Non-apoptotic pioneer neutrophils initiate an endogenous swarming response in a zebrafish tissue injury model

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Abstract

Neutrophils are rapidly recruited to inflammatory sites where they are able to coordinate their migration to form clusters, a process termed neutrophil swarming. The factors which initiate neutrophil swarming are not understood, requiring the development of new *in vivo* models. Using transgenic zebrafish larvae to study neutrophil migration, we demonstrate that neutrophil swarming is conserved in zebrafish immunity, sharing essential features with mammalian systems. We identified that one pioneer neutrophil was sufficient to induce neutrophil swarming after adopting a distinctive morphology at the wound site, followed by the coordinated migration of neutrophils to form a swarm. Using a FRET reporter of neutrophil apoptosis, we demonstrate that pioneer neutrophils do not undergo caspase-3 mediated apoptosis prior to swarming. These data provide some of the first evidence of endogenous neutrophil migration patterns prior to swarming and demonstrate that the zebrafish can be used to dissect the mechanisms modulating neutrophil swarm initiation.

Introduction

Inflammation is the coordinated response of immune cells to invading pathogens or endogenous danger signals. Sterile inflammation has evolved as a physiological response to noxious stimuli including mechanical trauma, ischemia, toxins and antigens in the absence of infection[1]. Neutrophils are one of the first responders to sterile inflammation, which rapidly home to inflamed tissue within hours of injury. Within inflamed tissue, neutrophils carry out specialised functions to destroy pathogens[2] and repair damage[3], ultimately leading to the restoration of tissue homeostasis. Neutrophils are recruited to an inflammatory stimulus through a series of well-defined molecular events which lead to their extravasation from the circulation into the tissue[4–6]. During their recruitment, neutrophils are primed by pro-inflammatory stimuli including growth factors, inflammatory cytokines and chemoattractants, a process which increases responsiveness to activating agents and enhances neutrophil function[7]. Within interstitial tissues, neutrophils are capable of integrating host- and pathogen-derived environmental signals, resulting in their polarisation and migration towards the initiating inflammatory stimulus[8]. However, the precise mechanisms by which neutrophils coordinate their migration and function within the complexity of inflamed interstitial tissue remain to be understood.

Advances in intravital imaging have increased our understanding of the spatiotemporal dynamics of neutrophil migration within interstitial tissue *in vivo*[9]. Neutrophils in the interstitium coordinate their migration patterns to form clusters in several models of sterile-inflammation and infection[9–13]. The parallels between these cellular behaviours and migration patterns seen in insects has led to use of the term “swarming”. A series of sequential phases leading to neutrophil swarming have been described; the initial migration of ‘pioneer’ or ‘scouting’ neutrophils proximal to the wound site (scouting) is followed by large scale synchronised migration of neutrophils from distant regions (amplification) leading to neutrophil clustering (stabilisation) and eventually resolution[9–12]. Communication between neutrophils during swarming is complex. Many chemoattractants including lipid and proteins mediate the response, with a dominant role for the lipid leukotriene B4 (LTB4) identified *in vivo*[10,11]. LTB4 produced by early responding neutrophils amplifies neutrophil tissue responses by signal relay to more distant tissue regions[11,14]. Less is understood about the initiating signals required for neutrophil attraction during the early stages of neutrophil swarming at sites of tissue damage.

Various chemoattractants from damaged cells and pathogens are present within an inflamed tissue during the early stages of inflammation, making the functional dissection of the signals required for neutrophil swarming challenging. The initial arrest and clustering of a small

number of early-recruited pioneer neutrophils precedes large scale migration of other neutrophils, leading to cluster growth[9,11]. In the context of tissue injury, cell death within the initial neutrophil cluster has been found to correlate with an amplification of neutrophil recruitment[11] and, in the context of infection, lysis of infected cells followed by parasite egress is associated with swarm formation[12]. Based on these migration patterns, it is likely that swarming neutrophils respond to an amplified signal initiated by pioneer neutrophils. The release of death signals including extracellular NAD+ from dying pioneer neutrophils has been implicated in swarm initiation[9], although the precise nature of these signals remains to be determined.

New models are required to study the migration patterns of endogenous neutrophils *in vivo* during early swarm formation to understand the precise signalling and tissue context required for swarm initiation. The zebrafish (*Danio rerio*) is a powerful model organism in which to study neutrophil function that is used extensively to study neutrophil migration towards and away from sites of sterile inflammation[15–17]. The optical transparency of transgenic zebrafish embryos allows for the tracking of endogenous GFP labelled neutrophils to wound sites within minutes following injury[18]. In this study, we use both inflammation and infection assays to demonstrate that neutrophil swarming is conserved in zebrafish immunity, highlighting the importance of this neutrophil behaviour across evolution. We define a three-stage sequence of migration events which leads to the swarming of endogenous neutrophils within the inflamed tissue and verify that LTB4 signalling is required for amplification of neutrophil recruitment. Importantly, we show that a single pioneer neutrophil is sufficient to induce a swarming response in a significant proportion of larvae and that this neutrophil adopts a phenotype distinct from other neutrophils within the inflamed tissue. We study neutrophil swarm initiation in a transgenic reporter of neutrophil apoptosis, and confirm that swarm initiating pioneer neutrophils are not apoptotic. Finally, we have identified that endogenous pioneer neutrophils can be imaged *in situ* prior to the swarming response, making the zebrafish an excellent model to dissect the signalling pathways which mediate swarm initiation.

Results

Neutrophil swarming is conserved in the zebrafish tissue damage response

Neutrophil swarming is characterised by the highly directed and coordinated movement of neutrophils followed by accumulation and clustering at sites of infection or injury[19]. To determine whether neutrophil swarming is conserved in zebrafish immunity we studied neutrophil mobilisation to inflammatory and infectious stimuli. Neutrophil responses to inflammatory stimuli were assessed by transecting the tail-fins of 3 days post fertilisation (dpf) *mpx*:GFP larvae, and tracking neutrophil migration using fluorescence microscopy during the recruitment phase (0-6 hours post injury, Supplementary Figure 1 and as previously described[18]). Analysis of migration patterns of neutrophils recruited to the wound site identified three outcomes: 1) persistent neutrophil swarming reminiscent of neutrophil swarming reported in mammalian systems[9–12] (Figure 1A-C, Supplemental Movie 1); 2) shorter lived transient neutrophil swarms which dissipated and reformed multiple times within the imaging period (Supplemental Figure 2, Supplemental Movie 2); 3) no coordinated migration leading to swarm formation (Supplemental Movie 3). Persistent swarming was defined as the formation of clusters which grew throughout the imaging period by the coordinated migration of individual neutrophils (Figure 1C). Persistent swarms were observed from 40 minutes post injury (Supplemental Figure 3A) and remained stable for 2.17 hours ± 0.32 (Supplemental Figure 3B). In our imaging experiments (n=14 larvae from 5 experimental repeats), persistent neutrophil swarms were observed in 50% of larvae, transient swarms (persisting for <1 hour) were seen in 14% of larvae, and no evidence of swarming behaviour within the imaging period in 36% of larvae (Figure 1D). During the imaging period, two stages of neutrophil recruitment were observed: the early migration of neutrophils proximal to the wound site (approximately closer than 350μm) within minutes following injury, followed by an influx of neutrophils from more distant sites (approximately further than 350μm) from around 60 minutes post injury (Figure 1E).

In mammalian neutrophil swarming biphasic neutrophil responses are modulated by the lipid LTB4[11]. We investigated the requirement for LTB4 in neutrophil chemotaxis towards the wound site in zebrafish using the CRISPR/Cas9 system. Biosynthesis of LTB4 in zebrafish occurs through fatty acid metabolism of arachidonic acid via common intermediates, resulting in the production of LTB4 by the enzyme leukotriene A4 hydrolase (LTA4H), encoded by the gene *lta4h* [20,21]. Zebrafish have three LTB4 receptors; the high affinity *blt1* receptor and two low affinity receptors *blt2a* and *blt2b*, of which neutrophils predominantly express *blt1* (Supplemental Figure 4A-B). Using Cas9 protein with guide RNAs (crRNAs) to target *lta4h* and *blt1,* early neutrophil recruitment (3hpi) and late neutrophil (6hpi) responses to the wound

site were assessed. A crRNA targeting the pigment gene tyrosinase (*tyr*)[22] was used for control injections and to allow for visual identification of successful knockdown. Knockdown of *tyr* produces an albino phenotype in zebrafish larvae (Supplemental Figure 5A) without affecting neutrophil development or the neutrophilic inflammatory response (Supplemental Figure 5B-C). Early neutrophil recruitment to the wound site at 3hpi was similar between control (*tyr),* *blt1* and *lta4h* crRNA injected larvae (Figure 1F), suggesting that LTB4 signalling is not required for early neutrophil responses. Interestingly at 6hpi, neutrophil recruitment in control (*tyr*) crRNA injected larvae increased as anticipated (Supplemental Figure 1), however, this increase in recruitment was not seen in *blt1* and *lta4h* crRNA injected larvae, which displayed significantly lower neutrophil counts at 6hpi compared to control larvae (Figure 1F). These results are in agreement with data from mouse[11] and human neutrophils[10], supporting a role for LTB4 signalling in neutrophil recruitment at the later stages.

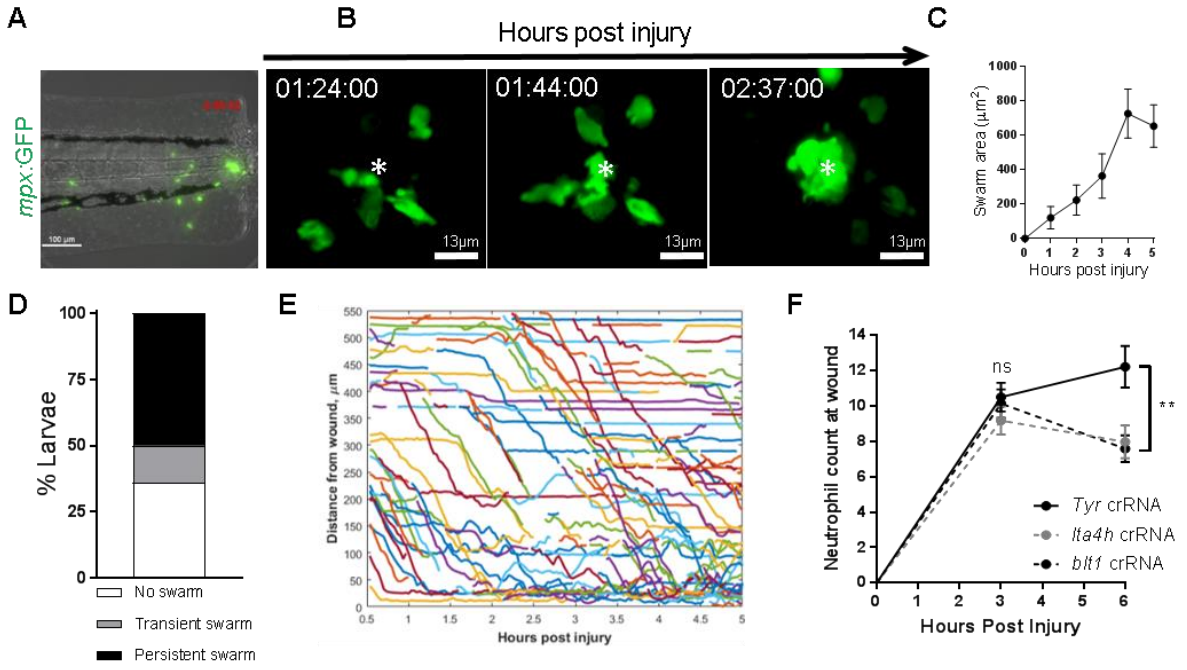

Figure 1. Neutrophil swarming is conserved in the zebrafish tissue damage response

A-C. Zebrafish neutrophils swarm at sites of tissue damage. **A**. Representative example (from 5 experimental repeats) of neutrophils swarming at the wound site following tail fin transection in 3dpf *mpx*:GFP larvae. Bright field highlights tail fin region. Time stamp shown is relative to the start of the imaging period at 30 minutes post injury and is h:mm:ss. **B**. 3D reconstruction time course illustrating neutrophils swarming at the wound site, where the swarm centre is highlighted by an asterix. Imaging was performed using a 40X objective spinning disk confocal microscope. Time stamps shown are relative to time post injury and are in hh:mm:ss. **C**. Area

of neutrophil swarms measured at the wound site during the 5 hour imaging period. Error bars shown are mean ± SEM, n=5 experimental repeats. **D**. Proportion of neutrophil swarming behaviour observed at the wound site within 5 hours following injury, n=5 experimental repeats. **E-F**. Relay signalling through LTB4 is required for neutrophil recruitment. **E**. Distance/time plot demonstrating the early recruitment of neutrophils proximal to the wound site (<350μm) followed by the later recruitment of more distant neutrophils. **F**. CRISPR/Cas9-mediated knockdown of LTB4 signalling reduces late neutrophil recruitment. Neutrophil counts at the wound site in control *tyr* crRNA injected larvae (black line), *lta4h* crRNA injected larvae (grey dotted line), and *blt1* crRNA injected larvae (black dotted line) at 3 and 6hpi. Error bars shown are mean ± SEM. Groups were analysed using an ordinary one-way ANOVA and adjusted using Tukey's multi comparison test. **p>0.008 n=45 from 3 independent experiments.

Neutrophil swarming is conserved in the zebrafish response to infection

After determining that swarming was a conserved component of the tissue damage response in zebrafish, neutrophil responses to infectious stimuli were assessed. *Staphylococcus aureus,* a gram positive bacteria which induces a neutrophil swarming response in mammalian neutrophils[23], was injected into the left otic vesicle of 2dpf *mpx*:GFP larvae. Injection of *S. aureus* induced robust neutrophil recruitment (21±2 neutrophils) to the otic vesicle at 6 hours post injury, which was not seen in larvae injected with a PBS control (1±0.3 neutrophils) (Figure 2A-B). To observe the migration patterns of zebrafish neutrophils in real time within infected otic vesicles, time-lapse imaging of neutrophil mobilisation towards *S. aureus* infection was performed from 1-hour post infection for 6 hours (Figure 2C). Neutrophils within otic vesicles infected with *S. aureus*, but not PBS, coordinated their migration to form swarms in tissue regions containing bacteria, which at 6hpi had an average volume of 48.1mm^3 (Figure 2D, Supplemental Movie 4). The identification of neutrophil swarming in response to inflammatory and infectious stimuli demonstrates that neutrophil swarming is a conserved component of zebrafish immunity. We therefore used the zebrafish model to study the early migration patterns of neutrophils prior to the swarming response.

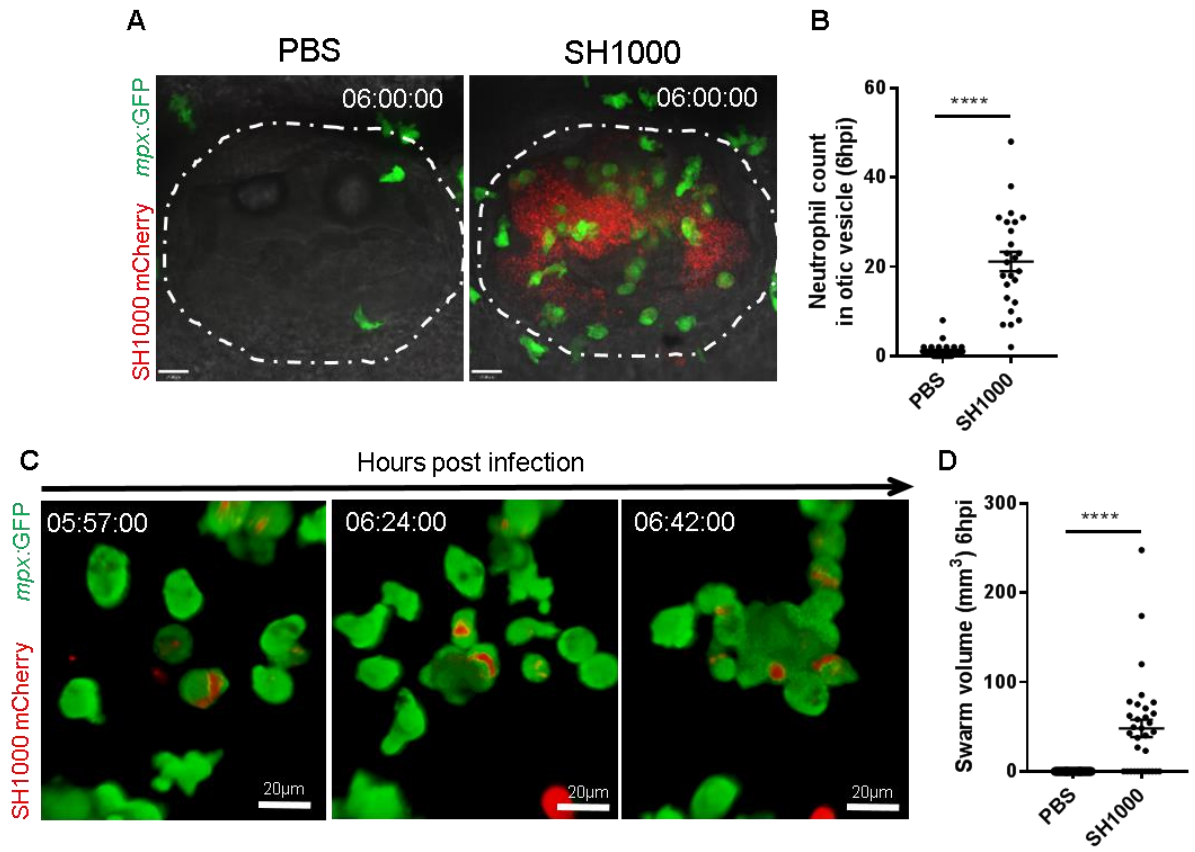

Figure 2. Zebrafish neutrophils swarm to *S. aureus* infection

A. Otic vesicles of 2dpf *mpx*:GFP larvae injected with a PBS vehicle control or 2500 cfu *S. aureus* SH1000 pMV158mCherry. Otic vesicles are highlighted by white dashed area. Time stamps shown are hh:mm relative to time post infection. **B**. Neutrophils mobilised to the otic vesicle at 6hpi. Error bars shown are mean ± SEM (n=32 larvae from 3 independent experiments). Error bars shown are mean ± SEM ****p>0.0001 from an un-paired t-test, n=32 from 3 independent repeats. **C-E**. Zebrafish neutrophils swarm at *S. aureus* infection. **C**. 3D reconstruction time course illustrating neutrophil swarming within otic vesicle of 2dpf *mpx*:GFP larvae injected with 2500 cfu S. aureus SH1000 pMV158mCherry. Imaging was performed using a 20X objective spinning disk confocal microscope. Time stamps shown are hh:mm:ss relative to time post injection. **D**. Volume of neutrophil swarms measured within otic vesicle at 6hpi. A volume of zero corresponds to no swarm observed. Error bars shown are mean ± SEM ****p>0.0001 from an un-paired t-test, n=32 from 3 independent experiments.

Neutrophil swarms are initiated by a pioneer neutrophil with distinct morphology

The factors which initiate neutrophil swarming are not well defined. Neutrophil swarms in mammals grow by large-scale migration towards 'pioneer neutrophils' in the context of both sterile inflammation and infection, which likely release additional chemoattractants to initiate the swarming response[9,11,12]. To understand whether a pioneer neutrophil is distinct to other early responding neutrophils with a specialised capability to initiate a swarm, the migration patterns of neutrophils in the time period preceding the swarming response were analysed by reverse chronological tracking of neutrophil migration to persistent swarms (Figure 3A). The presence of one individual neutrophil with a distinct morphology was identified in the tissue region which became the swarm centre in 100% of swarming events examined (Figure 3B-E). Based on its early recruitment and location at the swarm centre, this neutrophil is referred to as the pioneer neutrophil. Prior to swarming, the pioneer neutrophil remained stationary in the tissue region which became the swarm centre for on average 36 ± 7 minutes (Figure 3B). Pioneer neutrophils were rounded and non-motile, a distinct morphology which is illustrated by their higher circularity index and lower displacement compared to scouting neutrophils migrating at the wound site in the frame before swarming (Figure 3C-E). Strikingly in 100% of swarm initiation events examined, the pioneer neutrophil was the focal point of migration for swarming neutrophils, whilst non-swarming neutrophils migrated randomly within the wound region (Figure 3D-E, Supplemental Movie 5).

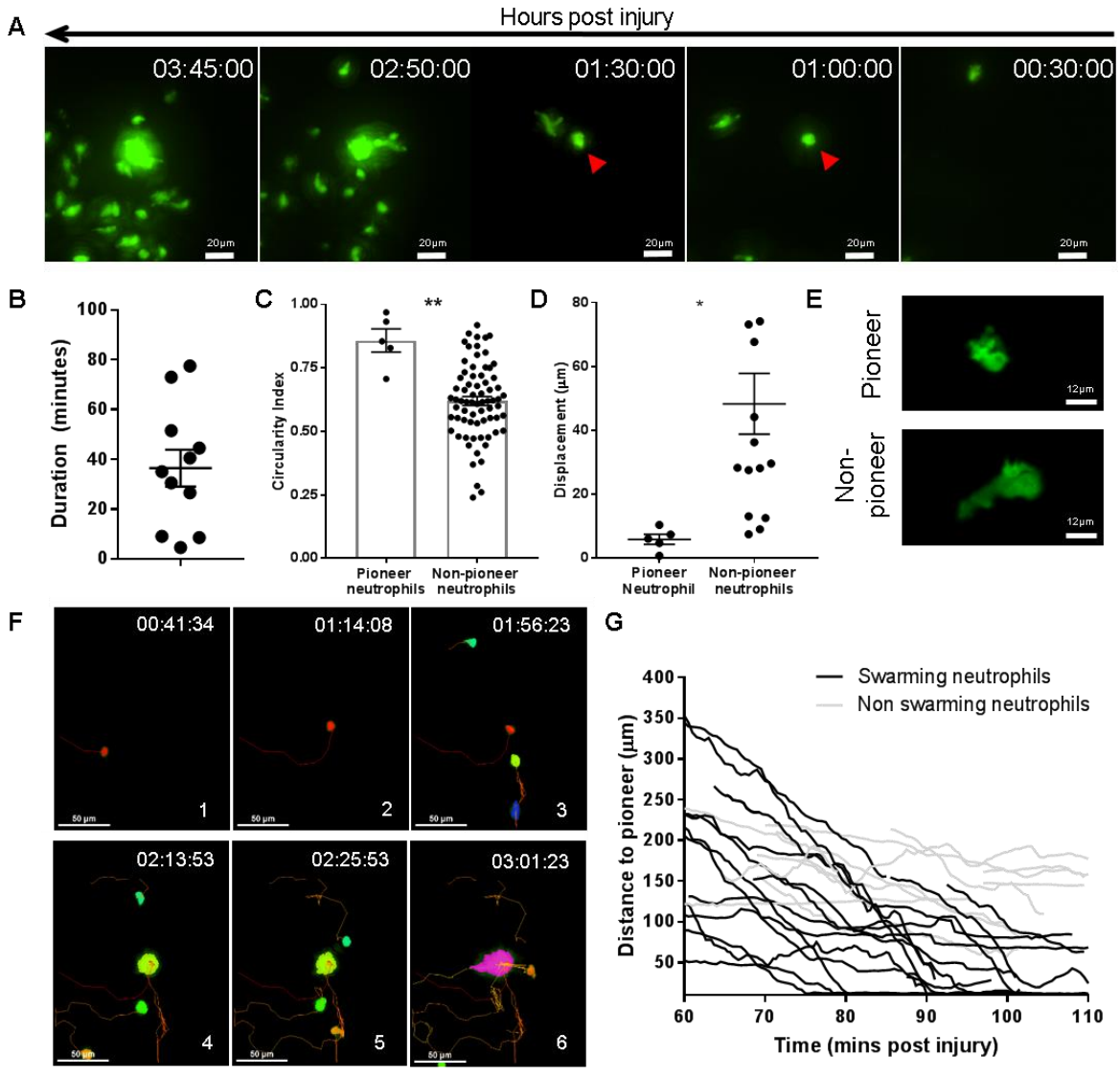

Figure 3. A pioneer neutrophil is the focal point of migration for swarming neutrophils

A. Reverse chronological time lapse sequence of a persistent neutrophil swarm where one individual neutrophil is visible in the swarm centre prior to neutrophil swarming (red arrows). Time stamps shown are hh:mm:ss relative to injury time. **B-E.** Quantification of pioneer neutrophil migration pattern in the frames preceding swarming. **B.** Duration pioneer neutrophil is observed in the swarm tissue region prior to swarming. **C-D.** The circularity index and displacement of pioneer neutrophils and scouting neutrophils migrating at the wound site in the same time period (n=5, unpaired t-test where * $p<0.05$ and ** $p<0.01$). **E.** Representative image (from 5 experiments) of pioneer and non-pioneer neutrophils. **F.** Chronological time lapse sequence of swarming neutrophil tracks. The migration of a pioneer neutrophil (red) to the wound site is observed (frames 1-2) followed by the directed migration of swarming neutrophils towards the pioneer, which is the focal point for migration (frames 3-5). The result

of migration is the aggregation of neutrophils to form swarms (frame 6). Tracks are coloured by time where red corresponds to early and yellow corresponds to late arriving neutrophils. **G.** Distance-time plot (DTP) of individual cell migration paths of swarming neutrophils (black tracks) and non-swarming neutrophils at the wound site in the same time period (grey tracks). Tracks are relative to pioneer neutrophil position; swarming neutrophils migrate to the pioneer neutrophil whilst non-swarming neutrophils do not (n= 4 independent experiments).

Neutrophil swarming responses to tissue damage occur in three sequential stages

To determine the relationship between the pioneer neutrophil and swarm initiation, neutrophil migration in the tail-fin at the entire population level was studied. Although there was variation from fish-to-fish in timing, all swarms formed by: 1) the early recruitment of neutrophils to the inflammatory site (scouting), 2) the behavioural change of a pioneer neutrophil at the wound site (initiation), followed by 3) the directed migration of neutrophils to the pioneer to form swarms (aggregation) (Figure 4, Supplemental Movie 6). Within minutes of injury, neutrophils began directed migration to the wound site (Figure 4A). This early scouting of neutrophils lasted on average 88 ± 24 minutes and is consistent with reports in zebrafish[18] and mammalian systems which describe the recruitment of neutrophils close to the inflammatory site in response to chemoattractant gradients[9]. Swarm initiation began when the pioneer neutrophil adopted its rounded, non-motile morphology having arrived at the wound site during the scouting phase (Figure 4B) and ended when the first neutrophil joined the swarm (on average 36 ± 7 minutes). During the aggregation phase, swarms developed through the directed migration of neutrophils, which lasted on average 183 ± 25, or until the end of the imaging period (Figure 4C). As proof of concept, a non-biased approach was used to study pioneer neutrophil migration. Pioneer neutrophils were tracked during the time period preceding the start of swarming, where a change in pioneer neutrophil behaviour was observed, correlating with the scouting and initiation phases (Figure 4D-E). These stages provide consistent phases with which to study pioneer neutrophil behaviour between larvae and are comparable to the swarm stages reported in mammals[9,10].

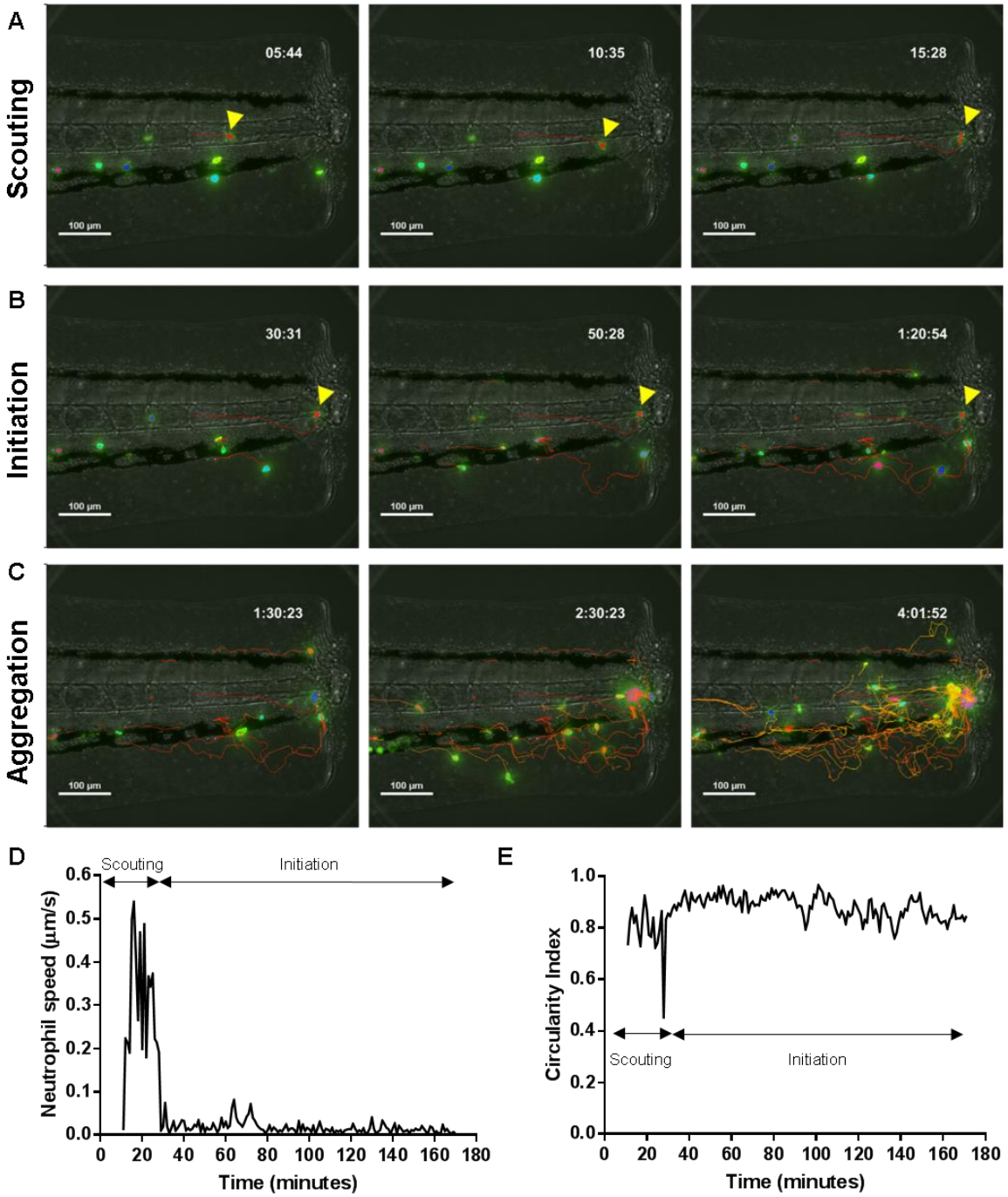

Figure 4. Neutrophil swarming responses to tissue damage occur in three sequential stages

Representative time-lapse sequence (from at least 20 independent observations) showing coordination of neutrophils to form swarms within the inflamed tail-fin. **A.** Stage 1; scouting. The recruitment of neutrophils proximal to the wound site occurs within minutes following tail fin transection. **B.** Stage 2; initiation. Pioneer neutrophils adopt a rounded, non-motile morphology at the wound site. **C.** Stage 3; aggregation. Neutrophils direct their migration to

the pioneer neutrophil which becomes to focal point for migration, resulting in swarm growth and neutrophil aggregation. Tracks are coloured by time where red corresponds to early and yellow corresponds to late arriving neutrophils. Time stamps are h:mm:ss relative to the start of imaging period at 30 minutes post injury. **D-E.** Non-biased approach to observe pioneer neutrophil behavioural change. **D.** Representative example of pioneer neutrophil migration speed prior to the swarming response. **E.** Representative example of pioneer neutrophil circularity index prior to the swarming response. Arrows correspond to the scouting and initiation phases. Data shown (D-E) corresponds to the pioneer neutrophil illustrated in the time course sequence (A-C).

Pioneer neutrophils adopt a distinct morphology at the wound site

We next investigated whether the morphology observed in pioneer neutrophils prior to swarming was distinct, or common, to all neutrophils upon arrival at the wound site. Tracks of neutrophils migrating to the wound site during the scouting and the initiation phases were extracted (Figure 5A-B) and parameters which describe neutrophil motility including speed, displacement and meandering index were analysed[15,16]. The speed, displacement and meandering index of pioneer neutrophils were significantly reduced in the initiation phase when compared to the scouting phase, whilst neutrophils migrating to the wound site within the same tissue region did not display this behavioural change (Figure 5C-E). These data demonstrate that pioneer neutrophils display a distinct morphology at the wound site prior to swarm formation, which is not seen in scouting neutrophils responding to chemoattractants produced at the wound edge. Taken together, these findings suggest that within the complexity of the inflamed tail-fin, specific guidance cues are produced from a single pioneer neutrophil which enables neutrophils to coordinate their migration to form swarms.

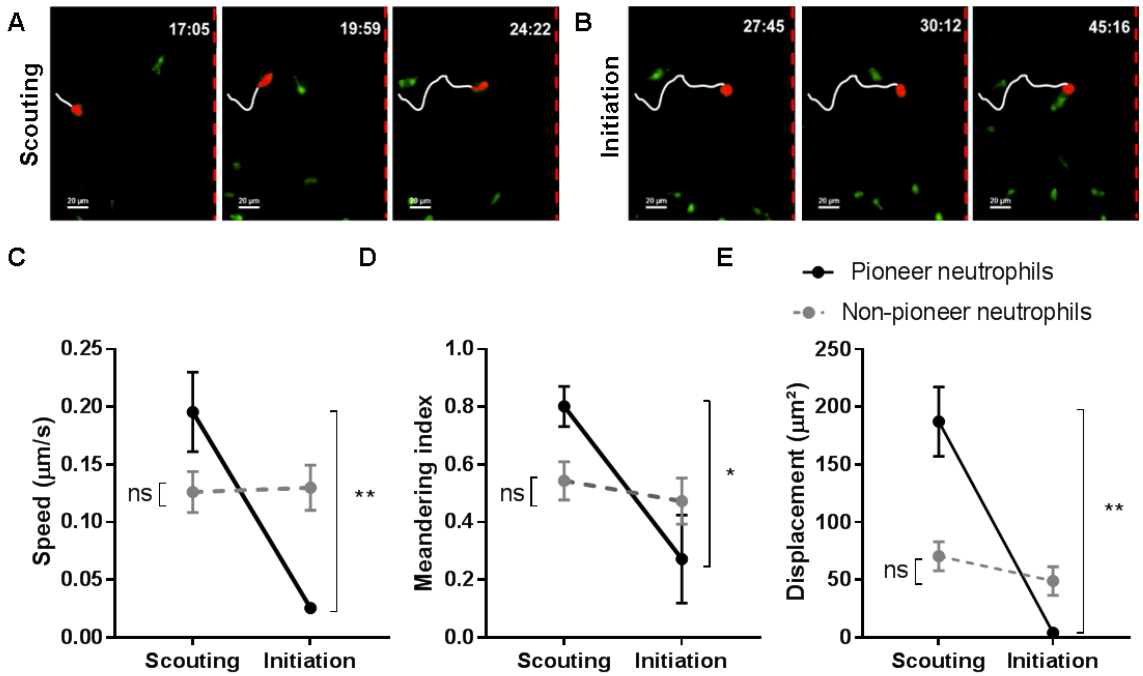

Figure 5. Pioneer neutrophils adopt a distinct morphology at the wound site

A-B. Representative example of tracking of pioneer neutrophil (highlighted) alongside neutrophils migrating within the same time period during the scouting (**A**) and initiation (**B**) stages. Wound edge is highlighted by red dashes. **C-E** Parameters to describe neutrophil migration were measured. **C.** Neutrophil speed. **D.** Neutrophil displacement (the linear distance each neutrophil travelled). **E.** Neutrophil meandering index (the displacement divided by the total length of the neutrophil track). Error bars are mean ± SEM. Groups were analysed using a paired t-test *p<0.05 **p<0.01, n=5 independent experiments.

Pioneer neutrophils are not apoptotic prior to swarming

Cell death signalling has been implicated in neutrophil swarm initiation[9,11], although the precise signals and mode of cell death remain to be determined. The rounded, non-motile morphology of pioneer neutrophils is characteristic of an apoptotic neutrophil phenotype previously reported[24]. Furthermore apoptotic cells secrete “find-me” and “eat-me” signals to promote the attraction of phagocytes for successful removal of apoptotic bodies[25], therefore we hypothesised that cell death signals released by apoptotic neutrophils could initiate the swarming response. Neutrophil apoptosis can be studied using the transgenic *Tg(mpx:CFP-DEVD-YFP)sh237* [24] zebrafish line (known as *mpx:*FRET*)* which expresses a genetically encoded Förster resonance energy transfer (FRET)[26] biosensor consisting of a caspase-3 cleavable DEVD sequence flanked by a CFP/YFP pair, under the neutrophil-specific *mpx* promoter. A loss of FRET signal in this system provides a read out of apoptosis specifically in

neutrophils *in vivo* in real time. Analysis of pioneer neutrophils prior to swarming within the tail-fin (Figure 6A) identified that despite the rounded, non-motile morphology observed in pioneer neutrophils, a FRET signal was present during both the scouting and initiation phases in all imaging studies where swarming was observed (Figure 6B, Supplemental Movie 7, n=6 neutrophils from 5 experimental repeats). Furthermore, when an apoptotic event was detected within the inflamed tail-fin, it was not followed by a neutrophil swarming response (Figure 6C, Supplemental Movie 8, n=2 neutrophils from 5 experimental repeats). These findings suggest that neutrophil apoptosis does not initiate neutrophil swarming, and that despite their morphology, pioneer neutrophils are not undergoing caspase-3 dependent apoptosis. Further study of neutrophil behaviour in this model using transgenic reporters of cell death will enable the dissection of the molecular cues which regulate swarm initiation.

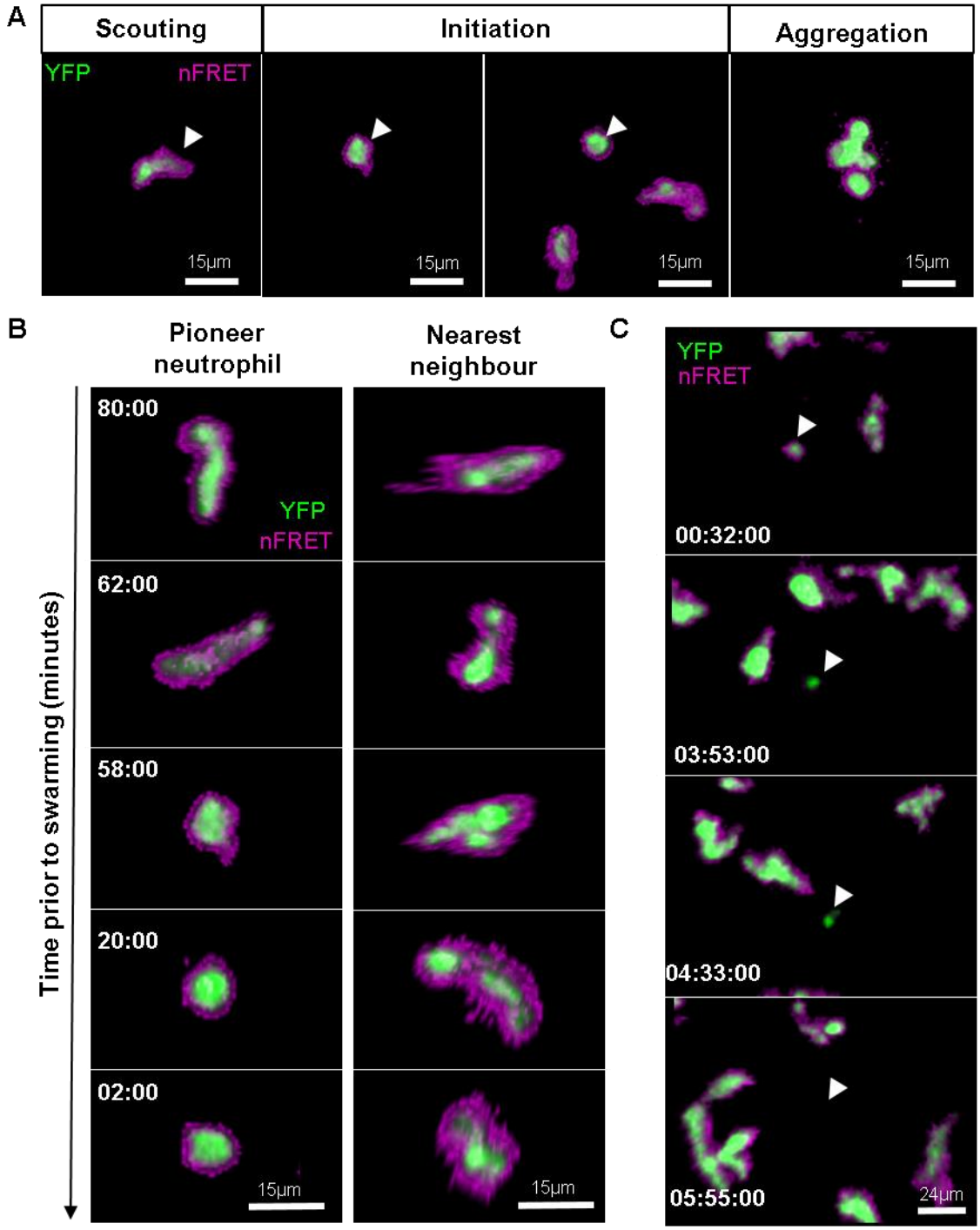

Figure 6. Pioneer neutrophils are not apoptotic prior to swarming

3dpf *mpx*:FRET larvae were injured and time lapse imaging was performed from 30 minutes post injury for 6 hours. Neutrophil signal from the acceptor (green) and nFRET (magenta) are shown to illustrate neutrophil apoptosis. **A.** Representative time lapse sequence (from six independent observations) illustrating a pioneer neutrophil at the wound site (white arrow) during the scouting and initiation phases prior to swarming in *mpx*:FRET larvae. **B.** Pioneer

neutrophils are not apoptotic. Representative example (from six independent observations) of a pioneer neutrophil and its nearest neighbour in the frames preceding neutrophil swarming. The initiation stage is observed 58 minutes prior to swarming (rounded pioneer neutrophil). nFRET signal is intact at all stages of migration prior to swarming in both the pioneer and nearest-neighbour non-pioneer neutrophil. Time stamps are mm:ss relative to the swarm start time. **C.** Apoptotic neutrophils do not initiate swarming. Representative example of neutrophil apoptosis (from 2 independent observations) at the wound site demonstrated by loss of FRET signal around 4 hours post injury, followed by the absence of neutrophil cluster formation in the same tissue region by the end of the imaging period. Time stamp is relative to injury time and is hh:mm:ss.

Discussion

In this study we investigated the migration patterns of neutrophils in the context of inflammation and infection and identified that neutrophil swarming behaviour is conserved in zebrafish immunity. We focused on neutrophil swarming in the context of injury-induced inflammation, where the zebrafish model allowed us to track endogenous neutrophils in a physiologically relevant tissue damage model *in vivo*. Here, we identified three stages of migration leading to swarming, where the altered behaviour of one individual neutrophil was sufficient to induce a swarming response in a significant proportion of larvae.

We utilised the optical transparency of zebrafish larvae to precisely track neutrophils over time, providing some of the first *in vivo* characterisation of endogenous neutrophil migration patterns in response to tissue injury since the identification of neutrophil swarming. Currently our knowledge of swarm initiation is based on mouse models using transfer of exogenous neutrophils into the ear dermis followed by focal laser injury. These studies adopt the transfer of neutrophils from a variety of donor backgrounds, enabling the screening of many candidate signalling pathways, and have thus been invaluable for understanding the molecular control of swarming at the later stages[11,12]. However, transplanted neutrophils bypass the early recruitment, priming and activation stages with which endogenous neutrophils undergo as they egress from the bloodstream, making the early stages of swarming difficult to dissect. The zebrafish model circumvents these limitations, enabling the visualisation of endogenous neutrophil migration from their physiological tissue niche.

Neutrophil responses to tissue injury are thought to occur in phases: the early recruitment of neutrophils (referred to as ‘scouting’) is followed by the large scale synchronised migration of neutrophils from distant regions (amplification), resulting in large scale tissue infiltration from the bloodstream[9–11]. We demonstrate in our model that neutrophil response to tail fin inflammation is bi-phasic; neutrophils proximal to the wound edge are recruited within minutes

following injury, whilst neutrophils from further away recruited between 2-6 hours following injury. This is reminiscent of the bi-phasic neutrophil response to focal tissue damage described in mice[9,11]. The time period of 6 hours required for recruitment in our system likely reflects the difference in assays adopted between our study and mammalian studies; the propagation of signals through the whole animal is required for neutrophil migration from tissue niches in zebrafish whilst in mammals, neutrophils are injected proximal to the damage site. In mammals, early neutrophil recruitment is modulated by signals released from damaged or necrotic cells which are likely to be damage-associated molecular patterns (DAMPs). These DAMPs include DNA, histones, interleukin-1α (IL-1α), N-formyl peptides and Adenosine triphosphate (ATP) (reviewed in [27]). These signals can be short-lived[17], so the production of longer term signals is required for sustained neutrophil recruitment[28]. LTB4 is a signal-relay molecule which acts over long distances to promote neutrophil recruitment to formyl peptides released from the centre of inflammatory sites[14]. We inhibited LTB4 signalling by targeting the LTA4H enzyme or the LTB4 receptor using CRISPR/Cas9 and found neutrophil responses were impaired only in the later stages of recruitment (3-6hpi). In zebrafish the CRISPR/Cas9 system is highly efficient, resulting in biallelic gene disruption in F0 zebrafish embryos which allows for direct phenotypic analysis of injected animals[22]. Based on the expression of lta4h and blt1 on neutrophils we propose that disruption of these genes will affect neutrophil production and detection of LTB4. Our data agree with those from mice studies, showing that the recruitment of neutrophils deficient in the LTB4 receptor is impaired only in neutrophils distant to the focal tissue injury, whilst those proximal to the wound site are recruited like wildtype neutrophils[11]. Evidence from zebrafish studies have demonstrated that following tail-fin transection, gradients of neutrophil chemoattractant signals are produced within minutes, which extend up to 200µm into the tail fin epithelium as a concentration gradient[17]. Hydrogen peroxide and chemokines such as CXCL8 are known to be important in neutrophil recruitment in zebrafish larvae[17,29]. We therefore propose that the initial recruitment of neutrophils to the wound site is dependent on the release of these chemoattractants, whilst signalling through LTB4 is required to attract neutrophils at later stages from more distant tissue regions.

Factors which are likely to influence the swarm outcome include the size of the initiating tissue damage, the presence of pathogens in the tissue, induction of secondary cell death and the number of neutrophils initially recruited[19]. Linear tail-fin transection avoids creating a focal source of neutrophil chemoattractants, migration towards which could mimic a swarming response without the requirement for neutrophil-neutrophil signalling. Based on our findings we propose that the formation of neutrophil swarms within a complex environment of diffusing chemotactic gradients at the wound site would be dependent on intercellular signalling between neutrophils. The zebrafish model therefore is a truly physiological system in which to

study the early events that determine the outcome of neutrophil swarming. Within the inflamed tail-fin tissue, we found that neutrophil swarms developed around one individual neutrophil. The single-cell resolution achieved in our study enabled us to make the striking observation that this pioneer neutrophil adopted a distinct morphology at the wound site prior to swarming. Other groups have found that within inflamed or infected interstitial tissue, the initial arrest of a small number of 'pioneer' or 'scouting' neutrophils precedes a later influx of neutrophil migration[11,12]. In these studies, it is unclear whether pioneer neutrophils are simply early responding 'scouting' neutrophils, or if they have a specialised capacity for swarm initiation. Based on our observations, we distinguished pioneer neutrophils from other scouting neutrophils and propose that pioneer neutrophils have specialised functions required for swarm initiation, whilst scouting neutrophils are simply early responders to chemoattractants produced by damaged cells or pathogens at the inflammatory site.

Neutrophil swarming at the wound site in our system occurred in three distinct stages, which are comparable to the sequential phases described in the swarming of neutrophils in intravenous/ intradermal transfer models in mice[9,30]. Ng et al. describe a three phase cascade of events to describe neutrophil migration towards laser induced or sterile needle induced tissue injury[9], which was further adapted into a five step attraction model[30]. In our linear tail-fin model we found that migration patterns leading to swarming shares features of both models. During the scouting phase we observed the chemotactic movement of neutrophils proximal to the wound site, sharing features with the scouting observed in mice and human neutrophils[9,10]. The initial recruitment of neutrophils proximal to the wound site is common to inflammation induced by infection or tissue injury, where these neutrophil 'scouts' are likely responding to gradients of chemoattractants produced by damaged cells or pathogens[9,10]. One pioneer neutrophil within the inflamed tail-fin was sufficient to initiate swarming in zebrafish larvae, sharing function with the pioneer neutrophils essential for swarm initiation in mice[30]. Due to the relatively few number of neutrophils present in zebrafish larvae (~300) in comparison with the thousands ($2–5x10^4$)[9] injected into the mouse ear, we propose that signals generated from just one pioneer neutrophil are sufficient to drive a swarming response in our system. The pioneer behavioural change was observed during the swarm 'initiation' phase. The initiation phase encapsulates the time period in which the pioneer neutrophil adopted a rounded non-motile morphology at the wound site, until the first swarming neutrophil makes contact. We propose that this stage is comparable to stage 2 'swarm amplification by cell death' reported in mice[30]. Following its arrest, we observed directed migration of neutrophils towards the pioneer during the aggregation phase which lasted until the end of the imaging period in many larvae. The aggregation phase corresponds to the aggregation phase reported by Lammermann[30], and the cluster 'stabilisation' phase described by Ng[9]. The

parallels between the migration patterns leading swarming in zebrafish with those reported in mice[9,30] and humans[10] suggests that the initiation of swarming is conserved between species. Furthermore, these stages provide measurable time periods for the comparison of neutrophil behaviour in future experiments to determine the signals released by pioneer neutrophils.

Based on the morphology of pioneer neutrophils we investigated whether neutrophil apoptosis generated the chemoattractant signals required to initiate a swarming response within the tail-fin. Interestingly caspase-3 was intact during the swarm initiation phase, indicating that swarm initiating pioneer neutrophils were not undergoing neutrophil apoptosis prior to swarming. Due to the requirement for live imaging to study pioneer neutrophils prior to swarming, it was not technically possible to confirm our results using staining assays such as TUNEL. However, other studies have found that results using the *mpx*:FRET transgenic line recapitulate TUNEL staining[24], suggesting this is a reliable way to read out neutrophil apoptosis. The successful application of the FRET transgenic reporter line to study apoptosis during swarm initiation identifies that it is possible to study neutrophil swarm initiation in different reporter lines, which will be useful in future to investigate other cell death signals important for swarm initiation. It has been proposed that within the interstitium, neutrophils must prioritise 'superior' chemoattractant gradients in order to home towards sites of necrosis or infection, sparing the surrounding viable tissue[27]. The presence of pathogens in the tissue or induction of secondary cell death are factors which influence neutrophil swarming[19]. Neutrophils integrate and prioritise chemoattractive cues where there appears to exist a hierarchical preference for bacterial derived end-point chemoattractants, such as formyl peptides, over endogenous intermediary gradients such as CXCL8 and LTB4[31,32]. The behavioural change observed in the swarm initiating pioneer, but not scouting neutrophils, suggests that pioneer neutrophils encounter a tissue environment which induces their behavioural change. Our findings suggest that pioneer neutrophils arrive at the wound site where they release chemoattractant molecules which are prioritised by a population of neutrophils, resulting in coordinated migration within the inflamed tissue region to form swarms (summarised in supplemental figure 7). The pioneer signals could be derived through the activation of a cell death pathway, the presence of a pathogen in the inflamed tissue, or a combination of both. Lysis of neutrophils corresponds with rapid migration of neutrophils within seconds in mice, suggesting a role for 'necrotaxis' in meditating neutrophil migration[12]. Furthermore, cell death and subsequent DNA release is observed at sites of alum injection associated with neutrophil swarming in mice, suggesting that cell death by NETosis may be important in swarming[33]. Pioneer neutrophils in our study appeared to be viable prior to swarming, suggesting that lysis is not an initiating factor in this model, although a programmed cell death process such as apoptosis or NETosis

473 is possible. Given that neutrophils respond to a multitude of chemoattractant signals, it is likely
474 that the release of multiple signals could be responsible for swarming behaviour.

475 Our findings in this study suggest that the zebrafish model of neutrophil swarming will be
476 extremely useful in dissecting the signalling which modulates early stages of neutrophil
477 swarming. Measuring and inhibiting intercellular signalling molecules is technically challenging
478 *in vivo*, posing significant barriers to dissecting the modulators of swarming at different stages.
479 Further elucidation of the nature of the pioneer neutrophil will require the development of new
480 technologies for the read-out of cell death phenomena and cytokine production *in vivo*. Using
481 a combination of transgenic zebrafish lines expressing cell-death read outs in neutrophils and
482 cell viability dyes, we will investigate pioneer neutrophil death as a potential mechanism for
483 swarm-initiation. Furthermore, the development of CRISPR interference technology and
484 neutrophil specific drivers of dead Cas9 by our group will enable us to inhibit genes of interest
485 in neutrophils specifically for loss-of-function studies, to identify the signals important in early
486 swarm initiation. These techniques will bypass limitations of other systems to allow the
487 dissection of early-swarming signals *in vivo.*

488 Understanding why swarms are initiated will be important for understanding the signals which
489 control the coordination of neutrophil migration within interstitial tissues. Our findings identify
490 that neutrophil swarm initiation at sites of tissue damage requires signals from one pioneer
491 neutrophil and that these signals can be dissected in future using the zebrafish model.

492

Materials and methods

Zebrafish husbandry and ethics

To study neutrophils during inflammation *Tg(mpx:EGFP)i114* (known as *mpx*:GFP) zebrafish larvae were in-crossed. All zebrafish were raised in the Bateson Centre at the University of Sheffield in UK Home Office approved aquaria and maintained following standard protocols[34]. Tanks were maintained at 28°C with a continuous re-circulating water supply and a daily light/dark cycle of 14/10 hours. All procedures were performed on embryos less than 5.2 dpf which were therefore outside of the Animals (Scientific Procedures) Act, to standards set by the UK Home Office**.**

Tail-fin transection assay

To induce an inflammatory response, zebrafish larvae at 2 or 3dpf were anaesthetised in Tricaine (0.168 mg/ml; Sigma-Aldrich) in E3 media and visualised under a dissecting microscope. Tail-fins were transected consistently using a scalpel blade (5mm depth, WPI) by slicing immediately posterior to the circulatory loop, ensuring the circulatory loop remained intact as previously described[18].

Widefield microscopy of transgenic larvae

For neutrophil tracking experiments, injured 3dpf *mpx*:GFP larvae were mounted in a 1% low melting point agarose solution (Sigma-Aldrich) containing 0.168 mg/ml tricaine immediately following tail fin transection. Agarose was covered with 500µl of a clear E3 solution containing 0.168 mg/ml tricaine to prevent dehydration. Time lapse imaging was performed from 0.5-5 hours post injury with acquisition every 30 seconds using 10 z-planes were captured per larvae over a focal range of 100µm using an Andor Zyla 5 camera (Nikon) and a GFP specific filter with excitation at 488nm. Maximum intensity projections were generated by NIS elements (Nikon) to visualise all 10 z-planes.

Confocal microscopy of transgenic larvae

For visualising neutrophil swarming at high magnification, larvae were mounted in a 1% low melting point agarose solution (Sigma-Aldrich) containing 0.168 mg/ml tricaine for imaging immediately after tail transection. Agarose was covered with 2000µl of clear E3 solution containing 0.168 mg/ml tricaine to prevent dehydration. Imaging was performed from 30 minutes post injury using a 20x or 40x objective on an UltraVIEWVoX spinning disc confocal laser imaging system (Perkin Elmer). Fluorescence for GFP was acquired using an excitation

wavelength of 488nm and emission was detected at 510nm, and fluorescence for mCherry was acquired using 525nm emission and detected at 640nm. Images were processed using Volocity™ software.

Tracking assays

Tracking of GFP labelled neutrophils was performed using NIS Elements (Version 4.3) with an additional NIS elements tracking module. A binary layer was added to maximum intensity projections to detect objects. Objects were smoothed, cleaned and separated to improve accuracy. A size restriction was applied where necessary to exclude small and large objects which did not correspond to individual neutrophils.

Distance-time plots

For wound plots the distances from the wound were obtained by processing neutrophil tracks under the assumption that the tail fin wound is a straight line parallel to the x-axis of the greyscale image. Neutrophil tracking data was extracted from NIS elements and imported into MatLab software. For distance to pioneer plots the pioneer centre was set as a reference point and tracking was performed to determine neutrophil distance to the reference point. Tracks were extracted from NIS elements and plotted manually using GraphPad Prism version 7.0.

Neutrophil specific expression of zebrafish genes

Gene expression was assessed using an RNA sequencing database from FACS sorted GFP positive cells from 5dpf zebrafish[35] (data deposited on GEO under accession number GSE78954). RPKM values for genes of interest were extracted. For single cell analysis gene expression values were extracted from the BASiCz (Blood atlas of single cells in zebrafish) cloud repository[36]. Cells of the neutrophil lineage were analysed for gene expression based of LTB4 signalling components.

CRISPR/Cas9 reagents

Synthetic SygRNA® (crRNA and tracrRNA) (Merck) in combination with cas9 nuclease protein (Merck) was used for gene editing. Transactivating RNAs (tracrRNA) and gene specific CRISPR RNAs (crRNA) were resuspended to a concentration of 20µM in nuclease free water containing 10mM Tris-hcl ph8. SygRNA® complexes were assembled on ice immediately before use using a 1:1:1 ratio of crRNA:tracrRNA:Cas9 protein. Gene-specific crRNAs to target the ATG region of *blt1* and *lta4h* were designed using the online tool CHOPCHOP (http://chopchop.cbu.uib.no/). We used the following crRNA sequences targeting the ATG

region of both genes, where the PAM site is indicated in brackets: *lta4h:* AGGGTCTGAAACTGGAGTCA(TGG), *blt1:* CAATGCCAATCTGATGGGAC(AGG).

Microinjection of SygRNA® into embryos

A 1nl drop of SygRNA®:Cas9 protein complex was injected into *mpx*:GFP embryos at the one-cell stage. Embryos were collected at the one cell stage and injected using non-filament glass capillary needles (Kwik-Fil™ Borosilicate Glass Capillaries, World Precision Instruments (WPI), Herts, UK). RNA was prepared in sterile Eppendorf tubes. A graticule was used to measure 0.5nl droplet sizes to allow for consistency of injections. Injections were performed under a dissecting microscope attached to a microinjection rig (WPI) and a final volume of 1nl was injected.

Genotyping and melting curve analysis

Site-specific mutations were detected using High Resolution Melting (HRM) Analysis which can reliably detect CRISPR/Cas9 induced indels in embryos [37,38]. Genomic DNA extraction was performed on larvae at 2dpf. Larvae were placed individually in 0.2ml PCR tubes in 90µl 50mM NaOH and boiled at 95° for 20 minutes. 10µl Tris-HCL ph8 was added as a reaction buffer and mixed thoroughly. Gene specific primers were designed using the Primer 3 web tool (http://primer3.ut.ee/). Sequences were as follows *lta4h_fw:* CGTGTAGGTTAAAATCCATTCGCA *lta4h_rev:* GAGAGCGAGGAGAAGGAGCT *blt1_fw:* GTCTTCTCTGGACCACCTGC *blt1_rev:* ACACAAAAGCGATAACCAGGA. HRM analysis (Bio-Rad) PCR reactions were made with 5µl Sybr™ Green master mix (Thermo Fisher), 0.5µl of each primer (10µM), 1µl gDNA and 3µl water to make a final reaction volume of 10µl. PCR reactions were performed in a LightCycler instrument (Bio-Rad) using 96-well plates. The two-step reaction protocol was as follows: 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 seconds, 58° for 30 seconds, 72° for 20 seconds. The second stage of the protocol was 95 °C for 30 seconds, 60 °C for 60 seconds, 65 °C for 10 seconds. The temperature then increased by 0.02 °C/s until 95 °C for 10 seconds. Melt curves were analysed using Bio-Rad software version 1.2. Succesful detection of CRISPR/Cas9 induced indels is illustrated in supplemental figure 6. Mutagenesis frequencies of 91% and 88% were detected for *lta4h* and *blt1* respectively.

Staphylococcus aureus preparation

Staphylococcus aureus strain SH1000 pMV158mCherry was used for all experiments[39]. An overnight bacterial culture was prepared by growing 1cfu of SH1000 pMV158mCherry in

10mLs of bovine heart medium (BHI) (Sigma Aldrich lot number 53286) and 10µLs of 5mg/mL tetracycline (Sigma-Aldrich) for 16-18 hours at 37°C. 500µLs of this overnight culture was then aliquoted into 50mLs of BHI (Sigma Aldrich, 53286) infused with 50µLs of 5mg/mL tetracycline (Sigma Aldrich) and grown until an optical density at 600nm of 0.5 was obtained. This culture was pelleted and resuspended in PBS (pH 7.4) (Fisher Scientific lot number 1282 1680) to a concentration of 2500cfu per nL.

Otic vesicle injection

2500cfu of Sh1000 pMV158mCherry was injected into the left otic vesicle of 2dpf *Tg(mpx:GFP)i114* larvae. A graticule was used to measure 0.5nl droplet sizes to allow for consistency of injections. Injections were performed under a dissecting microscope attached to a microinjection rig (WPI) and a final volume of 1nl was injected. For analysis of swarm volumes larvae were fixed in 4% paraformaldehyde in PBS and imaged using a spinning disk confocal microscope.

Förster resonance energy transfer imaging of neutrophil apoptosis

To visualise apoptotic events in the context of neutrophil swarming, 3dpf *Tg(mpx:CFP-DEVD-YFP)sh237* (known as *mpx*:FRET) were injured and mounted in a 1% agarose solution containing 0.168 mg/ml tricaine and covered with 500µl of a clear E3 solution containing tricaine to prevent dehydration.

FRET imaging was performed from 30 minutes post injury for 5 hours using a 20x objective lens on an UltraVIEWVoX spinning disc confocal laser imaging system (Perkin Elmer) with acquisition every 2 minutes. 10 z-planes were captured per larvae over a focal range of 100µm using the following filters: a donor CFP channel (440nm for excitation, 485nm for detection), an acceptor YFP channel (514nm for excitation and 587nm for detection), and a FRET channel (440nm for excitation and 587nm for detection). An Ultraview dichroic mirror passes 405,440,515,640 was used to increase imaging speed using these filter blocks. Volocity™ software was used to calculate normalised FRET values (nFRET). To compensate for the bleed through of the CFP and YFP fluorophores into the FRET channel, FRET bleed through constants were calculated. Control samples containing HeLa cells transfected with CFP alone or YFP alone were imaged using the same settings used for data acquisition of the *mpx*:FRET zebrafish reporter line. ROIs were drawn around a population of cells in the frame and Volocity™ software calculated FRET bleed through values as the mean intensity of the recipient channel (FRET) divided by the mean intensity of the source (CFP or YFP). These

FRET constants were then used by Volocity™ to calculate a normalised FRET value. Neutrophil apoptosis was observed by overlaying the YFP and nFRET channels.

Statistical analysis

Data were analysed using GraphPad Prism version 7.0. Paired *t* tests were used for comparisons between two groups and one-way ANOVA with appropriate post-test adjustment was used for comparisons of three or more groups.

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Competing Interests

The authors declare no conflict of interest.

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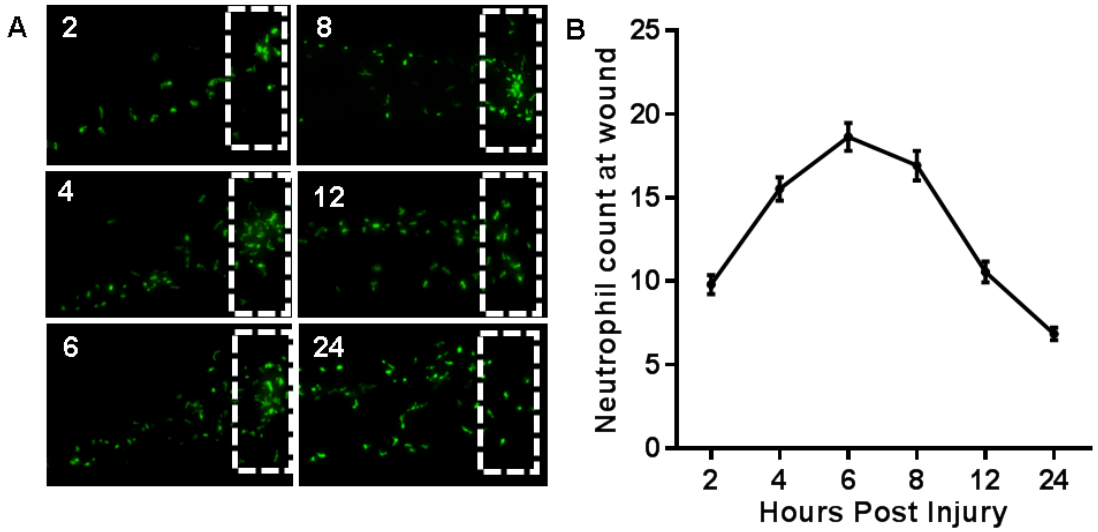
744 **Supplemental Figures**

745

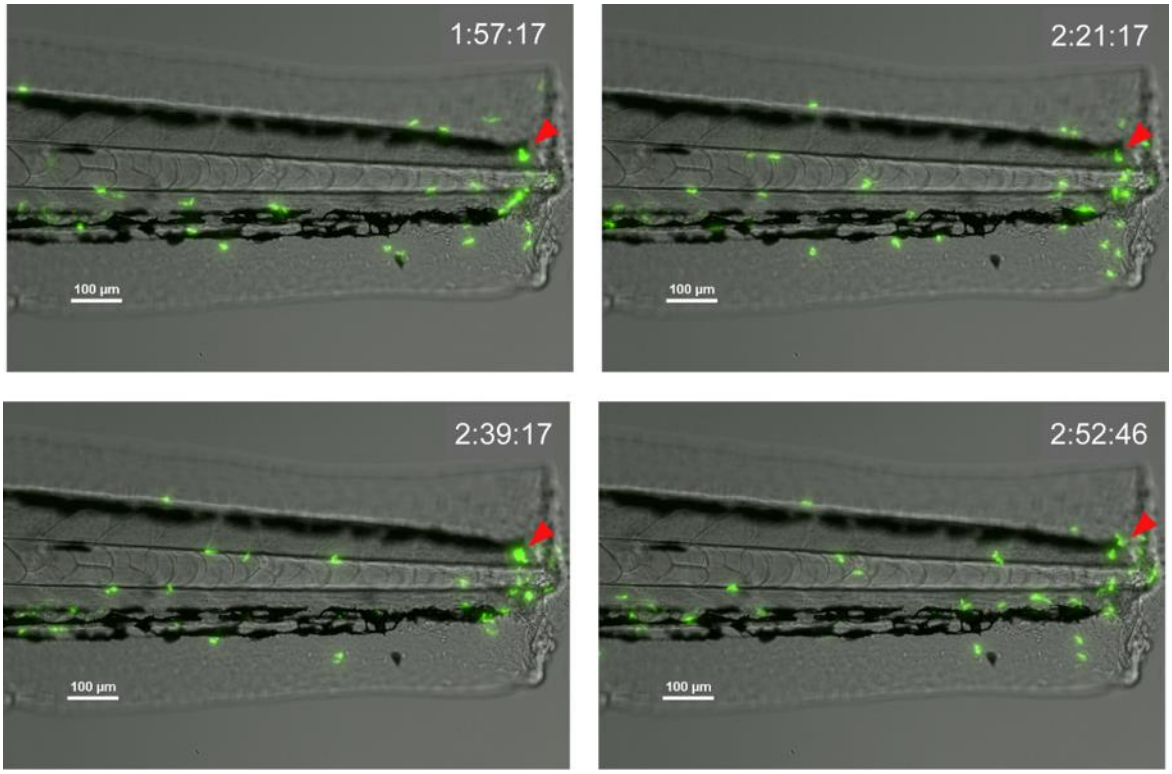


746

747 **Supplemental Figure 1. Dynamics of the neutrophil response to tissue injury**

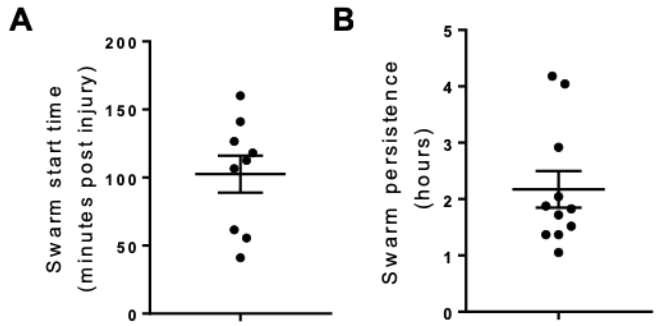
748 Following tail-fin transection of *mpx*:GFP transgenic larvae, the number of GFP neutrophils at
749 the site of injury were counted at 2, 4, 6, 8,12 and 24 hours post injury. **A.** Representative
750 images illustrating neutrophils in the tail fin region throughout the time course. **B.**
751 Quantification of neutrophil counts at the wound site throughout the time course. Data are
752 shown as mean ± SEM, n= 53 larvae from 3 independent experiments.

753



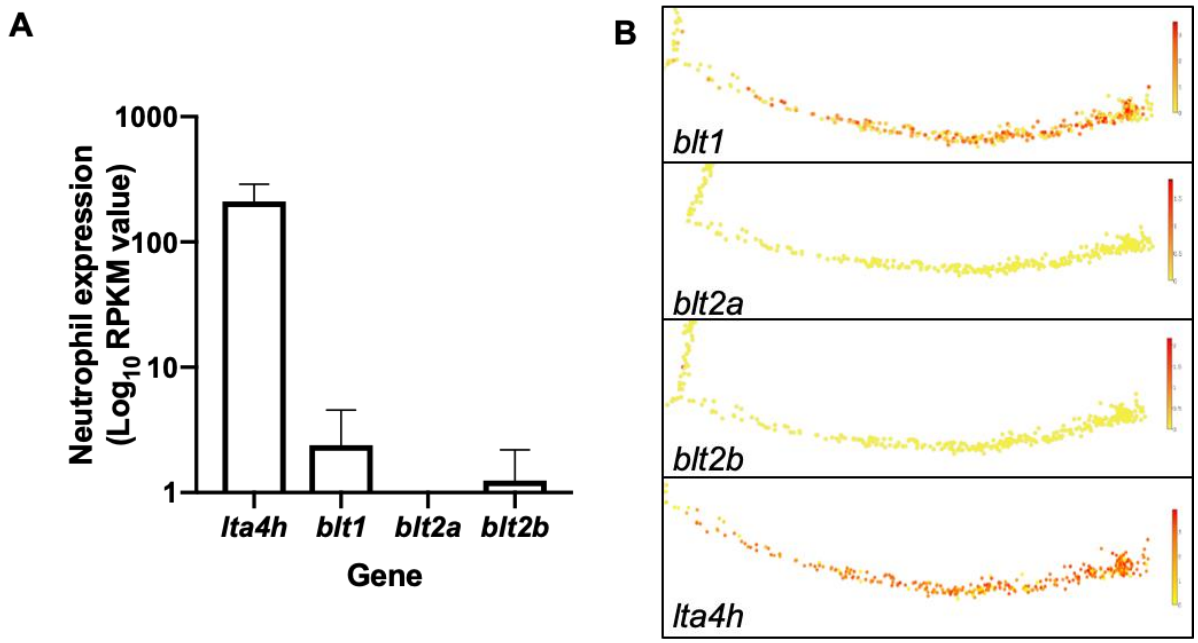
Supplemental Figure 2. Transient neutrophil swarms are observed within the inflamed tail fin

Time course of *mpx*:GFP transgenic zebrafish larvae following tail-fin transection illustrating short-lived (<1 hour) transient neutrophil swarming at the wound site. Phases of coordinated migration resulting in swarm formation (red arrow) were observed within the imaging period, followed by dissipation and re-formation. Time stamps shown in white (h:mm:ss) are relative to the start of imaging at 30 minutes post injury.



Supplemental Figure 3. Characterisation of persistent neutrophil swarms

A. Time (minutes post injury) in which persistent neutrophil swarms began to develop following tail-fin transection in zebrafish larvae (n=5 experimental repeats). **B.** Persistence time of neutrophil swarms measured during 5 hour imaging period (n=5 experimental repeats).



Supplemental Figure 4. Expression of LTB4 signalling components in zebrafish neutrophils

A. RNA sequencing of FACS sorted GFP positive cells from 5dpf *mpx*:GFP zebrafish larvae. RPKM values illustrate zebrafish neutrophil expression of *lta4h, blt1, blt2a* and *blt2b.* **B.** Single-cell gene expression profiles of LTB4 signalling components expressed in zebrafish neutrophils. Figure shows two-dimensional monocle plots illustrating individual neutrophil

776 expression profiles (circles) taken from FACS sorted cells from *mpx*:GFP positive transgenic
777 larvae. Gene expression is colour coded where red is high expression and yellow is no
778 expression. Data extracted from the Sanger BASiCz (Blood atlas of single cells in zebrafish)
779 cloud repository.

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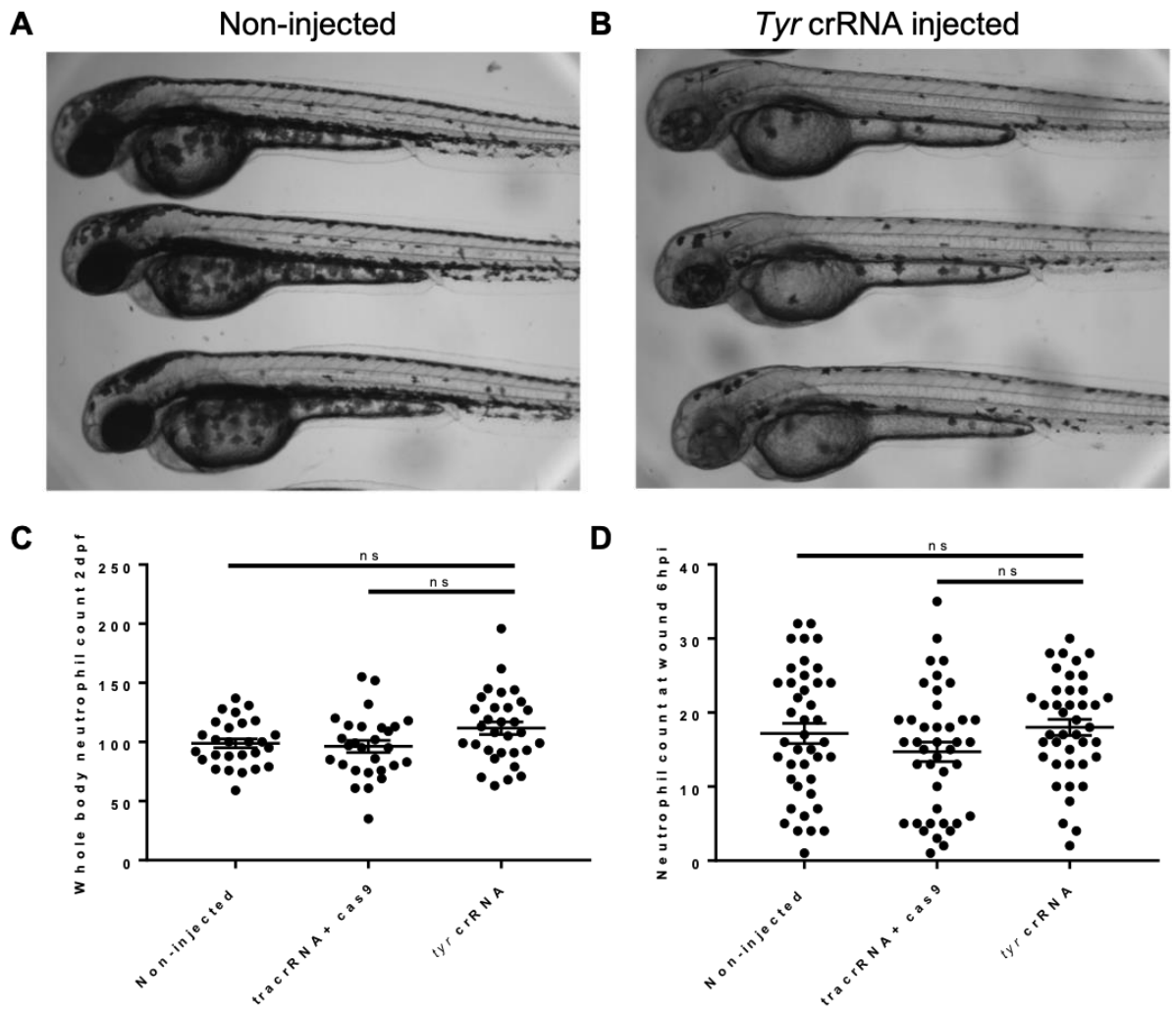

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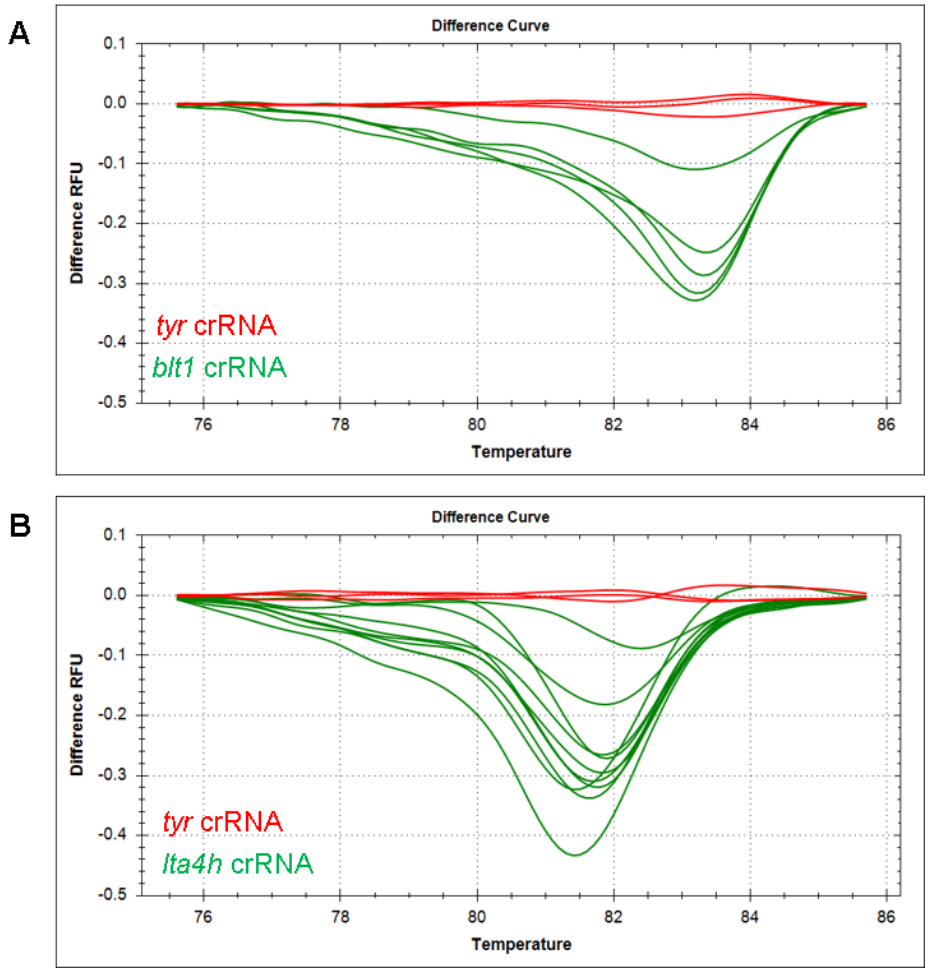
783 **Supplemental Figure 5. CRISPR/Cas9 knockdown of *tyrosinase* does not affect**
784 **neutrophil function**

785 **A-B.** Representative images of 2dpf *mpx*:GFP non-injected (**A**) and tyrosinase crRNA injected
786 (**B**) mosaic pigment phenotypes. **C.** Whole body neutrophil counts in non-injected, vehicle
787 control tracrRNA + cas9 protein injected and *tyrosinase* crRNA injected larvae. **D.** Neutrophils
788 recruited to the injury site at 6hpi in 2dpf non-injected, vehicle control tracrRNA + cas9 protein

789 injected and *tyrosinase* crRNA injected larvae. Error bars shown are mean ± SEM. Groups
790 were analysed using an ordinary one-way ANOVA and adjusted using Tukey's multi
791 comparison test, n=30 from 3 independent experiments.

792

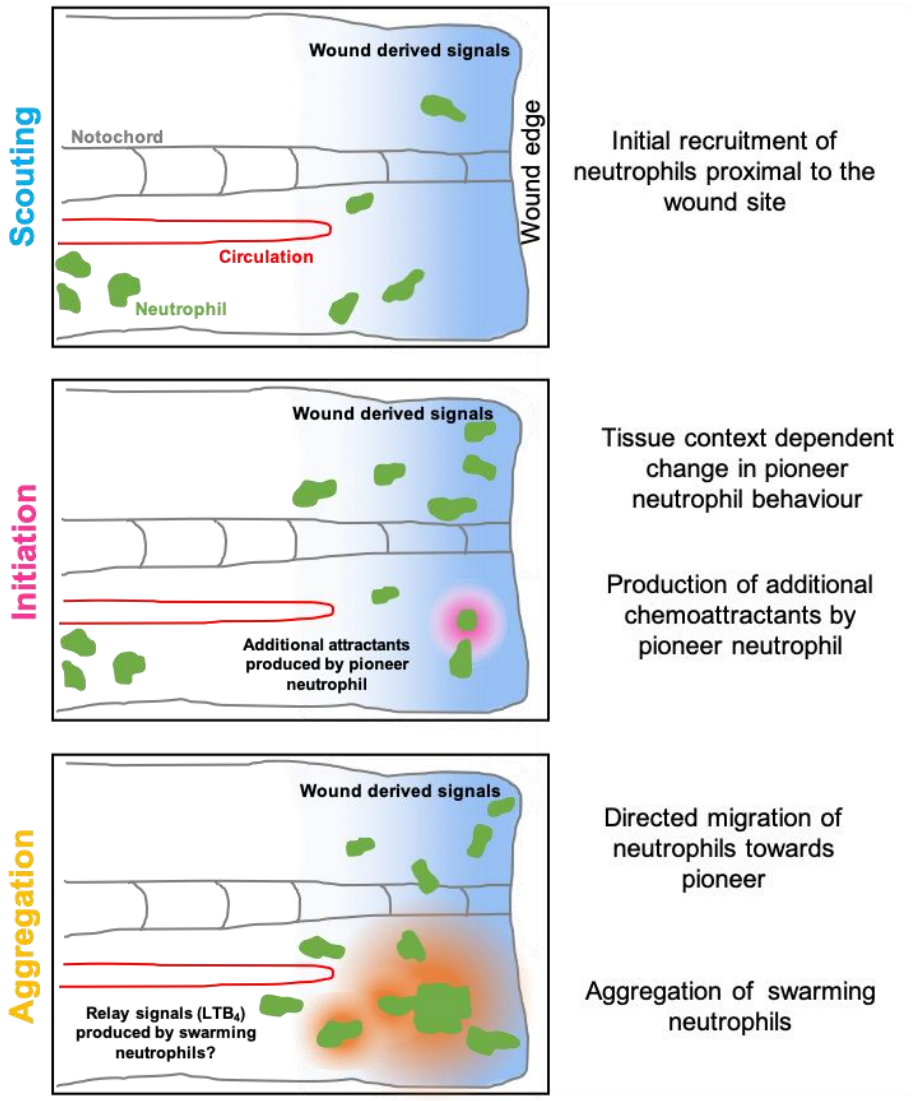


793

794 **Supplemental Figure 6. High resolution melt curve analysis for genotyping *blt1* and**
795 ***lta4h* CRISPR knockdown**

796 Genotyping example of successful CRISPR-induced indels by high resolution melt analysis
797 for *blt1* (**A**) and *lta4h* (**B**) injected larvae. Wild type curves (red) from three representative
798 control *tyrosinase* larvae and shifted, irregular melt curves (green) corresponding to mosaic
799 heteroduplex PCR fragments formed as a result of CRISPR/Cas9 mutations.

800



Supplemental figure 7. Proposed schematic of endogenous neutrophil swarm initiation at sites of tissue injury

Following tail fin transection neutrophils proximal to the wound site migrate towards wound-derived chemoattractants (scouting). Swarming is initiated when one pioneer neutrophil at the wound site changes behaviour and releases additional chemoattractant gradients which are responded to by a population of neutrophils (initiation), resulting in the directed migration of neutrophils to form a swarm (aggregation). The precise tissue context required for swarm initiation and the mediators released by the pioneer remain to be determined.