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 zebrafish neutrophils by using a microRNA (miRNA) would inhibit neutrophil migration and activation, which would reduce the immunological damage caused by systemic inflammation. We have generated a transgenic zebrafish line that overexpresses 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-matching sequence in the rac2 3′ UTR. Furthermore, miR-722 overexpressing 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, an miR-722 mimic protects zebrafish from lethal lipopolysaccharide challenge. Together, these results provide evidence for and the mechanism of an anti-inflammatory miRNA that restrains detrimental systemic inflammation.

KEY WORDS: Zebrafish, MicroRNA, miRNA, Systemic inflammation, Cell motility

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. Although 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 and extracellular traps, and produce a large amount of reactive oxygen species, which help with eliminating the threat of pathogens but can cause detrimental effects on the host. 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 (Seeramkumar 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 of 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 by 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 (miRNAs) are evolutionarily conserved, small non-coding RNAs that post-transcriptionally regulate protein synthesis (Fabian and Sonenberg, 2012). miRNA expression is controlled by specific promoters and regulated at the transcriptional and post-transcriptional levels. The 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 miRNA duplexes, containing both 5p and 3p strands. The duplex is then loaded into Argonaut (AGO) proteins, where the passenger strand is degraded, thus allowing the guide strand to direct the miRNA-induced silencing complexes to partial-complementary target sites (Guro 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 miRNAs bind to their target transcripts through 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 October 2017, 2588 human mature miRNAs have been identified and indexed in miRBase (www.mirbase.org), which are implicated in a wide variety of cellular processes and human diseases. miRNAs and anti-miRNAs 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 miRNAs. The miRNA expression profiles in various inflammatory conditions, including sepsis, have been documented (Guroi et al., 2016). However, the use of this information is currently limited to establishing miRNAs as biomarkers and diagnostic tools. The biological functions of these miRNAs and their therapeutic potential are merely starting to emerge. The majority of miRNA inflammation research is restricted to macrophages, with their roles in neutrophils being 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) each express a different profile of miRNAs. It is reasonable to speculate that miRNAs are potent regulators of neutrophil function and inflammation.

In the present study, we aim to identify a miRNA 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 Rad2P-containing complex, directly interacting with gp19phox and p67phox, and is responsible for the generation of superoxide ions during infection (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 the 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 Huttonlocher, 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 miRNA that suppresses the expression of Rac2, and demonstrate that partial Rac2 suppression attenuated the acute lethal inflammation under both sterile and non-sterile conditions.

**RESULTS**

**miR-722 overexpressing neutrophils are defective in motility and chemotaxis**

To test the efficacy of miRNAs as next-generation therapeutics that would restrain neutrophil migration and inflammation, we looked into miRNAs that can suppress rac2 expression. We performed bioinformatics analysis (TargetScanFish) and identified three miRNAs (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 of 69, possibly being a weaker regulator of rac2. Data compiled from previous miRNA 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 overexpressing this miRNA. In addition, the miR-722 level is below the detection limit by quantitative miRNA reverse-transcription (RT)-PCR (RT-qPCR) in sorted neutrophils. Last but not least, the seed-binding sequence is also present in the human rac2 3′UTR. Based upon aforementioned reasons, miR-722 was selected for further characterization.

First, we generated a transgenic zebrafish line that overexpresses miR-722 specifically in neutrophils (schematic in Fig. 1A). To facilitate the identification and characterization of cells expressing this miRNA, a 206 bp genomic DNA sequence flanking miRNA-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 miRNA, let-7e (Fig. 1B), confirming that miRNA 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-overexpressing neutrophils in two separate acute inflammation models: a localized bacterial infection and tail transection. Significantly fewer neutrophils were recruited in miR-722-overexpressing lines in both incidences (Fig. 1D; Movie 1). This phenotype was further confirmed in the offspring from separate founders (Fig. 1G-1), excluding the positional effect of the random genomic insertion by the Tol2 transposon method. Furthermore, the motility of the miR-722-overexpressing neutrophils was significantly hampered (Fig. 1F; Movie 2), which phenocopied the Rac2-deficient neutrophils (Deng et al., 2011; Rosowski et al., 2016), coinciding with the prediction that miR-722 downregulates rac2 expression in neutrophils.

**miR-722 directly suppresses zebrafish rac2 expression**

We then confirmed that miR-722 can directly suppress the zebrafish rac2 gene. The zebrafish rac2 3′UTR harbors 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 the zebrafish rac2 gene (Fig. 2B). Because reporter assays are based on enforced miRNA and transcript overexpression that can yield false-positive results, we measured the endogenous rac2 transcript level. In the miR-722-overexpressing zebrafish line, the rac2 mRNA level is significantly reduced (Fig. 2C), suggesting a direct destabilization of the rac2 transcript by miR-722 in neutrophils. Another neutrophil-specific gene, encoding lysozyme C, was not altered in the same sample, indicating the specificity of miR-722 towards rac2.

**Rac2 overexpression 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 overexpresses zebrafish rac2 followed by the SV40
3’UTR, which 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; Movie 3) resulting from miR-722 overexpression were all rescued by rac2 overexpression, pinpointing rac2 as a relevant miR-722 target in neutrophils.

**Neutrophil-specific miR-722 overexpression protects zebrafish from lethal systemic inflammation**

Neutrophils are a major cell type that causes tissue damage during severe inflammation. Thus, we tested whether miR-722-
overexpressing zebrafish were more resistant to lethal inflammatory challenges; in this experiment, we used a bacterial systemic infection model using the Gram-negative bacteria *Pseudomonas aeruginosa* PAK strain. The miR-722-overexpressing larvae showed increased survival compared with those overexpressing the vector control (Fig. 4A), despite the presence of similar bacterial burdens (possibly as a result of intact macrophage functions), excluding the possibility that the miR-722-overexpressing line had increased bactericidal activity (Fig. 4B). In addition, in both lines, there was an initial drop of neutrophil number 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-β family members (Fig. 4D,E). The pro-inflammatory cytokines, including TNF-α, IL-6 and IL-8, were also induced in the miR-722-overexpressing line, but not significantly. Interestingly, nos2b, an important pro-inflammatory gene that produces nitric oxide species, was not highly induced. Similar expression changes of *inos2b*, *il-10*, and *tgf-*β2 were observed in FACS-isolated neutrophils, consistent with neutrophil-restricted overexpression of miR-722 (Fig. 4F). Because zebrafish *nos2b* also harbors miR-722-binding sites, we next examined whether *rac2* overexpression mitigates the protective effect elicited by miR-722. Restoring *rac2* expression in the miR-722-overexpressing line increased susceptibility, to levels 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 the suppression of *rac2*. In wild-type larvae, the endogenous level of miR-722 was not upregulated during systemic inflammation (Fig. 4H).

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

### Fig. 2. miR-722 downregulates the zebrafish rac2 transcript through binding to seed complementary sequences in its 3′ UTR.

(A) Sequence of miR-722 and zebrafish rac2 3′UTRs. The seed sequence and its binding sites in the 3′UTRs are boxed. (B) Selective suppression of Renilla luciferase activity by miR-722 through binding to seed sequence in zebrafish rac2 3′UTRs. Results are presented as means±s.d. (N=3 independent experiments). *P* values were 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. Results are presented as means±s.d. (N=3 independent experiments with over 20 larvae each/experiment). *P* values were calculated with unpaired Student’s t-test.

### Fig. 3. Overexpression of rac2 rescues neutrophil recruitment in miR-722-expressing larvae.

(A) Tg(lyzC:miR-722/Dendra2)pu6 (miR-722) was crossed with Tg(mpx:mCherry-2A-Rac2) (Rac2) and the offspring were separated into four groups. For control, Tg(lyzC:miR-722/Dendra2)pu6 was crossed with Tg(mpx: mCherry) (mCherry). All experiments were performed with F2 larvae at 3 dpf. (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. (C,D) Quantification of neutrophil recruitment to tail wounding (C) or localized ear infection (D) in siblings separated into the four groups as depicted in A. One representative experiment of three independent biological repeats is shown (n=20 for each group). *P* values were calculated with unpaired two-way ANOVA.
miR-722 mimic protects against sterile inflammation

Finally, we injected an miR-722 mimic into zebrafish embryos at the one-cell stage to deliver miR-722 ubiquitously. As expected, neutrophil recruitment to the injury site was impaired in the larvae receiving miR-722 mimic as compared to the buffer-injected larvae, whereas 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 buffer- or the miR-722-inhibitor-injected larvae (Fig. 6B,C). One representative experiment of three biological repeats. (B,C) Means±s.d. (N=3 biological repeats with 10 larvae at each time point in each group). P values were calculated with the Gehan–Breslow–Wilcoxon test. (D,E) Relative abundance of transcripts of pro-inflammatory and anti-inflammatory cytokines to ef1a at 0 and 8 h. Results are presented as means±s.d. (N=3 biological repeats with 20 larvae in each group). P values were calculated with one-way ANOVA. (F) Neutrophils were sorted from larvae at 8 hpi and the relative transcript levels of the indicated genes to ef1a were quantified. One representative experiment of two independent biological repeats is shown. P values were calculated with paired Student’s t-test. (G) Tg(lyzC:miR-722/Dendra2)pu6 (miR-722) was crossed with Tg(mpox:mCherry-2A-Rac2) (Rac2) and the offspring were separated into the four groups as in Fig. 3A. Mortality was documented until 7 dpi. One representative experiment of three independent biological repeats (n=20 each group) is shown. P values were calculated with the Gehan–Breslow–Wilcoxon test. (H) Relative expression levels of miR-722 before or after intravenous injection with 25 ng of LPS or with 1000 CFU of Pseudomonas; means±s.d. (N=3 biological repeats with 10 larvae at each time point in each group). No statistical differences were found with unpaired one-way ANOVA.

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not reach statistical significance (Fig. 6F,G). The different host outcome with miR-722-mimic treatment between sterile and bacterial infection is possibly due to a threshold concentration lower than that of biological relevance or the short-lived effectiveness of the miR-722 mimic to have a sustained effect when battling live organisms that take days to clear (Fig. 4B).

**DISCUSSION**

Here, we have identified a miRNA, miR-722, that, when overexpressed in neutrophils, reduces neutrophil chemotaxis and protects the whole organism from both sterile and non-sterile inflammatory assaults. Our findings are of significant importance because 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 neutrophil release of reactive oxygen species and granular contents (major mediators of 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

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**Fig. 5. The miR-722-overexpressing line has increased resistance 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) is shown. P value was calculated with the Gehan–Breslow–Wilcoxon test. (B,C) Relative abundance of transcripts of pro-inflammatory inos2b and anti-inflammatory cytokines to ef1a at (B) 0 hpi and (C) 8 hpi; means±s.d. (N=3 biological repeats with 20 larvae in each group). P values were calculated with one-way ANOVA.

**Fig. 6. miR-722 mimic reduces neutrophilic inflammation and mortality from systemic LPS challenge.** Embryos were injected with buffer, miRNA mimic or an miR-722 inhibitor at the 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 (n=20 each group) is shown. 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) is shown. P values were calculated with the 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) is shown. P value was calculated with the 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) is shown. Not significant as determined with the Gehan–Breslow–Wilcoxon test. (F,G) Relative abundance of transcripts of pro-inflammatory inos2b and anti-inflammatory cytokines to ef1a at (F) 0 h and (G) 8 h after intravenous injection of 1000 CFU PAK. Results are presented as means±s.d. (N=3 biological repeats with 20 larvae in each group). P values were calculated with one-way ANOVA.
determined which is the most critical step in this process. Whether it is myelopoiesis, neutrophil exit from bone marrow, chemotaxis, adhesion to blood vessels, degranulation or releasing reactive oxygen or nitrite species requires 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 is 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). miRNAs can bind to the 3′UTR of their target genes and it is very practical to identify miRNAs that target Rac2 but not Rac1, making it possible to selectively suppress Rac2 expression. We have demonstrated that two different rac2-targeting miRNA mimics are equally potent in zebrafish. Many other miRNAs 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 miRNAs 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 miRNAs have to be carefully determined in humans to elicit the most favorable outcome.

We have selected a clinical strain of *P. aeruginosa* for our current study because of its prevalence in human sepsis patients (Gotts and Matthey, 2016) and because it is a well-characterized systemic infection model in zebrafish (Clatworthy et al., 2009). *Pseudomonas aeruginosa* is an opportunistic pathogen in humans, not a natural zebrafish pathogen, and it requires a much higher infection dose to cause significant mortality (2000-10,000 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 because 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. Treatment with the IL-1 receptor antagonist anakinra 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-null mutant also lived significantly longer than wild-type siblings in *B. cenocepacia* infection, with no differences in bacterial burden (Mesureur et al., 2017). In addition to live bacterial infection, we have also used *P. 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 number 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 that a substantial loss of neutrophil function is detrimental to the host.

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

Gcsfr (also known as Csfr3) morphants are more susceptible to *Salmonella* (Hall et al., 2012) and chikungunya virus (Palha et al., 2013). The caveat of this approach – whether Gcsfr depletion affects macrophage number, especially in older larvae – still needs to be determined. In addition, because Gcsfr-depleted larvae were more susceptible to infection than Runx1-depleted larvae (in which both neutrophil and macrophage numbers were reduced) (Hall et al., 2012), it is possible that Gcsfr regulates other aspects of neutrophil biology, not only neutrophil number.

An alternative approach for neutrophil depletion using the nitroreductase/metronidazole system was first performed by Prajnsr et al. (2012), where 50% of neutrophils were depleted without affecting macrophage number. 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 (Porchier et al., 2011). Our research is set apart from the existing literature in 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 overactivation of the immune system contributes to disease via miRNAs, particularly those targeting RAC2 expression. Human neutrophils have an estimated circulatory half-life of up to 90 h (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). miRNA administration in human therapeutic settings could, at least theoretically, be rapid enough to downregulate RAC2 expression in mature neutrophils and/or long-
lasing enough to downregulate RAC2 during neutrophil maturation in the bone marrow until their mobilization into the circulation. Owing 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-772 in treating existing inflammation. Nevertheless, we have demonstrated that miR-772 can be used as a prophylactic measure that alters the overall immune response during systemic inflammation, which may be relevant to conditions in humans, for example 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 with 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 miR-772 (M10004765) was PCR amplified using forward: 5′-AATCCAGAAGTGTGACGTCTGCT-3′, reverse: 5′-CTCTTTGCTGTCTCC-TCTCGGC-3′ primers, and inserted into the BbsI site in the intron of the vector modified from De Rienzo et al. (2012). 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 (plasmid 97101, Tol2-lyzC-Vector-Dendra2; plasmid 97130, Tol2-lyzC-miR-772-Dendra2). More than three founders (F0) for both Tg(lyzC:miR-722/Dendra2)p76 and Tg(LyzC:Dendra2)p75 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 (Gutman and Sive, 2009). Briefly, 2 or 3 dpf larvae were amputated posterior to the notochord, or inoculated with P. aeruginosa (PAK) into the left otic vesicle or into the ventricle region of the brain at 1000 CFU/embryo. The larvae were fixed in 4% paraformaldehyde at 1 h post-wounding or -infection. Neutrophils were stained with Sudan black and the number at the indicated regions were quantified.

**Dual luciferase reporter assay**

Zebrafish race2 3′UTR was amplified with One Step Ahead RT-PCR kit (Qiagen) from zebrafish mRNA using the following primers and inserted into pcRS2+GFP using EcoRI/Ncol sites: zRac2+; 5′-GTACAAGTGAGA-ATTCTGATACGGTCTGTCAGT-3′; zRac2-; 5′-ATTGCGGCCGCGGCCGAGCTGACGTCTGCAG-3′. Rac2 mutant 3′UTR constructs were generated using Infusion HD cloning kit (Clontech) with the following primers: zRac2 mut+; 5′-TTTTGGCAGAAAATGCGTTT- TTTTAAACTTAAGCTTGCCAC-CTTTTAAACTTAAGCTTGCCAC-3′; zRac2 mut-; 5′-CTTTTAAACTTAAGCTTGCCAC-CTTTTAAACTTAAGCTTGCCAC-3′. The mutations were confirmed by sequencing. The suppression of the reporter expression was measured. Reporter assay constructs were then cloned into pCISE2Check2 (Promega) at XhoI and NorI cloning sites using the following primers to amplify both wild-type and mutant 3′UTRs from pcRS2+ constructs. Psi-zRac2+; 5′-TGGCGAGTACG-CCTGAGAGATACACGATTCGTCACTGT-3′; Psi-zRac2-; 5′-TTTAAACTTAAGCTTGCCAC-GCA-3′. miR-722 expression vector was cloned by amplifying the 722 hairpin from the lyzC:miR-772 vector used to create the transgenic line and inserted into pcDNA3.1 at the HindIII/XhoI cloning sites using the following primers: pcDNA-722+; 5′-GGTTAAACCTTAAGCTTGCCAC-CATGGATAGGAAATTCG-3′; pcDNA-722-; 5′-AAAACCGGGCCCTC-TAGAGACCGGTACCCGGCTGC-3′. Plasmids were deposited to Addgene (plasmid 97158, pSi-check2-zRac2 3′UTR; plasmid 97159, pSi-check2-zRac2 - mut 3′UTR; plasmid 97160, pSi-check2-hRac2 3′UTR; plasmid 97161, pSi-check2-hRac2 - mut 3′UTR; plasmid 97163, miR-722-Dendra pCDNA).

Plasmids were co-transfected into HEK293 cells with Lipofectamine 3000 (Invitrogen). Cells were harvested after 48 h. Renilla luciferase activity was normalized with Photinus 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 1: LSM710, Zeiss) with a Plan-Apochromat 20×/0.8 M27 objective. The green and red channels were acquired sequentially with 0.1% power of the 488 nm laser and 0.4% of 611 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 overlaid 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 the MiRVANA miRNA purification kit (Thermo Fisher Scientific). miRNAs were reverse transcribed with Universal cDNA Synthesis Kit II (Exiqon). miRNA 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 was performed with FastStart Essential DNA Green Master (Roche). Primers are listed in Table S1. 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_sub.mht). A total of 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 RT-qPCR**

Larvae at 3 dpf from Tg(lyzC:miR-722/Dendra2)p76 and Tg(LyzC: Dendra2)p75 were injected with 1000 CFU P. aeruginosa (PAK) into the tail vein and incubated 8 hours post-infection (hpi). 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 RT-qPCR was performed with SuperScript® III Platinum® SYBR® Green One-Step qRT-qPCR Kit (Invitrogen), using LightCycler® 96 Real-Time PCR System (Roche Life Science).

**Survival assay**

Larvae at 3 dpf were injected with 1 ml of 25 ng/ml 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.

**miRNA mimic and inhibitor delivery**

All mimics and the miR-722 inhibitor were synthesized by Thermo Fisher Scientific. Embryos at the one-cell stage were injected with 1 ml 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, the Gehan–Breslow–Wilcoxon test was performed with a log-rank test and confirmed with Kaplan–Meier curve to ensure compatibility.

Competing interests
The authors declare no competing or financial interests.

Author contributions

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injection systems for exposure of zebrafish embryos to the natural pathogen Edwardsiella tarda. *BMC Immunol.* 12, 58.


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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How would you explain the main findings of your paper to non-scientific family and friends?

Control of the immune response is a delicate balance of activation to combat foreign pathogens, and keeping it in check to avoid damage to the host itself. Thus, maintaining just the right amount of immune activation is a difficult and delicate task. As such, the microRNAs (miRNAs) that serve as fine-tuners and moderate regulators of virtually all cellular processes seemed an appealing research target. We identified a novel miRNA, miR-722, which is expressed in low quantities in neutrophils, a type of immediate response innate immune cell. We then generated a zebrafish line that overexpressed miR-722 – tagged with a fluorescent protein – specifically in neutrophils. These fish demonstrated lower neutrophil recruitment to a tail wound and regional infection site, but exhibited improved survival rates during systemic infection and sterile inflammation. Diving into the mechanism, we found that miR-722 specifically targets and downregulates Rac2, a Rho-GTPase protein that has been shown to be important for cell motility and migration, and which can explain the decrease of neutrophil recruitment. To summarise, when we stimulated an immune response in fish, we found that overexpression of miR-722 in neutrophils can regulate inflammation and lessen the unwanted effects of over-inflammation.

What are the potential implications of these results for your field of research?

Our goal was to identify miRNAs that are not normally expressed in neutrophils and introduce them in order to identify potential regulators of immune recruitment and function. In science there is a great satisfaction in approaching a question with an unprecedented strategy and then deciphering the underlying biological mechanism.

What are the main advantages and drawbacks of the model system you have used?

Zebrafish is a fully sequenced model organism and its innate immune system is similar to that of humans, making it a good model to study acute inflammation scenarios. In addition, zebrafish larvae are transparent, making real-time imaging much easier and accessible for the study of immune cell properties and behaviour. Furthermore, the zebrafish is easy to genetically edit and the passage time is relatively short. However, although the innate immune system is over 98% conserved between zebrafish and humans, there are some receptors or immune components that may be different as zebrafish live in water and have unique methods of dealing with pathogens that humans would not normally encounter.

“In science there is a great satisfaction in approaching a question with an unprecedented strategy and then deciphering the underlying biological mechanism.”

What has surprised you the most while conducting your research?

It was a bit counterintuitive at first that hindering neutrophil recruitment (by overexpressing miR-722) during a systemic inflammation challenge improved survival rates, as you would think the pathogen would need to be cleared by these innate immune cells where neutrophils are the first line of defence to reach the inflammation site. We thus hypothesised that it was due to us only expressing miR-722 in neutrophils where the other innate immune cells were intact. When miR-722 was introduced into the whole embryo, survival rates were comparable during systemic bacterial challenge, but further improved during sterile inflammation. A closer look shed light on the mechanism; iNOS was lowered in neutrophils while anti-inflammatory cytokines were upregulated, suggesting that miR-722 induces a less activated state and thus may protect against undesired inflammation effects.
Describe what you think is the most significant challenge impacting your research at this time and how will this be addressed over the next 10 years?

The field of miRNA research is still blooming as more and more miRNAs are identified and characterised, and knowledge of the cellular processes in which they are involved is expanding as well. However, not all miRNAs are conserved between different organisms even though the nomenclature may be the same. Furthermore, the binding sites of miRNAs on the target mRNA may also not be conserved, leading to challenges in target validation and miRNA and target mRNA tissue expression profiles. Given the advancements and throughput of sequencing and methods to identify miRNA–mRNA hybrids, we hope that one day we may have an easy and accessible method to identify and validate miRNA–mRNA interactions, instead of relying on bioinformatics for target prediction.

What’s next for you?

My current work focuses on the dynamics and interactions of miRNAs in innate immune cells and how miRNAs affect immune cell behaviour and host–pathogen interaction. In the immediate future I will work to characterize more miRNAs and investigate their effects in neutrophils and macrophages. After identifying potential miRNA candidates, we may expand our work into the study of various pathogens, and how innate immune cell interactions affect infection outcome.

Reference