CXCR4 signaling regulates metastatic onset by controlling neutrophil motility and response to malignant cells

C. Tulotta, C. Stefănescu, Q. Chen, V. Torraca, A. H. Meijer & B. E. Snaar-Jagalska

Developing tumors interact with the surrounding microenvironment. Myeloid cells exert both anti- and pro-tumor functions and chemokines are known to drive immune cell migration towards cancer cells. It is documented that CXCR4 signaling supports tumor metastasis formation in tissues where CXCL12, its cognate ligand, is abundant. On the other hand, the role of the neutrophilic CXCR4 signaling in driving cancer invasion and metastasis formation is poorly understood. Here, we use the zebrafish xenotransplantation model to study the role of CXCR4 signaling in driving the interaction between invasive human tumor cells and host neutrophils, supporting early metastasis formation. We found that zebrafish cxcr4 (cxcr4b) is highly expressed in neutrophils and experimental micrometastases fail to form in mutant larvae lacking a functional Cxcr4b. We demonstrated that Cxcr4b controls neutrophil number and motility and showed that Cxcr4b transcriptomic signature relates to motility and adhesion regulation in neutrophils in tumor-naïve larvae. Finally, Cxcr4b deficient neutrophils failed to interact with cancer cells initiating early metastatic events. In conclusion, we propose that CXCR4 signaling supports the interaction between tumor cells and host neutrophils in developing tumor metastases. Therefore, targeting CXCR4 on tumor cells and neutrophils could serve as a double bladed razor to limit cancer progression.

Tumor-microenvironment interactions are crucial in cancer pathogenesis and several signals drive this communication. The composition of cancer microenvironments changes during cancer progression. Fibroblasts, endothelial and immune cells are main components of the tumor stroma, acting in concert with the extracellular matrix (ECM), growth factors, proteases and cytokines. The CXCR4-CXCL12 chemokine signaling axis sustains tumor cell growth and directs the formation of distant metastases. It is established that cancer cells expressing CXCR4 home to secondary organs where CXCL12 is highly secreted, mainly by mesenchymal stromal cells. Moreover, CXCL12 guides the migration of stromal cells that express CXCR4 and locally infiltrate the tumor, providing support by secretion of growth and angiogenic factors, as well as promoting metastasis through activation of epithelial-to-mesenchymal transition (EMT) via mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase/Protein kinase B (PI3K/AKT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathways.

A dual role in either supporting or inhibiting tumor progression has been linked with the immune system. CXCR4-CXCL12 signaling has been associated with the polarization towards an immune-suppressive microenvironment: the possible role of a CXCL12 shield that protects cancer cells from being recognized by cytotoxic lymphocytes and activates regulatory T-cells has recently been described. Polarization of macrophages towards a M2 phenotype has also been associated with tumor survival. Recent studies have pointed at the role of perivascular CXCR4-expressing M2 macrophages in creating tumor vascular networks after chemotherapy, leading to tumor relapse, and confirmed CXCR4 as M2 marker. It has been shown that CXCR4 can also be activated by alternative ligands like MIF (Macrophage Migration Inhibitory Factor). MIF signalling has been associated to inflammatory diseases. Upon binding to CXCR4 or CXCR2, MIF controls monocyte and T cell chemotaxis and its blockade leads to plaque regression in atherosclerosis. In zebrafish, MIF functions as a neurotrophin during the
development of the inner ear\(^8\). In cancer, MIF-CXCR4 signalling has been linked to Mesenchymal Stromal Cell (MSC) homing to tumours both in vitro and in vivo\(^9\). The FDA-approved CXCR4 antagonist AMD3100 inhibits MIF binding to CXCR4. However, because higher concentration of the antagonist is required to inhibit MIF binding to CXCR4 compared to CXCL12, it is likely that MIF binds to CXCR4 via a different mechanism compared to CXCL12 binding\(^11\). We previously showed that metastasis formation is inhibited in a cxcl12 zebrafish mutant, suggesting a pivotal role of the cxcl12-cxcr4 signaling axis in this process\(^12\).

Neutrophils are the most abundant white blood cells and the major first responders during inflammation\(^13\). In cancer, neutrophils are recruited to neoplastic sites and together with other immune cells have been shown to provide trophic signals that support tumor growth, angiogenesis, tumor cell motility and invasion of surrounding tissues\(^14\text{–}17\). Neutrophils have been classified in N1 (anti-tumor) and N2 (pro-tumor) types\(^18\text{–}20\). The polarization of neutrophils towards one or the other type is driven by a plethora of cytokines and chemokines that often direct the same polarization in macrophages. In particular, pro-inflammatory molecules such as interferon \(\beta\) (IFN\(\beta\)), interleukin-1\(\beta\) (IL1\(\beta\)) and tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) induce the polarization towards type 1 phenotypes, while interleukin 10 (IL-10) and transforming growth factor \(\beta\) (TGF\(\beta\)) are immunosuppressive and inhibitory of inflammation, skewing neutrophil polarity towards N2. Pro-tumoral and pro-angiogenic N2 neutrophils express high levels of vascular endothelial growth factor (VEGF), metalloprotease 9 MMP9 and CXCR4\(^20\). In addition, amongst different metalloproteases, MMP9 plays a key role in HSCs mobilisation from the bone marrow. CXCR4 expression is regulated by MMP9. Simultaneously MMP9 and CXCL12 expression is reciprocally regulated in bone marrow cells\(^23\).

Neutrophils have been reported to display overlapping as well as complementary functions with macrophages in infection and tumor relapse after chemotherapy\(^22\text{–}25\). Interestingly, tissue-resident macrophages, originated from the fetal liver during embryo development, and monocyte-derived macrophages, originated from hematopoietic stem and progenitor cells (HSPCs) in the adult bone marrow, work in concert to regulate recruitment of neutrophils in inflamed tissues, through epithelial layers\(^24\). Recent findings suggest that neutrophils work together with macrophages to regulate the hematopoietic niche\(^25\). The bone microenvironment represent a favorable site of metastatic growth for different tumor types, suggesting a possible involvement of the signals that regulate bone marrow and hematopoietic niche homeostasis\(^26\). Among those, CXCR4-CXCL12 signaling is a major candidate, considering its fundamental role in orchestrating HSPC and neutrophil retention in and mobilization from the bone marrow, with the involvement of the CXCL1/CXCL2-CXCR2 chemokine axis\(^27\text{–}29\).

The use of the zebrafish embryo as a xenotransplantation model has shown that hematogenously inoculated tumor cells home in the caudal hematopoietic tissue (CHT), where tumor growth and invasion take place, initiating early metastatic events\(^30\). The CHT is an intermediate site of hematopoiesis during zebrafish embryogenesis and is the functional analogue of the fetal liver in mammalian development\(^31\). Previous work from our group has suggested the role of neutrophils in preparing the metastatic niche by non-pathological transmigration from the CHT to the tail fin and vice versa. In their random motility, neutrophils form paths in the collagen, favoring tumor cell invasion\(^30\). We previously addressed the role of cell-autonomous CXCR4 signaling in early metastases in the zebrafish xenograft model\(^32\). Here, we address the role of the host-dependent CXCR4 signaling in driving the communication between tumor cells and neutrophils, during experimental metastasis formation in an in vivo zebrafish xenograft model.

### Results

**Myeloid cells support tumor early metastatic events.** Immune cells play dual roles during cancer progression. Inhibitory and supportive functions of the immune system have been associated with tumor growth and metastasis formation. Using the zebrafish embryo model we previously showed that myeloid cells, mainly neutrophils, support the establishment of tumor experimental micrometastasis, when the MAE-FGF2 transformed cell line was inoculated into the blood circulation of 2-day-old embryos\(^30\). Therefore, we used the same approach to investigate whether zebrafish myeloid cells exert similar tumor supportive functions, when other cell lines were implanted. In particular, we used the osteotropic triple negative breast cancer line MDA-MB-231-B, derived from bone metastases in a mouse xenograft model\(^33\). The zebrafish embryo model bears the great advantage of studying the contribution of the innate immune system during early metastasis formation separately from the adaptive immunity, which reaches full maturity in 3–4 week old juveniles\(^34\). To deplete both neutrophils and macrophages, we injected Pu.1/Spi1b morpholino (1 mM) into 1–2 cell stage embryos. Subsequently, the MDA-MB-231-B cell line was inoculated into the blood circulation of 2-day post fertilization (dpf) zebrafish embryos with GFP-expressing neutrophils. The reporter line \(Tg(mpx:GFP)\)\(^34\) was used to monitor neutrophil depletion, in view of the time-limited efficacy of gene knock-down obtained with morpholino anti-sense oligos. Macrophage depletion was not monitored as it already occurs with lower doses of the same morpholino (0.5 mM)\(^35\). Tumor phenotype assessment was performed 2-day post implantation (dpi) by quantifying tumor cell invasion in each larva. Depletion of myeloid cells in the Pu.1 morphants resulted in a reduced cancer cell invasion (68%) in the tail fin in proximity of the caudal hematopoietic tissue (CHT) (Fig. 1). As previously found, the CHT, a site of hematopoiesis analogous to the fetal liver during mammalian development, is a preferential site of early cancer metastasis formation in the zebrafish xenotransplantation model. In conclusion, myeloid cells support triple negative breast cancer early metastasis onset in zebrafish.

**Neutrophilic Cxcr4 signaling is involved in early tumor metastasis initiation.** Therapeutic targeting of CXCR4 on tumor cells could be an effective strategy to limit tumor cell growth and metastasis. However, CXCR4 signaling in the tumor microenvironment also plays a central role in cancer and further investigations are needed to fully understand its contribution.

In our model, teleost evolution has led to a \(c\text{cxcr4}\) gene duplication. \(c\text{cxcr4a}\) and \(c\text{cxcr4b}\) paralogues are expressed by different cell types, although redundant functions have been reported\(^36\). The CXCL12-CXCR4 signaling is
conserved between zebrafish and human: zebrafish Cxcr4 receptors display more than 60% identity with human CXCR4 and zebrafish Cxcl12 ligands have more than 65% identity with human CXCL12. We performed transcriptome analysis of GFP positive, FACS-sorted neutrophils from 5 dpf Tg(mpx:GFP)i114 larvae and RNA deep sequencing revealed high expression levels of the cxcr4 paralogues in neutrophils. In particular, cxcr4a and cxcr4b transcriptomic levels were higher in the GFP + fractions compared to the GFP − populations. Importantly, neutrophilic cxcr4b levels were at least 100-fold higher than neutrophilic cxcr4a, indicating that cxcr4b is the predominant human CXCR4 orthologue in zebrafish larval neutrophils (Fig. 2A). Therefore, to study if CXCR4 signaling in the tumor microenvironment supports cancer metastasis initiation, we engrafted the triple negative breast cancer cell line MDA-MB-231-B in the cxcr4b(−/−) mutant with deficient cxcr4b signaling.

Figure 1. Myeloid cell depletion impairs tumor cell invasion. (A) Relative tumor invasion was compared at 2 dpi in Pu.1 morphants, depleted of neutrophils and macrophages, and larvae injected with control morpholino (68% inhibition). Two-tailed un-paired t-test with Welch's correction (**p < 0.0001) was performed on a pool of two biological replicates (Control: n = 84, Pu.1: n = 67). Data are mean ± SEM. (B) Top panel shows MDA-MB-231-B cells forming a tumor mass and invading the tail fin tissue (bright field image, top right), while surrounded by GFP expressing neutrophils in 2 dpi Tg(mpx:GFP)ii14 injected with a control morpholino. In the bottom image, neutrophils are absent due to Pu.1 knockdown and a smaller tumor mass is formed compared to the control condition, resulting in impaired invasion of the local tissue (bright field, top right). Scale bar: 50 µm. Micrographs were acquired using a Leica MZ16FA fluorescent microscope coupled to a DFC420C camera.
compared to the wt siblings (Fig. 4A–C). We have previously shown that neutrophils prepare the metastatic niche by creating paths into the collagen, during the transmigration from the CHT to the tail fin. Hence, we hypothesized that path formation is linked to metalloprotease activity. Therefore, we quantified mmp9 expression in ody and wt siblings (whole body) and found decreased mRNA levels upon Cxcr4b inhibition (Fig. 4D).

Next, neutrophil number in the CHT and whole body was verified at 6 days post fertilization when metastasis formation was assessed. Neutrophil number was lower in the CHT of ody mutants compared to wt siblings (Fig. 4E,F). Moreover, at the same time point, the total body count of neutrophils was found to be lower (Fig. 4G), suggesting that Cxcr4b controls neutrophil development.

During zebrafish development, primitive and definitive waves of hematopoiesis can be distinguished. In a transition phase, between 24 and 36 hpf, neutrophils originate from the posterior blood island (PBI), which, with the onset of the definitive wave, is replaced by the CHT. Recent studies in zebrafish have revealed that CXCR4 signaling has a direct link with the development of HSPCs, mainly affecting their ability to colonize the CHT, which functions as an intermediate hematopoietic site. In the same study, the use of the CXCR4 antagonist...
AMD3100, between 48 and 72 hpf, decreased cmyb/runx+ HSPCs numbers. Because neutrophils develop first in the PBI, independently from the HSPCs, and subsequently in the CHT, dependently on the HSPCs with self-renewal potential, we investigated whether the development of neutrophils could be affected in a host with a non-functional Cxcr4b signaling. Neutrophil number was quantified during earlier stages of development (1 dpf), before HSPCs colonize the CHT and initiate the definitive wave of hematopoiesis. An increase in neutrophil number was found in the CHT of ody embryos, compared to wt siblings, whereas no difference was detected on whole embryo level (Fig. S1A–C). Subsequently, neutrophil number was quantified in the whole zebrafish embryo, as well as in the CHT region, between the dorsal aorta and caudal vein, starting from the end of the yolk extension, in 2 day old ccr4b−/− and ccr4b+/− Tg(mpx:GFP)i114 embryos. We identified an increase (31%) in neutrophil number in the CHT region of ody mutants compared to wt siblings (Fig. S1D,E) at 2 dpf. At the same time, no difference in total neutrophil number was observed (Fig. S1F).

These findings suggest that Cxcr4b controls neutrophil motility and development, in a putative HSPCs-dependent and independent manner.

The transcriptomic signature of Cxcr4b-deficient neutrophils links to defective cell motility.

In this study (Fig. 4A–C) we demonstrated that neutrophil motility is altered in physiological conditions when Cxcr4b signaling is impaired. In order to define the contribution of neutrophilic Cxcr4b signaling axis involved
in metastatic niche preparation and subsequent tumor cell invasion, RNA sequencing was performed from FACS-sorted GFP positive neutrophils after dissociation of cxcr4b+/+ and cxcr4b−/− Tg (mpx:GFP)i144 6 dpf larvae. An overall tendency towards upregulation of differentially expressed genes was found in neutrophils from ody mutants (61% upregulated genes vs 39% downregulated genes) (Fig. 5), when a cutoff was considered (p < 0.05 in DESeq and edgeR). More in details, in ody neutrophils 48% of the up-regulated genes showed an over 10-fold increase, whereas 57% of the down-regulated genes showed an over 10-fold decrease. Pathway analysis was performed in DAVID, after selecting 615 differentially expressed genes (p < 0.05 in DESeq and edgeR) and converting them to human orthologues with gPROFILER. Genes involved in focal adhesion and ECM-receptor interaction were found up-regulated in neutrophils, together with genes involved in axon guidance, suggesting impaired motility and anchoring properties (Table 1). In particular, integrins are involved in adhesion strengthening and arrest of leukocytes on the endothelium, during transendothelial migration41. Laminin, fibronectin and collagen are components of the extracellular matrix and increased transcription levels suggest a tighter adhesiveness and consequently challenged immune cell motility (Table 1). Members of the Roundabout signaling pathway (slit1b, sema4gb and srgap1), implicated among others in leukocyte chemotaxis and tumor angiogenesis42 were found to be up-regulated. Down-regulated genes were found to cluster in the metabolism of xenobiotics by cytochrome p450 pathway. Subsequently, pathway analysis was extended to differentially expressed genes identified through statistical analysis performed in RStudio using the package DESeq2 paired. Overall, the analysis performed in DESeq2 paired confirmed the enriched pathways identified with DESeq and edgeR. However, additional genes were identified, either belonging to previously described pathways (focal adhesion/ECM-Receptor interaction) or clustering in a new pathway (MAPK pathway) (Table 2). Furthermore, NETRIN-1 (zebrafish netrin1b), belonging to the family of laminin-secreted proteins and involved in neuronal chemotaxis43,44 and leukocyte migration45, was found up-regulated in cxcr4−/− neutrophils (Log2FoldChange = 2.6 and p = 0.00009).
NETRIN-1 has previously been linked with reduced neutrophil and macrophage infiltration in a kidney injury model. Taken together, our sequencing data support the above described results that suggest motility alteration in neutrophils bearing a \( \text{cxcr4b} \) mutation.

**Cxcr4b signaling affects the neutrophilic response to cancer cells during early metastasis formation.** Considering the involvement of Cxcr4b signaling in driving neutrophil motility and development in tumor-naive conditions, next we investigated the ability of neutrophils to respond to cancer cells in Cxcr4b \(-/-\) mutants. Generally, emergency hematopoiesis is initiated upon systemic infections and neutrophils leave the bone marrow in response to damage and danger signals, during inflammation and infection. Emergency hematopoiesis, dependent on Gcsf-Gcsfr signaling, has also been shown to occur in zebrafish larvae, resulting in expansion of HSPCs and mobilization of neutrophils from the CHT in response to lipopolysaccharide (LPS) injection or bacterial infection. Hence, the number of neutrophils in the CHT was quantified 3–6 hours after MDA-MB-231-B tumor cells were inoculated into the blood circulation of embryos at 2 dpf. We found that the acute response of neutrophils to tumor cell engraftment was characterized by a decreased number of neutrophils in the CHT in the wt siblings and \( \text{ody} \) embryos, compared to uninjected control groups (Fig. S2A,B). These results suggest that, at 2 dpf, the mobilization of neutrophils from the CHT in response to tumor engraftment is independent from Cxcr4b. As tumor early metastatic events in the CHT region were primarily affected in \( \text{ody} \) mutants at 4 dpi and the CHT colonization by HSPCs is known to occur at 2 dpf, neutrophil response to cancer cells was also assessed at 4 dpi (6 dpf). Like in 2 dpf embryos, we also observed a reduction of neutrophil number in the CHT of tumor-engrafted wt siblings at 6 dpf, compared to the uninjected controls. In contrast, neutrophil numbers were unchanged in tumor-engrafted \( \text{ody} \) mutants, compared to uninjected \( \text{ody} \) larvae (Fig. 6A). Therefore, Cxcr4b signaling is required for the mobilization of neutrophils from the CHT as well tumor-invasive phenotype at 6 dpf.

To further support the evidence that neutrophils display a different response towards cancer cells when Cxcr4b signaling is not functional, we quantified neutrophil motility in the metastatic region at 4 dpi (6 dpf). Neutrophils displayed a motility pattern characterized by lower speed and diminished average distance, in presence of MDA-MB-231-B in the wt siblings, compared to the uninjected controls (Fig. 6B,C,F,G). On the other hand, no differences in neutrophil speed and travelled distance were detected in \( \text{ody} \) larvae implanted with...
MDA-MB-231-B compared to engrafted wt siblings (Fig. 6D–G). In conclusion, Cxcr4b signaling impairment affects neutrophil response to cancer cells initiating early metastatic events.

Discussion

Chemokines are key mediators of directional cell migration and the CXCR4-CXCL12 chemokine axis is well known to display major roles in tumor progression, guiding tumor cell homing to CXCL12 expressing organs. Consequently, targeting the CXCR4 receptor expressed by cancer cells is a pharmacological approach that is currently explored in the clinic to limit tumor spreading and metastases. At the same time, it is important to consider the effect of CXCR4 signaling on the tumor microenvironment, especially in view of the antagonizing or supportive functions that myeloid cells are known to have on tumor progression. We previously showed that...
the zebrafish xenograft model is a powerful tool to study tumor-microenvironment interactions as CXCR4-based interspecies cross talk takes place and genetic and chemical inactivation of CXCR4 receptor on the engrafted human cancer cells block metastatic onset in zebrafish xenograft model. Moreover, the role of neutrophils in preparing the metastatic niche has been previously described by our group. We found that the non-pathological migration correlate with tumor cell invasion in the caudal hematopoietic tissue (CHT), functionally analogous to the fetal liver in mammalian embryo development. Hence, we hypothesized the involvement of CXCR4 signaling in controlling neutrophil motility and immune-tumor cell interactions involved in the initiation of early metastatic events and micrometastasis formation. First, we found that in zebrafish larvae neutrophils express high levels of cxcr4b, the homolog of human CXCR4 and paralog of zebrafish cxcr4a. Then, we used a cxcr4b homozygote

| Gene ID | Gene symbol | Gene name | DESeq | edgeR |
|---------|-------------|-----------|-------|-------|
| ENSDARG00000056624 | figf | c-fos induced growth factor | 3.9 | 3.0E-02 |
| ENSDARG00000099014 | col1a1b | collagen, type XI, alpha 1b | 1.8 | 6.0E-03 |
| ENSDARG0000019815 | fn1a | fibronectin 1a | 1.9 | 3.0E-02 |
| ENSDARG0000007950 | itga1b | integrin, alpha 1b | 4.3 | 3.0E-03 |
| ENSDARG0000033232 | itgb1b.1 | integrin beta 1b.1 | 1.2 | 4.0E-02 |
| ENSDARG0000102277 | lama1 | laminin, alpha 1 | 3.1 | 9.0E-03 |
| ENSDARG0000099390 | lama2 | laminin, alpha 2 | 4 | 1.0E-02 |
| ENSDARG0000018110 | pak4 | p21 protein (Cdc42/Rac)-activated kinase 4 | 1.5 | 2.0E-02 |
| ENSDARG0000038139 | pdgfb | platelet-derived growth factor beta polypeptide b | 6.8 | 8.0E-04 |
| ENSDARG0000078362 | tnc | tenascin C | 1.6 | 2.0E-02 |

Table 1. Enriched pathways in cxcr4b−/− neutrophils (analysis performed with DESeq and edgeR). Pathway analysis in Cxcr4b-deficient neutrophils. Genes selected with DESeq (p < 0.05) and edgeR (p < 0.05) analyses in RStudio (from 21621 to 615 genes) were converted to the human orthologues using g:PROFILER and uploaded in DAVID Bioinformatics. Resources 6.7 for pathway analysis. Up-regulation of genes involved in focal adhesion/ECM-Receptor interaction and axon guidance was identified, whereas down-regulation of genes in the metabolism of xenobiotic by P450 was found. Additional analysis was performed using DESeq2 paired (Table 2). The same pathways were identified with DESeq/edgeR (Table 1) and DESeq2 paired (Table 2) and the genes listed in Table 2 were in addition to genes described in Table 1. Enriched pathways indicate alteration in motility, as shown by the analysis performed with DESeq and edgeR and reveal members of the MAPK signaling to be differentially expressed.
mutant zebrafish (also known as odysseus or ody) and showed that engrafted human tumor cells failed to form micrometastases in the CHT region. Therefore, myeloid cell impairment or a non-functional Cxcr4b signaling led to experimental tumor micrometastasis inhibition.

Table 2. Enriched pathways in cxcr4b−/− neutrophils (analysis performed with DESeq2 paired). Pathway analysis in Cxcr4b-deficient neutrophils. Genes selected with DESeq (p < 0.05) and edgeR (p < 0.05) analyses in RStudio (from 21621 to 615 genes) were converted to the human orthologues using g:PROFILER and uploaded in DAVID Bioinformatics Resources 6.7 for pathway analysis. Up-regulation of genes involved in focal adhesion/ECM-Receptor interaction and axon guidance was identified, whereas down-regulation of genes in the metabolism of xenobiotic by P450 was found. Additional analysis was performed using DESeq2 paired (Table 2). The same pathways were identified with DESeq/edgeR (Table 1) and DESeq2 paired (Table 2) and the genes listed in Table 2 were in addition to genes described in Table 1. Enriched pathways indicate alteration in motility, as shown by the analysis performed with DESeq and edgeR and reveal members of the MAPK signaling to be differentially expressed.
Investigating a potential role of the host Cxcr4b signaling in the formation of early metastasis by affecting immune cell motility was the next approach. We found a downregulation in mmp9 mRNA levels in ody and a reduction in neutrophil motility in tumor-naïve cxcr4b deficient zebrafish embryos. These findings link with our previous work on the role of neutrophil physiological migration in tumor invasion in the tail fin. It has been reported that in addition to its function as a protease, mmp9 plays a role as a chemoattractant. Mmp9 chemotactic properties work in synergy with CXCL12. Therefore, inhibition of CXCR4 signaling could lead to impaired neutrophil motility and ability to respond to tumour cells also as a result of altered mmp9-driven chemotaxis. We next investigated whether Cxcr4b signaling affects neutrophil development. In mammals, CXCR4 and CXCR2 chemokine signaling axes regulate hematopoietic stem cell (HSC) retention in and mobilization from the bone marrow, respectively. CXCR4 chemical inhibition upon AMD3100 treatment results in mobilized HSCs. Furthermore, patients affected by WHIM syndrome, characterized by neutropenia and enhanced susceptibility to infection, bear a CXCR4-gain-of-function mutation that causes neutrophil retention in hematopoietic sites, in response to cognate ligand CXCL12, highly expressed in the bone marrow. These findings have been confirmed in a zebrafish model of WHIM syndrome, where neutrophils expressing constitutively active Cxcr4b were retained in the CHT and mobilized only upon cxcl12a knock down. We found that the number of neutrophils in the CHT in ody mutants was higher than in the wt siblings at 2 dpf. Because the overall neutrophil number was not affected by the cxcr4b mutation, a higher number of neutrophils in the CHT surprisingly suggested enhanced neutrophil retention. These findings show that receptor stimulation by cognate ligand is needed to activate cell motility, despite of chemotaxis towards Cxcl12. Retention and reduced motility of neutrophils with impaired Cxcr4b signaling at 2 and 3 dpf, respectively, support the hypothesis that neutrophil physiological behavior plays an important role in cancer micrometastasis formation at early stages.

Next we investigated if neutrophils played an important role in preparing the metastatic niche in later stages of tumor micrometastasis formation, when tumor cell invasion has taken place. Therefore, neutrophil number was counted in 6 dpf zebrafish larvae and, in contrast to the observations at 2 dpf, a reduction in neutrophils localized in the CHT was found in ody mutants, with tendency towards a reduced overall number in a whole larva. We hypothesize that the dichotomy in neutrophil numbers is linked to the role of CXCR4 signaling during hematopoiesis. Using the zebrafish embryo model, it has recently been shown that HSPCs colonize the hematopoietic tissue, interacting with mesenchymal cells and inducing modification in the perivascular niche. In the...
same study, the mesenchymal cells express cxcl12a, whereas cxcr4b expression is mainly found in the CHT region and treatment with the CXCR4 antagonist AMD3100 reduced the number of runx2+ hematopoietic progenitors. In line with their findings, we propose that the reduced number of neutrophils in the CHT of 6 dpf ody larvae relates to the reduced number of HSPCs and suggest that further investigations should be carried on to confirm this hypothesis. Importantly, a lower number of neutrophils in the CHT in 6 dpf ody mutants might result in a reduced niche modification due to a lower number of paths traced into the collagen by neutrophils themselves. On the other end, the increased number of neutrophils in earlier stages suggests the potential role of Cxcr4b in the primitive wave of hematopoiesis.

In agreement with our findings, Cxcr4b signature in tumor-naive zebrafish neutrophils confirmed Cxcr4b role in cell motility and adhesion. Upregulation of the integrins, as well as increased interaction with the ECM and alteration of the cytoskeleton reorganization were found in Cxcr4b deficient neutrophils. Members of the Roundabout signaling pathway were also differentially expressed. Roundabout signaling is associated with axon guidance mechanisms and its role in cancer metastasis has been reported. Importantly, Slit1b, found up-regulated in ody, functions as a repellent molecule that interferes with leukocyte chemotaxis and specifically blocks the ability of circulating neutrophils to migrate directionally. Moreover, we propose netrin1b as a candidate gene that links neutrophil ability to provide trophic signals to cancer cells. NETRIN-1 has been reported to reduce neutrophil infiltration in ischemic acute kidney injury by inhibiting COX-2 and PGE2 production. PGE2 has been identified as the trophic signal that sustains neoplastic transformation in a transgenic zebrafish cancer model.

After investigating the role of Cxcr4b in physiological neutrophil development and motility, we unraveled neutrophil behavior in presence of engrafted tumor cells, able to initiate early metastatic events. An acute response to engrafted cancer cells into the blood circulation of 2 dpf zebrafish embryos resulted in no alteration in Cxcr4b-deficient neutrophils. To assess neutrophil acute response in tumor-engrafted larvae, the number of mpox+ cells was counted in the CHT of zebrafish embryos few hours after tumor cell inoculation. As neutrophil number decreased in the CHT of engrafted wt or ody embryos compared to un.injected larvae, we propose that neutrophils mount an acute response upon tumor inoculation by leaving the CHT in line with previous observations of demand-driven granulopoiesis upon bacterial infection and that this response occurs in a Cxcr4b independent manner. On the other hand, an altered response was found at later stages. In 6 dpf (4 dpi) zebrafish larvae, tumor cells have formed a secondary tumor mass and initiated local tissue invasion. In response, wt siblings diminished the number of neutrophils in the CHT, increasing their mobilization. Mobilized neutrophils were found to migrate and in the surrounding of metastasizing cancer cells and to slow down and to interact with human malignant cells. In contrast, cxcr4b deficient neutrophils remained in the CHT and failed to localize in the surrounding of tailfin tumor micrometastases, suggesting a possibly diminished inflammatory response (Fig. 7).

In conclusion, we demonstrate that CXCR4 signaling plays a major role in neutrophilic innate immune response to early metastatic events and contributes to the establishment of tumor micrometastases. The development of CXCR4-targeted therapies directed to the tumor microenvironment is therefore essential.

Materials and Methods
Zebrafish husbandry. Zebrafish lines were kept in compliance with the local animal welfare regulations and European directives. The study was approved by the local animal welfare committee (DEC) of the University of Leiden (license number: 10612, protocol 14227). Zebrafish adults were maintained according to standard protocols (zfین.org), in a 10/14-hour dark/light cycle. Embryos were maintained at 28 °C in Egg water (60 μg/ml Ocean salt in distilled water), containing 0.003% PTU (1-phenyl-2-thiourea) to block pigmentation.

Zebrafish lines. Zebrafish reporter lines used in this study were Tg(mpg-GFP)11434 and Tg(Kdrl:EGFP)49161. The cxcr4b+mut line was outcrossed with each reporter line mentioned above. Homozygote cxcr4b+/- mutant embryos (odyssaeus or ody) were distinguished from wild type cxcr4b+/+ and heterozygote cxcr4b+/-/+ by phenotype (incomplete lateral line deposition) and genotype identification. Genotyping of adult fish by KASP assay was performed using the following primers A1 (forward) 5′-TGACGGTGGTCTTCAGTGCCTA-3′ and C1 (forward) 5′-CAAGAACTCCAAGGGTCAGACTCTA-3′ and confirmed by sequencing using previously described primers.

Cell culture. Breast MDA-MB-231-B dsRed (kindly provided by P. ten Dijke and Y. Drabsch, LUMC, Leiden, The Netherlands), MDA-MB-157 mCherry (ATCC®) and prostate PC3-M-Pro4-Luc2 (mCherry or tdTomato) (kindly provided by G. van der Pluijm, LUMC, Leiden, The Netherlands) and cancer cell lines were cultured in DMEM medium complemented with 10% fetal calf serum (FCS), at 37 °C in a humidified atmosphere with 5% CO2. Cell lines were regularly tested for mycoplasma with Universal Mycoplasma Detection kit (30–1012 k, ATCC). Pu.1 knock down. Pu.1 (Sp11b, 1 mM) and standard control morpholino injections (0.1 mM) were performed to deplete neutrophils and macrophages as previously described. Morpholino efficiency was assessed by counting number of Mpx+ neutrophils in the Tg(mpox:GFP)11434 zebrafish line.

Xenograft experiments. Tumor cells were inoculated in the blood circulation of 2 day post fertilization (dpf) zebrafish embryos as previously described.

Tumor burden. Zebrafish embryos engrafted with fluorescently labelled tumor cells were screened for correct engraftment 5–6 hours after inoculation into the blood circulation at 2 dpf, using a Leica MZ16FA fluorescent microscope coupled to a DFC420C camera. Larvae were positioned on a Petri dish with 1.5% agarose coating.
and tumor burden was quantified at 4 dpi, acquiring monographs of the metastatic site, in the CHT region. 
Laser scanning confocal microscopy was used to overlay the GFP and dsRed channels and snapshots were analyzed in Image-Pro Analyzer 7.0 (Media Cybernetics). For each larva tumor burden was calculated based on number of objects multiplied by mean area and mean intensity; generated with a macro designed by H. de Bont (Toxicology, LACDR, Leiden University) and previously used to quantify tumor migration and proliferation in zebrafish.  

**Neutrophil number and motility.** Neutrophil number was quantified by manual counting, using a Leica MZ16FA fluorescence microscope. Neutrophil basol motility was assessed using a Leica TCS SPE confocal microscope with a HC APO 20x DRY objective (0.7 N.A.). 3 dpf larvae were mounted on a 1% low melting point agarose layer, containing tricine and covering the glass surface of a Will-Co Dish® (Pelco®, Ted Pella, Inc). Egg water containing anesthetic was added on top of each larva. Time lapse was performed for 30 minutes, with 1 minute interval between frames. Maximum projections were generated, tail movements were corrected using Stack Reg plugin and neutrophil tracking was performed using the Manual Tracking plugin in ImageJ-Fiji. Neutrophil motility in response to metastatic cancer cells was quantified with a Nikon A1 confocal laser scanning microscope (Tokyo, Japan) using the 488 and 561 laser lines with 20 × (NA 0.75) lens. Images were acquired every minute during time lapse. Videos were analyzed using NIS-Elements AR and tracking performed for the first 30 frames in ImageJ, with Manual Tracking plugin.

**RNA isolation and real-time PCR.** Expression levels of mmp9 were quantified in 6 dpf cxcr4b larvae. RNA was isolated using TRIZOL extraction method, according to the manufacturer's instruction from a pool of zebrafish larvae (10 < n < 15). DNase treatment was performed using RQ1 RNase free-DNase (M6101 Promega). 1 μg input RNA was used for cDNA synthesis (i-Script ™ cDNA synthesis kit, Bio-Rad). Expression levels were measured by real-time PCR (iQ™ SYBR® Green Supermix, Bio-Rad), using the Chromo4™ Four-Color Real-Time PCR system. Relative fold changes of gene expression were calculated using the ΔΔCt method. The following primers were used: mmp9 forward 5′-AGTGGTGGTCCGTGGTTGAG-3′ and mmp9 reverse 5′-AGTGGTGGTCCGTGGTTGAG-3′. Peptidylprolyl isomerase A-like (pippal) was used as housekeeping gene (forward 5′-ACACTGAAACACGGAGGCAAAG-3′ and reverse RV 5′-CATCCACA ACCTCCGAAACAC-3′).

**cxcr4b transcriptomic signature in neutrophils: from larval dissociation to RNA sequencing analysis.** Zebrafish line cxcr4b larvae Tg(Kdr:EGFP) were used to isolate neutrophils from 6 dpf larvae. After harvesting, eggs were kept in Petri dishes (n < 100) at 28.5 °C to allow synchronized embryo development. Triplicates of GFP positive embryos (100–150 per replicate) were selected for dissociation, performed according to the instruction from a pool of zebrafish larvae (10 < n < 15). DNase treatment was performed using RQ1 RNase free-DNase (M6101 Promega). 1 μg input RNA was used for cDNA synthesis (i-Script ™ cDNA synthesis kit, Bio-Rad). Dissociation with 0.4 mg/ml collagenase/DPBS (Liberase TL, Roche, #05401020001) was alternatively used. Larvae were transferred directly from Egg water to collagenase solution. Dissociation was obtained mechanically with pipetting and 2 incubation steps at 28.5 °C for 10 min. 10% FCS was added and sample preparation was continued as described in sorting. Sorting was performed with a BD FACSort™ Cell Sorter (BD Biosciences, San Jose, CA, USA) with the BD FACSDiva software (version 6.1.3) and gates defined using GFP negative larvae. After sorting, samples were stored in QIAzol at –80 °C. RNA isolation was performed using miRNeasy Mini kit (# 217004 Qiagen). On-column DNase digestion was performed, using RNase-Free DNase Set (# 79254 Qiagen). Agilent Bioanalyzer 2100, RNA 6000 Pico kit (Agilent, Santa Clara) was used to assess RNA quality. cDNA synthesis and amplification were performed with SMARTer® Ultra ™ Low Input RNA Kit for Sequencing - v3 (Clontech) and cDNA quality validated, using Agilent 2100 BioAnalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit (#5067-4626, Agilent). cDNA shearing, library preparation and validation, and Illumina sequencing (HiSeq2000) were performed as described in by ZF-SCREENS (Leiden, The Netherland). Reads (18.684.327 is an average of 12 samples) were mapped to Ensembl transcripts (GRc10.80) and statistical analysis was performed using negative binomial distribution performed in R Studio, using DESeq, DESeq2 paired and EdgeR packages, available at Bioconductor.org. Pathway analysis was performed using DAVID Bioinformatics Resources 6.7. Identification of cxcr4a and cxcr4b expression levels in neutrophils by RNA sequencing shown in Fig. 2 was performed as described in.

**Statistics.** Statistical analysis was performed using GraphPad Prism (versions 5.0 and 6.0). Un-paired t-test was used in datasets of two groups and Welch's correction applied when group variances were significantly different (p < 0.05). One-way ANOVA with Bonferroni post hoc test was used in datasets of three or more groups (continuous variable) and Kruskal-Wallis with Dunn’s post hoc test was used to estimate significant difference in the case of counts (discrete variable).

**Data Availability**

Data generated or analysed during this study are included in this published article (and its Supplementary Information files). The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**

C.T., C.S. and Q.C. performed experiments and data analysis. V.T. contributed to immune cell sorting, optimization of RNA sequencing analytical methods and scientific discussions. A.H.M. gave valuable suggestions on data analysis and experimental design. C.T. and B.E.S.J. designed experiments and wrote the manuscript. All authors approved the final version of this manuscript.

**Additional Information**

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