Abstract

The oxygen sensing transcription factor HIF-1α plays a critical role in the regulation of myeloid cell function. The mechanisms of regulation are not well understood, nor the phenotypic consequences of HIF modulation in the context of neutrophilic inflammation. Species conservation across higher metazoans enables the use of the genetically tractable and transparent zebrafish embryo to study *in vivo* resolution of the inflammatory response. Using both a pharmacological approach known to lead to stabilization of HIF-1α, and selective genetic manipulation of zebrafish HIF-1α homologues we sought to determine the roles of HIF-1α in inflammation resolution. Both approaches reveal that activated Hif-1α delays resolution of inflammation following tail transection in zebrafish larvae. This delay can be replicated by neutrophil specific Hif activation, and is a consequence of both reduced neutrophil apoptosis, and increased retention of neutrophils at the site of tissue injury. Hif-activated neutrophils continue to patrol the injury site during the resolution phase, when neutrophils would normally migrate away. Site-directed mutagenesis of Hif *in vivo* reveals that hydroxylation of Hif-1α by Phd hydroxylases critically regulates the Hif pathway in zebrafish neutrophils. Our data demonstrate that Hif-1α regulates neutrophil function in complex ways during inflammation resolution *in vivo*.

Introduction

Neutrophilic inflammation is of fundamental importance in the innate immune response to bacterial and fungal infection in vertebrates, and can be initiated by sterile tissue injury. Irrespective of its aetiology, inflammation must resolve in a timely fashion to avoid damage to surrounding tissues.[1] Persisting, non-infectious inflammation is the hallmark of inflammatory diseases, a major cause of morbidity and mortality in the developed world. The resolution phase of inflammation is critical to the restoration of normal tissue function following an inflammatory response, and thus has a central role in determining the outcome of inflammation.[2] Despite the central place of failed resolution in the pathogenesis of inflammatory disease, much remains to be known about the cellular and molecular events involved.

While neutrophil apoptosis, and subsequent uptake and removal by macrophages (efferocytosis), is well documented as a disposal route for inflammatory neutrophils,[3-5] there is emerging evidence that other mechanisms may also contribute to certain types of inflammation resolution. In the lung, some neutrophils are lost into the airways and expectorated[6] and in rheumatoid arthritis, neutrophils may leave the inflammatory site while still alive and re-enter the circulation.[7] Neutrophils can also be removed by migration through tissues away from the infection site; a process termed retrograde chemotaxis, or reverse migration.[8-10] This process is most widely characterized in the zebrafish embryo and is less well studied in mammalian systems.

Each of these aspects of inflammation resolution may contribute to the removal of neutrophils, and be influenced by a range of extracellular signals encountered by neutrophils at the site of inflammation, including cytokines,[11,12] bacterial products,[12,13] and low oxygen tensions.[14-16] Physiological hypoxia is important since it induces both extended survival and preserved activity in neutrophils. Hypoxia exerts its effects in part through stabilization of Hypoxia Inducible Factor (HIF), with activation of the HIF pathway in murine myeloid cells by myeloid specific *Vhl* knockout leading to increased myeloid cell recruitment.[17] HIF signaling can be activated in normoxia by exposure of myeloid cells to bacterial products, demonstrating the fundamental importance of this pathway to immune cell function.[18] The effects of HIF-1α activation and silencing on resolution of neutrophilic inflammation have yet to be fully characterized, especially in an *in vivo* setting.

Three HIF-α isoforms have been identified in humans, of which HIF-1α is a key regulator of neutrophil function and lifespan.[17-19] HIF-1α stability is regulated by a group of oxygen sensitive enzymes: prolyl hydroxylases (PHDs) and factor inhibiting HIF (FIH). Oxygen dependent PHD activity

leads to degradation of HIF-1α via a ubiquitin ligase complex co-ordinated by the von Hippel Lindau (VHL) protein.[20-22] In contrast, hypoxia reduces PHD activity, stabilising HIF-1α, which joins a nuclear complex with the constitutively expressed HIF-β (or aryl hydrocarbon nuclear translocator, ARNT) and transduces the cellular response.[22-24] The HIF pathway can be manipulated pharmacologically using non-specific inhibitors of PHD enzymes, for example dimethyloxaloylglycine (DMOG), leading to stabilization of HIF-1α *in vitro*.[25] Such pan-hydroxylase inhibitors lack specificity, however, with potential widespread effects on cellular physiology.

Here, using a transgenic zebrafish line expressing a neutrophil specific reporter gene,[26] we investigate the effects of pharmaceutical and genetic manipulation of Hif-1α activity on neutrophil behaviour *in vivo*. We show for the first time that the resolution of neutrophilic inflammation *in vivo* is delayed following DMOG treatment in a whole organism model. We show this delay in resolution is a consequence both of a decrease in neutrophil apoptosis, and of increased retention of neutrophils at the site of inflammation. Furthermore, using dominant active and dominant negative variants of the two zebrafish homologues of HIF-1α (Hif-1αa and Hif-1αb) we show timely resolution of neutrophilic inflammation is dependent on prolyl hydroxylation of Hif-1αb, demonstrating the importance of the HIF pathway in determining the outcome of inflammation *in vivo*.

Methods

Fish husbandry

Two neutrophil specific fluorescent zebrafish lines were used: *Tg(mpx:GFP)i114* [26] and *Tg(lyz:GAL4.VP16)i252;Tg(UAS-E1b:Kaede)s1999t* subsequently termed *mpx*:GFP *and lyz*:Kaede. Zebrafish strains were maintained according to standard protocols.[27] Adult fish were maintained on a 14/10 hour light/dark cycle at 28°C in UK Home Office approved facilities in the MRC CDBG aquaria at the University of Sheffield.

Inflammation assay

Inflammatory responses were elicited in zebrafish embryos by tail transection as previously described.[26] Embryos were anesthetized at two or three days post-fertilization (dpf) by immersion in 0.168 mg/ml Tricaine (Sigma-Aldrich, St. Louis, MO) and transection of the tail was performed with a scalpel blade briefly immersed in the chemoattractant f-met-leu-phe (100nM, Sigma-Aldrich). Treatment with 100μM DMOG (or DMSO vehicle control) was performed by immersion at 4 hours post injury (hpi), or for recruitment assays for 2 hours before injury. Neutrophils were counted at the site of transection at 6, 24 and 48 hpi using a fluorescent dissecting stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) as described previously.[26] Where possible, counting was performed blind to experimental conditions.

Apoptosis assays

Rates of apoptosis were assessed blindly by TUNEL/TSA or by anti-active caspase-3/TSA staining, as previously described.[28,29]

Mpx:GFP embryos were injured at 3dpf (2dpf in the case of RNA injected embryos) and 10dpf. Embryos were treated with DMSO/DMOG at 4hpi, and fixed at 12hpi in 4% paraformaldehyde. TUNEL (ApopTag Red, Chemicon, Millipore, Billerica, MA) staining labeled apoptotic cells with red fluorescence and TSA (TSAplus kit, Fluorescence Systems, Perkin Elmer Inc., Waltham, MA) staining labeled neutrophils with fluorescein green fluorescence. Anti-active caspase-3 (R&D Systems, Minneapolis, MN) staining with a fluorescein secondary antibody (Alexa Fluor 488, goat anti-rabbit IgG, Invitrogen, Carlsbad, CA) gave green fluorescence. Neutrophils were labeled with red fluorescence using Cyanine-3 TSA (TSAplus kit, Fluorescence Systems, Perkin Elmer Inc.).

Neutrophils in the tail transection region were imaged on an UltraVIEWVoX spinning-disk confocal microscope (Perkin Elmer Inc.) and apoptosis assessed by the percentage of TSA positive neutrophils labeled with TUNEL or active caspase-3.

Development of a stable *Tg(lyz:GAL4.VP16)i252* transgenic line

The Tol2kit multisite Gateway-based transposon system was used to make a transgenic construct from which a stable line was raised.[30] 11 kilobases of the *lysosyme C* promoter (kindly provided by Phil Crosier),[10] was cloned into the p5E-MCS entry vector (Xho-I and Sma-I sites). An LR Clonase II Plus Enzyme (Invitrogen) Gateway reaction was performed with the resulting p5E-*lyz* along with, pME-Gal4VP16, p3E-polyA inserted into pDestTol2pA2 to produce *lyz*:Gal4VP16 construct. This construct was co-injected with tol2-transposase RNA into zebrafish one-cell stage embryos to create the *Tg(lyz:GAL4.VP16)i252* transgenic line.

Photoconversion of neutrophil specific Kaede protein

Tail transection of *lyz:*Kaede larvae was performed at 2 or 3dpf as previously described.[26] Embryos were raised to 6hpi and mounted in 1% low-melting point agarose (Sigma-Aldrich). A Perkin Elmer UltraVIEW PhotoKinesisTM device on an UltraVIEWVoX spinning disk confocal microscope (Perkin Elmer Inc.) was used to photoconvert the Kaede labeled cells using 120 pulses of the 405nm laser at 40% laser power (optimised in previous experiments, data not shown). Embryos were transferred to a Nikon Eclipse TE2000-U Inverted Compound Fluorescence Microscope (Nikon, Tokyo, Japan) where a Hamamatsu 1394 ORCA-ERA camera (Hamamatsu Photonics Inc. Hamamatsu City, Japan) was used to capture a timelapse series. Cells fluorescing in the red channel were then tracked over a 3.5 hour period.

Neutrophil tracking

Tracking analysis was performed in Volocity 5 (Improvision, Perkin Elmer Inc.), using the intensity of fluorescence to identify individual labeled neutrophils over time.

Morpholino knockdown of *arnt-1*

The *arnt-1* morpholino (Genetools, Philomath, OR) was used as previously reported.[31] A standard control morpholino (Genetools) was used as a negative control.

hif-1α cloning

Zebrafish 2dpf RNA purified using TRIzol (Invitrogen, Carlsbad, CA) was used for RT-PCR cloning of zebrafish *hif-1αa* and *hif-1αb* (using primers in Table S1) using Pfusion polymerase (Finnzymes, Espoo, Finland). These were cloned into TOPOBlunt (Invitrogen) and were subsequentially subcloned into pCS2+ (Invitrogen) for RNA synthesis and *in situ* hybridization expression studies.

Dominant negative forms of *hif-1αa* and *hif-1αb* were generated using primers amplifying DNA corresponding to amino acids 1-330 of human HIF-1α.[32] Dominant active forms of *hif-1αa* and *hif-1αb* were generated by successive rounds of site-directed mutagenesis.[33-35] In each round one of the hydroxylation sites were mutated into non-hydroxylatable amino acids as previously described in human HIF-1α.[33-35]

Dominant negative and dominant active RNAs were transcribed (mMessageMachine, Ambion, Life Technologies Corp., Carlsbad, CA) and micro-injected into zebrafish embryos at the one cell stage as previously described.[27,36] A *phd-3 in situ* hybridization was performed to assess the function of dominant *hif-1α* variants, using previously described methods.[36,37]

Development of a stable *Tg(UAS:da-hif-1αb-IRES-GFP)i218* transgenic line

Dominant active *hif-1αb* was cloned into the middle entry pME-MCS. An LR Clonase (Invitrogen) Gateway reaction was performed with p5E-UAS, pME-*da-hif-1αb* and p3E-IRES-EGFPpA inserted into pDestTol2pA2. The resulting plasmid was microinjected with tol2-transposase RNA into one-cell stage embryos. IRES-GFP expression was weak, so founders were screened by heart marker GFP

expression. The resulting *Tg(UAS:da-hif-1αb-IRES-GFP)i218* is subsequently referred to as *UAS:da-hif-1αb*.

Functional Hif expression was tested in *UAS:da-hif-1αb*, by injection with a cmv:Gal4VP16-IRES-nlsEGFP construct, made from the tol2kit as previously described.[30] Injected larvae were screened for expression of nlsEGFP at 3dpf and a *phd-3 in situ* hybridization was performed to assess levels of Hif-1α signaling. To investigate the effects of *da-hif-1αb* expression on the resolution of neutrophilic inflammation *UAS:da-hif-1αb* were crossed to *Tg(lyz:Gal4)i252* fish to generate double transgenic embryos identified by the presence of GFP heart marker and mCherry labeled leukocytes.

Statistical analysis

Data were analyzed (Prism 5.0, GraphPad Software, San Diego, CA) using unpaired, two-tailed t-tests for comparisons between two groups and one-way ANOVA (with Bonferonni post-test adjustment) for other data.

Results

DMOG treatment delays resolution of inflammation

Following tail transection in *mpx*:GFP embryos at 3dpf, neutrophil numbers can be assessed by counting fluorescent cells, visible in association with the site of injury. Neutrophils numbers act as a measure of the magnitude of the inflammatory response as they are its main cellular component. Neutrophils are recruited early, with numbers peaking around 6 hours,[26] followed by spontaneous resolution of this neutrophilic inflammation over 24 hours (Figure 1A). Macrophages are recruited later and persist longer.[38]

Because of the known effects of the pan-hydroxylase inhibitor DMOG on neutrophil apoptosis, we hypothesized that DMOG would delay inflammation resolution in the zebrafish tailfin model. Agents delaying neutrophil apoptosis have been shown to delay inflammation resolution in this model when added once neutrophils have been recruited.[28] We therefore added 100μM DMOG at 4hpi, and observed the effects on inflammation resolution. Treatment with DMOG caused a significant increase in neutrophil numbers at 24hpi (Figure 1B, 1C-D) compared to DMSO treated controls (Figure 1A, 1C-D) from the same clutch of embryos, but numbers had decreased to basal levels by 48hpi, despite persistence of Hif signaling at 48hpi, as assessed by *in situ* hybridization expression of the target gene *phd-3* (Figure S1A). There were no additional effects of DMOG on neutrophil distribution throughout the embryo (Figure 1E), nor any increase in total neutrophil number (Figure S1B). DMOG dose and timing of administration were assessed and found to be optimal (Figure S1C-D).

Recruitment of neutrophils was assessed at 6hpi and was unchanged by DMOG treatment (Figure 1F), confirming that the differences seen in percentage change in neutrophil number from 6 to 24 hours (a measure of inflammation resolution) in DMOG treated embryos, is independent of effects on neutrophil recruitment (Figure 1G).

Neutrophil counts do not take account of changes in the migration behaviour of neutrophils, which may be affected by hydroxylase inhibition. We therefore assessed the speed and meandering of neutrophil migration towards the site of tail transection by *in vivo* tracking of *mpx*:GFP neutrophils over 1 hour during the recruitment phase using 3D videotimelapse microscopy (Figure 1H). This tracking technique was sensitive enough to detect changes in neutrophil recruitment after treatment with the chemoattractant fMLP (Figure S2A-B). After DMOG treatment, however, no significant difference in neutrophil speed (Figure 1I) or meandering index (Figure 1J) was observed when compared to vehicle only controls during the recruitment phase.

DMOG treatment decreases neutrophil apoptosis and retains neutrophils at the inflammatory site

DMOG treatment increased the number of neutrophils at the site of tail transection at 24hpi, but the mechanisms behind this delay in resolution remained unknown. Neutrophils were never seen to leave

the zebrafish body through the wound margin or elsewhere, and it is reasonable to conclude that loss of neutrophils from the larva is not important in this model. We therefore assessed the effects of DMOG both on levels of neutrophil apoptosis at the transection site, and on migration of neutrophils away from it.

Neutrophil apoptosis was quantified using TUNEL staining to identify apoptotic cells, in combination with a fluorescent histochemical stain to identify neutrophils, as previously reported.[28,29,39] Post-mortem TSA staining was used in preference to GFP imaging, since we have previously shown GFP fluorescence is lost during neutrophil apoptosis.[28] In DMOG treated larvae there was a significant decrease in apoptotic neutrophils at the transection site when compared to that of DMSO treated larvae at 3dpf (Figure 1K). The rates of apoptosis seen are comparable to those in other studies of zebrafish neutrophil apoptosis,[28] and to published rates of detectable apoptosis in other models.[40] Because levels of detectable neutrophil apoptosis are low, these events are scarce in fixed embryos at a single timepoint, presumably reflecting the short lifespan of an apoptotic neutrophil *in vivo*. Therefore this experimental result was confirmed at 10dpf where numbers of neutrophils are higher using both TUNEL and anti-active caspase-3 staining (Figure S3A-B).

In order to investigate the migration of neutrophils away from the transection site, *lyz*:Kaede transgenic fish were used. *lyz*:Kaede fish use the lysozyme C promoter to drive GAL4, which drives expression of Kaede, a photoconvertible protein that fluoresces in the red channel following exposure to 405nm excitation.[41] This allows individual cells, or cells within a certain area, to be converted from green to red fluorescence and tracked over time. Tailfin transection was performed in *lyz*:Kaede transgenic zebrafish at 3dpf, and at 6hpi the neutrophils present at the transection site were photoconverted to red fluorescence and tracked over the next 3.5 hours (Figure 2A). Embryos treated with DMOG had significantly fewer red neutrophils moving away from the injury site than DMSO controls (Figure 2B-D). Not only were more neutrophils in DMSO treated embryos moving away from the injury site, but they were moving further away than those treated with DMOG (Figure S4A).

To further investigate neutrophil movement at the injury site after DMOG treatment, a small area of between 5-10 neutrophils located at the dorsal side of the midline near the wound was photoconverted at 6hpi (Figure 3A). It was observed that DMSO treated neutrophils tended to migrate away from the injury site whilst DMOG treated neutrophils favoured migration along the injury edge (Figure 3B). This was independent of measurements of speed and meandering, which remained unchanged after treatment (Figure 3C). Over 3.5 hours of tracking, neutrophils migrated out of the photoconverted region at the same rate in both DMSO and DMOG treated individuals (Figure 3D). Fewer neutrophils migrate away from the injury site in DMOG treated individuals than in DMSO controls (Figure 3E), whilst more neutrophils in DMOG treated embryos moved down the injury site ventral of the photoconverted region (Figure 3F). This is consistent with DMOG treated neutrophils patrolling the injury site, rather than moving away.

DMOG-induced delay in resolution is blocked by *arnt-1* morpholino and by dominant negative *hif-1αb*

Given the potential for non-specific effects of competitive hydroxylase inhibition by DMOG and the lack of selective inhibitors of individual PHD enzymes, we sought to investigate the HIF-1α dependence of hydroxylase inhibition. ARNT-1 (or HIF-1β) is a crucial binding partner of HIF-1α, and *arnt-1* morpholino[31] will block Hif signaling. In the morpholino experiments, tail transection was performed at 2dpf, to increase the likelihood that the morpholino would remain active during the experiment. The 2dpf tail transection model retains the same features as the 3dpf model used in other studies,[26,28,42] and was used for all morpholino and RNA injection experiments below. In control morpholino injected larvae, DMOG increased neutrophil numbers at 24hpi. Treatment with DMOG after *arnt-1* morpholino injection, however, caused no significant increase in neutrophil numbers at 24hpi (Figure 4A), supporting the Hif dependence of the DMOG effect.

A truncated form of human HIF-1α has been previously shown to block HIF-1α signaling in cell culture.[32] We generated analogous constructs for the zebrafish homologues, *hif-1αa* and *hif-1αb*. These

were expressed in all cells of the embryo by RNA injection at the one cell stage. We showed dominant negative *hif-1α* was able to block activation of the HIF pathway by DMOG as late as 56hpf, indicated by a decrease in *phd-3* expression (Figure S5).[43] Dominant negative *hif-1α* abrogated the DMOG induced increase in neutrophil numbers at the transection site at 24hpi (Figure 4B), with no effect on wholebody neutrophil numbers (Figure 4C). Dominant negative *hif-1αb* alone was sufficient to block the DMOG delay in resolution of neutrophilic inflammation (Figure 4B), with *hif-1αa* having no effect on inflammation (Figure 4B). TUNEL staining showed that there was no significant difference in levels of neutrophil apoptosis in embryos injected with dominant negative *hif-1α* (Figure 4D).

In order to investigate the effect on neutrophil retention at the inflammatory site, *lyz*:Kaede embryos were injected with dominant negative *hif-1αb* RNA and neutrophils at the inflammatory site were photoconverted. Neutrophil movement away from the wound was investigated at 6hpi after tail transection at 2dpf. In phenol red injected negative control embryos, DMOG treatment caused fewer neutrophils to leave the site of transection (Figure S4B), as was observed at 3dpf. However, in embryos injected with dominant negative *hif-1αb*, DMOG treated embryos had similar numbers of neutrophils migrating away from the site of transection as the DMSO treated individuals (Figure 4E-F). The neutrophils that did move away, moved similar distances away from the injury site in treatment groups (Figure S4C). Therefore dominant negative *hif-1α* is able to negate the DMOG-induced delay in resolution partially by restoring the normal pattern of neutrophil movement away from the wound edge.

Dominant active *hif-1αb* delays resolution of neutrophilic inflammation

Dominant active forms of Hif-1αa and Hif-1αb were generated by mutation of conserved proline hydroxylation sites for Phd and the asparagine hydroxylation site for Fih (Figure 5A). In mammalian studies, these mutations render HIF-1α resistant to hydroxylation by oxygen sensitive hydroxylases, leading to stabilization of HIF-1α.[33-35] In zebrafish larvae expressing dominant active *hif-1α* by RNA injection, Hif signaling was upregulated, shown by increased *phd-3* expression (Figure S5). In addition, an increase in neutrophil number at 24hpi was observed in dominant active *hif-1α*, DMSO treated embryos, with neutrophil numbers comparable to DMOG treated, mock-injected siblings (Figure 5B). As with dominant negative *hif-1α*, dominant active *hif-1αb* alone recapitulated the effects of DMOG on 24hpi neutrophil numbers present at the transection site (Figure 5B). Neither dominant active nor negative *hif-1αa* had any effect in these assays. *In situ* hybridization expression of wildtype *hif-1αa* and *hif-1αb* showed similar expression patterns, although in general *hif-1αa* appeared to be expressed at a lower level than *hif-1αb* (Figure S6).

Dominant active *hif-1α* injection was associated with increased wholebody neutrophil numbers at 2dpf (Figure 5C) suggesting activation of Hif signaling might regulate total neutrophil numbers *in vivo*. Since HIF is activated by infection, this effect might contribute to the increase in neutrophils seen during infections however, this increase in wholebody neutrophil number did not cause an increase in neutrophil recruitment to the injury site at 6hpi (Figure 5D). Like DMOG, dominant negative and dominant active forms of *hif-1α* caused no significant changes in neutrophil speed or meandering index during the recruitment phase (Figure S2C-D).

TUNEL staining showed that there was a significant decrease in levels of neutrophil apoptosis in embryos injected with dominant active *hif-1α* (Figure 5E), similar to that observed in DMOG (Figure 1K). Neutrophil migration away from the injury site was assessed as with *lyz*:Kaede. Fewer neutrophils move out of the injury site in dominant active *hif-1α* injected embryos compared to phenol red injected controls (Figure 5F).

Each hydroxylation site of Hif-1αb was mutated individually and RNA injected into *mpx*:GFP larvae to assess their effects on neutrophil retention at 24hpi. Mutation of either of the two Phd target proline residues was sufficient to significantly increase the neutrophil numbers at the site of transection at 24hpi to the same or greater level as DMOG treatment (Figure 5G). Mutation of the Fih target asparagine had no effect on neutrophil numbers at 24hpi (Figure 5G).

To confirm the effects of Hif activation act within the neutrophil itself, a stable transgenic line expressing dominant active *hif-1αb* under the UAS promoter was generated (*UAS:da-hif-1αb*). This line

was shown to express active Hif when driven by an injected cmv:GAL4 construct, demonstrated by increased *phd-3* expression (Figure S7). Dominant active *hif-1αb* was expressed in neutrophils by crossing *UAS:da-hif-1αb* with the *lyz:Gal4* line described above. Following tail transection at 3dpf, neutrophils were counted at 24hpi after fluorescent TSA. Neutrophil numbers at 24hpi are elevated in embryos with the heart marker (i.e. *da-hif-1α* positive) compared to heart marker negative siblings (Figure 5H). As with dominant active *hif-1α* RNA experiments, levels of apoptosis are significantly reduced in *da-hif-1α* positive transgenics compared to controls (Figure 5I).

Discussion

   In these studies we investigated the role of the HIF pathway in modulating neutrophilic inflammation *in vivo*. Although a number of *in vitro* studies have shown effects on neutrophil function and lifespan,[14-16,19] there has been no previous study of the effects of HIF signaling on neutrophil behaviour during inflammation *in vivo*. We found recruitment of neutrophils to the injury site was unaffected by hydroxylase inhibition, and in addition, the pattern of neutrophil localization was unaltered in uninjured embryos. Neutrophil migration properties including speed and meandering of neutrophils during the recruitment phase were also not affected. DMOG did, however, cause a delay in the resolution of inflammation in association with an increase in HIF signaling. HIF signaling pathways therefore modulate the resolution rather than recruitment phase of inflammation. The number of neutrophils at the transection site in DMOG treated embryos had reached basal levels by 48hpi, suggesting that the abrogation of hydroxylase activity delays rather than prevents the resolution of inflammation.

   There are two plausible explanations for the decrease in inflammation resolution observed in DMOG treated zebrafish embryos: neutrophils are actively retained at the site of injury and/or neutrophil apoptosis is suppressed. Movement of neutrophils away from sites of injury and infection, by reverse migration, has been reported in a number of zebrafish studies.[8-10] A similar phenomenon has been reported in some mammalian systems, although the underlying mechanisms are less well understood.[7] Cell tracking experiments were performed to assess movement of neutrophils away from the wound region. In DMOG treated embryos, fewer labeled neutrophils left the site of transection over a 3.5 hour period during the resolution phase of inflammation and neutrophils travelled a shorter distance away from the wound zone. These effects of DMOG were blocked by dominant negative and replicated by dominant active Hif constructs, indicating Hif signaling retained neutrophils in the wound zone during the resolution phase. By labeling a small population of cells at the injury site we were able to show that the same number of neutrophils remain in the labeled region in DMOG treated embryos in the resolution phase, and that neutrophils migrate with the same speed and meandering index. The difference between these populations is purely a difference in the direction of the migrating neutrophils. Blocking hydroxylases led to more neutrophils patrolling the injury site rather than moving away from the injury site. This change in migration direction may reflect a differential response of neutrophils to existing chemotactic gradients, perhaps as a result of altered chemokine receptor expression.[44] It is unlikely that global activation of Hif signaling causes a change in the chemical gradients at the injury site, as neutrophil specific expression of dominant Hif-1α led to persisting inflammation. The decrease in reverse migration after Hif activation is likely to be partly responsible for the decrease in the resolution of neutrophilic inflammation. To our knowledge, this is the first report of the modulation of a genetic pathway directly altering the migratory behaviour of neutrophils specifically during inflammation resolution. Whether this represents a global effect of HIF signaling on inflammation resolution, or whether it is specific to zebrafish will require careful experimentation in emerging models of *in vivo* inflammation in mammalian systems.

   In addition, DMOG treatment caused a modest but significant decrease in the levels of neutrophil apoptosis in the resolution phase of inflammation. This is likely also to contribute to the delay in resolution observed after hydroxylase inhibition. This effect was shown to be Hif-dependent as dominant active Hif downregulated apoptosis. Expressing dominant active Hif specifically in neutrophils was able to decrease neutrophil apoptosis showing this effect is mediated by the neutrophils themselves. Upregulated hypoxia has been previously demonstrated to decrease neutrophil apoptosis in mammalian

systems,[14-16,19] however this is the first time this effect has been observed *in vivo* in a whole organism. Dominant negative Hif expression did not detectably increase apoptosis levels compared to controls. This is likely to indicate that levels of Hif are low during the normal process of inflammation resolution, therefore further inhibition of this pathway does not significantly affect neutrophil apoptosis.

It is difficult to assess the relative contribution of apoptosis versus reverse migration to the clearance of neutrophils after Hif activation, because the number of apoptotic cells identified depends on unknown variables, such as the duration for which an apoptotic neutrophil can be detected before it is degraded following macrophage ingestion. However, these data suggest both are likely to play an important role in the clearance of neutrophils.

Given the potential for non-specific effects of competitive hydroxylase inhibition by DMOG, and the lack of selective inhibitors of individual PHD enzymes there were multiple possible modes of action of DMOG *in vivo*. DMOG has targets other than the HIF hydroxylases and PHD enzymes themselves have HIF independent effects, including on the NFκB pathway.[45] By manipulating gene expression we have been able to establish a direct link between the effects of hydroxylase inhibition and Hif-1α. Previous reports have shown that an *arnt-1* translation blocking morpholino is able to block Hif-1α signaling.[31] We found that morpholino mediated knock-down of Arnt-1, an obligate binding partner of Hif-1α, is able to block the delay in inflammation resolution caused by DMOG. In addition, we found that the pro-inflammatory effects of DMOG were replicated by dominant active *hif-1αb,* and blocked by dominant negative *hif-1αb*. Dominant active *hif-1αb* delayed the resolution of inflammation to a similar degree to that of DMOG treatment alone. Interestingly, dominant active *hif-1αb* also increased the whole body neutrophil number. This might be a feature of suppression of neutrophil apoptosis during hematopoiesis, or an increase in myeloid cell production following hypoxic stimulation. This effect is not seen with either DMOG or hypoxia itself (Figure S1B and data not shown), but prolonged exposure of developing larvae to DMOG or to direct hypoxia is toxic to embryos (data not shown). This agrees with the findings in *vhl* mutants, in which increased Hif signalling leads to increased leukocyte number.[43] By developing a stable *UAS:da-hif-1αb* transgenic line we were able to drive the expression of *da-hif-1αb* specifically in *lyz* expressing leukocytes. We were able to demonstrate that over-activation of the Hif-1α pathway in neutrophils alone was able to induce the delay in the resolution of neutrophilic inflammation observed when Hif-1α was upregulated across all tissues of the embryo.

The two zebrafish homologues of human HIF-1α had different effects on neutrophilic inflammation. Hif-1αb was able to modulate the resolution of neutrophilic inflammation: dominant active Hif-1αb was able to replicate the effect of hydroxylase inhibition while conversely dominant negative Hif-1αb almost completely blocked the DMOG effect. This is consistent with Hif-1αb being the hypoxia transcription factor responsible for the timely resolution of neutrophilic inflammation. Hif-1αa, by contrast, had no significant effect on neutrophil behaviour in the assays we performed. Interestingly Hif-1αa has less sequence homology to human of HIF-1α than Hif-1αb.[46] In particular, Hif-1αa lacks the first conserved proline site for Phd hydroxylation, although it does have the second conserved proline site, and the Fih asparagine. The loss of complete Phd hydroxylation in Hif-1αa may indicate a divergence of the role of Hif-1αa in evolution away from a role in transducing the hypoxic signal in inflammatory cells.

To assess the role of hydroxylation at different sites in Hif-1α, we assayed inflammation resolution in zebrafish larvae injected with a range of dominant Hif-1αb constructs, in which Phd and Fih hydroxylation sites were mutated alone and in combination. Mutation of either Phd hydroxylation site reproduced the delay of inflammation resolution phenotype, consistent with a stabilization of Hif, suggesting that either site may function independently, in keeping with the recent *in vitro* crystal structure of hydroxyproline recognition by pVHL.[47] These data are consistent with Phd hydroxylation of Hif-1αb being required for the timely resolution of neutrophilic inflammation. Whilst *in vitro* FIH/HIF interaction has been proposed to partially determine oxygen sensitivity, mutation of the Fih hydroxylation site, either alone or in combination with mutation of the Phd sites, had no detectable effect on inflammation resolution. This suggests that in zebrafish neutrophils, Phd enzymes are the critical regulators of Hif activity.

Our data show that Hif-1α delays the resolution of inflammation in a whole organism *in vivo* model. We show for the first time that this delay in resolution is caused by suppression of the movement of neutrophils away from the site of inflammation in favour of patrolling the inflamed area, and by a decrease in neutrophil apoptosis. Furthermore, we find that hydroxylation of Hif-1αb by Phd enzymes is required for the timely resolution of neutrophilic inflammation. By developing the stable *UAS:da-hif-1αb* transgenic line we are able to show that this depends on Hif signaling within the neutrophil. These data have important implications in understanding the role of HIF/hydroxylase signaling in innate immune responses and in addition provides an illustration of how zebrafish models might elucidate key regulators of inflammatory signaling pathways with relevance to human health.

Authorship
FJvE, SRW, MKW and SAR conceived the study and designed the experiments with PME; PWI and XW generated new transgenic reagents; PME, FJvE, PWI, MKW, SRW and SAR wrote the manuscript; CCRA assisted and advised on image processing; PME and GD performed the experiments. The authors declare no conflict of interest.

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Figure legends

Figure 1: DMOG delays resolution of neutrophilic inflammation. (A-B) Fluorescent neutrophil numbers in the *mpx*:GFP line were counted at 6, 24 and 48 hpi in anesthetized embryos. 6hpi is the timepoint of maximal neutrophil recruitment. By 24hpi neutrophilic inflammation has resolved in wildtype embryos. At 4hpi, fish were treated with 100μM DMOG (B) or with DMSO as vehicle control (A). Data shown are individual embryos for each line, n = 18 performed as 3 independent experiments. (C) Fluorescence photomicrographs of 8 zebrafish tails from (upper panel) control (DMSO) and (lower panel) DMOG treated larvae. Imaged at 24hpi, 2X magnification on a Nikon TE2000U inverted microscope at constant exposure. (D,E) Overlaid fluorescence and brightfield photomicrographs of injured 3dpf embryos at 6hpi and 24hpi (D) or uninjured 3dpf and 4dpf embryos (E) after treatment at 3dpf with DMSO and DMOG. Imaged at 2X magnification on a Nikon TE2000U inverted microscope at constant exposure. (F) 6hpi timepoint neutrophil counts, in DMSO and DMOG treated zebrafish embryos. Data shown are mean ± SEM, n = 18 performed as 3 independent experiments. (G) The resolution of the cellular component of inflammation is decreased in DMOG treated embryos, expressed as percentage change in neutrophil number between 6 and 24hpi. Data shown are mean ± SEM, n = 18 performed as 3 independent experiments. P values were calculated using one-way ANOVA and Bonferroni multiple

comparison test, *p<0.05, **p<0.01, and ***p<0.001. (H) A photomicrograph of a typical tracking experiment with arrows indicating the path of neutrophil movement over a 1 hour timelapse during the recruitment phase of inflammation (1-2hpi). No difference was observed in the speed of neutrophil migration (I) or meandering index (displacement/path length) (J) in DMOG treated larvae compared to DMSO controls. Data shown are mean ± SEM, n = 18 performed as 3 independent experiments. (K) TUNEL and TSA co-localisation shows the percentage of neutrophils at the injury site undergoing apoptosis. Data shown are mean ± SEM, n = 3 performed as independent experiments each containing 35-40 embryos per treatment group.

Figure 2: DMOG retains neutrophils in the region of tissue injury. (A) Photomicrographs of a 3dpf photoconverted, DMSO treated, *lyz*:Kaede embryo at 0 hours post conversion (hpc). The white dashed line indicates the border of the site of transection area. The cells to the right of the line were photoconverted, leaving them with red fluorescence rather than green. The lower fluorescent panel shows the area of interest where neutrophils migrated away from the injury site shown in subsequent figure (D). (B) Photomicrographs of the same embryo as in (A) at 0hpc and 3.5hpc. The red channel only is shown, as a binary image. Between 0 and 3.5hpc photoconverted cells have migrated away from the site of transection. (C) Photomicrographs of a DMOG treated embryo at 0 and 3.5hpc converted into black and white. Fewer photoconverted leukocytes have migrated away from the site of transection. (D) A plot showing the number of photoconverted leukocytes leaving the area of transection over 3.5hpc in DMSO and DMOG treated embryos. Data shown are mean ± SEM, n = 9 performed as 2 independent experiments. Line of best fit shown is calculated by linear regression. P value shown is for the difference between the 2 slopes.

Figure 3: DMOG treatment causes a change in direction of neutrophil movement independent of neutrophil speed and meandering. (A) Photomicrographs of a 3dpf photoconverted, DMSO treated, *lyz*:Kaede embryo at 0 hours post conversion (hpc). The white box indicates the area of photoconverted, neutrophils. The lower panel shows the regions to which neutrophils migrated in subsequent figures (D-F). (B) Photomicrographs of examples of DMSO (upper panel) and DMOG (lower panel) embryos with the tracks of neutrophils over the first 3.5hpc shown. The dashed line indicates the outline of the embryo. (C) Tracking of neutrophils over the first 3.5hpc in the resolution phase of inflammation showed no difference in the speed of neutrophil migration (upper panel) or meandering index (displacement/path length) (lower panel) in DMOG treated larvae compared to DMSO controls. (D) Leukocytes leave the photoconversion site at the same rate in DMSO and DMOG treated embryos over 3.5hpc. (E) The number of photoconverted leukocytes moving away from the wound is shown over 3.5hpc in DMSO and DMOG treated embryos. (F) More neutrophils remain and patrol the injury site in DMOG treated embryos, since a greater number of leukocytes move within the injury region, ventrally away from the patch area. (D-F) Data shown are mean ± SEM, n = 14 performed as 3 independent experiments. Line of best fit shown is calculated by linear regression. P values shown are the differences between the 2 slopes.

Figure 4: DMOG induced delay in inflammation resolution is blocked by genetic inhibition of the Hif-1α pathway. (A) The 24hpi neutrophil counts in the *mpx*:GFP line at 2dpf following injection with control and *arnt-1* morpholinos. DMSO and DMOG treatment was performed at 4hpi. Data shown are mean ± SEM, n = 12 performed as 2 independent experiments. (B-E) 177pg of dominant negative *hif-1α* RNA was injected into the 1 cell stage zebrafish *mpx*:GFP embryos and neutrophil counts were performed at 24hpi after tail transection at 2dpf. (B) Injection of dominant negative *hif-1αb* abrogated the increase in neutrophil number at the site of injury at 24hpi seen with DMOG treatment, whilst embryos injected with phenol red as a negative control, or dominant negative *hif-1αa* alone exhibited a significant increase in neutrophil number after DMOG treatment. Data shown are mean ± SEM, n = 24 performed as 2 independent experiments. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test, where * represented p<0.05, ** p<0.01, and ***, p<0.001. (C) Injection of dominant negative *hif-1α* variants led to no significant difference in total neutrophil number at 2dpf compared to

phenol red injected negative control embryos. Data shown are mean ± SEM, n = 36 performed as 3 independent experiments. (D) Injection of dominant negative *hif-1α* caused no significant change in percentage of neutrophils at the injury site co-labeling with TUNEL stain (12hpi injured at 2dpf). Data shown are mean ± SEM, n = 4 performed as independent experiments containing 5-25 embryos per injection group per repeat. (E-F) Dominant negative *hif-1α* can block the increased neutrophil retention at the site of injury caused by DMOG treatment. Embryos were injected with dominant negative *hif-1αb,* grown to 3dpf and imaged 3.5 hours after photoconversion, with only the red channel shown, as a binary image. (E, left panels) in DMSO treated larvae red-labeled neutrophils have migrated away from the site of transection. (E, right panel) in DMOG treated larvae, red-labeled neutrophils also move away from the site of transection. (F) The number of red (photoconverted) lysC labeled cells leaving the area of transection at 6hpi over a time period of 3.5hpc in DMSO and DMOG treated embryos at 2dpf that were injected at the 1 cell stage with dominant negative *hif-1αb*. Data shown are mean ± SEM, n = 14 performed as 3 independent experiments. Line of best fit shown is calculated by linear regression. P value shown is the difference between the 2 slopes.

Figure 5: Dominant active *hif-1αb* delays resolution of neutrophilic inflammation. (A) Partial protein alignment showing the conserved hydroxylation sites of Hif-1α. Dominant active *hif-1α* has the following non-hydroxylatable mutations: Hif-1αb P402A, P564G, N804A and Hif-1αa P493G, N678A (Hif-1αa lacks the first proline hydroxylation residue). (B-F) Dominant active forms of *hif-1α* RNA (177pg) were injected into the 1 cell stage zebrafish *mpx*:GFP embryos, tailfin transection performed at 2dpf, and neutrophils counted at 24hpi. (B) Dominant active *hif-1α* caused a significant increase in neutrophil number in the absence of DMOG treatment compared to phenol red injected negative controls. Dominant active *hif-1αb* alone was able to recapitulate the DMOG phenotype, whilst dominant active *hif-1αa* homologue did not. Data shown are mean ± SEM, n = 24 performed as 2 independent experiments. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test, where * represented $p<0.05$, ** $p<0.01$, and ***, $p<0.001$. (C) Injection of dominant active *hif-1α* variants led to a significant increase in total neutrophil numbers at 2dpf. Data shown are mean ± SEM, n = 36 performed as 3 independent experiments. (D) Injection of dominant active *hif-1α* variants did not alter the recruitment of neutrophils to the injury site after 6hpi when the tail was transected at 2dpf. Data shown are mean ± SEM, n = 24 performed as 2 independent experiments. P values were calculated using one-way ANOVA and Bonferroni multiple comparison test. (E) Injection of dominant active *hif-1α* led to a significant decrease in percentage of neutrophils at the injury site co-labeled with TUNEL apoptosis staining 12hpi when injured at 2dpf. Data shown are mean ± SEM, n = 4 performed as independent experiments containing 10-25 embryos per injection group per repeat. (F) The number of red (photoconverted) lysC labeled cells leaving the area of transection at 6hpi over a time period of 3.5hpc in phenol red and dominant active *hif-1α* injected embryos at 2dpf. Data shown are mean ± SEM, n = 14 performed as 3 independent experiments. Line of best fit shown is calculated by linear regression. P value shown is the difference between the 2 slopes. (G) Injection of *hif-1αb* with each hydroxylation site mutated individually shows that the asparagine hydroxylation site of Fih is not required for resolution of neutrophilic inflammation. Mutation of either proline hydroxylation site of Hif-1αb was sufficient to lead to the decrease in neutrophil inflammation resolution. Data shown are mean ± SEM, n = 24 performed as 2 independent experiments. (H) *Tg(lyz:Gal4)i252;Tg(UAS:da-hif-1αb-IRES-GFP)i218* positive embryos and wildtype siblings underwent tailfin transection at 3dpf. Data shown are 24hpi TSA positive neutrophil counts, mean ± SEM, n = 30 performed as 3 independent experiments. (I) *Tg(lyz:Gal4)i252;Tg(UAS:da-hif-1αb-IRES-GFP)i218* positive embryos had a lower rate of neutrophil apoptosis analyzed by TUNEL staining at 12hpi after tail transection at 3dpf. Data shown are mean ± SEM, n = 3 performed as independent experiments containing 9-40 embryos per treatment group per repeat.

Figure 1
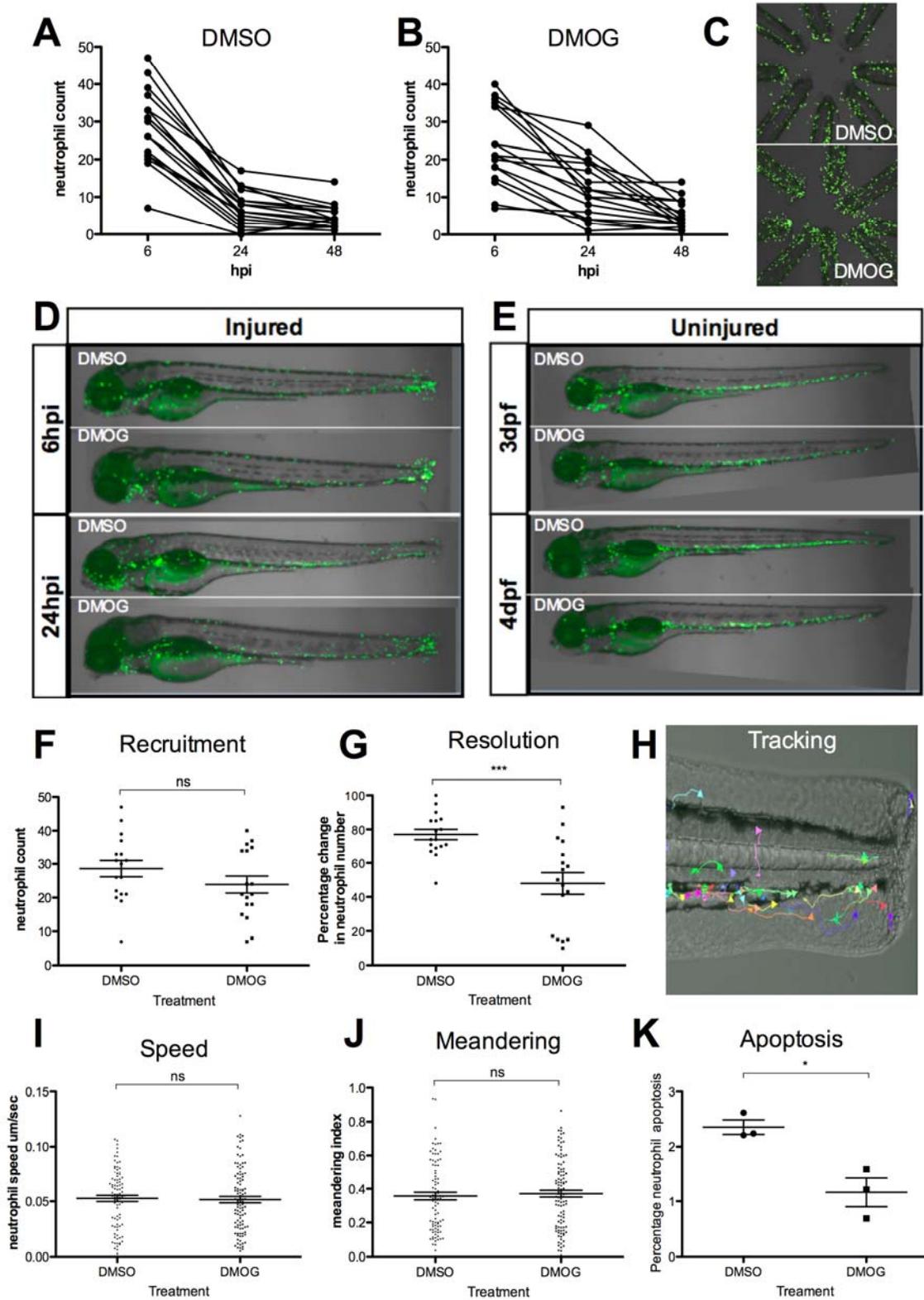
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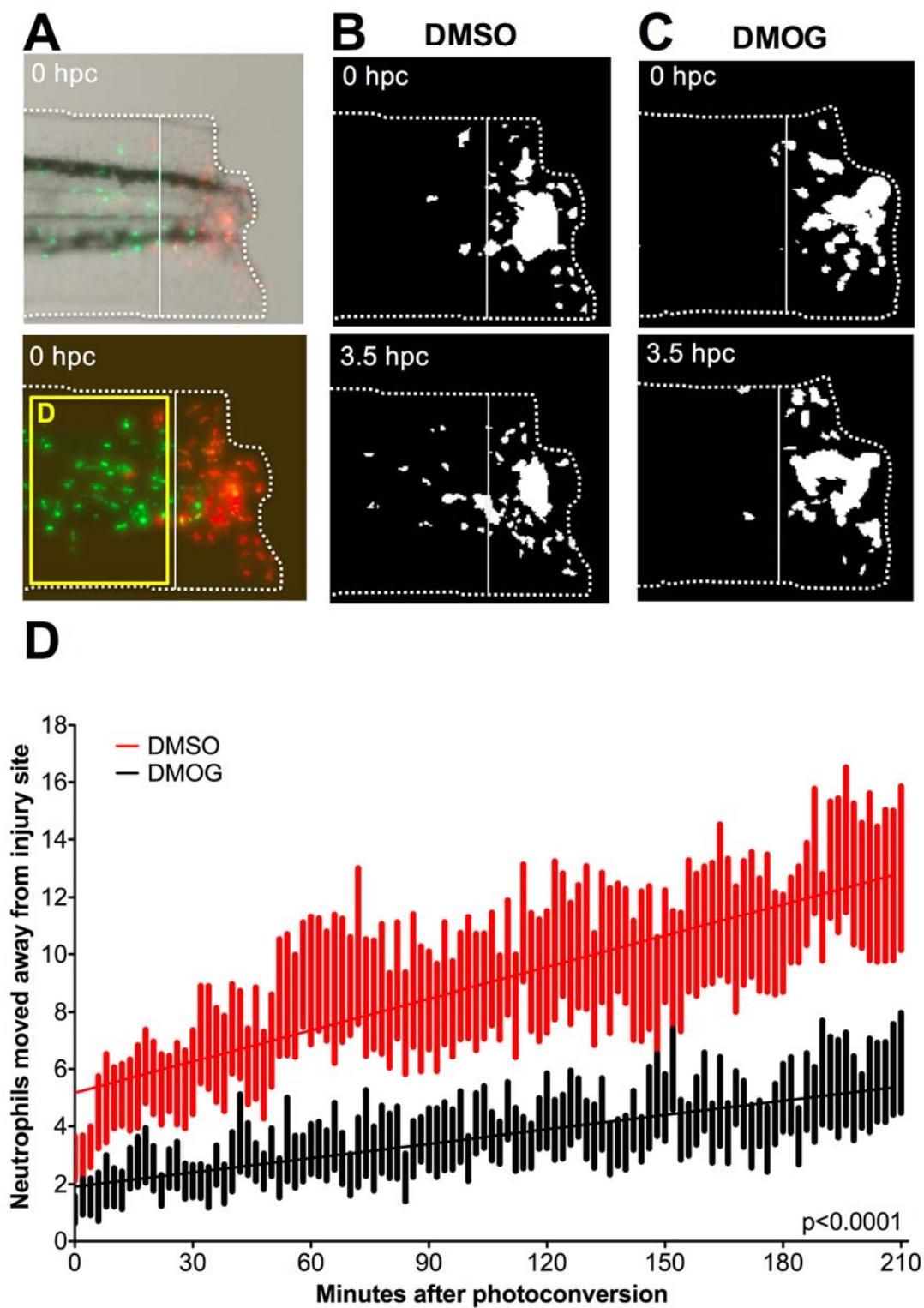
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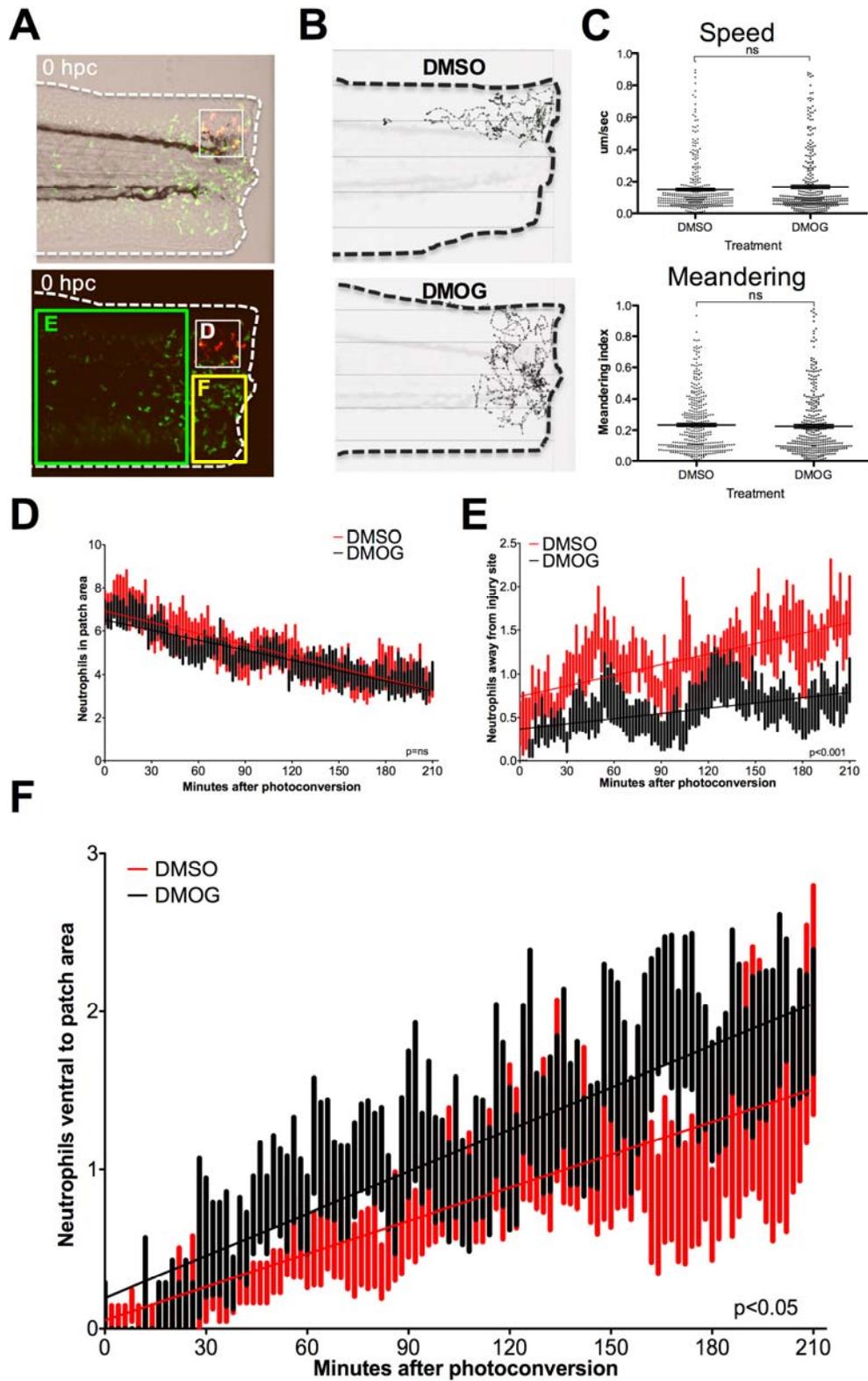
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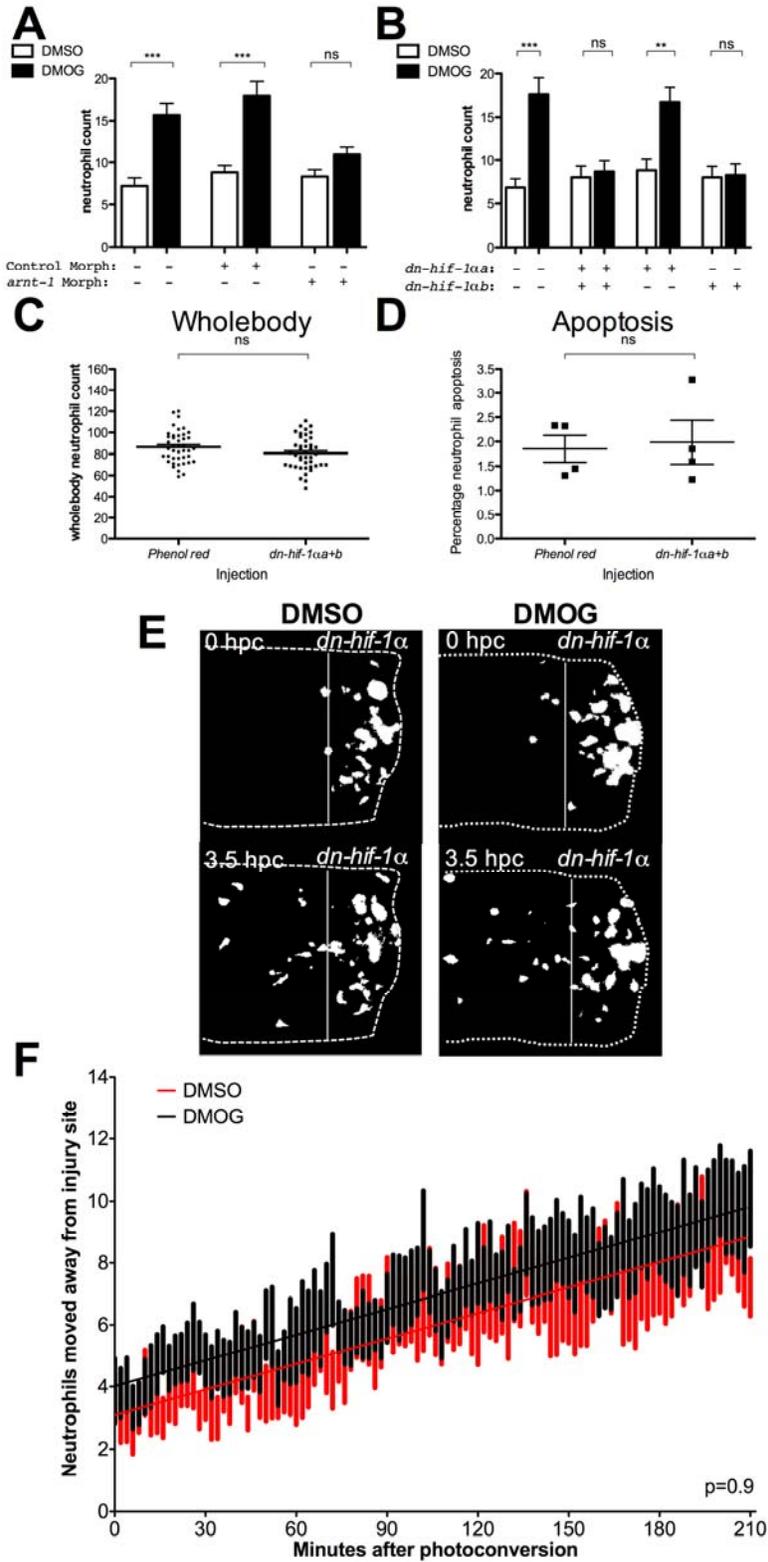
Figure 5

A

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                  :**::::::::*  ***********:*  .*******. :
Zebrafish HIF-1αA  ALTV-----AD   LDLEMLAPYIPM   HDCEVNAPVIG
Zebrafish HIF-1αB  ALTVLAPAAGD   LDLEMLAPYIPM   YDCEVNAPVQD
Fugu HIF-1α        ALTLLAPAAGD   LDLEMLAPYISM   DDCEVNAPLQG
Human HIF-1α       ALTLLAPAAGD   LDLEMLAPYIPM   YDCEVNAPIQG
Mouse HIF-1α       ALTLLAPAAGD   LDLEMLAPYIPM   YDCEVNAPIQG
Rat HIF-1α         ALTLLAPAAGD   LDLEMLAPYIPM   YDCEVNAPIQG
Chick HIF-1α       ALTVLAPAAGD   LDLEMLAPYIPM   YDCEVNAPIQG
Frog HIF-1α        SLTVLAPDAGD   LDLEMLAPYIPM   YDCEVNAPVHG
```

B

C Wholebody

D Recruitment

E Apoptosis

F

G

H

I Apoptosis

20