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Amphibian (*Xenopus laevis*) Macrophage Subsets Vary in Their Responses to the Chytrid Fungus *Batrachochytrium dendrobatidis*

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Abstract: The chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), infects amphibian skin, causing chytridiomycosis, which is a contributing cause of worldwide declines and extinctions of amphibians. Relatively little is known about the roles of amphibian skin-resident immune cells, such as macrophages, in these antifungal defenses. Across vertebrates, macrophage differentiation is controlled through the activation of colony-stimulating factor-1 (CSF1) receptor by CSF1 and interleukin-34 (IL34) cytokines. While the precise roles of these respective cytokines in macrophage development remain to be fully explored, our ongoing studies indicate that frog (*Xenopus laevis*) macrophages differentiated by recombinant forms of CSF1 and IL34 are functionally distinct. Accordingly, we explored the roles of *X. laevis* CSF1- and IL34-macrophages in anti-Bd defenses. Enriching cutaneous IL34-macrophages, but not CSF1-macrophages, resulted in significant anti-Bd protection. In vitro analysis of frog macrophage-Bd interactions indicated that both macrophage subsets phagocytosed Bd. However, IL34-macrophages cocultured with Bd exhibited greater pro-inflammatory gene expression, whereas CSF1-macrophages cocultured with Bd showed greater immunosuppressive gene expression profiles. Concurrently, Bd-cocultured with CSF1-macrophages, but not IL34-macrophages, possessed elevated expression of genes associated with immune evasion. This work marks a step forward in our understanding of the roles of frog macrophage subsets in antifungal defenses.

Keywords: *Batrachochytrium dendrobatidis*; chytridiomycosis; macrophage; antifungal; myelopoiesis; amphibian immune response; CSF1; IL34



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1. Introduction

The chytrid fungus *Batrachochytrium dendrobatidis* (Bd) is a leading cause of worldwide amphibian declines and extinctions [1–5]. The swimming zoospore stage colonizes the keratinized skin of adult amphibians and the keratinized mouthparts of tadpoles [1], forming germination tubes [6–8], and sending their contents into epidermal cells [7,8] to mature. The invading zoospores develop into urn-shaped zoosporangia in which new zoospores develop [1–3] and swim out to infect other areas of the skin or new hosts [6]. Bd infections remain confined to the amphibian skin [1–3,6], causing death by interfering with the essential ion transport across the skin, thereby leading to cardiac arrest [9–11].

Fungal pathogens typically have complex interactions with their hosts, and while host immune defenses may keep these pathogens at bay [12], many fungi have evolved

elaborate immune evasion strategies [13–15]. *Bd* is likewise believed to be capable of substantial immune evasion [1,3,16]. In turn, considerable research has shown that both the innate and adaptive arms of amphibian immunity are engaged during *Bd* infections [16–29]. Some notable hallmarks of amphibian anti-*Bd* defenses include antifungal products from symbiotic bacteria [27,30–36]; mucus and granular gland secretions containing antimicrobial peptides [16,23,28,29,37–40]; alkaloids [41–43]; and lysozymes [44,45]; as well as antibodies [33]. However, we know almost nothing about the contribution of skin-resident sentinel immune cells, such as macrophages (Mφs), to anti-*Bd* defenses.

Mφ-lineage immune cells are important to vertebrate immune responses, playing key roles in tissue surveillance, pathogen detection, and coordinating ensuing immune responses [46,47]. In mammals, Mφ-lineage cells known as Langerhans cells are critical to immune surveillance of skin epithelia [47], and Langerhans-like cells have been described in amphibians [48]. Relatively little is known about amphibian Mφ-lineage populations, and reagents to distinguish amphibian immune subsets corresponding to mammalian Mφ subtypes, such as Langerhans cells, are lacking. While numerous distinct Mφ-lineage subsets have been described [49,50], the differentiation and functionality of all vertebrate Mφs depend on colony-stimulating factor-1 receptor (CSF-1R), which is ligated by CSF-1 and interleukin-34 (IL34) cytokines [51]. IL34, but not CSF1, additionally ligates protein-tyrosine phosphatase zeta (PTPζ) and CD138 (Syndecan-1, reviewed in [52]). A growing body of literature indicates that Mφs differentiated by CSF1 and IL34 possess non-overlapping phenotypes and roles (reviewed in [52,53]). Our studies in the *Xenopus laevis* frog likewise indicate that Mφs differentiated by recombinant (r)CSF-1 and rIL-34 possess markedly distinct capacities to recognize and respond to disparate pathogens [54,55]. Moreover, *X. laevis* possess a substantial expression of both *csf1* and *il34* genes in their skin [56], indicating that both CSF-1- and IL-34-Mφs reside therein.

In this study, we investigated the distinct roles of *Xenopus laevis* CSF1- and IL34-differentiated Mφs during *Bd* infection, using both in vivo and in vitro approaches to assess their contribution to antifungal defense and host–pathogen interactions.

2. Materials and Methods

2.1. Animals

Adult *Xenopus laevis* (~1–2-year old) were purchased from Xenopus1 (Dexter, MI, USA). The animals were housed and handled under strict laboratory regulations of the Animal Research Facility at the George Washington University (GWU) per the GWU Institutional Animal Care and Use Committee regulations (approval number A2023-046).

2.2. *Batrachochytrium Dendrobatidis* JEL197

The *Bd* isolate JEL197 was cultured in 1% tryptone broth, supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Waltham, MA, USA) at 20 °C for 3–7 days. For in vivo infection studies, *Bd* cultures were filtered through sterile coffee filters to isolate and enumerate zoospores. For *Bd*-Mφ coculture, *Bd* thalli were enumerated by the trypan blue exclusion method and cocultured at ratios of 5 *Bd* thalli per Mφ.

2.3. Recombinant Cytokine Production

Recombinant (r)CSF1 and rIL34 were produced using previously described approaches [55]. Briefly, signal-peptide-cleaved transcripts corresponding to *X. laevis* CSF1 or IL34 were PCR-amplified and ligated into the pMIB/V5 His A insect expression vector (Invitrogen, Waltham, MA, USA) and used to transfect Sf9 insect cells with the transfection reagent cellfectin II (Invitrogen). Western blot analysis against the V5 epitope was performed on the supernatants from transfected Sf9 cells to confirm the expression of rCSF1

or rIL34, following the selection of positive transfectants using 10 µg/mL blasticidin. The cultures were then scaled up to 500 mL and grown until confluent (5–7 million cells/mL). The confluent cultures were pelleted to obtain supernatants, which were concentrated against polyethylene glycol flakes (8 kDa) at 4 °C to 100 mL volume. The concentrated supernatants were dialyzed overnight at 4 °C against 150 mM sodium phosphate, pH 8. Recombinant cytokines were isolated from the dialyzed supernatants via Ni-NTA agarose columns (Qiagen, Germantown, MD, USA). Bound proteins were washed six times with low-stringency wash buffer (0.5% Tween 20, 50 nM sodium phosphate, 500 mM sodium chloride, 40 mM imidazole). The recombinant CSF1 and IL34 were eluted in fractions with the elution buffer (as above, but with 250 mM imidazole), which were pooled and stored as aliquots at −20 °C. Protein concentrations were quantified by BCA (ThermoFisher, Waltham, MA, USA) assays. The purity of the recombinants was tested by Coomassie stains of proteins resolved on SDS-gels and Western blot against the V5 epitope (Figure S1).

The recombinant control (rctrl) was generated by transfecting Sf9 cells with an empty pMIB/V5 His A insect expression vector (Invitrogen), selecting positive transfectants with blasticidin, scaling up these cultures and processing the derived supernatants using the same approaches described above for rCSF1 and rIL34 production.

2.4. Subcutaneous Administration of rCSF1 or rIL34 and In Vivo Bd Challenge

To enrich cutaneous frog Mφs, the adult *X. laevis* were injected subcutaneously once between the dorsal skin and underlining muscle layer with rCSF1, rIL34 (5 µg in 20 µL APBS per animal), or equal volumes of the rctrl ($N = 4$ per treatment group). After 3 days, the animals were sacrificed by MS-222 overdose followed by decapitation, and their skins were isolated and fixed in 10% neutral buffered formalin. The skins were processed and embedded in paraffin, sectioned (5 µm thickness) by the GWU Pathology Core. The sections were then stained with α-Naphthyl Acetate (Non-Specific Esterase; Sigma, Darmstadt, Germany), according to the manufacturer's instructions, and optimized to frog tissues (diluted hematoxylin solution 1/3 in APBS) to visualize cutaneous Mφs.

After confirming Mφ enrichment quantitatively, the frogs were again injected dorsally with rCSF1, rIL34 (5 µg in 20 µL APBS/animal, based on prior work deriving peritoneal Mφs [56]) or equal volumes of the rctrl ($N = 7$ per treatment group, based on prior experiments of a similar nature), and after 3 days, the animals were challenged with Bd by water bath (10^7 Bd zoospores in 500 mL water). After 7 days of Bd-exposure (without water change), the animals were sacrificed, and their dorsal skins were excised and flash-frozen over dry ice in Trizol (Invitrogen) for later DNA isolation.

2.5. Bone Marrow-Derived Mφ Cultures and In Vitro Mφ-Bd Challenge

Amphibian serum-free (ASF) [57] medium supplemented with 10% fetal bovine serum, 0.25% *X. laevis* serum, 10 µg/mL gentamycin (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) was used for all Mφ cultures. Amphibian phosphate-buffered saline (APBS) has been described [37].

Frog bone-marrow-derived CSF1- and IL34-Mφ cultures were established using previously described methods [55]. Briefly, femur bones from the adult *X. laevis* were flushed with saline, and the bone marrow cells were enumerated by the trypan blue exclusion method and seeded at a density of 10,000 cells/well in a 96-well culture plate. Cells were treated with 250 ng/mL of either recombinant CSF1 or IL34 to generate CSF1- or IL34-Mφs, respectively, and incubated at 27 °C with 5% CO₂. After 5 days of culture [58], the cells were challenged with developing Bd thalli (5 fungal cells per Mφ) or mock challenged.

To assess Mφ responses when in coculture with Bd, CSF1- or IL34-Mφ cultures, generated as above, were incubated alone or with Bd (5 Bd cells/Mφ, based on preliminary

data) for 5 h before being collected for microscopy, RNA isolation (Trizol, Invitrogen), cDNA synthesis, and quantitative gene expression analyses.

2.6. Quantitative Gene Expression and Bd Load Analyses

The total RNA from the frog skin tissues, in vitro M ϕ cultures, and M ϕ -Bd cocultures were isolated using a Trizol RNA extraction protocol. For all experiments, the tissues and cells were collected into Trizol reagent (Invitrogen) and homogenized by passage through progressively higher gage needles. After homogenization, 500 ng of total RNA was used for cDNA synthesis using cDNA qscript supermix (Quantabio, Beverly, MA, USA), according to the manufacturer's instructions. The CFX96 Real-Time System and iTaq Universal SYBR green supermix (Quantabio) were used for qPCR analyses using the $\Delta\Delta CT$ method. All gene expression was examined relative to the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) endogenous control. All primers were validated prior to use. All primer sequences are provided in Supplemental Table S1.

DNA was isolated from the Trizol following RNA isolation. In brief, following phase separation and RNA extraction, back-extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1 M Tris pH 8.0) was added to the remaining Trizol layers, and the mixtures were centrifuged to isolate the DNA-containing aqueous phase. The DNA was precipitated overnight against isopropanol at -20°C , pelleted by centrifugation, washed with 70% ethanol, and resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). The DNA was further purified by phenol–chloroform extraction and resuspended in molecular-grade water.

Bd loads were quantified as previously described [59]. In brief, a standard curve was prepared by serial dilutions of Bd (JEL 197) zoospore DNA. This standard curve was used in absolute qPCR reactions, with 12 μg total input DNA per sample and using previously validated primers against the Bd ribosomal RNA internal transcribed spacer 1 (ITS1; Supplementary Table S1). All qPCR assays were performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and all experiments were analyzed via a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) and BioRad CFX Manager software (SDS, V 3.1).

2.7. Electron Microscopy

Electron microscopy was performed to visualize differences between the two M ϕ subsets with respect to the morphology and phagocytosis of Bd. The processing and imaging of the cells by transmission and scanning electron microscopy (TEM and SEM) was performed at the GWU Nanofabrication and Imaging Center (GWNIC). For TEM, the M ϕ or M ϕ -Bd cocultures were fixed as monolayers on six-well plates with 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer for one hour. The cells were treated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h. Following washes, the cells were *en bloc*-stained with 1% uranyl acetate in water overnight at 4°C . The samples were dehydrated via an ethanol series and embedded in epoxy resin using LX112. Inverted Beem capsules were placed into each tissue culture well to create *on face* block-faces for sectioning. Resin was cured for 48 h at 60°C . The 95 nm sections were post-stained with 1% aqueous uranyl acetate and Reynold's lead citrate. All imaging was performed at 80 KV in a Talos 200X transmission electron microscope (Thermo Fisher Scientific, Hillsboro, OR, USA).

For SEM, the M ϕ cultures were fixed with 2.5% glutaraldehyde/1% paraformaldehyde in sodium cacodylate buffer, followed by 1% OsO_4 , then dehydrated through an ethyl alcohol series. The coverslips were critical-point-dried and coated with 2 nm iridium. The cells were imaged using a Teneo Scanning Electron Microscope (Thermo Fisher Scientific).

2.8. Statistical Analyses

Statistical analyses were performed using GraphPad Prism 7.0 software. Data sets were assessed by one-way ANOVAs followed by post hoc Tukey's tests for multiple comparisons, and p -values < 0.05 were deemed as statistically significant.

3. Results

3.1. *X. laevis* IL34-Mφs, but Not CSF1-Mφs, Confer Anti-Bd Resistance In Vivo

Here, we explored the roles of *X. laevis* CSF1- and IL-34-Mφs in anti-Bd defenses. To this end, we enriched these subsets in *X. laevis* skins by subcutaneous rCSF-1 and rIL34 injections. Compared to the animals injected with the rctrl, the subcutaneous administration of rCSF1 and rIL34 resulted in significant enrichment of esterase-positive Mφs in the epidermal *X. laevis* skin (Figure 1A–D). Following challenge with Bd, the frogs with enriched IL34-Mφs possessed significantly lower fungal loads, whereas the animals enriched with CSF1-Mφs had skin Bd loads comparable to those seen in the rctrl-administered animals (Figure 1E). Irrespective of treatment, the skins of all the Bd-infected animals exhibited hallmark epidermal thickening, often accompanied by the presence of discharged zoospores (Figure 1F).

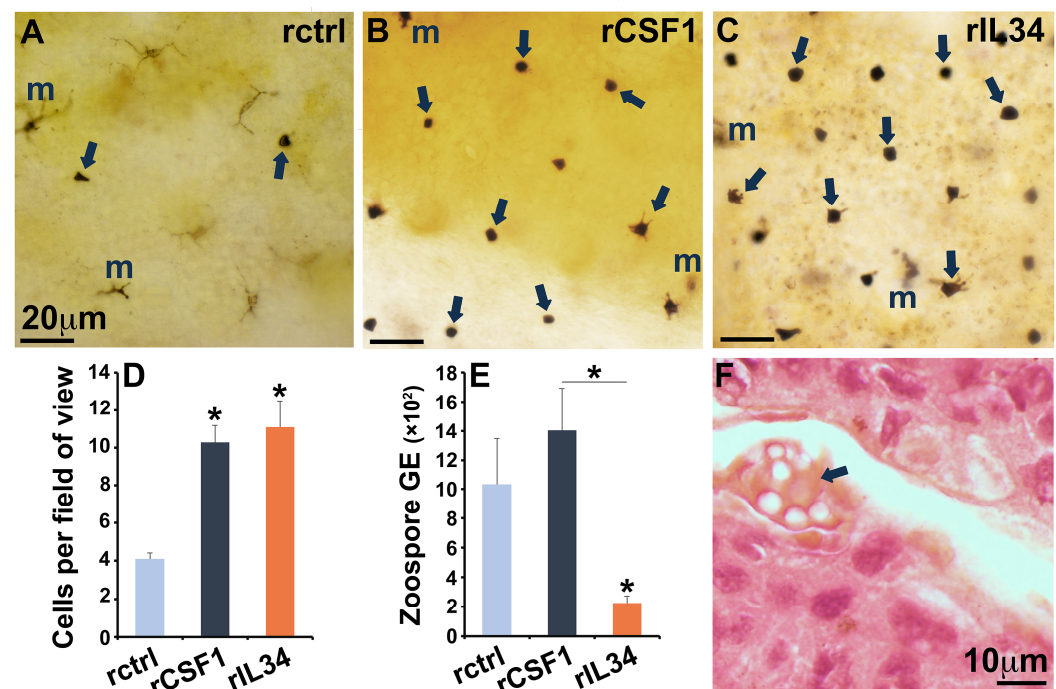


Figure 1. Enrichment of cutaneous CSF1- and IL34-Mφs and Bd challenge. Non-specific esterase (NSE) stain of epidermal skin layers from the (A) rctrl- and (B) rCSF1- and (C) rIL34-administered frogs. Arrows indicate myeloid (Mφs) cells, and melanocytes are denoted by 'm'. Images are representative of 4 animals per treatment group ($N = 4$). (D) Means $\pm$ SEMs of the number of NSE-positive cells per field of view of skin from frogs 3 days after subcutaneous administration of the rctrl or rCSF1 or rIL34 ($N = 4$ frogs/treatment group). (E) Bd loads (zoospore genomic equivalents, GE) in dorsal skins of frogs 3 days after control (rctrl)-, CSF1-, and IL34-Mφ-enrichment followed by 7 days of Bd challenge ($N = 7$ frogs/treatment group). (F) H&E stain of Bd (arrow)-infected skin. Asterisks above bars indicate statistical significance from the rctrl groups, and asterisks above lines are indicative of statistical differences between the treatment groups denoted by the lines, $p < 0.05$.

3.2. Both CSF1- and IL34-Mφs Phagocytose Bd In Vitro

To study the biology of amphibian Mφs, we previously optimized methods for generating *X. laevis* CSF1- and IL34-Mφ cultures in vitro from bone marrow-derived Mφ

precursors [58]. Presently, we used these cultures to discern how the frog M ϕ cultures respond to Bd. Our SEM and TEM analyses of the *X. laevis* CSF1- and IL34-M ϕ s corroborated our previous cytological comparisons [60], marking a striking difference in the morphologies of these two subsets (Figure 2A,B,F,G). Whereas the CSF1-M ϕ s appeared larger with substantially more ruffled membranes, the IL34-M ϕ s were relatively smaller and possessed many dendritic cell (DC)-like projections, consistent with their close relationship to frog DCs [61]. When we co-incubated CSF1- and IL34-M ϕ s with Bd, we found that both M ϕ populations were equally capable of ingesting and destroying Bd zoospores and zoosporangia (CSF1-M ϕ s: Figure 2C–E; IL34-M ϕ s: Figure 2H–J).

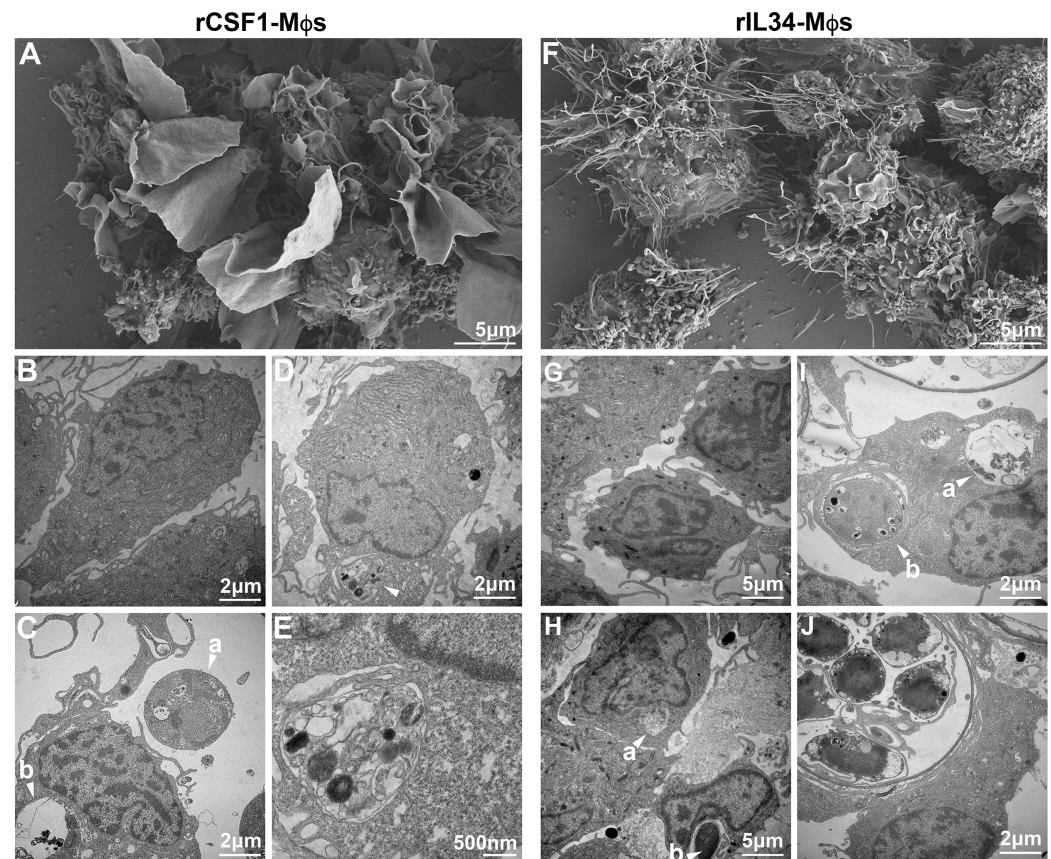


Figure 2. Scanning and transmission electron microscopy images of (A–E) CSF1- and (F–J) IL34-M ϕ s. CSF1- and IL34-M ϕ s were incubated alone (A,B and F,G, respectively) or with developing Bd thalli (C–E and H–J, respectively) for 5 h (5 Bd/M ϕ) before preparation and electron microscopy analyses. (A) SEM of CSF1-M ϕ s. (B) TEM of CSF1-M ϕ s. (C) TEM of CSF1-M ϕ s co-incubated with Bd. Arrows: (a) non-phagocytosed zoosporangium; (b) phagocytosed and degraded zoosporangium. (D) TEM of CSF1-M ϕ s co-incubated with Bd. Arrows: phagocytosed zoosporangium. (E) Higher magnification of zoosporangium-containing phagosome in (D). (F) SEM of IL34-M ϕ s. (G) TEM of IL34-M ϕ s. (H) TEM of IL34-M ϕ s co-incubated with Bd. Arrows: (a) phagocytosed Bd zoospore in the process of being degraded; (b) phagocytosed zoospore. (I) TEM of IL34-M ϕ s co-incubated with Bd. Arrows: (a) phagocytosed, degraded zoosporangium; (b) phagocytosed zoosporangium. (J) Higher magnification of mature zoosporangium-containing phagosome.

3.3. CSF1- and IL34-M ϕ s Cocultured with Bd Exhibit Disparate Polarization States

We next examined how Bd challenge affects the CSF1- and IL34-M ϕ expression of hallmark immune genes. Bd did not significantly alter the expression of NADPH oxidase catalytic domain genes (*nox2*, *nox2*) in either M ϕ subset but resulted in significantly increased inducible nitric oxide synthase (*inos*, catalyzes reactive nitrogen production) gene expression in both M ϕ subtypes (Figure 3). Notably, Bd elicited significantly increased

expression of the arginase-1 (*arg1*) and indoleamine 2,3 dioxygenase (*ido*) genes, which are archetypal of immunosuppressive Mφs [62], in CSF1-Mφs but not IL34-Mφs (Figure 3). Moreover, while Bd elicited increased expression of genes encoding the pro-inflammatory tumor necrosis factor (*tnf*) and the immunosuppressive interleukin-10 (*il10*) cytokines in both Mφ types, IL34-Mφs had significantly greater transcript levels for *tnf*, while CSF1-Mφs possessed significantly greater mRNA levels of *il10* (Figure 3).

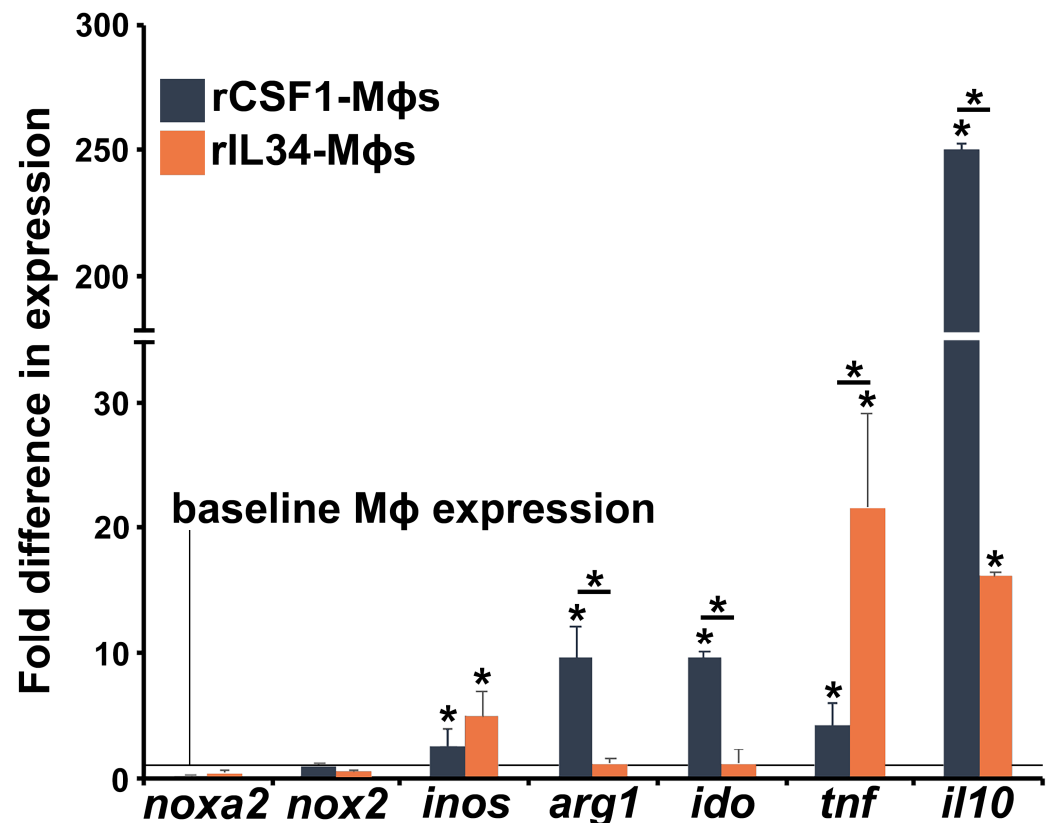


Figure 3. Consequences of Bd exposure on CSF1- and IL34-Mφ activation. CSF1- and IL34-Mφs were incubated with developing Bd thalli for 5 h (5 Bd/Mφ), and the cocultures were examined for their expression of immune genes relative to *gapdh* endogenous control. All data are represented relative to parallel respective CSF1- and IL34-Mφ cultures, incubated without Bd, depicted by the horizontal line. The results are means $\pm$ SEMs of immune gene expression from 6 independent CSF1- and IL34-Mφ cultures, derived from 6 individual frogs, $N = 6$. Asterisks above bars indicate statistical significance between baseline and Bd-elicited Mφ gene expression, and asterisks above lines are indicative of statistical differences between the treatment groups denoted by the lines, $p < 0.05$. The examined frog Mφ genes included *noxa2* and *nox2*-NADPH oxidase catalytic domains; *inos*—inducible nitric oxide synthase; *arg1*—arginase-1; *ido*—indoleamine 2,3 dioxygenase; *tnf*—tumor necrosis factor, and *il10*—interleukin-10.

3.4. Bd Gene Expression Differs When in Coculture with CSF1- or IL34-Mφs

Bd cells produce a plethora of immunosuppressive compounds. Chiefly among these are kynurenine [63], culminating from indoleamine 2,3 dioxygenase (IDO)-mediated tryptophan breakdown [64], as well as methylthioadenosine (MTA) [62] and spermidine [65], which are generated during ornithine breakdown by ornithine decarboxylase (ODC) and by spermidine synthase [66]. Interestingly, Bd co-incubated with IL34-Mφs exhibited significantly decreased expression of its *ido* gene, suggesting less production of inhibitory kynurenines. Bd cocultured with CSF1-Mφs possessed significantly increased expression of the Bd arginase-encoding *arg* gene, potentially leading to greater production of ornithine and inhibitory MTA and spermidine. We did not see significant changes in the expression

levels of spermidine synthase (*srm*; Figure 4). Bd cocultured with CSF1-Mφs, but not IL34-Mφs, also possessed a greater expression of antioxidant enzyme genes, catalase (*cat*), and superoxide dismutase (*sdm*; Figure 4).

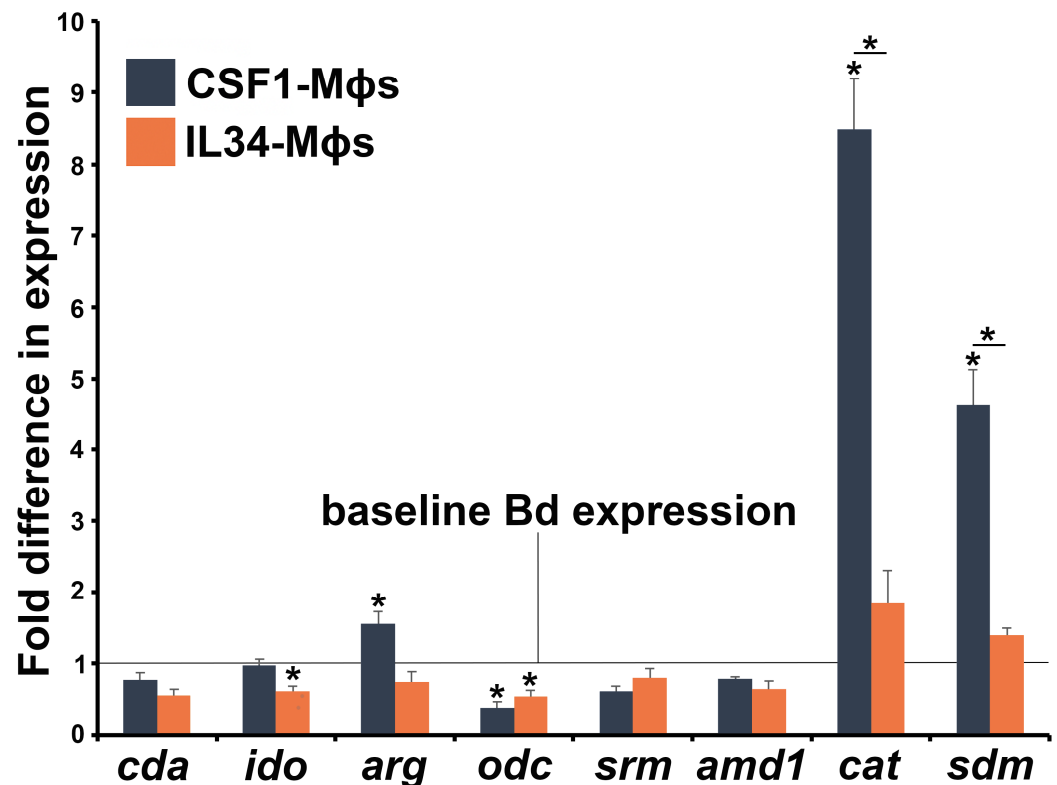


Figure 4. Consequences of Mφ-Bd coculture on Bd gene expression. Developing Bd thalli were incubated alone or in coculture with CSF1- and IL34-Mφs for 5 h (5 Bd/Mφ), and Bd was examined for expression of immune evasion genes relative to the Bd *gapdh* endogenous control. All data are represented relative to baseline Bd gene expression, depicted by the horizontal line. The results are means $\pm$ SEMs of Bd gene expression from 6 independent CSF1- and IL34-Mφ cocultures, with frog cells derived from 6 individual frogs, $N = 6$. Asterisks above bars indicate statistical significance between baseline and Mφ-elicited Bd gene expression, and asterisks above lines are indicative of statistical differences between the treatment groups denoted by the lines, $p < 0.05$. The examined Bd genes included *arg*—arginase; *amd*—adenosylmethionine decarboxylase; *cat*—catalase; *cda*—chitin deacetylase; *ido*—indoleamine 2,3 dioxygenase; *odc*—ornithine decarboxylase; *sdm*—superoxide dismutase; and *srm*—spermidine synthase.

4. Discussion

Tissue-resident Mφ-lineage cells serve as critical immune sentinels during fungal infections. They recognize fungal (among other) pathogens via a number of innate immune receptors, initiate antifungal responses, and directly eliminate the invading fungi through mechanisms such as phagocytosis and reactive radical-mediated clearance [67]. The CSF1R ligands, CSF1 and IL34 are important to the development of most if not all Mφ subsets [51]. With respect to cutaneous immunity, IL34 appears to be more critical to the maintenance of mammalian Langerhans cells under steady-state conditions [68], whereas CSF1 is believed to contribute to the differentiation of Langerhans cells during skin inflammatory responses [69], as reviewed in [52,53]. Langerhans-like cells have been described in several amphibian species [48,70,71]. These cells possess ATPase activity, express MHC class II, and stain positive with mammalian anti-langerin antibodies (CD207; hallmark marker of mammalian Langerhans cells [72]). Whether these cells are functional equivalents to mammalian Langerhans cells and what their roles are during Bd infections remains unexplored.

While we know that some of the functional dichotomy between these frog M ϕ subsets has been evolutionarily conserved, we also anticipate that the unique amphibian physiology will dictate key differences between frog CSF1- and IL34-M ϕ subsets and their mammalian counterparts. The subcutaneous administration of rCSF1 and rIL34 resulted in the enrichment of non-specific esterase-positive cells (marker of M ϕ -lineage) in frog epidermises, while in vitro-derived IL34-M ϕ s resembled mammalian dendritic cells, suggesting that as in mammals [68], the frog IL34 may play a role in the development of Langerhans-like cells at steady-state. Possibly, also akin to mammals [69], the frog CSF1 may be involved in generating Langerhans-like M ϕ s during inflammatory responses. It is worth mentioning that while the mammalian-specific anti-langerin antibody recognizes cells in amphibian skins [71], to our knowledge the gene encoding the amphibian version of this protein has not been identified. Moreover, other mammalian myeloid cells besides Langerhans cells express langerin [73–75]. As such, we refrain from calling the frog epidermal skin CSF1- or IL34-M ϕ s “Langerhans cells”, but in accordance with the findings presented here, we propose that these cell subsets play disparate roles during Bd infections of frog skin.

The continuum of M ϕ functional polarization is defined by the pro-inflammatory (M1) and the immunosuppressive (M2) polar extremes [62]. Our past work suggests that *X. laevis* CSF1-M ϕ s may be more inflammatory than IL34-M ϕ s in some contexts [55,76]. Moreover, there is a growing body of literature indicating that in mammals, IL34 is produced in tolerogenic contexts by cells such as T regulatory (Treg) cells [77] while mammalian IL34-M ϕ s adopt immunosuppressive phenotypes (reviewed in [53]). Conversely, our present work indicates that Bd coculture results in CSF1-M ϕ s adopting an M2-like transcriptional profile, including upregulated expression of hallmark M2 genes such as arginase-1 and interleukin-10. CSF1-M ϕ s cocultured with Bd also possessed increased gene expression of the indoleamine 2,3 dioxygenase (IDO) enzyme, which catalyzes tryptophan breakdown, the byproducts of which inhibit lymphocytes [78]. By contrast, Bd-challenged IL34-M ϕ s possessed significantly greater expression of the pro-inflammatory tumor necrosis factor cytokine, the mammalian counterpart of which is prominently involved in antifungal defenses [79]. There is the possibility that our in vitro M ϕ cultures do not reflect what happens to CSF1- and IL34-M ϕ s in vivo during Bd infections. However, our previous studies do indicate that these in vitro-derived CSF1- and IL34-M ϕ cultures recapitulate well the respective in vivo M ϕ subset responses to viral and mycobacterial pathogens [55,56,58,80]. Moreover, the more thoroughly characterized mammalian CSF1- and IL34-M ϕ polarization states are likewise context-dependent, and their respective pro- and anti-inflammatory phenotypes may be altered by additional immune stimuli (reviewed in [53]). Future time courses and ex vivo studies of these M ϕ subsets in the context of Bd infections will provide greater clarity for how the frog CSF1- and IL34-M ϕ s relate to their mammalian counterparts and how their activation states contribute to chytrid infection outcomes.

Phagocytosis and reactive oxygen responses are major mechanisms by which mammalian M ϕ s eliminate pathogenic fungi [67]. Both CSF1- and IL34-M ϕ s phagocytosed Bd zoospores in vitro, although only IL34-M ϕ s protected animals during in vivo infections. *X. laevis* IL34-M ϕ s possess significantly more robust reactive oxygen responses than CSF1-M ϕ s [56,58]. Coculture with Bd did not alter the IL34-M ϕ s gene expression of catalytic domains (*nox2*, *nox2*) of the NADPH oxidase complex responsible for ROS production. Conversely, following Bd-coculture, both frog M ϕ subsets upregulated their gene expression of iNOS, which catalyzes reactive nitrogen intermediate (RNI) production. While reactive oxygen responses are more prominently associated with M ϕ -mediated clearance of fungal pathogens, there are some contexts in which reactive nitrogen responses contribute to M ϕ antifungal responses [67]. Our *X. laevis* M ϕ model will be useful in future

in vitro, ex vivo, and in vivo studies to discern the extent to which M ϕ ROI, RNI, and other responses contribute to Bd clearance.

Akin to other fungal pathogens, Bd possesses many immune evasion strategies [81]. Amongst these, Bd produces immunosuppressive metabolites such as kynurenine, methylthioadenosine, and spermidine [63,65], catalyzed by the Bd-encoded indoleamine 2,3 dioxygenase, arginase, and ornithine decarboxylase enzymes, respectively. Bd also encodes superoxide dismutase and catalase enzymes, which protect it from myeloid cell-derived reactive oxygen species. Bd co-incubated with CSF1-M ϕ s, but not with IL34-M ϕ s, upregulated its arginase, superoxide dismutase, and catalase gene expression. Conversely, Bd cocultured with IL34-M ϕ s, but not with CSF1-M ϕ s, downregulated its *ido* gene expression. Presumably, Bd-CSF1-M ϕ interactions facilitate fungal immune evasion and possibly manifest in exacerbated infections at later times to those examined here. This Bd-CSF1-M ϕ immune suppression is not surprising, as our previous work indicates that this frog M ϕ subset is likewise prone to immune suppression by the Frog Virus 3 ranavirus [80] and *Mycobacterium marinum* [80]. On the other hand, the in vitro findings that IL34-M ϕ s are relatively less susceptible to Bd immune suppression and adopt more inflammatory phenotypes are consistent with our observation that enriching these cells in vivo results in greater anti-Bd protection. It is difficult to speculate what cues alert Bd to upregulate its expression of immunomodulatory genes when in CSF1-M ϕ but not IL34-M ϕ coculture. The observed differences in Bd gene expression in response to the two M ϕ subsets suggest that this fungus possesses mechanisms for sensing and adapting to distinct leukocyte subtypes. This notion may prove to be useful in future efforts to prevent chytridiomycosis and the spread of this fungal pathogen.

5. Conclusions

Mammalian M ϕ -lineage cells represent a critical line of defense against fungal pathogens [67]. Through the present work, we show that the same is true of the *X. laevis* frog IL34-M ϕ s but not CSF1-M ϕ s. Further studies of amphibian CSF1- and IL34-M ϕ s will undoubtedly provide new perspectives on the immune cell contributions to Bd susceptibility and resistance, as well as the evolutionarily diverged and converging pathways of M ϕ antifungal immunity.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/jof11040311/s1>: Table S1: list of primer sequences. Figure S1: Isolation of rCSF1 and rIL34. Recombinant (r)CSF-1 and rIL-34 were produced using an Sf9 insect cell expression system. The recombinant CSF-1 and IL-34 were eluted in fractions. The fractions containing the protein were pooled and concentrated, denoted as [rCSF1] and [rIL34]. Proteins were resolved by SDS-gels and Western blot against the V5 epitope.

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Abbreviations

The following abbreviations are used in this manuscript:

<i>arg</i>	arginase
<i>amd</i>	adenosylmethionine decarboxylase
Bd	<i>Batrachochytrium dendrobatidis</i>
<i>cat</i>	catalase
<i>cmd</i>	chitin deacetylase
CSF1	colony-stimulating factor-1
CSF1R	colony-stimulating factor-1 receptor
H&E	hematoxylin and eosin
<i>ido</i>	indoleamine 2,3 dioxygenase
<i>il10</i>	interleukin-10
IL34	interleukin-34
<i>inos</i>	inducible nitric oxide synthase
Mφ	macrophage
<i>odc</i>	ornithine decarboxylase
rctrl	recombinant control
<i>sdm</i>	superoxide dismutase
SEM	scanning electron microscopy
<i>srn</i>	spermidine synthase
TEM	transmission electron microscopy
<i>tnf</i>	tumor necrosis factor

References

- Berger, L.; Speare, R.; Daszak, P.; Green, D.E.; Cunningham, A.A.; Goggin, C.L.; Slocombe, R.; Ragan, M.A.; Hyatt, A.D.; McDonald, K.R.; et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9031–9036. [\[CrossRef\]](#)
- Longcore, J.E.; Pessier, A.P.; Nichols, D.K. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* **1999**, *91*, 219–227. [\[CrossRef\]](#)
- Pessier, A.P.; Nichols, D.K.; Longcore, J.E.; Fuller, M.S. Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). *J. Vet. Diagn. Investig.* **1999**, *11*, 194–199. [\[CrossRef\]](#)
- Scheele, B.C.; Pasmans, F.; Skerratt, L.F.; Berger, L.; Martel, A.; Beukema, W.; Acevedo, A.A.; Burrowes, P.A.; Carvalho, T.; Catenazzi, A.; et al. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. *Science* **2019**, *363*, 1459–1463. [\[CrossRef\]](#)
- Skerratt, L.F.; Berger, L.; Speare, R.; Cashins, S.; McDonald, K.R.; Phillott, A.D.; Hines, H.B.; Kenyon, N. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* **2007**, *4*, 125–134. [\[CrossRef\]](#)
- Berger, L.; Hyatt, A.D.; Speare, R.; Longcore, J.E. Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* **2005**, *68*, 51–63. [\[CrossRef\]](#)
- Greenspan, S.E.; Longcore, J.E.; Calhoun, A.J. Host invasion by *Batrachochytrium dendrobatidis*: Fungal and epidermal ultrastructure in model anurans. *Dis. Aquat. Organ.* **2012**, *100*, 201–210. [\[CrossRef\]](#)

8. Van Rooij, P.; Martel, A.; D'Herde, K.; Brutyn, M.; Croubels, S.; Ducatelle, R.; Haesebrouck, F.; Pasmans, F. Germ tube mediated invasion of *Batrachochytrium dendrobatidis* in amphibian skin is host dependent. *PLoS ONE* **2012**, *7*, e41481. [\[CrossRef\]](#)
9. Voyles, J.; Young, S.; Berger, L.; Campbell, C.; Voyles, W.F.; Dinudom, A.; Cook, D.; Webb, R.; Alford, R.A.; Skerratt, L.F.; et al. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* **2009**, *326*, 582–585. [\[CrossRef\]](#)
10. Voyles, J.; Berger, L.; Young, S.; Speare, R.; Webb, R.; Warner, J.; Rudd, D.; Campbell, R.; Skerratt, L.F. Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Dis. Aquat. Organ.* **2007**, *77*, 113–118. [\[CrossRef\]](#)
11. Marcum, R.D.; St-Hilaire, S.; Murphy, P.J.; Rodnick, K.J. Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus (Bufo) boreas boreas*. *Dis. Aquat. Organ.* **2010**, *91*, 17–21. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Wuthrich, M.; Deepe, G.S., Jr.; Klein, B. Adaptive immunity to fungi. *Annu. Rev. Immunol.* **2012**, *30*, 115–148. [\[CrossRef\]](#)
13. Rizzetto, L.; Cavalieri, D. Friend or foe: Using systems biology to elucidate interactions between fungi and their hosts. *Trends Microbiol.* **2011**, *19*, 509–515. [\[CrossRef\]](#)
14. Collette, J.R.; Lorenz, M.C. Mechanisms of immune evasion in fungal pathogens. *Curr. Opin. Microbiol.* **2011**, *14*, 668–675. [\[CrossRef\]](#)
15. Cheng, S.C.; Joosten, L.A.; Kullberg, B.J.; Netea, M.G. Interplay between *Candida albicans* and the mammalian innate host defense. *Infect. Immun.* **2012**, *80*, 1304–1313. [\[CrossRef\]](#)
16. Woodhams, D.C.; Bell, S.C.; Kenyon, N.; Alford, R.A.; Rollins-Smith, L.A. Immune evasion or avoidance: Fungal skin infection linked to reduced defence peptides in Australian green-eyed treefrogs, *Litoria serrata*. *Fungal Biol.* **2012**, *116*, 1203–1211. [\[CrossRef\]](#)
17. Bataille, A.; Cashins, S.D.; Grogan, L.; Skerratt, L.F.; Hunter, D.; McFadden, M.; Scheele, B.; Brannelly, L.A.; Macris, A.; Harlow, P.S.; et al. Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation. *Proc. Biol. Sci.* **2015**, *282*, 20143127. [\[CrossRef\]](#)
18. Ellison, A.R.; Savage, A.E.; DiRenzo, G.V.; Lanhammer, P.; Lips, K.R.; Zumudio, K.R. Fighting a losing battle: Vigorous immune response countered by pathogen suppression of host defenses in the chytridiomycosis-susceptible frog *Atelopus zeteki*. *G3* **2014**, *4*, 1275–1289. [\[CrossRef\]](#)
19. Ellison, A.R.; Tunstall, T.; DiRenzo, G.V.; Hughey, M.C.; Rebollar, E.A.; Belden, L.K.; Harris, R.N.; Ibanez, R.; Lips, K.R.; Zamudio, K.R. More than skin deep: Functional genomic basis for resistance to amphibian chytridiomycosis. *Genome Biol. Evol.* **2014**, *7*, 286–298. [\[CrossRef\]](#)
20. Grogan, L.F.; Cashins, S.D.; Skerratt, L.F.; Berger, L.; McFadden, M.S.; Harlow, P.; Hunter, D.A.; Scheele, B.C.; Mulvenna, J. Evolution of resistance to chytridiomycosis is associated with a robust early immune response. *Mol. Ecol.* **2018**, *27*, 919–934. [\[CrossRef\]](#)
21. McMahon, T.A.; Sears, B.F.; Venesky, M.D.; Bessler, S.M.; Brown, J.M.; Deutsch, K.; Halstead, N.T.; Lentz, G.; Tenouri, N.; Young, S.; et al. Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature* **2014**, *511*, 224–227. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Pask, J.D.; Woodhams, D.C.; Rollins-Smith, L.A. The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. *Glob. Change Biol.* **2012**, *18*, 1231–1238. [\[CrossRef\]](#)
23. Rollins-Smith, L.A.; Carey, C.; Longcore, J.; Doersam, J.K.; Boutte, A.; Bruzgal, J.E.; Conlon, J.M. Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. *Dev. Comp. Immunol.* **2002**, *26*, 471–479. [\[CrossRef\]](#)
24. Rollins-Smith, L.A.; Ramsey, J.P.; Reinert, L.K.; Woodhams, D.C.; Livo, L.J.; Carey, C. Immune defenses of *Xenopus laevis* against *Batrachochytrium dendrobatidis*. *Front. Biosci.* **2009**, *1*, 68–91. [\[CrossRef\]](#)
25. Rollins-Smith, L.A.; Woodhams, D.C.; Reinert, L.K.; Vredenburg, V.T.; Briggs, C.J.; Nielsen, P.F.; Conlon, J.M. Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Dev. Comp. Immunol.* **2006**, *30*, 831–842. [\[CrossRef\]](#)
26. Savage, A.E.; Zamudio, K.R. MHC genotypes associate with resistance to a frog-killing fungus. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 16705–16710. [\[CrossRef\]](#)
27. Woodhams, D.C.; Vredenburg, V.T.; Simon, M.-A.; Billheimer, D.; Shakhtour, B.; Shyr, Y.; Briggs, C.J.; Rollins-Smith, L.A.; Harris, R.N. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* **2007**, *138*, 390–398. [\[CrossRef\]](#)
28. Woodhams, D.C.; Ardipradja, K.; Alford, R.A.; Marantelli, G.; Reinert, L.K.; Rollins-Smith, L.A. Resistance to chytridiomycosis varies by amphibian species and is correlated with skin peptide defenses. *Anim. Conserv.* **2007**, *10*, 409–417. [\[CrossRef\]](#)
29. Woodhams, D.C.; Rollins-Smith, L.A.; Carey, C.; Reinert, L.; Tyler, M.J.; Alford, R.A. Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. *Oecologia* **2006**, *146*, 531–540. [\[CrossRef\]](#)
30. Brucker, R.M.; Baylor, C.M.; Walters, R.L.; Lauer, A.; Harris, R.N.; Minbiole, K.P. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* **2008**, *34*, 39–43. [\[CrossRef\]](#)

31. Brucker, R.M.; Harris, R.N.; Schwantes, C.R.; Gallaher, T.N.; Flaherty, D.C.; Lam, B.A.; Minbiole, K.P. Amphibian chemical defense: Antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* **2008**, *34*, 1422–1429. [[CrossRef](#)] [[PubMed](#)]
32. Harris, R.N.; James, T.Y.; Lauer, A.; Simon, M.A.; Patel, A. Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* **2006**, *3*, 53–56. [[CrossRef](#)]
33. Harris, R.N.; Brucker, R.M.; Walke, J.B.; Becker, M.H.; Schwantes, C.R.; Flaherty, D.C.; Lam, B.A.; Woodhams, D.C.; Briggs, C.J.; Vredenburg, V.T.; et al. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* **2009**, *3*, 818–824. [[CrossRef](#)]
34. Kueneman, J.G.; Bletz, M.C.; McKenzie, V.J.; Becker, C.G.; Joseph, M.B.; Abarca, J.G.; Archer, H.; Arellano, A.L.; Bataille, A.; Becker, M.; et al. Community richness of amphibian skin bacteria correlates with bioclimate at the global scale. *Nat. Ecol. Evol.* **2019**, *3*, 381–389. [[CrossRef](#)]
35. Loudon, A.H.; Holland, J.A.; Umile, T.P.; Burzynski, E.A.; Minbiole, K.P.; Harris, R.N. Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Front. Microbiol.* **2014**, *5*, 441. [[CrossRef](#)]
36. Rebollar, E.A.; Bridges, T.; Hughey, M.C.; Medina, D.; Belden, L.K.; Harris, R.N. Integrating the role of antifungal bacteria into skin symbiotic communities of three Neotropical frog species. *ISME J.* **2019**, *13*, 1763–1775. [[CrossRef](#)]
37. Ramsey, J.P.; Reinert, L.K.; Harper, L.K.; Woodhams, D.C.; Rollins-Smith, L.A. Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infect. Immun.* **2010**, *78*, 3981–3992. [[CrossRef](#)]
38. Rollins-Smith, L.A. The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochim. Biophys. Acta* **2009**, *1788*, 1593–1599. [[CrossRef](#)]
39. Rollins-Smith, L.A. The importance of antimicrobial peptides (AMPs) in amphibian skin defense. *Dev. Comp. Immunol.* **2023**, *142*, 104657. [[CrossRef](#)]
40. Rollins-Smith, L.A.; Doersam, J.K.; Longcore, J.E.; Taylor, S.K.; Shamblin, J.C.; Carey, C.; Zasloff, M.A. Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Dev. Comp. Immunol.* **2002**, *26*, 63–72. [[CrossRef](#)]
41. Daly, J.W.; Spande, T.F.; Garraffo, H.M. Alkaloids from amphibian skin: A tabulation of over eight-hundred compounds. *J. Nat. Prod.* **2005**, *68*, 1556–1575. [[CrossRef](#)]
42. Macfoy, C.; Danosus, D.; Sandit, R.; Jones, T.H.; Garraffo, H.M.; Spande, T.F.; Daly, J.W. Alkaloids of anuran skin: Antimicrobial function? *Z. Naturforsch. C J. Biosci.* **2005**, *60*, 932–937. [[CrossRef](#)]
43. Rodriguez, C.; Rollins-Smith, L.; Ibanez, R.; Durant-Archibold, A.A.; Gutierrez, M. Toxins and pharmacologically active compounds from species of the family Bufonidae (Amphibia, Anura). *J. Ethnopharmacol.* **2017**, *198*, 235–254. [[CrossRef](#)]
44. Ostrovsky, D.S.; Snyder, J.A.; Iwata, T.; Izaka, K.I.; Maglott, D.S.; Nace, G.W. Frog lysozyme. I. Its identification, occurrence as isozymes, and quantitative distribution in tissues of the leopard frog, *Rana pipiens*. *J. Exp. Zool.* **1976**, *195*, 279–290. [[CrossRef](#)]
45. Savage, A.E.; Kiemnec-Tyburczy, K.M.; Ellison, A.R.; Fleischer, R.C.; Zamudio, K.R. Conservation and divergence in the frog immunome: Pyrosequencing and de novo assembly of immune tissue transcriptomes. *Gene* **2014**, *542*, 98–108. [[CrossRef](#)]
46. Gordon, S.; Taylor, P.R. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **2005**, *5*, 953–964. [[CrossRef](#)]
47. Hovav, A.H. Mucosal and Skin Langerhans Cells—Nurture Calls. *Trends Immunol.* **2018**, *39*, 788–800. [[CrossRef](#)]
48. Carrillo-Farga, J.; Castell, A.; Perez, A.; Rondan, A. Langerhans-like cells in amphibian epidermis. *J. Anat.* **1990**, *172*, 39–45.
49. Guiducci, C.; Vicari, A.P.; Sangaletti, S.; Trinchieri, G.; Colombo, M.P. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. *Cancer Res.* **2005**, *65*, 3437–3446. [[CrossRef](#)]
50. Sacconi, A.; Schioppa, T.; Porta, C.; Biswas, S.K.; Nebuloni, M.; Vago, L.; Bottazzi, B.; Colombo, M.P.; Mantovani, A.; Sica, A. p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res.* **2006**, *66*, 11432–11440. [[CrossRef](#)]
51. Stanley, E.R.; Berg, K.L.; Einstein, D.B.; Lee, P.S.; Pixley, F.J.; Wang, Y.; Yeung, Y.G. Biology and action of colony--stimulating factor-1. *Mol. Reprod. Dev.* **1997**, *46*, 4–10. [[CrossRef](#)]
52. Freuchet, A.; Salama, A.; Remy, S.; Guillonnet, C.; Anegon, I. IL-34 and CSF-1, deciphering similarities and differences at steady state and in diseases. *J. Leukoc. Biol.* **2021**, *110*, 771–796. [[CrossRef](#)]
53. Munoz-Garcia, J.; Cochonnet, D.; Teletchea, S.; Moranton, E.; Lanoe, D.; Brion, R.; Lezot, F.; Heymann, M.F.; Heymann, D. The twin cytokines interleukin-34 and CSF-1: Masterful conductors of macrophage homeostasis. *Theranostics* **2021**, *11*, 1568–1593. [[CrossRef](#)] [[PubMed](#)]
54. Yaparla, A.; Docter-Loeb, H.; Melnyk, M.L.S.; Batheja, A.; Grayfer, L. The amphibian (*Xenopus laevis*) colony-stimulating factor-1 and interleukin-34-derived macrophages possess disparate pathogen recognition capacities. *Dev. Comp. Immunol.* **2019**, *98*, 89–97. [[CrossRef](#)]
55. Yaparla, A.; Popovic, M.; Grayfer, L. Differentiation-dependent antiviral capacities of amphibian (*Xenopus laevis*) macrophages. *J. Biol. Chem.* **2018**, *293*, 1736–1744. [[CrossRef](#)]

56. Grayfer, L.; Robert, J. Divergent antiviral roles of amphibian (*Xenopus laevis*) macrophages elicited by colony-stimulating factor-1 and interleukin-34. *J. Leukoc. Biol.* **2014**, *96*, 1143–1153. [\[CrossRef\]](#)
57. Robert, J.; Abramowitz, L.; Gantress, J.; Morales, H.D. *Xenopus laevis*: A possible vector of Ranavirus infection? *J. Wildl. Dis.* **2007**, *43*, 645–652. [\[CrossRef\]](#)
58. Grayfer, L.; Robert, J. Distinct functional roles of amphibian (*Xenopus laevis*) colony-stimulating factor-1- and interleukin-34-derived macrophages. *J. Leukoc. Biol.* **2015**, *98*, 641–649. [\[CrossRef\]](#)
59. Hauser, K.A.; Garvey, C.N.; Crow, R.S.; Hossainey, M.R.H.; Howard, D.T.; Ranganathan, N.; Gentry, L.K.; Yaparla, A.; Kalia, N.; Zelle, M.; et al. Amphibian mast cells serve as barriers to chytrid fungus infections. *Elife* **2024**, *12*, RP92168. [\[CrossRef\]](#)
60. Yaparla, A.; Koubourli, D.; Popovic, M.; Grayfer, L. Exploring the relationships between amphibian (*Xenopus laevis*) myeloid cell subsets. *Dev. Comp. Immunol.* **2020**, *113*, 103798. [\[CrossRef\]](#)
61. Hossainey, M.R.H.; Hauser, K.A.; Garvey, C.N.; Kalia, N.; Garvey, J.M.; Grayfer, L. A perspective into the relationships between amphibian (*Xenopus laevis*) myeloid cell subsets. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2023**, *378*, 20220124. [\[CrossRef\]](#) [\[PubMed\]](#)
62. Grayfer, L.; Kerimoglu, B.; Yaparla, A.; Hodgkinson, J.W.; Xie, J.; Belosevic, M. Mechanisms of Fish Macrophage Antimicrobial Immunity. *Front. Immunol.* **2018**, *9*, 1105. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Rollins-Smith, L.A.; Fites, J.S.; Reinert, L.K.; Shiakolas, A.R.; Umile, T.P.; Minbiole, K.P. Immunomodulatory metabolites released by the frog-killing fungus *Batrachochytrium dendrobatidis*. *Infect. Immun.* **2015**, *83*, 4565–4570. [\[CrossRef\]](#)
64. Lashgari, N.A.; Roudsari, N.M.; Shayan, M.; Niazi Shahraki, F.; Hosseini, Y.; Momtaz, S.; Abdolghaffari, A.H. IDO/Kynurenine: novel insight for treatment of inflammatory diseases. *Cytokine* **2023**, *166*, 156206. [\[CrossRef\]](#)
65. Rollins-Smith, L.A.; Ruzzini, A.C.; Fites, J.S.; Reinert, L.K.; Hall, E.M.; Joosse, B.A.; Ravikumar, V.I.; Huebner, M.I.; Aka, A.; Kehs, M.H.; et al. Metabolites Involved in Immune Evasion by *Batrachochytrium dendrobatidis* Include the Polyamine Spermidine. *Infect. Immun.* **2019**, *87*, 10–1128. [\[CrossRef\]](#)
66. Deng, K.; He, H.; Qiu, J.; Lorber, B.; Bryson, J.B.; Filbin, M.T. Increased synthesis of spermidine as a result of upregulation of arginase I promotes axonal regeneration in culture and in vivo. *J. Neurosci.* **2009**, *29*, 9545–9552. [\[CrossRef\]](#)
67. Brown, G.D. Innate antifungal immunity: The key role of phagocytes. *Annu. Rev. Immunol.* **2011**, *29*, 1–21. [\[CrossRef\]](#)
68. Wang, Y.; Colonna, M. Interleukin-34, a cytokine crucial for the differentiation and maintenance of tissue resident macrophages and Langerhans cells. *Eur. J. Immunol.* **2014**, *44*, 1575–1581. [\[CrossRef\]](#)
69. Nakamichi, Y.; Udagawa, N.; Takahashi, N. IL-34 and CSF-1: Similarities and differences. *J. Bone Miner. Metab.* **2013**, *31*, 486–495. [\[CrossRef\]](#)
70. Castell-Rodriguez, A.E.; Hernandez-Penaloza, A.; Sampedro-Carrillo, E.A.; Herrera-Enriquez, M.A.; Alvarez-Perez, S.J.; Rondan-Zarate, A. ATPase and MHC class II molecules co-expression in *Rana pipiens* dendritic cells. *Dev. Comp. Immunol.* **1999**, *23*, 473–485. [\[CrossRef\]](#)
71. Lombardo, G.P.; Miller, A.; Aragona, M.; Messina, E.; Fumia, A.; Kuciel, M.; Alesci, A.; Pergolizzi, S.; Lauriano, E.R. Immunohistochemical Characterization of Langerhans Cells in the Skin of Three Amphibian Species. *Biology* **2024**, *13*, 210. [\[CrossRef\]](#) [\[PubMed\]](#)
72. Kubo, A.; Nagao, K.; Yokouchi, M.; Sasaki, H.; Amagai, M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J. Exp. Med.* **2009**, *206*, 2937–2946. [\[CrossRef\]](#)
73. Idoyaga, J.; Suda, N.; Suda, K.; Park, C.G.; Steinman, R.M. Antibody to Langerin/CD207 localizes large numbers of CD8alpha+ dendritic cells to the marginal zone of mouse spleen. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 1524–1529. [\[CrossRef\]](#)
74. Sparber, F.; Dolowschiak, T.; Mertens, S.; Lauener, L.; Clausen, B.E.; Joller, N.; Stoitzner, P.; Tussiwand, R.; LeibundGut-Landmann, S. Langerin+ DCs regulate innate IL-17 production in the oral mucosa during *Candida albicans*-mediated infection. *PLoS Pathog.* **2018**, *14*, e1007069. [\[CrossRef\]](#)
75. Xuan, S.; Li, Y.; Wu, Y.; Adcock, I.M.; Zeng, X.; Yao, X. Langerin-expressing dendritic cells in pulmonary immune-related diseases. *Front. Med.* **2022**, *9*, 909057. [\[CrossRef\]](#)
76. Yaparla, A.; Grayfer, L. Isolation and Culture of Amphibian (*Xenopus laevis*) Sub-Capsular Liver and Bone Marrow Cells. *Methods Mol. Biol.* **2018**, *1865*, 275–281. [\[CrossRef\]](#)
77. Ni, B.; Zhang, D.; Zhou, H.; Zheng, M.; Wang, Z.; Tao, J.; Han, Z.; Ju, X.; Tan, R.; Gu, M. IL-34 attenuates acute T cell-mediated rejection following renal transplantation by upregulating M2 macrophages polarization. *Heliyon* **2024**, *10*, e24028. [\[CrossRef\]](#)
78. Yeung, A.W.; Terentis, A.C.; King, N.J.; Thomas, S.R. Role of indoleamine 2,3-dioxygenase in health and disease. *Clin. Sci.* **2015**, *129*, 601–672. [\[CrossRef\]](#)
79. Fei, M.; Bhatia, S.; Oriss, T.B.; Yarlagadda, M.; Khare, A.; Akira, S.; Saijo, S.; Iwakura, Y.; Fallert Junecko, B.A.; Reinhart, T.A.; et al. TNF-alpha from inflammatory dendritic cells (DCs) regulates lung IL-17A/IL-5 levels and neutrophilia versus eosinophilia during persistent fungal infection. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 5360–5365. [\[CrossRef\]](#)

80. Popovic, M.; Yaparla, A.; Paquin-Proulx, D.; Koubourli, D.V.; Webb, R.; Firmani, M.; Grayfer, L. Colony-stimulating factor-1- and interleukin-34-derived macrophages differ in their susceptibility to *Mycobacterium marinum*. *J. Leukoc. Biol.* **2019**, *106*, 1257–1269. [[CrossRef](#)]
81. Rollins-Smith, L.A.; Le Sage, E.H. Batrachochytrium fungi: Stealth invaders in amphibian skin. *Curr. Opin. Microbiol.* **2021**, *61*, 124–132. [[CrossRef](#)] [[PubMed](#)]

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