Factor Associated with Neutral Sphingomyelinase Activity Mediates Navigational Capacity of Leukocytes Responding to Wounds and Infection: Live Imaging Studies in Zebrafish Larvae

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Factor Associated with Neutral Sphingomyelinase Activity Mediates Navigational Capacity of Leukocytes Responding to Wounds and Infection: Live Imaging Studies in Zebrafish Larvae

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Factor associated with neutral sphingomyelinase activity (FAN) is an adaptor protein that specifically binds to the p55 receptor for TNF (TNF-RI). Our previous investigations demonstrated that FAN plays a role in TNF-induced actin reorganization by connecting the plasma membrane with actin cytoskeleton, suggesting that FAN may impact on cellular motility in response to TNF and in the context of immune inflammatory conditions. In this study, we used the translucent zebrafish larvae for in vivo analysis of leukocyte migration after morpholino knockdown of FAN. FAN-deficient zebrafish leukocytes were impaired in their migration toward tail fin wounds, leading to a reduced number of cells reaching the wound. Furthermore, FAN-deficient leukocytes show an impaired response to bacterial infections, suggesting that FAN is generally required for the directed chemotactic response of immune cells independent of the nature of the stimulus. Cell-tracking analysis up to 3 h after injury revealed that the reduced number of leukocytes is not due to a reduction in random motility or speed of movement. Leukocytes from FAN-deficient embryos protrude pseudopodia in all directions instead of having one clear leading edge. Our results suggest that FAN-deficient leukocytes exhibit an impaired navigational capacity, leading to a disrupted chemotactic response. The Journal of Immunology, 2012, 189: 1559–1566.

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live-imaging approaches. We used zebrafish embryos to image the wound- and infection-triggered inflammatory response. The data obtained showed that leukocyte motility is impaired after knock-down of FAN in zebrafish, although the total numbers of leukocytes in the organism are not altered. The wound inflammatory response is dramatically disrupted by perturbing the mechanism by which leukocytes orient toward wound signals.

Materials and Methods

Characterization of FAN in zebrafish

The genomic database ensemble (http://wwwensembl.org/Danio_rerio/index) was searched for FAN homologs. One potential FAN homolog was identified (transcript D1ensdarr000000073635). This was used to determine the full-length sequence for the zebrafish FAN (Supplemental Fig. 1). Sequence alignments of FAN from different species were made using Vector NTI (Inforigen).

Morpholino-oligonucleotide injections

Antisense morpholino-modified oligonucleotides against zebrafish FAN were targeted against the splice sites of the second intron (FAN-E2I2, 5'-GTAAGCTGCTCACCTGTTTGTCTCG-3', FAN-E2I1, 5'-GATGATGAAATTGCCGCAC-3', and Fan-rev1, 5'GAAGAAG-3'). The primers Fan-for1, 5'-ATGGCTTTCATCAC-3' and Fan-rev, 5' and 3'-actin-rev, 5'-ATG-GAGTGTGAATCGCGCAG-3' and 3'-actin-for, 5'-ACCATACCA-GAGTGCATCACG-3' were used. For the amplification of b-actin, the primers b-actin-forward, 5'-ATGGGCTTTTATCTACGGAAGAAG-3' and b-actin-reverse, 5'-GCTCCTGTGTACAAAA-3' was obtained from Gene Tools.

RNA isolation and RT-PCR

RNA from zebrafish embryos was isolated from embryos at different stages of development using the µMACS mRNA isolation kit (Miltenyi Biotech), according to the manufacturer’s protocol, by addition of a DNase I digest for 7 to 10 min to remove traces of genomic DNA. First-strand synthesis was performed by utilizing the Superscript III system (Invitrogen) using 300 ng mRNA and random hexamer primers. For RT-PCR, 7.5 ng first-strand synthesis and RedTaq DNA polymerase (Sigma-Aldrich) in a glass-bottom culture dish (MatTek) were used. RT-PCR was performed as follows: 2 min at 94°C; 15 (β-actin) and 30 (FAN) cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 1 min; 7 min at 72°C. For the amplification of FAN, the primers Fan-for1, 5'-ATGGGCTTTTATCTACGGAAGAAG-3' and Fan-rev1, 5'-CTAATACGTAGTTTCCACA-3' were used. For the amplification of β-actin, the primers β-actin-forward, 5'-ATGGGCTTTTATCTACGGAAGAAG-3' and β-actin-reverse, 5'-GCTCCTGTGTACAAAA-3' were used.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed, as described previously (15), with the only difference being that for this study no automated system was used.

Tail fin injury and confocal microscopy

The 2-days postfertilization (dpf) tg(PU1-Gal4-UAS-GFP; MPX-GFP; PU1-Gal4-UAS-RFP) (16, 17) embryos either injected with control MO or FAN-MO were anesthetized using tricaine (Sigma-Aldrich), and arranged on agarose plates for injection. Injection was achieved by injecting a Eppendorf Femtojet and micromanipulator.

Bacterial infection of the otic placode

Escherichia coli (DH5α) carrying the dsRed-expressing pGEMD3 plasmid (19, 20) were grown in standard Luria-Bertani medium containing ampicillin (50 µg/ml). For infections, a 3-ml overnight culture was centrifuged for 3 min at 4000 x g, supernatant was removed, and the pellet was washed three times with PBS. After washing, the pellet was resuspended in 500 µl PBS. The final injection solution contained the washed E. coli diluted in PBS containing 1/10 Phenol Red.

The 2-dpf tg(PU1-Gal4-UAS-GFP) embryos injected with either a control MO or FAN-MO were anesthetized using tricaine (Sigma-Aldrich) and photographed with a digital camera (DSFI1; Nikon).

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was standard two-tailed Student t test for two data sets using Prism (GraphPad). The p values <0.05 (*), <0.01 (**), and <0.001 (***++) were deemed as significant, highly significant, and most significant, respectively.

Results

Identification of zebrafish FAN

FAN is involved in TNF-mediated actin reorganization (6) and leukocyte recruitment (21). To test the function of FAN for leukocyte motility in vivo, we used the zebrafish (Danio rerio) as a model organism. The zebrafish embryo/larvae turned out to be a powerful model system to observe macrophage function in vivo (22–24). From this end, we first aimed to identify a FAN homolog in zebrafish. Zebrafish genome analysis revealed 5’ and 3’ parts of a single FAN homolog located on chromosome 7. FAN-specific PCR primer was designed, and the full-length gene was amplified from cDNA generated from zebrafish embryos 2 dpf. The zebrafish FAN gene encodes a protein with 911 aa and shows the typical signature of three functional domains, as follows: a weakly conserved PH domain (aa 191–286), a BEACH domain (aa 302–575), and a standard control morphology (5’-TCTTACCTCAGTTACAATTTATA-3’). The sequence alignment of zebrafish FAN (Supplemental Fig. 1). This high degree of conservation of FAN is served PH domain (aa 191–286), a BEACH domain (aa 302–575), and a standard control morphology (5’-TCTTACCTCAGTTACAATTTATA-3’). The sequence alignment of zebrafish FAN (Supplemental Fig. 1). This high degree of conservation of FAN is
and FAN knockdown embryos could be detected.

In situ hybridization for lysozyme revealed that FAN was already expressed during the first 6 days of development. To test whether the knockdown of FAN had any impact on total numbers of leukocytes in zebrafish, we injected antisense morpholinos (MO) targeted against the splice sites of the second intron and were injected into one cell stage embryos. The injection of MOs generated two shorter splice variants, whereas the wild-type transcript could not be detected in injected embryos (Fig. 1C). Sequence analysis of the two generated transcripts revealed a deletion of exons 2 and 3 parts of exon 4 within the shorter transcript. Both splice variants have a frameshift caused by the deletion of exons, resulting in a truncated protein.

To investigate whether the knockdown of FAN had any impact on total numbers of leukocytes in FAN morphants, in situ hybridization was performed using a probe against l-plastin, a marker for early leukocytes. In situ hybridization reveals that FAN morphant and control embryos have the same number of l-plastin–positive cells (Fig. 1D).

To determine the average number of macrophages, the number of neutrophils was subtracted from the total number of leukocytes. This shows a recruitment of 2.9 macrophages in control embryos compared with 2.3 macrophages in morphant embryos. In summary, the number of both macrophages and neutrophils is reduced in FAN-MO embryos. However, there is a stronger reduction of neutrophil recruitment than in macrophage recruitment (Fig. 2C), which is in line with the observations made by Montfort et al. (21).

In another set of experiments, a double-transgenic line (PU1-Gal4-UAS-GFP; MPX-GFP) was used to observe the number of leukocytes recruited to the wound. The total number of leukocytes is marked by PU1-Gal4-UAS-GFP line with the number of neutrophils responding to injury. To achieve this, neutrophils were visualized by Sudan black staining in PU1-Gal4-UAS-GFP transgenic fish. Again, the total number of leukocytes recruited to the wounds was reduced in morphant embryos compared with control embryos. In control embryos on average 4.5 ± 0.34 neutrophils reach the site of injury, whereas in morphant embryos only 3 ± 0.35 neutrophils reach the site of injury. The number of neutrophils in morphants was reduced to 0.7 ± 0.17 compared with 1.6 ± 0.34 in control embryos (Fig. 2C–E).

To determine the total number of leukocytes recruited to the wound was significantly reduced in FAN-MO–injected embryos compared with control embryos. On average only 1.9 ± 0.32 leukocytes reached the wound in FAN-MO–injected embryos, whereas 4.3 ± 0.39 and 4.7 ± 0.22 leukocytes were counted in the MO control and in wild-type embryos, respectively (Fig. 2B).

To reveal whether the reduced number of leukocytes at the site of injury affects the recruitment of both macrophages and neutrophils, we compared in a new set of experiments the total number of leukocytes labeled in the PU1-Gal4-UAS-GFP line with the number of neutrophils responding to injury. To achieve this, neutrophils were visualized by Sudan black staining in PU1-Gal4-UAS-GFP transgenic fish. Again, the total number of leukocytes recruited to the wounds was reduced in morphant embryos compared with control embryos. In control embryos on average 4.5 ± 0.34 leukocytes reach the site of injury, whereas in morphant embryos only 3 ± 0.35 leukocytes reach the site of injury. The number of neutrophils in morphants was reduced to 0.7 ± 0.17 compared with 1.6 ± 0.34 in control embryos (Fig. 2C–E). To determine the average number of macrophages, the number of neutrophils was subtracted from the total number of leukocytes. This shows a recruitment of 2.9 macrophages in control embryos compared with 2.3 macrophages in morphant embryos. In summary, the number of both macrophages and neutrophils is reduced in FAN-MO embryos. However, there is a stronger reduction of neutrophil recruitment than in macrophage recruitment (Fig. 2C), which is in line with the observations made by Montfort et al. (21).
FAN is required for oriented migration of leukocytes

The observed migration defect that results in fewer leukocytes reaching the side of injury might be due to a slower movement of leukocytes. Alternatively, the FAN knockdown might lead to a defect in the chemotactic migratory response, and therefore results in less leukocytes at the site of the wound. To further characterize the motility defect in FAN-MO–injected embryos and to determine whether this is secondary to a reduced velocity of leukocytes or to a defect in chemotactic migratory response, time-course series for 3 h after injury were performed and the movement of leukocytes was tracked. Speed analysis of the leukocytes showed that the overall speed of leukocytes in FAN-MO–injected embryos does not significantly differ from that in control and wild-type embryos (Fig. 3B). Thus, the lower number of leuko-
cytes that reaches the wound after 1.5 h (Fig. 2A, 2B) is not due to a reduction of velocity.

Because the speed of leukocytes in FAN knockdown embryos and siblings was comparable, we analyzed the movement of leukocytes in greater detail. The number of leukocytes that move toward the wound in control embryos is larger than that in FAN-MO–injected embryos. Tracking of the leukocytes revealed that in control embryos leukocytes move straight toward the wound and

![Image](image_url)

**FIGURE 3.** (A) Cell tracking from fluorescence movies up to 3 h after injury at the tail fin from 2-dpf embryos treated with control morpholino or FAN morpholino. A transgenic fishline (PU1-Gal4 UAS-GFP) was used for our experiments that has green fluorescent leukocytes. ×40 water objective. (B) Statistical illustration of the speed of leukocytes recruited to wounds in wild-type, control, and morphant embryos (Wt, n = 18, Ctrl, n = 18, MO, n = 26; p value Wt/MO < 0.1 n.s., *p value Ctrl/MO < 0.02). (C) Statistical illustration of the straightness of leukocytes recruited to wounds in wild-type, control, and morphant embryos (Wt, n = 18, Ctrl, n = 18, MO, n = 26; ***p value Wt/MO < 0.000003, ***p value Ctrl/MO < 0.00002).

The macrophage moves directly toward the wound and reaches it after 24 min. After reaching the wound, it stays there and does not move away from it. (C) Overview of cell tracking of a FAN morphant embryo. The white circle indicates the site of injury. (D) Detailed cell tracking of one leukocyte. The leukocyte does not move directly toward the wound, but performs protrusions in every direction before starting to move after 55 min. After 70 min, the macrophage reaches the wound, but directly starts to move away again. Single leukocytes were manually colored in red and blue by using ImageJ software. ×40 water objective.

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remain there after reaching it (Figs. 3A, 4A, 4B, Supplemental Video 1). In contrast, in FAN-MO–injected embryos, leukocytes do not start directly to move toward the wound; they extend protrusions in every direction before they start to move toward the wound. Furthermore, they do not move directly toward the site of injury, but rather move in another direction, ending up in a circular movement, never reaching the wound (Figs. 3A, 4C, 4D, Supplemental Video 2). In addition, FAN-MO leukocytes that reach the wound often leave it again, and some leukocytes stop on their way to the wound, turn around, and disappear again, which was never observed in control embryos (Fig. 3A, Supplemental Videos 1 and 2). To measure this movement of the leukocytes toward the wound, we calculated the straightness, which is the quotient of the total path length divided by the displacement (netto length) of a tracked leukocyte. Investigation of the straightness revealed a significant difference between FAN-MO–injected embryos and wild-type and control embryos (Fig. 3C). In control and wild-type embryos, the straightness of leukocyte movement is 0.59 ± 0.05 and 0.61 ± 0.04, whereas in FAN-MO–injected embryos it is reduced to 0.27 ± 0.04. This reduction results in fewer numbers of leukocytes at the site of injury after 1.5 h in FAN knockdown embryos.

Notably, the morphology of migrating leukocytes in FAN morphant embryos appears different from that of control embryos. In control embryos, leukocytes have the typical shape with several pseudopodia from their leading edge and a retracting part without pseudopodia (Fig. 5A). In FAN morphant embryos, leukocytes often do not have a clear leading edge and they extend pseudopodia in all directions (Fig. 5B). They retract these pseudopodia and then extend new pseudopodia in other directions without moving (Supplemental Fig. 2). In addition, leukocytes in FAN knockdown embryos have more pseudopodia compared with control embryos (74 per leukocyte versus 33 in control embryos), and these cells more frequently made the wrong choice of pseudopodia (50.8% versus 66.1% correct turns in control embryos) (Fig. 5C).

Taken together, to our knowledge, we describe for the first time in vivo the role of FAN in leukocyte motility. Leukocytes of FAN knockdown embryos do not move slower than their siblings in wild-type embryos, but have a reduced straightness, exhibit an abnormal shape with more pseudopodia in the wrong direction, and thus reach the site of injury less efficiently. These findings are in line with our previous observations in cultured FAN-deficient MEFs showing altered cell polarity in a wound-scratch test upon TNF treatment (6).

**Leukocyte migration upon infection in FAN knockdown embryos**

To test whether the difference in leukocyte migration is tissue or stimulus dependent, the otic placode of 2-dpf embryos was infected with red fluorescent *E. coli* and the numbers of leukocytes reaching the site of infection was counted 30 min postinfection (Fig. 6A–C). Statistical analysis demonstrates that in this infection model the number of leukocytes that reach the site of infection is significantly reduced in FAN morphant leukocytes. In control embryos, on average 15.25 ± 1.9 leukocytes reach the site of infection in wild-type, control, and morphant otic placodes postinfection with red fluorescent *E. coli* (Wt, n = 5, Ctrl, n = 5, MO, n = 5; **p** value Wt/MO <0.003, *p* value Ctrl/MO <0.01).
infection, whereas only 9 ± 0.85 leukocytes appear in FAN morphant embryos. This experiment provides evidence that the migration defect of leukocytes in morphant embryos is not tissue or stimulus dependent and implies a general role for FAN in the chemotactic migratory response.

Discussion

Evidence is accumulating suggesting that FAN might be involved in the motility of cells (6, 21). We recently reported that FAN links the TNF-R1 to the actin cytoskeleton and is essentially involved in filopodia formation in MEF cells (6). Furthermore, recruitment of neutrophils into the peritoneal cavity was reduced by >50% in FAN-deficient mice (21). Although these findings suggest a role for FAN in cell motility, formal proof was missing. The use of a transgenic zebrafish line with fluorescently tagged leukocytes and the translucency of the embryos have now allowed us to demonstrate in vivo the essential role of FAN in the navigation of leukocytes toward chemotactic cues emanating from tissue damage or infection. Zebrafish larvae provide an ideal miniature model of the human wound migratory cell response, because only 20–30 leukocytes are drawn to a wound, and each of these cells can be tracked by live imaging with fine spatial and temporal precision.

We first identified a single FAN homolog in the zebrafish genome located on chromosome 7, which encodes for a protein with 911 aa and is expressed throughout all analyzed stages of development until day 6. Sequence comparison between mouse and zebrafish FAN revealed a total homology of 70% and a consensus homology of 78.5%, which suggests an evolutionary conserved role in cellular processes. FAN was successfully targeted by morpholinos injected into zebrafish embryos at the one- to two-cell stage. One important observation indicated that, upon injury at the tail fin, the number of leukocytes that assemble at the site of injury was markedly reduced in FAN-MO–injected embryos compared with wild-type or control embryos left untreated. Tracking of individual leukocytes revealed that reduced numbers of leukocytes arrive at the site of injury. This was due to an impaired directionality of leukocytes in FAN morphants. The average speed, however, was not affected.

The rapid recruitment of macrophages and neutrophils is essential to coordinate wound closure. Recently, Niethammer and coworkers (26) reported that a gradient of hydrogen peroxide is an initial chemoattractant generated by a wounded tail fin in zebrafish larvae. In fact, a myriad of signaling molecules originate from a wound such as chemokines that attract leukocytes to a wound site. Notably, TNF is released upon injury or infection and leukocytes start to move toward the site of TNF (19), suggesting that FAN action in chemotactic responses is triggered by TNF. This is consistent with our previous observation that FAN-deficient murine embryonic fibroblasts (MEFs) display an impaired Golgi apparatus reorientation in a scratch-wound test (6). This reorientation of the Golgi apparatus is independent of chemokines. In addition, isolated FAN-deficient MEFs show impaired migratory response to TNF (our unpublished data), suggesting that impaired TNF/FAN signaling is responsible for this defect. TNF is known to have a major function in the initiation and amplification of the inflammatory response by inducing the expression of chemotactic cytokines and chemokines that are responsible for the migration of several immune cells such as macrophages (27). There has been substantial recent progress in understanding how neutrophils develop and respond to inflammatory cues in zebrafish (17, 22, 23, 26, 28). Notably, FAN seems to be selectively involved in TNF-induced chemokine expression, including CXCL-2 and CCL-2, which are chemoattractants for neutrophils and macrophages, respectively (21). Thus, the possibility remains that FAN morphants are unable to form an efficient chemoattractant gradient, which may contribute at least in part to decreased leukocyte recruitment to tail fin injury or to the infected otic placode.

To deal with the question how FAN might regulate the navigational capacity of leukocytes, it is instructive to consider the morphologic features of a cellular chemotactic response. Directional cues induce pseudopodia oriented toward the gradient, which includes selective retraction, oriented extension, and suppression of de novo pseudopodia formation (29). To move in the direction of a gradient, leukocytes must form many pseudopodia at the side of the cell that is facing the gradient. In this respect, it is important to note that leukocytes from FAN-deficient embryos protrude pseudopodia in all directions instead of having one clear leading edge. Leukocytes in FAN-deficient zebrafish larvae more often form pseudopodia at the wrong location, resulting in a loss of directionality. In addition, leukocytes from FAN-MO–injected embryos do not start directly to move toward the wound, which is probably also caused by multilateral protrusions observed before they start to move (Supplemental Video 2).

As to the molecular mechanisms regulating chemotaxis, studies in Dictyostelium have implicated many signaling pathways, including the phosphoinositide 3-kinase pathway activating AKT; the TorC2 pathway activating PKB1, a soluble guanly cyclase that is activated at the leading edge and produces cGMP; and phospholipase A 2, which has an unknown mechanism (30, 31). FAN signaling pathways may regulate pseudopodia formation in several ways. The observed defect in cell polarization and initiation of directed movement suggests problems in the cytoskeletal reorganization upon gradient sensing. The Rho family of small GTPases is known to play a central role in gradient sensing and cell polarization [reviewed by Charest and Firtel (32)]. Indeed, FAN mediates the TNF-induced modulation of the actin cytoskeleton through the small GTPase Cdc42 and VASP, which promotes F-actin bundling required for plasma membrane protrusions (6). We, therefore, assume that the loss of directionality in FAN knockdown leukocytes is due to impaired Cdc42 signaling. This is in line with the knockdown results of the Cdc42 effector molecule WASP. Cvejic et al. (22) could show that WASP knockdown macrophages have a reduced chemotactic index, resulting in a reduced number of macrophages reaching the site of injury.

Interestingly, FAN mediates the activation of nSMase by TNF (5, 33), which has been recently shown to involve the polycumb group protein EED (34). nSMase is a type C phospholipase-like phospholipase A2 that plays a crucial role for pseudopodia splitting and directional migration in Dictyostelium (30, 31). Thus, it will be interesting to investigate whether FAN regulates the navigational capacity of leukocytes through neutral SMase.

It is important to note that we observed similar navigational defects postinfection of the otic placode with red fluorescent E. coli. The reaction of FAN-deficient leukocytes to infection was impaired so that they cannot move directly toward the site of infection. Moreover, FAN-deficient leukocytes seem to have lost their orientation. Similar defective migratory phenotypes of leukocytes in two different tissues with two different stimuli suggest that FAN plays a general role in chemotaxis.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental figure legends

Supplemental Figure 1

Alignment of the predicted amino acid sequence of zebrafish FAN with those of human and mouse. Sequence comparison between mouse and zebrafish FAN revealed a total homology of 70% and a consensus homology of 78.5%.

Supplemental Figure 2

A) Time series of a wild-type leukocyte. The leukocyte starts moving directly toward the wound and extends protrusion only in the direction of motility. B) Time series of morphant leukocytes. The leukocytes extend protrusions in all directions and retract them again without moving. Even 47.30 minutes after injury the leukocytes have not started to move toward the tail fin wound.

Video 1: Leukocyte migration in wt zebrafish embryos: Leukocyte migration toward a laser wound in the tail fin of wt zebrafish embryos.

Video 2: Leukocyte migration in FAN-MO zebrafish embryos: Leukocyte migration toward a laser wound in the tail fin of FAN-MO zebrafish embryos.