Overexpression of microRNA-722 fine-tunes neutrophilic inflammation through inhibiting Rac2 in zebrafish

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Summary statement: Identification of a microRNA that suppresses Rac2 expression and regulates neutrophil migration and systemic inflammation

Key words: zebrafish, microRNA, systemic inflammation, cell motility
Abstract

Neutrophilic inflammation is essential for defending against invading pathogens, but can also be detrimental in many clinical settings. The hematopoietic-specific small Rho-GTPase Rac2 regulates multiple pathways that are essential for neutrophil activation, including adhesion, migration, degranulation and production of reactive oxygen species. This study tested the hypothesis that partially suppressing rac2 in neutrophils with a microRNA would inhibit neutrophil migration and activation, which will reduce the immunological damage caused by systemic inflammation. We have generated a transgenic zebrafish line that over-expresses microRNA-722 (miR-722) in neutrophils. Neutrophil motility and chemotaxis to tissue injury or infection are significantly reduced in this line. MiR-722 downregulates the transcript level of rac2 through binding to seed match in the rac2 3’UTR. Furthermore, miR-722 over-expressing larvae display improved outcomes in both sterile and bacterial systemic models, which correlates with a robust upregulation of the anti-inflammatory cytokines in the whole larvae and isolated neutrophils. Finally, the miR-722 mimics protect zebrafish from lethal LPS challenge. Together, we provide evidence and the mechanism of an anti-inflammatory microRNA that restrains detrimental systemic inflammation.

Introduction

How to dampen immune activation is a major challenge in modern medicine. Neutrophils are the most abundant white blood cells in the circulation and the first line of defense against infections. While essential for battling against pathogens, acute or chronic neutrophil activation drives immunopathology in numerous human diseases, including those directly involving an immune component (such as organ transplantation, sepsis and rheumatoid arthritis) and those that are not obviously linked (such as diabetes, neurodegenerative disease and cancer) (Borregaard, 2010; Nathan, 2006). Neutrophils release toxic granular contents including proteases, as well as extracellular traps and produce a large amount of reactive oxygen species, which help eliminate the threat of pathogens but can cause detrimental effects on hosts. Recent evidence suggests that neutrophils, in addition to mediating acute inflammation, are a critical regulator of the inflammatory landscape. They live longer than previously recognized (Pillay et al., 2010). In addition, they initiate
(Sreeramkumar et al., 2014), disseminate (Woodfin et al., 2011), and critically regulate the magnitude of inflammation (Warnatsch et al., 2015) while bridging innate and adaptive immunity (Abi Abdallah et al., 2011; Lim et al., 2015) in both sterile inflammation and infection. Thus, a successful strategy to prevent the infiltration of neutrophils is expected to significantly improve inflammatory conditions and reduce the risk of many modern diseases. As such, the microtubule destabilizing agent colchicine, a potent inhibitor for neutrophil motility and activation, is approved for treating acute inflammation in familial Mediterranean fever and gout patients (Cocco et al., 2010). However, colchicine and the broad-spectrum anti-inflammatory agent corticosteroids lack neutrophil specificity and are inevitably accompanied with adverse side effects (Cocco et al., 2010). There is an urgent need to develop anti-neutrophil therapies that would benefit a diverse population suffering from inflammatory ailments.

MicroRNAs are evolutionarily conserved, small non-coding RNAs that post-transcriptionally regulate protein synthesis (Fabian and Sonenberg, 2012). MiR expression is controlled by specific promoters and regulated at the transcriptional and post-transcriptional levels. The initial transcription of long primary transcripts are processed in the nucleus into short ~70 nt precursor miRNA hairpins, and further exported and processed in the cytoplasm into mature ~22 bp miR duplexes, containing both 5p and 3p strands. The duplex is then loaded into Argonout (AGO) proteins, where the passenger strand is degraded, which allows the guide strand to direct the miR Induced Silencing Complexes to partially complementary target sites (Gurol et al., 2016). Either or both of the 5p and 3p strands can act as the predominant functional strand, depending on the unstable terminus at the 5’ end or other unknown mechanisms. The majority of the microRNAs bind to their target transcript though complementarity in the 3’UTR to suppress gene expression, although in some cases, enhanced target gene expression was observed (Ma et al., 2010). The seed sequence (positions 2-8 of the mature miRNA) is the major determinant of target recognition, although the contribution of other nucleotides cannot be excluded (Helwak et al., 2013). As of April 2017, 2588 human mature microRNAs have been identified, which are implicated in a wide variety of cellular processes and human diseases. MicroRNAs and anti-microRNAs are recent additions to the clinician’s arsenal as next generation therapeutics to treat human
diseases (Broderick and Zamore, 2011; Hayes et al., 2014). Currently, miR-122 antagonists are in clinical trials for chronic hepatitis C infection (RG-101 by Regulus). Extensive effort has been made characterizing inflammation related microRNAs. The microRNA expression profiles in various inflammatory conditions, including sepsis have been documented (Gurol et al., 2016). However, the use of this information is currently limited to establishing microRNAs as biomarkers and diagnostic tools. The biological functions of these microRNAs and their therapeutic potential are merely starting to emerge. The majority of microRNA-inflammation research is restricted to macrophages, with their roles in neutrophils poorly characterized. Human peripheral blood neutrophils (Gantier, 2013; Landgraf et al., 2007; Ward et al., 2011) and activated tissue infiltrating neutrophils (Larsen et al., 2013) express a different profile of microRNAs. It is reasonable to speculate that microRNAs are potent regulators of neutrophil function and inflammation.

In the present study, we aim to identify a microRNA that would restrain hyperactive neutrophilic inflammation and to test its impact in acute systemic inflammation settings. Rac2, a member of the Rho small-GTPase family, is restricted to the hematopoietic lineage, which plays a principal role in regulating the actin cytoskeleton and neutrophil biology. By using neutrophils isolated from Rac2 knockout mice (Roberts et al., 1999), in combination with studying a dominant negative form of Rac2 in zebrafish neutrophils in vivo (Deng et al., 2011), multiple parallel pathways of Rac2 effector functions have been discovered. Rac2 is required for neutrophil motility and chemotaxis by regulating actin polymerization at the leading edge in a positive feedback loop with PI3K (Yoo et al., 2010). Rac2 is required for adhesion and retention of neutrophils in the hematopoietic tissue, yet not required for their release from this tissue (Deng et al., 2011). In addition, Rac2 is an essential subunit for the phagocyte NADPH oxidase complex, directly interacting with gp19\textsuperscript{phox} and p67\textsuperscript{phox}, and is responsible for the generation of super oxide ions during infections (Jonzon and Bindslev, 1991). Furthermore, Rac2 is required for the degranulation of primary granules in neutrophils (Abdel-Latif et al., 2004). It is our expectation that suppressing Rac2 activity in neutrophils will greatly reduce the number of infiltrating neutrophils in the tissue and alleviate patients from over-inflammatory burdens. However, benefits of Rac2 as therapeutic targets have not been previously explored, probably due to a lack of a Rac2 specific inhibitor as well
as the fact that Rac2 deficiency results in primary immune deficiency and poor wound healing (Williams et al., 2000).

The zebrafish is a fully sequenced vertebrate model organism with a conserved innate immune system (Deng and Huttenlocher, 2012). The ease of genetic manipulation and the optical transparency of zebrafish larvae made them ideal model organisms to observe the behavior of phagocytes in a non-invasive way and to dissect related molecular mechanisms. Here we provide the first microRNA that suppresses the expression of Rac2 and demonstrated that partial Rac2 suppression attenuated the acute lethal inflammation under both sterile and non-sterile conditions.

**Results**

**MiR-722 over-expressing neutrophils are defective in motility and chemotaxis.**

To test the efficacy of microRNAs as next-generation therapeutics that would restrain neutrophil migration and inflammation, we looked into microRNAs that can suppress rac2 expression. We performed bioinformatics analysis (TargetScanFish) and identified three microRNAs (miR-194, miR-722 and miR-129) that are predicted to bind to the 3'UTR of both transcript variants of the zebrafish Rac2 gene. miR-722 and miR-129 share the same seed sequence and bind to a perfect seed matching site in the rac2 3'UTR with a context + score percentile above 90. MiR-194 binds to a separate site with a partial seed match and a context + score percentile at 69, possibly a weaker regulator of rac2. Data compiled from previous microRNA sequencing experiments suggest that miR-722 is intergenic and predominantly produces a mature 3p strand that harbors the rac2 binding sequence (www.miRbase.org). In contrast, both the mature 5p and 3p strands of miR-129 are detected, which potentially complicates the biological consequence of over-expressing this microRNA. In addition, miR-722 level is below the detection limit by quantitative microRNA RT-PCR in sorted neutrophils. Last but not least, the seed binding sequence is also present in human RAC2 3'UTR. Based upon aforementioned reasons, miR-722 was selected for further characterization.
First, we generated a transgenic zebrafish line that over-expresses miR-722 specifically in neutrophils (schematic in Fig. 1A). To facilitate the identification and characterization of cells expressing this microRNA, a 206 bp genomic DNA sequence flanking microRNA-722 was cloned into an intron that allows co-expression of miR-722 with a green fluorescent reporter protein, Dendra2. Three founders each of the zebrafish that express the vector control or miR-722 were obtained. We observed specific upregulation of both the precursor and mature forms of miR-722 in the transgenic animals, without alterations in the level of miR-223 or a ubiquitously expressed microRNA let-7e (Fig. 1B), confirming that microRNA biogenesis in neutrophils is intact. In addition, similar numbers of neutrophils were present in both lines (Fig. 1C), indicating that miR-722 does not impair neutrophil biogenesis or survival. We next examined the recruitment of miR-722 over-expressing neutrophils in two separate acute inflammation models: a localized bacterial infection and tail transection. Significantly fewer neutrophils were recruited in miR-722 over-expressing lines in both incidences (Fig. 1D, E and Movie S1). This phenotype was further confirmed in the offspring from separate founders (Fig. 1 G, H and I), excluding the positional effect of the random genomic insertion by the tol2 transposon method. Furthermore, the motility of the miR-722 over-expressing neutrophils was significantly hampered (Fig. 1F and Movie S2), which phenocopied the Rac2-deficient neutrophils (Deng et al., 2011; Rosowski et al., 2016), coinciding with the prediction that miR-722 down-regulates rac2 expression in neutrophils.

**MiR-722 directly suppresses zebrafish Rac2 expression.**

We then confirmed that miR-722 can directly suppress zebrafish Rac2 gene. The zebrafish rac2 3'UTRs harbor a miR-722 binding site with perfect seed sequence match (Fig. 2A). We performed reporter assays to validate the direct translational suppression by miR-722. Expression of miR-722 significantly suppressed the relative luciferase activity, which was dependent on the seed sequences in zebrafish Rac2 gene (Fig. 2B). Since reporter assays are based on enforced microRNA and transcript over-expression that can yield false positive results, we measured the endogenous rac2 transcript level. In the miR-722 over-expressing zebrafish line, the rac2 mRNA is significantly reduced (Fig. 2C), suggesting a direct destabilization of
the rac2 transcript by miR-722 in neutrophils. Another neutrophil specific gene, lysozyme C, was not altered in the same sample, indicating the specificity of miR-722 towards rac2.

**Rac2 over-expression rescues miR-722 induced phenotypes.**

To further validate that rac2 is a major target of miR-722 in neutrophils, we performed a rescue experiment using a transgenic zebrafish line that over-express zebrafish rac2 followed by the SV40 3’UTR that is resistant to miR-722 mediated suppression (Deng et al., 2011). A line that expresses mCherry alone was used as a control. Clutch mates were used in this experiment to minimize the impact of genetic variation in different lines (Fig. 3A). Consistent with our data that miR-722 directly downregulates endogenous rac2 expression, defects in neutrophil motility (Fig. 3B) or their recruitment to tissue injury (Fig. 3C) or infection (Fig. 3D and Movie S3) resulted from miR-722 over-expression were all rescued by rac2 over-expression, pinpointing rac2 as a relevant miR-722 target in neutrophils.

**Neutrophil specific miR-722 over-expression protects zebrafish from lethal systemic inflammation.**

Neutrophils are a major cell type which causes tissue damage during severe inflammation. Thus, we tested whether miR-722 over-expressing zebrafish were more resistant to lethal inflammatory challenges, for example, a bacterial systemic infection model using the Gram-negative bacteria Pseudomonas aeruginosa PAK strain. The miR-722 over-expressing larvae survived better (Fig. 4A), despite with similar bacterial burdens (possibly as a result of intact macrophage functions), excluding the possibility that the miR-722 over-expression line had increased bactericidal activity (Fig. 4B). In addition, in both lines, there was an initial drop of neutrophil numbers upon infection, which later recovered (Fig. 4C). This increased resistance coincided with a more robust upregulation of the anti-inflammatory cytokines, including IL-10 and the TGF-beta family members (Fig. 4D, E). The pro-inflammatory cytokines, including TNF-alpha, IL-6 and IL-8 were also induced in the miR-722 over-expressing line, but not significantly. Interestingly, nos2b, an important pro-inflammatory gene that produces nitric oxide species was not highly induced. Since zebrafish nos2b also harbors miR-722 binding sites, we next examined
whether the rac2 over-expression will mitigate the protective effect elicited by miR-722. Restoring rac2 expression in the miR-722 over-expression line increased susceptibility, comparable to the wild-type larvae, from the acute systemic Pseudomonas infection (Fig. 4G), suggesting that miR-722 protects zebrafish from lethal inflammatory challenge via suppressing rac2. In wild-type larvae, the endogenous level of miR-722 was not induced during systemic inflammation (Fig. 4H).

We also developed a sterile systemic inflammation model by injecting lipopolysaccharide (LPS) into the zebrafish intravenously. The vector control over-expressing larvae succumbed to over-inflammation within 6 days post injection. In comparison, miR-722 over-expressing larvae survived significantly better (Fig. 5A). Similar changes of the pro- and anti-inflammatory cytokines were observed as with the bacterial infection (Fig. 5B, C).

MiR-722 mimic protects against sterile inflammation.

Finally, we injected miR-722 mimics into zebrafish embryos at 1-cell stage to deliver miR-722 ubiquitously. As expected, neutrophil recruitment to the injury site was impaired in the larvae receiving miR-722 mimics as compared to the buffer injected while recruitment was more robust in miR-722 inhibitor injected larvae (Fig. 6A). We observed significantly increased resistance to lethal LPS challenge in the miR-722 mimic injected larvae, compared with the buffer or the miR-722 inhibitor injected larvae (Fig. 6B), suggesting that miR-722 is a potential prophylactic measure in sterile inflammation. MiR-129-5p, which is conserved between zebrafish and human, shares the same seed sequence of miR-722. The seed match is present in the 3'UTRs of both the zebrafish rac2 and human RAC2 genes. To test the broader translational value of Rac2 targeting microRNAs, hsa-miR-129-5p mimics were delivered into the zebrafish embryos. Larvae which received the miR-129 mimic, but not a non-rac2 targeting miR-223 mimic, were more resistance to LPS challenge (Fig. 6B, C). To demonstrate that the miR-722 mimic also elicits its protective role via inhibiting rac2 in neutrophils, the mimic was injected into a line that expressed the miR-722 resistant rac2 in neutrophils. Indeed, the protective role of miR-722 was abrogated in the rac2 over-expressing line, but not in a line that express the mCherry
control in neutrophils (Fig. 6D). Taken together, our results suggest that rac2 inhibiting microRNA mimics can improve the outcome in sterile inflammation.

On the contrary, a protective effect from miR-722 mimics in the *Pseudomonas* sepsis model was not observed (Fig. 6E). Similar amounts of neutrophils were recruited to localized PAK infection (data not shown). In addition, despite that the anti-inflammatory cytokines were upregulated in infected larvae that received miR-722 mimic, the fold changes were variable between experiments that did not reach statistical significance (Fig. 6 F, G). The different host outcome with miR-722 mimic treatment between the sterile and bacterial infection is possibly due to lower than biologically relevant threshold concentration or short-lived effectiveness of the miR-722 mimic to have a sustained effect when battling live organisms that take days to cleared (Fig. 4B).

**Discussion**

Here we have identified a microRNA, miR-722, that when over-expressed in neutrophils, reduces neutrophil chemotaxis and protects the whole organism from both sterile and non-sterile inflammatory assaults. Our findings are of significant importance as we have identified a leukocyte-specific manner to restrain the systemic inflammatory response and have a direct impact on numerous human diseases, including those directly involving an immune component such as rheumatic arthritis and those that are not obviously linked such as diabetes, neurodegenerative disease and cancer.

Here we have demonstrated that rac2 is a major target of miR-722 in neutrophils. Rac2 regulates multiple steps in neutrophil-mediated tissue damage, including reducing neutrophil adhesion to the capillary (causing ischemic damage) and their release of reactive oxygen species and granular contents (major mediators for the secondary organ damage). In light of the detrimental roles neutrophils play, it is not surprising that Rac2 inhibition improves the outcome in our systemic inflammation models. It remains to be determined which is the most critical step in this process.
Whether it is myelopoiesis, neutrophil exit from bone marrow, chemotaxis, adhesion to blood vessel, degranulation or releasing reactive oxygen or nitrile species require further investigation. It certainly is possible that multiple steps have to be spontaneously inhibited to elicit a protective effect in treating undesired inflammation. The therapeutic potential of Rac2 inhibition has not been explored previously, probably due to the difficulty in developing a specific chemical inhibitor for Rac2 that would not inhibit the closely related family member Rac1, which is expressed in all cells and developmentally essential (Duquette and Lamarche-Vane, 2014). Rac1 and Rac2 proteins are very similar in their structure and function, although they harbor different functions in neutrophils (Zhang et al., 2009). MicroRNAs can bind to the 3'UTR of their target genes and it is very practical to identify microRNAs that target only RAC2 but not RAC1, making it possible to selectively suppress RAC2 expression. We have demonstrated that two different rac2 targeting microRNA mimics are equally potent in zebrafish. Many other microRNAs are predicted to target the RAC2 gene in humans, but not the RAC1 gene, for example, miR-6090 and miR-6726 (targetscan), which warrant further characterization.

It is interesting that Rac2 knockout animals or zebrafish expressing a dominant negative form of Rac2 in neutrophils are more susceptible to infections (Deng et al., 2011; Roberts et al., 1999; Rosowski et al., 2016). In contrast, miR-722 overexpression in neutrophils protected zebrafish from lethal challenge of Pseudomonas infection. This discrepancy is possibly due to the fact that microRNAs are fine-tuners that modulate the protein expression level post transcriptionally. In our study, we observed a partial inhibition of Rac2 expression and impaired/delayed neutrophil chemotaxis. We reasoned that neutrophils still preserve some of the effector functions, yet the magnitude of the inflammation and the bystander tissue damage are decreased which translates into a favorable balance of the pro-inflammatory and anti-inflammatory cytokines that promotes the resolution of inflammation. Along the same line, therapeutic doses and delivery methods of Rac2 targeting microRNAs have to be carefully determined in humans to elicit the most favorable outcome.
We have selected a clinical strain of *Pseudomonas aeruginosa* for our current study because of its prevalence in human sepsis patients (Gotts and Matthay, 2016) and it is a well characterized systemic infection model in zebrafish (Clatworthy et al., 2009). *Pseudomonas aeruginosa* is an opportunistic pathogen in human, not a natural zebrafish pathogen and it requires a much higher infection dose to cause significant mortality (2000-10000 cfu) in immune competent larvae, compared with natural fish pathogens, such as *Edwardsiella tarda* (van Soest et al., 2011) and *Streptococcus iniae* (Harvie et al., 2013). It has long been appreciated that neutrophils cause tissue damage while eliminating bacterial infections (reviewed in (Weiss, 1989)). However, it is difficult to separate these two functions, since similar mechanisms, such as reactive oxygen species, proteases and extracellular traps contribute to both processes. To date, solid evidence that inflammation contributes to mortality in a *Pseudomonas* blood infection model is not available, although it is an attractive hypothesis based on the literature. Our work has associated the improved survival with increased production of anti-inflammatory pre-resolving cytokines, but not with bacterial burden, providing the first evidence that inflammation is relevant to mortality in this model.

There are several recent examples that anti-inflammatory intervention increases zebrafish survival without altering the bacterial burden. The interleukin-1 receptor antagonist anakinra treatment enhanced zebrafish survival in *Shigella flexneri* or *Burkholderia cenocepacia* infection, without affecting the bacterial load (Mazon-Moya et al., 2017; Mesureur et al., 2017). The Myd88 mutant also lived significant longer than the wild-type siblings in *Burkholderia cenocepacia* infection, with no differences in bacterial burden (Mesureur et al., 2017). In addition to live bacterial infection, we have also used *Pseudomonas aeruginosa* LPS to induce mortality that is caused by sterile inflammation. We observed similar phenotypes with both live bacteria and a purified bacterial cell wall component, indicating that miR-772 regulates the inflammation process to favor host survival.

So far, reduction of neutrophil numbers has not been associated with increased survival in zebrafish infection models. Several primary neutrophil deficiency models have been established and many are associated with increased susceptibility to
infections (reviewed in (Harvie and Huttenlocher, 2015)). In the WHIM and LAD models, neutrophil recruitment to wounding or infection is completely abolished, indicating that neutrophils provide protective immunity and a substantial loss of neutrophil function is detrimental to the host.

A partial reduction of neutrophil number can be achieved by disrupting the GCSFR/Csf3r, using either a morpholino (Liongue et al., 2009) or a recently generated mutant (Pazhakh et al., 2017), or with the *Escherichia coli* nitroreductase/metronidazole system (Pisharath et al., 2007).

The GCSFR/Csf3r morphants are more susceptible to *Salmonella* (Hall et al., 2012) and Chikungunya Virus (Palha et al., 2013). The caveat of this approach is whether GCSFR depletion affects macrophage numbers, especially in older larvae, still needs to be determined. In addition, since Gcsfr-depleted larvae were more susceptible to infection than Runx1-depleted larvae (where both neutrophil and macrophage numbers were reduced) (Hall et al., 2012), it is possible that GCSFR regulates other aspects of neutrophil biology, not restricted to neutrophil numbers.

The alternative approach for neutrophil depletion using the nitroreductase/metronidazole system was first performed by Prajsnar *et al* where 50% of neutrophils were depleted without affecting macrophage numbers (Prajsnar et al., 2012). Although the larvae are more susceptible, neutrophils were discovered as a privileged intraphacyte niche for disseminated *Staphylococcus* infection, highlighting the multifaceted role of this phagocyte. In a more recent study, over 95% depletion of neutrophils did not affect zebrafish survival during *Burkholderia* infection (Mesureur et al., 2017). The caveat of this approach is the risk of non-specific alteration in the immune system caused by un-natural phagocyte death.

Nevertheless, neutrophil depletion has been proven to be beneficial in many murine inflammation models, including infections (reviewed in (Mocsai, 2013)). To be more specific, mice depleted of FcεRI+ neutrophils were less susceptible to experimental cerebral malaria after infection with *Plasmodium berghei*, without reducing the parasite burden in blood (Porcherie et al., 2011). Our research sets apart from the
existing literature that a fine-tuning of neutrophil function, rather than total neutrophil depletion or loss-of-function was achieved.

In summary, we have provided a proof-of-concept strategy in treating conditions in which over-activation of the immune system contributes to disease with microRNAs, particularly those targeting RAC2 expression. Human neutrophils have an estimated circulatory half-life of up to 90 hours (Pillay et al., 2010; Tak et al., 2013). Although this measurement may be explained alternatively as the half-life of neutrophil progenitors, a population of older neutrophils survive for several days in the body in other model organisms (Cheretakis et al., 2006; Vincent et al., 1974). MicroRNAs in human therapeutic settings could at least theoretically be rapid enough to downregulate Rac2 expression in mature neutrophils and/or long-lasting enough to downregulate Rac2 during neutrophil maturation in the bone marrow until their mobilization into the circulation. Due to current technical hurdles that prevent us from effectively delivering miR-772 into neutrophils in the larvae, we have not been able to show the efficacy of miR-722 in treating existing inflammation. Nevertheless, we have demonstrated that miR-722 can be used as a prophylactic measure that alters the overall immune response during systemic inflammation, which may be relevant to conditions in humans such as a means to prevent overt inflammation elicited during organ transplantations. With the combination of a yet-to-be optimized efficient phagocyte specific delivery system, we provide an alternative concept in restraining unresolving neutrophilic inflammation.

Materials and Methods

Generation of transgenic zebrafish lines:

The zebrafish experiment was conducted in accordance to the internationally accepted standards. The Animal Care and Use Protocol was approved by The Purdue Animal Care and Use Committee (PACUC), adhering to the Guidelines for Use of Zebrafish in the NIH Intramural Research Program (Protocol number: 1401001018). A 206 bp genomic DNA sequence flanking microRNA-722 (MI0004765) was PCR amplified using forward: 5’-AATCAGGACTGTGTTGCTGTCT-3’, reverse: 5’-CCTCTTCGTCTTCTCTCGGC-3’ and inserted into the BbsI site in the intron of the vector modified from (De Rienzo et
GFP was replaced with Dendra2 and then cloned into the Tol2 backbone containing the lyzC promoter and SV40 polyA. The plasmids were deposited to Addgene. More than 3 founders (F0) for both Tg(lyzC:miR-722/Dendra2)pu6 and Tg(LyzC:Dendra2)pu7 were obtained as described in the AB background (Deng et al., 2011). Experiments were performed with F2 larvae produced by F1 fish.

**Zebrafish neutrophil recruitment assay:**

Zebrafish wounding and infection were performed as described (Deng et al., 2011). Hindbrain injection was done as described in (Gutzman and Sive, 2009). Briefly, 2 or 3 dpf larvae were amputated posterior to the notochord, inoculated with *P. aeruginosa* (PAK) into the left otic vesicle or into the ventricle region of the brain at 1000 CFU/embryo respectively. The larvae were fixed in 4% paraformaldehyde at 1 hour post wounding or infection. Neutrophils were stained with Sudan black and the number at the indicated regions were quantified.

**Dual luciferase reporter assay:**

Zebrafish rac2 3'UTR was amplified with One Step Ahead RT-PCR kit (Qiagen) from zebrafish mRNA using the following primers and inserted into pCS2+GFP using EcoRI/NotI sites: zRac2+: 5'-GTACAAGTGAGAATTCAGATACACGATTCGTCACTG-3'; zRac2-: 5'-ATTGGCGGCCGCGCCGCCAGTTGTACAGTTTATTTTTGC-3'; Rac2 mutant 3'UTR constructs were generated using Infusion HD cloning kit (Clontech) with the following primers: zRac2 mut+: 5'-TTTTGGCAGAAAATGCGTTTTTTAAACTGTACAACTGGCGGCC-3'; zRac2 mut-: 5'-CATTTTCTGCCAAAAATAATTCCATAC-3'. The mutations were confirmed by sequencing.

The suppression of the reporter expression was measured. Reporter assay constructs were then cloned into psiCHECK2 (Promega) at XhoI and NotI cloning sites using the following primers to amplify both wild-type and mutation 3'UTRs from pCS2+ constructs. Psi-zRac2+: 5'-TAGGCGATCGCTCGAGAGATACACGATTCGTCACTGT-3'; Psi-zRac2-: 5'-TTGCGGCCAGCGCCGCCGCAACAGTTGTACAGTTATTTTTGCC-3'; Psi-zRac2 Mut-: 5'-TTGCGGCCAGCGCCGCCGCAACAGTTGTACAGTTAAAAACGCA-3'. MiR-722 expression vector was cloned by amplifying the 722 hairpin from the lyzC:miR-722
vector used to create the transgenic line and inserted into pcDNA3.1 at the HindIII/XbaI cloning sites using the following primers: pcDNA-722+: 5’-GTTTAAACTTAAGCTTGCCACCATGGATGAGGAAATCGC-3’; pcDNA-722-: 5’ – AAACGGGCCCTCTAGAGACCGGTACCCCCGGGCTGC-3’;

Plasmids were co-transfected into HEK293 cells with Lipofectamine 3000 (Invitrogen). Cells were harvested after 48h. Renilla luciferase activity was normalized with photynus luciferase activity, which were sequentially determined using a dual luciferase reporter assay (Promega) and a plate reader (BioTek). Three independent biological repeats were performed for each 3'UTR.

**Confocal Imaging:**

Larvae at 3 dpf were settled on a glass-bottom dish. Time-lapse fluorescence images were acquired with a laser-scanning confocal microscope (Movie S1, LSM710; Zeiss) with a Plan-Apochromat 20x/0.8 M27 objective. The green and red channels were acquired sequentially with 0.1% power of the 488nm laser and 0.4% of 561 nm laser respectively with a 200 μm pinhole at a speed of 1.27 μs/pixel and averaged (line 2). The fluorescent stacks were flattened using the maximum intensity projection and overlayed with a single slice of the bright field image. Neutrophil speed was quantified using ImageJ plug-in MTrackJ (Meijering et al., 2012).

**RT-qPCR:**

Total RNA was purified using MiRVANA miRNA purification kit (ThermoFisher). MicroRNAs were reverse transcribed with Universal cDNA Synthesis Kit II (Exiqon). MicroRNA RT-qPCR was performed with ExiLENT SYBR® Green master mix (Exiqon) using LightCycler® 96 Real-Time PCR System (Roche Life Science). Primers used in this study are: miR-223-3p (205986), dre-let-7e-5p (2106780), dre-miR-722 (2107521) and dre U6 (206999). Messenger RNAs were reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-qPCR were performed with FastStart Essential DNA Green Master (Roche). Primers are listed in Supplemental table 1. All primers amplified a single product according to the melt-curve analysis. The relative fold change is calculated following instructions provided by Real-time PCR Minor with correction of the primer efficiencies (http://ewindup.info/miner/data_submit.htm). 10-20 larvae were used in each repeat
to generate an average value that was used to calculate the final mean ± s.d. from three independent experiments.

**FACS of dissociated embryo neutrophils and one-step qRT:**

Larvae at 3 dpf from Tg(lyzC:miR-722/Dendra2)pu6 and Tg(LyzC:Dendra2)pu7 were injected with 1000 CFU *P. aeruginosa* (PAK) into the tail vein and incubated to 8hpi. Neutrophils were sorted out by FACSARIA II with the 488 laser as described (Deng et al., 2011). Neutrophil RNA was extracted as described above and one-step qRT was performed with SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitogen), using LightCycler ® 96 Real-Time PCR System (Roche Life Science).

**Survival assay:**

Larvae at 3 dpf were injected with 1nl of 25ng/nl LPS or 1000 CFU *P. aeruginosa* (PAK) into the tail vein and incubated individually in 96 well plates. Survival was tracked for 7 days or when one group reached 100% mortality. Representative experiments of at least three independent repeats (n>=20 larvae in each experiment) were shown.

**MicroRNA mimic and inhibitor delivery:**

All mimics and the miR-722 inhibitor were synthesized by ThermoFisherScientific. Embryos at the one-cell stage were injected with 1nl of 15 µM dre-miR-722 mimic (#4464066), dre-miR-722 inhibitor (#4464084), hsa-miR-129-5p mimic (#4464084), 1 µM dre-miR-223 mimic (#4464066) or buffer as a control. Tail wounding and survival assays were carried out as described above but at 2 dpf.

**Statistical Analysis:**

Statistical analysis was carried out by PRISM 6 (GraphPad). Unpaired Student’s t-test (comparing two groups), one-way ANOVA (when comparing to single group), or two-way ANOVA (for multiple comparisons) were utilized in neutrophil recruitment assays and the reporter assays. For RT-qPCR, each gene was normalized to the reference gene and compared with paired Student’s t-test. For survival assays, Gehan-Breslow-Wilcoxon test was performed with a log-rank test and confirmed with Kaplan-Meier curve to ensure compatibility.
**Authorship**: DQ designed research. AH, DW and TG performed experiments and analyzed data. WZ and XZ generated essential reagents. JL performed experiments. DQ and AH wrote the manuscript.

**Competing interests**: The authors declare no competing financial interests.

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References


Figure 1. Neutrophil recruitment and motility is hindered in miR-722 over-expressing zebrafish line.

(A) A Schematic of Tol2-lyzC:miR-722/Dendra2 plasmid, injected into wild-type AB zebrafish embryos to generate the transgenic line Tg(lyzC:miR-722/Dendra2)pu6 (miR-722). Tg(lyzC:Dendra2)pu7 was generated using the same configuration without the miR-722 insertion (vector). All experiments were performed with F2 larvae at 3dpf. (B) Relative expression level of precursor and mature miR-722, miR-223, and let-7e.
let-7e (normalized to U6 expression) in vector and miR-722 lines determined by RT-qPCR, mean ± s.d. (N = 3 biological repeats with 10 larvae at each time point in each group). P value was calculated with unpaired Student’s t-test. (C) Representative images and quantification of total neutrophils in vector and miR-722 lines. One representative result of three independent experiments was shown (n=20). Scale bar: 500 μm. (D) Representative images and quantification of neutrophil recruitment to localized ear infection in vector or miR-722 larvae at 1 hour post infection. The infected ear is indicated with the circle. One representative result of three independent experiments was shown (n=20). P value was calculated with unpaired Student’s t-test. Scale bar: 100 μm. (E) Representative images and quantification of neutrophil recruitment to tail transection site in vector or miR-722 larvae at 1 hour post injury. Neutrophils in the boxed region were quantified. One representative result of three independent experiments was shown (n=20). P value was calculated with unpaired Student’s t-test. Scale bar: 100 μm. (F) Tracks and quantification of neutrophil motility in vector or miR-722 larvae. Results were pooled from three independent larvae (n=60). P value was calculated with unpaired Student’s t-test. Scale bar: 50 μm. Quantification of total number of neutrophils (G), neutrophils recruited to the ear 1 h post P. aeruginosa infection (H) and to the wound 1 hour post tail transection (I). One representative result of three independent experiments was shown (n=20). No statistical difference among the results from separate founders were observed with unpaired one-way ANOVA.
Figure 2. miR-722 down regulates the zebrafish Rac2 transcript through binding to seed complementary sequences in the 3'UTR.

(A) Sequence of miR-722 and zebrafish rac2 3'UTRs. The seed sequence and its binding sites in 3'UTRs are boxed. (B) Selective suppression of Renilla luciferase activity by miR-722 through binding to seed sequence in zebrafish rac2 3'UTRs. Result was presented as mean ± s.d. (N = 3 independent experiments). P value was calculated with paired Student’s t-test. (C) Relative expression level of rac2 and lyzC mRNA (normalized to ef1a) in vector and miR-722 larvae determined by RT-qPCR. Result was presented as mean ± s.d. (N = 3 independent experiments with over 20 larvae each/experiment). P value was calculated with unpaired Student’s t-test.
Figure 3. Over-expression of *rac2* rescues neutrophil recruitment in the miR-722 expressing larvae.

(A) *Tg(lyzC:miR-722/Dendra2)*<sup>pu6</sup> was crossed with *Tg(mpx:mCherry-2A-Rac2)* (Rac2) and the offspring were separate into four groups. For control, *Tg(lyzC:miR-722/Dendra2)*<sup>pu6</sup> was crossed with *Tg(mpx:mCherry)* (mCherry). All experiments were performed with F2 larvae at 3dpf. (B) Tracks and quantification of neutrophil motility in indicated lines. Results were pooled from three independent larvae. *P* values were calculated with unpaired Student’s *t*-test. Scale bar: 100 μm.

Quantification of neutrophil recruitment to tail wounding (C) or localized ear infection (D) in siblings separated into four groups as depicted in (A). One representative experiment of three independent biological repeats were shown (*n*=20 for each group). *P* values were calculated with unpaired two-way ANOVA.
Figure 4. The miR-722 over-expression line is more resistant to bacterial induced systemic inflammation.

F2 larvae from Tg(lyzC:miR-722/Dendra2)^pu6 (miR-722) and Tg(lyzC:miR-722/Dendra2)^pu6 (vector) at 3 dpf were injected intravenously with 1000 CFU of Pseudomonas. (A) Mortality, (B) colony forming unit (CFU) and (C) total neutrophil number in the vector or miR-722 lines were documented till 7 days post infection (dpi). One representative experiment of three biological repeats was shown in (A). (B, C) mean ± s.d. (N = 3 biological repeats with 10 larvae at each time point in each group). P value was calculated with Gehan-Breslow-Wilcoxon test. (D, E) Relative abundance of transcripts of pro-inflammatory and anti-inflammatory cytokines at 0h and 8h post infection (hpi). Result was presented as mean ± s.d. (N = 3 biological repeats with 20 larvae in each group). P value was calculated with one-way ANOVA. (F) Neutrophils were sorted from larvae at 8 hpi and the relative transcript levels of indicated genes were quantified. One representative experiment of two independent biological repeats was shown. P value was calculated with paired Student’s t-test. (G) Tg(lyzC:miR-722/Dendra2)^pu6 (miR-722) was crossed with Tg(mpx:mCherry-2A-
(Rac2) and the offspring were separate into four groups as in Fig. 3A. Mortality was documented till 7 dpi. One representative experiment of three independent biological repeats (n = 20 each group) were shown. $P$ value was calculated with Gehan-Breslow-Wilcoxon test. (H) Relative expression levels of miR-722 before or after intravenous injection with 25ng of LPS or with 1000 CFU of Pseudomonas, mean ± s.d. (N = 3 biological repeats with 10 larvae at each time point in each group). No statistical differences with unpaired one-way ANOVA.
Figure 5. The miR-722 over-expression line is more resistant to sterile systemic inflammation.

F2 larvae at 3 dpf were injected intravenously with 25 ng LPS. (A) Mortality of the vector and miR-722 lines. One representative experiment of three independent biological repeats (n=20 each group) was shown. $P$ value was calculated with Gehan-Breslow-Wilcoxon test. (B-C) Relative abundance of transcripts of pro-inflammatory inos2b and anti-inflammatory cytokines at (B) 0h and (C) 8h post injection (hpi), mean ± s.d. (N = 3 biological repeats with 20 larvae in each group). $P$ value was calculated with one-way ANOVA.
Figure 6. miR-722 mimic reduces neutrophilic inflammation and mortality from systemic LPS challenge.

Embryos were injected with buffer, microRNA mimics, or a miR-722 inhibitor at one-cell stage and experiments were performed with larvae at 2 dpf. (A) Neutrophil recruitment to the injury site at 1 h post tail transection. One representative experiment of three independent biological repeats were shown (n = 20 for each group). P values were calculated with unpaired one-way ANOVA. (B-C) Survival of larvae with (B) buffer, miR-722 mimic, or inhibitor or (C) buffer, miR-129 mimic, or miR-223 mimic upon systemic LPS challenge. One representative experiment of three independent biological repeats (n ≥20 each group) was shown. P values were calculated with Gehan-Breslow-Wilcoxon test. (D) Survival of Tg(mpx:mCherry) and Tg(mpx:mCherry-2A-Rac2) larvae with miR-722 mimic upon systemic LPS challenge. One representative experiment of three independent biological repeats (n =20 each group) was shown. P value was calculated with Gehan-Breslow-Wilcoxon test. (E) Survival of larvae intravenously injected with PAK at 2 dpf. One representative experiment of three independent biological repeats (n =20 each group) was shown. Not significant as determined with Gehan-Breslow-Wilcoxon test. (F-G) Relative abundance of transcripts of pro-inflammatory inos2b and anti-inflammatory cytokines at (F) 0h and (G) 8h after intravenous injection of 1000 CFU PAK. Results are presented as mean ± s.d. (N = 3 biological repeats with = 20 larvae in each group). P value was calculated with one-way ANOVA.
**Movie S1. Neutrophil recruitment to ear infection in vector and miR-722 larvae.**

Lateral view of larvae with neutrophils expressing vector or miR-722, responding to a localized ear infection. Delayed recruitment of neutrophils were observed in the miR-722 line. Scale bar: 100 µm.
Movie S2. Neutrophil random motility in vector and miR-722 larvae.

Lateral view of neutrophil random migration in the mesenchymal tissues of the head. Note reduced proportion and speed of neutrophil migration in miR-722 larvae. Scale Bar: 50 µm.
**Movie S3. Rac2 rescues the motility defect in the miR-722 larvae.**

Lateral view of neutrophil random migration in the mesenchymal tissues of the head. Note the increased proportion and speed of larvae expressing Rac2, but not the mCherry control, in the miR-722 background. Scale Bar: 50 µm.
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