The SH2-domain-containing inositol 5-phosphatase (SHIP) limits the motility of neutrophils and their recruitment to wounds in zebrafish

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Summary

Neutrophil recruitment to sites of injury or infection is essential for host defense, but it needs to be tightly regulated to prevent tissue damage. Phosphoinositide 3-kinase (PI3K), which generates the phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P_3], is necessary for neutrophil motility *in vivo*; however, the role of SH2-domain-containing 5-inositol phosphatase (SHIP) enzymes, which hydrolyze PI(3,4,5)P_3 to phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2], is not well understood. Here we show that SHIP phosphatases limit neutrophil motility in live zebrafish. Using real-time imaging of bioprobes specific for PI(3,4,5)P_3 and PI(3,4)P_2 in neutrophils, we found that PI(3,4,5)P_3 and PI(3,4)P_2 accumulate at the leading edge while PI(3,4)P_2 also localizes to the trailing edge of migrating neutrophils *in vivo*. Depletion of SHIP phosphatases using morpholino oligonucleotides led to increased neutrophil 3D motility and neutrophil infiltration into wounds. The increase in neutrophil wound recruitment in SHIP morphants was rescued by treatment with low dose PI3Kγ inhibitor, suggesting that SHIP limits neutrophil motility by modulating PI3K signaling. Moreover, overexpression of the SHIP phosphatase domain in neutrophils impaired neutrophil 3D migration. Taken together, our findings suggest that SHIP phosphatases control neutrophil inflammation by limiting neutrophil motility *in vivo*.

Key words: Neutrophil, SHIP, Inflammation, SH2-domain-containing 5-inositol phosphatase, Zebrafish

Introduction

Neutrophil infiltration into sites of infection or tissue injury is essential for host defense. On the other hand, tight control of neutrophil accumulation and activation is necessary to prevent tissue damage and chronic inflammation. Despite substantial progress in identifying mechanisms that initiate neutrophil wound attraction (Deng et al., 2012; McDonald et al., 2010; Niethammer et al., 2009; Yoo et al., 2010; Yoo et al., 2011), how neutrophil infiltration is regulated in live animals is not well defined.

It is well established that chemoattractant induced G protein-coupled receptor signaling induces phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P_3]/phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] concentration at the leading edge during single cell chemotaxis *in vitro* (Parent, 2004; Servant et al., 2000) and neutrophil motility *in vivo* (Yoo et al., 2010). Leading edge activation of phosphoinositide 3-kinase (PI3K) results in the rapid accumulation of PI(3,4,5)P_3/PI(3,4)P_2, polarized F-actin polymerization and directed motility (Barberis and Hirsch, 2008; Hawkins and Stephens, 2007; Yoo et al., 2010). The PI3K product PI(3,4,5)P_3 can be hydrolyzed to PI(3,4)P_2 by SH2-domain-containing inositol 5-phosphatase (SHIP) (Rohrschneider et al., 2000). In support of an important role for SHIP in controlling inflammation, knockout mice show robust leukocyte infiltration into lungs (Helgason et al., 1998). Moreover, bone marrow-derived mast cells from SHIP-deficient mice, show increased cytokine induced PI(3,4,5)P_3 levels and Akt activation (Liu et al., 1999). By contrast, SHIP1-null neutrophils *in vitro* have impaired polarization and motility, suggesting that SHIP1 is necessary for neutrophil motility (Nishio et al., 2007). These reports suggest opposite roles for SHIP in regulating neutrophil motility *in vitro* and inflammation *in vivo*, indicating that further studies are needed to delineate the *in vivo* role of SHIP in regulating neutrophil motility.

The zebrafish, *Danio rerio*, has emerged as a powerful vertebrate model to study cell motility and immunity (Huttenlocher and Poznansky, 2008; Kanther and Rawls, 2010). The optical transparency of zebrafish embryos together with tissue specific promoter driven gene expression allow for high-resolution real time imaging at both the subcellular and single cell level in live animals. Here, we show that SHIP plays an important role in limiting leukocyte motility and infiltration in live zebrafish embryos. Depletion of SHIP 5′-phosphatases increased neutrophil wound attraction and random motility through a PI3K-dependent pathway. Moreover, ectopic expression of the SHIP1 phosphatase domain impaired neutrophil migration. Taken together, our findings suggest that SHIP phosphatases serve as a brake that limit neutrophil motility and inflammation, at least in part through their effects on PI3K signaling.

Results and Discussion

PI(3,4,5)P_3/PI(3,4)P_2 signaling in neutrophils *in vivo*

PI(3,4,5)P_3/PI(3,4,)P_2 localizes to the leading edge of neutrophils *in vitro* (Servant et al., 2000). We have also demonstrated, using

the PH domain of Akt/PKB (PH-Akt), that PI(3,4,5)P_3/PI(3,4)P_2 localizes to the leading edge and occasionally the tail of neutrophils migrating *in vivo* (Yoo et al., 2010). The PH-Akt probe detects both PI(3,4,5)P_3 and PI(3,4)P_2 (Frech et al., 1997; Gray et al., 1999). To further define the endogenous dynamics and inter-conversion of these phosphoinositides *in vivo*, we used the *mpx* promoter to transiently express the PI(3,4,5)P_3-specific bioprobe GRP1-PH and the PI(3,4)P_2-specific bioprobe TAPP1-PH (Furutani et al., 2006) in zebrafish neutrophils. Using ratiometric imaging of neutrophils randomly migrating in the head, we found that TAPP1-PH [detecting PI(3,4)P_2] concentrated both at the leading edge and the tail of motile neutrophils (Fig. 1A; supplementary material Movie 1). By contrast, GRP1-PH [detecting PI(3,4,5)P_3] accumulated only at the leading edge of neutrophils (Fig. 1B; supplementary material Movie 2). Accordingly, ratiometric imaging comparing the accumulation of the PI(3,4,5)P_3 to PI(3,4)P_2 probes revealed that more PI(3,4,5)P_3 than PI(3,4)P_2 accumulated at the neutrophil leading edge (Fig. 1C; supplementary material Movie 3). We have previously shown by ratiometric imaging of the farnesylated membrane-bound probe EGFP-F, membrane signal periodically accumulated at the tail but not at the front of neutrophils (Yoo et al., 2010). Therefore, the cell front signal observed is unlikely due to membrane accumulation. These findings indicate that there are local sub-cellular pools of phospholipids that can be distinguished temporally and spatially during neutrophil migration *in vivo*.

SHIP expression and localization in zebrafish leukocytes

The dynamic accumulation of PI(3,4)P_2 during neutrophil motility prompted us to investigate the function of SHIP phosphatases during neutrophil motility. In mammals, *SHIP1* expression is restricted to the hematopoietic lineage (Liu et al., 1998) and *SHIP2* is expressed broadly (Muraille et al., 1999). According to the PubMed database, zebrafish expresses one copy of SHIP1 (accession no. XP_001923007) that shares 61% identify with both human SHIP1a and SHIP1b (supplementary material Fig. S1). There are two SHIP2 paralogs in zebrafish, SHIP2a and SHIP2b. SHIP2a is most closely related to human SHIP2 (65% overall identity) and is broadly expressed in zebrafish (Jurynec and Grunwald, 2010). RT-PCR analysis using mRNA extracted from FACS-sorted leukocytes or whole zebrafish at 3 days post fertilization (dpf) using *Tg*(*mpx:dendra2*) (Mathias et al., 2009; Yoo and Huttenlocher, 2011), demonstrated that zebrafish myeloid cells express *SHIP1* and *SHIP2a*, but not *SHIP2b* (Fig. 2A). Purity of the FACS-sorted myeloid cell population was confirmed by RT-PCR (Fig. 2B).

Previous studies have reported that SHIP1 and SHIP2 are localized at the leading edge in MDCK cells (Koch et al., 2005). To determine where SHIP phosphatases localize in motile neutrophils, we transiently expressed human EGFP-SHIP1 in *Tg*(*mpx:mCherry*) zebrafish embryos (Yoo et al., 2010), using the neutrophil specific lysozyme C (*lyz*) promoter (Kitaguchi et al., 2009). Human SHIP1, like PI(3,4)P_2, localized both at the front and tail of motile neutrophils (Fig. 2C; supplementary material Movie 4). We further characterized the subcellular localization of zebrafish EGFP-SHIP1 and EGFP-SHIP2a in neutrophils *in vivo*. In accordance with our findings with human SHIP1, both zebrafish SHIP1 and SHIP2a transiently localize to the cell front and at times can be seen in the tail of motile neutrophils *in vivo* (supplementary material Fig. S2; Movie 5). Neutrophil-specific protein expression using the *lyz* promoter was confirmed by crossing the transgenic line *Tg*(*lyz:TagRFP*), which we generated based on the published *lyz* promoter sequence (Kitaguchi et al., 2009), with *Tg*(*mpx:dendra2*) (Yoo and Huttenlocher, 2011) (supplementary material Fig. S3).

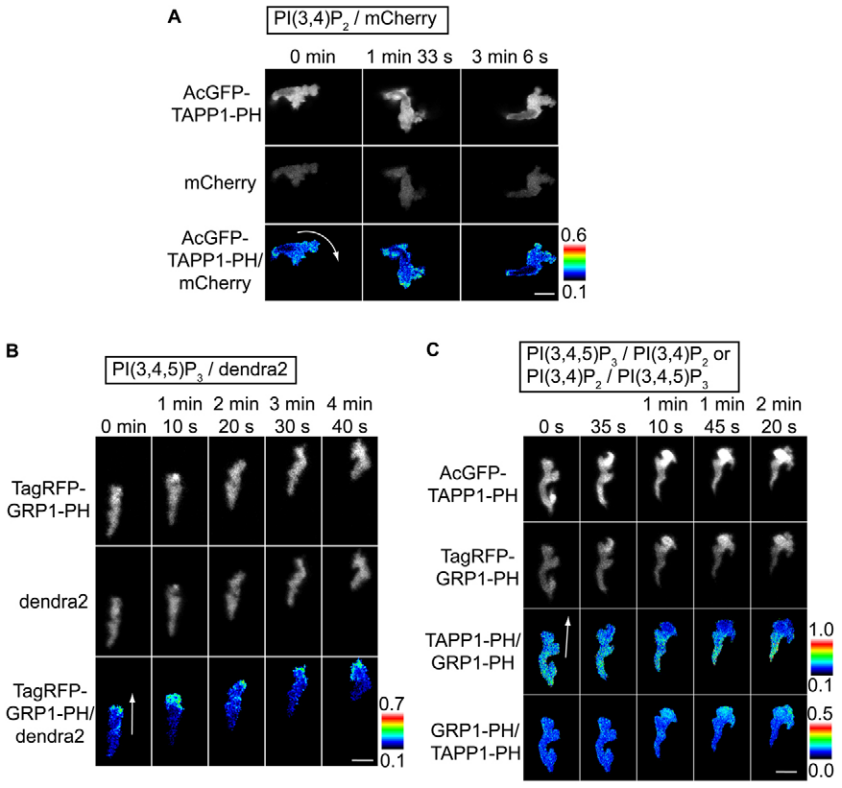

Fig. 1. Ratiometric imaging of PI(3,4,5)P_3 and PI(3,4,)P_2 in motile neutrophils in zebrafish. Representative images are presented as maximum intensity projections. (**A**) Time lapse ratiometric imaging of PI(3,4)P_2 (TAPP1-PH/mCherry) using a 60×NA1.10 objective with 270 μm pinhole and 1.01 μm step size. (**B**) Time-lapse ratiometric imaging of PI(3,4,5)P_3 (GRP1-PH/dendra2) and (**C**) PI(3,4,5)P_3 to PI(3,4)P_2 (GRP1-PH/TAPP1-PH) or PI(3,4)P_2 to PI(3,4,5)P_3 (TAPP1-PH/GRP1-PH), using a 20×NA0.75 objective with 344 μm pinhole and 3.89 μm step size. White arrow indicates the direction of neutrophil migration. The numerical values of ratiometric analysis are shown in the scales. Scale bar, 10 μm.

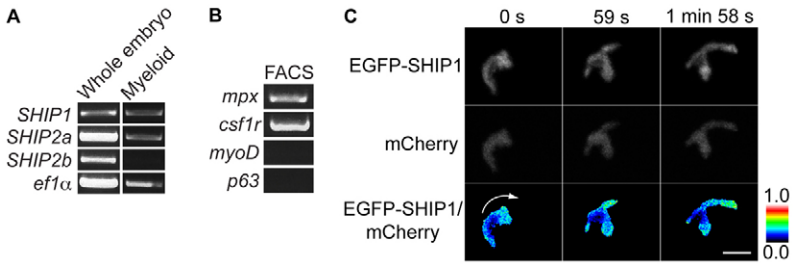

Fig. 2. Expression and localization of SHIP *in vivo*. (**A**) Expression of SHIP isoforms (*SHIP1*, *SHIP2a* and *SHIP2b*) from whole embryo or FACS sorted myeloid cells from *Tg*(*mpx:dendra2*) determined by RT-PCR. (**B**) Expression of specific markers of FACS-sorted myeloid cells from *Tg*(*mpx:dendra2*) fish by RT-PCR with markers that are expressed (*mpx, csf1r*) or not expressed (*myoD*, *p63*) in myeloid cells. (**C**) Time-lapse ratiometric imaging of human EGFP-SHIP1/mCherry using *lyz:EGFP-SHIP1* injected into *Tg*(*mpx:mCherry*) showing localization of SHIP1 at the front and rear of motile neutrophils. Images were acquired using a 20×NA0.75 objective with 241 µm pinhole and 2.78 µm step size; representative images are presented as maximum intensity projections. White arrow indicates the direction of migration. The numerical value of ratiometric analysis is shown in the scale. Scale bar, 10 µm.

SHIP phosphatases impair recruitment of neutrophils to wounds

To determine if SHIP phosphatases regulate neutrophil motility *in vivo*, we used SHIP-targeted splice donor blocking morpholinos (MOs) to deplete SHIP1 and SHIP2a. RT-PCR confirmed SHIP knockdown (Fig. 3A). We found that depletion of both SHIP1 and SHIP2a (Fig. 3B) but not single SHIP1/2a (data not shown) increased neutrophil wound recruitment at 0.5 and 1 hour post wound (hpw) in 2.5 dpf zebrafish. The effect of SHIP depletion was confirmed using a second set of morpholinos, which targeted *SHIP1* (*SHIP1* MO2) and *SHIP2a* (*SHIP2a* MO1) (Fig. 3C). It has been reported that there are increased total neutrophils in SHIP-deficient mice (Rauh et al., 2004). By contrast, total neutrophil numbers were decreased in *SHIP* morphants (supplementary material Fig. S4), despite having more neutrophils infiltrate into wounds. Moreover, we also found that macrophage recruitment to wounds was increased in the *SHIP* morphants (supplementary material Fig. S5), suggesting that SHIP phosphatases limit both

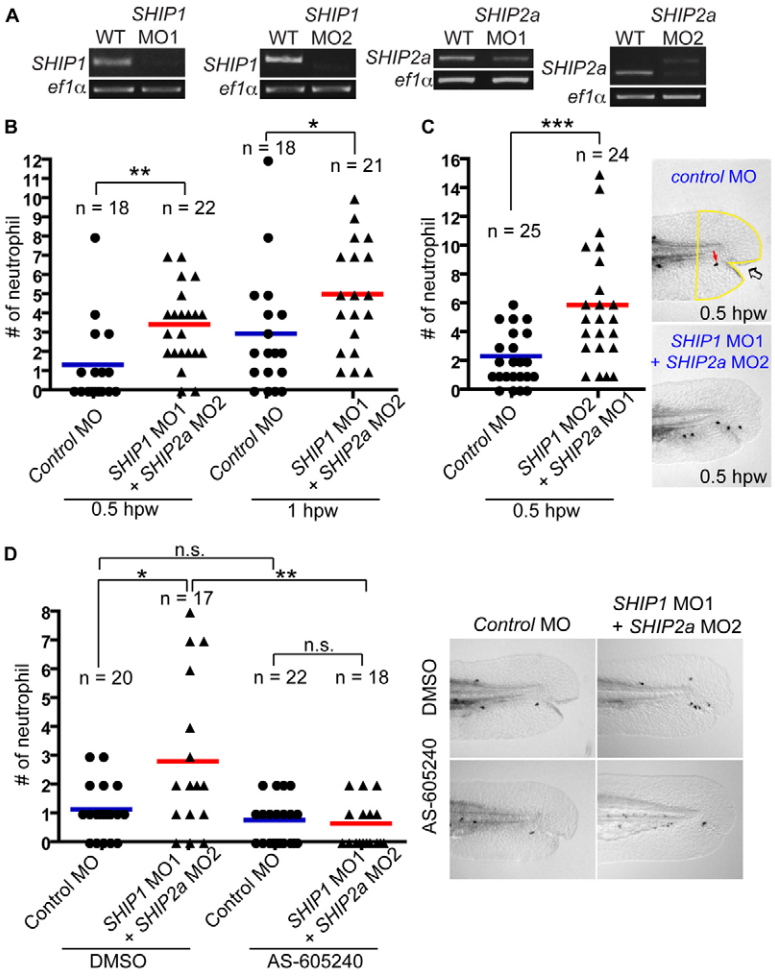

Fig. 3. Neutrophil wound recruitment in *SHIP* morphants and rescue by PI3Kγ inhibition. (**A**) RT-PCR analysis of *SHIP1* and *SHIP2a* morphants. (**B**) Quantification of neutrophils at wounds in control and *SHIP1* (MO1) and *SHIP2a* (MO2) morphants at 0.5 and 1 hour post wounding (hpw). Data are representative of three experiments. (**C**) (Left panel) Quantification of neutrophils at wounds in control and *SHIP1* (MO2) and *SHIP2a* (MO1) morphants. Data are representative of at least three experiments. (Right panel) Representative image of Sudan Black-stained embryos. Lateral view of the tail fin of embryos at 2.5 dpf. The yellow outline indicates the area where the number of neutrophils were counted. Red arrow on control panel indicates a neutrophil after Sudan Black staining. Open arrow indicates site of wounding. *n*=number of embryos wounded and counted. **P*<0.05, ***P*<0.01, ****P*<0.001 (two-tailed, unpaired *t*-test). (**D**) PI3Kγ inhibitor (AS-605240) (1 µM) reverses the effects of *SHIP1* and *SHIP2a* double knockdown on neutrophil recruitment. Data are representative of three experiments. *n*=number of embryos wounded and counted. **P*<0.05, ***P*<0.01 (one-way ANOVA with Bonferroni post-test). (Right panel) Representative image of Sudan Black-stained embryos used for neutrophil recruitment analysis. Lateral view of the tail fin of embryo at 2.5 dpf.

neutrophil and macrophage wound recruitment. After initial recruitment, neutrophils migrate repeatedly towards and away from the wound in a process called reverse migration (Mathias et al., 2006; Yoo and Huttenlocher, 2011). *SHIP* morphants did not show a significant difference in reverse migration or resolution of inflammation as compared to control (data not shown).

Previous studies have reported that depletion of SHIP1 increases PI(3,4,5)P_3 accumulation in leukocytes (Harris et al., 2011; Nishio et al., 2007; Vedham et al., 2005). To test the possibility that SHIP phosphatases modulate neutrophil wound recruitment by affecting PI(3,4,5)P_3 levels, we inhibited PI(3,4,5)P_3 generation in *SHIP* morphants using low dose PI3Kγ inhibitor. It is known that PI3Kγ inhibition impairs PI(3,4,5)P_3/PI(3,4)P_2 generation and neutrophil motility *in vivo* (Yoo et al., 2010). We found that treatment with low dose PI3Kγ inhibitor did not affect neutrophil wound recruitment in control zebrafish. However, low dose PI3Kγ inhibitor was sufficient to rescue neutrophil wound recruitment in *SHIP* morphants to control levels (Fig. 3D). These findings suggest that depletion of SHIP increases neutrophil recruitment through a PI3K-dependent pathway. However, we cannot rule out the possibility that the generation of PI(3,4)P_2 may also be important for SHIP effects on neutrophil wound attraction. Since PI(3)K activity and leading edge PI(3,4,5)P_3 is indispensable for neutrophil motility *in vivo* (Yoo et al., 2010), it is interesting to speculate that SHIP hydrolyzes PI(3,4,5)P_3 into PI(3,4)P_2, particularly in the front of neutrophils, and thereby limits neutrophil motility.

SHIP phosphatases limit neutrophil motility *in vivo*

It is possible that neutrophil wound recruitment is enhanced in *SHIP* morphants due to increased neutrophil motility in *SHIP* morphants. To test this possibility, we performed real time imaging of neutrophil random motility in the head region in control and *SHIP* morphants at 2.5 dpf. We found that there was increased neutrophil random motility in *SHIP* double morphants compared to control (Fig. 4A–C; supplementary material Movie 6).

To determine if the SHIP phosphatase activity is sufficient to alter neutrophil motility, we ectopically expressed a membrane-bound form of the human SHIP1 phosphatase domain (aa364–826) in zebrafish neutrophils (Freeburn et al., 2002). Transient expression was achieved using the *lyz* promoter driving both the constitutively active SHIP1 phosphatase and EGFP expression with the viral 2A peptide system which allows multiple protein products to be expressed from a single transgene (Provost et al., 2007). Transient expression of this construct in *Tg*(*mpx:mCherry*) embryos allowed for mosaic expression of the phosphatase domain labeled with EGFP and mCherry to be compared to control neutrophils that expressed mCherry alone. Live imaging of neutrophil random motility in the head of 3 dpf embryos showed that ectopic expression of the SHIP1 phosphatase domain impaired neutrophil random motility as compared to control neutrophils (Fig. 4D–E; supplementary material Movie 7). By contrast, ectopic expression of EGFP control did not impair neutrophil motility (Fig. 4F). Further analysis demonstrated that there was no significant difference in the meandering index (displacement/track length) measured in the *SHIP* knockdown embryos and the embryos overexpressing the SHIP1 phosphatase domain (data not shown), suggesting that SHIP functions to limit neutrophil speed but not directional migration or persistence. Taken together, our findings suggest that SHIP limits neutrophil motility through the modulation of PI3K signaling. Moreover,

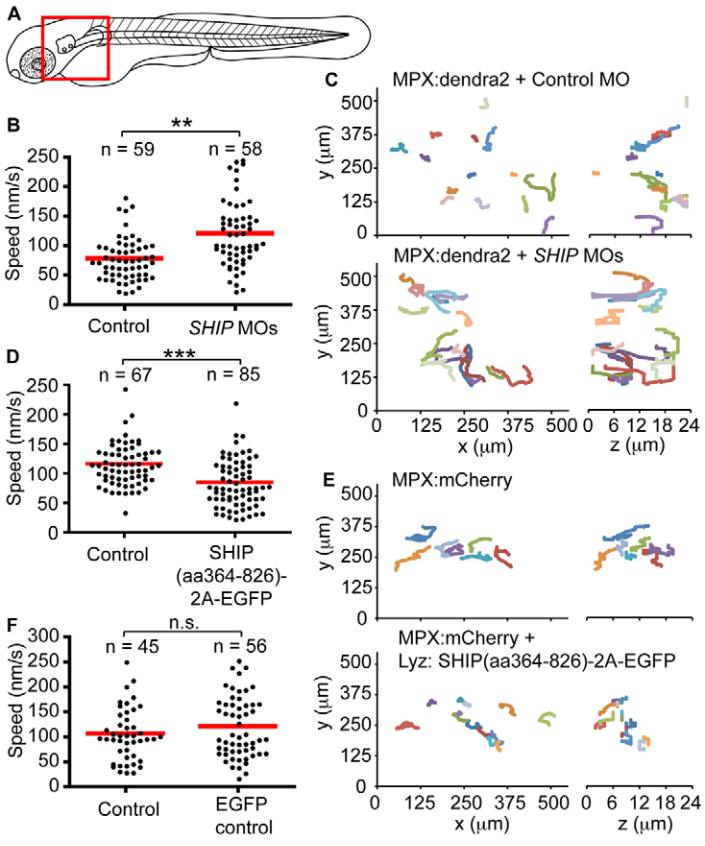

Fig. 4. SHIP regulates neutrophil motility. (**A**) Schematic showing region (red box) where random motility was quantified. (**B**) Scatter plot showing the mean speed of *Tg*(*mpx:dendra2*) neutrophils from control or *SHIP* morphants at 2.5 dpf. Neutrophils were tracked in 3 dimensions (3D) using the Image J software and the MTrackJ plugin. *SHIP* morphants showed increased neutrophil speed compared with controls. Data were collected from 4 individual movies for controls and morphants. $^{**}P<0.01$ (two-tailed, unpaired *t*-test). (**C**) Cell migration tracks plotted in 3D and viewed in the xy-plane (left) or the zy-plane (right). (**D**) Graph showing mean velocity of 3D tracked neutrophils from control or SHIP phosphatase domain [SHIP(aa364–826)-2A-EGFP] expressing neutrophils in *Tg*(*mpx:mCherry*) at 3 dpf. Data were collected from 7 individual movies. $^{***}P<0.001$ (two-tailed, unpaired *t*-test). (**E**) Similar to C, the tracks were plotted in 3D and viewed in the xy-plane (left) or the zy-plane (right). (**F**) As a control for (D), mean velocity of neutrophils expressing EGFP compared with neutrophils in the same embryo that do not express EGFP (Control). Data were collected from 7 individual movies. *n*=number of neutrophils counted.

our findings demonstrate that the effects of ectopic expression of SHIP1 are cell autonomous, since SHIP1 expression in neutrophils alone was sufficient to impair migration.

It is well established that localized generation of PI(3,4,5)P_3 is critical for neutrophil motility. PI(3,4,5)P_3 can activate Rac through the recruitment of Rac guanine nucleotide exchange factors (Nishikimi et al., 2009) and it is known that Rac2 is required for neutrophil motility *in vivo* (Deng et al., 2011). SHIP prevents PI(3,4,5)P_3 accumulation and it is possible that SHIP phosphatases limit neutrophil motility by affecting Rac2 activity at the leading edge. In addition to the role that SHIP plays in dampening PI(3,4,5)P_3 signaling, it is also possible that SHIP alters neutrophil motility through the generation of PI(3,4)P_2 which can act as a second messenger by binding pleckstrin homology(PH)-containing proteins such as TAPPs (Marshall et al., 2002), Akt, PDK-1 and PKC family members (reviewed by Rameh and Cantley, 1999).

Our findings that SHIP impairs leukocyte motility are consistent with previous reports that bone marrow-derived SHIP-deficient macrophages exhibit spontaneous and enhanced migration (Vedham et al., 2005) and the increase in myeloid infiltration into vital organs in SHIP$^{-/-}$ mice (Helgason et al., 1998). A previous report showed that SHIP-deficient neutrophils have impaired cell polarity and migration *in vitro*, suggesting a role for SHIP in regulating neutrophil motility (Nishio et al., 2007). However, this did not explain the SHIP-deficient inflammation phenotype observed *in vivo*. This discrepancy may be due to the differences between *in vitro* 2D and *in vivo* 3D motility, which sometimes yield contradictory results. For example, integrins are required for leukocyte migration on 2D surfaces but not in 3D interstitial tissues (Lämmermann et al., 2008). Moreover, MEK-cofilin signaling controls T-cell migration in 3D but not 2D environments (Klemke et al., 2010). Therefore, the use of *in vivo* model systems is crucial to increase our understanding of how specific pathways regulate leukocyte motility and inflammation in live animals.

Our findings suggest that SHIP is a key brake that limits neutrophil motility *in vivo*. An important unanswered question is how and when this "brake" is released to allow for motility. Hydrogen peroxide is generated at wounds and mediates rapid wound detection in zebrafish (Niethammer et al., 2009). Moreover, we have recently shown that the Src family kinase Lyn acts as a redox sensor that can mediate leukocyte wound attraction (Yoo et al., 2011). In addition to activating Lyn kinase, hydrogen peroxide can also inactivate phosphatases, such as SHIP, through the oxidation of specific cysteine residues (Paulsen and Carroll, 2010; Poole and Nelson, 2008). Although Lyn can activate SHIP under some conditions (Baran et al., 2003), it is intriguing to speculate that in the presence of hydrogen peroxide, Lyn activation of SHIP phosphatases (the "brake") is prevented. A future challenge is to understand how these positive and negative regulatory pathways are coordinated and balanced to mediate proper leukocyte wound attraction and prevent untoward inflammation *in vivo*.

Materials and Methods

Tail fin wounding, whole-mount immunolabeling and Sudan Black staining

All animal experiments were performed according to approved guidelines. Embryos pretreated with 0.5% DMSO, AS-605240 (1 μM) for 1 hour at 2.5 dpf were anesthetized by 0.2 mg/mL tricaine and wounded at the tail fin with a 33 gauge needle. *Tg*(*mpx:dendra2*) embryos were fixed in 1.5% formaldehyde in 0.1 M PIPES, 1 mM $MgSO_4$, and 2 mM EGTA overnight at 4℃ and immunolabeled for L-plastin as previously described (Mathias et al., 2009; Yoo and Huttenlocher, 2011). Wild-type embryos were fixed in 4% formaldehyde overnight at room temperature and stained with Sudan Black as described previously (Le Guyader et al., 2008).

RT-PCR

RT-PCR was performed using mRNA from FACS-sorted myeloid cells using *Tg*(*mpx:dendra2*) zebrafish embryos as previously described (Mathias et al., 2009; Yoo and Huttenlocher, 2011). Primers used for checking expression of different *SHIP* isoforms are as follow: *SHIP1*-RT-F: 5′-CACAGCCAATCAAATCCCACATGAC-3′; *SHIP1*-RT-R: 5′-GCTTCGTGTGATGGAAGGTCAAC-3′; *SHIP2a*-RT-F: 5′-CTGTGCAAACCTTCGAGGTGAAAC-3′; *SHIP2a*-RT-R: 5′-CCACCCATTCTTTATCGCACACAG-3′; *SHIP2b*-RT-F: 5′- GAGAATTTGAAAGGAGGCGCGTC-3′; *SHIP2b*-RT-R: 5′-CTCTGAAACTTCACCAACTGCACG-3′.

Primers used for checking MO knockdown efficiency are as follow: *SHIP1* MO1-F: 5′-GGCACCATGGCAATATCACTCGG-3′; *SHIP1* MO1-R: 5′-CCCATTCTGGACCTGTTCTGCATC-3′; *SHIP1* MO2-F: 5′-GTCTCCTGGAGCTGGAAGACTAAG-3′; *SHIP1* MO2-R: 5′-GTCATGTGGGATTTGATTGGCTGTG-3′; *SHIP2a* MO1/MO2-F: 5′-CTTCGCCAGTGCAAAGAAAAGGG-3′; *SHIP2a* MO1/MO2-R: 5′-CTCTCTCCAGGTTCAGCTGGTC-3′.

DNA, RNA and morpholino injection

All DNA expression vectors contain either the zebrafish myeloperoxidase (*mpx*) promoter or lysozyme C (*lyz*) promoter for neutrophil expression (Kitaguchi et al., 2009; Mathias et al., 2006), minimal Tol2 elements for efficient integration (Urasaki et al., 2006), and an SV40 polyadenylation sequence (Clontech Laboratories, Inc.). Constructs with each of the following in the backbone were constructed: TagRFP-GRP1-PH, AcGFP-TAPP1-PH (Furutani et al., 2006), human EGFP-SHIP1 (accession BC113582), zebrafish EGFP-SHIP1 (accession XM_001922972), zebrafish EGFP-SHIP2a (accession DQ272661) and TM-SHIP1(aa364–826)-2A-EGFP [TM, plasma membrane targeting signal (Lyn kinase targeting sequence); human SHIP1b accession BC113582], The primers *SHIP1*F, 5′-AGCAGATCTATGTTTTCCCCCCAGCCGTG-3′ and *SHIP1*R, 5′-GCTTCTAGATCACTTTAGCTGTCGACCCATCTTCAG-3′ were used to amplify the full-length coding sequence of zebrafish SHIP1 from WT AB strain cDNA at 3 dpf, which was then tagged with EGFP. The primers *SHIP2a*F, 5′-AGCGAATTCGTGTGAGTCTCCGCGATGGC-3′ and *SHIP2a*R, 5′-GCTACTAGTGGAATTCCTCAGGTCAGATTCACCAGTGC-3′ were used to subclone *SHIP2a* from *SHIP2a* cDNA and tagged with EGFP.

Expression of single construct was obtained by injection of 3 nL solution containing 12.5 ng/μL of DNA plasmid and 17.5 ng/μL *in vitro* transcribed (Ambion) Tol2 transposase mRNA into the cytoplasm of one-cell stage embryos. Expression of two constructs was obtained by mixing 6.25 ng/μL of each DNA plasmid. Two non-overlapping antisense splice-blocking morpholino oligonucleotides (MOs) of zebrafish *SHIP1* and *SHIP2a* were synthesized by Gene Tools: *SHIP1* MO1, 5′-TTGGACTGTTCAGATGTACCTGGTT-3′ which corresponds to the putative exon 3/intron 3 boundary upstream of the phosphatase domain of zebrafish *SHIP1*; *SHIP1* MO2, 5′-ATGACTTAAGACATCTCACCCATGT-3′ which corresponds to the putative exon 12/intron 12 region within the phosphatase domain of zebrafish *SHIP1*; *SHIP2a* MO1, 5′-TGTGTTGTTGTGTTCCTGACCGAGT-3′ which corresponds to the exon 13/intron 13 of zebrafish *SHIP2a*; and *SHIP2a* MO2, 5′-CCCAGAATGCCGTGTTTCACCTGTA-3′ which corresponds to the exon 15/intron 15 region within the phosphatase domain of zebrafish *SHIP2a*. Morpholinos were resuspended in Danieau buffer at a stock concentration of 1 mM. Final MO concentrations were injected into the cytoplasm of one-cell stage embryos. Individual concentrations used: 100 μM, 3 nl *SHIP1* MO1; 250 μM, 3 nl *SHIP1* MO2; 25 μM, 3 nl *SHIP2a* MO1; 200 μM, 3 nl *SHIP2a* MO2. Standard control MO from Gene Tools 5′-CCTCTTACCTCAGTTACAATTTATA-3′ was used as controls. Injected embryos were cultured in E3 medium at 28.5℃.

Live imaging

Embryos at 2–3 dpf were anesthetized using 0.2 mg/mL tricaine on a glass-bottom dish for live imaging. Time-lapse fluorescence images were acquired using a confocal microscope (FluoView FV1000, Olympus) using a NA 0.75/20×objective or a NA 1.10/60×water immersion objective. Sequential line scanning was used for each fluorescence channel. Z-stacked and ratiometric images were generated by FluoView FV1000 software (Olympus). After the ratio analysis with background subtraction was performed, a median filter was used to generate an image for visualization. For supplementary material Fig. S2 and Movie 5, a spinning disk confocal system (Yokogawa CSU-X) mounted on a Zeiss Observer Z.1 inverted microscope was used. A Photometrics Evolve EMCCD camera and a NA1.3/60×water immersion objective was used to acquire the Z-series images with a 0.4 μm step size and 300 EM gain. Maximum intensity projection images were made using the Zen 2011 (blue edition) software (Carl Zeiss). Ratio images were generated by using the AxioVison SE64 Rel 4.8 Physiology measurement function with confidence mapping.

Statistics

Experimental results were analyzed with Prism version 4 (GraphPad Software) statistical software. Statistical significance was determined with the unpaired Student *t* test or one-way ANOVA with Dunnett post-test. The resulting *P* values are included in the figure legends for each experiment; *n*=the number of embryos or neutrophils quantified.

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