How tissue damage is detected to induce inflammatory responses is unclear. Most studies have focused on damage signals released by cell breakage and necrosis. Whether tissues use other cues in addition to cell lysis to detect that they are damaged is unknown. We find that osmolarity differences between interstitial fluid and the external environment mediate rapid leukocyte recruitment to sites of tissue damage in zebrafish by activating cytosolic phospholipase a2 (cPLA2) at injury sites. cPLA2 initiates the production of non-canonical arachidonate metabolites that mediate leukocyte chemotaxis through a 5-oxo-ETE receptor (OXE-R). Thus, tissues can detect damage through direct surveillance of barrier integrity, with cell swelling probably functioning as a pro-inflammatory intermediate in the process.

Epithelial tissue injury is associated with cell damage, disruption of cell–cell interactions, and direct exposure of cells inside the tissue to the outside environment. Leukocytes detect and migrate towards epithelial breaches over distances of several hundred micrometres within minutes. Leakage of damage-associated molecular patterns (DAMPs) from broken cells leads to leukocyte necrotaxis and is at present the best characterized paradigm for tissue damage detection. By contrast, detection mechanisms that provide surveillance at the tissue barrier level remain poorly studied. For skin injuries, collapse of the transepithelial electrostatic potential has been proposed to produce an instructive, electrostatic signal, but how this could steer leukocytes to the site of injury is unclear. Epithelia can maintain large concentration differences between the extracellular space of tissues and the external environment. The skin of freshwater organisms, such as zebrafish, as well as the mucosal surfaces of the oral cavity, oesophagus and potentially the lung of land mammals, are exposed to hypotonicity. These epithelial barriers separate interstitial fluid, which contains ions and metabolites in the high mOsm range (~270–300 mOsm, which is the common extracellular toxicity of vertebrates), from the external environment, which contains ions in the low mOsm range (fresh water, ~10 mOsm; saliva, ~30 mOsm). This leads to significant differences in chemical potential of specific ions and osmotic pressure across the epithelium, and disruption of these gradients could potentially be sensed by cells at the injury site.

The immune system of zebrafish closely resembles that of mammals. The optical transparency of their larvae enables non-invasive imaging and interrogation of tissue damage detection mechanisms by live microscopy in the intact animal. Zebrafish tail fin wounds are initially (that is within 40–60 min after injury) detected by neutrophils and macrophages. Migration of these myeloid leukocytes to wounds can be monitored by light transmission microscopy. To test if changes in ion concentrations or osmotic pressure are detected by leukocytes following epithelial barrier injury, we generated tail fin wounds in zebrafish larvae incubated in either hypotonic medium (resembling fresh water) or medium adjusted to the tonic composition and toxicity characteristic of vertebrate interstitial fluid by addition of NaCl. We observed a concentration-dependent (Fig. 1a,b and Supplementary Video S1) and reversible (Supplementary Fig. S1a,b and Video S2) inhibition of leukocyte recruitment to the wound site, associated with decreased migration velocity and path-length (Fig. 1a), without obvious signs of impaired animal health under these conditions. To test if leukocyte migration inhibition was caused by increased external medium toxicity or by ion-specific effects, we replaced Cl− and Na+ ions with choline or gluconate, respectively, and used sucrose and mannose as non-ionic osmolytes. Whereas isotonic NaCl exerted the most pronounced anti-inflammatory effect, all conditions that reduced the osmolarity difference between tissue and external medium inhibited recruitment (Fig. 1c). This indicates that exposure of the zebrafish tail fin to low toxicity is required to trigger rapid recruitment of leukocytes after wounding.

Hypotonic exposure results in cell swelling through osmotic water influx. Subnanometre-sized ionic osmolytes (for example Na+ and Cl−) easily diffuse through a micrometre-sized wound. Thus, cells inside the tail fin are bound to experience osmotic pressure changes following wounding in hypotonic bathing medium. The precise amount of interstitial hypotonicity and cell swelling will depend on wound size, wound-opening time, cell distance from the wound and efficiency of regulatory volume decrease mechanisms. In extreme cases, osmotic cell swelling may lead to injury or lysis, which could promote leukocyte recruitment through DAMP signalling. To test this idea, we wounded zebrafish in hypo- or isotonic medium supplemented with the small, membrane impermeable DNA dye Sytox Orange.
Figure 1  Hypotonicity is required for rapid leukocyte recruitment to larval zebrafish tail fin wounds. (a) Recruitment of leukocytes to incisinal tail fin wounds imaged in zebrafish larvae by light transmission microscopy. During wounding and subsequent imaging, larvae were kept either in normal, hypotonic E3 embryo medium (‘Control’, containing 5 mM NaCl) or in embryo medium that had been adjusted to the common extracellular tonicity of vertebrates (~270–300 mOsm) by the addition of 140 mM NaCl (‘Isotonic (NaCl)’, 145 mM NaCl). Left: representative leukocyte tracks capturing all visible cell movements within 40 min after injury. Graph: mean number of leukocytes reaching the wound within t = 40 min after injury. Table: quantification of mean velocity (v), path length (l), wound directionality (D_d) and path persistence (D_p). (b) Mean leukocyte recruitment to larval tail fin wounds within 40 min after injury plotted versus salt concentration of the medium. (c) Mean leukocyte recruitment within 40 min after injury as a function of different isotonic medium compositions. ‘Control’, 5 mM NaCl. ‘Mannitol’, control + 270 mM mannitol. ‘Sucrose’, control + 270 mM sucrose. ‘NaGluc’, control + 135 mM sodium gluconate. ‘ChCl’, control + 135 mM choline chloride. ‘NaCl’, control + 135 mM NaCl. (d) HyPer imaging of wound margin H_{2}O_{2} production in response to wounding in hypotonic medium (h), isotonic medium (i) or isotonic medium + 100 μM of the NADPH oxidase inhibitor diphenyliodonium chloride (i/DPI). Top: representative HyPer-ratio images. Red, high [H_{2}O_{2}]. Blue, low [H_{2}O_{2}]. Bottom: normalized HyPer ratio as a function of time after wounding. The number of larvae used for the analyses is given in parentheses on the graphs. Error bars, s.e.m. ** t-test P < 0.005. *** t-test P > 0.0005. Scale bar, 100 μm.

which enters cells following plasma-membrane damage. DNA binding enhances Sytox Orange fluorescence ~500-fold, highlighting damaged or lysed cells. As expected, we frequently detected cell damage along the wound margin, but the toxicity of the medium did not significantly affect the amount of detected damage (Supplementary Fig. S1c). Orthogonally, we wounded fish in the presence of ATP or epithelial cell extract to test if exogenous DAMPs could bypass isotonic inhibition of leukocyte recruitment. Neither of these treatments reconstituted leukocyte recruitment after isotonic injury (Supplementary Fig. S1d). Isotonicity also did not significantly alter dual oxidase (DUOX) activity at the wound margin (Fig. 1d), which was previously identified as a physiological cue necessary for initial leukocyte recruitment^{13–16}. In conclusion, our data do not provide evidence that cell lysis or cytoplasmic leakage instruct initial leukocyte recruitment in our system, and suggest that other pathways in addition to DUOX activation are required.

Cell swelling activates a wide variety of ion channels, tyrosine kinases and lipases including PLA2 in different cell types^{17}. PLA2 enzymes regulate inflammation and cell volume homeostasis by releasing fatty acids such as arachidonic acid (AA) from the sn-2 position of
**Figure 2** Hypotonicity locally activates cPLA2 at the wound site. (a) Confocal imaging of injury induced cPLA2–mKate2 translocation to nuclear membranes in live zebrafish larvae. Left: representative maximal intensity projection showing cPLA2–mKate2 localization before (top) or 30 s after (bottom) laser injury of the tail fin. Note that the cell cytoplasm and cell periphery are not visible, because cPLA2 localizes exclusively to the nucleus. These magnifications were derived from tissue regions near the (prospective) injury site. Far right images: superposition of nuclear histone H2A–GFP (green) and cPLA2–mKate2 (red) fluorescence. Right: representative intensity profile plots of H2A–GFP (green) and cPLA2–mKate2 (red) derived from neighbouring image data (dashed lines). Scale bars, 10 μm. (b) Top: full field of view images of cPLA2–mKate2 fluorescence 30 s post wounding (pw) in larval tail fins subjected to laser injury in hypotonic medium (h), isotonic medium (i) or hypotonic medium supplemented with 500 μM Gd3+ (h + Gd3+). Bottom: average cPLA2–mKate2 translocation density projected onto normalized wound coordinates at indicated conditions (see Methods for details). Colour scale, relative translocation densities (white, high; red, low; black, none). Scale bar, 100 μm. (c) Average number of nuclei per animal that show cPLA2–mKate2 translocation in response to wounding at indicated conditions. The number of larvae (n) used for the analyses is given in parentheses on the graphs. Error bars: s.e.m. Student’s t-test: ∗∗P < 0.01; ∗∗∗P < 0.005. (d) Average number of nuclei per animal with cPLA2–mKate2 translocation in response to wounding under indicated conditions as a function of distance from the wound margin.
phospholipids, but how cell swelling activates PLA2 remains unclear. Secreted (sPLA2) and Ca2+-independent (iPLA2) members of the PLA2 family accept phospholipid substrates of broadly varying sn-2 fatty acid chain length. We therefore focused on cPLA2, which specifically releases the inflammatory mediator AA (ref. 17). Semi-quantitative RT–PCR on RNA pools from FACS-sorted cell populations of transgenic larvae expressing the far-red fluorescent protein mKate2 in leukocytes indicated uniform cpla2 message expression in leukocyte-enriched and leukocyte-depleted cell fractions (Supplementary Fig. S2a).

cPLA2 translocation to the nuclear membrane, where key enzymes of AA metabolism (for example cyclooxygenases and lipoxygenases) localize, is associated with phospholipid hydrolysis at that site, and provides a microscopically tractable, steady-state readout for cPLA2 activation in live cells. cPLA2 was amino-terminally fused to mKate2 and the reporter messenger RNA was injected into one-cell stage embryos. Spinning disk confocal microscopy of live larvae revealed that cPLA2–mKate2 was localized within the nucleus regardless of expression level (Fig. 2a, upper panel), resembling the endogenous cPLA2 localization in proliferating cells. Following tail fin wounding with an ultraviolet laser, cPLA2–mKate2 translocated to the nuclear membrane within seconds (Fig. 2a, lower panel, and Supplementary Video S3). The localization and translocation of endogenous cPLA2 was also confirmed by immunofluorescence staining (Supplementary Fig. S2a). We typically observed ~80% translocation in the first two or three rows of wound margin cells and a gradually decreasing translocation frequency over a distance of ~100–150 μm into the tissue. The larval fin epithelium consists of various cell types and we observed translocation in at least two distinct cell populations, probably epithelial and fibroblast-like cells, as judged by inspection of nuclear shapes (Fig. 2a, first image column; compare round with irregular shaped nuclei).

To determine whether cPLA2 translocation depends on cell volume, fish were wounded in isotonic medium to inhibit osmotic cell swelling. cPLA2–mKate2 translocation was severely inhibited under these conditions (Fig. 2b–d). Conversely, we blocked regulatory volume decrease with gadolinium (Gd3+) or ethylene glycol tetra-acetic acid (EGTA) as previously described to augment osmotic cell swelling. Gd3+ exposure enhanced wound margin swelling, as judged by visual inspection of light transmission time-lapse videos (Supplementary Video S4), and significantly enhanced cPLA2–mKate2 translocation around the injury site (Fig. 2b, right versus left panel, Fig. 2c,d). By contrast, EGTA treatment completely inhibited cPLA2–mKate2 translocation, even in response to hypotonic wounding (Supplementary Video S5). This is consistent with cPLA2 translocation being Ca2+ dependent. Following bath supplementation with further Ca2+ to overcome EGTA-mediated extracellular Ca2+ sequestration (after cells were already swollen), cPLA2–mKate2 rapidly translocated to nuclear membranes within the complete field of view (Supplementary Video S5). Simultaneous imaging of cPLA2–mKate2 translocation and cytoplasmic Ca2+ influx in EGTA treated fish revealed a tight spatiotemporal correlation of the two events (Fig. 3a,b). Wound-induced Ca2+ waves have been observed in different tissues across phyla, but their downstream effectors have remained largely unclear. Tail fin injury rapidly increases cytoplasmic Ca2+ at the wound site irrespective of medium tonicity (Fig. 3c and Supplementary Video S6), whereas cPLA2 translocation only occurs under hypotonic conditions. This suggests that, in this physiological context, CA2+ alone is not sufficient to induce cPLA2 activation, which is different from what has been reported in cell culture experiments. Such a co-requirement for Ca2+ and swelling may function as a physiological AND gate to suppress erroneous inflammatory outputs in response to non-correlated fluctuations of [Ca2+] or cell volume in the absence of a wound.

To test if cPLA2 is required for leukocyte recruitment to tail fin wounds, we depleted functional cpla2 message using a splice interfering morpholino. This generated a truncated cpla2 mRNA, and strongly inhibited leukocyte recruitment by impairing both migration velocity and directionality (Fig. 4a and Supplementary Video S7). Morphant embryos had no morphological defects even at high morpholino concentrations (2–4 pmol per embryo). Leukocyte recruitment in response to bath application of the chemoattractant LTB4 was unperturbed (Fig. 4b), excluding non-specific and/or permissive effects of cpla2 knockdown. Likewise, leukocyte recruitment in cpla2 morphants was partially rescued by co-injection of mRNA coding for cPLA2–mKate2 (Supplementary Fig. S2c), which also confirms the functionality of our cPLA2 reporter. Non-selective pharmacological inhibition of PLA2 activity with N-(p-aminocinnamoyl) anthranilic acid (ACA) inhibited leukocyte recruitment (Supplementary Fig. S2d), corroborating the morpholino experiments. Together with our translocation data, this suggests that hypotonic swelling activates cPLA2 in cells at the wound margin by a CA2+-dependent mechanism, and raises the possibility that AA metabolites are involved in leukocyte recruitment.

To explore this possible role of AA metabolites, we attempted to rescue leukocyte recruitment to wounds in the absence of cPLA2 activation, using isotonic medium. AA in the fish medium caused a concentration-dependent rescue of leukocyte recruitment (Fig. 4c), which was similar to the endogenous wound response in terms of speed and radius of leukocyte recruitment. To test if non-specific, biophysical effects (alterations of membrane fluidity, amphiphilic modulation of channel activation, and so on) contribute to AA-induced leukocyte activation, we tested the effect of two structurally related AA downstream metabolites, 5(S)- and 15(S)-HETE, which have an additional hydroxyl group at different positions when compared with AA. The wound response was reconstituted with 5(S)-HETE, but not by its positional isomer 15(S)-HETE (Fig. 4d). Thus, AA and its metabolite 5(S)-HETE mediate leukocyte migration by a molecule-specific signalling mechanism. Preferential entry through the epithelial barrier breach, or local wound metabolism into different chemotactic species, may explain the ability of these cell-permeable lipids to generate spatially instructive wound cues following uniform application in fish bathing solution.

The small tissue size rendered biochemical quantification of local AA production and oxidation in wounded tail fin tips impossible. AA oxidation can occur through enzymatic or non-enzymatic routes. A gross pharmacological characterization of the enzymes known to act on AA indicated a possible role for arachidonate 5-lipoxygenase (ALOX5), but not cyclooxygenases or leukotriene A4 hydrolase (LTA4H; Supplementary Fig. S3). Concordant with studies showing leukotriene precursor production by non-myeloid cells of the skin, and ubiquitous alox5 mRNA expression in embryonic zebrafish, we detected alox5 message in both leukocytes and leukocyte-depleted tissue (Supplementary Fig. S3f). These experiments indicated the
Nuclear translocation ratio

| Time (min) | 0 | 10 | 20 | 30 | 40 |
|------------|---|----|----|----|----|
| 0          | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 |
| 1          | 0.8 | 0.9 | 1.0 | 1.1 | 1.2 |
| 2          | 1.2 | 1.3 | 1.4 | 1.5 | 1.6 |

\(\text{Nuclear translocation ratio}\)

Percentage of cells with translocation

| Time (min) | 0 | 10 | 20 | 30 | 40 |
|------------|---|----|----|----|----|
| 0          | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| 1          | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |
| 2          | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 |
| 3          | 1.3 | 1.4 | 1.5 | 1.6 | 1.7 |

\(\text{Percentage of cells with translocation}\)

Ca\(^{2+}\) signal (GEX-GECO1 405 nm/488 nm ratio)

| Time (min) | 0 | 10 | 20 | 30 | 40 |
|------------|---|----|----|----|----|
| 0          | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| 1          | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |
| 2          | 0.9 | 1.0 | 1.1 | 1.2 | 1.3 |
| 3          | 1.3 | 1.4 | 1.5 | 1.6 | 1.7 |

\(\text{Ca}^{2+}\) signal (GEX-GECO1 405 nm/488 nm ratio)

- **Figure 3** Extracellular Ca\(^{2+}\) is required for cPLA2 activation. (a,b) Parallel confocal microscopy imaging of cPLA2-mKate2 and cytosolic Ca\(^{2+}\) signals using the GEX-GECO1 Ca\(^{2+}\) indicator in live zebrafish larvae. Larvae were wounded manually in isotonic medium without Ca\(^{2+}\), supplemented with 1 mM EGTA, and mounted in a small volume of low melting isotonic agarose. Hypotonic medium without Ca\(^{2+}\), supplemented with 1 mM EGTA, was added on top of the isotonic agar pad at \(t = 0\) min. cPLA2-mKate2 fluorescence (montage) and GEX-GECO1 405 nm/488 nm excitation ratio images were acquired over the indicated time without (a) or with (b) readout of CaCl\(_2\) to reach a final [Ca\(^{2+}\)]\(_{\text{intra}}\) of 0.3 mM at 5 min. Left: cPLA2-mKate2 perinuclear translocation as a function of time, measured by automatic perinuclear/nuclear fluorescence ratio calculation (see Methods for details). Points represent individual nuclei with the threshold of translocation ratio empirically set to 1.05 (red). Right: cytosolic Ca\(^{2+}\) signal of the same cells (left axis) and the percentage of cells with a translocation ratio over 1.05 (right axis, red) as a function of time. Scale bars, 10 \(\mu\)m. (c) Cytosolic Ca\(^{2+}\) measurements in larval tail fins of live zebrafish expressing the genetically encoded calcium indicator GCaMP3 during ultraviolet-laser induced wounding at 19 s, under hypotonic (h) or isotonic (i) conditions. Scale bars, 100 \(\mu\)m.

- The ALOX5 product 5(S)-HETE can undergo oxidation to 5-KETE (also termed 5-oxo-ETE) by 5-hydroxyeicosanoid dehydrogenase in various cell types, including myeloid and epithelial cells\(^{34}\). The non-canonical eicosanoid 5-KETE is a potent neutrophil and eosinophil chemoattractant in mammals\(^{35}\), but its physiological role remains little understood. Confirming its chemoattractive function in zebrafish, addition of 5-KETE to isotonic tail fins restored dynamic parameters and recruitment of leukocytes to hypotonic control levels (Fig. 5a and Supplementary Video S8). In human cells, 5-KETE signals through a G-protein coupled receptor termed OXE-R. To investigate if OXE-R signalling contributes to the zebrafish wound response, we identified in the zebrafish genome a predicted G-protein coupled receptor gene orthologous to human OXE-R (OXER1). Henceforth referred to as oxer1 (GPR81-4, ENSDARG00000087084). FACS and semiquantitative RT-PCR revealed that oxer1 mRNA was robustly expressed in both leukocytes and leukocyte depleted tissue (Supplementary Fig. S4d). oxer1 knockdown with a translation blocking morpholino inhibited leukocyte recruitment to tail fin wounds by reducing leukocyte migration velocity and directionality (Fig. 5b and Supplementary Video S9), whereas it did not significantly affect embryo survival or gross morphology even at high morpholino concentrations (~2 pmol per embryo). To probe for potential morpholino off-target effects and permissive functions of OXE-R, we quantified leukocyte migration in unwounded fish in response to bath application of LTB4, produced strong migration in both wild-type (wt) and oxer1 morphant fish (Fig. 5c). By contrast, migration in response to bath application of 5-KETE was blocked only in oxer1 morphants, and not in wt embryos (Fig. 5d). Receptor desensitization by prolonged preincubation with exogenous 5-KETE significantly reduced hypotonic (that is control) wound recruitment of leukocytes (Supplementary Fig. S4a). Leukocyte chemotaxis to isotonic wounds in response to bath application of AA was also inhibited in oxer1 morphants (Supplementary Fig. S4b). Together, these data suggest that zebrafish OXE-R mediates 5-KETE and AA responses, and rapid, endogenous leukocyte recruitment to tail fin wounds.

- Activation of the epithelial NADPH oxidase DUOX at the wound site is necessary for rapid leukocyte chemotaxis\(^{39}\). Isotonic inhibition of cPLA2 activation, as well as cpl2 and oxer1 knockdowns, inhibit leukocyte recruitment, but not NADPH oxidase activity at the wound margin (Fig. 1d and Supplementary Fig. S4c). This indicates that DUOX activation alone is not enough to trigger leukocyte chemotaxis, and we now show that osmotically induced production of AA is also required. Oxo-eicosanoids, such as 5-KETE, are preferentially produced in response to oxidative stress, for example caused by H\(_2\)O\(_2\) or cytoplasmic NADPH depletion, respectively\(^{34}\). Thus, it is possible that DUOX activity, which generates extracellular H\(_2\)O\(_2\) by consuming cytoplasmic NADPH, mediates tissue damage detection by promoting production of oxidized, chemoattractive AA metabolites at the wound site, in addition to directly signalling to leukocytes through extracellular H\(_2\)O\(_2\) (refs 14,36,37 and Supplementary Fig. S5).
Figure 4 cPLA2 is required for rapid leukocyte recruitment to larval zebrafish tail fin wounds. (a) Average recruitment and migratory parameters of leukocytes within 40 min after tail fin wounding of wt and cpla2 morphant larvae. MO, morpholino oligonucleotide. bp, base pairs. Inset: RT–PCR on RNA derived from wt and cpla2 morphant larvae using cpla2-specific primers. (b) Migratory parameters ($v$, $l$) of leukocytes tracked for 60 min in unwounded wt or cpla2 morphant larvae in response to bath application of LTB$_4$. (c) Leukocyte recruitment to isotonic tail fin incisions at indicated concentrations of AA within 40 min. (d) Leukocyte recruitment (within 40 min) in response to tail fin incisions in hypotonic (h) or isotonic (i) medium tonicities (‘Ton’) in the presence or absence of AA, 5(S)-HETE or 15(S)-HETE. Indicated compounds (‘Comp’) were used at 5μM. The number of larvae used for the analyses is given in parentheses on the graphs. Error bars, s.e.m. Using Student’s $t$-test: *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. Scale bars, 100 μm.

We have demonstrated the existence of an osmotic signalling circuit that directly monitors tissue barrier integrity. In contrast to current danger signalling paradigms, our findings suggest that tissues can sense injury even in the absence of dead cells by harnessing cell swelling as a pro-inflammatory intermediate. Some human pathologies, for example cystic fibrosis, cause defective cell volume homeostasis and inflammation. Tissue necrosis (for example during ischemia) produces excessive cell swelling and leukocyte recruitment. Leukocyte necrotaxis has been typically linked to cytoplasmic leakage. Given our results, leakage-independent contributions of necrotic cell swelling, which generally precedes necrotic lysis, warrant closer investigation.

Osmotic surveillance of barrier integrity probably evolved in freshwater organisms to ensure reliable detection and safeguarding
Figure 5 OXE-R is required for rapid leukocyte recruitment to larval zebrafish tail fin wounds. (a) Average recruitment and migratory parameters of leukocytes within 60 min after isotonic tail fin wounding of wt larvae in the presence or absence of 2 μM 5-KETE in the supernatant medium (see Methods for details). (b) Average recruitment and migratory parameters of leukocytes within 40 min after hypotonic tail fin wounding of wt and oxer1 morphant larvae. (c) Migratory parameters \( (v, l) \) of leukocytes tracked for 60 min in unwounded wt or oxer1 morphant larvae in response to bath application of LTB₄. (d) Migratory parameters of leukocytes tracked for 60 min in unwounded wt or oxer1 morphant larvae in response to bath application of 5-KETE. The number of larvae used for the analyses is given in parentheses on the graphs. Error bars, s.e.m. Using Student’s t-test: *P < 0.05; **P < 0.005; ***P < 0.0005. Scale bars, 100 μm.

of epithelial breaches. These present major infection risks, but can easily occur with minimal cell death. Notably, major parts of the upper digestive tract, and potentially also the lungs³⁴ of land living mammals, are covered with hypotonic fluid. Human saliva is initially isotonic, and becomes desalted by ductal passage. Although the tonicity of lung fluid is debated⁴⁰, it is clear that our bodies establish a freshwater-like envi-
We thank T. Mitchison, A. Hall, M. Overholtzer and A. Kapus for their valuable contributions.

The physiological purpose of this remains unclear, but it is conceivable that luminal hypotonicity presents part of an ancestral barrier defence mechanism of wet epithelia that proved useful during evolution.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

P.N. conceived the project. B.E. and P.N. designed the experiments. B.E., P.N., S.K. and T.N.-Z. carried out the experiments. P.N. and B.E. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

General fish procedures. Adult wt and transgen (AB or casper41) background zebrasfish and larvae were maintained according to institutional animal healthcare guidelines as described42. For wounded assays, 2.5–3 days post-fertilization (dpf) larvae were anaesthetized in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) containing 0.2 mg ml−1 tricaine (Sigma) before wounding. To prevent pigment formation, larvae were maintained in E3 containing 0.2 mM N-phenylthiourea (Sigma).

Bright field and fluorescence imaging. For all imaging experiments larvae were embedded in 1% low melting agarose (low) and maintained in E3 or isotonic, E3 medium supplemented with indicated treatments (indicated osmolites), both containing the indicated compounds or drugs and 0.2 mg ml−1 tricaine. To image leucocyte recruitment, up to 30 AB larvae were placed in a glass bottom dish (Makite), mounted in agarose and cut at the ventral tail fin using a 0.25 mm tungsten needle (Fine Science Tools). Every minute starting at ~35 min pw, light transmission images were acquired of all larvae. For quantification of wound margin necrosis, agarose and bathing medium (isotonic or hypotonic) was supplemented with 1 μM Sytox Orange (Life Technologies).

Fluorescence was excited using light-emitting diode light and a 549/15 excitation filter (Lumencor) and emission was acquired using a 632/60 emission filter (Chroma). Imaging of H2O2 production was carried out as previously described.39 All images were acquired at room temperature (~26 °C) using NIS-Elements software (Nikon). To analyse cells, a 435 nm ultraviolet MicroPoint laser (Andor) was used for confocal microscopy as previously described,40 subcloning the bactin2, hsp70 or hsp promoter,41 full-length hyper, gcam3 (ref. 42) or gcam3-mKate2 and an SV40 polyadenylation sequence into the pDestTo2C2 vector backbone with minimal tole elements. The lysC:Gcam3-mKate2 plasmid drives the expression of the gcam3 cytosolic Ca2+ sensor fused to the mKate2 protein.

Generation of transgenic lines. A solution containing 25–25 pg of the bactin2:hyPer, hsp70:GCaMP3 or lysC:GCaMP3-mKate2 plasmid and transposase mRNA was injected into the cytosol of one-cell stage casper or AB embryos. Injected larvae with mosaic cardiac eGFP (enhanced green fluorescent protein) expression were raised to sexual maturity and screened by crossing with wt fish to identify larvae with mosaic cardiac eGFP (enhanced green fluorescent protein) expression. Anesthetized 2.5 h, and were imaged 2 min using a 435 nm ultraviolet MicroPoint laser (Andor). For confocal microscopy imaging of cytosolic Ca2+ signals, either the TH/hsp70:GCaMP3 line was used or GEX-GECO1, which was injected as mRNA (1 ng) into one-cell stage casper embryos. To image GCaMP3, 2–3 dpf embryos were heat shocked at 37 °C for 1 h, and were imaged 2–3 h later by exciting laser light at 488 nm and collecting emission using a 535/20 bandpass filter (Chroma). Imaging was acquired every 5 s for a period of 3–40 min. Wounding was carried out at ~30 s using a 435 nm ultraviolet MicroPoint laser (Andor). For confocal microscopy imaging of cytosolic Ca2+ signals, either the TH/hsp70:GCaMP3 line was used or GEX-GECO1, which was injected as mRNA (1 ng) into one-cell stage casper embryos. To image GCaMP3, 2–3 dpf embryos were heat shocked at 37 °C for 1 h, and were imaged 2–3 h later by exciting laser light at 488 nm and collecting emission using a 535/20 (Chroma) bandpass filter. GEX-GECO1 fluorescence was excited using the 405 and the 488 laser line and emission was collected using the 535/20 bandpass filter. For calcium-switch experiments, anesthetized 2.5–3 dpf larvae that had been injected at the one-cell stage with mRNA encoding cPLA2–mKate2 and GEX-GECO1 (1 ng) were preincubated for 5 min in isotonic Ca2+−free E3 supplemented with 1 mM EGTA (Sigma). The larvae were wounded using a tungsten needle, embedded in agar and then mounted on the microscope stage using ~50 μl of E3 medium with or without immersion. While still transiently acquired 3 ml of hypotonic E3 containing 1 mM EGTA was added on top of the agar. CaCl2 was added back to the solution at 5 min to reach a final [Ca2+]i of 3.0 mM.

Immunofluorescence. Anaesthetized intact and wounded larvae were fixed 10 min in 4% paraformaldehyde (Sigma) at 4 °C overnight. Embryos were rinsed four times for 10 min each in PBS, permeabilized for 5 min in 0.25% triton-EDTA solution and then rinsed again four times for 10 min each in PBSTx (PBS, 1% Triton X-100). Embryos were blocked in 5% goat serum (Sigma) in PBSTx for 1 h and then incubated with the primary, anti-phospholipase A2 antibody (Abcam 135825) diluted 1:4000 in blocking reagent overnight at 4 °C. Following four washes in PBSTx for 1 h each, the embryos were incubated with the secondary antibody, Alexa 488 goat anti-rabbit IgG (Life Technologies) diluted 1:1,000 in blocking reagent, for 2 h at room temperature and then rinsed four times for 1 h each in PBSTx. Imaging was carried out with the same illumination and acquisition settings for all samples on the spinning disk confocal microscope described above, and similar results were obtained in three independent experiments (Supplementary Fig. 5b).

Image processing and data analysis. Leucocyte recruitment was determined by counting all migrating cells that arrived at the wound margin within 43 min pw as judged from the 1 min / frame time-lapse movies of light transmission microscopy images. Cells that already resided at the wound margin at the beginning of the time-lapse sequence (~3 min pw) were not counted.

To quantify cPLA2–mKate2 translocation to the nuclear membrane, maximal intensity projection images were generated using Fiji42 and the coordinates of the centre of mass of those cells that showed translocation within 5 min pw were measured at the time of wounding. To normalize the relative positions of these cells when compared with the wound margin, coordinates of the centre of the approximately circular wounds and their diameters were measured in all experiments together with the angle between the fin margin and the x axis of the images. The distances of the nuclei from this normalized wound margin were calculated and nucleus coordinates were then transformed, placing them at their original distance from a normalized 60 μm wide wound in the middle of a horizontal tail fin. Relative translocation densities were calculated by counting the average number of cells showing nuclear translocation per experiment in the whole field of view, divided into 9 × 9 μm units, the size of an average cell. cPLA2–mKate2 translocation was also quantified by counting the average number of cells showing translocation within the whole field of view. To assess the extent of perineuronal translocation in time-series experiments, nuclei were automatically detected using the ‘analyse particle’ function of Fiji after water-shedding the binary masks of the thresholded images. A translocation ratio was calculated by dividing the average fluorescence intensity of the outer 0.5 μm band of the nuclei by the average inner intensity. Sytox Orange signals were quantified by measuring the fluorescent area of Sytox Orange positive nuclei at the wound margin. HyPer ratio images were calculated as previously described.43 To calculate GEX-GECO1 ratio images, smoothed (one-pixel thickness, background subtracted and thresholded EXmax and EXmean images were divided using Fiji. To quantify Z/A signals in individual moving cells (Fig. 3), regions of interest automatically identified for nuclear translocation measurements were used, and the average value on the ratio images of a 1 μm band around the nuclei was measured (that is the perinuclear cytoplasmic region). To assign the measurements in each frame along the time series to the individual moving cells, tracks of region of interest displacement were calculated using the Simple Tracker algorithm in Matlab (MathWorks).

Leukocyte trajectory analysis. Trajectory analysis was carried out on the leukocyte time-lapse data that were used to quantify wound recruitment of leucocytes or on 60 min time-lapse data of unwounded larvae. Trajectories were generated by marking the approximate centre of mass of those cells that moved to the tail fin, and could be identified with adequate reliability using the MTrackJ plugin of Fiji. Only those cells that described a path of at least 50 m units, the size of an average cell. cPLA2–mKate2 translocation to the nuclear membrane, maximal intensity projection images were generated using Fiji and the coordinates of the centre of mass of those cells that showed translocation within 5 min pw were measured at the time of wounding. To normalize the relative positions of these cells when compared with the wound margin, coordinates of the centre of the approximately circular wounds and their diameters were measured in all experiments together with the angle between the fin margin and the x axis of the images. The distances of the nuclei from this normalized wound margin were calculated and nucleus coordinates were then transformed, placing them at their original distance from a normalized 60 μm wide wound in the middle of a horizontal tail fin. Relative translocation densities were calculated by counting the average number of cells showing nuclear translocation per experiment in the whole field of view, divided into 9 × 9 μm units, the size of an average cell. cPLA2–mKate2 translocation was also quantified by counting the average number of cells showing translocation within the whole field of view. To assess the extent of perineuronal translocation in time-series experiments, nuclei were automatically detected using the ‘analyse particle’ function of Fiji after water-shedding the binary masks of the thresholded images. A translocation ratio was calculated by dividing the average fluorescence intensity of the outer 0.5 μm band of the nuclei by the average inner intensity. Sytox Orange signals were quantified by measuring the fluorescent area of Sytox Orange positive cells at the wound margin. HyPer ratio images were calculated as previously described.43 To calculate GEX-GECO1 ratio images, smoothed (one-pixel thickness, background subtracted and thresholded EXmax and EXmean images were divided using Fiji. To quantify Z/A signals in individual moving cells (Fig. 3), regions of interest automatically identified for nuclear translocation measurements were used, and the average value on the ratio images of a 1 μm band around the nuclei was measured (that is the perinuclear cytoplasmic region). To assign the measurements in each frame along the time series to the individual moving cells, tracks of region of interest displacement were calculated using the Simple Tracker algorithm in Matlab (MathWorks).

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the centre of mass of the wound (W), and $d_{cw}$ being the distance between the track endpoint and W.

**Pharmacological treatments, morpholino injection and RT–PCR.** Pharmacological inhibition was achieved by preincubating larvae for 40–60 min in E3 or isotic E3 supplemented with the following compounds: 20 µM ACA (Sigma), 20 µM MK866 (Calbiochem), 20 µM EDBC (Santa Cruz), 100 µM bestatin (Cayman), 20 µM Zileuton (Cayman) and 100 µM DFI (Sigma). Mounting agarose and imaging medium (E3 or isotic E3) also contained the same concentration of the indicated compounds. AA (Cayman) and its metabolites, 5(S)/HETE, 15(S)/HETE, 5-KETE and LTB4 (Cayman), either were preincubated for 40-60 min or, when used in conjunction with inhibitors, were added to the imaging medium after wounding, when acquisition started. Leukocyte recruitment was followed for 60 min in the latter case or when metabolites were applied to unwounded fish. Dimethylsulphoxide was used as a solvent for all non-water-soluble compounds, with a maximal final concentration of 1% in the media.

One-cell stage larvae were injected (2.3 nl) with the splice blocking morpholino (Gene Tools) cpla2 MO1, 5'-AAGGGTCACTTACTATAATGTGGGA-3' (1 mM), and translation blocking morpholinos oxxr1 MO 5'-GATGTTACCTATTGAGAACGGT-3' (1 mM), itadl MO 5'-AGCTAGGGTCTGAAACTGGAGTC-AT-3' (0.25 mM; ref. 48), cpla2 MO2 5'-ATGCTCAACTATAATGTTGGACATT-3' (1 mM). The latter gave the same phenotype as the cpla2 MO1 (data not shown). All depicted experiments were carried out using cpla2 MO1. To confirm knockdown efficiency of the cpla2 splice morpholino, RNA was prepared from 2.5–3 dpf larvae using the RNeasy mini kit (Qiagen) followed by one-step RT–PCR (Qiagen) using the following primers: cpla2 forward, 5'-CCATCTGTGACCTCAAAGTGC-3'; cpla2 reverse, 5'-CGTCCACCTTGGGTAAATG-3'. The sequenced RT–PCR product showed that cpla2 MO mediated splice perturbation produced a 23 bp insertion in the cpla2 mRNA, introducing a premature stop codon into the resulting splice-mutant mRNA, coding for a 21 amino acid truncated translation product. Knockdown efficiency was further confirmed by immunofluorescence staining of endogenous cpla2 in wt and morphant larvae (Supplementary Fig. S2b).

To confirm that the oxxr1 MO phenotype is not due to off-target effects as a result of p53-dependent apoptosis, we also tested it in combination with inhibitors, either were applied by preincubation for 40 min. The sequenced RT–PCR product showed that cpla2 MO mediated splice perturbation produced a 23 bp insertion in the cpla2 mRNA, introducing a premature stop codon into the resulting splice-mutant mRNA, coding for a 21 amino acid truncated translation product. Knockdown efficiency was further confirmed by immunofluorescence staining of endogenous cpla2 in wt and morphant larvae (Supplementary Fig. S2b).

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**Preparation of clarified cytoplasm extract.** Caco-2 cells (~80 million) were trypsinized and resuspended in 1 ml isotic E3 medium (E3 supplemented with 140 mM NaCl). The cells were sonicated then ultracentrifuged at 100,000g for 15 min. The clear supernatant was used in a 1:1 dilution with isotic low melting agarose to embed 3 dpf larvae for wound healing experiments, resulting in a solution with ~10–20% cytosolic content of Caco-2 cells.

**Statistics.** All error bars indicate standard errors of means (s.e.m.). All P-values have been derived by an unpaired, two-tailed t-test assuming unequal variances (heteroscedastic) using Excel (Microsoft). No statistical method was used to predetermine sample sizes. The experiments were not randomized and the investigators were not blinded to allocation during experiments or outcome assessment.

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**Figure S1** Isotonic inhibition of leukocyte recruitment is reversible and is not a result of necrosis or cytoplasmic leakage. (a) Scheme of Experimental design. Larvae are wounded within a small volume (100-200 µl) of isotonic low melting agarose, wounded and imaged for 10 min. 3 ml of ‘isotonic’ (‘i’, 145 mM NaCl) or hypotonic medium (‘h’, 5 mM NaCl) are then added on top of the isotonic agar pad, and wound recruitment of leukocytes within t = 60 min after medium addition is quantified by light transmission microscopy (i.e. allowing 20 min for equilibration of salt concentration throughout the agar pad as compared to our standard t = 40 min assays). Rapidly migrating cells (which correspond to leukocytes) are highlighted with coloured tracks. (b) Left panel, representative time-lapse images of wound recruitment after shifting the medium that the larvae were wounded in from isotonic to either hypotonic medium (‘i → h’ shift) or isotonic medium (‘i → i’ shift). Right panel, quantification of leukocyte recruitment within 60 min after tonicity shift. Number of larvae (n) used for the analyses is given in parentheses on the graphs. Error bars, SEM. ***, t-test p<0.0005. Scale bar, 100 µm. (c) Staining of necrotic cells with Sytox orange. Larvae were wounded either in hypotonic (h) or isotonic medium supplemented with 1 µM Sytox Orange. Necrosis was quantified as area of Sytox-fluorescent cells at the wound site at t = 40 min after injury. Scale bar, 100 µm. (d) Leukocyte recruitment (within 40 min) in response to tail fin incisions in hypotonic (‘h’) or isotonic (‘i’) medium in the presence or absence of 5 µM AA, 0.5 and 5 mM ATP or cytoplasm extract from Caco-2 cells (see Methods section for details). Number of larvae (n) used for the analyses is given in parentheses on the graphs. Error bars, SEM. ***, t-test p<0.0005.
Figure S2 (a) cpla2 mRNA expression in leukocyte vs. non-leukocyte tissue. Leukocyte/non-leukocyte total mRNA was generated by FACS sorting of dissociated, transgenic zebrafish larvae expressing a red fluorescent protein (mKate2) under the control of the lysC promoter. cpla2 mRNA expression between these samples was compared by semi-quantitative RT-PCR. (b) Immunofluorescence staining of cPLA2 in intact and wounded wt, cpla2 morphant and cPLA2-mKate2 overexpressing larvae (expression of the latter shown in red). Scale bar, 10 µm. (c) Average leukocyte recruitment after hypotonic tail fin wounding of wt, cpla2 morphant, or cpla2 morphant larvae that have been coinjected with mRNA encoding cPLA2-mKate2 for rescue. Data for the wt and cpla2 morphant larvae derive from the same data set as shown in Fig. 4a. (d) Average leukocyte recruitment after hypotonic tail fin wounding in the presence of 20 µM non-selective PLA2 inhibitor N-(p-Amylcinnamoyl) anthranilic Acid (ACA). Number of larvae (n) used for the analyses is given in parentheses on the graphs. Error bars, SEM. ***, t-test p<0.0005.
Figure S3 Lipoxygenases, but not prostaglandins or canonical leukotrienes are involved in wound recruitment of leukocytes. Average recruitment of leukocytes after hypotonic tail fin wounding in the presence of (a) 20 µM MK-886 (ALOX inhibitor, more selective for ALOX5), 20 µM EDBC (ALOX inhibitor, more selective for ALOX12/15), (b) 100 µM Bestatin (LTA₄ inhibitor), or 20 µM Zileuton (ALOX inhibitor more selective for ALOX5). (c) Average recruitment of leukocytes after tail fin wounding at indicated tonicity in the presence/absence of 15 µM AA and 100 µM acetylsalicylic acid (ASA, cyclooxygenase inhibitor). (d) Average recruitment of leukocytes after isotonic tail fin wounding in the presence/absence of 15 µM AA and 100 µM Bestatin. (e) Average recruitment of leukocytes after hypotonic tail fin wounding of wt or lta4h morphant larvae, using a previously published translation targeting morpholino. (f) alox5 mRNA expression in leukocyte vs. non-leukocyte tissue. Leukocyte/non-leukocyte total mRNA was generated by FACS sorting of dissociated, transgenic zebrafish larvae expressing a red fluorescent protein (mKate2) under the control of the lysC promoter. alox5 mRNA expression between these samples was compared by semi-quantitative RT-PCR. Number of larvae (n) used for the analyses is given in parentheses on the graphs. Error bars, SEM. *, t-test p< 0.05. **, t-test p< 0.005. ***, t-test p<0.0005.
**Figure S4** (a) Average recruitment and migratory parameters of leukocytes after hypotonic wounding of larvae that had, or had not been pretreated with 5-KETE. For OXE-R desensitization, larvae were soaked for 90 min in 2 µM 5-KETE prior to wounding. 5-KETE was washed out, and leukocyte recruitment to hypotonic wounds was measured in treated and non-treated samples. (b) Average recruitment of leukocytes within 40 min after isotonic tail fin wounding of *wt* or *oxer1* morphant larvae in the presence of 5 µM AA in the medium. (c) Average recruitment of leukocytes within 40 min after hypotonic tail fin wounding of *p53* morphant and *p53*-*oxer1* morphant embryos. (d) *oxer1* mRNA expression in leukocyte vs. non-leukocyte tissue. Leukocyte/non-leukocyte total mRNA was generated by FACS sorting of dissociated, transgenic zebrafish larvae expressing a red fluorescent protein (mKate2) under the control of the lysC promoter. *oxer1* mRNA expression between these samples was compared by semi-quantitative RT-PCR. (e) HyPer imaging of wound margin H$_2$O$_2$ production in response to wounding *wt*, *cpla2* morphant and *oxer1* morphant larvae. Upper panel, representative HyPer-ratio images. Red, high [H$_2$O$_2$]. Blue, low [H$_2$O$_2$]. Lower panel, normalized HyPer-ratio as a function of time after wounding. Number of larvae (n) used for the analyses is given in parentheses on the graphs. Error bars, SEM. NS, t-test p > 0.05. *, t-test p < 0.05. **, t-test p < 0.005. ***, t-test p < 0.0005. Scale bar, 100 µm.
Figure S5 (a) Two paradigms of tissue damage detection. Left panel, classic ‘cell-integrity paradigm’: Passive leakage of cytoplasmic DAMPs (damage associated molecular patterns) from broken cells produces leukocyte necrotaxis. Right panel, ‘tissue integrity paradigm’: Epithelial barrier breakage induces cell swelling and de novo production of chemoattractants that attract leukocytes. (b) Scheme of proposed regulatory circuits. Black arrows, mechanisms proposed by this study. Grey arrows, mechanisms proposed by previous studies (see references 14, 29, 34, 37). Dashed grey arrows, speculative mechanisms.
Supplementary Videos

**Video S1 - Hypotonicity is required for rapid leukocyte recruitment to larval zebrafish tail fin wounds**
This video shows leukocyte recruitment after tail fin wounding of *wt* larvae in hypotonic (control) or isotonic (145mM NaCl) medium. Imaging starts ~3 min pw (60 sec/frame). Scale bar: 100 µm.

**Video S2 - Isotonicity reversibly inhibits rapid leukocyte recruitment to larval zebrafish tail fin wounds**
This video shows leukocyte recruitment after tail fin wounding of *wt* larvae in isotonic medium that was shifted to hypotonic medium (‘i®h’ shift) or isotonic medium (‘i®i’ shift). Imaging starts ~3 min pw (30 sec/frame). Scale bar: 100 µm.

**Video S3 - Hypotonicity locally activates cPLA2 at the wound site**
This video montage shows cPLA2-mKate2 translocation to the nuclear membrane induced by UV-laser wounding in hypotonic (hypo), isotonic (iso) or hypotonic medium supplemented with 500 µM Gd³⁺ (hypo+Gd³⁺). Laser wounding at ~1 min (15 sec/frame). Scale bar: 100 µm.

**Video S4 - Gd³⁺ exposure enhances wound margin swelling**
This video shows leukocyte recruitment after hypotonic tail fin wounding of *wt* larvae in the presence or absence of 500 µM Gd³⁺. Imaging starts ~3 min pw, Gd³⁺ added at 0 min (60 sec/frame). Scale bar: 100 µm.

**Video S5 - Extracellular Ca²⁺ is required for cPLA2 activation**
This video montage shows cPLA2-mKate2 translocation to the nuclear membrane induced by calcium-switch in hypotonic medium. Hypotonic Ca²⁺-free medium supplemented with 1mM EGTA was added at 0 min to the larvae that were prewounded in isotonic medium with EGTA. Larve were imaged for 40 min with (A, C) or without (B) readdition of Ca²⁺ at 5 min. Videos from different experiments. Low magnification scale bar: 100 µm, high magnification 10 µm, (15 sec/frame).

**Video S6 - Tail fin injury rapidly increases cytoplasmic [Ca²⁺] at the wound site irrespective of medium tonicity**
This video shows the cytosolic Ca²⁺ signal induced by UV-laser wounding of larval zebrafish tail fins maintained in hypotonic (h) or isotonic (i) medium. Laser wounding at ~19 sec (3 sec/frame). Scale bar: 100 µm.

**Video S7 - cPLA2 is required for rapid leukocyte recruitment to larval zebrafish tail fin wounds**
This video shows leukocyte recruitment in *cpla2* morphant larva (*cpla2 MO*) vs. *wt* larva 3dpf. Imaging starts ~3 min pw (60 sec/frame). Scale bar: 100 µm.

**Video S8 - 5-KETE is a chemoattractant for leukocytes in zebrafish**
This video shows leukocyte recruitment after isotonic tail fin wounding of *wt* larvae in the presence or absence of 2 µM 5-KETE (5-oxo-ETE). Imaging starts ~3 min pw, 5-KETE added at 0 min (60 sec/frame). Scale bar: 100 µm.

**Video S9 - OXE-R is required for rapid leukocyte recruitment to larval zebrafish tail fin wounds**
This video shows leukocyte recruitment in *oxer1* morphant larva (*oxer1 MO*) vs. *wt* larva 3dpf. Imaging starts ~3 min pw (60 sec/frame). Scale bar: 100 µm.