Live imaging of neutrophil motility in a zebrafish model of WHIM syndrome.

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Running Title: Zebrafish Model of WHIM Syndrome
Abstract

CXCR4 is a G-protein coupled chemokine receptor that has been implicated in the pathogenesis of primary immunodeficiency disorders and cancer. Autosomal dominant gain-of-function truncations of CXCR4 are associated with warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome, a primary immunodeficiency disorder characterized by neutropenia and recurrent infections. Recent progress has implicated CXCR4-SDF1 signaling in regulating neutrophil homeostasis but the precise role of CXCR4-SDF1 interactions in regulating neutrophil motility in vivo is not known. Here, we utilize the optical transparency of zebrafish to visualize neutrophil trafficking in vivo in a zebrafish model of WHIM syndrome. We demonstrate that expression of WHIM mutations in zebrafish neutrophils induces neutrophil retention in hematopoietic tissue, impairing neutrophil motility and wound recruitment. The neutrophil retention signal induced by WHIM truncation mutations is SDF1 dependent, since depletion of SDF1 using morpholino oligonucleotides restores neutrophil chemotaxis to wounds. Moreover, localized activation of a genetically encoded, photoactivatable Rac GTPase is sufficient to direct migration of neutrophils that express the WHIM mutation. The findings suggest that this transgenic zebrafish model of WHIM syndrome may provide a valuable tool to screen for agents that modify CXCR4-SDF1 retention signals.
Introduction

Stromal cell-derived factor 1 (SDF1, CXCL12) mediated activation of the chemokine receptor CXCR4 is important for both normal and pathological processes including primordial germ cell migration, HIV pathogenesis, invasive migration of cancer cells and leukocyte trafficking. Therefore, there is substantial interest in understanding how CXCR4-SDF1 signaling regulates cell motility and how these mechanisms can be targeted to treat human disease. CXCR4 signaling is attenuated by receptor internalization, which is regulated by phosphorylation events and binding of regulatory proteins to the cytoplasmic tail. The functional importance of CXCR4 internalization is highlighted by the dominantly inherited primary immunodeficiency, WHIM syndrome, where truncations of CXCR4 lead to altered signaling and gain-of-function.

WHIM syndrome is characterized by warts, hypogammaglobulinemia, infections and myelokathexis, a severe chronic neutropenia. Substantial evidence supports the importance of CXCR4 signaling in regulating neutrophil homeostasis and release from the bone marrow (BM). It has been postulated that the neutropenia in WHIM patients results from both neutrophil retention in the BM and enhanced neutrophil apoptosis of retained neutrophils. Direct evidence to support this hypothesis has been provided by a mouse model of WHIM syndrome induced by the ectopic expression of WHIM truncation mutations of CXCR4 in hematopoietic stem cells that show impaired neutrophil release into the blood and increased rates of apoptosis in the BM. Previous reports indicate that neutrophils from WHIM patients demonstrate increased signaling and chemotaxis in response to SDF1. However, there have been some reports that suggest that the C-terminus of CXCR4 can both positively and negatively
regulate cell motility,\textsuperscript{8,9,14} and alternatively, may be involved in modulating the precise targeting of cells in vivo.\textsuperscript{15}

Despite the importance of CXCR4-SDF1 signaling, there are few animal models of WHIM syndrome amenable to imaging or screening for drugs that modulate CXCR4-SDF1 function in vivo. Modeling WHIM syndrome is particularly attractive since CXCR4 signaling is important to many disease processes and is a direct result of aberrant chemokine signaling. Therefore, developing a model of WHIM syndrome in a system that allows the direct visualization of motility and chemotactic events in vivo would be a beneficial tool to understand disease pathogenesis. Our current understanding of WHIM syndrome is mostly derived from in vitro experiments\textsuperscript{8,9,13} or in vivo mouse models where observing chemotactic events is difficult.\textsuperscript{12} The zebrafish, \textit{Danio rerio}, is an ideal system to visualize neutrophil migration in vivo.\textsuperscript{16} Additionally, there has been substantial recent progress in understanding how neutrophils develop\textsuperscript{17,18} and respond to inflammatory cues in zebrafish.\textsuperscript{16,19,20}

Here we describe the generation of a zebrafish model of the primary immunodeficiency syndrome, WHIM syndrome. WHIM zebrafish are neutropenic and WHIM neutrophils demonstrate impaired recruitment to wounds and tissue inflammation, recapitulating the human disease. Exploiting the optical clarity of zebrafish larvae we use real-time imaging to directly observe neutrophil motility in vivo. Expression of WHIM truncation mutations of CXCR4 in zebrafish neutrophils induces neutrophil retention in hematopoietic tissue and mediates a retention signal that is SDF1 dependent. These findings support the power of zebrafish to model primary immune disorders and provide a valuable tool to screen for agents that modify CXCR4-SDF1 retention signals.
Materials and Methods

Transient expression in zebrafish and generation of CXCR4b-GFP and WHIM-GFP transgenic lines

All protocols using zebrafish in this study were approved by the University of Wisconsin-Madison Research Animal Resources Center. All DNA expression vectors contain either the zebrafish MPO promoter for neutrophil expression\textsuperscript{16} or a CMV promoter, minimal Tol2 elements for efficient integration,\textsuperscript{21} and an SV40 polyadenylation sequence (Clontech Laboratories, Inc). Constructs with each of the following in the backbone were constructed: zCXCR4b-GFP, zCXCR4b-WHIM-GFP, mCherry, SDF1a-2A-mCherry and Dendra2 (Evergen). Expression of constructs was obtained by injection of 0.5-1 nl of 25 ng/\(\mu\)l plasmid with 45 ng/\(\mu\)l in vitro transcribed (Invitrogen) Tol2 transposase mRNA into the yolk of one-cell staged embryos just below the cell. Injected embryos were raised to sexual maturity and screened by crossing with wild-type fish to identify founders. F1 embryos were screened at 2-3 day post fertilization (dpf) for GFP expression in the caudal hematopoietic tissue (CHT) and raised to sexual maturity. Experiments were done on progeny of F1 incrosses. Transgenic lines from at least two separate founders were generated for each injected construct. All results reported here are from experiments performed on one of the transgenic lines but many experiments were replicated on other lines to ensure consistency between founders. All experiments were done at least three times and images of fixed larvae are representative of at least 20 larvae.

Tailfin wounding, whole-mount immunolabeling and Sudan Black staining

For wounding assays larvae were anesthetized in E3 containing 0.1mg/ml tricaine and wounded in the ventral tailfin with a 25-gauge needle, or their tails were transected using a surgical blade.
After wounding, live larvae were embedded in 1% low-melt agarose for imaging or fixed for further processing. Larvae were fixed in 1% formaldehyde in PBS overnight at 4°C and double-immunolabeled for MPO and L-plastin as described\textsuperscript{22} or fixed in 4% formaldehyde overnight at 4°C and stained with Sudan Black as reported.\textsuperscript{18}

**Whole-mount in situ hybridization**

Plasmids containing \textit{mpo}, \textit{sdf1a}, and \textit{sdf1b} were linearized using restriction enzymes (SalI for \textit{mpo} and NotI for \textit{sdf1a} and \textit{b}) to serve as template for transcription of anti-sense probes. Digoxigenin-labeled RNA probes were transcribed for \textit{sdf1a} and \textit{sdf1b} using T3 RNA polymerase. For double whole-mount in situ hybridization (WISH), fluorescein-labeled antisense \textit{mpo} RNA probe was generated with T7 RNA polymerase. WISH was performed as described.\textsuperscript{23} Following staining, embryos were placed in 80% glycerol for imaging.

**Morpholino oligonucleotide microinjection**

MO (Gene Tools) were resuspended in 1x Danieau buffer at a stock concentration of 1 mM. Final MO concentrations were injected (0.5-1 nl) into the yolk of 1-2 cell-staged embryos.

**Statistics**

Experimental results were analyzed using the Prism 4 (GraphPad Software) statistical software package. Statistical significance was determined using unpaired student’s t-test (to compare two groups) or one-way ANOVA followed by Tukey’s multiple comparison post-test (to compare multiple groups) at a 95% confidence interval. The resulting p-values are included in the figure legends for each experiment. n = the number of larvae or neutrophils quantified.
Results

Hematopoietic expression of CXCR4b and SDF1a in zebrafish larvae

The zebrafish genome encodes two SDF1 ligands, SDF1a and SDF1b, and two SDF1 receptors, CXCR4a and CXCR4b. The expression pattern of CXCR4 in zebrafish hematopoietic cells has not previously been defined. To determine if CXCR4 is expressed in zebrafish neutrophils, we performed RT-PCR for both CXCR4a and CXCR4b on RNA isolated from neutrophils and macrophages sorted from MPO:Dendra2 transgenic larvae at 3 dpf by fluorescence activated cell sorting (Figure S1A).22,24,25 As previously reported, cells with low levels of fluorescence represent macrophage-like cells and express the receptor CSF1R. In contrast, cells with high levels of fluorescence represent neutrophil-like cells and express MPO (Figure 1A).22 We found that both macrophages and neutrophils express CXCR4b but not CXCR4a. In contrast, both CXCR4a and CXCR4b are expressed in whole kidney marrow isolated from adult zebrafish (Figure 1A). These findings suggest that the primary SDF1 receptor in neutrophils from zebrafish larvae is CXCR4b.

To determine the spatial distribution of the CXCR4 ligand, SDF1, in zebrafish larvae, we performed WISH for SDF1a or SDF1b mRNA at 2 and 3 dpf. Interestingly, we observed concentration of SDF1a mRNA in regions of neutrophil production including in the head (Figure 1B), the caudal hematopoietic tissue (CHT, region of larval neutrophil development located in the anterior ventral tailfin) and along the pronephric duct (Figure 1C, S1F and S1G) as previously reported26. These areas of high SDF1a expression correlated with two distinct populations of neutrophils observed in zebrafish during this stage of development (Figure 1D). SDF1b was also expressed in the head and ventral body at 2 dpf but staining was generally more
diffuse (Figure S1B and S1C). Taken together, these findings suggest that zebrafish neutrophils develop or accumulate in regions with high SDF1a expression in zebrafish larvae.

**Generation of WHIM-GFP transgenic zebrafish line**

To model the human disorder, WHIM syndrome, in zebrafish we expressed the homologous CXCR4 receptor truncation mutations in zebrafish neutrophils using the MPO promoter. Alignment of the C-terminal tail of zebrafish CXCR4b with human CXCR4 revealed a high level of conservation, especially in serine residues that are critical for the proper internalization of CXCR4 after binding to SDF1 (Figure 2A). By truncating 19 amino acids from the C-terminus of CXCR4b, we generated a truncation mutation analogous to mutations described in patients with WHIM syndrome (Figure 2A). To determine if this mutation affects receptor internalization we expressed both wild-type CXCR4b-GFP and the truncation mutant (hereafter referred to as WHIM-GFP) in HEK cells and treated the cells with human SDF1. As expected both CXCR4b-GFP and WHIM-GFP localized to the cell membrane of transfected HEK cells (Figure S2A). Treatment with SDF1 induced internalization of CXCR4b-GFP into intracellular vesicles, but not GFP or WHIM-GFP, indicating that the WHIM truncation impairs CXCR4b internalization after ligand binding (Figure 2B).

To develop a zebrafish model of WHIM syndrome we generated a stable transgenic line by expressing WHIM-GFP specifically in neutrophils using the MPO promoter (Figure 2C). The MPO:WHIM-GFP transgenic line will hereafter be referred to as WHIM-GFP. In accordance with our findings reported with the MPO:GFP transgenic line we observed GFP+ neutrophils in the CHT in WHIM-GFP larvae (Figure 2D, 2E and S2B). WHIM-GFP localized to the cell membrane in individual cells in the CHT (Figure 2G), as compared to the diffuse

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localization of GFP alone (Figure 2F). In contrast to the previously published MPO:GFP16 transgenic we did not observe ectopic expression of WHIM-GFP in tissues other than hematopoietic cells (Figure S2B).

**Neutrophil development in WHIM-GFP larvae**

To determine if expression of the WHIM-GFP receptor affects the distribution of neutrophils during zebrafish development, wild-type or WHIM-GFP larvae were fixed at 2,3,7 or 13 dpf and stained with Sudan Black to highlight neutrophils.18 At 3 dpf, we observed normal neutrophil development in the CHT in both wild-type and WHIM-GFP larvae (Figure 3A and 3B). However, WHIM-GFP larvae demonstrated neutrophil aggregates at 3 dpf (Figure 3B arrows), which were even more apparent at 7 dpf (Figure 3C and 3D arrows). Neutrophils were not detected ventrally over the gut (Figure 3C arrows and 3D) or along the dorsal ridge of WHIM-GFP larvae as seen in controls (Figure 3C arrowheads and 3D). Strikingly, at 13 dpf, WHIM-GFP larvae demonstrated persistent neutrophil aggregates in the CHT (Figure 3F arrows) and in the midline region (Figure 3F arrowheads), while wild-type zebrafish no longer had neutrophils in the CHT (Figure 3E).

Sudan Black staining also revealed neutrophil aggregates in the heads of 2 and 3 dpf WHIM-GFP larvae (Figure S3C,S3D,3K, and 3L arrows) in contrast to wild-type larvae that demonstrate a diffuse distribution of neutrophils (Figure S3A,S3B,3G, and 3H). These neutrophil aggregates persisted on the ventral side of the head at 7 (Figure S3H arrow) and 13 (Figure S3J arrow) dpf. Interestingly, less prominent neutrophil aggregates were also observed in the head of transgenic larvae that ectopically express wild-type CXCR4-GFP in neutrophils (Figure S4A and S4B). This is consistent with a previous study that reported similar phenotypes
with overexpression of wild-type and WHIM-truncated CXCR4 in mouse models. These findings indicate that the WHIM truncation is a gain-of-function mutation in vivo.

By 7 dpf, neutrophil development was also apparent in the kidney in wild-type zebrafish (Figure 3M), which is the primary site of adult hematopoiesis. Interestingly, in WHIM-GFP transgenic larvae the hematopoietic tissues of the developing kidney had greater neutrophil accumulation compared to control larvae (Figure 3N arrow). This difference remained prominent at 13 dpf (Figure 3O and 3P). However, no differences in neutrophil development were observed among wild-type, MPO:GFP or GFP siblings of WHIM-GFP larvae. Additionally, no differences in apoptosis of neutrophils were observed by TUNEL staining in WHIM-GFP and control larvae (K.B.W, unpublished data). Together, these findings suggest that WHIM-GFP larvae have neutrophil aggregates at sites of SDF1a expression including the CHT and ventral head. Furthermore, WHIM-GFP larvae have neutrophil aggregates in the kidney, the site of neutrophil development in adult zebrafish, reminiscent of the human disorder WHIM syndrome.

**CXCR4-SDF1 signaling mediates neutrophil arrest and aggregation in the head**

To determine if neutrophils aggregate in regions of high SDF1a expression in the head, we compared SDF1a expression by WISH with Sudan Black staining. At 2 dpf a ventral view of the head revealed two bilateral patches of SDF1a expression above the yolk sac (Figure S3E and S3F arrows) that converge at 3 dpf to form an area of concentrated SDF1a expression on the ventral side of the head (Figure 3I, 3J and S1Gii, arrow). The areas of SDF1a expression closely paralleled the location of neutrophil aggregates in the head of WHIM-GFP larvae at both 2 (Figure S3C-S3F) and 3 (Figure 3I-3L) dpf. In contrast to SDF1a, at 3 dpf SDF1b was expressed
on the ventral side of the head (Figure S1D and S1E) in areas without neutrophil aggregation, suggesting that neutrophils accumulate in areas with high SDF1a but not SDF1b expression. To confirm this in the context of an individual larva, we performed double WISH with probes for SDF1a (purple) and mpo (red). Indeed, in WHIM-GFP larvae at 3 dpf, we found that mpo expression co-localized with a patch of SDF1a expression on the ventral head (Figure S3K and S3L).

The formation of neutrophil aggregates in areas of high SDF1a expression suggests that WHIM-GFP neutrophils are retained by CXCR4b-SDF1a interactions in vivo. To determine if SDF1a mediates neutrophil aggregation in the head, endogenous SDF1a was depleted using morpholino oligonucleotides (MO). Injection of high concentrations of SDF1a MO (between 1 mM and 200 μM) resulted in gross morphological and neutrophil developmental defects (Figure S5A and S5B), while injection of a lower concentration (110 μM) allowed for near normal neutrophil development (Figure S5C). Injection of the lower concentration of SDF1a (Figure 4C and 4D) but not SDF1b (Figure 4E and 4F) or control (Figure 4A and 4B) MO restored a diffuse distribution of neutrophils in the head, indicating that CXCR4b-SDF1a signaling mediates neutrophil aggregate formation. In order to characterize how CXCR4b-SDF1a interactions regulate neutrophil aggregation in the head, we monitored neutrophil motility in vivo using live fluorescence microscopy in uninjected and SDF1a morphant MPO:GFP or WHIM-GFP transgenic larvae. Control neutrophils (MPO:GFP) in the head exhibit spontaneous, random migration in zebrafish larvae at 2-3 dpf. Live imaging shows rapid polarization and persistent migration of GFP+ control neutrophils in the head at 3 dpf (Figure 4H and Movie S1). Knockdown of SDF1a in MPO:GFP larvae did not affect migration of MPO:GFP neutrophils (Figure 4I and Movie S1). Strikingly, in WHIM-GFP larvae, neutrophils in the head
demonstrate random protrusions but impaired persistent motility resulting in arrested migration and retention in regions of high SDF1a expression in the head (Figure 4J and Movie S1). Injection of the SDF1a MO partially restored directed neutrophil motility and decreased neutrophil aggregation in WHIM-GFP larvae (Figure 4K and Movie S1), indicating that SDF1a mediates the neutrophil retention signal. The 3D velocity of neutrophils in WHIM-GFP larvae was significantly impaired compared to control neutrophils (Figure 4G). Injection of the SDF1a MO, however, partially rescued the migration velocity of WHIM-GFP neutrophils (Figure 4G). Taken together these results suggest that CXCR4 WHIM mutations impair persistent neutrophil motility in the head of zebrafish larvae through interactions with endogenously expressed SDF1a.

To determine if expression of SDF1a is sufficient to attract WHIM-GFP neutrophils and induce neutrophil aggregation we transiently expressed SDF1a-2A-mCherry in a mosaic fashion in WHIM-GFP larvae. The self-cleaving, viral 2A peptide\(^\text{29}\) allows for expression of untagged, secreted SDF1a while marking the secreting cells with mCherry. WHIM-GFP neutrophils could be found in close association with SDF1a expressing cells in a variety of different tissues of the head, body and yolk sac (Figure 4L), with occasional clusters of WHIM-GFP neutrophils forming in regions with ectopic expression of SDF1a (Figure 4Li). In contrast, expression of mCherry alone failed to attract WHIM-GFP neutrophils (Figure 4L). Live imaging revealed that the association of WHIM-GFP neutrophils and SDF1a expressing cells was dynamic and persistent, often with long periods of close contact between the cells (Movie S2). Together these results suggest that regionalized expression of SDF1a is necessary and sufficient to form WHIM-GFP neutrophil aggregates.
WHIM-GFP transgenic larvae are neutropenic

A hallmark of the human disorder, WHIM syndrome, is peripheral neutropenia. To determine if WHIM-GFP larvae are neutropenic in the vasculature, we used live imaging to scan blood flow for GFP+ cells. In MPO:GFP larvae, neutrophils can be observed in the blood stream by 3 dpf. By quick acquisition of a small section of the dorsal aorta we quantified the number of bright GFP+ cells per minute of blood flow (Figure 5A). In 3 and 4 dpf MPO:GFP transgenic larvae the number of neutrophils in the blood varied from 6 neutrophils/min. to 32 neutrophils/min. with an average of approximately 21 neutrophils/min. of blood flow (Figure 5B and Movie S3). In contrast, GFP+ cells were absent in the blood of WHIM-GFP transgenic larvae at 3-4 dpf (Figure 5B and Movie S3) and 7 dpf (K.B.W, unpublished data). These findings demonstrate that we have generated a neutropenic model of WHIM syndrome in zebrafish.

Impaired chemotaxis of WHIM-GFP neutrophils to wounds

Wounding the fins of zebrafish larvae results in the rapid recruitment of neutrophils, and this recruitment can be visualized by live imaging. Interestingly, we observed impaired neutrophil chemotaxis to wounds in the ventral tailfin of WHIM-GFP larvae (Figure 5C and Movie S4). To quantify the recruitment defect we used a fixed wounding assay with Sudan Black staining to visualize neutrophils at the wound. At 2 hours post wound (hpw) there was robust recruitment of neutrophils to the wounds in control larvae with few detectable neutrophils recruited to the wounds of WHIM-GFP larvae (Figure 5D-5F). A time course showed impaired neutrophil recruitment to wounds at all time points between 1 and 24 hours post wounding (Figure 5G).
To determine if WHIM-GFP neutrophils could respond to stronger inflammatory stimuli we examined neutrophil recruitment in response to tail transection and in the context of chronic inflammation. We have previously reported two mutant lines, spint1a\textsuperscript{hi2217} and clint1\textsuperscript{hi1520}, that demonstrate epidermal hyperproliferation and robust chronic neutrophil recruitment into the tailfin\textsuperscript{19,31}. Using a MO targeting clint1 to recapitulate the clint1\textsuperscript{hi1520} phenotype, we observed neutrophil recruitment into the tailfins of control larvae but not in WHIM-GFP larvae (Figure 6A and Movie S5). Similarly, transient expression of WHIM-GFP in neutrophils of spint1a\textsuperscript{hi2217} larvae\textsuperscript{19} impaired neutrophil recruitment to the tailfin (Movie S6). WHIM-GFP neutrophils also failed to respond to tail transections (Figure 6B and Movie S7). Interestingly, although neutrophil chemotaxis to tail transections was impaired, wounding induced increased protrusion and localized motility of WHIM-GFP neutrophils in the CHT (Movie S7) of transgenic larvae. Taken together, these findings suggest that even in the presence of robust inflammatory stimuli, neutrophils demonstrate impaired mobilization and recruitment into inflamed tissues in the WHIM transgenic.

To determine if ectopic expression of wild-type CXCR4 receptor also affected neutrophil recruitment to wounds, we quantified neutrophil numbers at wounds in transgenic larvae that express wild type CXCR4-GFP in neutrophils. Although neutrophil recruitment to wounds was reduced with ectopic expression of wild-type CXCR4\textsuperscript{b} (Figure S4F), neutrophils remained responsive to wounding in contrast to WHIM-GFP larvae (Figure S4C-S4F, Movie S8 and S9). It is possible that this difference in recruitment may be due to variation in receptor expression levels or alternatively increased activity of the WHIM truncation as compared to wild-type receptor. In any case, the findings indicate that the WHIM truncations induce a gain of function in CXCR4 signaling that impairs neutrophil motility in response to inflammatory signals in vivo.
Previously, we reported that larval inflammatory macrophages, a subset of macrophages, express low levels of GFP in MPO:GFP transgenic larvae, are highly phagocytic, have an elongated morphology, respond to wounds in the tailfin and express L-plastin but not MPO.\textsuperscript{22} This population of cells will hereafter be referred to as macrophages. In WHIM-GFP larvae, macrophages do not express detectable levels of the WHIM transgene (K.B.W, unpublished data). To determine if macrophage recruitment was also affected in the WHIM-GFP transgenic, we examined macrophage chemotaxis to the wound by quantifying macrophage number using double immunolabeling for MPO and L-plastin as recently reported.\textsuperscript{22} In control larvae, both L-plastin\textsuperscript{+}/MPO\textsuperscript{+} neutrophils (Figure 6C bottom row, arrows) and L-plastin\textsuperscript{+}/MPO\textsuperscript{-} macrophages (Figure 6C bottom row, arrowheads) were recruited to the wounds. In 100% of WHIM-GFP larvae examined, L-plastin\textsuperscript{+}/MPO\textsuperscript{-} macrophages (arrowheads) were present at the wounds, while no L-plastin\textsuperscript{+}/ MPO\textsuperscript{+} neutrophils (arrows) were detected (Figure 6C top row). This is an interesting observation since neutrophils are the first cells recruited to wounds in vivo. Our findings suggest that impaired neutrophil recruitment does not affect the later recruitment of macrophages.

**Depletion of SDF1a rescues neutrophil motility in WHIM-GFP larvae**

Previous studies have suggested that enhanced CXCR4-SDF1 signaling mediates neutrophil retention in the BM in patients with WHIM syndrome. However, current models are not amenable to directly testing this hypothesis. To determine if SDF1a mediates neutrophil retention in the zebrafish model of WHIM syndrome, we depleted endogenous SDF1a using MO. Following injection of a control MO, WHIM-GFP neutrophils showed impaired chemotaxis to wounds (Figure 7B) or tail transection (Figure 7E). In contrast, we found that
after injection of the SDF1a MO, WHIM-GFP neutrophils showed robust recruitment to wounds (Figure 7A,7C and Movie S10) and tail transections (Figure 7F). Quantification revealed a significant rescue in the ability of neutrophils to respond to wounding (Figure 7D) or tail transection (Figure 7G) after injection of the SDF1a MO. Taken together, the findings provide the first direct evidence showing that SDF1a is necessary for the neutrophil retention signal induced by CXCR4 WHIM truncations. Moreover, the findings suggest that intrinsic cell motility in the absence of SDF1a is not affected by truncation of CXCR4, but that the mutation increases the sensitivity of neutrophils to endogenous SDF1a, thereby inducing a retention signal that impairs neutrophil recruitment.

### Localized activation of Rac is sufficient to direct migration of WHIM-GFP neutrophils

Rac GTPases are critical regulators of SDF1-induced directed migration in hematopoietic stem cells, T-cells, cancer cells and endothelial cells.\textsuperscript{32-35} Interestingly, previous studies have reported altered SDF1-induced F-actin polymerization in WHIM patient neutrophils, suggesting that WHIM neutrophils may have defects in polarized F-actin polymerization.\textsuperscript{8} To determine if polarized, Rac-induced, F-actin polymerization is sufficient to rescue the directed migration of neutrophils in WHIM-GFP larvae, we utilized a genetically encoded Rac1 GTPase, which can be photoactivated reversibly and repeatedly by a 458 nm light,\textsuperscript{36} and can direct the migration of neutrophils in vivo.\textsuperscript{28} Photoactivation of Rac at the cell edge of WHIM-GFP neutrophils induced pseudopod protrusion and directed cell migration in WHIM-GFP larvae (Figure 7H and 7I) similar to control neutrophils.\textsuperscript{28} Repeated photoactivation of Rac was sufficient to direct the migration of WHIM-GFP neutrophils away from cell aggregates in the CHT (Figure 7J and Movie S11) and into the tailfin (approximately 85 μm in this example). After photoactivation
was stopped, the neutrophil rapidly migrated back to the cell aggregates in the CHT (Figure 7J and Movie S12). Together the findings suggest that polarized F-actin polymerization induced by Rac activation is sufficient to rescue the directed migration of WHIM-GFP neutrophils in vivo.

Discussion

In this study we report a zebrafish model of a primary immunodeficiency syndrome, WHIM syndrome. We utilize the optical transparency of zebrafish to visualize how CXCR4-SDF1 signaling affects neutrophil trafficking in vivo. We have shown that expression of WHIM mutations in zebrafish neutrophils induces peripheral neutropenia and neutrophil retention in hematopoietic tissues, reminiscent of the human disorder. Live imaging reveals that constitutive CXCR4-SDF1 signaling impairs persistent, directed neutrophil motility thereby retaining neutrophils in regions of high SDF1a expression and inducing neutrophil aggregates. The neutrophil retention signal induced by WHIM truncation mutations is SDF1a dependent, since depletion of SDF1a using MO restores neutrophil mobilization. The finding that SDF1a is required for neutrophil retention in the WHIM transgenic provides the first in vivo evidence to suggest that constitutive signaling through CXCR4-SDF1 mediates neutrophil retention in hematopoietic tissue contributing to peripheral neutropenia. Taken together, these findings support the utility of the zebrafish system to understand chemokine signaling through CXCR4-SDF1 and to identify putative targets that modify CXCR4-SDF1 functions in vivo.

CXCR4 and SDF1 have been studied during developmental cell migration, including migration of primordial germ cells, development of the lateral line and movements of endodermal cells in gastrulation.2,37,38 Here our findings suggest that neutrophils are located in areas where SDF1a mRNA is highly expressed including the CHT and regions of the head in
wild-type larvae. Previous studies have demonstrated that endogenous SDF1a is essential for normal targeting of primordial germ cells in zebrafish.\textsuperscript{2} In contrast, we found that depletion of endogenous SDF1a mRNA by MO did not affect the targeting of neutrophils in control zebrafish, suggesting that there may be redundancy with other factors that also mediate neutrophil retention in hematopoietic tissues in vivo. However, when higher concentrations of SDF1a MO were used we found that neutrophil development was impaired with fewer neutrophils present in the CHT, suggesting that endogenous SDF1a may affect targeting of hematopoietic stem cells that mediate neutrophil development in zebrafish, consistent with previous reports in the literature.\textsuperscript{39}

This is, to our knowledge, the first study to visualize the consequences of altered chemokine signaling on neutrophil trafficking in vivo using live imaging. Previous studies have reported that truncations of the CXCR4 receptor that impair receptor internalization do not alter chemotaxis of primordial germ cells, but rather affect the precision of migration by inducing prolonged “runs” and less tumbling type migration in vivo.\textsuperscript{15} In contrast to the findings with PGC migration, we found that neutrophils in the WHIM transgenic displayed prominent random membrane protrusions but impaired persistent motility in vivo, resulting in retention within areas of high SDF1a expression. Our findings indicate that truncation of the C-terminal tail increases CXCR4 sensitivity to endogenous SDF1, supporting previous reports that suggest WHIM truncations block proper internalization and desensitization of the CXCR4 receptor.\textsuperscript{8,9,13} Taken together, the findings suggest that constitutive CXCR4-SDF1 signaling induces neutrophil stopping or retention within areas of concentrated SDF1a, including hematopoietic tissues, thereby impairing neutrophil trafficking into the vasculature or to tissue wounds.
There has been substantial recent interest in defining mechanisms that stop cell motility and contribute to retention within tissues. Perhaps the best studied example of stop signals have been in the context of T cell motility stopping in response to contact with antigen presenting cells with cognate antigen.\textsuperscript{40} Neutrophils have also been reported to display stopped migration in response to specific factors, including TNFα.\textsuperscript{41} Stop signals have also been implicated in chemokine-mediated signaling in vitro. For example, exposure of neutrophils to one chemokine can impair the migration to other chemokines in vitro.\textsuperscript{42} Here, we demonstrate the effects of competing signals on neutrophil motility in vivo. Constitutive signaling through CXCR4-SDF1 impairs neutrophil recruitment to wounds despite competing gradients of hydrogen peroxide produced at the wound.\textsuperscript{43} Interestingly, polarized Rac activation was sufficient to overcome this retention signal and induce directed migration of WHIM-GFP neutrophils. Taken together, our findings suggest that neutrophil retention signals likely play an essential role in affecting neutrophil trafficking in vivo.

Zebrafish provide a powerful system to model human diseases and allow unique insight into disease pathogenesis since events can be visualized in vivo by real-time imaging. This has been demonstrated in studies observing leukocyte trafficking in the context of chronic inflammation mutant lines,\textsuperscript{19,31,44} bacterial infection\textsuperscript{45} and during granuloma formation in zebrafish mycobacterial infection.\textsuperscript{46} Additionally, depletion of WASp, the protein mutated in the immune disorder Wiskott Aldrich Syndrome has demonstrated defects in wound inflammation and clot formation.\textsuperscript{47} The zebrafish model of WHIM syndrome presented here provides novel insight into the pathogenesis of neutropenia in patients with WHIM syndrome. The findings demonstrate that the WHIM truncations induce a gain of CXCR4 function that requires the presence of SDF1α. Human studies and mouse models of WHIM syndrome supported the
hypothesis that the WHIM mutation induced neutrophil retention in the BM causing neutropenia. We now provide direct evidence that WHIM-induced neutrophil retention is mediated by altered signaling that can be rescued by depletion of SDF1a and blocking CXCR4-SDF1 signaling. WHIM patients are commonly treated with G-CSF, which is thought to mobilize neutrophils from the BM by down-regulating expression of CXCR4 on neutrophils and reducing SDF1 expression in the BM.\textsuperscript{48,49} Since the zebrafish homolog of G-CSF has been identified and characterized,\textsuperscript{50} the zebrafish model of WHIM syndrome provides an ideal system to study how G-CSF affects neutrophil trafficking in vivo in WHIM syndrome.

Here we utilized the optical transparency of zebrafish to visualize how CXCR4-SDF1 signaling affects neutrophil trafficking in vivo. Our work has demonstrated that constitutive signaling through the CXCR4 receptor induces neutrophil retention in hematopoietic tissue that impairs neutrophil motility and recruitment to inflamed tissues. The neutrophil retention signal is mediated by CXCR4-SDF1 signaling, since depletion of SDF1 restores neutrophil motility and wound recruitment. We propose that the WHIM transgenic zebrafish will provide a powerful in vivo tool to screen for agents that modify CXCR4-SDF1 signaling and may therefore have therapeutic potential.

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**Authorship**

Contribution: K.B.W. designed and performed experiments, analyzed data and wrote the paper; J.M.G., J.C.S. and S.Y. performed experiments; A.H. designed experiments, analyzed data and wrote the paper.

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**References**


Figure Legends

Figure 1. Expression of CXCR4b and SDF1a in zebrafish larvae. (A) RT-PCR of csf1r (macrophage marker), mpo (neutrophil marker), eflα (loading control), CXCR4a and CXCR4b from MPO:Dendra2 high (hi) and low (lo) populations. WKM = whole kidney marrow from adult wild-type fish. (B,C) Whole-mount in situ hybridization of SDF1a expression in 2 dpf larvae, lateral view. Note SDF1a expression in the head (B) and CHT (C, arrowheads). p = pronephric duct, cht = caudal hematopoietic tissue. (D) Whole-mount Sudan Black staining to visualize neutrophils in 2 dpf larvae, lateral view. Note neutrophil accumulation in areas of SDF1a expression in the head (box) and CHT (arrowheads). Bar = 200 μm in B-D.

Figure 2. Generation of WHIM-GFP transgenic larvae. (A) Alignment of the C-terminal tails of human CXCR4 with zebrafish CXCR4b and WHIM-truncated zebrafish CXCR4b. Note conservation of serine residues. Arrow marks an identified WHIM truncation mutation. (B) Fluorescence images of HEK cells expressing GFP (first column), CXCR4b-GFP (second column) or WHIM-GFP (third column) after incubation with human SDF1 (bottom row) or vehicle control (top row). (C) Schematic of Tol2-MPO:zCXCR4b-WHIM-GFP vector injected to generate WHIM-GFP transgenic lines. (D) Schematic of 3 dpf zebrafish larvae. Boxed region is approximate area magnified in E. (E) Fluorescence image of the CHT region of a 3 dpf WHIM-GFP larva. (F,G) High magnification image of GFP expressing neutrophils from the CHT of a MPO:GFP (F) and a WHIM-GFP (G, blow up of box in E) larva. Note membrane expression in G. Bar = 50 μm in E; 20 μm in B; 10 μm in F,G.
Figure 3. Neutrophil development in WHIM-GFP larvae. (A-F) Sudan Black staining in the CHT of WT (A,C,E) or WHIM-GFP (B,D,F) larvae at 3 (A,B), 7 (C,D) and 13 (E,F) dpf. Lateral view, anterior to the left. Arrows point to neutrophils over the gut (C) or in clumps in the CHT (B,D,F). Arrowheads point to neutrophils along the dorsal ridge (C) or midline (F). (G-L) Lateral view (G,I,K) or ventral view (H,J,L) of the head of WT (G-J) or WHIM-GFP (K,L) 3 dpf larvae stained with Sudan Black (G,H,K,L) or for SDF1a expression (I,J) by WISH. Arrows point to area of dark SDF1a expression (I,J) or areas of neutrophil accumulation (K,L) on the ventral side of the head under the jaw. (M-P) Sudan Black staining to reveal neutrophils in the kidney (arrows) in 7 (M,N) and 13 (O,P) dpf WT (M,O) or WHIM-GFP (N,P) larvae. Lateral view, g = gut. Bar = 200 μm in A-P.

Figure 4. Neutrophil retention in the head is SDF1a dependent. (A-F) Sudan Black stained WHIM-GFP larvae injected with either control (A,B), SDF1a (C,D) or SDF1b (E,F) MO. Lateral (A,C,E) or ventral (B,D,F) view of the head at 3 dpf; arrows point to neutrophil accumulation. (G) The mean velocity of tracked neutrophils from the ventral head of uninjected or SDF1a morphant MPO:GFP or WHIM-GFP larvae, ** = p-value <0.001,* = p-value < 0.01. (H-K) Neutrophil migration in the ventral head of MPO:GFP (H), SDF1a morphant MPO:GFP (I), WHIM-GFP (J) or SDF1a morphant WHIM-GFP (K) larvae was tracked in three dimensions. The tracks are plotted in 3D space and viewed in the xy-plane (left) or zy-plane (right). Units are in μm on each axis. Only tracks of neutrophils that lasted for at least 14 min. and only the first 14 min. of longer tracks are included. Tracks were taken from Movie S1 with additional tracks in SDF1a morphant larvae from additional movies not shown. (L) WHIM-GFP larvae at 2 or 3 dpf injected with Tol2-CMV:SDF1a-2A-mCherry at the 1-cell stage. Three
examples of WHIM-GFP neutrophils (green) in close association with cells expressing SDF1α-2A-mcherry (red) in the body (i), head (ii) and yolk sac (iii). Cells expressing mCherry alone did not recruit WHIM-GFP neutrophils. Bar = 200 μm in A-F; 25 μm in L.

**Figure 5. WHIM-GFP neutrophils fail to enter the blood stream and respond to wounding in the ventral tailfin.** (A) Schematic of the tail of 3 dpf larvae and sample GFP frame from Movie S3; red box is area of dorsal aorta where time-lapse imaging was performed. DA = dorsal aorta outlined by white lines, arrows indicates direction of blood flow, CHT = caudal hematopoietic tissue where GFP+ neutrophils not in circulation can be seen. White arrowheads indicate neutrophils in the circulation. (B) Quantification of neutrophils in the blood of MPO:GFP and WHIM-GFP 3-4 dpf transgenic larvae, * = p-value < 0.01. Each dot represents a separate larva whose blood was analyzed by time-lapse imaging for 1 min. as in Movie S3. (C) Time lapse imaging of the wound response in WHIM-GFP transgenic larvae (from Movie S4), GFP fluorescence overlaid with DIC image at indicated time points. (D,E) Sudan Black staining to reveal neutrophils at wounds in the ventral tailfin in 3 dpf control (D) or WHIM-GFP (E) 2 hpw. (F) Quantification of neutrophil recruitment to wounds in fixed larvae as in D and E,* = p-value < 0.001, n = the number of individual larva wounded and counted, control = GFP- siblings of WHIM-GFP larvae. (G) Time course of neutrophil wound recruitment in MPO:GFP and WHIM-GFP transgenic larvae, error bars = s.e.m., ** = p-value <0.001, * = p-value <0.01. n = 20-25 larvae at each time point. Bar = 200 μm in C-E; 20 μm in A.

**Figure 6. WHIM-GFP neutrophils fail to respond to tail transections or chronic inflammatory signals.** (A) Sudan Black staining of tails from Control (left column) or WHIM-
GFP (right column) larvae injected with clint-ex1 MO to induce epidermal hyperproliferation and chronic inflammation in the tail. (B) Sudan Black staining of tail transections in Control (left column) or WHIM-GFP (right column) larvae. (C) Confocal imaging at wounds in WHIM-GFP (top row) or Control (bottom row) larvae at 3 dpf immunolabeled with a rabbit antibody to MPO and a FITC-conjugated anti-rabbit Fab Fragment (left column) followed by a rhodamine-red-conjugated rabbit antibody to L-plastin (middle column). Overlapping signals are yellow in the overlay (right column). Arrows are MPO⁺, L-plastin⁺ neutrophils, arrowheads are MPO⁻, L-plastin⁺ macrophages, white * indicates location of the wound. Control = GFP siblings of WHIM-GFP larvae. Representative images of at least 20 larvae in each condition. Bar = 200 μm in A,B; 100 μm in C.

**Figure 7. Depletion of SDF1a and photoactivation of Rac are sufficient to restore WHIM-GFP neutrophil directed migration in vivo.** (A) Time-lapse imaging (from Movie S10) of GFP fluorescence showing WHIM-GFP neutrophils responding to a wound (*) in the ventral tailfin in a 3 dpf SDF1a morphant larvae. White arrows indicate WHIM-GFP neutrophils. (B,C,E,F) Sudan Black staining of neutrophil response to wounding (*) or line) in the ventral tailfin (B,C) or to tail transection (E,F) of 3 dpf WHIM-GFP transgenic larvae injected with control (B,E) or SDF1a (C,F) MO. (D) Quantification of WHIM-GFP neutrophil response in wounded 3 dpf morphant larvae fixed 2 hpw as in B,C, * = p-value <0.01  (G) Quantification of WHIM-GFP neutrophil response to tail transection in 3 dpf morphant larvae fixed 2 hours post transection as in E,F, * = p-value <0.05. (H-J) Laser stimulation with a 458 nm light induces directed migration of WHIM-GFP neutrophils also expressing mCherry-PA-Rac from the CHT. Repeated photoactivation was used to direct a single WHIM-GFP/mCherry-PA-Rac neutrophil
away from the cell aggregate in the CHT into the tailfin. (H) Z-stack images from the indicated time points in Movie S11 were summed into a single two-dimensional image, and then consolidated into a semi-one-dimensional line. Stars indicate time points and position of laser stimulations. (I) Two examples of the WHIM-GFP/mCherry-PA-Rac neutrophil protruding after stimulation from Movie S11. Black circles indicate position of laser stimulation; white circles are included as reference points. (J) Composite DIC image of the posterior CHT, blood vessels and tailfin from Movies S11 and S12 overlaid with the track (black line) of the directed WHIM-GFP/mCherry-PA-Rac neutrophil migration away from and return to the CHT. The starting and stopping points of photoactivation are indicated. Note that after termination of photoactivation the neutrophil immediately returns to the neutrophil aggregate in the CHT. Similar observations were made in three different experiments with three different larvae. CHT = caudal hematopoietic tissue, BV = blood vessel. Arrows indicate direction of migration. Bar = 200 μm in B,C,D,F; 100 μm in A; 40 μm in J; 20 μm in I.
Figure 1

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A, C, E

G

MPO:GFP

H, I

WHIM-GFP

J, K

MPO:GFP + SDF1a MO

L

mCherry
Figure 5

A

B

C

D

E

F

G

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Live imaging of neutrophil motility in a zebrafish model of WHIM syndrome

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