



Published in final edited form as:

*Int J Dev Biol.* 2014 ; 58(10-12): 757–766. doi:10.1387/ijdb.140271jr.

## Mechanisms of amphibian macrophage development: characterization of the *Xenopus laevis* colony-stimulating factor-1 receptor

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### Abstract

Macrophage-lineage cells are indispensable to vertebrate homeostasis and immunity. In turn, macrophage development is largely regulated through colony-stimulating factor-1 (CSF1) binding to its cognate receptor (CSF1R). To study amphibian monopoiesis, we identified and characterized the *X. laevis* *CSF1R* cDNA transcript. Quantitative analysis revealed that *CSF1R* tissue gene expression increased with *X. laevis* development, with greatest transcript levels detected in the adult lung, spleen and liver tissues. Notably, considerable levels of *CSF1R* mRNA were also detected in the regressing tails of metamorphosing animals, suggesting macrophage involvement in this process, and in the adult bone marrow; corroborating the roles for this organ in *Xenopus* monopoiesis. Following animal infections with the ranavirus Frog Virus 3 (FV3), both tadpole and adult *X. laevis* exhibited increased kidney *CSF1R* gene expression. Conversely, while FV3-infected tadpoles increased their spleen and liver *CSF1R* mRNA levels, the FV3-challenged adults did not. Notably, FV3 induced elevated bone marrow CSF1R expression, and while stimulation of tadpoles with heat-killed *E. coli* had no transcriptional effects, bacterial stimulation of adult frogs resulted in significantly increased spleen, liver and bone marrow CSF1R expression. We produced the *X. laevis* CSF1R in recombinant form (rXCSF1R) and determined, via *in vitro* cross-linking studies, that two molecules of rXCSF1R bound the dimeric rXCSF1. Finally, administration of rXCSF1R abrogated the rXCSF1-induced tadpole macrophage recruitment and differentiation as well as bacterial and FV3-elicited peritoneal leukocyte accumulation. This work marks a step towards garnering greater understanding of the unique mechanisms governing amphibian macrophage biology.

### Keywords

Xenopus; monopoiesis; CSF1; macrophage development; ranavirus

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## Introduction

Macrophage-lineage cells are indispensable to homeostasis and host defenses of all vertebrate species. Monopoiesis, or the development and differentiation of macrophage lineage cells is chiefly mediated by the colony stimulating factor-1 (CSF1; macrophage colony-stimulating factor, MCSF) cytokine and growth factor (Garceau *et al.*, 2010; Hanington *et al.*, 2007; Pixley and Stanley, 2004; Wang *et al.*, 2008). CSF1 functions as a homo-dimer, ligating the high-affinity tyrosine kinase CSF1 receptor (CSF1R) (Dai *et al.*, 2002), the cell surface expression of which is restricted predominantly to mononuclear phagocytes and their derivative cell populations (Guilbert and Stanley, 1980; Lichanska *et al.*, 1999).

In mammals, the expression levels of the CSF1R increase progressively with macrophage development, from lower levels by myeloid precursors to greater levels by monocytes and further elevated levels by terminally differentiated macrophages (Stanley *et al.*, 1997). Furthermore, the ligation of CSF1R by CSF1 is critical not only to macrophage proliferation, differentiation and survival, but also for effective antimicrobial and anti-tumor host responses orchestrated by these cells (Bober *et al.*, 1995; Korbassi *et al.*, 1987; Munn and Cheung, 1995; Sweet and Hume, 2003). Thus, CSF1R gene expression serves as a reliable marker in the study of monoipoiesis during animal development and host immune responses.

Despite the fact that more ancestral vertebrate species possess the CSF1/CSF1R monoipoietic systems, the evolutionary origins of these pathways remain poorly understood. CSF1 is present in birds (Garceau *et al.*, 2010), amphibians (Grayfer and Robert, 2013), and bony fish (Hanington *et al.*, 2007); and CSF1Rs have been identified, in birds (Garceau *et al.*, 2010) and teleosts (Barreda *et al.*, 2005; Honda *et al.*, 2005; How *et al.*, 1996; Parichy *et al.*, 2000; Pettersen *et al.*, 2008). However, it appears that the bony fish CSF1 ligand-receptor axis is distinct to that of mammals (Hanington *et al.*, 2007; Wang *et al.*, 2008; Wang *et al.*, 2014). Indeed, fish species possess multiple distinct gene copies of both the CSF1 ligand and receptor, which contrasts to single copies seen across higher vertebrates. Owing to the key position occupied by amphibians in vertebrate evolution, it would be interesting to determine whether the *Xenopus* CSF1 ligand-receptor system is more reminiscent of those seen in fish, mammals or alternatively a hybrid intermediate. Moreover, amphibian macrophage development remains to be fully characterized. Notably, monoipoiesis of most vertebrate species occurs within designated hematopoietic tissues/organs such as the bone marrow of birds and mammals (Garceau *et al.*, 2010; Tushinski *et al.*, 1982) and the teleost head kidney (Belosevic *et al.*, 2006; Neumann *et al.*, 2000). By contrast, although the *Xenopus* liver periphery clearly functions as primary site of hematopoiesis (Hadjj-Azimi *et al.*, 1987; Hadji-Azimi *et al.*, 1990; Lane and Sheets, 2002), our recent findings indicate that rather than the subcapsular liver, the bone marrow functions as the primary site of *X. laevis* macrophage development and contains CSF1 responsive macrophage precursors (Grayfer and Robert, 2013).

Infections of amphibians by ranaviruses (family *Iridoviridae*) and the resulting population die-offs are now believed to be a significant contributing factor to amphibian declines

(Chinchar, 2002; Chinchar *et al.*, 2009; Williams *et al.*, 2005). A growing body of literature indicates that amphibian macrophage-lineage cells are critical players in both anti-ranaviral immunity and the ranavirus infection strategies (reviewed in Grayfer *et al.*, 2012). Thus, greater insight into amphibian macrophage development may contribute to bridging the gap in our understanding of the evolution of vertebrate monopoiesis and to studying ranaviruses and developing preventative measures against these emerging pathogens.

Accordingly, to begin to delineate the roles of CSF1R in amphibian macrophage development and anti-RV immunity, we identified the *X. laevis* CSF1R, characterized the expression of this gene in developing, bacterially and virally challenged *X. laevis* and assessed the biological efficacies of a recombinant form of the *X. laevis* CSF1R protein. This manuscript marks the first characterization of an amphibian colony-stimulating factor-1 receptor.

## Results

### Identification and amino acid alignment analysis of the *X. laevis* CSF1R

To investigate amphibian macrophage development, we identified the full cDNA transcript encoding the *X. laevis* principal macrophage growth factor receptor, the colony-stimulating factor-1 receptor (CSF1R) by means of conventional and RACE PCR. The extracellular portion of the putative *X. laevis* CSF1R exhibits low amino acid sequence identity with the CSF1R molecules of other vertebrate species. However, all of these molecules have in common conserved and structurally important cysteine residues and the presence of five putative immunoglobulin-like domains (D1–D5; Fig. 1). Akin to the other vertebrate CSF1Rs, the putative *X. laevis* receptor possesses a disrupted intracellular domain composed of an ATP binding motif, a kinase insert domain as well as a major tyrosine kinase catalytic domain (Fig. 1). Notably, in contrast to the poor amino acid sequence conservation across the extracellular portions of the vertebrate CSF1Rs (including that of *X. laevis*), the intracellular components of these respective proteins exhibit many stretches of evolutionarily conserved residues, particularly throughout the tyrosine kinase catalytic domains (Fig. 1).

### Phylogenetic analysis of vertebrate CSF1R proteins

Phylogenetic analysis of the putative vertebrate CSF1R protein sequences indicated a clear evolutionary relationship, wherein the fish CSF1Rs formed a separate clade, ancestral to the amphibian CSF1Rs, which in turn branched independently and ancestral to other vertebrate CSF1Rs (Fig. 2). The orthology of the *Xenopus* sequences is supported by high bootstrap values (Fig. 2). Notably, the avian and reptilian CSF1R molecules branched closer together, in a separate clade from the marsupial and mammalian CSF1R proteins (Fig. 2). The zebrafish KitR, which is structurally related to CSF1R, was used as an out-group to root the tree (Fig. 2).

### Quantitative analysis of CSF1R gene expression in tissues of tadpoles, metamorphic and adult *X. laevis*

Since CSF1R is primarily expressed by macrophage-lineage cells, we next wanted to examine the quantitative gene expression of the *X. laevis* CSF1R in tadpoles (Stg. 54; (Thors

*et al.*, 1982a; Thors *et al.*, 1982b)), metamorphs (Stg. 64) and adults (2 years old) and hence delineate the distribution of macrophage populations during development (Fig. 3). CSF1R gene expression in tadpole and adult muscle was low, whereas CSF1R mRNA levels were significantly greater in regressing tail-muscle tissues of metamorphosing animals (Fig. 3), consistent with the accumulation of active macrophages involved in tail reabsorption (Nishikawa *et al.*, 1998). The spleen, liver and lung CSF1R gene expression increased with development, where the metamorph and adult liver and lung CSF1R transcript levels were significantly greater than those seen in the respective tadpole tissues (Fig. 3). Interestingly, the lung CSF1R mRNA levels were also significantly greater than those seen in tadpoles and metamorphs (Fig. 3). Finally, marginal but detectable levels of CSF1R transcripts were detected in the adult bone marrow (Fig. 3).

### **Quantitative analysis of CSF1R gene expression in tissue of *X. laevis* tadpoles and adult frogs immunologically challenged with FV3 and heat-killed *E. coli***

To infer on macrophage distribution upon immunological challenge, we examined the expression of CSF1R following infection with the ranavirus Frog Virus 3 (FV3; *Iridoviridae*) or after injections with heat-killed *E. coli* (Fig. 4). In tadpoles, the kidney, which is the central site of FV3 replication, exhibited a modest but significant increase in the CSF1R gene expression at 3 days post FV3 infection (dpi), followed by a decrease at 6 dpi (Fig. 4A). In contrast, a substantial increase of CSF1R gene expression was observed in tadpole spleens (the primary amphibian immune tissue) at 6 (but not 3) dpi (Fig. 4A). The hematopoietic tadpole liver exhibited significant transcriptional increases in CSF1R at both 3 and 6 dpi (Fig. 4A). Interestingly, challenge of tadpoles with heat-killed *E. coli* had no bearing on CSF1R gene expression (Fig. 4C).

As in tadpoles, FV3 infection of adult frogs resulted in increased kidney CSF1R gene expression at 3 dpi and a subsequent decrease at 6 dpi (Fig. 4B). In contrast to the increased CSF1R gene expression in tadpole spleens following FV3 infection, no significant increase of CSF1R gene expression was observed in adult spleens following ranaviral challenge (Fig. 4B). Furthermore, the adult liver CSF1R gene expression decreased following FV3 infection, significantly so at 6 dpi (Fig. 4B). The bone marrow CSF1R gene expression was significantly increased at both 3 and 6 dpi (Fig. 4B), supporting our previous findings that the amphibian bone marrow may serve as a site of monopoiesis (Grayfer and Robert, 2013),

Intriguingly and again in contrast to the tadpole expression patterns; six days following heat-killed *E. coli* stimulation of adult frogs, these animals possessed significantly elevated spleen, liver and bone marrow CSF1R gene expression, where the magnitude of the bone marrow upregulation of this gene far exceeded any other CSF1R transcriptional changes reported here (Fig. 4C).

### **In vitro rXICSF1 and rXICSF1R binding studies**

To confirm that the *Xenopus* CSF1R is the cognate receptor for the monopoietic CSF1 ligand, we produced recombinant rXCSF1R and rXCSF1 molecules using an insect expression system, and assessed ligand-receptor binding *in vitro* using the disuccinimidyl suberate (DSS, 2.5 mM final concentration) crosslinker to stabilize protein interactions and

western blot analysis against the V5 tags on these recombinant proteins (Fig. 5). Since the mammalian CSF1 interacts with the D2 and D3 domains of its cognate CSF1R (Chen *et al.*, 2008), we engineered the (r*XI*)CSF1R to comprise of the D2 and D3 extracellular domains of the *X. laevis* receptor. Western blot analysis of the crosslinked 25 kDa r*XI*CSF1 ligand revealed a band shift to 50 kDa; indicative of r*XI*CSF1 dimerization (Fig. 5). Following DSS crosslinking, r*XI*CSF1R could no longer be detected by western blot, which is typical of non-interacting proteins (Fig. 5). Notably, when the r*XI*CSF1 and r*XI*CSF1R were coincubated and crosslinked with DSS, a band shift to 130 kDa was observed, indicating that the dimerized r*XI*CSF1 interacted with two 40 kDa molecules of r*XI*CSF1R in solution (Fig. 5). The crosslinking of r*XI*CSF1R in the presence of bovine serum albumin (BSA) did not yield a banding pattern, indicating lack of non-specific interactions (Fig. 5).

### **r*XI*CSF1R abrogates the r*XI*CSF1-mediated recruitment and differentiation of tadpole peritoneal macrophages**

To confirm the biological roles of r*XI*CSF1 and r*XI*CSF1R, we examined the ability of the recombinant ligand to elicit and differentiate tadpole peritoneal macrophages, and the capacity of the CSF1R D2-D3 extracellular domain-containing r*XI*CSF1R to abrogate this process by antagonizing the effects induced by r*XI*CSF1 (Fig. 6). Twenty-four hours following intraperitoneal injection of tadpoles with the r*XI*CSF1 resulted in significant accumulation of peritoneal macrophages (Fig. 6A). In contrast, administration of r*XI*CSF1R induced a reduction in the numbers of tadpole resident peritoneal phagocyte populations (Fig. 6A). Furthermore, a four-fold excess of r*XI*CSF1R co-injected with r*XI*CSF1 abrogated the r*XI*CSF1-mediated macrophage elicitation (Fig. 6A).

Microscopy analysis of cytospin preparations stained by Giemsa revealed that peritoneal leukocytes obtained from tadpoles injected with vector control were comprised primarily of smaller mononuclear phagocytes, whereas r*XI*CSF1 treatment resulted in the accumulation/differentiation of considerably larger, ruffled and highly vacuolated macrophages (Fig. 6B). On average, cells from vector control administered animals consisted of only 14.9% large, ruffled and vacuolated macrophages, whereas these larger cells comprised 69.6% of the r*XI*CSF1-elicited tadpole peritoneal phagocytes (Fig. 6C). Notably, peritoneal phagocytes derived from animals that had been co-injected with r*XI*CSF1 and r*XI*CSF1R had significantly lower proportions (32.1%) of these morphologically distinct macrophage populations as compared to the r*XI*CSF1-cultures (Fig. 6C). Interestingly, peritoneal phagocytes isolated from tadpoles administered r*XI*CSF1R alone had significantly lower numbers of large, ruffled macrophages (6.4%) than even those seen in the vector control cultures (Fig. 6C), suggesting that resident peritoneal macrophages also rely on native CSF1 for survival.

Given that the administration of r*XI*CSF1R significantly affected the differentiation and abundance of resident tadpole peritoneal macrophages (see Fig. 6), we next assessed how r*XI*CSF1R treatment would impact the tadpoles' capacity to recruit leukocytes into the peritoneum following heat-killed *E. coli* or FV3 challenge (Fig. 7A). Notably, the numbers of peritoneal leukocytes elicited one day after stimulation with heat-killed *E. coli* were dramatically decreased by co-injection with r*XI*CSF1R (Fig. 7A). Similarly, macrophage

infiltration induced one day after FV3 infection was significantly decreased by co-injecting r*X*CSF1R with the FV3 (Fig. 7A). These findings suggest that the macrophage-suppressive effects conferred by r*X*CSF1R administration (Fig. 6) culminate in decreased capacities of resident mononuclear phagocytes to recruit additional leukocyte populations in response to immune stimuli.

Since we have shown that *X. laevis* macrophages derived by the recombinant ligand r*X*CSF1 increases tadpoles susceptibility to FV3 (Grayfer and Robert, 2014) and that converging evidence indicates that ranaviruses rely on macrophages to disseminate inside their hosts (reviewed in Grayfer *et al.*, 2012), we postulated that ablation of tadpole resident peritoneal macrophages by r*X*CSF1R should also reduce FV3 dissemination into tadpole organs. Accordingly, tadpoles were injected with FV3 alone, or in combination with r*X*CSF1R and virus loads in various tissues were assessed the following day (Fig. 7B). In support of our previous findings, the r*X*CSF1R administration significantly reduced FV3 loads in kidney and spleen, but not liver (Fig. 7B). This suggests that peritoneal CSF1-dependent phagocytes are critical for the dissemination of this virus.

## Discussion

This manuscript represents the first characterization of an amphibian CSF1R. Despite the poor conservation of the overall amino acid sequence among vertebrate CSF1R molecules, all CSF1R gene products, including the *X. laevis*, share hallmark features including 5 putative immunoglobulin domains, structurally conserved cysteine residues as well as a disrupted tyrosine kinase domain. The extracellular portions of CSF1R molecules exhibit more divergence, possibly reflecting evolutionary drift to facilitate the binding of respective cognate CSF1 ligands, which also display low amino acid sequence conservation (Grayfer and Robert, 2013). By contrast, the intracellular catalytic tyrosine kinase domains of these respective CSF1R proteins are remarkably well conserved, perhaps marking the evolutionary functional necessity for retaining these protein sequences. The evolutionarily diverged CSF1R amino acid sequences of distinct vertebrate classes are also reflected in their phylogenetic relationships, wherein the fish, amphibian, avian, reptilian and mammalian receptors all branched in respective separate clades. Indeed the CSF1R catalytic domains have been evolutionarily conserved across vertebrates, suggesting conservation in downstream CSF1R cell signaling and presumably the resulting biological outcomes. However, many other aspects of lower vertebrate macrophage biology appear to be distinct from what has been documented in mammals. This includes varying teleost CSF1 ligand and receptor gene copy numbers (Hanington *et al.*, 2007; Wang *et al.*, 2008; Wang *et al.*, 2014; Williams *et al.*, 2002) and unique physiological localization of amphibian monopoiesis (Grayfer and Robert, 2013). It remains to be determined whether these differences arise from and/or are dependent on CSF1-CSF1R functions which are different to those of mammals. Thus, it is possible that the monopoietic roles conferred by CSF1-CSF1R of distinct lower vertebrate species are at least partially unique to those described in mammals.

Whereas birds and mammals possess a single CSF1 gene expressing alternatively spliced transcripts, fish possess at least two distinct CSF1 genes that do not appear to produce alternatively spliced products (Hanington *et al.*, 2007; Wang *et al.*, 2008; Wang *et al.*, 2014).

Presently, it is unknown whether the functions of the different fish CSF1 genes correspond to those mediated by the respective alternatively spliced mammalian transcript products. Furthermore, at least some fish species, including Fugu, possess 2 distinct CSF1R genes (Williams *et al.*, 2002). Therefore, it is quite possible that similar to the fish type II IFN system (Aggad *et al.*, 2012; Grayfer and Belosevic, 2009; Grayfer *et al.*, 2010; Shibasaki *et al.*, 2013; Yabu *et al.*, 2011), the multiple teleost CSF1 receptor and ligand gene products may exhibit complex interactions, distinct from the single ligand, single receptor strategy of higher vertebrates. In our recent study of the *X. tropicalis* and *X. laevis* CSF1 genes (Grayfer and Robert, 2013), we did not identify additional CSF1 gene copies in *X. laevis* and *X. tropicalis* genomes, nor did we detect additional CSF1R genes during the studies described here. It is noteworthy that despite our best efforts, using both conventional and RACE PCR approaches, we were unable to identify alternatively spliced *X. laevis* CSF1 transcripts (data not shown). Although more investigation are needed, it appears that alternatively spliced CSF1 and/or CSF1R genes are absent or at least of minor importance in *Xenopus*. It is possibly that the unique *Xenopus* monopioietic strategy requires a single non-alternatively spliced *Xenopus* CSF1 and a single CSF1R.

The highest level of CSF1R gene expression was observed in the lung and liver of *X. laevis* adults. Presumably these expression patterns reflect the presence of alveolar macrophages (Lin *et al.*, 1989) and Kupffer cells (Amemiya *et al.*, 2011), wherein the mammalian counterparts of both of these macrophage-lineage populations express high CSF1R levels. Notably, CSF1R gene expression increased with *X. laevis* development in the majority of tissues examined, including kidney, spleen, liver, lung and skin. This may represent the accumulation of resident macrophages, reflecting complex growing biological necessities for these cells with frog development and maturation. Alternatively, it is possible that the level of CSF1R gene expression per cell increases during development. In addition, the CSF1R gene expression increased in the muscle tissue of regressing tails from metamorphosing animal. Indeed, macrophages have been previously demonstrated to play crucial roles in tail and body muscle remodeling during metamorphosis (Nishikawa *et al.*, 1998), where our present observations corroborate with these earlier findings. It will be interesting to examine the differences in CSF1 responsiveness of macrophage precursors in tadpoles and adult *X. laevis*, since they are both CSF1-responsive (Grayfer and Robert, 2013; Grayfer and Robert, 2014), whereas macrophage development in adult involves the bone marrow that is absent in tadpoles (Grayfer and Robert, 2013).

Following FV3 infection, both tadpoles and adults exhibited increased kidney CSF1R gene expression, which is interesting considering that the kidney is the primary site of FV3 replication (Gantress *et al.*, 2003; Robert *et al.*, 2007). In addition, macrophages are intimately involved in both immunity and the infection strategy of FV3 (Morales *et al.*, 2010; Grayfer *et al.*, 2012). Therefore, it seems reasonable to speculate that CSFR gene expression increases due to the infiltration of kidney tissues by macrophage-lineage cells. Intriguingly, the spleen and liver CSF1R gene expression patterns induced by FV3 infection were markedly different between tadpoles and adults, possibly underlining their distinct monopioietic strategies. In light of our recent findings that the *X. laevis* bone marrow serves as the prime source of macrophage precursors (Grayfer and Robert, 2013), together with our present observation that FV3 and heat-killed *E. coli* both elicit increased bone marrow

CSF1R expression may indicate that in response to immunological challenges, adult *Xenopus* increase monopoietic activity at the level of the bone marrow. By contrast, tadpoles do not possess bone marrow and thus, presumably orchestrate monopoiesis in the hematopoietic liver. This would explain the increased CSF1R gene expression in FV3-infected tadpole, but not adult liver tissues. In comparison to adults, the tadpole spleen may likewise be more prominently involved in macrophage development and immunity as reflected in substantially more upregulated CSF1R gene expression within this tadpole tissue following FV3 challenge. It is interesting that heat-killed *E. coli* did not elicit significant CSF1R expression changes in any of the tadpole tissues examined, whereas following this inflammatory stimulus adults exhibited substantially more robust CSF1R gene expression increases in the spleen, liver and bone marrow than observed following FV3 infection. These disparities may reflect differences in pathogen pattern recognition receptor expression between tadpoles and adults, and a physiological necessity for more prominent adult monopoietic responses to inflammatory, rather than viral challenges. A greater understanding of *Xenopus* macrophage development and immune responses will no doubt shed light on this present enigma. We emphasize that in our present work *E. coli* was used as an inflammatory stimulus rather than a direct point of comparison with the more relevant FV3 viral challenge. Future *X. laevis* infection studies using relevant bacterial pathogens will lend to our understanding of the differences in macrophage involvement during different host responses.

The mammalian dimeric CSF1 binds exclusively to the D2 and D3 domains of the CSF1R, dimerizing the receptor (Chen *et al.*, 2008). Our *in vitro* binding studies indicate that the D2 and D3 domains of a recombinant amphibian CSF1R are also sufficient to bind the recombinant homodimerized CSF1 ligand. More detailed studies will be needed to elucidate the modalities and stoichiometry of these interactions. Notably, the rXCsf1R very effectively inhibited rXCsf1-mediated tadpole elicitation and development of macrophages into cells morphologically resembling mature, differentiated populations. Furthermore, rXCsf1R administration also ablated the recruitment of peritoneal leukocytes elicited by heat-killed *E. coli* and the FV3. Since tadpoles administered with rXCsf1R exhibited diminishing numbers of mature resident peritoneal macrophages, the compromised capacities of rXCsf1R-treated tadpoles to recruit leukocytes into the peritoneum upon ip immune challenges likely reflects a functional impairment of peritoneal macrophages, which would normally chemo-attract additional immune populations in response to immune stimuli. Notably, teleost fish appear to have evolved an additional regulatory mechanism of monopoiesis and inflammation whereby they produce an alternatively spliced, soluble CSF1R (Barreda *et al.*, 2005; Rieger *et al.*, 2014a; Rieger *et al.*, 2014b). Intriguingly, this moiety only possesses the D1 and D2 domains of the membrane-bound CSF1R and yet this molecule is highly biologically active and inhibits fish macrophage proliferation and inflammatory responses (Barreda *et al.*, 2005; Rieger *et al.*, 2014a; Rieger *et al.*, 2014b). It will be interesting to learn which specific domains are involved in the interactions of the respective teleost CSF1 ligand(s), soluble and membrane bound CSF1Rs and whether a similar system is present in amphibian species.

Converging evidence indicate that macrophage-lineage cells are utilized by ranaviruses as a means of dissemination within their hosts (Grayfer *et al.*, 2012). In addition, CSF1-derived



macrophages render tadpoles more susceptible to FV3 (Grayfer and Robert, 2014). Here, we show that intraperitoneal administration of rXICSF1R reduces resident phagocyte numbers and impairs some of their functions such as leukocyte recruitment. The treatment with rXICSF1R also results in decreased dissemination of FV3 from the inoculation site of the peritoneum to the kidney (central site of ranavirus replication) and the spleen (central immune organ), whereas it does not affect low viral dissemination into the liver. These observations underline the complexity of the roles of mononuclear phagocytes as both mediators of anti-RV immune responses and culprits in the progression of these infections.

The CSF1-CSF1R axis represents the focal point of vertebrate monopoiesis and it has become apparent that evolutionarily more ancestral species such as teleosts and amphibians may possess varying strategies for CSF1-mediated macrophages development, distinct from those described in mammalian species. Further research into CSF1 and CSF1R macrophage biology of lower vertebrates will yield new insights into the evolutionary basis of monopoiesis and permit the development of more effective preventative measures against pathogens that infiltrate macrophage-lineage cells of more ancient species.

## Materials and Methods

### Animals

Outbred pre-metamorphic (stage 54, (Thors *et al.*, 1982a; Thors *et al.*, 1982b)) tadpoles, metamorphic (stage 64) and adult (2 years old) frogs were obtained from our *X. laevis* research resource for immunology at the University of Rochester (<http://www.urmc.rochester.edu/mbi/resources/Xenopus-laevis/>). All animals were handled under strict laboratory and University Committee on Animal Research regulations (Approval number 100577/2003-151).

### Identification and analysis of *X. laevis* colony-stimulating factor-1 receptor

The identification of the *X. laevis* CSF1 was described previously (Grayfer and Robert, 2013). A fragment of the *X. laevis* CSF1R cDNA transcript was identified using primers against the predicted *X. tropicalis* CSF1R sequence (Acc. no.: BC082504). Subsequently, RACE PCR was performed in accordance with manufacturers' directions (Clontech) to identify the complete *X. laevis* CSF1R cDNA transcript (Acc. No.: KM400585). All primer sequences are available upon request.

Protein sequence alignments were performed using the Clustal W software (<http://www.ebi.ac.uk/clustalw/>). Signal peptide regions were identified using the SignalP 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>). Protein motif and domain predictions were achieved using the ELM (<http://elm.eu.org/>) and SMART (<http://smart.embl-heidelberg.de/>) servers. Phylogenetic analysis was performed by Clustal X software using the neighbor joining method and bootstrapped 10,000 times, with values expressed as percentages.

### Production of rXICSF1 and rXICSF1R

The production of the rXICSF1 has been described previously (Grayfer and Robert, 2013). The rXICSF1R was produced by transfecting Sf9 insect cells (cellfectin II, Invitrogen) with

the pMIB/V5 HisA insect expression vector (Invitrogen) containing the *X. laevis* CSF1R sequence corresponding to the IG2 and IG3 domains of the extracellular portion of the protein. Transfected Sf9 supernatants were confirmed for rXCSF1R expression, positive transfectants were scaled up to 500 mL in liquid cultures and grown for 6 days in blasticidin (10 µg/mL)-containing medium. Resulting supernatants were dialyzed overnight at 4°C against 150 mM sodium phosphate, concentrated against polyethylene glycol flakes (8 kDa), dialyzed overnight at 4°C against 150 mM sodium phosphate and passed through Ni-NTA agarose columns (Qiagen). Columns were washed with 2×10 volumes of high stringency wash buffer (0.5% Tween 20; 50 mM Sodium Phosphate; 500 mM Sodium Chloride; 100 mM Imidazole) and 5× with low stringency wash buffer (as above, but with 40 mM Imidazole). The rXCSF1R was eluted in fractions using 400 mM Imidazole. The purity of the eluted fractions was confirmed by silver-stain and the presence of rXCSF1R was assessed by western blot against the V5 epitope on the recombinant protein. Fractions containing the rXCSF1R were pooled and the protein concentration determined by the Bradford Protein Assay (BioRad). A protease inhibitor cocktail (Roche) was added to the purified protein, which was then aliquoted and stored at -20°C until use.

The vector control was produced by transfecting Sf9 cells with an empty expression vector and treating the resulting supernatants akin to and in parallel to the rXCSF1 production.

### Cell culture media

The amphibian (ASF) culture medium used in these studies has been previously described (Robert *et al.*, 2007). All cell cultures were established using ASF supplemented with 10% fetal bovine serum, 2.5% heat-inactivated *X. laevis* serum, 20 µg/mL kanamycin and 100 U/mL penicillin / 100 µg/mL streptomycin (Gibco). Amphibian PBS (APBS) has been previously described (Robert *et al.*, 2007).

### Isolation of rXCSF1-elicited tadpole macrophages

Tadpoles at developmental stages 54 (Stg. 54; (Thors *et al.*, 1982a; Thors *et al.*, 1982b)) were intraperitoneally (ip) injected with 10 µL of the vector control, rXCSF1 (250 ng), rXCSF1R (1000 ng) or a combination of rXCSF1 (250 ng) and rXCSF1R (1000 ng) in 10 µL volumes. After 24 hrs, peritoneal macrophages were collected by lavage with 50µL volumes of APBS. Cells were enumerated using trypan blue exclusion, collected on glass slides by cytospin and stained with Giemsa (Fluka).

### Frog Virus 3 stocks and animal infections

Fathead minnow cells (FHM; American Type Culture Collection, ATCC No.: CCL-42) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/mL) and streptomycin (100 µg/mL) with 5% CO<sub>2</sub> at 30°C. FV3 was grown by a single passage on FMH cells, purified by ultracentrifugation on a 30% sucrose cushion and quantified by plaque assay on BHK monolayer under an overlay of 1% methylcellulose (Morales *et al.*, 2010).

Tadpoles and adult frogs were infected by ip injections with previously-established (Gantress *et al.*, 2003; Hanington *et al.*, 2007; Robert *et al.*, 2005) infection doses of 1×10<sup>4</sup>

and  $5 \times 10^6$  plaque forming units (PFU) of FV3, respectively. Three and six days post infection (dpi) animals were euthanized by immersion in 0.5% tricaine methane sulfonate (MS-222), tissues removed and processed for RNA isolation.

For determination of FV3 loads, tadpoles were ip injected with  $1 \times 10^4$  PFU of FV3 alone, or in combination with 1  $\mu$ g of rXICSF1R. After 24 hrs, tadpoles were sacrificed, their tissues excised and processed for plaque forming assay analysis to determine respective infectious FV3 burdens.

### Heat-killed *Escherichia coli* stimulation

*E. coli* (XL1-blue, Stratagene, La Jolla, Ca.) cultured overnight at 37°C, were boiled for 1 hour, pelleted by centrifugation and resuspended in one-tenth of the initial volume (approximately  $1 \times 10^8$  bacteria/mL) of APBS. Tadpoles and adult frogs were injected ip with 10 and 100  $\mu$ l of this heat-killed (hk) bacterial preparation.

### Quantitative gene expression analysis

Total RNA was extracted from frog tissues using the Trizol reagent following the manufacturer's directions (Invitrogen). All cDNA synthesis was performed using the iScript cDNA synthesis kit according to manufacturers' directions (Bio-Rad, Hercules, CA) using 500 ng of total DNase treated (Ambion) RNA.

Relative qRT-PCR gene expression analyses was performed via the  $\Delta\Delta$ CT method using validated *X. laevis* CSF1R-specific primers, with expression examined relative to the GAPDH endogenous control and normalized against the lowest observed expression. All experiments were performed using the ABI 7300 real-time PCR system and PerfeCTa® SYBR Green FastMix, ROX (Quanta). Expression analysis was performed using ABI sequence detection system software (SDS). All primers were validated prior to use. All primer sequences are available upon request.

### In vitro rXICSF1 and rXICSF1R cross-linking studies

One microgram each of rXICSF1, rXICSF1R, rXICSF1 + rXICSF1R and BSA + rXICSF1R were incubated in APBS (100  $\mu$ l final volume) for 30 min, cross-linked for 30 min using 2.5 mM disuccinimidyl suberate (DSS, final concentration, Thermo Scientific). Cross-linking was terminated for 15 min by the addition of 50 mM Tris (final concentration). The reactions were then resolved and visualized using SDS-PAGE and western blot against the V5 epitopes on the recombinant proteins and developed using ECL (Pierce) on X-ray film (Eastman Kodak Co.)

### Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) and Tukey's post hoc test, using Vassar Stat (<http://faculty.vassar.edu/lowry//anova1u.html>) statistical program. Probability level of  $P < 0.05$  was considered significant.

## Acknowledgments

This work was supported by National Institute of Health (R24-AI-059830) and National Science Foundation (IOB-074271) grants to J.R. L.G. was supported by a National Sciences and Engineering Research Council of Canada Postdoctoral Fellowship and a Life Sciences Research Fellowship from the Howard Hughes Medical Institute. We thank Tina Martin for animal husbandry. This manuscript was improved by the insightful suggestions of one anonymous reviewer.

## Abbreviations used in this paper

|              |                             |
|--------------|-----------------------------|
| <b>CSF-1</b> | colony-stimulating factor-1 |
| <b>FV3</b>   | frog virus 3                |

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structural cysteines are in white text within black boxes and the transmembrane domain is also in bold face. As indicated overhead; the *X. laevis* CSF1R ATP binding and tyrosine kinase major catalytic domains are in white text within black boxes while the kinase insert domain is indicated by an overhead line.

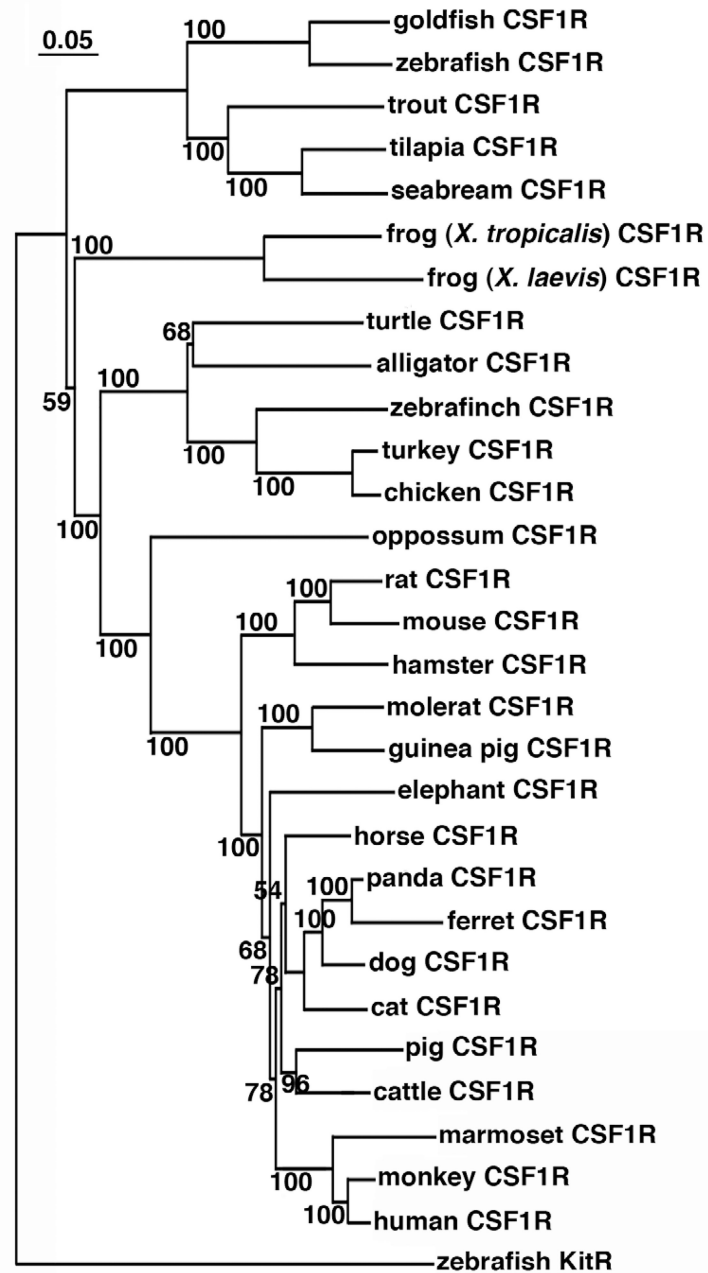
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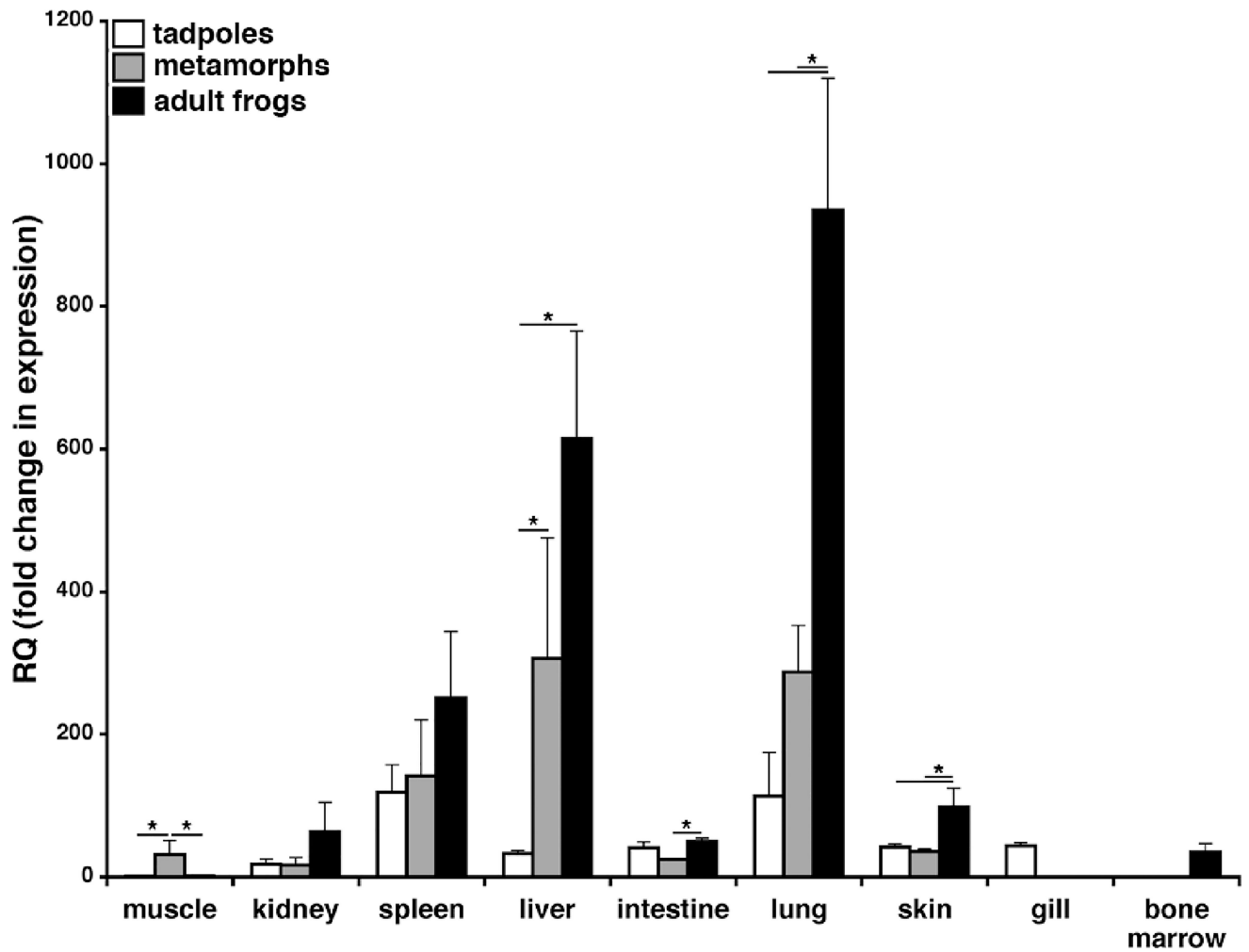
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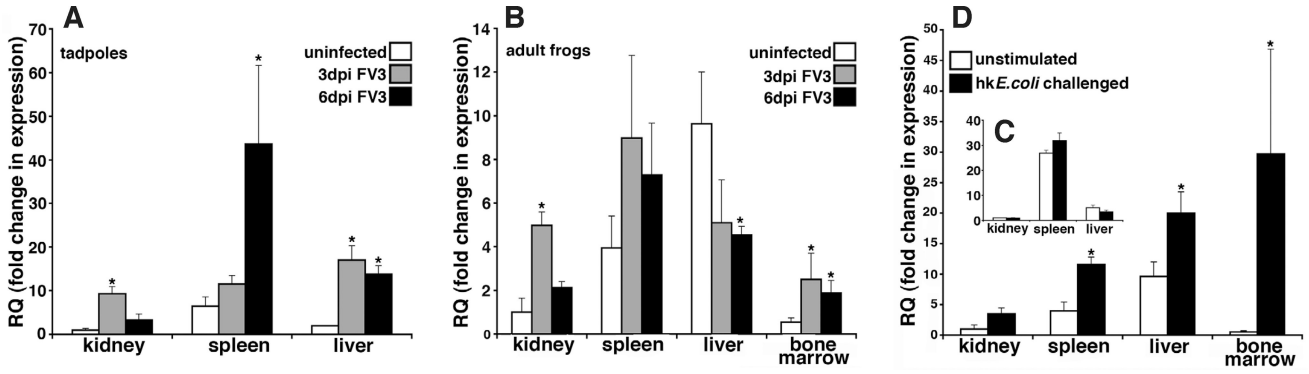
**Fig. 2. Phylogenetic analysis of teleost, amphibian, reptile, avian and mammalian colony-stimulating factor-1 receptor (CSF1R) molecules**

The phylogenetic tree was constructed from multiple deduced amino acid sequence alignments using the neighbor joining method and bootstrapped 10,000 times (denoted as %). The zebrafish KitR was used as outgroup to root the tree.

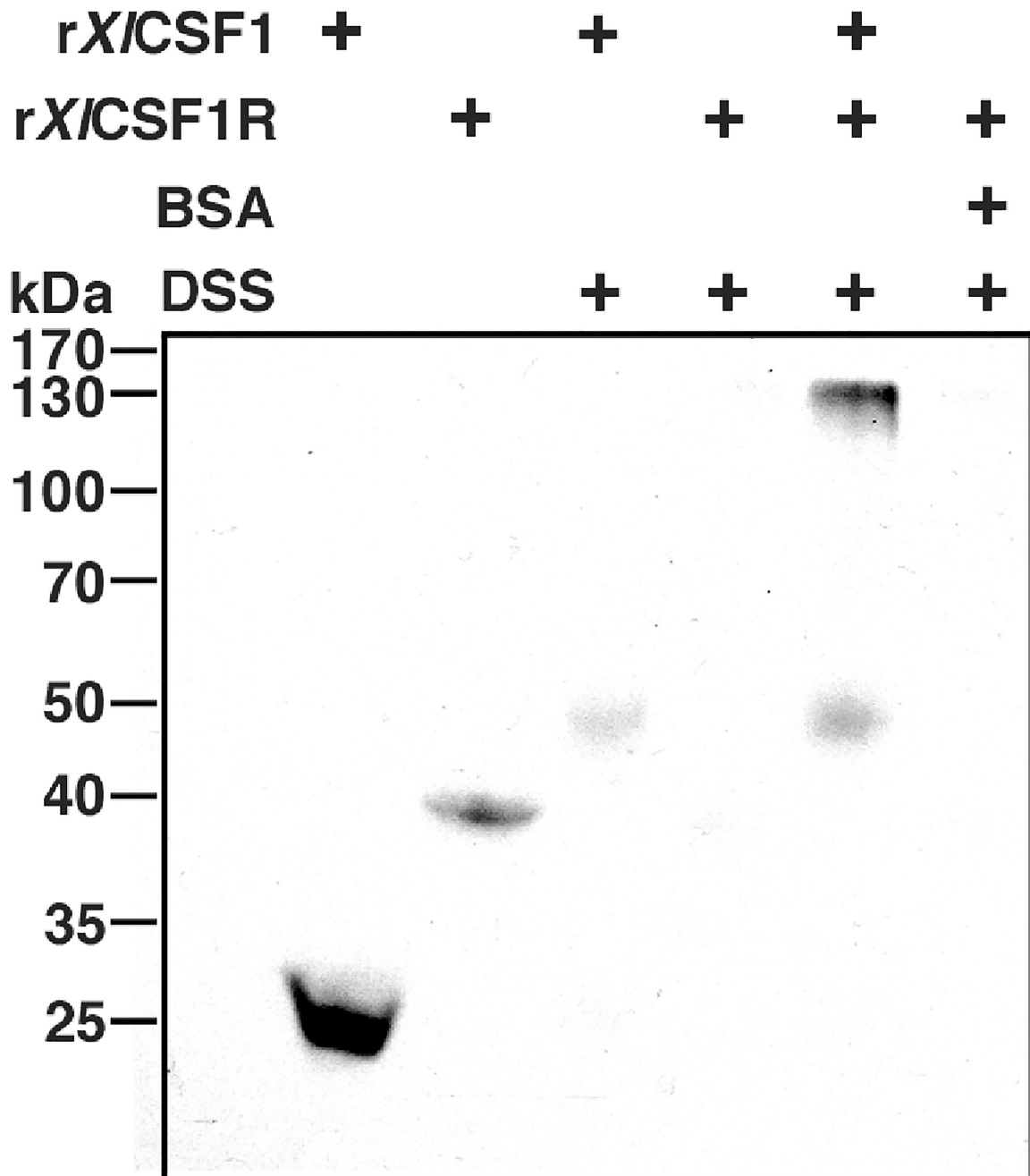


**Fig. 3. Quantitative *colony-stimulating factor-1 receptor (CSF1R)* tissue gene expression analysis of tadpoles (Stg. 54), metamorphic (Stg. 64) and adult (2 years-old) frogs**

Tissues from three individuals (N=3) were used for all expression studies, the expression was performed via the delta<sup>delta</sup> CT method using *X. laevis* CSF1R-specific primers. The expression examined relative to the GAPDH endogenous control and normalized against the tadpole muscle tissue expression. Results are means  $\pm$  SEM, N=4 and the (\*) above lines denotes significant difference between tissues indicated by the lines, P<0.05.

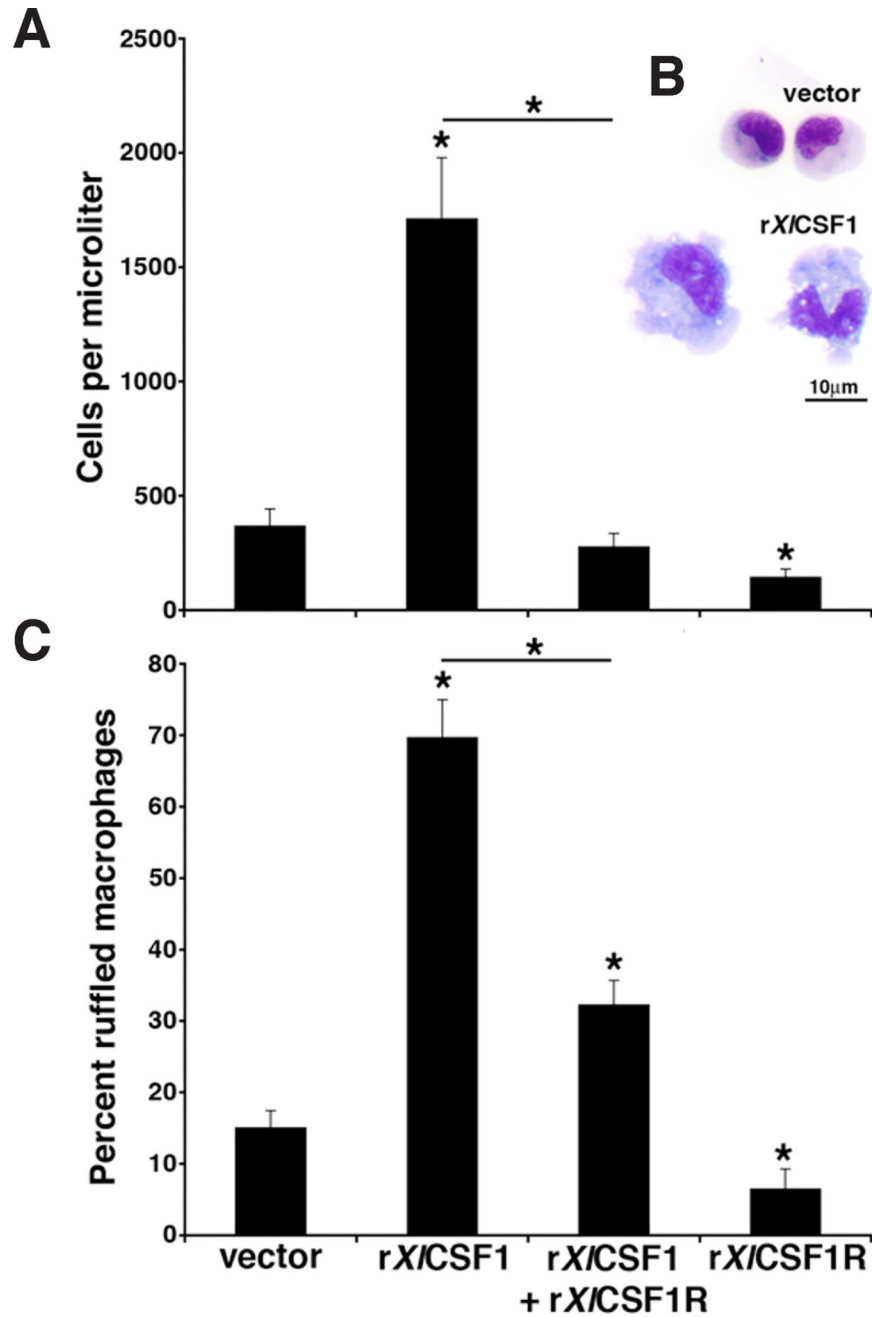


**Fig. 4. Quantitative analysis of colony-stimulating factor-1 receptor (CSF1R) tissue gene expression in Frog Virus 3 (FV3) and heat-killed *E. coli* challenged tadpoles and adult *X. laevis*** Tadpoles (Stg. 54) and adult frogs (2 years-old) were infected by ip injections with  $1 \times 10^4$  and  $5 \times 10^6$  PFU of FV3, respectively. Alternatively, tadpoles and adult frogs were injected respectively with 10 $\mu$ l and 100 $\mu$ l of heat-killed (hk) *E. coli*. (A) FV3-infected tadpole CSF1R gene expression. (B) FV3-infected tadult frog CSF1R gene expression. (C) Heat-killed *E. coli*-stimulated tadpole CSF1R gene expression. (D) Heat-killed *E. coli*-stimulated adult frog CSF1R gene expression. All gene expression analysis was performed via the delta<sup>delta</sup> CT method using *X. laevis* CSF1R-specific primers, the expression examined relative to the GAPDH endogenous control and normalized against respective uninfected kidney expression. Results are means  $\pm$  SEM,  $N=4$  and the (\*) above lines denotes significant difference between tissues indicated by the lines,  $P < 0.05$ .



**Fig. 5. *In vitro* rX/CSF1 and rX/CSF1R cross-linking studies**

One microgram each of rX/CSF1, rX/CSF1R, rX/CSF1 + rX/CSF1R and BSA + rX/CSF1R were incubated in APBS (100µl final volume) for 30 min and cross-linked for 30 min using 2.5 mM DSS, final concentration. Cross-linking reactions were terminated by addition of 50 mM Tris (final concentration). Reactions were resolved and visualized using SDS-PAGE and western blot against the V5 epitopes on the recombinant proteins.



**Fig. 6. The rX/CSF1R abrogates the rX/CSF1-mediated tadpole macrophage recruitment and differentiation**

(A) Tadpoles were injected with vector control, 250ng of rX/CSF1, 1000ng of rX/CSF1R or a combination of rX/CSF1 (250ng) and rX/CSF1R (10000ng). After 24hrs peritoneal phagocytes were lavaged and enumerated. Results are means  $\pm$  SEM,  $N=6$  and the (\*) above lines denotes significant difference between treatment groups indicated by the lines,  $P<0.05$ .

(B) Morphological analysis of Giemsa-stained vector-control and rX/CSF1R derived cultures. Scale bar = 10µm. (C) Cultures from (A) were Giemsa-stained, enumerated for the presence of morphologically differentiated macrophages as exemplified in (B) by the

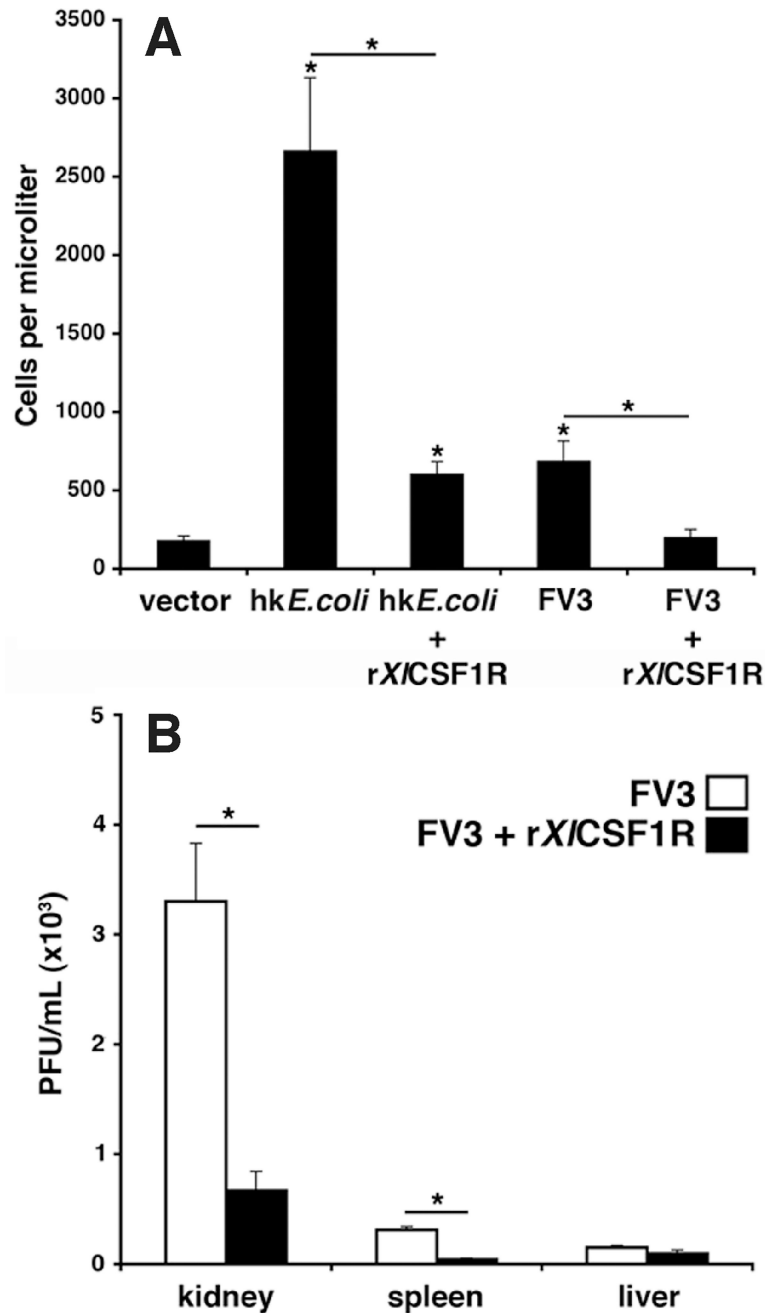
r.XCSF1R-derived cells. Results are expressed as percent means  $\pm$  SEM from ten random fields of view. The (\*) above lines denotes significant difference between treatment groups indicated by the lines,  $P < 0.05$ .

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**Fig. 7. The rX/CSF1R abrogates heat-killed *E. coli* and Frog Virus 3 elicited recruitment of tadpole peritoneal leukocytes and reduces FV3 dissemination**  
**(A)** Tadpoles were injected with vector control, heat-killed (hk) *E. coli*, FV3 ( $10^4$  PFU), a combination of hk *E. coli* and rX/CSF1 ( $1\mu\text{g}$ ) or a combination of FV3 ( $10^4$  PFU) and rX/CSF1R ( $1\mu\text{g}$ ). After 24hrs peritoneal phagocytes were lavaged and enumerated. Results are means  $\pm$  SEM,  $N=4$ . (\*) denotes statistical difference from the vector control and the (\*) above lines denotes significant difference between treatment groups indicated by the lines,  $P<0.05$ . **(B)** Tadpoles were injected with  $1 \times 10^4$  PFU of FV3 alone, or in combination with  $1\mu\text{g}$  of rX/CSF1R. Following 24 hrs, tadpoles were sacrificed; their tissues were isolated and examined for infectious FV3 burdens by plaque assays. Results are means  $\pm$  SEM,  $N=3$ . (\*)

above lines denotes significant difference between treatment groups indicated by the lines,  $P < 0.05$ .

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