Developmental and Comparative Immunology

A comparison of amphibian (Xenopus laevis) tadpole and adult frog macrophages

--Manuscript Draft--

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Abstract:	The amphibian declines are compounded by emerging pathogens that often preferentially target distinct amphibian developmental stages. While amphibian immune responses remain relatively unexplored, macrophage (Mφ)-lineage cells are believed to be important to both amphibian host defenses and to their pathogen infection strategies. As such, a greater understanding of tadpole and adult amphibian Mφ functionality is warranted. Mφ biology is interdependent of interleukin-34 (IL-34) and colony-stimulating factor-1 (CSF-1) cytokines and we previously showed that CSF-1- and IL-34-derived Mφs of the Xenopus laevis frog are morphologically, transcriptionally, and functionally distinct. Presently, we directly compared the cytology and transcriptomes of X. laevis tadpole and frog CSF-1- and IL-34-Mφs. Our results indicate that tadpole and frog CSF-1-Mφs possess greater non-specific esterase activity, typically associated with Mφ-lineage cells. By contrast, both tadpole and frog IL-34-Mφs have greater specific esterase activity, which is typically attributed to granulocyte-lineage cells. Our comparisons of tadpole CSF-1-Mφ transcriptomes with those of tadpole IL-34-Mφs indicate that the two tadpole populations possess significantly different transcriptional profiles of immune and non-immune genes. The frog CSF-1-Mφ gene expression profiles are likewise significantly disparate from those of frog IL-34-Mφs. Compared to their respective tadpole Mφ subtypes, frog CSF-1- and IL-34-Mφs exhibited greater expression of genes associated with antigen presentation. Conversely, compared to their frog Mφ counterparts, tadpole CSF-1- and IL-34-Mφs possessed greater levels of select Fc-like receptor genes. Presumably, these cytological and transcriptional differences manifest in distinct biological roles for these respective tadpole and frog Mφ subtypes.
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Response to Reviewers:	

THE GEORGE WASHINGTON UNIVERSITY
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Biological Sciences

12/30/2022

Dear Dr. Dixon,

I am writing on behalf of all authors to re-submit to the journal of Developmental and Comparative Immunology our revised manuscript titled "A comparison of amphibian (*Xenopus laevis*) tadpole and adult frog macrophages" by Muhammad Riadul Haque Hossainey, Amulya Yaparla, Zarafsha Uzzaman, Tyler Moore and Leon Grayfer, to be considered for possible publication.

Please find that in response to the reviewers' comments, we have revised our manuscript into a short communication. We feel that we have successfully addressed all reviewer concerns, thereby improving this work.

Many thanks for your further consideration.

Best wishes,

Leon Grayfer, PhD
Associate Professor,
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DCI-D-22-00307

We thank the reviewers for their helpful comments and suggestions. We feel that we have successfully addressed all reviewers' comments and incorporated all suggestions into our revised manuscript, thereby improving its content.

We agree with the reviewers' that the original version of our manuscript was not a complete story. Accordingly, please find that we have removed the microbial-challenge data and have revised the cytology and transcriptomic data of our original manuscript into a short communication.

Please find the point-by-point address of individual comments below.

Reviewer #1: In the manuscript (DCI-D-22-00307), the authors tried to do a comparison of macrophages from amphibian (Xenopus laevis) tadpole and adult frog by transcription analysis. However, the manuscript appears immature and has severe limitations. The following are major comments:

1. The topic of the manuscript is ambiguous. In this study, the authors did not use primary macrophages alone to do comparison analysis, whereas CSF-1- and IL-34-macrophages were used as cell models. However, the manuscript did not introduce the necessary of treatment with CSF-1 and IL-34 to distinguish the macrophages from tadpole and adult frog. In other words, are the similarities and differences of macrophages from tadpole and adult frog subjected to CSF-1 and IL-34 stimulation or treatment? Moreover, bacterial challenge experiments did not explain the immune significance of amphibian macrophages at distinct growth stages.

We thank the reviewer for their comments and opinion regarding our manuscript. Please note that since there is no established method for culturing tadpole macrophages *in vitro*, we instead used a previously published approach of enriching peritoneal tadpole macrophages with recombinant growth factors (rCSF1 or rIL34) before isolating these respective cell subsets by lavage. To directly compare the tadpole macrophages to their adult counterparts, we used the same peritoneal macrophage enrichment and lavage approach to derive adult CSF1- and IL34-macrophages. Please note that we used this approach in several previous publications and have characterized and optimized the utility of deriving polarized tadpole and froglet CSF1- and IL34-differentiated macrophages through these means.

Grayfer, L. and J. Robert, Divergent antiviral roles of amphibian (*Xenopus laevis*) macrophages elicited by colony-stimulating factor-1 and interleukin-34. J Leukoc Biol, 2014. 96(6): p. 1143-53.

Grayfer, L. and J. Robert, Distinct functional roles of amphibian (*Xenopus laevis*) colony-stimulating factor-1- and interleukin-34-derived macrophages. J Leukoc Biol, 2015. 98(4): p. 641-9.

We used the peritonea-derived tadpole and froglet CSF1- and IL34-macrophages to directly compare the respective macrophage subtest between pre- and post-metamorphic animals. To do this, we performed transcriptomic and cytological studies. Because pre- and post-metamorphic

amphibians exhibit notoriously disparate immune efficacies, we felt that a direct comparison of the transcriptional and cytological differences would represent a means of focusing in on where these immune differences may be coming from.

Please find that we removed the microbial-challenge and corresponding immune gene expression data from our revised manuscript.

Please find that we have significantly revised our manuscript to highlight the experimental design, the motivation behind the experiments and the value of the findings.

2. The main results of the manuscript were gene expression profiles in Fig. 2-Fig. 6. However, the expression patterns at transcriptional level cannot provide any helpful information on functional role of the immune cells, which do not contribute anything new to our understanding of macrophage characterization from amphibian (Xenopus laevis) at different growth stages. In addition, the logical relationship among those target genes whose expression was detected in Fig.4-Fig. 6 is unclear.

We agree that it is often difficult to deduce functionality from complex transcriptional data such as ours. However, in our previous studies we established several notable functional differences between amphibian CSF1- and IL34-macrophages. Our present transcriptional comparisons drastically corroborate these previous findings, as they indicate that tadpole CSF1-macrophages are significantly different from tadpole IL34-macrophages and the same is true of the adult macrophage counterparts. Moreover, our comparisons of tadpole CSF1-macrophages with adult CSF1-macrophages and tadpole IL34-macrophages with the adult counterparts reveal notable differences in antigen presentation and chemotaxes genes, inferring functional differences between the tadpole and adult macrophage subsets.

Please find that we have highlighted these differences in the revised highlights, abstract and results & discussion sections of our revised (short communication) manuscript.

Please find that we have revised our manuscript to better explain the impetus behind our experimental design.

Please find that we removed Figures 4-6 of our original manuscript, combined the transcriptomic data into one paper and one supplemental figure and revised our manuscript into a short communication.

3. The highlights in the manuscript were confusing. In detail, no data in this study supported the conclusion "Macrophage biology depends on IL-34 and CSF-1"; It is odd that "We compared tadpole and frog IL-34- and CSF-1-macrophages" was regarded as a highlight; It is expected that "Tadpole and frog macrophages have different transcriptional profiles", which did not provide any new information on amphibian macrophages; "Tadpole and frog macrophages possess similar gene expression responses to pathogens" did not contribute new to our understanding of immune response in amphibian macrophages.

Please see the above comments and note that we have revised our highlights section accordingly.

4. There were originally many subtypes of macrophages induced by rCSF-1-and rIL-34, but the authors did not described which subtype was detected in the manuscript. Unexpectedly, the authors did not introduce how to isolate peritonea-derived macrophages, especially in tadpole.

Please find that in our revised manuscript, we acknowledge that as we previously noted, CSF1- and IL34 macrophages likely comprise of heterogenous populations. As discussed in our revised manuscript, the paucity of *X. laevis*-specific antibodies has prevented us from exploring this possible heterogeneity further.

Please find that our description of macrophage isolation is described in the methods section of our revised manuscript.

5. The obvious morphological difference between CSF-1- and IL-34-Macrophages (see Fig.1B) is impressed. This finding should be explained and discussed.

Please find that we have expanded on these results in our revised manuscript.

Reviewer #2: This manuscript deals with an interesting dataset which could be 'exploited' better than currently the case. At present the manuscript is highly descriptive without a clear research question. Also, at the end of the introduction (L65) please make more clear what is the aim of the current study, how does it add to and what is new in comparison to the previous work. The full RNAseq datasets should be made available.

We thank the reviewer for their kind and encouraging words and helpful suggestions. Please find that we have revised our manuscript to better highlight the questions being posed.

Please also find that we have revised this work into a short communication, focusing on the cytology and transcriptomics of tadpole and frog macrophage populations.

Please find that we have also made the full RNAseq dataset available as a supplemental file.

The 'highlights' should be rephrased and more specific, e.g. 'macrophage biology depends on IL-34 and CSF-1' is too broad. The last conclusion in the abstract at L38-42, is formulated too one-dimensional, please rephrase.

Please find that we have changed the highlights to be more informative of our manuscript's content.

Please re-read and double-check the phrasing in the Introduction section and the complete manuscript, e.g.; i) L48 The first sentence does not at appear to be correct, ii) the word 'these' in sentence 2 does not refer to anything obvious in the previous sentence, etc.

Thank you. Please find that we have revised our introduction section.

Introduction: reference to inos and tnfa for M1 and il10 and arginase-1 for M2 is too simplified and debatable. Please check the current opinion on markers for polarized macrophages and include comparative reference to work in e.g. chickens and fish. For example, in the latter, arginase-2 rather than arginase-1 has been discussed as M2 marker.

Please find that we removed the content of the introduction dealing with M1/M2 types in our revised short communication.

M&M: L87-104 Bacteria are grown at three different temperatures (20, 25, 30 oC) and heat killed, but also used alive shown in the Results, which is not clear from the M&M section. How do these bacteria grown at different temperatures behave at the same (?) and if so, which one (L111, temperature not specified) temperature used to grow the cells? I assume the dynamics of live bacteria will be very different under such different circumstances, how does this influence comparisons. All primers were validated prior to use (L130); this is a nice but meaningless statement with further details.

Please find that all data pertaining to microbial challenge and the corresponding immune gene expression analyses has been removed from what is now a short communication.

In the results and discussion section datasets are compared; transcripts of macrophages from tadpoles, with transcripts of macrophages from adults, collected after injection of csf, or injection of il-34. Also compared are macrophage transcripts of csf-injected, or il-34-injected animals, between tadpoles, or between adults. The questions addressed are very different (tadpole versus adult, or csf-versus il-34), and the results should be discussed separately and (more) clearly.

We thank the reviewer for this suggestion. Please find that we have revised our manuscript accordingly.

For the results, the authors frequently make a difference between 'transcriptionally unique' and 'transcriptionally different', this requires further specification. The discussion should be aligned with a clear research question defined at the end of the introduction.

We thank the reviewer for noting this. Please find that we have gone through our manuscript and reinforced such statements and framed our results within more concrete research questions.

Highlights

- Cytology and transcriptomics of tadpole & frog CSF-1- & IL-34-Mϕs were compared
- Tadpole & frog CSF-1-Mϕs are larger & have greater non-specific esterase activity
- Tadpole & frog IL-34-Mϕs are smaller & have greater specific esterase activity
- Tadpole & frog CSF-1- & IL-34-Mϕs differ in their immune gene expression
- Frog CSF-1- & IL-34-Mϕs express more antigen presentation genes than tadpole counterparts
- Tadpole CSF-1- & IL-34-Mϕs express greater levels of some Fc-like receptor genes

A comparison of amphibian (*Xenopus laevis*) tadpole and adult frog macrophages

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+ these authors contributed equally to this work

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Abstract

The amphibian declines are compounded by emerging pathogens that often preferentially target distinct amphibian developmental stages. While amphibian immune responses remain relatively unexplored, macrophage (Mϕ)-lineage cells are believed to be important to both amphibian host defenses and to their pathogen infection strategies. As such, a greater understanding of tadpole and adult amphibian Mϕ functionality is warranted. Mϕ biology is interdependent of interleukin-34 (IL-34) and colony-stimulating factor-1 (CSF-1) cytokines and we previously showed that CSF-1- and IL-34-derived Mϕs of the *Xenopus laevis* frog are morphologically, transcriptionally, and functionally distinct. Presently, we directly compared the cytology and transcriptomes of *X. laevis* tadpole and frog CSF-1- and IL-34-Mϕs. Our results indicate that tadpole and frog CSF-1-Mϕs possess greater non-specific esterase activity, typically associated with Mϕ-lineage cells. By contrast, both tadpole and frog IL-34-Mϕs have greater specific esterase activity, which is typically attributed to granulocyte-lineage cells. Our comparisons of tadpole CSF-1-Mϕ transcriptomes with those of tadpole IL-34-Mϕs indicate that the two tadpole populations possess significantly different transcriptional profiles of immune and non-immune genes. The frog CSF-1-Mϕ gene expression profiles are likewise significantly disparate from those of frog IL-34-Mϕs. Compared to their respective tadpole Mϕ subtypes, frog CSF-1- and IL-34-Mϕs exhibited greater expression of genes associated with antigen presentation. Conversely, compared to their frog Mϕ counterparts, tadpole CSF-1- and IL-34-Mϕs possessed greater levels of select Fc-like receptor genes. Presumably, these cytological and transcriptional differences manifest in distinct biological roles for these respective tadpole and frog Mϕ subtypes.

Key words: macrophage, interleukin-34, colony-stimulating factor-1, amphibian, tadpole

Introduction

Macrophage (Mϕ) lineage cells are indispensable to the physiology and immunity of all vertebrates, including amphibians [1]. While Mϕ polarization is highly dynamic [2, 3], the differentiation and functionality of all vertebrate Mϕs depend on the colony-stimulating factor-1 receptor (CSF-1R), which is ligated by CSF-1 and interleukin-34 cytokines [4]. Our recent work indicates that the *X. laevis* tadpole and adult Mϕs differentiated by recombinant (r)CSF-1 and rIL-34 possess markedly distinct capacities to recognize and respond to various pathogens [5-8]. Our past studies indicate that IL-34-derived Mϕs of both tadpoles and adult frogs are considerably more resistant to viral infections than tadpole and adult CSF-1-Mϕs [5, 6, 9]. Our work also suggests that tadpole and adult IL-34-Mϕs share the expression of some immune genes while differing in the expression of others, as is the case with tadpole and adult CSF-1-Mϕs [5, 6]. However, these tadpole and adult frog counterpart Mϕ subsets have hitherto not been directly compared. To reconcile possible differences in tadpole and adult frog Mϕs, here we compare tadpole and adult frog CSF-1- and IL-34-Mϕ cytology and transcriptional profiles.

Materials and Methods

Animals and cell culture conditions

Outbred, Nieuwkoop and Faber (NF) stage -54 tadpoles and approximately 1 years-old adult *X. laevis* were purchased from Xenopus1 (Dexter, MI), housed, and handled under strict laboratory regulations of Animal Research Facility at the George Washington University (GWU) and as per the GWU Institutional Animal Care and Use Committee regulations (Approval number 15-024).

All cell cultures were established in amphibian serum-free medium supplemented with 10% fetal bovine serum, 0.25% *X. laevis* serum, 10 μg/ml gentamycin (Thermo Fisher Scientific), 100

U/ml penicillin, 100 μg/ml streptomycin (Gibco). Amphibian phosphate buffered saline (APBS) that was used while isolating the cells has been previously described [8].

X. laevis rCSF-1 and rIL-34 were produced using an insect expression system by a previously described methods [8].

Peritoneal macrophage isolation and cytology

Tadpoles (stage NF 54) and adult frogs (1-2 years-old) were injected intraperitoneally with 2.5μg/g body weight of rCSF-1 or rIL-34 (5-6 animals per treatment, *N*=5-6). Three days later (based on our previous results [5, 6]) animals were lavaged with APBS and the isolated Mϕs were enumerated using trypan blue live/dead exclusion. Cells were either used in RNAseq studies or spun onto glass slides using a cytocentrifuge, fixed with 10% neutral buffered formalin (VWR) and stained with Naphthol AS-D Chloroacetate (Specific Esterase; Sigma) or α-Naphthyl Acetate (Non-Specific Esterase; Sigma) according to the manufacturer's instructions.

RNA sequencing and analyses

Towards RNA sequencing analysis, total RNA was isolated from tadpole and frog CSF-1- and IL-34-Mϕs, as described above and submitted to University of Maryland Genomic Resource Center for library preparation, sequencing, and analysis. RNA libraries were prepared by using poly A selection and paired-end sequencing was performed using Illumina. Reads were mapped to the *X. laevis* v9.1 genome (Xenbase, http:// www.xenba se. org/ entry/). The alignments were generated by HISAT2 and Samtools was used to generate alignment statistics. The read counts for each transcript were generated by HTSeq and the reads were either normalized as CPM (count per million reads), or RPKM (Reads Per Kilobase Million).

The raw counts generated by HTSeq were used as input for differential expression analysis using DESeq2 Bioconductor packages. The number of differentially expressed genes for each comparison of interest with significance were defined using a P-adjusted (padj) value cutoff of <=0.05 and a log2 fold change (LFC) ≥ [1]. For each comparison, volcano plots were created to visualize the relationship of the LFC in expression of each gene between the two conditions against the padj value of the gene.

The complete dataset of the differentially expressed genes is provided as supplemental data.

Results and Discussion

Comparison of rCSF-1- and rIL-34-elicited tadpole and adult frog peritoneal macrophages

Akin to their mammalian counterparts, the *X. laevis* CSF-1 and IL-34 are chemo-attractive to Mϕ progenitors and derived populations [5, 6]. In past studies, we exploited this property of the respective growth factors to inject tadpoles [5] and adult *X. laevis* [6] intraperitoneally (ip) with recombinant (r)CSF-1 or rIL-34, thereby accumulating Mϕs into the animal peritonea and differentiating them towards CSF-1- or IL-34-Mϕ biases, respectively. To directly compare tadpole and adult frog CSF-1- and IL-34-Mϕs, in the present study we administered rCSF-1 and rIL-34 (2.5μg/g body weight) into tadpole and adult frog peritonea and three days later isolated the recruited Mϕs by lavage. Notably, while tadpoles possess considerably fewer total leukocytes compared to adult frogs [10], we observed comparable per μl numbers of CSF-1- or IL-34-Mϕs accumulating in tadpole and adult frogs following the respective cytokine administrations (Fig. 1A). Notably, rIL-34 resulted in greater accumulation of tadpole peritoneal Mϕs than elicited by rCSF-1, whereas the opposite effect was seen in adult frogs (Fig. 1A). The mammalian CSF-1 and

IL-34 exhibit distinct interactions with the CSF-1R [11], with the mammalian IL-34 possessing greater affinity for the receptor than CSF-1 [12, 13]. Moreover, IL-34 also ligates receptor protein tyrosine phosphatase-ζ (PTPRζ), which in mammals is expressed by neuronal progenitor cells [14]. While our expression studies indicate that frog macrophages do not express PTPRζ (unpublished observation), possibly the frog IL-34 and/or CSF-1 ligate additional Mϕ/progenitor-expressed receptors and/or have unique interactions with the frog CSF-1R, explaining the observed differences in tadpole and adult frog peritoneal Mϕ accumulation (Fig. 1A) and our previously observed disparate functionalities of these respective Mϕ subsets.

We previously showed that *X. laevis* bone marrow derived CSF-1- and IL-34-Mϕs possess distinct enzymology [15]. Specifically, we found that IL-34-Mϕs possessed greater specific esterase (SE) activity whereas CSF-1-Mϕs had greater non-specific esterase (NSE) staining. We found the same to be true of the tadpole and adult frog CSF-1- and IL-34-Mϕs, wherein both tadpole and adult IL-34-Mϕs had greater SE activity whereas the tadpole and adult CSF-1-Mϕs showed greater NSE staining (Fig. 1B). Across vertebrates, SE activity is associated with granulocyte-lineage cells [16], whereas mononuclear phagocytes are known for their NSE activity [16-18]. Our past studies suggest that amphibian IL-34-Mϕs share many features with granulocytes [19], which is corroborated by the observation that both tadpole and frog IL-34-Mϕs possessed greater SE activity. Moreover, the morphology of tadpole and adult CSF-1-Mϕs more closely resembled 'classical' mammalian Mϕ morphology and perhaps the greater NSE activity of these cells reflects their differentiation towards what has been more traditionally thought of as a Mϕ. Indeed, both tadpole and adult CSF-1-Mϕs tended to be larger and possess more ruffled membranes than the counterpart IL-34-Mϕs (Fig. 1B). Together, these findings indicate that not

143 only do CSF-1 and IL-34 result in morphologically and enzymatically distinct *X. laevis* Mϕ
144 subsets, but that these differences appear to be consistent across developmental stages.
145 Our previous studies suggest that peritonea-derived IL-34- and CSF-1-Mϕs are each likely
146 comprised of heterogenous Mϕ subtypes [6]. While our ongoing work, including the present
147 studies, has helped to delineate differences between amphibian CSF-1- and IL-34-Mϕ subsets, the
148 paucity of *X. laevis*-specific antibodies has prevented us from exploring the possible heterogeneity
149 within each of these Mϕ populations further.
150
151 *Tadpole and frog CSF-1- and IL-34-Mϕs differ in their transcriptional profiles*
152 To get a better sense of the differences between tadpole and adult frog CSF-1 and IL-34-Mϕs,
153 we performed RNA sequencing analyses of these cell subsets (Fig. 2A&B, Fig. S1A&B). Our
154 results indicated that tadpole IL-34-Mϕs are very transcriptionally distinct from tadpole CSF-1-
155 Mϕs (Fig. 2A, Fig. S1A) and that the same was true for adult frog IL-34- and CSF-1-Mϕs (Fig.
156 2B, Fig. S1B).
157 Amongst the highest expressing genes in tadpole CSF-1-Mϕs were the gene encoding the
158 CXCL2 chemokine, which is involved in immunoregulatory and inflammatory processes [20-22],
159 the gene encoding for hepcidin-1 (*hepc*), which is important for Mϕ recycling of iron [23], and the
160 gene encoding secreted protein acidic and cysteine rich (*sparc*), which is involved in tissue
161 remodeling, cell matrix cross-talk [24], (Fig. 2A, Fig. S1A). Conversely, tadpole IL-34-Mϕs
162 possessed higher transcript levels for sialic acid binding Ig like lectin 10 (*siglec10*), which
163 regulates immune cell function during various immune events [25] and may be utilized as a marker
164 of this Mϕ subset in the future; two genes the products of which are involved in DNA repair

(*rrm2b,* [26] and *rad54*, [27]); and a gene involved in Wnt signaling and associated with fibrosis (*sfrp4*, [28]).

While primarily associated with T cells, Mϕs have been shown to produce lytic perforin molecules [29], which is consistent with frog CSF-1-Mϕs possessing greater expression levels of perforin-1 (*prf1*) genes (Fig. 2B, Fig. S1B).

Frog IL-34-Mϕs had greater expression levels of an interleukin-4 induced protein (*il4i1*), which has been shown to dictate macrophage differentiation [30] (Fig. 2B, Fig. S1B). Macrophages have also been shown to respond to interleukin-2 [29], which coincides with adult frog IL-34-Mϕs possessing elevated levels of the IL-2 receptor beta (*il2rb*; Fig. 2B, Fig. S1B).

Our previous studies collectively indicate that both tadpole and adult frog IL-34-Mϕs are functionally distinct from the respective tadpole and frog CSF-1-Mϕs [5, 6, 9], and this is substantiated by the marked transcriptional differences between tadpole IL-34- and CSF-1-Mϕs and between the corresponding frog Mϕ types. Our comparisons of exclusively differentially expressed immune genes between tadpole CSF-1- and IL-34-Mϕs and between frog CSF-1- and IL-34-Mϕs echo these past observations, as both tadpole and frog CSF-1-and IL-34-Mϕs possess distinct expression profiles of putative cell surface markers, antimicrobial genes and genes encoding cytokine and chemokine ligands and receptors (Tables S1 & S2, respectively). Notably, both tadpole and adult frog IL-34-Mϕs also expressed genes typically associated with granulocytes, such as trypsin and chymotrypsin (tadpole IL-34-Mϕs) as well as neutrophil cytosolic factor (frog IL-34-Mϕs; Tables S1 & S2, respectively). This is consistent with our previous findings that frog IL-34-Mϕs share many features with frog granulocytes [15] and our

186 current observations that tadpole and frog IL-34-Mϕs possess robust SE activity (Fig. 1B), which
187 is typically seen in granulocytes [16].
188
189 *Tadpole Mϕ subtypes are transcriptionally different from the frog Mϕ counterparts*
190 To gain insight into possible differences between pre- and post-metamorphic Mϕ subsets, we
191 compared the transcriptomes of tadpole IL-34-Mϕs with frog IL-34-Mϕs and tadpole CSF-1-Mϕs
192 with the frog CSF-1-derived counterparts (Fig. 2C&D, Fig. S1C&D). In a comparison of tadpole
193 and adult frog IL-34-Mϕs, some of the more notable genes broadly expressed in the adult IL-34-
194 Mϕs included major histocompatibility complex (*mhc*) *2a* and *mhc2b*, colony stimulating factor-2
195 receptor alpha (*csf2ra*) and *cxcl8b* (Fig. 2C, Fig. S1C). Conversely, some notable genes expressed
196 at greater levels by tadpole IL-34-Mϕs compared to the adult counterparts include an Fc-receptor
197 like gene-5 (*fcrl5*), macrophage stimulating receptor 1 (*mst1r*) and perforin-1 (*prf1*; Fig. 2C, Fig.
198 S1C).
199 When comparing the tadpole and adult frog CSF-1-Mϕs, the notable genes expressed more
200 broadly in frog CSF-1-Mϕs included *mhc1a*, *mhc2a* and the chimeric chemokine-like receptor 1
201 (*cmklr1*; Fig. 2D, Fig. S1D). By contrast, the notable immune genes expressed at greater levels by
202 tadpole CSF-1-Mϕs included phospholipase a2 (*pla2*) and cathepsin k (*catk*; Fig. 2D, Fig. S1D).
203 Together these analyses support previous reports that *X. laevis* tadpole leukocytes express
204 lower MHC class I and II [31]. The robust differences in the transcriptional profiles of each of the
205 examined Mϕ subtypes suggest that tadpole and adult CSF-1- and IL-34-Mϕs likely have many
206 non-overlapping functional differences.
207 Comparisons of exclusively immune genes that were differentially expressed between tadpole
208 and adult IL-34-Mϕs or between tadpole and frog CSF-1-Mϕs (Tables S3 & S4, respectively),

revealed far longer lists of differentially expressed genes than those seen when comparing tadpole or frog Mϕ subsets (Tables S1 & S2, respectively). This suggests that there are far more immune differences between the same or similar Mϕ subsets from pre- and post-metamorphic animals than there are between the tadpole CSF-1- and IL-34-Mϕs or between frog CSF-1- and IL-34-Mϕs. These differences include much greater numbers of differentially expressed cell surface marker genes, antimicrobial genes as well as genes encoding cytokines/chemokine ligands and receptors (Tables S3 & S4, respectively). Not surprisingly and as described above, frog IL-34- and CSF-1-Mϕs possessed greater expression levels of antigen presentation genes (Tables S3 & S4), corroborating the previously established notion that post-metamorphic frogs have greater antigen presentation capacities [10]. However, it was notable that tadpole IL-34- and CSF-1-Mϕs expressed greater levels of Fc-like receptor genes (Tables S3 & S4), possibly reflecting an as-of-yet undefined link between tadpole Mϕs and their adaptive immune responses.

Concluding remarks

Future studies of tadpole and adult frog Mϕs that consider other variables such as the respective developmental stage physiologies, hematopoiesis strategies and microbiomes, will undoubtedly grant more clarity to why and under what conditions amphibian tadpoles and adult frogs utilize conserved and diverged immune mechanisms. Because Mϕ-lineage cells are so important to vertebrate physiology and immunity, we believe that future studies that contrast Mϕ subsets during different tadpole and adult frog immune and physiological processes will be important to elucidating the biological reasons governing these similarities and differences.

Acknowledgments

MRHH, AY and ZU thank the Wilbur V. Harlan endowment for summer research support. This work was supported by a grant from National Science Foundation-IOS:1749427 to LG. We thank the anonymous reviewers whose helpful comments and suggestions improved the scope of this communication.

References:

1. Grayfer, L. and J. Robert, *Amphibian macrophage development and antiviral defenses.* Dev Comp Immunol, 2016. 58: p. 60-67.
2. Guiducci, C., et al., *Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection.* Cancer Res, 2005. 65(8): p. 3437-46.
3. Saccani, A., et al., *p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance.* Cancer Res, 2006. 66(23): p. 11432-40.
4. Stanley, E.R., et al., *Biology and action of colony--stimulating factor-1.* Mol Reprod Dev, 1997. 46(1): p. 4-10.
5. Grayfer, L. and J. Robert, *Divergent antiviral roles of amphibian (Xenopus laevis) macrophages elicited by colony-stimulating factor-1 and interleukin-34.* J Leukoc Biol, 2014. 96(6): p. 1143-53.
6. Grayfer, L. and J. Robert, *Distinct functional roles of amphibian (Xenopus laevis) colony-stimulating factor-1- and interleukin-34-derived macrophages.* J Leukoc Biol, 2015. 98(4): p. 641-9.
7. Yaparla, A., et al., *Amphibian (Xenopus laevis) macrophages differentiated by colony-stimulating factor-1 and interleukin-34 possess distinct pathogen recognition capacities.* Cytokine, 2018. in review.
8. Yaparla, A., M. Popovic, and L. Grayfer, *Differentiation-dependent antiviral capacities of amphibian (Xenopus laevis) macrophages.* J Biochem, 2018. 293(5): p. 1736-1744.
9. Yaparla, A., M. Popovic, and L. Grayfer, *Differentiation-dependent antiviral capacities of amphibian (Xenopus laevis) macrophages.* J Biol Chem, 2018. 293(5): p. 1736-1744.
10. Robert, J. and Y. Ohta, *Comparative and developmental study of the immune system in Xenopus.* Dev Dyn, 2009. 238(6): p. 1249-70.
11. Nakamichi, Y., N. Udagawa, and N. Takahashi, *IL-34 and CSF-1: similarities and differences.* J Bone Miner Metab, 2013. 31(5): p. 486-95.
12. Bain, C.C., et al., *Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors.* Mucosal Immunol, 2013. 6(3): p. 498-510.
13. Wei, S., et al., *Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells.* J Leukoc Biol, 2010. 88(3): p. 495-505.
14. Nandi, S., et al., *Receptor-type protein-tyrosine phosphatase zeta is a functional receptor for interleukin-34.* J Biol Chem, 2013. 288(30): p. 21972-86.
15. Yaparla, A., et al., *Exploring the relationships between amphibian (Xenopus laevis) myeloid cell subsets.* Dev Comp Immunol, 2020. 113: p. 103798.
16. Rindler, R., et al., *Naphthol AS-D chloroacetate esterases in granule extracts from human neutrophil leukocytes.* Blut, 1971. 23(4): p. 223-7.
17. Neumann, N.F., D.R. Barreda, and M. Belosevic, *Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures.* Fish Shellfish Immunol, 2000. 10(1): p. 1-20.
18. Yourno, J., et al., *Monocyte nonspecific esterase. Enzymologic characterization of a neutral serine esterase associated with myeloid cells.* J Histochem Cytochem, 1986. 34(6): p. 727-33.

19. Yaparla, A., E.S. Wendel, and L. Grayfer, *The unique myelopoiesis strategy of the amphibian Xenopus laevis.* Dev Comp Immunol, 2016. 63: p. 136-43.
20. Belperio, J.A., et al., *CXCR2/CXCR2 ligand biology during lung transplant ischemia-reperfusion injury.* J Immunol, 2005. 175(10): p. 6931-9.
21. Sekido, N., et al., *Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8.* Nature, 1993. 365(6447): p. 654-7.
22. Souza, D.G., et al., *Repertaxin, a novel inhibitor of rat CXCR2 function, inhibits inflammatory responses that follow intestinal ischaemia and reperfusion injury.* Br J Pharmacol, 2004. 143(1): p. 132-42.
23. Sullivan, J.L., *Macrophage iron, hepcidin, and atherosclerotic plaque stability.* Exp Biol Med (Maywood), 2007. 232(8): p. 1014-20.
24. Termine, J.D., et al., *Osteonectin, a bone-specific protein linking mineral to collagen.* Cell, 1981. 26(1 Pt 1): p. 99-105.
25. Macauley, M.S., P.R. Crocker, and J.C. Paulson, *Siglec-mediated regulation of immune cell function in disease.* Nat Rev Immunol, 2014. 14(10): p. 653-66.
26. Iqbal, W., et al., *RRM2B Is Frequently Amplified Across Multiple Tumor Types: Implications for DNA Repair, Cellular Survival, and Cancer Therapy.* Front Genet, 2021. 12: p. 628758.
27. Maranon, D.G., et al., *NUCKS1 promotes RAD54 activity in homologous recombination DNA repair.* J Cell Biol, 2020. 219(10).
28. Tinazzi, I., et al., *SFRP4 Expression Is Linked to Immune-Driven Fibrotic Conditions, Correlates with Skin and Lung Fibrosis in SSc and a Potential EMT Biomarker.* J Clin Med, 2021. 10(24).
29. Li, H., et al., *Macrophage precursor cells produce perforin and perform Yac-1 lytic activity in response to stimulation with interleukin-2.* J Leukoc Biol, 1994. 56(2): p. 117-23.
30. Yue, Y., et al., *IL4I1 Is a Novel Regulator of M2 Macrophage Polarization That Can Inhibit T Cell Activation via L-Tryptophan and Arginine Depletion and IL-10 Production.* PLoS One, 2015. 10(11): p. e0142979.
31. Rollins-Smith, L.A., et al., *Involvement of thyroid hormones in the expression of MHC class I antigens during ontogeny in Xenopus.* Dev Immunol, 1997. 5(2): p. 133-44.

346 **Figure legends**

347 **Figure 1.** Peritonea-derived tadpole and adult frog CSF-1- and IL-34-Mϕs possess disparate
348 enzymology. *X. laevis* tadpole (N.F.=54) and adult frogs (~1 year-old) were intraperitoneally
349 injected with recombinant control (r-ctrl) or 2.5μg of rCSF-1 or rIL-34 and harvested by lavage
350 with 0.5 ml of APBS, 3 days later. The cells were (A) enumerated and (B) cytologically examined
351 following staining with NASDCl-specific esterase (SE; left panel) or α-Naphthyl Acetate (non-
352 specific esterase; NSE; right panel) stains. The results in (A) are means ± SE for cells derived from
353 6 individual animals (*N*=6), per treatment group. The results in (B) are representative of peritoneal
354 cells derived from 6 individual, per treatment group. The letters above head bars indicate statistical
355 groups, with each letter representing a distinct statistical grouping, $p < 0.05$.
356
357 **Figure 2.** Transcriptional differences between tadpole CSF-1- and IL-34-Mϕs, frog CSF-1- and
358 IL-34-Mϕs, tadpole and frog IL-34-Mϕs and tadpole and frog CSF-1-Mϕs. Comparisons of
359 differentially expressed genes between *X. laevis* tadpole and adult frog Mϕ subsets were performed
360 by using DESeq2. The heatmaps depict top 30 significantly differentially expressed genes between
361 (A) tadpole CSF1-Mϕs and IL-34-Mϕs, (B) frog CSF1-Mϕs and IL-34-Mϕs, (C) tadpole and frog
362 IL-34-Mϕs, and (D) tadpole and frog CSF1-Mϕs. Each cell type was derived from 3 individual
363 animals (*N*=3/cell type). Gene names that contain '.s' or '.l' suffixes, indicate transcripts from
364 short or long arms of *X. laevis* chromosomes, respectively. Sequences without annotations were
365 listed as model IDs starting with 'loc' and without significant hits were marked with asterisk (*)
366 sign(s). All heatmaps were visualized using 'pheatmap' package in R (4.0.2 version).
367

Supplemental Figure 1. Transcriptional differences between tadpole CSF-1- and IL-34-Mϕs, frog CSF-1- and IL-34-Mϕs, tadpole and frog IL-34-Mϕs and tadpole and frog CSF-1-Mϕs. The volcano plots show the global transcriptional change between (A) tadpole CSF1-Mϕs and IL-34-Mϕs, (B) frog CSF1-Mϕs and IL-34-Mϕs, (C) tadpole and frog IL-34-Mϕs, and (D) tadpole and frog CSF1-Mϕs. Each cell type was derived from 3 individual animals (*N*=3/cell type). For the volcano plots, the x-axis represents the log2 fold change of each gene and the y-axis represents the -log10 of its adjusted p-value. Genes with a padj value of less than 0.05 and a log2 fold change >1 are the upregulated genes and indicated by red dots while genes with a padj value of less than 0.05 and a log2 fold change <-1 are called downregulated genes and indicated by blue dots. The grey dots represent statistically non-significant genes. Gene names that contain '.s' or '.l' suffixes, indicate transcripts from short or long arms of *X. laevis* chromosomes, respectively.

Figure 1

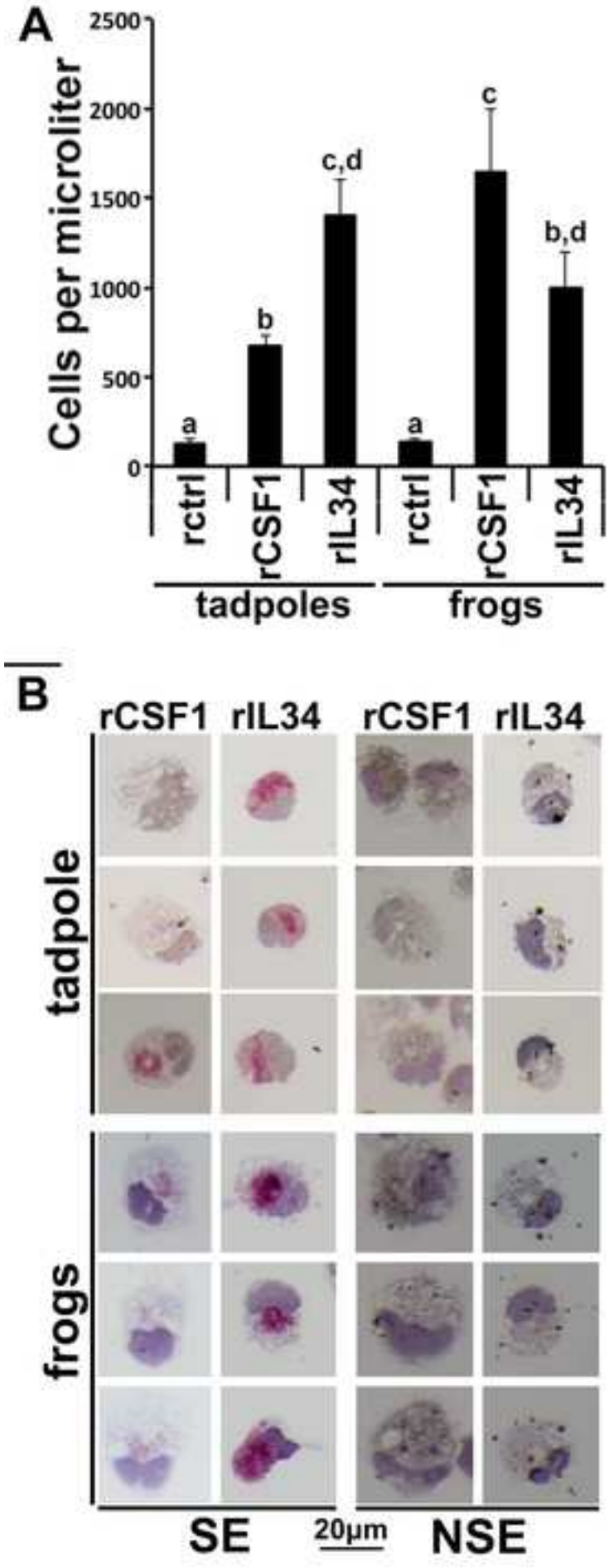

Figure 2

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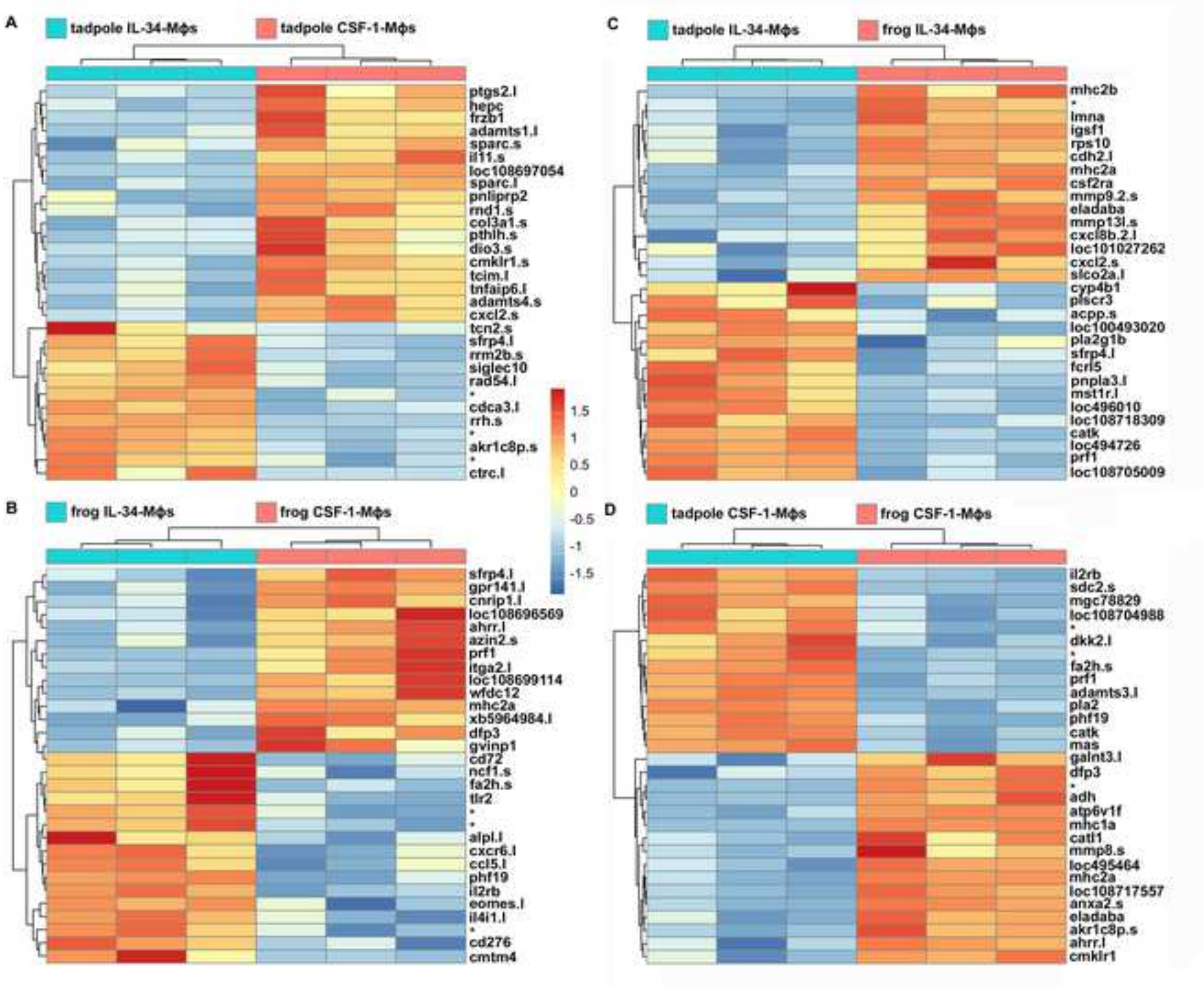

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Supplementary Material

DEGs_frog CSF-1_vs_frog IL-34-Macs.xlsx