Amphibian macrophage development and antiviral defenses

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Abbreviations: Arg-1, arginase-1; CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; CFU, colony forming units; FV3, Frog Virus 3; HSCs, hematopoietic stem cells; IL-34, interleukin-34; M-CSF, macrophage-colony-stimulating factor, NF, Nieuwkoop and Faber; PWM-SCM, pokeweed mitogen-stimulated spleen cell medium; rXl, recombinant *Xenopus laevis*

Abstract

Macrophage lineage cells represent the cornerstone of vertebrate physiology and immune defenses. In turn, comparative studies using non-mammalian animal models have revealed that evolutionarily distinct species have adopted diverse molecular and physiological strategies for controlling macrophage development and functions. Notably, amphibian species present a rich array of physiological and environmental adaptations, not to mention the peculiarity of metamorphosis from larval to adult stages of development, involving drastic transformation and differentiation of multiple new tissues. Thus it is not surprising that different amphibian species and their respective tadpole and adult stages have adopted unique hematopoietic strategies. Accordingly and in order to establish a more comprehensive view of these processes, here we review the hematopoietic and monopoietic strategies observed across amphibians, describe the present understanding of the molecular mechanisms driving amphibian, and in particular *Xenopus laevis* macrophage development and functional polarization, and discuss the roles of macrophage-lineage cells during ranavirus infections.

1. Introduction

It is becoming evident that akin to other vertebrate species, amphibians rely heavily on macrophage-lineage cells not only for immune defense, but also for homeostasis and tissue remodeling/resorption (Haislip et al., 2011; Nishikawa et al., 1998). Whereas committed macrophage precursors of other vertebrates reside within designated hematopoietic sites (Bartelmez et al., 1989; Garceau et al., 2010; Kriegler et al., 1994), amphibian species appear to vary in their respective hematopoietic strategies and do not always harbor macrophage precursors within designated sites used by other blood cells for development. This is particularly interesting when considering that many amphibians possess two distinct developmental stages: a typically aquatic tadpole stage and a more terrestrial adult one, each with distinct physiological and immunological requirements. Although metamorphosis is generally rudimentary, or even cryptic in urodelian species (e.g. salamanders, newts), in anuran species it is a major developmental transition between two distinct immune systems [reviewed in (Flajnik et al., 1987; Robert and Ohta, 2009)]. In addition, the different ecological niches occupied by tadpole and adult stages are presumably populated by different pathogens, thus representing unique immunological pressures. As such, metamorphosis is likely to have a profound influence on macrophage development and biology. Macrophage-lineage cells are of particular relevance when considering the alarming increase in the morbidity and mortality of amphibian populations worldwide caused by ranavirus infections [large DNA viruses of the family *Iridoviridae* (Chinchar, 2002; Chinchar et al., 2009; Williams et al., 2005)]. Indeed, there is increasing evidence implicating amphibian macrophages in persistence, evasion and dissemination of ranaviruses, and possibly differences in the interaction of these pathogens with tadpole and adult

macrophages [discussed below and reviewed in (Chen et al., 2011; Grayfer et al., 2012)]. Thus, it is imperative that we garner greater insights into the ontogeny and functionality of these amphibian innate immune effectors.

The embryonic origins of amphibian hematopoietic precursors have been described in detail elsewhere (Ciau-Uitz et al., 2014; Ciau-Uitz et al., 2010) and will be addressed in passing here. This review addresses the current understanding of amphibian hematopoiesis with a focus on myelopoiesis, and it highlights recent notable findings pertaining to the roles of amphibian macrophages during ranavirus infections.

2. Diversified sites of hematopoiesis in amphibians

Vertebrate blood cell precursors differentiate within designated sites of hematopoiesis. Typically, avian and mammalian committed myeloid-lineage progenitors arise from the bone marrow pluripotent populations (Bartelmez et al., 1989; Garceau et al., 2010; Kriegler et al., 1994), whereas teleost fish utilize the head kidney as their designate site of hematopoiesis (Belosevic et al., 2006; Neumann et al., 2000). In amphibians, the sub-cortical (peripheral) liver is generally considered to be the principal hematopoietic site from early development (Chen and Turpen, 1995; Hadji-Azimi et al., 1987; Hadji-Azimi et al., 1990; Nogawa-Kosaka et al., 2011). However, recent findings, combined with older literature suggest that in fact different amphibian species may actually localize their blood cell development to different organs and tissues (Akulenko, 2012; Brunst, 1958; Carver and Meints, 1977; Durand et al., 2000; Golub et al., 2004; Hadji-Azimi et al., 1987; Hadji-Azimi et al., 1990; Lane and Sheets, 2002). In this regard, it is noteworthy that although the amphibian bone marrow is relatively rudimentary and has been largely overlooked as a potential site of hematopoiesis (Hadji-Azimi et al., 1987; Hadji-Azimi et

al., 1990), it appears that certain amphibian species utilize this site for blood cell development (discussed below).

2.2 *Urodela*

Hematopoiesis in urodela (salamanders and newts) is thought to occur within the liver and spleen of both larvae and adults (Brunst, 1958; Durand et al., 2000; Golub et al., 2004). In contrast, to our knowledge there are currently no published studies implicating the bone marrow of urodeles in blood cell development. Notably, urodelians are invaluable animal models for hematopoiesis research owing to their life-long regenerative capacity, which anurans only possess during a short period before the onset of metamorphosis [reviewed in (Godwin and Rosenthal, 2014)]. Recent work by Lopez *et al.* (2014) has demonstrated that axolotl spleen cells are very good producers of hematopoietic colony stimulating factors and are a major source of pluripotent hematopoietic stem cells (HSCs). Stimulation of axolotl splenic HSCs with the pokeweed mitogen-stimulated spleen cell medium (PWM-SCM) gives rise to a predominant fraction of erythroid-lineage cells as well as mixed mononuclear and polymorphonuclear myeloid progenitor populations (Lopez et al., 2014). The detection of PWM-SCM-responsive colony forming units strongly suggests that hematopoiesis in axolotls is confined to spleen and liver but absent from bone marrow, thymus, and kidney tissues (Lopez et al., 2014). This is further underlined by the observation that GFP⁺ spleen- and liver-derived hematopoietic cells, adoptively transferred into γ -irradiated albino axolotl recipients differentiate only within the liver and spleen tissues (Lopez et al., 2014). Furthermore, when two axolotls are connected by probiosis, thus sharing their circulating HSCs, GFP⁺ cells from one animal home into and populate the liver and spleen of the other animal. Strikingly, irradiated but not extraneously

reconstituted animals suffer severe anemia, confirming that the liver and spleen indeed serve as sources of hematopoietic precursors and sites of axolotl blood-cell development (Lopez et al., 2014). Although these investigations do not completely rule out the possibility that other axolotl tissues may be contributing to hematopoiesis, they strongly suggest that the reconstituted liver and spleen cell populations are sufficient to circumvent cell depletion resulting from γ -irradiation.

2.3 *Anura*

The anuran tadpole and adult life stages exhibit distinct physiology and ecological niches that likely include exposure to different potential pathogens. As such it is not surprising that the blood cell development of these life stages is also distinct.

2.3.1 *Tadpole hematopoiesis*

In *Xenopus*, the embryonic anterior blood island (equivalent to the mammalian yolk sac) that forms soon after neurulation (NF stage 20, 22-23 hours post-fertilization) serves as the initial source of myeloid cells, whereas erythroid and lymphoid blood cell lineages originate from the adjacent posterior ventral blood island (Ciau-Uitz et al., 2000; Kau and Turpen, 1983; Maeno et al., 1985). At this early developmental stage (NF stage 30), primitive myeloid cells called ‘myelocytes’ comprise the initial blood cells to differentiate within the *Xenopus* embryo and can be seen migrating throughout the developing organism (Costa et al., 2008). Even before development of functional vasculature, these myelocytes are readily recruited to sites of injury or pathogenic challenge, possibly serving as transient innate immune effector cells. The long-term

functional roles, modes of action, and ultimate fates of myelocytes during later animal life remain to be defined.

Early on during *Xenopus* larval development (NF stage 40-46) and subsequent to the establishment of blood circulation, the liver becomes the primary hematopoietic site, responsible for the formation of erythrocytes, leukocytes and B cells (Chen and Turpen, 1995). At this early developmental stage, when the spleen is still absent, expression of a fluorescence marker under the control of cell type-specific promoters in transgenic animals has revealed that these larvae possess complex and diverse populations of myeloid cells including granulocytic and monocytic lineages (Paredes et al., 2015).

Based on *Xenopus* studies, the sub-cortical (peripheral) liver is generally considered to be the principal anuran hematopoietic site from early development (Chen and Turpen, 1995; Nogawa-Kosaka et al., 2011). However, hematopoiesis in tadpoles of the European common frog, *Rana temporaria*, appears to be restricted to pronephric interstices of kidneys, and does not occur in liver at all (Meseguer et al., 1985). Similarly, tadpoles of the edible common frog *Rana esculenta* employ their trunk pronephrous tissue for granulopoiesis (Frank, 1988; Frank, 1989). Interestingly, during embryogenesis and early larval development of the related leopard frog, *Rana pipiens*, the pronephrous tissue is also involved in hematopoiesis, although myeloid, lymphoid and erythroid lineages are also generated in the liver (Carpenter and Turpen, 1979). More specifically, precursors of these lineages are localized in the endothelia-lined sinusoids found within the sub-capsular liver as well as deeper within the hepatic tissues (Turpen et al., 1979). The respective contribution of kidney and liver to *R. pipiens* hematopoiesis is presently not clear. It is also notable that, as described below, the bone marrow of post-metamorphic *Rana* spp. may serve as an important site of hematopoiesis (Carver and Meints, 1977). We believe that

it would be useful to reexamine some of this older work, using more advanced molecular techniques to delineate the exact contributions of respective *Rana spp.* tissues to the development of distinct blood cell lineages at distinct developmental stages.

2.3.2 Adult hematopoiesis

Since urodelians appear to lack bone marrow-mediated hematopoiesis, anurans may represent the first vertebrate phylogenetic group to involve bone marrow in blood cell development. Immunohistological studies of the adult American bullfrog (*Rana catesbeianus*) have revealed the presence of active hematopoiesis within the vertebrae, femur and finger bone marrow as well as in their kidneys, but not in spleen, liver, gastrointestinal system, lungs tegument and heart tissues (de Abreu Manso et al., 2009). Unlike salamanders, there is no evidence of hematopoietic activity within the spleen and liver tissues of this species. Moreover, bone marrow and kidney HSCs of *L. catesbeianus* serve as precursors to heterophils, basophils, eosinophils, monocytes, erythrocytes and lymphocytes (de Abreu Manso et al., 2009). These observations are interesting since they indicate that the bullfrogs hematopoiesis takes place not only in the kidney, as in some anuran tadpoles (discussed above) and bony fish, but also in the bone marrow, similar to endothermic vertebrates. Other variations on the hematopoietic sites across anuran species are exemplified by the marsh frog *Pelophylax ridibundus*, whose hematopoiesis occurs in the bone marrow as in the bullfrog, but unlike the bullfrog also in the liver (Akulenko, 2012). As an additional layer of complexity to the already poorly understood process of anuran hematopoiesis, the *P. ridibundus* bone marrow and the liver erythropoiesis and myelopoiesis appear to be subject to seasonal variations (Akulenko, 2012).

It will be interesting to delineate the respective contribution of these anuran hematopoietic sites to steady state as well as immunologically and physiologically elicited blood cell development.

3. Monopoiesis in anura

3.1 The roles of anuran bone marrow

With respect to monoopoiesis, or the development of macrophage lineage cells, it is noteworthy that in mammals, surface expression of the colony stimulating factor-1 receptor (CSF-1R) on progenitor cell populations is considered a hallmark of commitment to the macrophage lineage (Tagoh et al., 2002). The transcripts and protein levels of the CSF-1R increase to detectable levels from early precursor stages (detected as macrophage colony forming units) through macrophage development and maturation (Bonifer and Hume, 2008; Kryszinska et al., 2007; Rosa et al., 2007). Before this commitment, CSF-1 stimulation is insufficient to drive HSCs down the macrophage developmental pathway (Bartelmez et al., 1989; Kriegler et al., 1994), whereas commitment to the mononuclear phagocyte lineage and the coordinated CSF-1R expression renders these progenitors responsive to CSF-1 stimulation and further macrophage-lineage specific differentiation (Cecchini et al., 1994; Stanley et al., 1978).

Interestingly, although the sub-cortical liver clearly functions as the primary site of hematopoiesis in adult *X. laevis* (Hadji-Azimi et al., 1987; Hadji-Azimi et al., 1990; Lane and Sheets, 2002), our recent findings strongly suggest that committed macrophage precursor populations are located within the bone marrow (Grayfer and Robert, 2013). This is evidenced by the ability of a recombinant *X. laevis* CSF-1 (rX/CSF-1) to stimulate bone marrow cells to proliferate, to form colonies, and to differentiate into cells bearing hallmark macrophage

morphology and intense CSF-1R gene expression. By contrast, at least under our experimental conditions, rX/CSF-1 treatment of *X. laevis* sub-capsular liver-derived cells does not result in any observable changes. Furthermore, whereas a distinct bone marrow cell population exhibits rX/CSF-1 binding, cells derived from the adult *X. laevis* peripheral liver fail to bind to this monopoietic growth factor.

Interestingly, upon infection with the ranavirus Frog Virus 3 (FV3, discussed in detail in section 4), *X. laevis* adults exhibit increased CSF-1R gene expression in kidneys, which is consistent with the recruitment of mononuclear phagocytes (bearing the CSF-1R) to this site of prominent FV3 replication (Grayfer et al., 2015). Moreover, while FV3-infected *X. laevis* tadpoles increase their splenic and hepatic CSF-1R gene expression, the FV3-challenged adult frogs do not (Grayfer et al., 2015), suggesting that these tadpole and adult organs have distinct roles in monopoiesis. Indeed, although FV3 does not substantially disseminate into the bone marrow (Grayfer and Robert, 2013), CSF-1R gene expression significantly increases within this site during infection (Grayfer et al., 2015). This is consistent with an active involvement bone marrow in *X. laevis* monopoiesis. The robust upregulation of CSF-1R mRNA levels in the bone marrow, induced by stimulation with heat-killed *E. coli* further substantiates this possibility (Grayfer et al., 2015). Based on the literature and our recent studies, we predict that several adult *X. laevis* organs including the spleen, liver and bone marrow are involved in monopoiesis. We propose that the final steps of these processes are restricted to the bone marrow, while the sub-capsular liver and/or spleen tissues supply the pre-committed HSCs that give rise to definitive myeloid populations.

It is intriguing that the development of other adult *X. laevis* immune cells, such as B lymphocytes, occurs in the spleen and liver tissues but not within the bone marrow (Hadji-Azimi

et al., 1990; Marr et al. 2007). This implies that, for presently unknown physiological reasons, the final steps of *X. laevis* monoopoiesis are segregated into the bone marrow and away from the development of other blood cell subsets. It is particularly notable that more terrestrial anuran amphibian species, such as those belonging to the genus *Rana*, utilize the bone marrow for erythropoiesis (Carver and Meints, 1977), whereas the *Xenopus* liver periphery is utilized towards this end (Nogawa-Kosaka et al., 2011). Presumably, involvement of the bone marrow in blood cell development represents a co-evolutionary adaptation, paralleled with the progressive adaptation towards terrestrial life and marked by a move from hepatic to bone marrow-mediated hematopoiesis. In this regard, it is notable that the *X. laevis* bone marrow is devoid of a central venous system and must rely on sub-endothelial veins to drain this site (Tanaka, 1976). By contrast, the femoral venous systems of terrestrial anurans, such as that of the American bullfrog (*Rana catesbiana*), possess significantly more developed sub-endothelial venous plexus, which are connected to primary arteries within the bone marrow. This vascularization is reminiscent of that seen in the mammalian bone marrow and coincides with more pronounced bone marrow-mediated hematopoietic activity (Tanaka, 1976). This suggests that from a phylogenetic perspective, the increased roles of bone marrow in vertebrate hematopoiesis are perhaps more dependent on the development of effective marrow vascularization rather than the formation of marrow fat. This is intuitive, considering that more developed vasculature would further facilitate hormonal intercommunication of any hematopoietic bone marrow with the rest of the organism and would be more amicable to HSC egress from this site.

The notion that the evolution of sinusoidal architecture lends to more advanced hematopoiesis can be extended to hepatic hematopoiesis as well. Fascinatingly, a comparative histological study involving 46 amphibian species revealed that urodelians and anurans exhibit

progressively more developed hepatic vascularization combined with more intricate hematopoietic sites within the connective tissues and in the perihepatic subcapsular liver (Akiyoshi and Inoue, 2012). Notably, while the more aquatic anuran species of the *Bombina* and *Xenopus* genera exhibit hepatic hemaopoietic tissue surrounding this vascular architecture, other, more terrestrial anuran species do not appear to possess hepatic hematopoietic tissues (Akiyoshi and Inoue, 2012). Whether the above reflects amphibian physiological adaptations and/or phylogenetic origins (i.e., the *Xenopus*, *Bombina* and *Rana* belong to very distinct phylogenetic clades), the association between developed tissue vascularization and hematopoiesis in the liver or the bone marrow merits further investigation.

3.2 Evolution of colony stimulating factor-1

Monopoiesis is controlled through binding of the principal macrophage growth factor, colony-stimulating factor-1 (CSF-1, macrophage-colony-stimulating factor, M-CSF; Garceau et al., 2010; Hanington et al., 2007; Pixley and Stanley, 2004; Wang et al., 2008) to its cognate receptor (CSF-1R; Dai et al., 2002), which is almost exclusively expressed on committed macrophage precursors and derivative phagocyte populations (Guilbert and Stanley, 1980; Lichanska et al., 1999). It is noteworthy that birds and mammals possess a single, alternatively spliced mRNA transcript, giving rise to membrane-bound and secretory forms of CSF-1 (Manos, 1988; Rettenmier and Roussel, 1988). By contrast, teleost fish possess two distinct CSF-1 genes that do not appear to be alternatively spliced (Wang et al., 2008). It is presently unknown whether the distinct fish molecules encoded by these two CSF-1 genes have assumed the respective biological roles conferred by the mammalian CSF-1 splice variants.

In mammals, CSF-1R gene expression increases progressively during mononuclear phagocyte development (Stanley et al., 1997), while the ligation of CSF-1R by CSF1 is critical to macrophage biology and immunity (Bober et al., 1995; Karbassi et al., 1987; Munn and Cheung, 1995; Sweet and Hume, 2003). Thus, CSF-1R gene expression serves as a reliable marker in the study of macrophage development and biology.

Although, the respective CSF-1R genes of diverged vertebrate species share poor sequence identities (especially in the extracellular domains), all the gene products, including those of *X. laevis* possess hallmark CSF-1R features including 5 putative immunoglobulin domains, structurally conserved cysteine residues and a disrupted tyrosine kinase domain (Grayfer et al., 2015). Notably, the intracellular catalytic tyrosine kinase domains of these respective CSF-1R proteins are remarkably conserved, presumably owing to the evolutionary pressure to retain signaling molecule binding sites, which CSF-1R shares with many other intracellular pathways. In contrast, the extracellular portions of CSF-1R molecules have significantly diverged with evolutionary time. This likely reflects selective pressure to facilitate the binding of respective cognate CSF-1 ligands, which also display low amino acid sequence conservation. Notably, the fish, amphibian, avian, reptilian and mammalian CSF-1Rs all phylogenetically branch in respectively separate clades. As many aspects of macrophage biology appear to be distinct across divergent species, it may well prove that the distinct CSF-1 ligand and receptor systems contribute to these differences.

Inspection of the fully sequenced genome of *X. laevis* and *X. tropicalis* has revealed that unlike fish, there is only one CSF-1 gene per *Xenopus* genome (Grayfer and Robert, 2013; Grayfer et al., 2015). This difference is further supported by analysis of vertebrate CSF-1 gene synteny. In zebrafish, the two CSF-1 genes are located on distinct chromosomes, each flanked by

syntenic genes, which are also found within the locus of the single mammalian CSF-1 gene and within a single *X. tropicalis* gene scaffold (Grayfer and Robert, 2013). However, in contrast with mammals, no CSF-1 transcript splice variants could be identified in *X. laevis*. All attempts, including conventional and RACE-PCR on cDNA templates derived from healthy as well as immunologically challenged tadpole and adult *X. laevis* detected only single transcripts (Grayfer and Robert, 2013). It is notable that the N-terminal 150 residues of the mammalian CSF-1 proteins, which are retained irrespective of splice variation, are sufficient to confer the biological activity mediated by this molecule (Koths, 1997; Taylor et al., 1994). Since it appears that *Xenopus* have adopted an unusual monopoietic strategy compared to other vertebrate species (i.e. macrophage developing in the bone marrow, away from general hepatic hematopoiesis), it is possible that the presence of a single CSF-1 gene, not subject to alternative splicing is also a *Xenopus* peculiarity. As a representative amphibian species, *X. laevis* holds an evolutionarily intermediate position between teleosts, which possess two un-spliced CSF-1s and mammals, encoding a single spliced CSF-1 molecule. Thus, it is conceivable that amphibians have adopted some hybrid of these two respective CSF-1 producing strategies. This notion can be resolved by the characterization of CSF-1 genes in other amphibians, especially terrestrial frogs such as *Rana* spp..

3.3 Interleukin-34 as possible sources of macrophage functional heterogeneity

The functional heterogeneity of macrophage-lineage cells is presently defined by two polarization states:. The classically activated/inflammatory (M1) macrophages produce multiple proinflammatory mediators and participate in an array of antimicrobial responses; whereas alternatively activated/regenerative (M2) macrophages produce immunosuppressive and

angiogenic compounds and facilitate the resolution of inflammatory sites [reviewed in (Zhou et al., 2013)]. It should be emphasized that these M1/M2 functional subsets represent opposite boundaries of a continuum of possible functional states. Notably, the mammalian CSF-1 contributes to the this functional heterogeneity of macrophage-lineage cells by polarizing macrophages towards the M2 phenotype (Hamilton et al., 2014), whereas the teleost fish CSF-1 appears to skew macrophages towards an M1-like state [reviewed in (Hodgkinson et al., 2015)]. Intriguingly, the interleukin-34 (IL-34) cytokine, which bears no sequence identity with CSF-1, also binds to, and activates with CSF-1R in mammals, thereby contributing to monopoiesis (Chihara et al., 2010; Liu et al., 2014; Ma et al., 2012; Droin and Solary, 2010; Lin et al., 2008; Wei et al., 2010). IL-34 appears to be crucial to the development and regulation of mammalian osteoclasts (Baud'huin et al., 2010; Chen et al., 2011), microglia (Greter et al., 2012), Langerhans cells (Greter et al., 2012; Wang and Colonna, 2014), and B cell-stimulating myeloid cells (Yamane et al., 2014). We speculate that since CSF-1 serves as one facet of macrophage functional polarization (Hamilton et al., 2014), IL-34 may additionally increase the functional spectrum of mononuclear phagocytes.

3.4 X. laevis tadpole colony-stimulating factor-1- and interleukin-34-derived macrophages

The identification of CSF-1 and IL-34 genes in *Xenopus* has provided us with an opportunity to investigate the involvement of these cytokines in promoting macrophage functional heterogeneity in a non-mammalian species. The respective role of CSF-1 and IL-34 was examined in *X. laevis* by producing recombinant forms of these proteins (rX/CSF-1, rX/IL-34) and assessing their roles in tadpole monopoiesis (Grayfer and Robert, 2014). We found that peritoneal macrophages derived by intraperitoneal injection of rX/CSF-1 possess significantly

greater phagocytic capacities than those derived with rX/IL-34. The peritoneal macrophages elicited with rX/CSF-1 are also more susceptible to infection with FV3 than cells elicited with rX/IL-34 (Grayfer and Robert, 2014). Indeed, despite being similarly permissive to viral entry during *in vitro* infection, rX/IL-34-derived macrophages are more potent in preventing viral replication than rX/CSF-1-derived phagocytes (Grayfer and Robert, 2014). Moreover, when *X. laevis* tadpoles are administered with rX/CSF-1 prior to FV3 challenge, they more rapidly succumb to FV3 infections (Grayfer and Robert, 2014). In contrast, pre-stimulation of tadpoles with rX/IL-34 prior to FV3 infection significantly extends animal survival times (Grayfer et al., 2014). Presumably, the administration of rX/CSF-1 expands and polarizes a subset of tadpole macrophages, which in turn enhances tadpole susceptibility to this pathogen. Conversely, rX/IL-34 enriches tadpoles for macrophage population(s) that are resistant to FV3, as observed *in vitro*. Consistent with this notion, tadpole macrophages derived with rX/IL-34, but not those derived with rX/CSF-1, express higher levels of a type I interferon gene, which is critical for *X. laevis* anti-ranaviral defenses (Grayfer et al., 2014).

Considering the importance of *Xenopus* IL-34 in the polarization of potent anti-FV3 macrophages, it is intriguing that FV3 infection does not trigger IL-34 gene expression in tadpole kidneys (the main site of FV3 replication), whereas this pathogen upregulates CSF-1 gene expression at this site.. This suggests that tadpole susceptibility to FV3 may in part stem from the absence of, or inadequate recruitment of this important innate immune cell population to the active sites of infection within their kidneys.

3.5 *X. laevis* adult colony-stimulating factor-1- and interleukin-34-derived macrophages

As described above, CSF-1 contributes to macrophage heterogeneity, while the roles of IL-34 remain to be fully defined. To extend upon our IL-34/CSF-1 studies in tadpoles and in an effort to delineate possible roles of IL-34 in ectothermic vertebrates, we characterized adult *X. laevis* bone marrow- and peritoneum-derived rX/CSF-1 and rX/IL-34 macrophages. As in tadpoles, these cytokines promote morphologically and functionally distinct adult *X. laevis* macrophage populations (Grayfer and Robert, 2015). Adult bone marrow and peritoneal macrophages derived by rX/CSF-1 stimulation are more phagocytic and express greater levels of the iNOS gene, which is reflected in substantially more robust nitric oxide production by these cells. In contrast, adult *X. laevis* rX/IL-34-derived peritoneal macrophages exhibit greater transcript levels of arginase-1 as well as the NADPH oxidase catalytic subunits, p67^{phox} and gp91^{phox}, which is consistent with the greater respiratory burst capacities of these cells. Moreover, akin to tadpole macrophages, adult *X. laevis* rX/CSF-1 macrophages are highly susceptible to FV3, whereas the rX/IL-34 macrophages possess potent anti-FV3 activity. Interestingly, the adult *X. laevis* rX/IL-34 macrophages do not express substantially greater type I interferon levels, whereas their anti-FV3 activity appears to be dependent of their robust reactive oxygen responses.

Considering our findings regarding the dichotomous roles of frog CSF-1 and IL-34 cytokines, it is interesting that the binding sites of the mammalian IL-34 and CSF-1 on CSF-1R are distinct and result in unique ligand-receptor conformations and downstream signaling events (Chihara et al., 2010; Liu et al., 2014; Ma et al., 2012). It is equally noteworthy that these cytokines exhibit differential tissue expression across a range of vertebrate species (Chemel et al., 2012; Eda et al., 2011; Grayfer and Robert, 2014; Greter et al., 2012; Hwang et al., 2012;

Wang et al., 2013; Wei et al., 2010). All these findings are consistent with CSF-1 and IL-34 promoting disparate macrophage functions in *X. laevis* studies.

In mammals, M1 inflammatory macrophages are characterized by their elevated capacities to undergo respiratory burst and nitric oxide antimicrobial responses; whereas the M2 polarized phagocytes are characterized by their elevated arginase activity (Gordon and Martinez, 2010; Gordon and Taylor, 2005; Mantovani et al., 2002; Mills, 2012). Since macrophage polarization is highly dynamic and reversible (Guiducci et al., 2005; Saccani et al., 2006), these somewhat rigidly defined criteria undoubtedly represent extremes of an as-of-yet poorly defined spectrum of macrophage functional plasticity. It is thus compelling that the same cell-signaling pathways often culminate in M1 and M2 macrophage types [reviewed by (Wang et al., 2014)]. Although not easily categorized according to the M1/M2 classification, *X. laevis* CSF-1- and IL-34-derived macrophages have distinct transcriptional profiles and antimicrobial capacities. For example, enhanced arginase-1 or arginase-2 activity antagonizes the pro-inflammatory nitric oxide response and serves as a marker of M2 alternatively polarized macrophages in mammals and fish, respectively (Joerink et al., 2006a; Joerink et al., 2006b). Intriguingly, the *X. laevis* IL-34-derived macrophages possess elevated arginase-1 gene expression and yet also exhibit potent respiratory burst responses, typical of mammalian M1 macrophages. It is notable that IL-34 is found across all vertebrates (Garceau et al., 2010; Gow et al., 2012; Gow et al., 2013; Wang et al., 2013). In fact, this cytokine may even predate CSF-1, considering the presence of IL-34 and absence of CSF-1 in the elasmobranch genome (Venkatesh et al., 2014). We believe that further investigation into the respective roles of CSF-1 and IL-34 in non-mammalian vertebrate macrophage monopoiesis will enrich and redefine our present understanding of the evolutionary basis and functional heterogeneity seen across vertebrate macrophage populations.

4. Roles of amphibian macrophages during ranavirus infections

Ranaviruses (family *Iridoviridae*) are large double stranded DNA viruses that have become major amphibian pathogens (Chinchar, 2002; Chinchar et al., 2009; Williams et al., 2005). Extensive population die-offs caused by these infectious agents are thought to significantly contribute to the decline of amphibian species worldwide [reviewed in Chinchar, 2002; Grayfer et al., 2015]. Increasing evidence suggests that amphibian macrophage-lineage cells are critical to anti-ranaviral immunity as well as to the infection and dissemination strategies. In particular, recent findings indicate that the ranavirus Frog Virus 3 (FV3) infects, persists and disseminates by infiltrating frog mononuclear phagocytes (Morales et al., 2010; Robert et al., 2007) and that distinct macrophage-lineages are involved in viral clearance (Andino et al., 2012; Grayfer and Robert, 2014; Morales et al., 2010).

4.1 Amphibian macrophage vectors of ranaviral persistence and dissemination

FV3 infection of *X. laevis* provides an excellent experimental platform for studying the ranavirus-amphibian host immune interactions. Notably, converging lines of evidence from our past and current work suggest multiple important roles for *X. laevis* macrophages during FV3 infections. For example, FV3 becomes quiescent within *X. laevis* adult macrophages and is detectable up to several months subsequent to the clearance of the primary infection (Robert et al., 2007). This is of relevance considering that terminally differentiated long-lived macrophages represent an excellent outlet for pathogen persistence and dissemination.

Indeed, this notion has been by-and-large empirically supported, whereby transmission electron microscope analysis of FV3-infected *X. laevis* peritoneal leukocytes, unequivocally

revealed icosahedral viral particles in peritoneal leukocytes bearing macrophage morphology (Morales et al., 2010). These FV3-infected phagocytes exhibit accumulated pools of assembled viral particles, suggesting that frog macrophages serve as FV3 reservoirs, likely towards dissemination and reminiscent of the HIV-macrophage relationship, wherein large pools of formed viral particles accumulate intracellularly, but are not released until the infected macrophages come into contact with T cells, which are the primary cellular HIV targets (Coiras et al., 2009; Goodenow et al., 2003; Gousset et al., 2008; Groot et al., 2008).

Intraperitoneal inoculation of adult *X. laevis* with FV3 results in leukocyte recruitment, with macrophage-like cells comprising a large proportion of these cells (Morales et al., 2010). These phagocytes express both hallmark macrophage inflammatory genes (TNF α , IL-1 β and Arginase-1; Morales et al., 2010) as well as the reliable macrophage-lineage marker, CSF-1R (Grayfer and Robert, unpublished observations). Interestingly, while the FV3 genome is detectable within these peritoneal phagocytes for up to 21 days post infection, the virus appears to become predominantly transcriptionally silent as FV3 early and late genes are no longer detectable at later infection times (Morales et al., 2010). Presumably, the viral genome is quiescently maintained within frog phagocytes and may be transcriptionally reactivated, pending appropriate physiological cues. Furthermore, we have recently found that *in vitro* FV3-infected *X. laevis* macrophages may harbor transcriptionally quiescent, infectious virus for as long as several months (Grayfer and Robert, unpublished observations).

4.2 Reactivation of macrophage-residing quiescent Frog Virus 3

With infrequent exceptions, *X. laevis* peritoneal phagocytes typically do not possess detectable FV3 DNA or transcripts one month after viral infection (Robert et al., 2014). By

contrast, FV3 genomic DNA and active viral gene expression are detected in as much as sixty-seven percent of previously infected frogs subsequent to their inflammatory stimulation by ip administration of heat-killed *E. coli* (Robert et al., 2014). Moreover, immunofluorescence microscopy using Abs against an FV3 gene product required for FV3 replication (53R) and a known *X. laevis* macrophage marker [HAM56; (Nishikawa et al., 1998)] indicates that indeed these reactivated peritoneal populations consist of macrophage-lineage cells bearing productive FV3 replication (Robert et al., 2014).

As evidenced in the above findings and described in greater detail above, anurans possess macrophage populations that are highly susceptible to FV3 (CSF-1-derived) as well as mononuclear phagocyte subsets that are important towards the clearance of this pathogen (IL-34-derived).

5. Concluding remarks

It is evident from the literature described above that much remains to be elucidated regarding the monopoietic strategies of amphibian species and the roles of distinct macrophage populations during amphibian immune responses. In the face of the global amphibian decline, it is imperative to more fully examine the disparate hematopoietic/monopoietic strategies in phylogenetically and physiologically divergent amphibian species. We believe that this will bring us closer to understanding why there is such variability in susceptibility/resistance to pathogens like ranaviruses across amphibian species. Likewise, a better characterization of differences in the antiviral efficacies of macrophage populations between susceptible and resistant amphibian hosts, including the proportions of CSF-1 and IL-34-derived phagocytes,

should decipher the respective contributions of these effector cell populations to anti-ranaviral defenses.

To date research into amphibian immunity mainly represents work performed in few key animal models (e.g., *Xenopus*, axolotl). We hope that through this review we have successfully conveyed the breadth of distinct hematopoietic and immunological strategies seen in different amphibian species. It is our conviction that study of macrophage function should be extended to non-model amphibian species in order to close the gap in our understanding of the complex roles of macrophage in immune defenses against ranaviruses.

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