Original Research

Granulocyte Colony-Stimulating Factor Mediated Regulation of Early Myeloid Cells in Zebrafish

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Abstract

Background: Colony-stimulating factor 3 (CSF3), more commonly known as granulocyte colony-stimulating factor (G-CSF), acts via a specific cell surface receptor CSF3R (or G-CSFR) to regulate hematopoiesis, with a particularly key role in the myeloid cell lineage where it impacts the development and function of neutrophilic granulocytes. Zebrafish possess a conserved CSF3R homologue, Csf3r, which is involved in both steady-state and emergency myelopoiesis, as well as regulating early myeloid cell migration. Two CSF3 proteins have been identified in zebrafish, Csf3a and Csf3b. Methods: This study investigated the roles of the Csf3a and Csf3b ligands as well as the downstream Janus kinase (JAK) and phosphatidylinositol 3-kinase (PI3K) pathways in mediating the effects of Csf3r in early myeloid cell development and function using gene knockdown and pharmacologic approaches. Results: This study revealed that both Csf3a and Csf3b contribute to the developmental and emergency production of early myeloid cells, but Csf3a is responsible for the developmental migration of early neutrophils whereas Csf3b plays the major role in their wounding-induced migration, differentially participated in these responses, as did several downstream signaling pathways. Both JAK and PI3K signaling were required for developmental production and migration of early myeloid cells, but PI3K signaling was required for emergency production and initial migration in response to wounding, while JAK signaling mediated retention at the site of wounding. Conclusions: This study has revealed both distinct and overlapping functions for Csf3a and Csf3b and the downstream JAK and PI3K signaling pathways in early myeloid cell production and function.

Keywords: CSF3; CSF3R; G-CSF; G-CSFR; myelopoiesis; migration; wound healing

1. Introduction

Granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3 (CSF3), is the principal cytokine regulating neutrophil development and function [1]. It plays a crucial role in myelopoiesis and particularly the emergency response to infection [2,3], and has also been found to be involved in the process of wound healing [4]. Recombinant G-CSF is used widely in the clinic as an important therapeutic agent in the treatment of neutropenia in patients undergoing immunosuppressive chemotherapy or suffering from certain congenital disorders [5].

G-CSF acts via a specific cell-surface receptor, called G-CSFR or CSF3R, expressed in a range of hematopoietic cells with a predominance in the myeloid lineage, as well as in some non-hematopoietic cells [3]. Binding of G-CSF triggers receptor homodimerization and the activation of various intracellular signaling cascades including the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [2,3]. However, it remains unclear how individual pathways regulate the broad spectrum of biological activities promoted by G-CSF.

The zebrafish has emerged as a powerful model to study hematopoiesis, with strong conservation between zebrafish and mammals [6,7]. The zebrafish G-CSFR orthologue, Gcsfr or Csf3r, has been shown to be involved in both steady-state and emergency myelopoiesis, as well as regulating early myeloid cell migration [8–10]. Two G-CSF ligands have been identified in zebrafish, named Csf3a and Csf3b. These have similar predicted structure and ligand/receptor interaction sites but display differential spatial and temporal expression patterns as well as biological properties [11]. This study sought to further our understanding of ligand specificity in early myelopoiesis and myeloid cell migration mediated via zebrafish Csf3r through gene knockdown, including developmental migration of primitive myeloid cells and emergency myelopoiesis, and for the first time investigate the role of downstream signaling pathways in these processes using specific inhibitors.

2. Materials and Methods

2.1 Fish Husbandry and Manipulations

Wild-type, Tg (mpx::GFP) [12] or Tg (lyz::DsRed) [13] fish were maintained using standard husbandry
practices. Embryos at the 1–8 cell stage were injected into the yolk with approximately 1 nl anti-sense morpholinos diluted to 50 nM (4.1 ng) in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM CaCl2, 5.0 mM HEPES; pH 9). Morpholinos (Gene Tools, Philomath, OR) used were: csf3a Mo: 5'－AAAAAACCCTTGGACTCAACGAGC and csf3b Mo: 5'－CAGGGTTAATGTTGAATTACGTT [11], along with a csf3r Mo: 5'－TTGTCTTACAGATCGCCAGTTC and a scrambled control. (scr Mo): 5'－CCTCTTACCTCAGTTAATTTATA. Embryos were also treated with inhibitors to JAK2 (AG490, 50 µM) and PI3K (LY294002, 30 µM) at 16 hpf for developmental studies.

2.2 In Vivo Analyses

Tg (mpx:GFP) and Tg (lyz:DsRed) embryos were visualized by fluorescence microscopy to allow quantification of myeloid cell numbers and relative migration. To simulate an emergency hematopoietic response, 2 dpf embryos were injected with 5 µg/mL LPS and analyzed 8 hours later, as described [8]. Wound-healing assays were performed by transecting the end of the caudal tail fin of 3 dpf embryos with a scalpel after anesthesia with 0.1% benzocaine, as described [13] and monitored for 8 h. Inhibitors to JAK2 (AG490, 80 µM), PI3K (LY294002, 60 µM) and Src (PP2, 5 µM) were added 1 h prior to injection or wounding, as appropriate.

2.3 Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and qRT-PCR

Total RNA was extracted from ~50 zebrafish embryos using Trizol reagent (Life Technologies Australia, Mulgrave, Vic, Australia), converted into cDNA using iScript (Biorad Laboratories, Gladesville, NSW, Australia), and used as a template for PCR with Taq polymerase (Invitrogen Australia, Mt Waverley, Vic, Australia) in an iCycler thermocycler (Biorad Laboratories) and the following primers: csf3a: 5'－CGTGTGGTCCTGCTCTTTCC and 5'－AGACACGCAATTTACACAGAGC, csf3b: 5'－AAGGATCCATCCACGCAGCACCC and 5'－CTCGAGGGTTCGTCTTAGTTG, csf3r: 5'－CAGGAACAAATAATACAGGGGG and 5'－TAAAGCAGACGTCAACAAAAAT, mpo: 5'－CTGGGGACCTTACTAATGATGG and 5'－CCTGGAATGGTCCAGGTTC, lyz: 5'－CATGATTCGGAGGTCGTCGGCATGT and 5'－ATCTCGAGGGTCTCGAGCTGGGAGGC and the control actb (beta-actin) 5'－TGGCATCACACCTTCTAC and 5'－AGACACATCCAGGAGGG. Negative control templates were water and samples in which with the reverse transcriptase was omitted. Quantitative RT-PCR was performed using SYBR Green, with expression normalized to actb and fold change calculated as $2^{−∆∆Ct}$, where Ct represents the threshold cycle, ∆Ct the difference in Ct between test and control genes, and ∆∆Ct the difference in ∆Ct between experimental and control groups [14].

2.4 Statistical Analysis

Statistical analysis were performed using Graph Pad Prism version 8 (San Diego, CA, USA). Student’s t-tests were performed on the data, using the Holm-Sidak multiple comparison test where necessary.

3. Results

3.1 G-CSFR Pathways Involved in Early Myelopoiesis

We have previously shown that the zebrafish G-CSFR plays a key role in early myelopoiesis, with ablation of csf3r resulting in decreased numbers and reduced migration of primitive myeloid cells over the yolk sac [8]. To determine the contributions of the CsF3a and CsF3b ligands, embryos were injected with the published splice-site targeting morpholinos csf3a Mo or csf3b Mo [11], respectively, along with csf3r Mo [8]. Each robustly inhibited splicing, but produced no visible effect compared to a control scrambled morpholino (scram Mo) by light microscopy (data not shown). Knockdown of either csf3a or csf3b in Tg (mpo:GFP) embryos [12] resulted in a significant decrease in mpo+ myeloid cells at 22 hpf compared to control embryos, albeit significantly less than that observed following csf3r knockdown (Fig. 1A–E). This was confirmed by injection into Tg (lyz:DsRed) embryos [13], where both morpholinos significantly reduced the number of lyz+ myeloid cells, although again less than with csf3r morphants of this response (Fig. 1I–M). In contrast, knockdown of csf3a (or csf3r), but not csf3b, was able to significantly decrease the migration of mpo+ cells over the yolk sac at 22 hpf (Fig. 1A–D,F). This suggests the two ligands have differential effects on early myeloid cell migration, but not production where they both make a contribution.

Key downstream G-CSFR pathways were investigated by treating embryos with specific inhibitors. Both the JAK2 inhibitor AG490 and the PI-3K inhibitor LY294002 resulted in significantly lower numbers of mpo+ cells at 22 hpf (Fig. 1G) and of lyz+ cells at 48 hpf (Fig. 1N) compared to control DMSO-treated embryos. Both inhibitors also reduced migration of mpo+ cells in treated compared to DMSO control embryos (Fig. 1H).

3.2 G-CSFR Pathways Involved in Response to Wounding

Neutrophils play a key role in the response to injury, and we have demonstrated that zebrafish CsF3r mediates retention of myeloid cells at sites of injury [8]. To determine the contribution of CsF3a and CsF3b, a tail fin wounding assay [8] was performed in Tg (mpo:GFP) and Tg (lyz:DsRed) embryos. Tg (mpo:GFP) embryos injected with scr Mo displayed an increase in mpo+ cells at 2 hours post wounding (hpw), which then declined (Fig. 2A–C,M). In csf3a and csf3b morphants the magnitude of this response
was reduced, but this only reached statistical significance for csf3b from 2–6 hpw (Fig. 2D–I,M). The extent of reduction was greatest in csf3r morphants, which was significant across all time-points (Fig. 2J–M). The lyz+ cell population peaked at 6 hpw in scr Mo-injected Tg (lyz::DsRed) embryos (Fig. 2N). The magnitude was again reduced in csf3a and csf3b morphants, which reached statistical significance from 4–8 hpw, with csf3r morphants showing the largest effect across all time points (Fig. 2N).
Embryos treated with DMSO produced similar kinetics to scr Mo-injected embryos for both mpo+ (Fig. 3A–C,J) and lyz+ (Fig. 3K) cells. Interestingly, the JAK2 inhibitor failed to influence the initial recruitment kinetics of either the mpo+ (Fig. 3D–E,J) or lyz+ (Fig. 3K) cells, but showed decreased numbers of each at later timepoints (Fig. 3F–J–K). In contrast, the PI3K inhibitor impacted the early response (Fig. 3G–H,J–K), but its effects were abrogated at later timepoints (Fig. 3I–K).

3.3 G-CSFR Pathways Involved in Emergency Myelopoiesis

We previously showed that zebrafish csf3r contributed to the ‘emergency’ myelopoiesis stimulated by bacterial lipopolysaccharide (LPS) [8]. LPS stimulated csf3a expression by >4-fold but csf3b by a lesser extent (<2-fold) (Fig. 4A). LPS injection also resulted in a significant increase in the expression of both mpo (Fig. 4B) and lyz (Fig. 4C). This increase was abrogated in both csf3a and csf3b morphants, with co-injection of both morpholinos completely blocking their induction by LPS (Fig. 4B,C). This was confirmed in Tg (lyz::DsRed) embryos, which showed a significant LPS-mediated increase in lyz+ cells in embryos injected with scr Mo (Fig. 4D,E,J), which was reduced in those injected with csf3a Mo (Fig. 4F–G,J), with no significant increase in those injected with both csf3a Mo and csf3b Mo (Fig. 4H–J).

LPS also significantly increased the number of mpo+ (Fig. 5A,B,I) and lyz+ (Fig. 5J) cells in DMSO treated embryos. Treatment with AG490 failed to prevent the LPS-induced increase in either population (Fig. 5C,D,I,J), despite fewer cells overall. In contrast, LY294002 blocked the increase in both mpo+ (Fig. 5E,F,I) and lyz+ (Fig. 5J) cells. The Src kinase Lyn has been suggested to lie upstream of PI3K [15] and contribute to G-CSFR-mediated proliferation [16]. Therefore, embryos were also treated with the Src kinase inhibitor PP2, which also blocked LPS-induced increased in mpo+ (Fig. 5G–I).

4. Discussion

The zebrafish has emerged as an invaluable model to study all aspects of neutrophil biology [8,17,18], and associated diseases [10,19,20]. Since G-CSFR signaling is central to many of these, it is important to understand the relative roles of the two zebrafish ligands, Csfs3a and Csfs3b.

Zebrafish Csfs3r has been shown to be required for the robust production of various early myeloid cell populations and the developmental migration of mpo+ cells over the zebrafish yolk sac [8,10], with Csfs3b previously implicated in some aspects [21]. The data presented here suggest that both Csfs3a and Csfs3b contribute independently to the generation of mpo+ neutrophils and lyz+ leukocytes, since ablation of either reduced these cell populations. Csfs3a ablation had a greater effect, suggesting it blocks signaling by both ligands. In contrast, only Csfs3a was required to me-

![Fig. 3. Role of alternative G-CSFR pathways in response to wounding.](A–I) Fluorescent microscopy images of representative Tg (mpo::GFP) embryos pre-treated with either DMSO (A–C), AG490 (D–F) or LY294002 (G–I) at the indicated times post wounding. (J,K) Quantitation of recruitment of mpo+ (J) and lyz+ (K) cells to the wounding site at 0, 2, 4, 6 and 8 hours post wounding (hpw) in Tg (mpo::GFP) and Tg (lyz::DsRed) embryos, respectively, that had been pre-treated with the indicated inhibitors, showing mean and S.E.M. along with statistical significance of inhibitor in comparison to DMSO control (*: p < 0.05; **; p < 0.001; ***: p < 0.0001; n = 15+).
expression of csf3a and csf3b that has been reported [11]. Both the JAK and PI3K signaling pathways were required to mediate both of these developmental roles.

Fig. 4. Role of alternative G-CSFR ligands in emergency myelopoiesis. (A–C) qRT²-PCR analysis of wild-type embryos (A) or those injected with scr, csf3a, csf3b or csf3a and csf3b (csf3a/b) Mo without (-) or with (+) LPS treatment as indicated, for expression of csf3a and csf3b (A), mpo (B) or lyz (C) showing mean and S.E.M (n = 3). (D–I) Fluorescent microscopy images of representative Tg (lyz::DsRed) embryos injected with either scr Mo (D,E), csf3a Mo (F,G) or csf3a/b Mo (H,I), either without (-) (D,F,H) or with (+) (E,G,I) LPS treatment. (J) Quantitation of lyz+ cells in Tg (lyz::DsRed) embryos injected with the indicated morpholinos either without (-) with (+) LPS treatment showing individual embryos as well as mean and S.E.M.. For panels A–C and J statistical significance is indicated (*: p < 0.05; **: p < 0.001; ***: p < 0.0001; ns, not significant). For panels B and C, black indicates relative to scr Mo -LPS and red indicates relative to scr Mo +LPS.

Emergency myeloid cell production is a pivotal aspect of the immune response [22]. This can be stimulated in zebrafish embryos by infection [23] or injection of bacterial LPS [8], and has been shown to require Csfr3r [8]. Enforced expression of either Csfr3a or Csfr3b has been shown to stimulate the production of various early myeloid cells, including both the mpo+ and lyz+ populations [11]. Our data using LPS injection to stimulate emergency myelopoiesis would suggest that while both play a role, Csfr3a exerts a greater influence, being induced to a higher extent by LPS and with its ablation abrogating the effects of LPS marginally more compared to Csfr3b. Csfr3a has been shown to possess higher colony forming unit (CFU)-promoting abilities than Csfr3b potentially reflecting higher biological activity [11], which may also be relevant in this context. PI3K signaling was found to be essential for emergency myelopoiesis. This pathway has previously been implicated in G-CSFR-mediated cell proliferation [15,24]. Interestingly, the Src kinase inhibitor PP2 also abrogated emergency myelopoiesis, consistent with research indicating a role for the Src kinase Lyn in G-CSFR-mediated proliferative responses [16] and suggesting a previously-described Lyn-PI3K pathway [15] may be involved. The absence of a requirement for JAK signaling in emergency myelopoiesis is concordant with data showing that JAK2 is not crucial for G-CSFR-mediated cell growth [25].

Neutrophils play a key role in the response to injury, with G-CSF treatment shown to improve wound healing in mice and humans [4,26]. The role of G-CSFR pathways...
was investigated using a tail fin wounding assay. The results demonstrated that the initial recruitment of mpo+ neutrophils was mainly driven by PI3K. This is in agreement with other research demonstrating that PI3K mediates the enhancement of neutrophil motility induced by G-CSF [27], and is necessary for the directed migration of zebrafish neutrophils in response to wounding [19]. Other research has suggested that wounding-mediated neutrophil migration in zebrafish is dependent on Cxcl18 and Cxcr2 [28,29]. How these chemokines cross talk with Csf3r/PI3K signaling remains to be determined. In contrast, the retention of neutrophils required JAK signaling, potentially related to the ability of JAK2 to enhance G-CSFR expression [30]. We showed that Csf3b is the more important upstream ligand consistent with previous work showing that Csf3b is strongly induced by wounding and plays a role in wounding-induced migration [21]. The lyz+ leukocyte population behaved similarly with respect to initial recruitment, although Csf3a along with PI3K also contributed to retention, suggesting different signaling requirements in these cells.

5. Conclusions

Together the results of this paper provide evidence for both overlapping, redundant and specific roles for Csf3a and Csf3b during development, ‘emergency’ myelopoiesis and wound healing, which is consistent with their differential but overlapping spatial and temporal expression patterns and biochemical properties. The downstream JAK and PI3K signaling pathways showed similar relationships, with both involved developmentally, but with PI3K more important in emergency production and initial recruitment to wounding, while JAK signaling played a greater role in later recruitment. Whether Csf3a and Csf3b differentially use these pathways remains to be determined.

Abbreviations

CSF, colony-stimulating factor; dpf, days post fertilization; ERK, extracellular signal regulated kinase; G-CSF, granulocyte colony-stimulating factor; hpf, hours post fertilization; JAK, Janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; R, receptor; STAT, signal transducer and activator of transcription.

Author Contributions

CL and ACW designed the research study. ABM, FB, RS, ML and CL performed the research. ABM, CL and ACW analyzed the data. ABM and ACW wrote the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

National and institutional guidelines for the care and use of laboratory animals were followed in all studies, which were approved by the Deakin University Animal Ethics Committee (G28-2013 and G23-2016).

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Conflict of Interest

The authors declare no conflict of interest.

References


