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journal homepage: www.sciencedirect.com/journal/comparative-immunology-reportsGranulocytes accumulate in resorbing tails of metamorphosing *Xenopus laevis* amphibians

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ABSTRACT

Amphibian metamorphosis represents a dramatic example of post-embryonic development. In the anuran *Xenopus laevis* frog, this process involves extensive changes to larval tissues, structures, and physiology to produce its adult form. As a long-standing model to study tissue remodeling, both amphibian metamorphosis and mammalian development are under the control of thyroid hormone. Successful remodeling though, also requires precise temporospatial regulation of immune activation. Yet there is much to learn about the immune components linked to metamorphosis. In turn, granulocytes are a class of innate immune cells recently touted for their participation in processes beyond classical immune defenses, including in pathological and non-pathological tissue remodeling. In this manuscript, we explore the roles of granulocytes in perhaps the most conspicuous anuran metamorphic event: tadpole tail reabsorption. We characterize granulocyte infiltration into the tail as metamorphosis progresses. Although some granulocyte subpopulations exist in both *Xenopus* and mammals, our previous work has identified additional *Xenopus*-specific populations. Thus, here we further explored subpopulation dynamics through distinct stages of natural metamorphosis, their likely roles during this process, and their relationship with thyroid hormone. As endocrine disruptors continue to threaten species across the animal kingdom, the work described here offers much-needed insight into immune contributions to endocrine-linked development.

Introduction

Amphibian metamorphosis involves impressive remodeling of larval physiology and typically coincides with the transition from aquatic to terrestrial life [1]. This process is particularly extensive in anuran amphibians (frogs and toads). The adult animal hardly resembles their juvenile forms. Tadpole tails and gills are lost, the skin is completely restructured, and new limbs develop [1]. Despite often being thought of as completely distinct processes, amphibian metamorphosis resembles mammalian perinatal development in many respects. For example, during the mammalian perinatal period their skin also keratinizes, limbs elongate, and like amphibians, mammals must also transition from the aqueous environment of the amniotic sac to a terrestrial one. And although separated by vast evolutionary time, amphibian metamorphosis and higher vertebrate development both rely on thyroid hormone (T_3) [1].

The metamorphosis of the anuran *Xenopus laevis* is a long-standing model to study tissue remodeling [3,4]. Recent reports show the

profiles of certain developmental genes across several metamorphic *Xenopus* tissues are recapitulated in the corresponding mammalian tissues in the first weeks after birth, both in a T_3 -dependent manner [5]. Concurrently, disruptions to T_3 levels during early animal development are linked to serious developmental issues with long-term health consequences across taxa from mammals to lower vertebrates [6,7]. As T_3 inhibitors have become ubiquitous environmental contaminants [8,9], it is imperative that we better understand such T_3 -dependent tissue changes.

While the prominent roles of T_3 in metamorphosis have been broadly investigated, the intimate involvement of immune components in this process is a newer area of research [10,11]. Since immune systems fundamentally work to distinguish 'self' from 'non-self', it follows that immune components would be integral to amphibian metamorphosis. During metamorphosis, the animal changes its 'self' and must regulate its immune system to tolerate its 'new self' [11]. Moreover, many metamorphic events require inflammatory processes to both remove larval tissues as well as to stimulate/recruit other cells to generate adult

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ones [11].

Foundational studies on this topic focused on circulating leukocyte profiling during metamorphosis [12], while more recent studies have identified some functions of macrophages and non-immune cells [13]. However, granulocyte-lineage leukocytes have been largely overlooked as potential contributors to this category of development. Recent research identifying novel immune and non-immune roles as well as complexities of these cells suggest they are likely important in orchestrating metamorphosis [14–16]. In fact, granulocyte-lineage cells are essential in other contexts of tissue remodeling such as wound repair and regeneration [14–16]. The four classical granulocytes subtypes (neutrophils, eosinophils, basophils, and mast cells) are all able to readily phagocytose and release extracellular matrix-altering compounds, as well as recruit and activate diverse cell types [17–19].

Cells resembling the four classical granulocytes have been identified in *X. laevis* [20]. Granulocyte colony stimulating factor (G-CSF, a.k.a. colony stimulating factor-3; CSF3) differentiates and recruits neutrophil-like cells, which we showed are important to infection outcomes in these animals [21]. CXCL8 (interleukin-8, IL-8) is a hallmark inflammatory CXC chemokine, first identified in mammals for its potent granulocyte recruitment [22]. Most mammals encode single CXCL8 genes, which belong to a group of chemokines possessing the Glu-Leu-Arg (ELR) inflammatory motif [23]. Except for *Gadiformes* (cod, haddock) bony fish CXC chemokines generally lack an ELR motif and instead possess XLR motifs (X(other residue)-Leu-Arg; DLR in salmonids) [24]. Such CXCL8 genes have been identified across a range of bony fish species, many of which encode multiple CXCL8 isoforms [25]. Our own studies indicate *X. laevis* encode both ELR-containing and ELR-lacking CXCL8 isoforms: CXCL8a and CXCL8b, respectively [15,26]. Of these, CXCL8a appears to be linked to *X. laevis* inflammatory responses, while CXCL8b recruits a unique population(s) of granulocytes associated with tissue remodeling. Thus, investigating the roles *X. laevis* granulocytes in the context of metamorphosis is a natural transition from this work. Accordingly, the present studies explore the roles of granulocyte-lineage cells during *X. laevis* metamorphosis.

Materials and methods

Animals

Outbred mixed-sex pre-metamorphic tadpoles (Nieuwkoop and Faber (NF) Stage 54), pro-metamorphic tadpoles (NF Stage 61), tadpoles in climax of metamorphosis (NF Stage 64), and post-metamorphic *X. laevis* froglets were purchased from Xenopus 1 (Dexter, Michigan, USA). Animals were housed and handled under strict laboratory and IACUC regulations (Approval number 15–024).

Xenopus laevis recombinant G-CSF, CXCL8a, and CXCL8b production

The production of *X. laevis* recombinant (r)G-CSF, rCXCL8a, and rCXCL8b has been previously described [15,26]. Briefly, the *X. laevis* G-CSF, CXCL8a, and CXCL8b sequences representing the signal peptide-cleaved transcripts were ligated into the pMIB/V5 His-A insect expression vector (Invitrogen) and transfected into Sf9 insect cells (cellfectin II, Invitrogen). Recombinant protein production was confirmed by western blot and the positive transfectants were selected using 10 µg/ml blasticidin. The expression cultures were scaled up as 500 ml liquid cultures, grown for 5 days, pelleted, and the supernatants collected. These were dialyzed overnight at 4 °C against 150 mM sodium phosphate, concentrated against polyethylene glycol flakes (8 kDa) at 4 °C, dialyzed overnight at 4 °C against 150 mM sodium phosphate, and passed through Ni-NTA agarose columns (Qiagen). Columns were washed with 2 × 10 volumes of high stringency wash buffer (0.5 % Tween 20; 50 mM Sodium Phosphate; 500 mM Sodium Chloride; 100 mM Imidazole) and 5 × 10 volumes of low stringency wash buffer (as above, but with 40 mM Imidazole). Recombinant cytokines were eluted

using 250 mM imidazole and were confirmed by western blot against the V5 epitopes on the proteins and the protein concentrations were determined by Bradford protein assays (BioRad). Halt protease inhibitor cocktail (containing AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A; Thermo Scientific) was added to the purified proteins, which were then stored at –20 °C in aliquots until use. The recombinant control (r-ctrl) was generated by transfecting Sf9II cells with an empty pMIB/V5 His-A expression vector and processing the cell supernatants using the same approaches described above for the recombinant cytokines.

Tadpole triiodothyronine (T_3) treatments

Tadpoles were reared in water containing T_3 (10 nM final concentration) or in diluted solvent control (NaOH) alone for 5 days (Yao et al., 2017). Water was changed daily for the duration of the experiments.

rCXCL8-attracted granulocyte isolation

Granulocytes were obtained according to previously validated methods (Koubourli et al., 2018). Outbred mixed-sex pre-metamorphic tadpoles (NF Stage 54), pro-metamorphic tadpoles (NF Stage 61), tadpoles in climax of metamorphosis (NF Stage 64), and post-metamorphic *X. laevis* froglets were injected with rCXCL8a or rCXCL8b (1 µg/g body weight). Cells were collected 4 h later by lavage with 2 × 25 µl of A-PBS. Cells were counted, pooled by treatment, cytocentrifuged onto glass slides and stained with Giemsa histological stain to examine proportions of leukocyte subsets present in the distinct lavages.

Histology

X. laevis tails were isolated with sterile razors and immediately fixed in 10 % neutral buffered formalin (VWR, Radnor, Pennsylvania, USA) for 24 hours. Tails were processed, embedded in paraffin, and sectioned (5 µm) by the GWU Pathology Core or Georgetown University Histopathology and Tissue Shared Resource Center. Sections were then stained using a Naphthol AS-D Chloroacetate (specific esterase) kit (Sigma) according to the manufacturer's instructions and optimized to frog tissues. Granulocytes were quantified by averaging the number of specific esterase-positive cells (bright pink) per field of view (FOV) and by counting the total number of positive cells in whole tail sections to ensure differences were not due to differences overall area. Red blood cells occasionally stain faintly pink above background levels with specific esterase but are differentiated from granulocytes via morphology.

RNA isolation and quantitative gene expression analyses

For all experiments, tadpole and metamorphic whole tails were homogenized by passage through progressively higher gauge needles in Trizol reagent (Invitrogen, Carlsbad, California, USA), flash frozen on dry ice, and stored at –80 °C until RNA isolation. RNA isolation was performed using Trizol (Invitrogen) according to the manufacturer's directions.

Quantitative analysis of *X. laevis* gene expression has been described [27]. All cDNA syntheses were performed using iScript cDNA synthesis kits in accordance with manufacturer's directions (Bio-Rad, Hercules, CA) and using 500 ng of total RNA. Quantitative qRT-PCR analysis was performed using 2.5 µl of the derived cDNA templates. All gene expression analyses was performed relative to the *gapdh* endogenous control.

All experiments were performed using the CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, California, USA) and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories). The BioRad CFX Manager software (SDS) was employed for all expression analysis. All primers were validated prior to use (Supplemental Table 1).

Statistical analysis

Statistical analyses were performed using student's *t*-test, one-way analysis of variance (ANOVA), or two-way ANOVA with *post hoc* Tukey *t*-test using Vassar Stats and Graph Pad statistical programs. Probability level of $p < 0.05$ was considered significant.

Results

Granulocytes accumulate within regressing tails of metamorphosing *Xenopus laevis*

The complex process of metamorphosis can be subclassified into distinct stages according to the status of key events (*e.g.*, extent of tail resorption). We first evaluated granulocyte prevalence in tails from pre-metamorphic tadpoles (premet; NF Stage 54), pro-metamorphic tadpoles (promet; NF Stage 61), tadpoles in climax of metamorphosis (climax; NF Stage 64), and post-metamorphic *X. laevis* froglets by assessing specific esterase activity, a marker of granulocyte-lineage cells [28]. Specific esterase staining revealed tails from pro-metamorphic animals contained significantly more granulocyte-lineage cells than those from pre-metamorphic tadpoles (Fig. 1). Moreover, we observed more than a 4.5 magnitude increase in granulocyte-lineage cells in the tails of tadpoles in climax of metamorphosis compared to tails of pro-metamorphic animals. Total specific esterase-positive cells in whole tails increased as

metamorphosis progressed (Fig. S1). We noted these cells within tail muscle tissue of both pro-metamorphic and climaxing metamorphic animals (Fig. 1E and H & I, respectively).

Hallmark granulocyte genes are dynamically expressed in regressing tails

We followed up our histological studies with expression analyses of hallmark granulocyte genes within tails of metamorphosing *X. laevis*. We previously demonstrated *X. laevis* granulocyte colony-stimulating factor (G-CSF), CXCL8a, and CXCL8b chemoattract at least partially distinct granulocyte populations [21,26]. Moreover, granulocyte lineage commitment, development, and functionality also depend on G-CSF [29]. Our present expression analyses of metamorphosing *X. laevis* tails indicated all three granulocyte genes (*gcsf*, *cxcl8a*, *cxcl8b*) increased with progression of metamorphosis (Fig. 2A). Compared to pre-metamorphic tadpole tails (NF 54), *cxcl8a* expression significantly increased in regressing tails of pro-metamorphic animals (NF 61) and did not further change during climax of metamorphosis (NF 64; Fig. 2A). The mRNA levels of *cxcl8b* and *gcsf* were elevated in pro-metamorphic tadpole tails and continued to increase into climax of metamorphosis (Fig. 2A).

We additionally examined the tails of pre-metamorphic tadpoles, pro-metamorphic tadpoles, and tadpoles in midst of metamorphic climax for expression of genes associated with granulocyte production of lipid mediators, including cyclooxygenase (*cox*)–1 and –2,

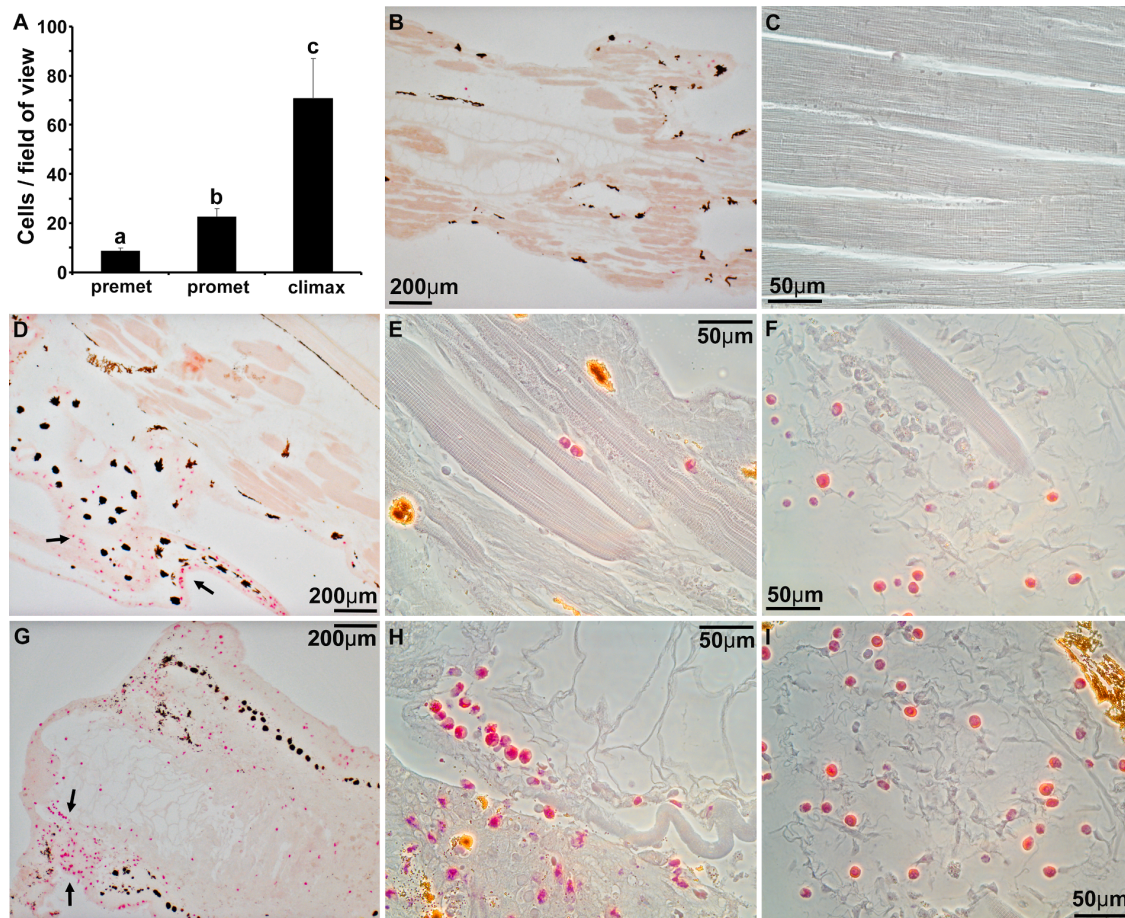


Fig. 1. Granulocyte-lineage cells infiltrate the regressing tail as *X. laevis* progresses through metamorphosis. (A) The number of specific esterase (SE)-positive cells per field of view (FOV, $N = 5$) from *X. laevis* whole tails increased from pre-metamorphic (premet; NF 54) tadpoles (B, C), to pro-metamorphic (promet; NF 61) tadpoles (D, E, D), and tadpoles in climax (climax; NF 64) of metamorphosis (G, H, I).

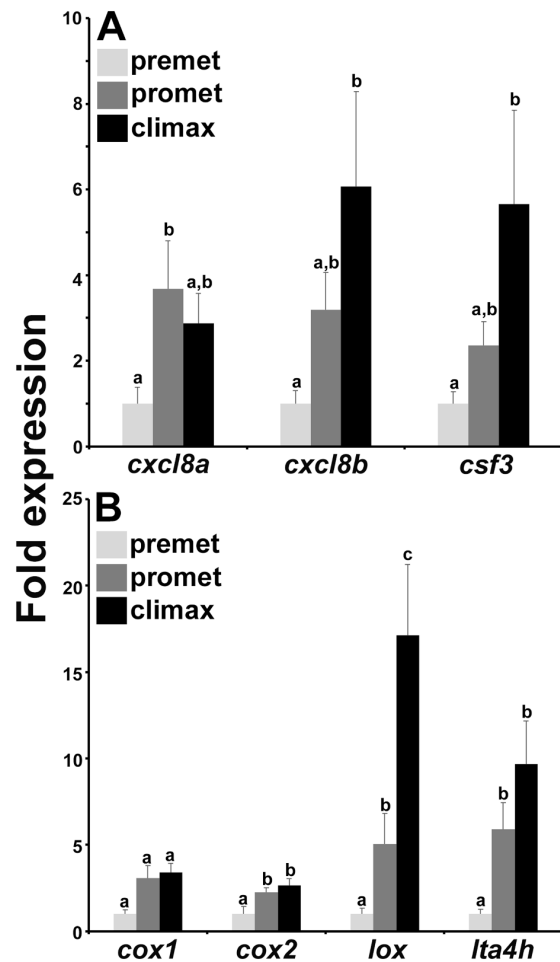


Fig. 2. *X. laevis* granulocyte-related genes are dynamically expressed in regressing tails of metamorphosing animals. Expression of key granulocyte chemokines (A) and inflammatory mediators (B) was compared between whole tails from pre-metamorphic tadpole (premet; NF 54), pro-metamorphic tadpoles (promet; NF 61), and tadpoles in climax of metamorphosis (climax; NF 64). All gene expression analyses was performed relative to the *gapdh* control. Results represent means $\pm$ SE ($N = 6$); letters denote statistical differences between treatment groups within each gene, $p < 0.05$.

lipoygenase (*lox*), and leukotriene A4 hydrolase (*lta4h*) (Fig. 2B). Of these, expression of *cox2*, *lox*, and *lta4h* were significantly elevated during metamorphosis (Fig. 2B).

Granulocytes populate tadpole tails in response to exogenous T_3

Since T_3 is critical for the initiation and progression of metamorphosis [3,4], we next examined whether granulocyte infiltration into tadpole tails could be recapitulated by exposing *X. laevis* pre-metamorphic tadpoles to exogenous T_3 . As anticipated, T_3 treatment resulted in a pronounced increase in specific esterase-positive cells within these tadpole tails (Fig. 3A-C).

The localization of specific esterase-positive cells also differed between solvent control- and T_3 -exposed animals (Fig. 3). In control animals, granulocytes were largely restricted to the lateral edges of the tail, near or within the skin. After T_3 treatment, these cells were still found in lateral regions but also in the blood vessels supplying the tail somatic muscle and embedded within the muscle tissues themselves. We also observed sporadic pockets of granulocytes within the notochords of

regressing tails (Fig. 3). As T_3 -induced metamorphosis does not completely recapitulate natural amphibian progression through this process, the lesser magnitude of change in granulocyte counts compared to natural metamorphosis is not unexpected (Figs. 3C & 1, respectively).

The tails of T_3 -treated tadpoles were evaluated for expression of *gcsf*, *cxcl8a*, *cxcl8b* as well as the gene encoding the G-CSF receptor (*gcsfr*) (Fig. 3D). While *gcsfr* and *cxcl8a* expression were unaffected, *gcsf* and *cxcl8b* were significantly elevated in T_3 -treated animals (Fig. 3D).

Our previous studies indicate the *X. laevis* CXCL8b isoform recruits an immunosuppressive/remodeling population(s) of granulocytes, which express functionally associated genes like arginase-1 (*arg1*) and interleukin-10 (*il10*) [15,26]. Because tissue-specific expression of *cxcl8b* implies recruitment of such granulocyte subset(s) thereto, *arg1* and *il10* expression was also assessed in control and T_3 -treated animals. While we did not detect changes to *arg1* expression, the tails of T_3 -exposed animals expressed more transcripts for the *il10* immunosuppressive cytokine gene (Fig. 3D).

CXCL8b-attracted granulocytes from metamorphosing *Xenopus laevis* express thyroid hormone activating enzymes and thyroid hormone receptor b

The relationship between thyroid hormones and innate immune cells is not unidirectional [30,31]. In mammals, neutrophils, macrophages, and monocytes participate in thyroid hormone metabolism, both in converting the prohormone thyroxine (T_4) into the active T_3 hormone or T_3 back into this prohormone form [31]. Although macrophages are the chief mammalian innate immune cells that produce deiodinase 2 (*dio2*), the enzyme responsible for local T_3 activation [31], we speculated the CXCL8b-attracted tissue remodeling granulocytes may serve this function in amphibians during metamorphosis. To answer this question, we injected the peritonea of pre-metamorphic tadpoles (premet), pro-metamorphic tadpoles (promet), tadpoles in climax of metamorphosis (climax), and post-metamorphic froglets with recombinant (r)CXCL8a and rCXCL8b or a recombinant control (r-ctrl) and four hours later isolated the recruited cells via peritoneal lavage towards cytology and gene expression analyses.

As anticipated, compared to r-ctrl-administered animals, rCXCL8a and rCXCL8b resulted in significant accumulation of leukocytes in the peritonea of animals in all stages of development (Fig. 4A). Leukocyte accumulation in response to rCXCL8a did not differ from cell numbers elicited by rCXCL8b for any examined stage of development (Fig. 4A). While the peritonea of r-ctrl-treated animal contained greater proportions of mast cells and fewer neutrophils, the rCXCL8a- and rCXCL8b-recruited leukocytes comprised of 50 % or more of neutrophils, depending on developmental stage (Fig. 4B). Except for froglet rCXCL8b-recruited cells, the leukocytes elicited by rCXCL8a and rCXCL8b into the peritonea of all developmental stages had greater proportions of cells with monocyte/macrophage morphology compared to the respective r-ctrl leukocyte populations (Fig. 4B).

Interestingly, rCXCL8b-recruited granulocytes isolated from pro-metamorphic *X. laevis* expressed significantly greater levels of *dio2* compared to rCXCL8a-attracted granulocytes (Fig. 5). In fact, pro-metamorphic tadpole rCXCL8b-attracted leukocytes possessed significantly greater *dio2* expression than seen in rCXCL8b- or rCXCL8a-attracted granulocytes derived from pre-metamorphic tadpoles, tadpoles in climax of metamorphosis, or post-metamorphic froglets (Fig. 5).

Expression of thyroid hormone receptors is known to promote *dio2* expression in the presence of thyroid hormone [32]. The expression of thyroid hormone receptor a (*thra*) did not significantly differ across cells isolated from pre-metamorphic, pro-metamorphic, climaxing, or post-metamorphic animals, irrespective of r-ctrl, rCXCL8b or rCXCL8a treatment (Fig. 6A). By contrast, the thyroid hormone receptor b (*thrb*) gene expression was significantly elevated in the rCXCL8b-elicited leukocytes isolated from pro-metamorphic animals (Fig. 6B), in line with their greater *dio2* expression (Fig. 5).

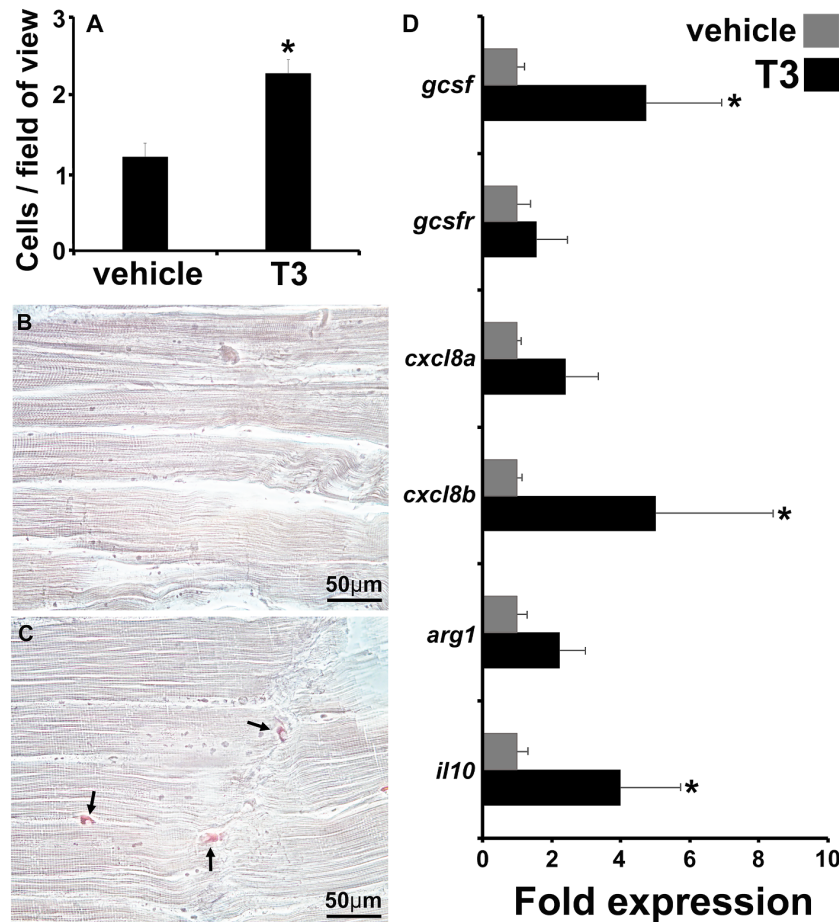


Fig. 3. T₃-exposed tadpoles recapitulate granulocyte tail infiltration and expression of key granulocyte genes. The number of specific esterase-positive cells in the tail per field of view ($N = 4$) increases after T₃ treatment (A, C) compared to solvent control treatment (A, B). Expression of key granulocyte genes were analyzed in whole tails from T₃- or solvent-treated *X. laevis* tadpoles (NF 54; D). All gene expression analyses was performed relative to the *gapdh* control. Results are means $\pm$ SE ($N = 6$); asterisks (*) denote statistical differences between treatment groups, $p < 0.05$.

Discussion

As research models, amphibians offer unparalleled platforms to study the mechanisms controlling metamorphosis. ‘Amphibian’ stems from the Greek ‘both (amphi)’ – ‘lives (bios)’ [33]. Appropriately, for many amphibians, metamorphosis marks the transition from aquatic to terrestrial life, or at least the acquisition of changes suited for terrestrial life [1]. Despite long-standing fascination with biological metamorphosis that has inspired mythology for centuries and countless literary works [34,35], researchers have only recently started to address the many scientific questions inspired by this process. To this end, the present work demonstrates the presence of multiple granulocyte populations in the regressing tail of metamorphosing *X. laevis*.

Granulocytes were long considered to be exclusively involved in antimicrobial innate immune responses [36]. However, this view has since been challenged [37], and significant attention has been dedicated to understanding granulocytes in pathological (e.g., wound healing) and non-pathological (e.g., development) tissue remodeling [14,17]. The work here explores granulocyte contributions to amphibian metamorphosis, a type of non-pathological tissue remodeling mechanistically related to wound healing (reviewed in [38]). We observed granulocytes accumulating in regressing tails as animals progressed through metamorphosis, which coincided with dramatic loss of tail muscle tissues. We suspect these granulocytes represent several distinct populations,

traversing *X. laevis* tails during distinct stages of metamorphosis and likely conferring disparate effector functions through time. This is supported by the observed differences in expression of granulocyte subpopulation-specific chemokines and growth factors between tadpole, early, and late metamorphic tails. The results from natural metamorphosis, together with comparable T₃-induced changes in tail granulocyte proportions and granulocyte chemokine gene expression, indicate granulocyte-lineage cells intimately participate in tail regression during *X. laevis* metamorphosis.

While we did not detect major morphological differences among granulocytes infiltrating metamorphosing animal tails, our previous studies indicate that *X. laevis* CXCL8a-, CXCL8b-, and G-CSF-recruited granulocytes may not be readily distinguished by cytology. Presently, we also observed that rCXCL8a and rCXCL8b recruited heterogeneous populations of granulocytes, predominated by neutrophil-like cells, but also some monocytes/macrophages. Nonetheless, these subpopulations do differ in their gene expression and functionalities [15,26]. Thus, as supported by our peritoneal lavage studies, several granulocyte subtypes likely extravasate into regressing tails with unique temporal and spatial patterns. We previously demonstrated that CXCL8a recruits fairly archetypal granulocytes with canonical inflammatory features, and which serve antimicrobial roles [15,26]. In contrast, CXCL8b-recruited granulocytes have characteristics of and are functionally associated with immune suppression and tissue remodeling [15,26]. The

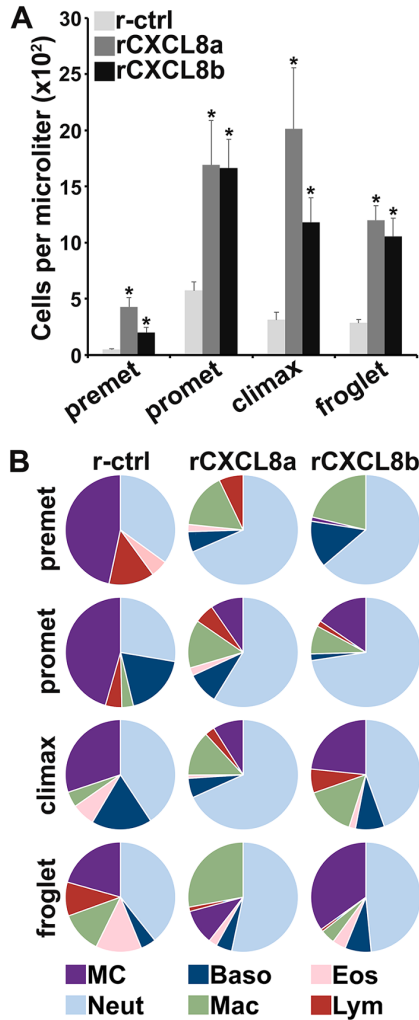


Fig. 4. Peritoneal administration of rCXCL8a and rCXCL8b to developing *X. laevis* results in recruitment of mixed granulocyte populations. Outbred mixed-sex pre-metamorphic tadpoles (premet; NF 54), pro-metamorphic tadpoles (promet; NF 61), tadpoles in climax of metamorphosis (climax; NF 64), and post-metamorphic *X. laevis* froglets ($N = 6$ per developmental stage) were injected with rCXCL8a or rCXCL8b ($1 \mu\text{g/g}$ body weight). Cells were collected 4 h later by lavage, counted, and pooled by treatment for cytological analysis. (A) Means $\pm$ SE ($N = 6$) of isolated leukocyte cell counts. Asterisks (*) denote statistical differences between r-ctrl and rCXCL8a or rCXCL8b treatment groups, $p < 0.05$. (B) Proportions of discernable immune subtypes within isolated peritoneal leukocytes. MC: mast cells; Baso: basophil; Eos: eosinophils; Neut: neutrophils; Mac: monocytes/macrophages; Lym: lymphocytes.

spatiotemporal patterns of granulocyte tail infiltration during metamorphosis are likely to be more nuanced than the rather heterogeneous subtypes experimentally recruited by rCXCL8a and rCXCL8b into peritonea of developing animals. We anticipate that the distinct expression kinetics of *cxcl8a* and *cxcl8b* genes in regressing *X. laevis* tails coincide with very specific and dynamic gradients of these chemokines, serving to recruit very targeted and controlled numbers and population(s) of granulocytes and likely other leukocytes.

It is exceedingly evident the endocrine and immune systems are profoundly interconnected, and we suspected there are additional dimensions to the relationships between *X. laevis* granulocytes and thyroid hormone. Importantly, thyroid hormone is generally produced in its prohormone form (T_4), which can then be converted to the active T_3

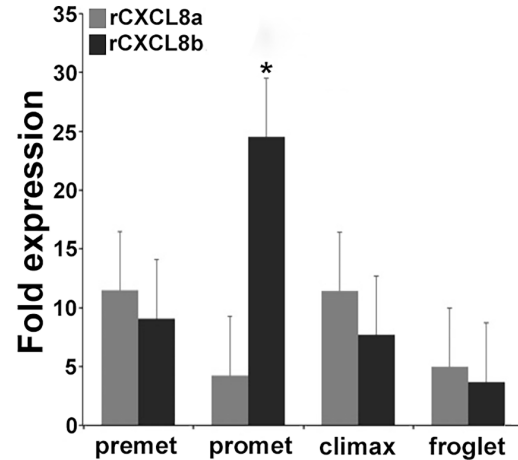


Fig. 5. Pro-metamorphic rCXCL8b-attracted granulocytes express a primary T_3 -activating enzyme, *dio2*. Peritoneum-derived rCXCL8a and rCXCL8b granulocytes were compared across *X. laevis* life stage (premet, NF 54; promet, NF 61; climax, NF 64; post-metamorphic froglets) for expression of the T_3 -activating enzyme, *dio2*. The gene expression of *dio2* was compared relative to the *gapdh* housekeeping gene. Results are means $\pm$ SE ($N = 6$); asterisk (*) denotes statistical difference between chemokine and across life stage, $p < 0.05$.

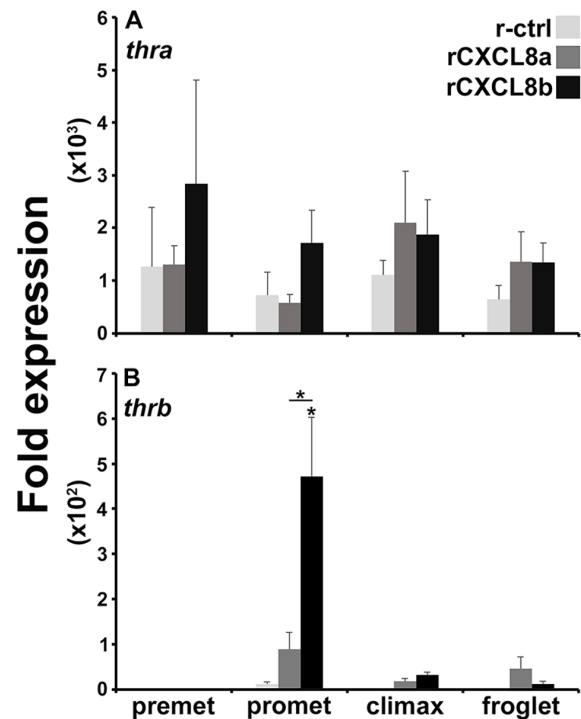


Fig. 6. Pro-metamorphic tadpole rCXCL8b-attracted granulocytes express greater levels of thyroid hormone receptor b. Peritoneum-derived rCXCL8a and rCXCL8b granulocytes were compared across *X. laevis* life stage (premet, NF 54; promet, NF 61; climax, NF 64; post-metamorphic froglets) for expression of (A) thyroid hormone receptor a (*thra*) and (B) *thrb*. All gene expression was compared relative to the *gapdh* housekeeping gene. Results are means $\pm$ SE ($N = 6$); asterisk (*) denotes statistical difference from r-ctrl group for the corresponding life stage; asterisk over a bar (*) indicates statistical differences between the treatment groups indicated by the bar, $p < 0.05$.

form by deiodinase enzymes. Deiodinase-2 (dio2) is responsible for the local conversion of T₄ to T₃ [31]. Interestingly, dio2 also functions in tissue remodeling during skeletal muscle regeneration in a T₃-dependent manner, albeit in stem cells [39]. Our results indicate rCXCL8b-recruited granulocytes derived from early metamorphic *X. laevis* robustly express *dio2* as well the thyroid hormone receptor b (*thrb*) but not *thra*. Although *dio2* is primarily expressed in mammalian macrophages, monocytes, and dendritic cells [31], it is intuitive that tissue-remodeling *X. laevis* CXCL8b-recruited granulocytes would be major sources of *dio2*. Future research of these processes will likely yield greater understanding into the functional significance of *thrb* but not *thra* expression by CXCL8b-recruited cells.

While rCXCL8b-elicited peritoneal leukocytes were predominantly composed of granulocytes and chiefly neutrophil-like cells, we did observe cells with monocyte/macrophage morphology within these recruited populations. As we do not currently have the means to separate these sub-populations, we cannot say with certainty which of the immune subtypes within these rCXCL8b-recruited leukocytes were responsible for the observed *dio2* and *thrb* expression.

Expression analyses of key granulocyte proinflammatory genes in regressing tails offer further insight into the immune status of this tissue across metamorphosis. Genes associated with cyclooxygenase (cyclooxygenase-2) and lipoxygenase (Leukotriene A4 hydrolase, Arachidonate 5-lipoxygenase) pathways were differentially expressed in distinct phases of metamorphosis. Leukotriene A4 hydrolase activates a potent neutrophil chemoattractant (i.e., LTB₄) [40], presumably contributing to the observed granulocyte accumulation within the regressing tail tissues. It is likewise noteworthy the *cox-2* gene product, though typically associated with inflammation induction, is also critically important for neural development and adaption (reviewed in [41]). Thus, in the context of anuran tail regression, elevated *cox-2* may contribute to the notochord degeneration and spinal cord remodeling that occurs [42].

The anatomist J.F. Gundersnatsch first recorded that thyroid extracts could induce the transition from tadpole to frog in the early 20th century [10]. In the decades since his studies, much has been learned about the mechanisms involved in this process across the animal kingdom. Indeed, development driven by T₃ is a highly conserved phenomenon [2]. However, the precise action of species-specific immune cell components linked to T₃ is poorly understood. Given both the importance of the immune system in remodeling tissue during development and the alarming increase of endocrine-disrupting chemicals in the environment, it is critically necessary to understand both systems and how they function together [43].

CRedit authorship contribution statement

Kelsey A. Hauser: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Muhammad R.H. Hossainey:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Dustin T. Howard:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Daphne V. Koubourli:** Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Namarta Kalia:** Methodology, Investigation, Formal analysis, Data curation. **Leon Grayfer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cirep.2024.200139.

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