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IMMUNOLOGY

PGE$_2$ production at sites of tissue injury promotes an anti-inflammatory neutrophil phenotype and determines the outcome of inflammation resolution in vivo

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Neutrophils are the first immune cells recruited to a site of injury or infection, where they perform many functions. Having completed their role, neutrophils must be removed from the inflammatory site—either by apoptosis and efferocytosis or by reverse migration away from the wound—for restoration of normal tissue homeostasis. Disruption of these tightly controlled physiological processes of neutrophil removal can lead to a range of inflammatory diseases. We used an in vivo zebrafish model to understand the role of lipid mediator production in neutrophil removal. Following tailfin amputation in the absence of macrophages, neutrophillic inflammation does not resolve, due to loss of macrophage-dependent handling of eicosanoid prostaglandin E2 (PGE$_2$) that drives neutrophil removal via promotion of reverse migration. Knockdown of endogenous PGE synthase gene reveals PGE$_2$ as essential for neutrophil inflammation resolution. Furthermore, PGE$_2$ is able to signal through EP4 receptors during injury, causing an increase in Alox12 production and switching toward anti-inflammatory eicosanoid signaling. Our data confirm regulation of neutrophil migration by PGE$_2$ and LXA$_4$ (lipoxin A$_4$) in an in vivo model of inflammation resolution. This pathway may contain therapeutic targets for driving inflammation resolution in chronic inflammatory disease.

INTRODUCTION

Inflammation is a critical process that maintains normal tissue homeostasis following injury or infection, by removing potential pathogens and beginning the process of wound repair and healing. Neutrophils are the first immune cells recruited to a site of injury or infection, where they carry out a number of functions (*1*). They destroy foreign pathogens, control the spread of infection, and thereby minimize tissue damage. Neutrophils engulf bacteria and cell debris at the site of inflammation through phagocytosis and release antimicrobial molecules by degranulation. Along with the release of reactive oxygen species, this activity aids in the destruction of pathogens (*2*) and permits neutrophil penetration into otherwise inaccessible tissues (*3*). Having completed their role, neutrophils must then be removed from the inflammatory site for successful inflammation resolution to occur. If this tightly controlled physiological process is disrupted, then failure of inflammation resolution can lead to inflammatory diseases such as chronic obstructive pulmonary disease.

There are several mechanisms by which neutrophils can be removed from sites of inflammation. Caspase-dependent neutrophil apoptosis and subsequent efferocytosis by macrophages have been shown to contribute to the successful resolution of inflammation in mouse (*4*), human (*5*), and zebrafish (*6*). Natural killer cells can also induce granulocyte apoptosis as a proresolving mechanism (*7*).

Neutrophil apoptosis is tightly coupled to uptake by macrophages to prevent tissue damage (*8*). Macrophages phagocytose pathogens and apoptotic cells and contribute to inflammation resolution (*9*), tissue remodeling, and regeneration (*9*, *10*); however, their influence on inflammation resolution is incompletely defined.

More recently, it has been demonstrated that neutrophils can also undergo reverse migration away from sites of inflammation, which, in some circumstances, might contribute to the dissipation of the inflammatory burden and hence to inflammation resolution (*11*, *12*). In the zebrafish, reverse migration is anti-inflammatory and is suppressed by proinflammatory stimuli such as hypoxia (*13*). Variations on this process have also been seen in mouse models (*14*, *15*) and in human neutrophils (*16*, *17*). Understanding the molecular mechanisms that govern neutrophil removal, either via efferocytosis or via reverse migration, is essential to developing new therapeutics for inflammation resolution.

Macrophages are well known to be key mediators in determining the outcome of the inflammatory response (*18*). Macrophages and neutrophils secrete a range of pro- and anti-inflammatory cytokines, dependent on the local inflammatory environment. Studies in mammalian systems (*19*, *20*) have shown that upon uptake of apoptotic cells, macrophages regulate pro- and anti-inflammatory cytokine production (*21*). When fed with apoptotic neutrophils, lipopolysaccharide-stimulated human monocyte-derived macrophages down-regulate key proinflammatory cytokines and up-regulate anti-inflammatory mediators transforming growth factor–β1 (TGFβ1), prostaglandin E2 (PGE$_2$), and platelet activating factor (PAF). Prostaglandins, such as PGE$_2$, are not stored in cells, but rather produced following arachidonic acid metabolism and therefore can be produced by almost all cells in the body (*22*). PGE$_2$ is the most abundant prostaglandin in humans (*23*) generated via the action of cyclooxygenase enzymes. While PGE$_2$ is known to have proinflammatory

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properties under defined conditions (24), it has also been shown to have anti-inflammatory functions (25). In zebrafish cancer models, inhibition of PGE_2 production led to a change in macrophage behavior and enhanced proinflammatory activity (26), consistent with PGE_2 directing macrophages toward an anti-inflammatory phenotype (27). In human peripheral blood neutrophils, PGE_2 switches lipid mediator biosynthesis from predominantly proinflammatory leukotriene B_4 [LTB_4; 5-lipoxygenase (5-LO)–initiated pathway] to lipoxin A_4 (LXA_4), which reduces neutrophil infiltration into exudates (28). These studies support a role for PGE_2 in influencing immune cell phenotype and led us to ask whether macrophages might be altering local lipid mediator signaling as a mechanism for influencing inflammation resolution in vivo.

Using a zebrafish model of in vivo inflammation resolution, we examined the potential role of the lipid mediator PGE_2 in driving inflammation resolution. We demonstrate that macrophage uptake of apoptotic cells is necessary for successful inflammation resolution; PGE_2 is produced and acts via EP4 receptors to drive inflammation resolution by reverse migration.

RESULTS

Macrophage clearance of apoptotic cells is necessary for successful resolution of neutrophilic inflammation in vivo

Inflammation requires the presence and activity of both neutrophils and macrophages. To test whether neutrophil dynamics were influenced by the presence of macrophages, we ablated macrophages using tissue-specific bacterial nitroreductase expression and by treatment with metronidazole as previously described (29, 30). Injury was performed on 3 days post fertilization (dpf) macrophage-ablated larvae, and neutrophil and macrophage numbers were assessed at the time points indicated (Fig. 1A). In control larvae, neutrophil numbers peaked between 4 and 6 hours post injury (hpi), with numbers returning to basal levels by 24 hpi, demonstrating spontaneous resolution of inflammation. Macrophages were recruited to the site of injury at a lower rate, with numbers plateauing at 12 hpi and remaining high until at least 24 hpi. In metronidazole-treated larvae, macrophages were not seen at the wound throughout the inflammatory time course, confirming successful ablation. Neutrophil numbers at the wound site were significantly increased at 24 hpi in macrophage-depleted larvae compared to control larvae, suggesting failed inflammation resolution (Fig. 1, A and B). In contrast to wound-site neutrophils, whole-body neutrophil counts were unaltered (Fig. 1C). These data demonstrate that macrophages are necessary at the site of tissue injury for normal inflammation resolution to occur.

In many models of inflammation, including the zebrafish, apoptosis of neutrophils contributes to inflammation resolution (6, 31). It might therefore be expected that macrophage depletion would leave apoptotic neutrophils at the wound site. To test whether the persistence of apoptotic corpses explained the increase in neutrophil numbers, we ablated macrophages by treating transgenic zebrafish with metronidazole at 7 dpf, injuring the tailfin as before at 8 dpf and then fixing at 24 hpi in 4% paraformaldehyde for TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) staining. Older larvae were used to increase cell numbers involved in the inflammatory response and to increase our ability to detect apoptotic events. We costained for endogenous peroxidase activity of neutrophils using a modification of the tyramide signal amplification (TSA) system (6). Double-stained cells corresponding to apoptotic neutrophils were then counted in macrophage-depleted larvae. Although the number of neutrophils was higher in the metronidazole-treated fish, the percentage of these neutrophils that were apoptotic was not different in the metronidazole-treated group (Fig. 1D), suggesting that persisting apoptotic neutrophil corpses did not account for the increase in neutrophil numbers in the absence of macrophages. During analysis of these images, we did notice a striking and unexpected increase in TUNEL-positive, TSA-negative cells in macrophage-depleted larvae (Fig. 1E), corresponding to apoptotic cells from nonneutrophil lineages. Representative images of stained cells are shown in Fig. 1F. This finding demonstrates that macrophages have an important role in removing apoptotic cells arising from tissue injury and that neutrophil persistence is not due to uncleared apoptotic neutrophils. This suggested to us that the majority of neutrophils might be removed from the wound by an alternative mechanism, such as reverse migration (12).

Inflammation resolution is not exclusively dictated by direct macrophage-neutrophil interaction

Neutrophil-macrophage interaction has been observed during the inflammatory response (32) and, under some circumstances, this has been shown to alter neutrophil migratory behavior in ways that could explain the observed failure of inflammation resolution in the absence of macrophages. We therefore sought to analyze neutrophil migration before and after interaction with macrophages to see whether that interaction caused neutrophils to migrate away from the wound, thereby dissipating the inflammatory burden. We tracked individual transgenically labeled macrophages and neutrophils, monitoring their interaction with other inflammatory cells. We tracked neutrophils and macrophages during an inflammatory response and plotted their x,y coordinates (Fig. 2A). Interaction between red (macrophage) and green (neutrophil) tracks was identified, and for each interaction, we identified whether contact between a macrophage and a neutrophil induced a significant change in behavior of the neutrophil. Direct cell contact was defined as when membranes of both cell types could be seen to touch. Interaction with a macrophage made no significant difference in the neutrophil meandering index (Fig. 2B), suggesting that interaction did not lead to a change in migratory behavior. Moreover, analysis of vector maps of neutrophils before and after interaction with a macrophage showed no clear difference between the two vector maps. The vector maps show the overall direction a neutrophil takes during the analyzed period (Fig. 2C). When analyzed, the percentage of reverse-migrated neutrophils that did not interact with a macrophage was 71%, compared to 65% of neutrophils reverse-migrating following a macrophage interaction (Fig. 2D). There was no significant difference in reverse migration behavior following macrophage interaction ($P = 0.78$, Fisher's exact test). Direct contact between macrophages and neutrophils is therefore not sufficient to completely explain the observed changes in neutrophil direction, suggesting that an additional soluble factor is involved.

The eicosanoid PGE_2 is necessary for timely inflammation resolution

Previous studies in vitro have shown that, following uptake of apoptotic cells, macrophages can alter both their profile of cytokine production and their phenotype, from pro- to anti-inflammatory (21). It has been reported that levels of TGFβ1 and PGE_2 increase following
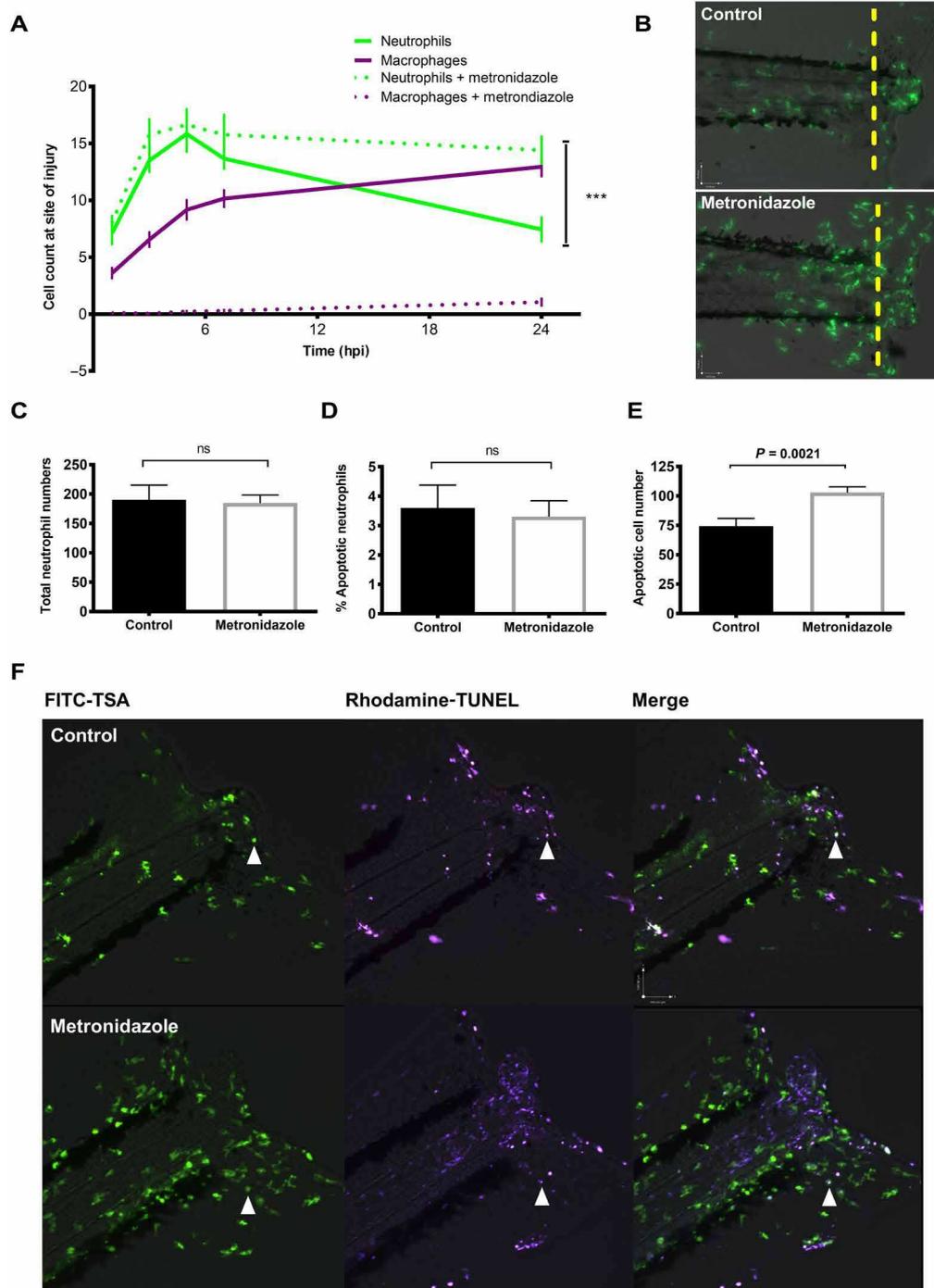
Fig. 1. Macrophage clearance of apoptotic cells is necessary for successful resolution of neutrophilic inflammation in an in vivo zebrafish model. (**A**) Neutrophil and macrophage counts at the wound in triple transgenic *Tg(cfms:Gal4)i186;Tg(UAS:nfsB-mCherry)i149;Tg(mpx:EGFP)i114* larvae in the presence or absence of metronidazole. Neutrophil numbers are significantly higher at the wound in the absence of macrophages at 24 hpi, ***$P < 0.0001$. Statistics: Two-tailed nonpaired *t* test comparing neutrophil counts at 24 hpi with or without macrophage present. All data are $n = 18$ from three individual experiments plotted as means ± SEM. (**B**) Representative photomicrographs at 24 hpi showing increased neutrophil numbers at the site of injury in metronidazole-treated larvae compared to control. Wound area classed as area to the right of the yellow dashed line. Images were taken using ×10 magnification on a TE2000U inverted microscope (Nikon). (**C**) Total neutrophil numbers are not affected in the absence of macrophages. Data are $n = 17$ individual larvae. (**D**) Dual fluorescein isothiocyanate (FITC)–TSA and Rhodamine-TUNEL–positive cell counts in 8 dpf larvae at 24 hpi in the presence or absence of metronidazole show no significant difference (ns) in the percentage of apoptotic neutrophils. (**E**) Total apoptotic cell counts of TUNEL-positive cells in 8 dpf larvae at 24hpi. Macrophage-depleted larvae have significantly more apoptotic bodies at the wound, $P = 0.0021$. All data are presented as means ± SEM, $n = 12$, from two individual experiments for (D) and (E). (**F**) Representative photomicrographs of 8 dpf larvae at 24 hpi treated with either dimethyl sulfoxide (DMSO) control or metronidazole and dual-stained with FITC-TSA to label neutrophils and Rhodamine-TUNEL to label apoptotic cells at the wound (white arrow head). Images were taken using ×10 magnification on a TE2000U inverted microscope (Nikon).
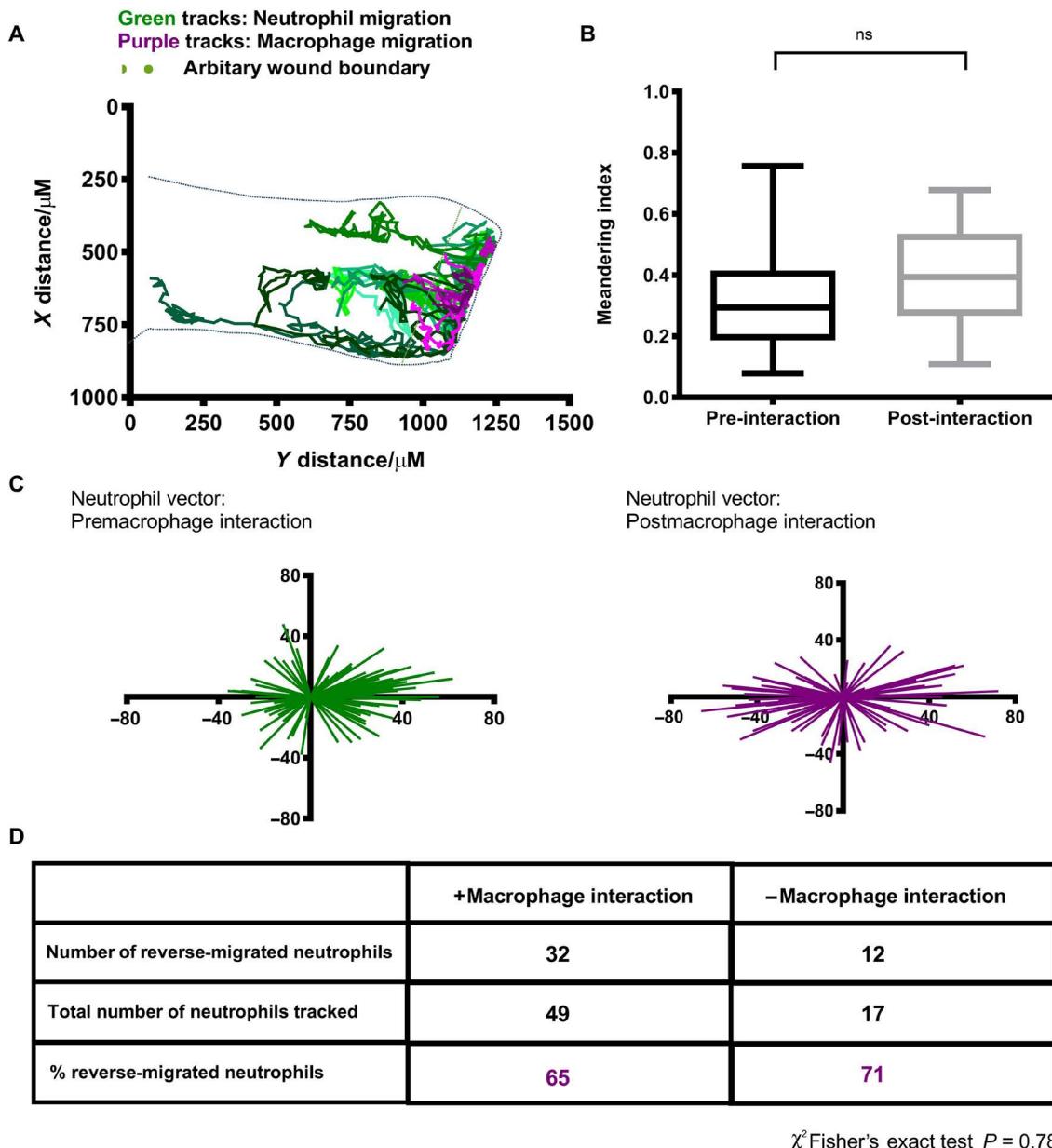
Fig. 2. Inflammation resolution is not exclusively dictated by direct macrophage-neutrophil interaction. (**A**) Neutrophil (eight green lines) and macrophage (six magenta lines) tracks during an inflammatory response are plotted using their *x,y* coordinates. Larval caudal fin outline superimposed over tracks to indicate position at wound. (**B**) The meandering index (migration pattern) of neutrophils before and after contact with a macrophage shows no significant difference. Number of cells tracked: 14. Two-tailed paired *t* test. (**C**) Representative vector maps of overall neutrophil direction pre (green) and post macrophage (magenta) interaction. (**D**) Neutrophils (65%) that interacted with a macrophage reverse-migrated, whereas 71% neutrophils without a macrophage interaction also reverse-migrated away from the wound site. Chi-squared Fisher's exact test indicated no significant difference in reverse migration with or without macrophage contact. $P = 0.78$.

phagocytosis of apoptotic cells, whereas levels of cytokines such as interleukin-10 and tumor necrosis factor–α fail to increase further. When PGE$_2$ production was blocked by indomethacin, TGFβ1 levels remained low, suggesting that PGE$_2$ might be an upstream regulator of macrophage phenotype following uptake of apoptotic cells (*21*). We therefore sought to establish whether PGE$_2$ could be a potential mediator in enhancing inflammation resolution following tail injury in vivo.

Microsomal PGE synthase, Ptges, is necessary for the conversion of prostaglandin H$_2$ into PGE$_2$ (*33*). PGE$_2$ levels can be significantly

reduced when Ptges expression is inhibited through the use of a morpholino antisense oligo (*34*). We used a previously published morpholino to knock down *ptges* to test the effect of reduced PGE$_2$ levels on neutrophil behavior during inflammation. Reverse transcription polymerase chain reaction (PCR) demonstrated a significant reduction in correctly spliced *ptges* transcript at 4 dpf (fig. S1). Neutrophil counts throughout the inflammatory response revealed a significant increase in neutrophil recruitment to the wound site. At the later time points, during the resolution phase, significantly more neutrophils were retained at the site of injury (Fig. 3A). Since

PGE$_2$ has been shown to be critical in hematopoiesis (35), we looked at total neutrophil number in *ptges* morphants. Knockdown of *ptges* significantly reduced the number of neutrophils in the whole zebrafish (Fig. 3B), demonstrating that the increase in neutrophil numbers seen during inflammation is not due to an overall increase in neutrophils within the zebrafish. These data demonstrate that Ptges activity plays an important role in the resolution of inflammation.

Genetic knockdown of *ptges* by morpholino has indicated that PGE$_2$ production is essential for successful inflammation resolution. Because of the well-documented nonspecific effects of some morpholinos (36), we wanted to confirm our findings using a second approach. To address this, we have used CRISPR/Cas9 technology to support our findings (37). We designed a guide RNA (gRNA) that targets the ATG of *ptges*. Successful mutation by Cas9 protein deletes an MwoI restriction site, allowing the efficiency of the guide to be assessed and the larvae to be genotyped (fig. S4) with an uncut mutant band present at 293 base pairs (bp). Using our tailfin injury model, we have injected *Tg(mpx:EGFP)i114* embryos at the one-cell stage with transactivating crRNA (tracrRNA) and Cas9 protein alone or in combination with a control gRNA, targeting *tyrosinase* (*tyr*) or a gRNA targeting *ptges*. *Tyrosinase* is the gene required for converting tyrosine into the pigment melanin, enabling visual identification of successful Cas9 knockdown (38). Genetic knockdown of *ptges* using Cas9 protein also leads to persisting neutrophils at the wound site compared to *tyr*-injected larvae (Fig. 3C), phenocopying the *ptges* morphant. Representative images were taken of each group to show this (Fig. 3D). These data strongly support the essential role of PGE$_2$ for successful neutrophil removal from wound sites.

PGE$_2$ is sufficient to drive inflammation resolution

PGE$_2$ has both anti- (39) and proinflammatory effects dependent on the timing of its production and concentration (24, 40). To test whether PGE$_2$ is sufficient to drive inflammation resolution, we immersed 3 dpf transgenic zebrafish larvae in a range of PGE$_2$ concentrations at 8 hours after tailfin transection, after peak neutrophil recruitment had occurred. Wound neutrophil numbers were then assessed at 12 hpi to look for accelerated inflammation resolution. Twelve hours post injury is a time point where untreated larvae still have relatively high neutrophil numbers, and therefore, an enhanced reduction in neutrophil numbers would be detectable. Neutrophil numbers were significantly decreased in a dose-dependent manner when treated with PGE$_2$, with 1 μM chosen as the optimal concentration (Fig. 3E). Exogenous PGE$_2$ was able to rapidly enhance neutrophil removal from the site of injury to return neutrophil numbers to basal levels, thereby promoting successful inflammation resolution. As PGE$_2$ reduced neutrophil numbers at the site of injury, we tested whether PGE$_2$ accelerated neutrophil apoptosis at the wound site. Three days post fertilization larvae were injured, treated with PGE$_2$, and fixed at 12 hpi for dual TUNEL (apoptosis) and TSA (neutrophil) staining. There was no difference in apoptotic cell numbers in larvae treated with PGE$_2$ compared to control (fig. S2A). This suggests that neutrophils are removed through a mechanism other than apoptosis.

Since exogenous PGE$_2$ could enhance inflammation resolution in our model, we investigated whether PGE$_2$ could rescue the persisting neutrophil phenotype observed with macrophage ablation. Zebrafish larvae with nitroreductase-expressing macrophages and green fluorescent protein (GFP)–expressing neutrophils were treated with metronidazole at 2 dpf, injured at 3 dpf, and treated with PGE$_2$

at 8 hpi. Cell counts were performed at 12 and 24 hpi. PGE$_2$ was able to significantly reduce neutrophil numbers at the wound in metronidazole-treated larvae at 12 hpi compared to metronidazole-only larvae, with means of 4.6 ± 0.4 and 9.6 ± 0.8, respectively (fig. S2B). At 24 hpi, neutrophil numbers remained significantly reduced (Fig. 3F). These data demonstrate that PGE$_2$ can drive inflammation resolution in the absence of macrophages, thus correcting the resulting inflammatory phenotype.

Neutrophil migration pattern, but not speed, is modulated by PGE$_2$

During inflammation resolution, neutrophils can be removed through multiple mechanisms, including neutrophil apoptosis (41) and reverse migration (13, 42). Macrophage depletion did not result in a detectable difference in apoptotic neutrophil numbers compared to control, due to either residual macrophage activity or the infrequency of neutrophil apoptosis in the zebrafish model. Nonetheless, PGE$_2$ can still reduce neutrophil numbers in these larvae, implying that neutrophils involved in the inflammatory response are being removed from the site of injury through a mechanism other than efferocytosis. We therefore investigated whether PGE$_2$ could enhance inflammation resolution by acceleration of neutrophil reverse migration in tail-transected zebrafish larvae. Using well-established photoconversion protocols (43, 44) in transgenic zebrafish expressing the photoconvertible fluorophore *kaede* in neutrophils, we labeled neutrophils at the wound site at 8 hours after tailfin transection and observed the effects of addition of PGE$_2$. Neutrophils at the wound site were tracked during the resolution phase of inflammation for 2 hours, from 10 to 12 hpi. Red, photoconverted neutrophils could be seen migrating away from the site of injury more readily in PGE$_2$-treated larvae (Fig. 4, A to C). PGE$_2$ significantly increased the number of neutrophils moving away from the wound area (Fig. 4D). This equates to there being a 50% difference in the number of reverse-migrated neutrophils in the PGE$_2$-treated fish at 140 min after conversion, with a significant *P* value of 0.0015. These data show that PGE$_2$ drives inflammation resolution by enhanced reverse migration away from the injury site.

We then asked whether this enhanced migration from the wound could be due to an increased migration speed of the neutrophils or to them leaving the site of injury sooner. Neutrophil migration speed away from the site of injury was measured in the same reverse migration assays described above (Fig. 4E). There was no significant difference in migration speed, implying that PGE$_2$ may enhance release of neutrophils from their patrolling behavior at the wound site. The timing of PGE$_2$ release is therefore critical to allow neutrophils to perform their role and then drive them away without compromising the inflammatory response.

Signaling through EP4 receptors contributes to inflammation resolution

PGE$_2$ can signal through four prostanoid receptors: EP1, EP2, EP3, and EP4 (45). The expression and distribution of these receptors vary between different tissues and cell types. EP4 is considered a stimulatory receptor and is expressed on human macrophages, with studies showing that blockage of EP4 signaling inhibits cytokine release by macrophages (46). All four EP receptors are present in zebrafish, with multiple paralogs of each receptor (47). EP4b is the most abundant in adult zebrafish and is closest to human EP4 phylogenetically (48). To test whether PGE$_2$ could be signaling in vivo through EP
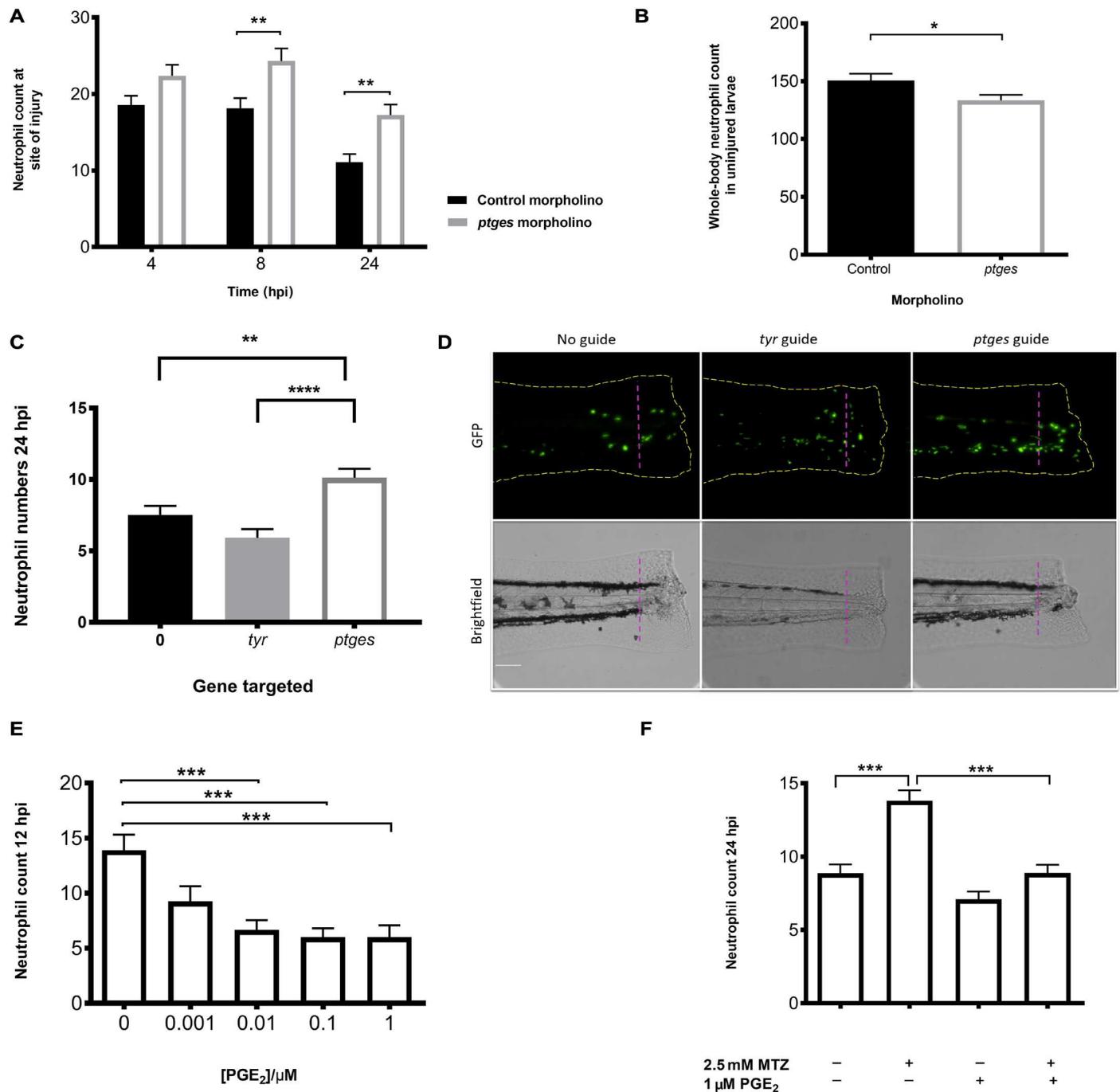
Fig. 3. In vivo effects of PGE$_2$ on neutrophils during an inflammatory response through genetic manipulation and PGE$_2$ supplementation. (A) Morpholino knockdown of endogenous *ptges* shows the necessity of PGE$_2$ during inflammation resolution, with a significant increase in neutrophil numbers at the wound site at 8 and 24 hpi. *$P < 0.01$, two-way analysis of variance (ANOVA) with Bonferonni's posttest. Data are n = minimum 30 from three experimental repeats. **(B)** Total neutrophil numbers in unstimulated larvae are reduced ($P < 0.05$, n = 24 larvae) in the absence of *ptges*, indicating that the increase during an inflammatory response is not due to increased neutrophils numbers overall. **(C)** Recapitulation of morphant phenotype in crispant. Three days post fertilization larvae with CRISPR/Cas9-mediated knockdown of *ptges* display significantly more neutrophils at the wound site at 24 hpi compared to a control-injected guide group targeting *tyr*. Injured crispants phenocopy *ptges* morphants. **$P < 0.01$, ****$P < 0.0001$, one-way ANOVA with Bonferroni posttest. Data are plotted as means ± SEM with a combined minimum of 40 larvae per group from three experimental repeats. **(D)** Representative images of crispant larvae at 24 hpi. Area to the right of the magenta dashed line indicates the wound site where GFP neutrophils are counted. Brightfield images demonstrate successful *tyr* knockdown as a reduction in pigmentation is visible. **(E)** Dose-response showing increasing concentrations of PGE$_2$ significantly drive neutrophilic inflammation resolution. PGE$_2$ was added at 8 hpi with neutrophil counts performed at 12 hpi. Neutrophil numbers at the site of injury are significantly reduced between 0.01 and 1 μM. **(F)** In the absence of macrophages, exogenous PGE$_2$ is able to significantly reduce neutrophil numbers back to basal levels at 24hpi. ***$P < 0.001$, one-way ANOVA with Bonferroni posttest. Minimum of 32 larvae from three repeats. MTZ, metronidazole.

receptors, we used a previously published antagonist, AH23848, to inhibit EP4 receptor signaling (49). As we were assessing endogenous PGE$_2$ signaling during a natural inflammatory response, where neutrophil numbers reduce from around 6 hpi, we performed 24 hpi counts for inhibitor assays to be able to detect neutrophil persistence over normal basal levels. Neutrophil counts at 24 hpi showed a significant increase in number when EP4 signaling was blocked, suggesting that endogenous PGE$_2$ may act via this receptor. Addition of PGE$_2$ at standard doses did not lead to significant abrogation of this effect (Fig. 5F). These data suggest that PGE$_2$ signals predominantly through the EP4 receptor, contributing significantly to inflammation resolution. However, it remained unclear how EP4 signaling might lead to altered neutrophil behavior.

Up-regulation of lipoxygenase by PGE$_2$ is necessary for inflammation resolution

The metabolism of the membrane lipid arachidonic acid is a complex pathway, producing not only prostaglandins but also other eicosanoids such as proresolving lipoxins and proinflammatory leukotrienes. Lipoxygenases (LOXs) are essential for the production of these metabolites, with select isozymes being key for particular pathways. Previous studies have shown that arachidonate 12-lipoxygenase (12-LO) or 15-lipoxygenase (15-LO) activity, in combination with arachidonate 5-lipoxygenase (5-LO), can produce lipoxins, and 5-LO activity alone forms leukotrienes (50). Increasing concentrations of PGE$_2$ inhibit 5-LO translocation from the cytoplasm to the nucleus, abrogating leukotriene synthesis (51) and skewing the balance between pro- and
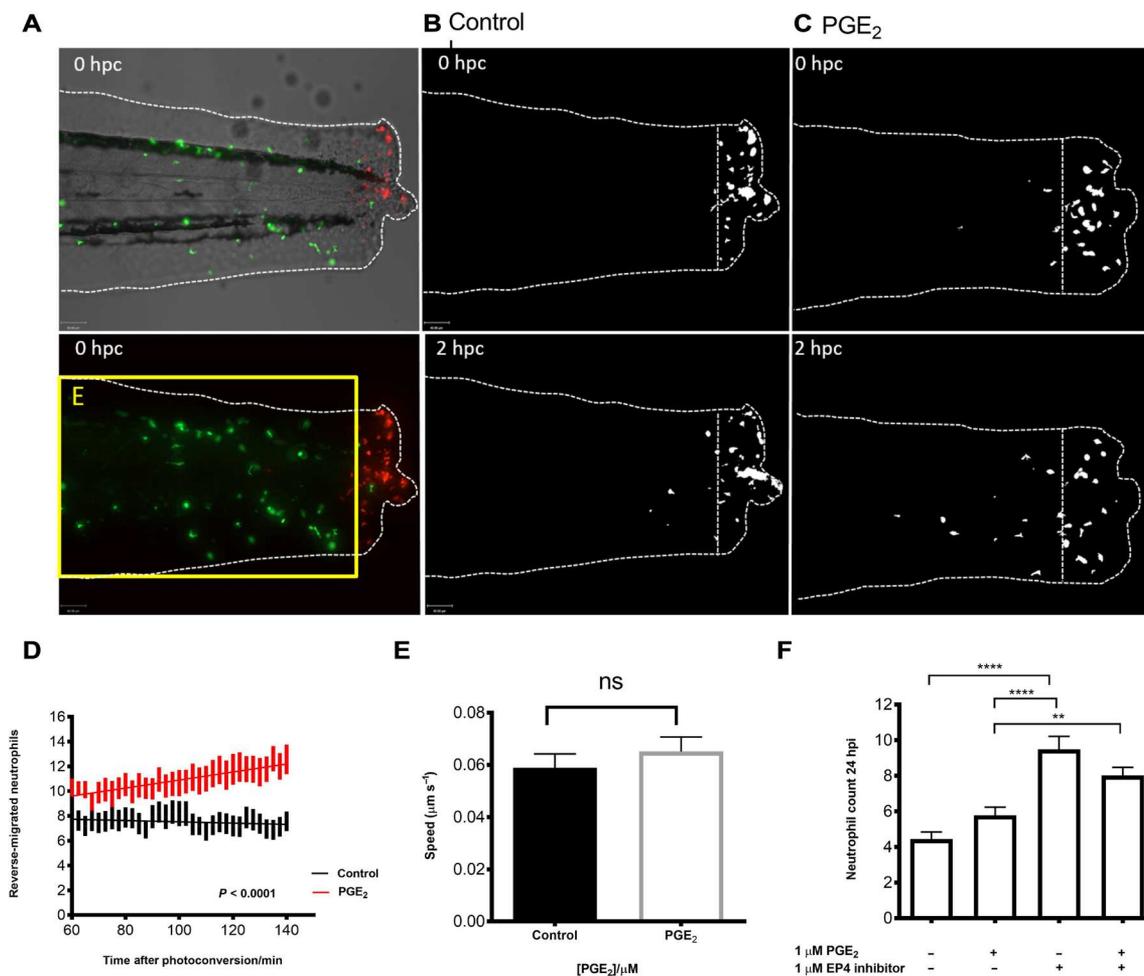
Fig. 4. PGE$_2$ drives accelerated reverse migration through EP4 receptor signaling. (**A**) Representative photomicrographs of control or PGE$_2$-treated 3 dpf *Tg(mpx:gal4)sh267;Tg(UAS:kaede)i222* larvae following photoconversion. The area to the right of the white dashed vertical line indicates wound site, where cells were photoconverted from green to red fluorescence. The yellow box E indicates the area into which the photoconverted cells migrate and corresponds to data in (D). (**B**) The red channel only is shown as a binary image of a control larva at 0 and 2 hours post conversion (hpc). Very little migration away from the wound site occurs between 10 and 12 hpi. (**C**) Binary images of the red channel of a PGE$_2$-treated larva at 0 and 2 hpc. At 12 hpi, neutrophils have migrated away from the wound site, when treated with PGE$_2$. (**D**) Plot showing the number of neutrophils moving away from the wound over 10 to 12 hpi, preincubated with or without PGE$_2$ from 8 to 9 hpi. PGE$_2$-treated neutrophils migrate away from the site of injury between 10 and 12 hpi more readily. Line of best fit shown is calculated by linear regression. *P* value shown is for the difference between the two slopes. (**E**) The speed of neutrophils moving away from the site of injury is not significantly different in the presence of exogenous PGE$_2$, indicating that neutrophils migrate away sooner rather than at a greater speed in PGE$_2$-treated larvae. (**F**) Neutrophil counts at 24 hpi show a significant increase in neutrophil number when EP4 signaling is blocked using the antagonist AH23848. **$P < 0.01$, ****$P < 0.0001$. Addition of PGE$_2$ does not lead to significant abrogation of this effect, implying PGE$_2$ signals through the EP4 receptor to promote neutrophil removal. All data are presented as means ± SEM, from $n = 18$ larvae for (D) and (E) and $n = 53$ for (F) from three experimental repeats. Images were taken using ×10 magnification on a TE2000U inverted microscope (Nikon).

anti-inflammatory mediators toward increased lipoxin synthesis—a process termed "lipid mediator class switching" (*28*). Zebrafish have the three key LOX genes for lipid mediator synthesis: *alox12*, *alox5*, and *alox15b* genes (*52–54*). To assess the contribution of lipoxin production during inflammation resolution in the zebrafish, we immersed injured transgenic larvae in PD146176, a specific 15-LO inhibitor (*55*). Again, as we hypothesized that there would be a delay in inflammation resolution, we performed 24 hpi counts for inhibitor assays to be able to detect neutrophil persistence over normal basal levels. We found that inhibiting 15-LO activity during inflammation led to a significant increase in neutrophil numbers at 24 hpi compared to control larvae (Fig. 5A), indicating persisting inflammation at the wound site. This effect was dose-dependent (Fig. 5B). Addition of exogenous PGE_2 following 15-LO inhibition was unable to abrogate this effect, with neutrophil numbers remaining high at the wound site 24 hpi (Fig. 5C), supporting a mechanism of PGE_2 acting upstream of 15-LO in this pathway.

The oxygenation of arachidonic acid by LOXs can occur at varying positions along the fatty acid carbon chain (*50*) and can determine the functionality of the protein according to which carbon is oxygenated and which amino acid residue is present at specific sites. In zebrafish, LOX genes are annotated in Ensembl as *alox12* and *alox15b*; we predict that zebrafish Alox12 should function predominantly in the role of a 15-LO due to the presence of phenylalanine (F) at position 353 and a bulky valine (V) at position 418 [in com-

parison to the rabbit leukocyte 12-LO protein sequence (*56*) and associated with predominantly 15-LO activity in mammalian LOXs (*57*); fig. S3]. We therefore assessed alteration of expression of *alox12* (the zebrafish putative 15-LO) by quantitative PCR (qPCR) during injury-induced inflammation. There was a significant linear increase in 12-LO expression up to 6 hpi (Fig. 5D), demonstrating that inflammation caused by injury induces a change in LOX activity. This suggests a plausible mechanism for the induction of reverse migration through increased lipoxin production during inflammation resolution.

Lipoxin A$_4$ drives inflammation resolution via reverse migration

LXA_4 is a proresolving product of arachidonic acid metabolism that inhibits neutrophil chemotaxis, transmigration, superoxide generation, and the production of proinflammatory cytokines (*58*). Because *alox12* expression was increased during injury-induced inflammation, we next determined whether its key product LXA_4 plays a role in inflammation in the zebrafish model. First, we added exogenous LXA_4 to the injured larvae and assessed neutrophil number at 6 hpi. Our data demonstrate a protective effect of LXA_4 acting to inhibit neutrophil chemotaxis, significantly reducing neutrophil recruitment to the wound site (Fig. 6A). This provides evidence that exogenous LXA4 is able to promote a response in our in vivo model. If PGE_2 production following injury increases *alox12* activity during inflammation resolution, and thereby increases endogenous LXA_4 production, we would
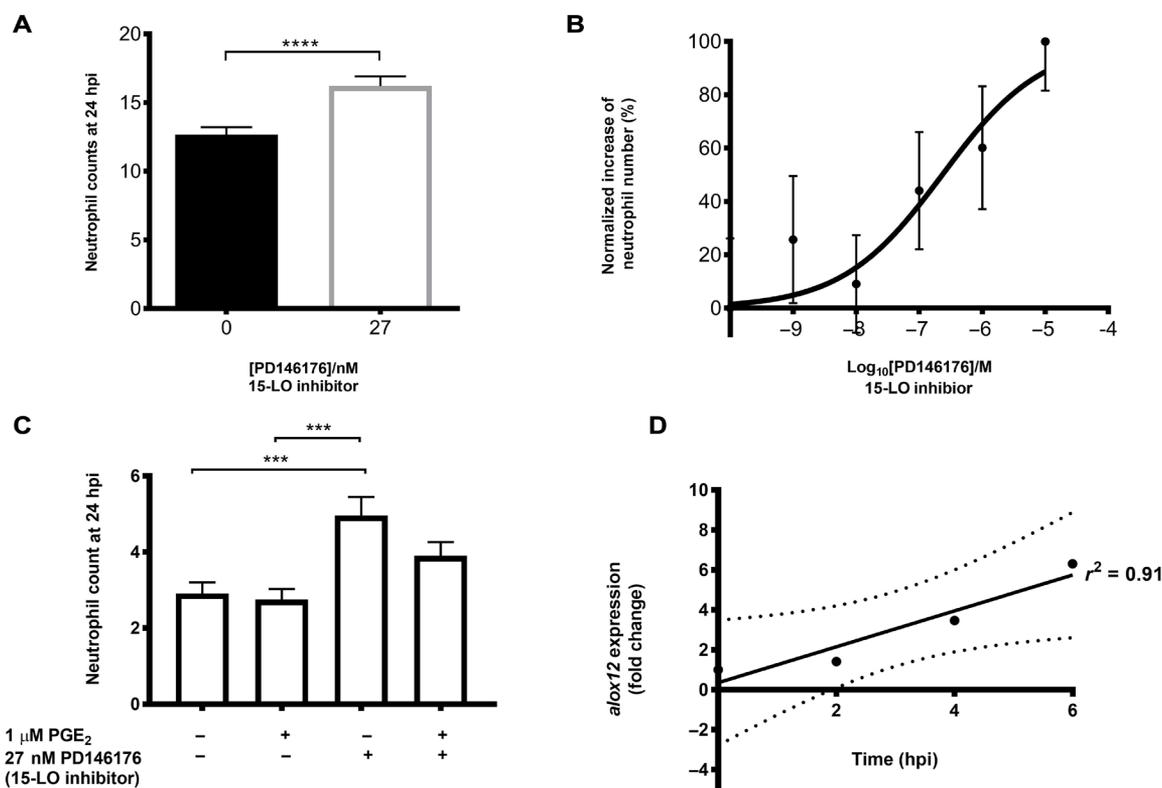
Fig. 5. EP receptor signaling and downstream activation of LOXs by PGE$_2$ lead to lipid mediator class switching and inflammation resolution. (**A**) Downstream activity of 15-LO was inhibited using the antagonist PD146176, also causing a significant delay in neutrophil removal from the wound site at 24 hpi. ****$P < 0.0001$. Data represent three replicates with a minimum of 30 fish per replicate. T test was performed. (**B**) Varying doses of PD146176 increase neutrophil numbers at the wound site at 24 hpi in a dose-dependent manner. (**C**) Exogenous PGE$_2$ is unable to significantly decrease neutrophil numbers in the presence of the 15-LO inhibitor PD146176 at 24 hpi. ***$P < 0.001$. (**D**) qPCR expression analysis of *alox12* following injury shows a significant increase up to the peak of inflammation. Fold change of expression to the reference gene represents the means ± SEM of four replicate experiments per time point, with a minimum of 50 larvae per replicate, therefore $n = 200$ individual larvae. $P = 0.04$, $r^2 = 0.91$.

predict that LXA$_4$ could allow removal of neutrophils already present at the wound site due to loss of sensitivity to local wound signals. To assess this, we performed reverse migration experiments in the presence of exogenous LXA$_4$. LXA$_4$ caused neutrophils to move away from the wound earlier than in untreated larvae (Fig. 6B), without altering neutrophil migration speed (Fig. 6C) or the path that the neutrophils take (Fig. 6D). Our data are best explained by proposing that production of endogenous LXA$_4$ by Alox12 following injury-induced PGE$_2$ production is the mechanism that promotes inflammation resolution in vivo.

DISCUSSION

Restoration of tissue homeostasis following infection or injury requires successful resolution of inflammation. Inflammation resolution should not occur before the threat is ablated, nor should inflammation persist longer than necessary. The process is therefore tightly regulated. Neutrophils and macrophages as a population are well positioned to assess the threat to the organism and determine the duration of the inflammatory response. Once the resolution is decided, neutrophils can either die at inflammatory sites by apoptosis and be cleared by macrophages (5) or can be removed from inflammatory sites by altered migratory patterns (reverse migration) (42). If this tightly controlled inflammatory process is disrupted, failure of inflammation resolution can lead to the tissue damage seen in chronic inflammatory diseases (59). The molecular mechanisms controlling the decisions on duration of inflammation are complex and yet to be fully determined. Existing evidence indicates that lipid mediator secretion is likely to be important in signaling inflammation resolution (60), but it is unknown which mediators regulate neutrophil removal from sites of inflammation in vivo. We have shown, for the first time, that the lipid mediator signaling molecule PGE$_2$ is essential for determining the outcome of neutrophil inflammation in response to tissue injury. Moreover, our data suggest that alterations in LOX activity lead to production of LXA$_4$. This lipid mediator class switching appears to be the critical mechanistic step by which macrophage PGE$_2$ release after uptake of apoptotic cells promotes inflammation resolution in vivo.

Previous in vitro studies have suggested that phagocytosis of apoptotic neutrophils by macrophages might contribute toward successful inflammation resolution (21, 61). Ingestion of apoptotic cells by macrophages promotes an anti-inflammatory environment, suppressing proinflammatory cytokine production and promoting LXA$_4$ production (21). While mice lacking macrophages and neutrophils show healing without excessive inflammation or scar formation, in macrophage-only depleted mice, neutrophils populated the wound area, with impaired wound morphology and delayed healing (62). We have shown that in the absence of macrophages, apoptotic bodies accumulate at the wound site, resulting in neutrophil persistence and prolonged inflammation. This finding demonstrates the importance of understanding macrophage-neutrophil interactions during inflammation resolution to prevent further damage to healing tissues.

Recent work has shown that reverse migration is a contributor to neutrophil removal from inflammatory sites (13, 15, 42, 63). It has been shown that macrophage recruitment and redox-SFK (Src family kinase) signaling play key roles in neutrophil-mediated inflammation resolution (32). These studies also suggest a direct contact mechanism between neutrophils and macrophages, which may direct neutrophil reverse migration; however, in the absence of macrophage contact, neutrophils are still able to reverse-migrate, suggesting that other, unknown factors may be involved. Tracking analysis of macrophage and neutrophil contact in our model of inflammation demonstrates that direct contact is not an essential requirement for reverse migration. Furthermore, our studies support the importance of a soluble factor, the lipid mediator PGE$_2$, for successful inflammation resolution. Our data suggest that this lipid mediator is a key signaling molecule between macrophages and neutrophils during inflammation, leading to production of LXA$_4$, enhancing neutrophil reverse migration and, hence, speeding inflammation resolution.

PGE$_2$ is known to be involved in many cellular processes, including cell proliferation, apoptosis, angiogenesis,
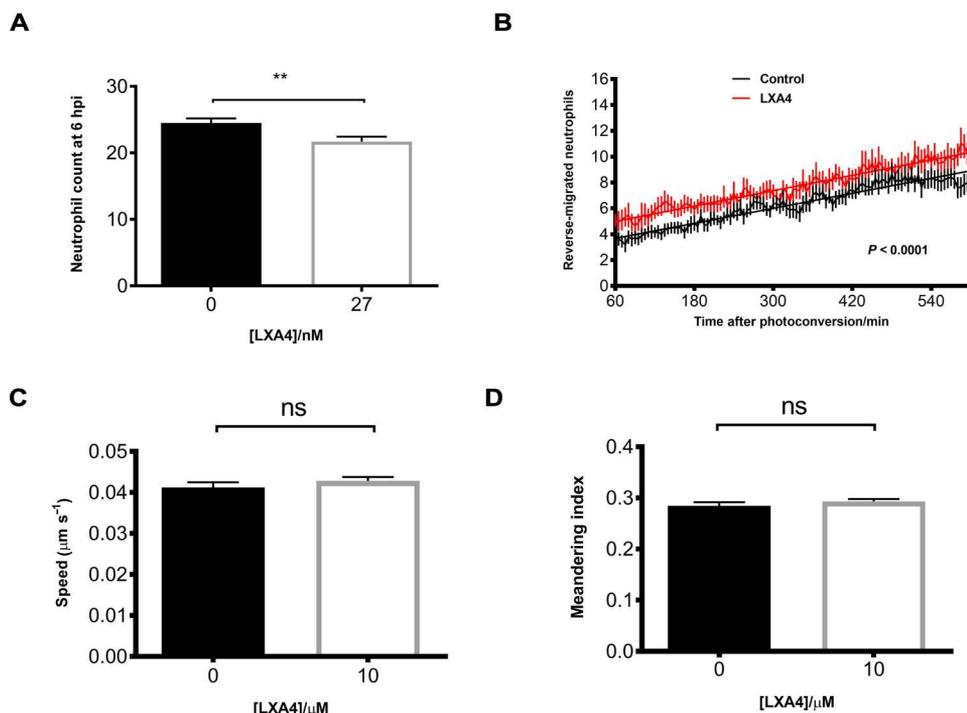
Fig. 6. LXA$_4$ drives inflammation resolution via reverse migration. (**A**) Injection of LXA$_4$ into zebrafish larvae causes a small but significant reduction in neutrophil numbers at the wound site at 6 hpi. n = 120 larvae. (**B**) Plot showing the number of neutrophils reverse-migrating away from the wound over a time course of 6 to 15 hpi, preinjected at 4 hpi with or without LXA$_4$. LXA$_4$-treated neutrophils migrate away from the site of injury sooner than control neutrophils. Line of best fit shown is calculated by linear regression. P value shown is for the difference between the two slopes. n = 21 larvae. (**C**) The speed of neutrophils moving away from the site of injury was measured and is not significantly different in the presence of exogenous LXA$_4$. (**D**) The path the neutrophils take (meandering index) when moving away from the wound was also not significantly different when supplemented with LXA$_4$. All data are presented as means ± SEM, from three experimental repeats.

inflammation, and immune surveillance (*64*). The production of PGE$_2$ from prostaglandin synthases is essential for the development of organisms past the gastrulation stage (*34*) and is crucial for hematopoietic stem cell homeostasis (*35*). However, to our knowledge, PGE$_2$ has not been shown to be necessary for resolution of neutrophilic inflammation. We have shown by genetic and pharmacological approaches that the production of PGE$_2$ is essential for inflammation to resolve and that PGE$_2$ is sufficient to drive neutrophil removal from wound sites. PGE$_2$ can promote neutrophil removal from wound sites in the absence of macrophages, implying an important connection between macrophages and the lipid mediator PGE$_2$. This could prove to be a useful target for therapeutic intervention in chronic inflammatory disease.

PGE$_2$ acts via EP receptors present on a range of cell types, including neutrophils, macrophages, and epithelial and endothelial cells. Neutrophils predominantly express EP2 and EP4 (*65*), both of which, upon activation, cause an increase in adenosine 3′,5′-monophosphate (cAMP) levels within the cell. An increase in intracellular cAMP can "reprogram" the cell to switch from 5-LO products to the production of 15-LO products (*28*). Our studies show PGE$_2$ signaling through EP4 receptors to be important in the resolution of neutrophilic inflammation and that this activity is mediated via 15-LO products, most likely the anti-inflammatory mediator LXA$_4$.

Human polymorphonuclear leukocytes in vitro markedly increase expression of 15-LO mRNA in the presence of PGE$_2$ (*28*). Our data from whole animals show a significantly increased expression of the annotated zebrafish 12-LO mRNA following tailfin injury relative to time. Human blood neutrophils exposed to PGE$_2$ inhibit LTB$_4$ biosynthesis and demonstrate reduced neutrophil migration (*66*). Our data confirm the regulation of neutrophil migration by PGE$_2$ and LXA$_4$ in an in vivo model of inflammation resolution. Neutrophils more readily migrate away from the wound site when exposed to exogenous PGE$_2$ or LXA$_4$. The addition of PGE$_2$ after the maximum recruitment of neutrophils to the wound is able to markedly enhance the reverse migration of neutrophils. The mechanism of this step is still unknown, but PGE$_2$ alteration of LO activity would fit with its known role in inhibiting neutrophil recruitment (*67*). In our studies, exogenous LXA$_4$ causes neutrophils to leave the wound sooner. LXA$_4$ has been shown to inhibit chemokine signaling in neutrophils in vitro (*68*), suggesting a mechanism by which LXA$_4$ facilitates neutrophil reverse migration from sites of inflammation. Mathematical modeling data from in vivo neutrophil migration following zebrafish tailfin wounding suggest that neutrophil reverse migration is a phenomenon of stochastic redistribution of neutrophils in the tissues (*69*). This suggests a model whereby neutrophils are retained at wounds by a molecular "retention signal" and that decay of this signal leads to reverse migration (*12*). Chemokine receptor desensitization would be a strong candidate for explaining how these retention signals might be initiated and subsequently decayed. LXA$_4$ interference with these signals might be a potential mechanism by which PGE$_2$ ultimately alters neutrophil migration patterns, leading to inflammation resolution.

In this context, an in vivo reporter would be useful to further elucidate the presence and activity of PGE$_2$. Understanding the molecular mechanisms involved in neutrophil removal from wound sites, specifically during reverse migration, would greatly improve our understanding of inflammatory disease and provide potential strategies and targets for much needed therapeutic intervention.

MATERIALS AND METHODS

Maintenance and breeding of zebrafish
To visualize both macrophages and neutrophils within the same zebrafish larva, triple transgenic fish *Tg(cfms:Gal4)i186;Tg(UAS: nfsB-mCherry)i149;Tg(mpx:EGFP)i114*, as previously described (*29*), were incrossed. *Tg(mpx:gal4)sh267;Tg(UASkaede)i222* zebrafish were used for photoconversion and neutrophil tracking experiments, subsequently termed *mpx:kaede*. *Tg(mpx:EGFP)i114* zebrafish were used to study neutrophils during inflammation. Zebrafish strains were maintained according to standard protocols. Adult fish were maintained on a 14:10-hour light/dark cycle at 28°C in UK Home Office–approved facilities in The Bateson Centre aquaria at the University of Sheffield, under AWERB (Animal Welfare and Ethical Review Bodies) and UK Home Office–approved protocols.

Inflammation assay
Inflammation was induced in zebrafish embryos by tail transection as described previously (*70*). Embryos were anesthetized at 3 or 8 dpf by immersion in tricaine (0.168 mg/ml; Sigma-Aldrich), and tail transection was performed using a microscalpel (World Precision Instruments). Neutrophils and macrophages were counted at the site of transection at various time points including 2, 4, 6, 8, and 24 hpi using a fluorescence dissecting stereomicroscope (Leica).

In vivo cell-specific ablation
Metronidazole (Sigma-Aldrich) was dissolved to a concentration of 2.5 mM in E3 with 0.2% DMSO. Solutions of metronidazole were made fresh on the day of use with vigorous agitation until fully dissolved. *Tg(cfms:Gal4)i186;Tg(UAS:nfsB-mCherry)i149;Tg(mpx:EGFP)i114* were immersed in metronidazole or E3 and 0.2% DMSO at 2 or 7 dpf overnight at 28°C for 15 hours. Larvae were wrapped in foil during the experiment. At 3 or 8 dpf, larvae were checked for macrophage depletion on a fluorescence dissecting stereomicroscope (Leica).

Neutrophil and apoptotic cell labeling
Rates of neutrophil apoptosis were assessed by dual staining with Rhodamine-TUNEL and FITC-TSA. Transgenic zebrafish larvae were injured at 8 dpf and fixed at 24 hpi in 4% paraformaldehyde. TUNEL (ApopTag Red; Millipore Corp.) staining labeled apoptotic cells with red fluorescence, and TSA (TSAplus kit; Fluorescence Systems, PerkinElmer Life and Analytical Sciences) staining labeled neutrophils with fluorescein green fluorescence. Neutrophils at the wound were imaged on an Eclipse TE2000-U inverted compound fluorescence microscope (Nikon), and neutrophil-specific apoptosis was assessed by the percentage of TSA-positive neutrophils labeled with TUNEL. Red TUNEL–positive–only cells were assessed for total apoptosis rates.

Cell tracking
To assess neutrophil migration behavior before and after interaction with a macrophage, transgenic larvae *Tg(mpx:EGFP)i114* were crossed to *Tg(mpeg1:mCherry-CAAX)sh378*. Three days post fertilization larvae were injured, and images were taken every 3 min between 0 and 12 hpi using an Eclipse TE2000-U inverted compound fluorescence microscope (Nikon). Direct contact was defined as when cell membranes could be seen to touch. Comparisons of migration behavior were made over a 30-min period before and after interaction.

Compound treatment of zebrafish larvae

Following tail transection at 3 dpf, zebrafish larvae were screened at 4 hpi for neutrophil recruitment. Selected larvae were immersed in 1 μM PGE$_2$ (Sigma-Aldrich) at 8 hpi with counts performed at 12 hpi to look for accelerated inflammation resolution and 24 hpi for correction of phenotype assays. Concentrations of PGE$_2$ ranged from 0.01 to 10 μM for dose-response assays. The EP4 antagonist AH23848 (Cayman Chemicals) was used at a final concentration of 1 μM. Fish were incubated at 2 dpf, and tail transection was performed at 3 dpf. PD146176, a 15-LO inhibitor (Sigma-Aldrich), was injected at 6 hpi into the Duct of Cuvier to a final concentration of 27 nM in 2 dpf larvae. LXA$_4$ (Cayman Chemicals) was injected into the Duct of Cuvier to a final concentration of 27 nM at 2 dpf, followed by tail transection and 6 hpi counts.

Reverse migration assay

Tail transection of *Tg(mpx:gal4)sh267;Tg(UAS:kaede)i222* larvae was performed at 3 dpf. Embryos were raised to 8 hpi, incubated in 1 μM PGE$_2$ for 1 hour, and mounted in 0.8% low-melting-point agarose (Sigma-Aldrich). An UltraVIEW PhotoKinesis device on an UltraVIEW VoX spinning disk confocal microscope (PerkinElmer Life and Analytical Sciences) was used to photoconvert Kaede-labeled cells at the wound site using 120 pulses of the 405-nm laser at 40% laser power. Embryos were transferred to an Eclipse TE2000-U inverted compound fluorescence microscope (Nikon), where a 1394 ORCA-ERA camera (Hamamatsu Photonics Inc) was used to capture a time-lapse series with 2.5-min intervals for 2 hours. Tracking analysis of red-fluorescing neutrophils was performed in Volocity 6 (Improvision; PerkinElmer Life and Analytical Sciences), using the intensity of fluorescence to identify individually labeled neutrophils over time. LXA$_4$ was injected at 4 hpi at a final concentration of 27 nM, neutrophils at the wound site was photoconverted at 5 hpi, and larvae were time-lapsed between 6 and 15 hpi. Speed data were calculated using tracking time lapses, where larvae were incubated in 10 μM LXA$_4$. We refer to the track a neutrophil takes as the meandering index of the neutrophil, where a direct movement in a straight line from A to B is given the value of 1. Tracks that are not a straight line fall between zero and 1.

Morpholino studies

All morpholinos were obtained from GeneTools LLC. *ptges* splice blocking morpholino is described by Feng *et al.* (*26*). Standard control morpholino (CoMo; 0.5 nl of 0.4mM) or *ptges* morpholino was injected into embryos at the one-cell stage previously described (*26*). Injected larvae were incubated in 5 μM PGE$_2$ at the onset of epiboly to compensate for the PGE$_2$ required for normal development up to 42 hours post fertilization (hpf). PGE$_2$ was then withdrawn from the larvae at 48 hpf. At 3 dpf, larvae were injured, and neutrophil counts were performed at 4, 8, and 24 hpi. Knockdown was assessed in the same fish at 48 hpf and 4 dpf using reverse transcription PCR, and primer sequences were PTGESF1 5′-CTCGGGAGCGACATACAGTT-3′ and PTGESR1 5′-AGCAGATATGCAACGCTGTG-3′. *ptges* splice blocking morpholino leads to deletion of exon 2 and a loss of 83 bp.

Quantitative PCR

Three days post fertilization larvae were injured, and total RNA was extracted using TRIzol at various time points after injury. Complementary DNA synthesis was performed using SuperScript II (Thermo Fisher Scientific), and qPCR experiments using a DyNAmo Flash SYBR green qPCR kit (Thermo Fisher Scientific) were carried out. ΔCT was calculated using *elongation factor 1 alpha* as a reference gene. Relative expression levels were plotted after determining ΔΔCt by normalizing to a single sample with a high ΔCT value. Primer sequences for *alox12* gene were alox12F ccacggaatatcaccagatgga and alox12R attcagagtcgttgtgggtatc.

gRNA design and CRISPR/Cas9 injection

The online web tool CHOPCHOP was used to design a specific gRNA spanning the ATG of *ptges*. Purified gRNA, Cas9 protein, and tracrRNA were purchased from Sigma-Aldrich. The *ptges* guide sequence is gauagaggcucaagaugcucguuuuagagcuaugcuguuuug, targeting sequence gatagaggctcaagatgctcggg of the gene. Each embryo was injected with 1 nl of 6.6 nM gRNA, tracrRNA, and Cas9 protein at the one-cell stage. Genomic DNA was extracted from injected larvae, and PCR amplification was performed, generating a 345-bp product spanning the ATG of the *ptges* gene (ENSDARG00000020136). Primers used were ptges guide F1 gccaagtataatgaggaatggg and ptges guide R1 aatgtttggattaaacgcgact. Genotype was determined by digestion with MwoI. Wild-type PCR products produce 184-, 109-, and 52-bp bands. Mutant forms produce 293- and 52-bp bands.

Statistical analysis

Data were analyzed (Prism 7.0; GraphPad Software) using unpaired, two-tailed *t* tests for comparisons between two groups and one-way ANOVA (with appropriate posttest adjustment) for other data. A χ^2 Fisher's exact test was used to assess statistical significance in Fig. 2. Linear regression analysis was performed on qPCR data in Fig. 6.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/9/eaar8320/DC1

Fig. S1. Reverse transcription PCR demonstrates successful exon deletion.

Fig. S2. PGE$_2$ drives resolution of inflammation in the absence of macrophages by 12 hpi and does not alter neutrophil apoptosis.

Fig. S3. Amino acid positions 353 and 418 determine LOX functionality.

Fig. S4. Genotyping method for *ptges* crispant.

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Citation: C. A. Loynes, J. A. Lee, A. L. Robertson, M. J. Steel, F. Ellett, Y. Feng, B. D. Levy, M. K.B. Whyte, S. A. Renshaw, PGE2 production at sites of tissue injury promotes an anti-inflammatory neutrophil phenotype and determines the outcome of inflammation resolution in vivo. *Sci. Adv.* **4**, eaar8320 (2018).

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