Supplemental Information

Neutrophil Swarming in Damaged Tissue Is Orchestrated by Connexins and Cooperative Calcium Alarm Signals

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A

B

C

D

E

F

G

H

**Neutrophil number**

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**Cluster size (µm²)**

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**Normalised radial speed (µm/min)**

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**Laser Wound**

**Mechanical Wound**
Figure S1. Role of neutrophil biosynthesis of LTB4 and dynamics of neutrophil swarming in zebrafish wounds, related to Figure 1.

A RT-PCR of lta4h from mRNA extracted from 3 dpf wild type (AB) zebrafish. The arrow denotes the presence of an alternative transcript in larvae injected with a splice-blocking lta4h morpholino; NI = Non-injected, Ctr = Control morpholino, lta4h = injected with the splice-blocking lta4h morpholino.

B Sudan black staining of neutrophils in wild type (WT) and lta4h morpholino-injected 3dpf larvae amputated with a scalpel and fixed 3h post-wounding. The dotted lines represent the area in which neutrophils were counted. Scale bar = 100μm.

C, D Quantification of the total number of neutrophils recruited to wounds. NI = Non-injected, Ctr MO = Control morpholino, lta4h = injected with the splice-blocking lta4h morpholino. C 81-89 larvae per group pooled from 3 experiments. D 54-68 larvae per group pooled from 3 experiments. p value <0.0001. Kruskal-Wallis test with Dunn’s post-test.

E Projections from two photon (left) and spinning disk confocal images (right) showing neutrophils from Tg(mp5x:GFP)i114 larvae migrating from the caudal hematopoietic tissue (CHT) towards a laser wound (LW; dotted line) at the ventral fin-CHT boundary (VF/CHT) or mechanical ventral fin wound (MW) respectively. The red dotted line indicates area occupied by the neutrophil cluster. Scale bar = 25μm.

F Evolution of cluster size over time post wounding for neutrophils in Tg(mp5x:GFP)i114 larvae. n=4 larvae with laser wound, n=14 larvae with mechanical fin wound from 2 and 7 experiments respectively. Mann Whitney test.

G Evolution of neutrophil radial speed over time post laser wounding (LW) for neutrophils in Tg(mp5x:GFP)i114 or Tg(lyz:GCamp6F) larvae. Instantaneous speed values for individual neutrophils were divided by the mean instantaneous speed value of the corresponding embryo to normalise differences in speed across embryos (see also methods). Error bars represent
standard error of the mean from data binned every 7.5 min. n=1201-1719 cell-steps per bin from n=10 larvae.

H Evolution of neutrophil radial speed over time post mechanical fin wounding for neutrophils in Tg(mpox:GFP)114 or Tg(lyz:GCamp6F) larvae. Error bars represent standard error of the mean from data binned every 7.5 min from n=1248-1800 cell-steps per bin from 14 larvae.
Figure S2. Nuclear localisation of 5-LO in zebrafish neutrophils and kinetics of neutrophil death in the first hour post-wounding, related to Figures 2 and 3

A-D Laser scanning confocal projections of Tg(mpx:GFP)$^{114}$xTg(lyz:tRFP-5LO) larvae stained with antibodies against GFP (green) and tRFP (magenta) and co-labelled with nuclear DAPI staining (white) A and C represent examples from two different larvae (Scale bar=25µm). B and D represent zoomed-in images corresponding to insets in A and C respectively (Scale bar=10µm).

E Time-lapse series of two-photon confocal projections images of neutrophils Tg(lyz:GCamp6F) larvae (green) incubated in PI (magenta). Time post-wounding is indicated in minutes. Dotted line indicates laser wound (LW). CHT: caudal hematopoietic tissue. VF: ventral fin. Scale bar=50µm.

F Zoomed in example of an apoptotic neutrophil. The green arrow indicates a neutrophil with an apoptotic shape in the GCamp6F channel. The magenta arrows indicate the nucleus of this neutrophil subsequently up taking PI. Scale bar = 10µm.

G,H Percentage of dead or alive neutrophils over time (G) or averaged through the first hour post-wounding (H). Data are means of 8 larvae in 7 independent experiments. Mann-Whitney test.
Figure S3. Effect of SKF96365, 2-APB, U-75302 and EGTA on neutrophil dynamics, related to Figure 4

A Time-lapse sequence of two-photon confocal image projections showing neutrophils in Tg(lyz:GCamp6F)xTg(lyz:tRFP-5LO) larvae, without treatment (ctr) or in the presence of 20 µM SKF96365, 25 µM 2-APB, 3 µM U-75302 (and control dilutions of Ethanol to be compared with the latter two). Scale bars = 50µm and 20µm, for zoomed-out and zoomed-in images respectively. CW: calcium wave. Time after laser wounding (LW) is shown in minutes. CHT: caudal hematopoietic tissue. VF: ventral fin.

B Normalised GCamp6F levels in control or SKF96365-treated larvae. n=5 control, n=4 SKF96365-treated larvae from 3 experiments. GCamp6F intensity was normalised as in Figure 1D. Mann-Whitney test.

C-D Neutrophil radial speed (C) or speed (D) over time post-laser wounding in control or SKF96365-treated larvae. n=1226-1527 cell-steps per bin from 5 control larvae and n=968-1219 cell-steps per bin from 4 SKF96365-treated larvae from 3 experiments. Mann-Whitney test.

E Normalised GCamp6F levels in Ethanol control, 2-APB and U-75302-treated larvae. n=5 control, n=4 2-APB-treated larvae and n=8 U-75302-treated larvae from 2, 2 and 3 independent experiments respectively. One-way ANOVA with Dunnet’s multiple comparison post-test.

F-G Neutrophil radial speed (F) or speed (G) over time post-laser wounding in Ethanol control, 2-APB and U-75302-treated larvae. n=1464-1743 cell-steps per bin from 5 control larvae, n=679-771 cell-steps per bin from 4 2-APB-treated larvae and n=2712-3135 cell-steps per bin from 8 U-75302-treated larvae from 2, 2 and 3 independent experiments respectively. Kruskal-Wallis with Dunn’s multiple comparison test (black stars; ctr vs U-75302, grey stars; ctr vs 2-APB).
H  Spinning-disk confocal projection images from neutrophils in Tg(lyz:GCamp6F)xTg(lyz:tRFP-5LO) post-mechanical fin wound (MW) and EGTA treatment. Time after wounding is indicated within images. Time after EGTA addition is indicated above. The light blue dotted line indicates area occupied by the neutrophil cluster. Scale bar = 50µm.

I  Normalised GCamp6F levels in clustering cells (light blue line; corresponding to cells within the wound area occupied by clustering neutrophils shown in J) or migrating cells (dark blue line; corresponding to cells outside the wound area) over time. Red dotted line indicates time of EGTA addition. n=288-810 cell-steps per bin for migrating cells, n=40-144 cell-steps per bin for clustering cells from 5 larvae in 2 independent experiments.

J  Normalised neutrophil speed in migrating cells over time. Red dotted line indicates time of EGTA addition. n=170-677 cell-steps per bin from 5 larvae in 2 independent experiments.
A

PBS  LTB4

B

Relative GCamp6F intensity (inside/outside OC)

C

Relative GCamp6F intensity (inside/outside OC)

D

E

F

GCamp6F

F

G

H

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Figure S4. Effect of chemoattractant injection or transplantation of chemokine-secreting cells on neutrophil calcium dynamics, related to Figures 4 and 5

A Confocal spinning disk projection images of neutrophils in Tg(lyz:Gcamp6F) around the otic cavity (OC) of 3 dpf larvae 50 min after local injection of 30 nM LTB4, 30 nM Cxcl8a, 200 µM non-hydrolysable ATP-γS and control and PBS control. The otic cavity is delimited by dotted lines. Scale bar = 50µm.

B and C Relative GCamp6F levels in Ethanol (EtOH) control, PBS control, LTB4-injected Cxcl8a-injected and ATP-injected Tg(lyz:GCamp6F) larvae. Levels of GCamp6F in neutrophils within the otic cavity (OC) are expressed relative to the levels of cells outside the wound cavity in the first image of the movie. n=5 PBS, n=5 EtOH, n=4 LTB4, n=5 ATP and n=5 Cxcl8a larvae from 2, 3, 2, 2 and 2 independent experiments respectively.

D Confocal spinning disk projection images of neutrophils in Tg(lyz:Gcamp6F) 3 dpf larvae (in green) moving around a transplant of HEK293T cells expressing Cxcl8a-mCherry (magenta). The transplantation zone is delimited by dotted lines and neutrophils within this area are indicated with white arrows. Scale bar = 25µm.

E Quantification of GCamp6F intensity in neutrophils on the Cxcl8a transplant (ON) and beyond (OFF). Data are means of n=4 independent embryos.

F Spinning-disk confocal projection images of neutrophils in Tg(lyz:GCamp6F)xTg(lyz:tRFP-5LO) larvae 90 minutes after mechanical ventral fin wound (MW) in the presence of 50 µM CBX. Representative control shown in Figure 4F, for comparison. MW: mechanical wound. CHT: caudal hematopoietic tissue. VF: ventral fin. Scale bar = 50µm.

G Mean normalised GCamp6F intensity in Tg(lyz:GCamp6F)xTg(lyz:tRFP-5LO) larvae treated or not with CBX. GCamp6F intensity was normalised as in Figure 1D and E. Data are
means from n=9 control larvae and n= 5 CBX-treated larvae from 3 and 2 imaging sessions respectively. Mann-Whitney test.

H Percent of translocating neutrophils out of all neutrophils recruited into the ventral fin over 90 min of imaging starting 10 min post-wounding. Data are means of n=7 control larvae and n=5 CBX-treated larvae from 3 and 2 imaging sessions respectively. Mann-Whitney test.
**Figure S5. Neutrophil Cx43 expression and knockdown in zebrafish larvae and effects on neutrophil dynamics, related to Figure 5**

A Neutrophil speed over time post-laser wounding in control, CBX-treated and cx43 MO-treated larvae from Tg(mpx:GFP)\textsuperscript{i114} and Tg(lyz:GCamp6F) larvae. n=1201-1719 cell-steps per bin from 12 control, n=1268-1535 cell-steps per bin from 8 CBX-treated and n=1308-1554 cell-steps per bin from 11 cx43 MO-injected larvae from 8, 3 and 4 experiments respectively. Mann-Whitney test (grey stars indicate significance for both Ctr vs CBX and Ctr vs cx43 MO; black stars indicate significance for just Ctr vs CBX).

B RT-PCR for cx43 and cx43.4 expression from cDNA samples of FACS-sorted GFP\textsuperscript{+} (+) or GFP\textsuperscript{-} cells (-) from Tg(mpx:GFP)\textsuperscript{i114} 4.5 dpf larvae.

C-D Laser scanning confocal projections of Tg(mpx:GFP)\textsuperscript{i114} larvae stained with antibodies against GFP (green) and Cx43/Cx43.4 (magenta). C represents example of a cluster overview from one larva (Scale bar = 25\textmu m) with insets showing a closer view of a subset of clustering cells (Scale bar = 10\textmu m). D shows additional examples of neutrophils from 5 wounded and 4 unwounded larvae stained with anti-Cx43 and from 1 wounded larva stained with IgG ctr. Scale bar=10\textmu m. Neutrophils are marked with a number. In the examples from wounded larvae, cells 1-5 are at the wound margin and cell 6 is in the CHT. In the examples of unwounded larvae, cells 1-5 are in the CHT. The IgG control example is at the wound margin of a wounded larva. Cartoon shows area of mechanical wounding (MW) and imaging. Arrows indicate example regions with Cx43 stain on the periphery of the neutrophil.

E Western Blot showing the expression of Cx43 and Cx43.4 in 3dpf larvae of wild type AB (WT) and morphant zebrafish larvae. Amount injected for each morpholino in pmol is shown.

F Image of different phenotypes of 3dpf zebrafish larvae injected with cx43/cx43.4 combination MOs. The black arrow indicates the mildest phenotype with detectable difference in eye size that was selected for subsequent experiments.
A) Images show the effects of various substances (EtOH, LTB4, PBS, ATP, Cxcl8a) on Neutrophil number in different conditions (Ctr, cx43 MO).

B) Graph showing Neutrophil number across different conditions with significant differences indicated by *** (p < 0.001) and ns (not significant).

C) Similar graph for different conditions, showing significant differences with ** (p < 0.01) and ns.

D) Graph for Neutrophil number showing significant differences with * (p < 0.05) and ns.

E) Images comparing Ctr Sibling, cx43 DN, WT, and cx43 MO conditions.

F) Graph showing Neutrophil number across different conditions with significant differences indicated by *** and **.

G) Graph showing Speed (µm/min) over time (min) with significant differences indicated by *.

H) Images comparing WT, NF279, cx43 MO, and NF279 + cx43 MO conditions.

I) Graph showing Neutrophil number across different conditions with significant differences indicated by ****.
Figure S6. Recruitment of neutrophils in response to chemoattractant injection and effect of Cx43 inhibition on wound recruitment with or without co-inhibition of ATP signalling, related to Figure 5

A Sudan black staining of neutrophils in transgenic wild type AB larvae 45 min following injection of Ethanol control, PBS control, 30 nM LTB4, 30 nM Cxcl8a and 200 µM non-hydrolysable ATP-γS in the otic vesicle, with or without cx43 MO injection. The dotted lines represent the periphery of the cavity where neutrophils were counted. Scale bar = 50µm.

B-D Number of neutrophils recruited to the otic vesicle at 45 min. n=30-56 larvae per condition, across 2 independent experiments. Kruskal-Wallis test with Dunn’s post-test.

E Sudan black staining of neutrophils in transgenic Tg(lyz:cx43DN-T2A-mCherry) larvae (cx43DN) and their negatively screened siblings (Sibling) or larvae injected with cx43/cx43.4 combination of morpholinos (cx43 MO), fixed at 3h after wounding. The dotted lines represent the area in which neutrophils were counted. Scale bar = 50µm.

F Number of neutrophils recruited to wounds in the different conditions pooled from three independent experiments. cx43 DN, n=32 larvae; negative siblings (Ctr sibling), n=22 larvae; WT, n=37 larvae; cx43 MO, n=30 larvae. Kruskal-Wallis test with Dunn’s post-test.

G Neutrophil speed over time post-laser wounding in Tg(lyz;GCamp6F)xTg(lyz:cx43DN-T2A-mCherry) zebrafish larvae, positive (cx43 DN) or negative for the Cx43 DN-T2A-mCherry transgene (control siblings: Ctr sib). n=1330-1602 cell-steps per bin from 5 control siblings, n=2573-3050 cell-steps per bin from 7 Cx43 DN larvae from 3 experiments. Mann-Whitney test.

H Sudan black staining of neutrophils in wild type larvae injected or not with cx43/cx43.4 combination of morpholinos (cx43 MO) and treated or not with 10µM NF279 and fixed 3h after wounding. Scale bar =100µm.
Quantification of neutrophil number at wounds in the different conditions. Data pooled from 2 independent experiments. WT (non-injected and not treated with drug), n=60 larvae; NF279, n=40; cx43 MO, n=32; cx43 MO with NF279, n=25. One-way ANOVA with Tukey’s multiple comparison test.
Figure S7. Zebrafish tail fin wound colonisation assay with *P. aeruginosa*, related to Figure 7

**A** 3 dpf larvae were injured at the tail fin with a scalpel in the presence of titrating doses of *P. aeruginosa* in the bath. Concentration-dependent effects on survival in wounded larvae are shown.

**B** Same titration in non-wounded larvae. Injury-independent death is observed only at doses below $10^9$ CFU/ml. Data represent one experiment with 20 larvae per condition.