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Biology of amphibian granulocytes - From evolutionary pressures to functional consequences

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ABSTRACT

Granulocyte-lineage cells are important innate immune effectors across all vertebrates. Named for conspicuous secretory granules, granulocytes have historically been studied for their antimicrobial roles. Although versions of these cells are found in all vertebrate species examined to date, disparate environmental and physiological pressures acting on distinct vertebrate classes have shaped many of the facets dictating granulocyte biology. Immune pressures further determine granulopoietic constraints, ultimately governing granulocyte functions. For amphibians that inhabit pathogen-rich aquatic environments for some or all their lives, their unique granulocyte biologies satisfy many of their antimicrobial needs. Amphibians also occupy an intermediate position in the evolution of vertebrate immune systems, using combinations of primitive (*e.g.,* subcapsular liver) and more recently evolved (*e.g.,* bone marrow) tissue sites for hematopoiesis and specifically, granulopoiesis. The last decade of research has revealed vertebrate granulocytes in general, and amphibian granulocytes in particular, are more complex than originally assumed. With dynamic leukocyte phenotypes, granulocyte-lineage cells are being acknowledged for their multifaceted roles beyond immunity in other physiological processes. Here we provide an overview of granulopoiesis in amphibians, highlight key differences in these processes compared to higher vertebrates, and identify open questions.

1. Introduction

A class of immune cells collectively known as granulocytes, which have potent antimicrobial activities, are integral to the immune defenses of all vertebrates (Owen et al., 2013). As their name suggests, granulocytes contain several types of granules with myriad bioactive molecules, allowing for the specialized functions of the four classical granulocyte subtypes: neutrophils, eosinophils, basophils, and mast cells (MCs). These myeloid-lineage cells include both tissue-resident and circulating populations that readily infiltrate damaged or infected tissues. In higher vertebrates, neutrophils, basophils, and eosinophils are recruited to tissue during heightened immune responses, while MCs are maintained within healthy tissue (Owen et al., 2013). Recent research has yielded insight into granulocyte immune roles, non-immune roles, and phenotypic complexities that continue to energize interest in this field (Groeneweg and Hidalgo, 2020). Ultimately, granulocyte development intrinsically underlies functionality, and granulopoiesis is a tightly regulated process involving diverse growth factors, cytokines, hematopoietic niches, and cellular interactions (Lee et al., 2020; Schulz et al., 2021; Zhang et al., 2019; Tanaka, 1976).

A comprehensive understanding of amphibian granulocyte ontogeny and their functional capacities is necessary to answer biological questions that have far-reaching implications. For example, global amphibian declines are compounded by emerging pathogens like chytrid fungi and ranaviruses, both of which subvert and overcome the immune systems of their hosts. By extension, the stability of the delicate, interdependent ecosystems occupied by amphibians are also being threatened (Fortin et al., 2005; Cortéz-Gómez et al., 2015). In defining the precise roles of amphibian immune strategies mediated by granulocytes, we gain much-needed insight into the defense successes and pitfalls against emerging pathogens. As non-classical research models, amphibians offer unique perspectives on vertebrate immune evolution. By studying amphibian immunity, we cultivate appreciation for the evolutionarily conserved immune processes and those that have diverged to meet the unique physiological needs of amphibians (Boehm, 2012). Furthermore, identifying diverged immune strategies offers alternative approaches to address the limitations of our own immune systems. Here, we review current knowledge regarding amphibian

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granulopoiesis and granulocyte biology. We also highlight outstanding gaps in our understanding of amphibian granulocyte development and where future research efforts might be best directed.

2. Tissue sites of amphibian granulopoiesis

Tissue locations of granulopoiesis vary extensively across amphibian species, life stages, and even seasons (Jordan and Speidel, 1923) (principal locations summarized in Fig. 1A). Because the switch from larval to adult stages in most amphibians is marked by metamorphosis, during which striking development and tissue reorganization take place, it is unsurprising that the locations and the mechanisms of granulocyte development sometimes shift as well. Similarly, these animals undergo substantial physiological alterations with seasonal changes, taking advantage of favorable environmental circumstances and minimizing deleterious ones (*e.g.,* spawning habitats, food availability) (Jordan, 1919). Such changes have likewise been shown to alter granulopoietic tissues (Jordan and Speidel, 1923). The kidney, liver and/or bone marrow may participate in amphibian granulocyte development.

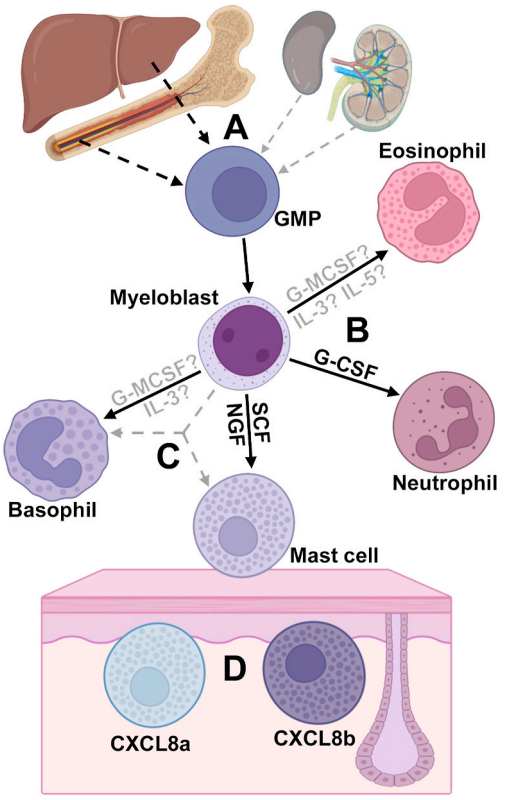

Fig. 1. Tissue sources and fates of granulocyte precursors differ across amphibian species and developmental stages. (**A**) Granulocyte Macrophage Precursors (GMPs) are thought to arise primarily from urodele and caecilian liver tissues. Spleens serve as additional sources of myeloid progenitors. GMPs are found in the pronephric tissue and/or the subcapsular (peripheral) liver throughout life of some anuran amphibians and are thought to home to the bone marrow of most post-metamorphic anuran amphibians. The kidneys of some anuran species may also serve as granulopoietic reserves following hibernation. (**B**) GMPs give rise to myeloblasts which may be differentiated into neutrophils with G-CSF or into eosinophils, which in mammals are produced by GM-CSF, IL-3, and IL-5. GM-CSF, IL-3 and IL-5 have not yet been identified in amphibians. (**C**) In mammals, myeloblasts may also be differentiated into basophils following stimulation with GM-CSF and IL-3 or into MCs following stimulation via SCF or NGF. Whether MCs differentiate as a tissue-resident subset of basophils or arise as a unique granulocyte lineage is still debated. (**D**) At least in *X. laevis*, subset(s) of skin-resident MCs appear to be homed/retained therein by CXCL8a and CXCL8b chemokines. Figure generated using BioRender.

However, each amphibian species leverages a unique subset of these sites for this purpose (Yaparla et al., 2016, 2020; Jordan and Speidel, 1930; Manning, 1991).

Unlike higher vertebrates wherein granulopoiesis occurs in the bone marrow (BM), all hematopoiesis, and granulopoiesis by extension, is absent from the BM of the more ancestral amphibians such as apoda (caecilians) and urodeles, with very few exceptions (Zapata et al., 1982; Duellman and Trueb, 1994; Curtis et al., 1979). Urodeles instead primarily employ their livers and spleens for this purpose (Frangioni and Borgioli, 1987). On the other hand, caecilians use their thymus, liver, and spleen tissues (Zapata et al., 1982). Granulopoiesis in many of the anurans (frogs and toads) is segregated to pronephric tissue and/or the subcapsular (peripheral) liver throughout life and the BM once developed during metamorphosis (Akulenko, 2012; Kanesada, 1956) (Fig. 1A). In several anuran species, the kidney serves as a granulopoietic reserve for a brief period after hibernation when BM hematopoietic stem cells have been depleted (Kanesada, 1956). Granulopoietic strategies are perhaps even more complex in another post-metamorphic anuran frog, *Xenopus laevis*. Multipotent precursors differentiate into common myeloid progenitors (CMPs) in the subcapsular liver and then travel through the bloodstream to the BM in response to BM-derived chemotactic factors (Yaparla et al., 2020). In these animals, initial commitment to the granulocyte lineage likely occurs during migration to the BM, and it is the BM microenvironment that further supports CMP differentiation. We suspect CMPs both finalize commitment to the granulocyte lineage and mature in the BM niche, which simultaneously restricts differentiation into other myeloid subtypes (Yaparla et al., 2020). Although only the femur BM has been explored in *X. laevis* (Yaparla et al., 2020), granulopoietic activity has been noted in the vertebrae, finger, and femur BM of the bullfrog *(Lithobates catesbeianus)* (de Abreu Manso et al., 2009). Within the hematopoietic BM itself, blood cell progenitors are contained in cell cords composed of reticular fibers and loosely organized collagen. Similar structures are found in the renal tubules of urodeles and larval anurans (Zapata et al., 1982).

An attractive hypothesis to account for these observations posits the advent of amphibian BM-mediated granulopoiesis coevolved with increased BM vascularization, a characteristic that allowed for the transition from aquatic to terrestrial life (Yaparla et al., 2020; Zapata et al., 1982; Padial et al., 2014). Without the protection from radiation provided by water, hematopoietic stem cells are left particularly susceptible to harmful solar rays. BM offers the safest microenvironment for these cells, with considerable radioprotective properties (Zapata et al., 1982). This theory is further bolstered by studies with the exclusively terrestrial salamander, *Plethodon glutinosus,* in which the BM is the primary source of granulocytes (Curtis et al., 1979). Considering the newly uncovered intricacies of anuran granulopoiesis, it would be beneficial to reexamine the adult granulopoietic organs of other amphibians. Here, we present current knowledge concerning amphibian granulocyte differentiation at a more *granular* level, describing the development of the subtypes that have been identified to date. Amphibian granulocytes have been primarily described based on their gross morphology and Wright-Giemsa staining (Wright and Whitaker, 2001). Thus, although amphibians possess leukocytes bearing morphological likeness to the four mammalian classical granulocyte subtypes, these cell subsets may not necessarily be functionally equivalent (Wright and Whitaker, 2001).

3. Granulocyte development and functionality

3.1. Neutrophils (& heterophils)

As specialized phagocytes and the most abundant of the granulocytes (in mammals), neutrophils are perhaps the most studied subtype. Some amphibians possess neutrophils and the functionally analogous heterophil, and other amphibians only have heterophils (Fingerhut et al., 2020). Although early reports used these terms interchangeably, the

granules of heterophils stain with eosin, a property absent in neutrophil granules (Curtis et al., 1979; Fingerhut et al., 2020).

Mammalian neutrophils contain at least four distinct granule types: primary/azurophilic granules, secondary/specific granules, tertiary granules, and secretory vesicles (Lacy, 2006). The primary granules of mammalian neutrophils contain toxic mediators such as myeloperoxidase, elastin, defensins and cathepsins, whereas their secondary and tertiary granules contain lactoferrin and matrix metalloproteinase-9 (Lacy, 2006). The secondary/specific granules have been documented in neutrophils from several amphibian species (Curtis et al., 1979). These granules increase in number, size, and electron density as these cells mature. Unlike the specific granules of mammalian neutrophils, mature specific inclusions in amphibians are associated with peroxidase activity (Curtis et al., 1979). The presence and quantity of primary/azurophilic granules in neutrophils across amphibian species is more variable (Curtis et al., 1979).

At least within the urodelian order, developing neutrophils are categorized in one of four morphologically distinct stages (Hightower, 1978). Originally described by Hightower and Harr in 1975, they are classified as either neutrophilic myeloblasts or neutrophilic early, maturing, or late myelocytes (Hightower, 1978). Both myeloblasts and early myelocytes localize mainly to the subcapsular liver. Immature neutrophil nuclei will sometimes acquire a curved or band shape (Hadji-Azimi, 1987). As neutrophilic myeloblasts differentiate further, they develop multilobed nuclei. Extensive lobulation is thought to largely underlie the remarkable migratory capabilities of these cells to distal sites of inflammation (Manley et al., 2018). As these cells migrate interstitially, segmented nuclei present less steric hindrance compared to round ones (Manley et al., 2018). As they differentiate, urodele neutrophils migrate to deeper layers of the subcapsular liver, closer in proximity to the hepatic parenchyma (Hightower, 1978). Mature neutrophils will then enter the bloodstream by way of the liver sinusoids, from which they easily infiltrate infected tissues and perform critical effector functions (Hightower, 1978).

The intriguing work from Hightower (1978) noted variations in newt neutrophil cell cycle timing compared to other animals (Hightower, 1978). In fact, newt neutrophils spend significantly more time in each of the cell cycle stages than mammalian neutrophils and take an average of 9 days to mature compared to just the 36-h neutrophil maturation window recorded in mice (Hightower, 1978). The evolutionary underpinnings and functional consequences of the diverged urodele neutrophil differentiation kinetics are not well understood.

What does seem to be conserved between vertebrates and across amphibians is the particular responsiveness of granulocyte precursors (myeloblasts) to granulocyte colony-stimulating factor (G-CSF, colony stimulating factor-3, CSF-3; Fig. 1B), a critical cytokine in granulocyte commitment and especially in neutrophil maturation (Demetri and Griffin, 1991; Koubourli et al., 2017a; Pinheiro et al., 2020). G-CSF acts through a cognate cell-surface receptor, G-CSFR on these precursor cells. Expression levels of G-CSFR increase with progressive neutrophil maturation, up to three-fold greater expression in mature neutrophils compared to immature neutrophils (Demetri and Griffin, 1991). Our work using the anuran *X. laevis* confirmed that a recombinant (r)G-CSF effectively generates mature neutrophils with hyper-segmented nuclei and robust expression of neutrophil transcription factors (higher *klf6, c/ebpdelta, c/ebpzeta, c/ebpepsilon* expression compared to other granulocyte subtypes; Yaparla et al., 2016; Khoyratty et al., 2021; Bjerregaard et al., 2003). G-CSF/G-CSFR signaling has dual roles: to differentiate and maintain neutrophil populations within the BM and to readily chemoattract these cells to sites of inflammation.

Many of the immune roles of amphibian neutrophil-like granulocytes, namely microbicidal activities, correspond to those attributed to their mammalian counterparts. Among these is the ability of amphibian neutrophils and neutrophil-like cells to readily phagocytose particles of assorted size and origin (Titon et al., 2022; Cary et al., 2014). Amphibian and mammalian granulocytes have also been linked to antiviral responses (Galani and Andreakos, 2015; Koubourli et al., 2017b, 2018). In fact, *X. laevis* G–CSF–derived granulocytes appear to be important antiviral effectors during Frog Virus 3 (FV3) ranavirus infections (Koubourli et al., 2017a). Neutrophils also exert their immunological effects by releasing their granular contents, of which serine proteases represent a considerable proportion (Fu et al., 2018). In attempting to understand the general biological roles of the most abundant human neutrophil serine protease, proteinase 3, a recent study cloned the *Xenopus* ortholog xPR-3 (Fu et al., 2018). Based on cleavage site analysis, hPR-3 and xPR-3 are both suspected to function as potent elastases (Fu et al., 2018), directly targeting pathogens and altering tissue structure at sites of inflammation (Gabay et al., 1989; Rao et al., 1991).

Mammalian neutrophils are also known for their capacities to form antimicrobial extracellular traps (NETs), made primarily of neutrophil DNA (Brinkmann et al., 2004). To our knowledge and as noted by others (Niedźwiedzka-Rystwej et al., 2019), while neutrophils of all vertebrate species are suspected to be capable of NET formation, this process has not been described in amphibian (or reptilian) granulocytes.

3.2. Eosinophils

Some believe amphibians represent the evolutionary point at which neutrophils and eosinophils diverged in their immune roles (Pinheiro et al., 2020). Interestingly, most amphibians have comparable proportions of these granulocyte subsets, a property likely related to the prevalence of parasites, viruses, and bacteria in their environment (Pinheiro et al., 2020). In endothermic vertebrates, on the other hand, neutrophils usually far outnumber eosinophils, presumably because these animals tend to be in relatively less direct contact with pathogen-rich environments compared to amphibians (Baccari et al., 2011). Although eosinophils appear to be more prevalent in amphibians than other animals, literature regarding the cytokines and growth factors responsible for amphibian eosinophil development is sparse. This is likely in part attributed to the fact that those cytokines important for eosinophil differentiation in higher vertebrates (*e.g.,* interleukin (IL)-3, IL-5, and granulocyte macrophage colony-stimulating factor, GM-CSF, colony stimulating factor-2, CSF-2; Fig. 1B) seem to be missing or perhaps are not annotated in many amphibian genomes (Hauser et al., 2020). The latter supposition is supported by the identification of the genes encoding the receptors for these ligands in several amphibian genomes.

Given that eosinophils are also found in the amphibian subcapsular liver, myeloblasts probably give rise to this amphibian granulocyte subset in addition to neutrophils. In anurans, immature eosinophilic granules are distinguished by the presence of small ubiquitous vesicles, which supply the granules with materials needed for further development (Frank, 1988). Later in maturation, these vesicles fuse with the granular membranes, creating a distinctive wavy appearance. The contents of mature eosinophilic granules vary across amphibian orders. Urodeles and some anuran eosinophils have round granules with dense, uniform contents (Frank, 1988). Other anurans have granules with crystalloid cores (Surbis, 1978).

Across vertebrates, eosinophils are classically associated with immune defenses against parasite infections (reviewed in (Hogan et al., 2008)). Several observations corroborate this is also the case in amphibians. For example, circulating eosinophils levels are elevated in salamanders infected with intestinal *protozoans*, while eosinophils are also recruited to the kidneys of juvenile grass frogs infected with flatworms (Davis and Golladay, 2019; Mitchell, 1982). Beyond antiparasitic immunity, amphibian eosinophils are believed to participate in several tissue remodeling contexts, including metamorphosis (Stacy and Ackerman, 2021). Eosinophil levels peak at metamorphic climax and return to baseline levels in post-metamorphic animals (Davis and Golladay, 2019). Since mammalian eosinophils respond to tissue injury and are involved in tissue repair, similar processes likely occur to modulate

tissue remodeling and repair during amphibian metamorphosis (Hota et al., 2013).

3.3. Basophils

The hallmark growth factors associated with mammalian basophil development overlap with those responsible for their eosinophil development (*i.e.,* IL-3, GM-CSF; Fig. 1C) and have hitherto not been identified in amphibian genomes. The relative frequencies of basophils vary dramatically between amphibian species. In some, they are the most numerous leukocytes, in other species they constitute less than 1% of peripheral blood leukocytes, and in others no circulating basophils have been detected (Cannon and Cannon, 1979; Cowden, 1965). This, compounded by their physical fragility, presents a set of challenges impeding study of their development (Cannon et al., 1988). A cytochemical investigation of the urodele *Amphiuma mean* by Cowden (1965), however, proved exceptionally fruitful, demonstrating the presence of basophils in their spleens (Cowden, 1965). Cowden showed these cells go on to mature in circulation and accumulate inclusion materials, as evidenced by their acid mucopolysaccharide content, like heparin (Cowden, 1965). The latter discovery appears to be a pattern of development consistent across many amphibians and vertebrates. Although heparin has long been medically employed as an anticoagulant, its biological function is suspected to be directly antimicrobial.

Early studies on amphibian basophils identified two subtypes, described as small basophils and the violet cells (Van Oordt, 1966; Kerr, 1966; Pasteels, 1960). Both have been described in *X. laevis* tadpoles and the larvae of the large newt, *Pleurodeles waltl* (Kerr, 1966; Pasteels, 1960). A detailed morphological report noted the presence of both basophil types in the pituitary of the common toad, *Bufo bufo*, with these cells appearing and developing during metamorphosis (Van Oordt, 1966). The small basophils, which are suspected to produce thyroid-stimulating hormone and are therefore also likely intimately linked to metamorphosis, reach maturation rapidly after they first appear in early pro-metamorphosis. Strangely, the nuclei of these small basophils undergo a pattern of expansion and contraction. In late pro-metamorphosis, nuclei are measurably reduced in size, increase again at the peak of remodeling, and by the end of metamorphic climax these cells and their nuclei are again compact and small. Throughout the first week post-metamorphosis, the small basophil nuclei continue to shrink. The other type of basophil, 'violet cells,' tend to be smaller and more elongated than small basophils, contrary to its naming convention (Van Oordt, 1966). Across vertebrates, basophils are the scarcest peripheral leukocyte (Titon et al., 2022), and basophils and MCs (see below) also remain the least characterized vertebrate granulocyte subsets (Frank, 1988).

3.4. Mast cells

Compared to the aforementioned 'traditional' granulocytes, there is continued debate concerning MC ontogeny, even in rigorously studied mammalian systems (Valent et al., 2020). Often considered 'tissue basophils,' it is unknown whether MCs represent a unique stage of basophil development or arise from an entirely different lineage (Fig. 1C). That this controversy is unsettled is somewhat surprising given ubiquitous MC tissue distribution and diverse function in processes and physiological systems (Valent et al., 2020).

Immature MCs have several identifiable characteristics shared by mammals and amphibians (Baccari et al., 2011). Namely, they are mostly round, alcianophilic cells with limited orthochromatic (blue) granules after toluidine blue staining (Pinelli et al., 2010). Generally, mature MCs become metachromatic (violet) and elongated. Of course, there are always exceptions in biology, and as such, MCs can be elongated or round in mature form in some anuran species (Baccari et al., 2011; Esposito et al., 2002). Moreover, immature amphibian MCs are marked by a centrally located nuclei with chromatin condensations and a few electron-dense, homogenous pro-granules originating from the Golgi (Krylova, 2010). These pro-granules become more morphologically heterogeneous with characteristic MC inclusions. This differs from the granules in fully developed mammalian MCs, which are more homogenous in shape (Krylova, 2010). The incredible MC morphological diversity that exists within amphibious individuals is almost certainly influenced by the physiologically unique tissue microenvironments in which they develop, and which ultimately determine their functions.

MCs are notoriously difficult to study in amphibians and other lower vertebrates, largely owing to their species-specific staining properties (Baccari et al., 2011). MCs are classically identified by the metachromasia revealed with basic aniline or thiazine dyes (*e.g.,* toluidine blue) (Chieffi Baccari et al., 1998). This staining property is attributed to sulfated glycosaminoglycans granule contents such as heparin (Chieffi Baccari et al., 1998). Within amphibian skin, we identified MCs with unequivocal metachromatic staining (Hauser et al., 2020). However, we also identified what we believe is an additional MC subtype in this mucosal barrier tissue. These MCs lack characteristic metachromasia (Hauser et al., 2020) and are discussed further in the following section.

There is a well-studied association between MCs and nerve fibers in frogs and toads (Baccari et al., 2011). MCs are critically important to the early stages of nerve development in tadpoles, forming a network around Schwann cell-axon complexes. Numbers of immature MCs detected in the nascent brain of larval amphibians increase throughout development and mature in tandem with metamorphic climax (Manning, 1991). The mature MCs will often localize between perineural layers, likely helping to maintain the blood-brain-barrier. During this period of extensive remodeling, MCs also assist in forming the nerve-tissue barrier of the tongue perineurium. MCs are further found within the sciatic nerves, branchial nerves, endoneurium, and epineurium of these animals, localized largely to the meningeal lining and densely packed within the anterior and posterior plexuses of the brain (Baccari et al., 2011). While these studies elegantly describe amphibian MC development-CNS interactions, they also underline the need to understand granulocytes outside of the amphibian central nervous system.

Two key cytokines contribute to amphibian MCs differentiation: nerve growth factor (NGF) and stem cell factor (SCF) (Baccari et al., 2003, 2011) (Fig. 1C). Named for its neurotrophic capabilities, NGF additionally regulates homeostasis in several systems and is produced by many cell types. As it pertains to the present discussion, NGF is sufficient to induce MC development in *Rana esculenta* following a granule maturation process similar to that seen in bird and mammalian MCs (Baccari et al., 2003). It is unlikely that NGF acts as a proliferative factor but rather as a trophic factor responsible for both MC precursor differentiation at early stages of tadpole life (Nieuwkoop and Faber, NF: 26–29) and for the maturation of immature MCs in older tadpoles (NF 31) (Baccari et al., 2003; Nieuwkoop et al., 1975). However, these findings should be considered with caution as the referenced studies used a recombinant human NGF rather than a species-specific NGF (Baccari et al., 2003).

SCF (c-kit ligand, mast cell growth factor, steel factor)-mediated MC differentiation seems to be an evolutionarily ancient strategy (Dobson et al., 2008; Crivellato and Ribatti, 2010). SCF receptor (SCFR) expression has been verified on MCs of teleost fish, birds, and mammals, and countless studies support the *in vitro* and *in vivo* MC differentiation capabilities attributed to SCF (Baccari et al., 2011). We recently produced *X. laevis* SCF in recombinant form and validated its utility in making amphibian MCs from granulocyte/macrophage precursors isolated from the BM [manuscript in prep]. Amphibian SCF-differentiated bone marrow-derived MCs (BMMCs) have characteristic MC cytology and gene expression profiles. After 9 days, most BM cultures incubated with rSCF were positively stained for specific-esterase (indicative of granulocyte-lineage cells) and had mono-morphonuclear properties typical of mammalian MCs. Electron microscopy revealed BMMCs have electron-dense granules and extensive folding of the plasma membrane almost indistinguishable from mammalian MCs (Goldmann and Medina,

2013). Our transcriptomic analyses confirm the 'MC' identify of these cells wherein *scfr* and an MC transcription factor, *mitf,* transcript levels are higher in unstimulated BMMCs compared to more conventional granulocytes generated with rG-CSF. However, frog MCs do not entirely match the gene expression profiles associated with mammalian MCs. Mast cell carboxypeptidase and tryptase are MC-specific proteins that contribute to their central immune roles, yet the expression of these genes did not differ between the *X. laevis* rSCF-derived MCs and the rG–CSF–generated granulocyte cultures [manuscript in prep].

MCs are typically categorized as mucosal- or connective tissue-type, and their phenotypes reflect the tissue in which they mature (Kitamura et al., 2006; Chiu and Lagunoff, 1971). Distinct tissues supply the chemical and physical factors that contribute to MC differentiation (Kitamura et al., 2006). SCF is chemotactic to MCs in higher vertebrates (Nilsson et al., 1994) and our recent work suggests this is likewise the case in amphibians [manuscript in prep]. We successfully enriched skin MCs in *X. laevis* via subcutaneous injections with rSCF. It is possible this enrichment is due exclusively to rSCF-mediated maturation of MC precursors already resident in the skin rather than recruitment of precursors from distal sites. The increase of MCs after rSCF treatment more likely results from a combination of MC recruitment and maturation of resident MC precursors. Nonetheless, that SCF can expand MCs in a mucosal tissue such as frog skin is notable given mammalian MC recruitment to mucosal tissues relies on CXC_3CR1 (Gurish et al., 2001), which is absent from or has not been identified in frog genome(s).

3.5. Novel amphibian granulocyte-lineages or MC subsets?

In addition to the four amphibian granulocyte subtypes reminiscent of those in higher vertebrates, amphibians may possess unique granulocyte population(s) tied to their expanded chemokine repertoires (Koubourli et al., 2018). For instance, although mammals encode one isoform of the granulocyte chemokine, CXCL8 (IL-8), *Xenopus* encode two isoforms: CXCL8a and CXCL8b (Koubourli et al., 2018). Both the mammalian CXCL8 and *Xenopus* CXCL8a contain an ELR (glutamic acid-leucine-arginine) motif at the N-terminus, which is characteristic of inflammatory cytokines (Koubourli et al., 2018; Nilsson et al., 1994). This motif is absent in the *Xenopus* CXCL8b isoforms (Koubourli et al., 2018). Studies from our laboratory demonstrated that CXCL8a and CXCL8b chemoattract two functionally distinct granulocyte populations, both via CXCR1 and CXCR2 engagement. Since only CXCL8a contains the ELR motif, we suspect these unique populations are recruited due to disparate ligand-CXCR1/2 interactions (Koubourli et al., 2018; Hauser et al., 2020).

We found *X. laevis* CXCL8a and CXCL8b are constitutively expressed in healthy tadpole and adult *X. laevis* skin, liver, kidney, and intestine tissues and are dynamically expressed across immune and non-immune perturbations (Koubourli et al., 2018). This was particularly perplexing because MCs are considered the only granulocyte-lineage cells populating healthy tissue, while all other granulocytes are recruited to tissue during inflammatory responses (Hauser et al., 2020). Histological analyses revealed an additional population of *X. laevis* skin granulocytes (specific esterase-positive, toluidine blue-negative) that are spatially and transcriptionally distinct from classical skin MCs (toluidine blue-positive). We noted skin granulocytes were homed and retained to this tissue by CXCL8a and CXCL8b (Fig. 1D). Moreover, these resident skin granulocytes appear to be present in many other amphibian species, both urodeles and anurans of pre- and post-metamorphic stages (Fig. 2). Although distinct from the toluidine blue-positive MCs, these cells do share some characteristics with conventional MCs. For instance, tadpole and adult skin granulocytes express several MC markers (*e.g.,* MC carboxypeptidase and histidine decarboxylase). Our initial analyses

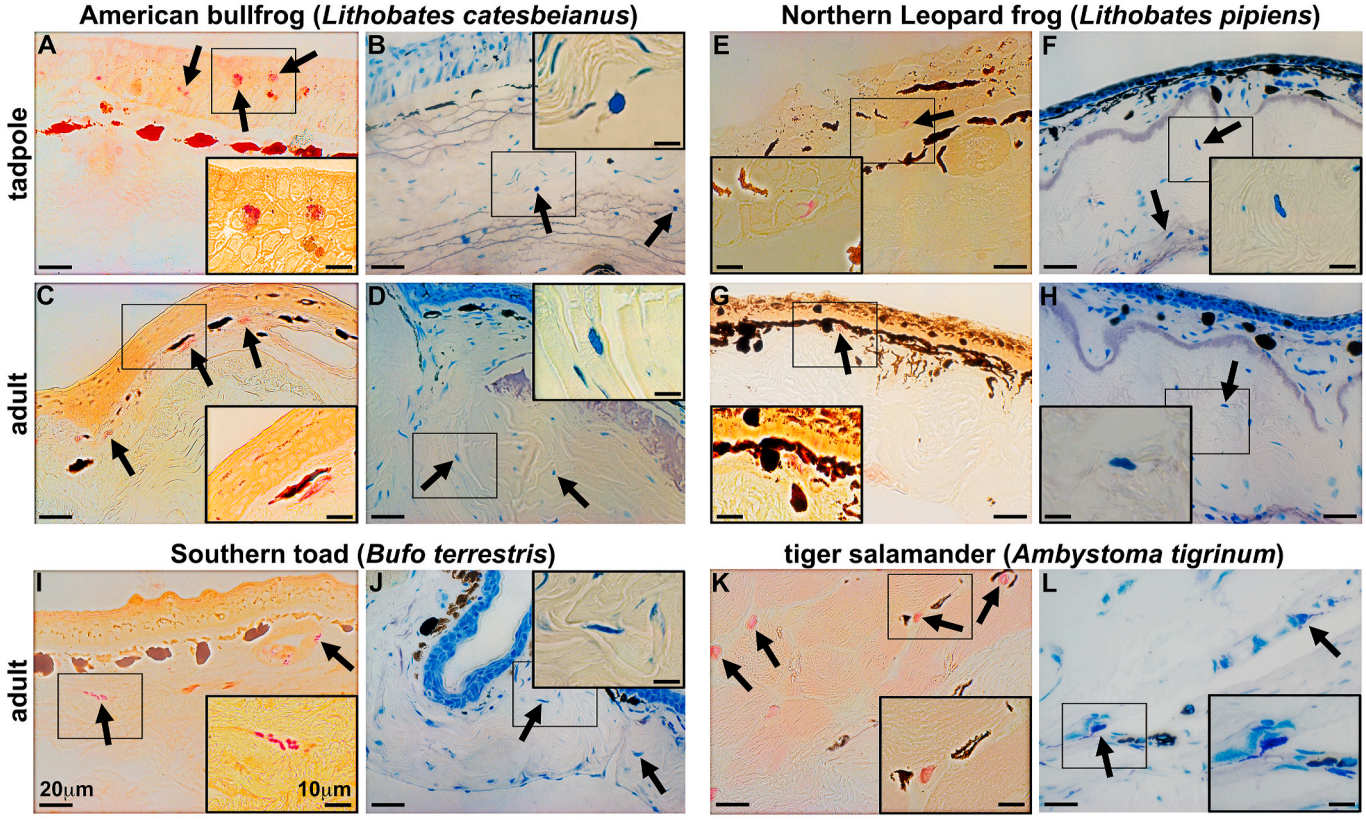

Fig. 2. Larval and adult urodele and anuran skins contain unique granulocyte-lineage cells. Specific esterase-positive (granulocytes) cells (A, C, I, E, G, K) and toluidine blue-positive (mast cells) cells (B, D, J, F, H, L) in larval (A, B, E, F) and adult (C,D,G-L) cutaneous tissue from healthy amphibians. Arrows indicate esterase and toluidine blue-positive cell. Inset panels are magnified views of the boxed regions within respective images.

suggested these skin granulocytes and MCs originate from separate lineages (Hauser et al., 2020). Rather, our more recent work indicates these cells are in fact a subpopulation of skin MCs [manuscript in prep].

4. Concluding remarks and future outlooks

Although much has been revealed about the biology of amphibian granulocyte lineages, we anticipate there is considerably more to learn about these remarkable cells in amphibian physiology and immunity. As bioinformatic, genomic, and molecular approaches to study the immune systems of non-traditional organisms are refined, we can continue to explore amphibian granulocyte ontogeny and functionality. In turn, new insights into converged and divergent features of vertebrate granulopoiesis will grant abundant opportunities to improve ecological and human health.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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References

Akulenko, N., 2012. Haemopoietic system of the anurans: the role of bone marrow and liver. Вестник зоологии 46 (4), 347–354, 347–354.
Baccari, G.C., et al., 2003. Induced maturation of frog mast cells by nerve growth factor during ontogenesis. Microsc. Res. Tech. 62 (5), 439–450.
Baccari, G.C., et al., 2011. Mast cells in nonmammalian vertebrates: an overview. Int. Rev. Cell. Mol. Biol. 290, 1–53.
Bjerregaard, M.D., et al., 2003. The in vivo profile of transcription factors during neutrophil differentiation in human bone marrow. Blood 101 (11), 4322–4332.
Boehm, T., 2012. Evolution of vertebrate immunity. Curr. Biol. 22 (17), R722–R732.
Brinkmann, V., et al., 2004. Neutrophil extracellular traps kill bacteria. Science 303 (5663), 1532–1535.
Cannon, M.S., Cannon, A., 1979. The blood leukocytes of Bufo alvarius: a light, phase-contrast, and histochemical study. Can. J. Zool. 57 (2), 314–322.
Cannon, M.S., et al., 1988. Cytochemistry of the blood basophil of Bufo marinus. J. Herpetol. 389–393.
Cary, T.L., Ortiz-Santaliestra, M.E., Karasov, W.H., 2014. Immunomodulation in post-metamorphic northern leopard frogs, Lithobates pipiens, following larval exposure to polybrominated diphenyl ether. Environ. Sci. Technol. 48 (10), 5910–5919.
Chieffi Baccari, G., et al., 1998. In situ characterization of mast cells in the frog Rana esculenta. Cell Tissue Res. 292 (1), 151–162.
Chiu, H., Lagunoff, D., 1971. Histochemical comparison of frog and rat mast cells. J. Histochem. Cytochem. 19 (6), 369–375.
Cortéz-Gómez, A., et al., 2015. Ecological functions of neotropical amphibians and reptiles: a review. Univ. Sci. 20 (2), 229–245.
Cowden, R.R., 1965. Quantitative and qualitative cytochemical studies on the Amphiuma basophil leucocyte. Z. für Zellforsch. Mikrosk. Anat. 67 (2), 219–233.
Crivellato, E., Ribatti, D., 2010. The mast cell: an evolutionary perspective. Biol. Rev. 85 (2), 347–360.
Curtis, S.K., Cowden, R.R., Nagel, J.W., 1979. Ultrastructure of the bone marrow of the salamander Plethodon glutinosus (Caudata: plethodontidae). J. Morphol. 159 (2), 151–183.
Davis, A.K., Golladay, C., 2019. A survey of leukocyte profiles of red-backed salamanders from Mountain Lake, Virginia, and associations with host parasite types. Comp. Clin. Pathol. 28 (6), 1743–1750.
de Abreu Manso, P.P., de Brito-Gitirana, L., Pelajo-Machado, M., 2009. Localization of hematopoietic cells in the bullfrog (Lithobates catesbeianus). Cell Tissue Res. 337 (2), 301–312.
Demetri, G.D., Griffin, J.D., 1991. Granulocyte Colony-Stimulating Factor and its Receptor.
Dobson, J.T., et al., 2008. Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination. Blood. J. Am. Soc. Hematol. 112 (7), 2969–2972.
Duellman, W.E., Trueb, L., 1994. Biology of Amphibians. JHU press.
Esposito, B., et al., 2002. Mast cells in Wallerian degeneration: morphologic and ultrastructural changes. J. Comp. Neurol. 445 (3), 199–210.
Fingerhut, L., Dolz, G., de Buhr, N., 2020. What is the evolutionary fingerprint in neutrophil granulocytes? Int. J. Mol. Sci. 21 (12), 4523.
Fortin, D., et al., 2005. Wolves influence elk movements: behavior shapes a trophic cascade in Yellowstone National Park. Ecology 86 (5), 1320–1330.
Frangioni, G., Borgioli, G., 1987. Periodic changes in the organs involved in the erythropoiesis of anemic newts. J. Exp. Zool. 243 (3), 409–416.
Frank, G., 1988. Granulopoiesis in tadpoles of Rana esculenta. Survey of the organs involved. J. Anat. 160, 59.
Fu, Z., et al., 2018. Extended cleavage specificity of human neutrophil elastase, human proteinase 3, and their distant ortholog clawed frog PR3—three elastases with similar primary but different extended specificities and stability. Front. Immunol. 2387.
Gabay, J.E., et al., 1989. Antibiotic proteins of human polymorphonuclear leukocytes. Proc. Natl. Acad. Sci. USA 86 (14), 5610–5614.
Galani, I.E., Andreakos, E., 2015. Neutrophils in viral infections: current concepts and caveats. J. Leukoc. Biol. 98 (4), 557–564.
Goldmann, O., Medina, E., 2013. The expanding world of extracellular traps: not only neutrophils but much more. Front. Immunol. 3, 420.
Groeneweg, L., Hidalgo, A., 2020. Emerging roles of infiltrating granulocytes and monocytes in homeostasis. Cell. Mol. Life Sci. 77 (19), 3823–3830.
Gurish, M.F., et al., 2001. Intestinal mast cell progenitors require CD49dβ7 (α4β7 integrin) for tissue-specific homing. J. Exp. Med. 194 (9), 1243–1252.
Hadji-Azimi, I., 1987. Atlas of adult Xenopus laevis laevis hematology. Dev. Comp. Immunol. 11, 807–874.
Hauser, K., et al., 2020. Discovery of granulocyte-lineage cells in the skin of the amphibian Xenopus laevis. FACETS 5 (1), 571–597.
Hightower, J.A., 1978. The cell cycle of the neutrophilic granulocyte in a Urodele amphibian. Copeia 86–92.
Hogan, S.P., et al., 2008. Eosinophils: biological properties and role in health and disease. Clin. Exp. Allergy 38 (5), 709–750.
Hota, J., Das, M., Mahapatra, P.K., 2013. Blood cell profile of the developing tadpoles and adults of the ornate frog, Microhyla ornata (Anura: microhylidae). Int. J. Zool. 2013.
Jordan, H., 1919. The histology of the blood and the red bone-marrow of the leopard frog, Rana pipiens. Am. J. Anat. 25 (4), 436–480.
Jordan, H., Speidel, C., 1923. Blood cell formation and distribution in relation to the mechanism of thyroid-accelerated metamorphosis in the larval frog. J. Exp. Med. 38 (5), 529.
Jordan, H., Speidel, C., 1930. The hemocytopoietic effect of splenectomy in the salamander, Triturus viridescens. Am. J. Anat. 46 (1), 55–90.
Kanesada, A., 1956. A phylogenetical survey of hemocytopoietic tissues in submammalian vertebrates. Bull. Yamaguchi Med. Sch. 4 (1).
Kerr, T., 1966. The development of the pituitary in Xenopus laevis Daudin. Gen. Comp. Endocrinol. 6 (3), 303–311.
Khoyratty, T.E., et al., 2021. Distinct transcription factor networks control neutrophil-driven inflammation. Nat. Immunol. 22 (9), 1093–1106.
Kitamura, Y., Oboki, K., Ito, A., 2006. Molecular mechanisms of mast cell development. Immunol. Allergy Clin. 26 (3), 387–405.
Koubourli, D.V., et al., 2017a. Immune roles of amphibian (Xenopus laevis) tadpole granulocytes during Frog Virus 3 ranavirus infections. Dev. Comp. Immunol. 72, 112–118.
Koubourli, V.D., et al., 2017b. Immune roles of amphibian (Xenopus laevis) tadpole granulocytes during Frog Virus 3 ranavirus infections. Dev. Comp. Immunol. 72, 112–118.
Koubourli, D.V., et al., 2018. Amphibian (Xenopus laevis) interleukin-8 (CXCL8): a perspective on the evolutionary divergence of granulocyte chemotaxis. Front. Immunol. 9, 2058.
Krylova, M., 2010. Mast cells of lymph hearts during ontogenesis of frogs Rana temporaria. Tsitologiia 52 (9), 749–759.
Lacy, P., 2006. Mechanisms of degranulation in neutrophils. Allergy Asthma Clin. Immunol. 2 (3), 98–108.
Lee, D., Kim, D.W., Cho, J.-Y., 2020. Role of growth factors in hematopoietic stem cell niche. Cell Biol. Toxicol. 36 (2), 131–144.
Manley, H.R., Keightley, M.C., Lieschke, G.J., 2018. The neutrophil nucleus: an important influence on neutrophil migration and function. Front. Immunol. 9, 2867.
Manning, M., 1991. Histological organization of the spleen: implications for immune functions in amphibians. Res. Immunol. 142 (4), 355–359.
Mitchell, J., 1982. The effect of host age on Rana temporaria-Gorgoderina vitelliloba interactions. Int. J. Parasitol. 12 (6), 601–604.
Niedźwiedzka-Rystwej, P., Repka, W., Tokarz-Deptuła, B., Deptuła, W., 2019. In sickness and in health"–how neutrophil extracellular trap (NET) works in infections, selected diseases and pregnancy. J. Inflamm. 16 (1), 1–8.
Nieuwkoop, P.D., 1975. In: Nieuwkoop, P.D., Faber, J. (Eds.), Normal Table of Xenopus laevis. North-Holland, Amsterdam.
Nilsson, G., et al., 1994. Stem cell factor is a chemotactic factor for human mast cells. J. Immunol. 153 (8), 3717–3723.
Owen, J.A., Punt, J., Stranford, S.A., 2013. Kuby Immunology. WH Freeman, New York, NY, USA.
Padial, J.M., Grant, T., Frost, D.R., 2014. Molecular systematics of terraranas (Anura: brachycephaloidea) with an assessment of the effects of alignment and optimality criteria. Zootaxa 3825 (1), 1–132, 1–132.
Pasteels Jr., J., 1960. Experimental study of different categories of chromophilic elements of the adult hypophysis of Pleurodeles waltili, of their function and of their control by the hypothalamus. Arch. Biol. 71, 409–471.

Pinelli, C., et al., 2010. Mast cells in the amphibian brain during development. J. Anat. 216 (3), 397–406.
Pinheiro, D., et al., 2020. In-silico analysis of myeloid cells across the animal kingdom reveals neutrophil evolution by colony-stimulating factors. Elife 9, e60214.
Rao, N.V., et al., 1991. Characterization of proteinase-3 (PR-3), a neutrophil serine proteinase. Structural and functional properties. J. Biol. Chem. 266 (15), 9540–9548.
Schulz, C., Petzold, T., Ishikawa-Ankerhold, H., 2021. Macrophage regulation of granulopoiesis and neutrophil functions. Antioxidants Redox Signal. 35 (3), 182–191.
Stacy, N.I., Ackerman, S.J., 2021. A tribute to eosinophils from a comparative and evolutionary perspective. J. Allergy Clin. Immunol. 147 (3), 1115–1116.
Surbis, A.Y., 1978. Ultrastructural Study of Granulocytes of Bufo Marinus. Florida Scientist, pp. 45–52.
Tanaka, Y., 1976. Architecture of the marrow vasculature in three amphibian species and its significance in hematopoietic development. Am. J. Anat. 145 (4), 485–497.
Titon, S.C.M., et al., 2022. Optimizing studies of phagocytic activity by flowsight cytometry in amphibians. South Am. J. Herpetol. 23 (1), 58–66.
Valent, P., et al., 2020. Mast cells as a unique hematopoietic lineage and cell system: from Paul Ehrlich's visions to precision medicine concepts. Theranostics 10 (23), 10743.
Van Oordt, P., 1966. Changes in the pituitary of the common toad, Bufo bufo, during metamorphosis, and the identification of the thyrotropic cells. Z. für Zellforsch. Mikrosk. Anat. 75 (1), 47–56.
Wright, K.M., Whitaker, B.R., 2001. Amphibian Medicine and Captive Husbandry. Krieger Publishing Company.
Yaparla, A., Wendel, E.S., Grayfer, L., 2016. The unique myelopoiesis strategy of the amphibian Xenopus laevis. Dev. Comp. Immunol. 63, 136–143.
Yaparla, A., Reeves, P., Grayfer, L., 2020. Myelopoiesis of the Amphibian Xenopus laevis is segregated to the bone marrow, away from their hematopoietic peripheral liver. Front. Immunol. 10, 3015.
Zapata, A., et al., 1982. Lymphoid organs and blood cells of the caecilian Ichthyophis kohtaoensis. Acta Zool. 63 (1), 11–16.
Zhang, P., et al., 2019. The physical microenvironment of hematopoietic stem cells and its emerging roles in engineering applications. Stem Cell Res. Ther. 10 (1), 1–13.