verify. Here, we focused on the neutrophil recruitment in the early acute phase. Collectively, these data suggest that mpx may regulate injury-induced neutrophil directional migration, at least in part by regulating cxcl8 expression.

In addition, time-lapse analysis was performed to assess dynamic neutrophil behavior. Our results showed that the track length, speed, and velocity of neutrophil migration to the wound site in abah treatment were not different from those in control group. At 0.5 h after tissue injury, H2O2 produced by the wound regulates the initial neutrophils percepting and migrating into the wound. After 0.5 hpa, inflammatory factors released by immune cells and endothelial cells mediate a cascade inflammatory response, and induce more neutrophils to migrate to the wound area with an increase in migration velocity, speed, and forward migration distance. In this study, our results showed that the H_2O_2 level maintained its normal expression at a peak of 0.5 h after zebrafish tail fin amputation, which was consistent with previous research (data not shown). However, abah treatment had no inhibitory effect on neutrophil movement characteristics. The possibilities for no differences are that our results used a time window of 0.75 hpa and 1.5 hpa for statistical analysis, which may be relatively early for observing differences in migration behavior between the abah treatment group and the control group. Meanwhile, half an hour may be a relatively short time period for statistical analysis of neutrophil migration. A longer time period may be needed to observe this process for more accurate statistical analysis. On the other hand, this study showed extensive effects by a pharmacological inhibitor of mpx activity on the entire body, especially systemic inflammatory response. Thus, we do not preclude other signal paths being induced by various factors, although it was a fact that cxcl8 expression decreases after the inhibition of mpx activity in injury-induced inflammation response. In fact, mpx has the effect of cascade amplification on inflammatory cells during inflammation. Cytokines and chemokines play a more important role. In addition, we believe that the phenotypes we observed in our study likely represent mpx inactivity without any toxic effect.

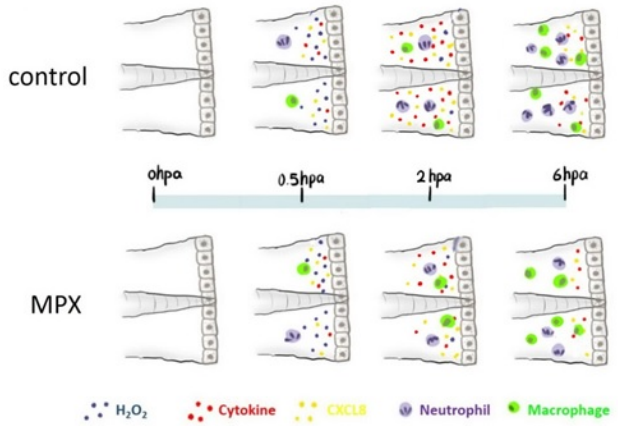

Figure 7: A schematic of regulatory mechanism of mpx on neutrophil migration. A hypothesis is that at 30 min after tail fin amputation, wound tissue produces a normal H_2O_2 signal, inducing initial neutrophils and macrophages to rapidly migrate to the wound site, releasing inflammatory cytokine and chemokine. Due to mpx inhibition, inflammatory and chemotactic signals that regulated neutrophil attenuate; subsequently, the number of neutrophils decreases.

To summarize the above results, a hypothesis is proposed in the sketch of Figure 7. Fifteen to 30 minutes after tail fin injury, a H_2O_2 signal is produced normally and induces initial neutrophils and macrophages to migrate rapidly to the wound site, releasing inflammatory cytokine and chemokine. Due to mpx inhibition, inflammatory and chemotactic signals that regulate neutrophils attenuate, and subsequently the number of neutrophils decreases. Thus functional inactivation with inhibitor after innate immune development could have great value to suppress the immune response, additional studies are required to identify the specific target of abah in neutrophils.

These results further confirm that there is a great opportunity to observe the function of mpx *in vivo* inflammation using a tg (*lyz*:GFP) transgenic zebrafish model, and that inhibitor-targeted mpx may become a powerful therapy to control inflammatory pathology. By using transgenic zebrafish lines, we can analyse the phenotypic effects of human cells to detect pathogenesis in the future. This can help screen for more effective inhibitors to treat chronic myeloid leukaemia, as well as aiding the search for new drugs to regulate neutrophil migration in inflammatory response.

Gene	Name	Nucleotide	Use
beta-actin	fwd	5'-CATTGGCAATGAGCGTTTC-3'	RT-PCR
beta-actin	rev	5'-TACTCCTGCTTGCTGATCCAC-3'	RT-PCR
Cxcl8	fwd	5'-AATGAGGGTGAAGCTCTACCTCCAC-3'	RT-PCR
Cxcl8	rev	5'-CACAGTGATAACAGTCCATTCGCAA-3'	RT-PCR
Cxcr3.2	fwd	5'-AGACGAGACGTGCCTAACATC-3'	RT-PCR
Cxcr3.2	rev	5'-ACACGATGACTAAGGAGATGA-3'	RT-PCR
Scyba	fwd	5'-TGTGGGATGACACTACCAGTGAA-3'	RT-PCR
Scyba	rev	5'-GACCGGTGTGCTTTATAAGCTTGT-3'	RT-PCR
Cxcl8	fwd	5'-CCACACACACTCCACACACA-3'	qPCR
Cxcl8	rev	5'-CCACTGAATTGTCCTTTCATCA-3'	qPCR
Ef1a	fwd	5'-CTTCTCAGGCTGACTGTGC-3'	qPCR
Ef1a	rev	5'-CCGCTAGCATTACCCTCC-3'	qPCR

Table 1: Primers.

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Disclosure

The authors declare no conflict of interest.

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