Neutrophil contributions to the induction and regulation of the acute inflammatory response in teleost fish

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ABSTRACT

Neutrophils are essential to the acute inflammatory response, where they serve as the first line of defense against infiltrating pathogens. We report that, on receiving the necessary signals, teleost (Carassius auratus) neutrophils leave the hematopoietic kidney, enter into the circulation, and dominate the initial influx of cells into a site of inflammation. Unlike mammals, teleost neutrophils represent <5% of circulating leukocytes during periods of homeostasis. However, this increases to nearly 50% immediately after intraperitoneal challenge. In teleosts, a neutrophic phase (18 h after injection) precedes the peak influx of neutrophils into the circulation, and contributes to both the induction and the resolution of inflammation. Neutrophils, which precede the peak influx of neutrophils into the challenge site (18 h after injection), were shown to migrate through the sinusoids into the surrounding tissue, driving the inflammation. Neutrophils at the site of inflammation alter their phenotype throughout the acute inflammatory response, and contribute to both the induction and the resolution of inflammation. However, neutrophils isolated during the proinflammatory phase (18 h after injection) produced robust respiratory burst responses, released inflammation-associated leukotriene B4, and induced macrophages to increase reactive oxygen species production. In contrast, neutrophils isolated at 48 h after infection (proresolving phase) displayed lower levels of reactive oxygen species, released the proresolving lipid mediator lipoxin A4, and downregulated reactive oxygen species production in macrophages before the initiation of apoptosis. Lipoxin A4 was a significant contributor to the uptake of apoptotic cells by teleost macrophages and also played a role, at least in part, in the downregulation of macrophage reactive oxygen species production. Our results highlight the contributions of neutrophils to the promotion and the resolution of teleost fish inflammation and provide added context for the evolution of this hematopoietic lineage.


Introduction

Neutrophils are important innate effector cells that dominate the initial influx of leukocytes to a site of inflammation [1–3]. On activation within the inflammatory site, neutrophils become powerful killers, release toxic intracellular granules [4, 5], produce ROS [5, 6], and deploy neutrophil extracellular traps [5, 7]. This notion is consistent with the early descriptions of the microphage by Metchnikoff [8], which he identified as highly phagocytic leukocytes important in the defense against microorganisms. Historically, it has been these proinflammatory roles that have served as our focus for this immune cell; their contributions to the resolution of inflammation have been limited to apoptotic cell death and subsequent clearance by macrophages. However, more recently, we, and others, have shown that both human and murine neutrophils internalize ACs and actively contribute to the decreased production of ROS in other leukocytes [9–11]. Acquisition of this mechanism would presumably allow mammals a novel alternative for the control of inflammation.

In humans, the generation of neutrophils is a key activity of the hematopoietic compartment, in which approximately \(5 \times 10^6\) to \(10 \times 10^9\) new neutrophils are generated each day [11]. Among others, these contribute to the establishment of a storage pool of mature neutrophils within the bone marrow [12–14]. The mobilization and recruitment of neutrophils is dependent on signals that originate from the site of inflammation. A diverse panel of stimuli, including pathogen-associated molecular patterns and damage-associated molecular patterns activate the tissue-resident cells to release proinflammatory mediators (e.g., TNF-α, IFN-γ, and IL-1β) and neutrophil-specific chemottractants (e.g., CXCL-8) and lipid mediators (e.g., LTB4) [15–17]. Within minutes of tissue injury, neutrophils begin “rolling” along the walls of venules surrounding the affected site [18]. An interaction between

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neutrophil L-selectin and vascular E- and P-selectins then allows for extravasation [18]. These mediators also activate neutrophils, increasing their capacity for phagocytosis and antimicrobial effector mechanisms on recruitment to the inflammatory site [19].

The characterization of immune cell populations in a range of animal models suggests that neutrophils arose among the osteichthyans about 420 million years ago, before the divergence of the last common ancestor for ray- and lobe-finned fish [20–22]. Because teleosts represent the most basal animal group to contain bona fide neutrophils, they represent a viable platform to better understand the origins of their contributions to inflammation. However, significant gaps remain regarding the effector and potential regulatory contributions of teleost neutrophils at the inflammatory site and the mechanisms that promote and control their effective recruitment from hematopoietic tissues. In the present study, we examined the contributions of neutrophils to the acute inflammatory response in teleost fish using a self-resolving zymosan peritonitis model. This model allowed both biochemical and cellular analyses of local inflammatory responses, which resembled the PMN-rich exudates of human disease. Using this model, we investigated the mobilization of neutrophils and the regulation of inflammatory processes during an acute inflammatory response. We report that neutrophils play a significant role in the proinflammatory and resolution phases of the acute inflammatory response in teleost fish. Consistent with mammalian studies, our data show that in goldfish, a large pool of mature neutrophils rapidly exit the hematopoietic tissue, enter circulation, and infiltrate the inflammatory site. We found that neutrophil infiltration into the peritoneum peaked at 18 hpi. Once at the inflammatory site, we found that teleost neutrophils possess the capacity to mediate divergent pro- and anti-inflammatory responses. Teleost neutrophils isolated at 18 hpi, a period in which the inflammatory site exhibited a classic proinflammatory phenotype, displayed increased ROS production compared with neutrophils isolated at 48 hpi, a point at which the phenotype had shifted to the proresolving phase. Neutrophils from this proresolution time point also demonstrated a greater ability to modulate macrophage function. These neutrophils were able to mediate divergent responses, both increasing and decreasing ROS production in macrophages after stimulation with zymosan or ACs, respectively. We found LXA4 to be a significant contributor to the uptake of ACs by teleost macrophages. In addition, LXA4 played a role, at least in part, in the downregulation of ROS production in macrophages. Our results highlight the evolving roles of neutrophils in both the promotion and the regulation of the inflammatory response.

**MATERIALS AND METHODS**

**Ethics statement**

All animals were maintained according to the guidelines of the Canadian Council on Animal Care, and the University of Alberta Animal Care and Use Committee approved the protocols (protocol no. 769; Biosciences). The fish were held at 18°C in a flow-through water system with constant aeration. All fish were housed in the Aquatic Facility of the Department of Biologic Sciences, University of Alberta, in a simulated natural photosystem.

**Animals**

Goldfish (Carassius auratus L.) 10–15 cm long were purchased from Mount Parnell (Merricksburg, PA, USA) and obtained from Aquatic Imports (Calgary, AB, Canada). The fish were held at 18°C in a flow-through water system with constant aeration. All fish were housed in the Aquatic Facility of the Department of Biologic Sciences, University of Alberta, in a simulated natural photosystem.

**Isolation of hematopoietic leukocytes**

Goldfish total kidney leukocytes were isolated as previously described [10, 23]. In brief, goldfish kidneys were macerated through mesh screens with incomplete MGFL-15 medium [24]. Debris was allowed to settle, and the supernatant was removed. The leukocytes were washed twice with incomplete MGFL-15 medium via centrifugation for 10 min at 311 g and 4°C. To isolate kidney neutrophils, total kidney leukocytes were layered onto 51% Percoll (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 25 min at 400 g and 4°C. The pellet containing RBCs and neutrophils was collected, and RBCs were lysed with ACK lysis buffer (Lonza, Basel, Switzerland) for 3 min. The remaining neutrophils were washed twice with incomplete HBSS (no calcium or magnesium) via centrifugation for 10 min at 311 g and 4°C and resuspended in complete HBSS (100 U/ml penicillin, 100 µg/ml streptomycin, and 5% carp serum, calcium, and magnesium) for subsequent assays.

**Isolation of peritoneal leukocytes**

Peritoneal cells were isolated from goldfish injected with 2.5 mg of zymosan by lavaging the fish with 1× PBS (no calcium, no magnesium). The cells were spun down and washed with 1× PBS before use, as previously described [10]. To isolate neutrophils, exudates were subsequently layered onto 51% Percoll and centrifuged for 25 min at 400 g and 4°C. The Buffy coat was removed and discarded. The pellet containing RBCs and neutrophils was collected, and RBCs were lysed with ACK lysis buffer (Lonza) for 3 min. The remaining neutrophils were washed twice with incomplete HBSS (no calcium or magnesium) via centrifugation for 10 min at 311 g and 4°C and resuspended in complete HBSS for subsequent assays. Neutrophil viability was consistently >95% according to annexin V/PI staining, and the level of purity for isolated neutrophils was 95–97%. The small percentage of contaminating cells (primarily lymphocytes) was removed during the flow cytometric analysis through gating based on the forward and side scatter characteristics.

**Goldfish PKM cultures**

PKMs were generated as previously described [24, 25]. In brief, isolated goldfish kidney leukocytes were seeded in 15 ml of complete MGFL-15 medium (MGFL-15 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml gentamicin, and 10% newborn calf serum [ Gibco, Burlington, ON, Canada]) and 5% carp serum) and 5 ml of cell-conditioned medium from previous cultures. PKM cultures were developed over 7 d at 20°C and were used at the proliferative phase of culture development. This growth phase is dominated by significant proliferation of macrophage progenitors coupled to their differentiation into mature macrophages [26, 27].

**Preparation of zymosan and apoptotic neutrophils**

Unlabeled zymosan particles (Molecular Probes, Burlington, ON, Canada) were labeled overnight with 250 ng/ml FITC (Sigma-Aldrich) with continuous shaking at 4°C in carbonate buffer (0.1 M sodium carbonate, 0.1 M sodium bicarbonate; pH 9.6). After staining, zymosan-FITC was washed twice with 1× PBS (no calcium or magnesium). Apoptotic neutrophils were generated by injection of 2.5 mg of zymosan into the goldfish peritoneum. Cells were removed by lavage, and neutrophils were isolated on a 51% Percoll gradient. Apoptotic neutrophils were generated by incubating cells for 24 h in the presence of 10 µg/ml cycloheximide (Sigma-Aldrich). Subsequent to this procedure, the neutrophils were consistently 80–90% apoptotic, as...
determined by annexin V/PI staining. The treated cells were washed twice in 1× PBS/− and stained for 1 h with wheat germ agglutinin Alexa Fluor 555 (Molecular Probes). ACs were then washed twice with 1× PBS/−. Both zymosan and apoptotic neutrophils were used at a 3:1 (particle/cell) ratio in the experiments. This ratio was chosen based on previous results, in which zymosan was capable of inducing strong proinflammatory responses, and ACs downregulated ROS production from stimulated to basal levels [23].

**In vivo effects of zymosan**

Goldfish were injected i.p. with 2.5 mg of zymosan (Sigma-Aldrich) in 100 µl of 1× PBS/−. The goldfish were killed after 0, 8, 12, 18, 24, 36, 48, or 72 h, and the cells were harvested by peritoneal lavage to determine the time point with maximal cell infiltration based on hemocytometer counts, respiratory burst, and phagocytosis. Within these time points, the changes in cellular numbers were largely associated with cellular infiltration. The cells were used in the assays as described in the subsequent sections.

**Cytochemical stains**

For all cytochemical stains, 5 × 10^5 cells were spun onto glass slides at 55 g for 6 min at room temperature using a cytospin centrifuge (Shandon Instruments, Waltham, MA, USA), and fixation and staining for Sudan Black was performed according to the manufacturer’s protocols. In brief, for Sudan Black staining (Sigma-Aldrich), the cells were fixed for 1 min in an acetone-glutaraldehyde solution. The cells were then stained for 5 min with Sudan Black, followed by a 5-min counterstain with hematoxylin. Images were generated using a DM1000 microscope (Leica, Wetzler, Germany) using a bright field 100× objective (×1000 magnification), and acquired using QCapture software (QImaging, Surrey, BC, Canada).

**Respiratory burst**

Respiratory burst assay was performed as previously described with minor modifications [10]. The cells were harvested and collected into 5-ml polystyrene, round bottom tubes (BD Falcon, Mississauga, ON, Canada). DHR (Molecular Probes) was added to the cells at a final concentration of 10 µM and incubated for 5 min to allow the cells to take up the DHR. PMA (Sigma-Aldrich) was then added at a final concentration of 100 ng/ml. The cells were further incubated for 30 min to allow oxidation of the DHR. All samples were appropriately staggered with respect to timing to accommodate the transient state of oxidized DHR fluorescence. Live cells were gated according to the forward scatter and side scatter parameters. DHR fluorescence was detected in the FITC channel, with positive cells having a shift >1 log compared with the unstimulated controls.

**Gene expression**

Gene expression was performed as previously described [28]. In brief, total RNA was extracted from peritoneal inflammatory cells and reverse-transcribed into cDNA using SMARTScribe Reverse Transcriptase (Clontech, Mountain View, CA, USA). cDNA was then analyzed by quantitative PCR on ABI 7500 system (Applied Biosystems, Carlsbad, CA, USA). The samples were then acquired on a FACScan Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s protocols. In brief, 50-µl aliquots were loaded in the microplate in duplicate, followed by 50 µl of LTB4-HRP or LXA4-HRP and incubated for 1 h. After incubation, the microplate was washed 3 times with wash buffer. After washing, 150 µl of 3.5,3.5-tetramethylbenzidine substrate was added to each well and incubated for 15 min. The microplate was then read at 650 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). The data were analyzed by determining the ratio of sample binding to the percentage of maximal binding (blank well with control lipid) according to a standard curve.

**Phagocytosis of ACs**

ACs were added at a 3:1 ratio to primary kidney macrophages after stimulation and incubated for 2 h. The cells were then washed twice in 1× PBS/− and fixed in 1% formaldehyde and maintained at 4°C overnight. Data were acquired on an ImageStream MkII multispectral imaging flow cytometer (Amnis Corporation, EMD Millipore, Seattle, WA, USA) and analyzed using INSPIRE software (Amnis Corporation, EMD Millipore, Seattle, WA, USA). At least 1 × 10^6 cells were acquired. Phagocytosis was analyzed as previously described [29].

**Assessment of LTB4 and LXA4 production via ELISA**

ELISAs were performed as previously described [30], with minor modifications. In brief, goldfish were injected intraperitoneally with 2.5 mg of zymosan. At each time point, neutrophils were isolated, incubated for 1 h in peritoneal exudate, and then resuspended in 1× PBS/−. The samples were then analyzed for the presence of LTB4 or LXA4 (Oxford Biomedical Research, Rochester Hills, MI, USA) according to the manufacturer’s protocols. In brief, 50-µl aliquots were loaded in the microplate in duplicate, followed by 50 µl of LTB4-HRP or LXA4-HRP and incubated for 1 h. After incubation, the microplate was washed 3 times with wash buffer. After washing, 150 µl of 3.5,3.5-tetramethylbenzidine substrate was added to each well and incubated for 15 min. The microplate was then read at 650 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). The data were acquired using analysis of variance and Tukey’s post hoc tests in Prism software (GraphPad Software, La Jolla, CA, USA).

**Statistical Analysis**

ImageStream data were analyzed using IDEAS software (Amnis; EMD Millipore), as described previously [29]. FACScanto II data were analyzed using FACSDiva software, as described previously [29]. Statistical analyses were performed using analysis of variance and Tukey’s post hoc tests in Prism 6 software (GraphPad Software, La Jolla, CA, USA).

**RESULTS**

**Kinetic analysis of teleost acute inflammation using an in vivo peritonitis model**

Our goal in the present study was to define the acute inflammatory response in teleosts to better understand the
evolutionary origins of neutrophil-driven mechanisms of immunity. As a first step to defining the proinflammatory phase of acute inflammation in teleost fish, we examined the kinetics of cellular infiltration into an inflammatory site, the production of antimicrobial ROS, and the expression of proinflammatory cytokines using an in vivo zymosan peritonitis model. We found recruitment of leukocytes into the peritoneum as early as 8 hpi, with peak infiltration observed at 18 hpi. The total cell numbers at the site of inflammation remained elevated through 36 h compared with noninjected fish (0 h; Fig. 1A). By 72 hpi, the number of cells present at the inflammatory site had returned to basal levels. Consistent with the kinetics of cellular infiltration, ROS production increased significantly between 8 and 36 h, with a peak at 18 hpi (Fig. 1B).

Cytokine expression analysis was performed by quantitative PCR using the primers in Table 1. RNA was isolated from total leukocytes within the peritoneal cavity and examined for the expression of TNF-α2, IL-1β1, and IFN-γ, cytokines known to be important in the inflammation process [31, 32]. Overall, we identified high local expression of TNF-α2, IL-1β1, and IFN-γ within the first 24 hpi, with a peak at 8–12 hpi and a return to basal levels by 48 hpi (Fig. 1C). Although individual animals were derived from an out-bred population, we found significant homogeneity among the kinetics of proinflammatory cytokine gene expression profiles across all the fish. Characterization of the leukocyte populations within the exudate identified neutrophils as the dominant subset, representing nearly 50% of all infiltrating leukocytes at the site of inflammation at 18 hpi (Fig. 1D).

Teleost inflammatory neutrophils exhibit robust antimicrobial responses compared with their hematopoietic counterparts

On entry into an inflammatory site, mammalian neutrophils are rapidly activated from the naive phenotype seen in the hematopoietic compartment and exhibit potent histotoxic capabilities, including phagocytosis and downstream killing mechanisms [33–35]. To determine whether teleost neutrophils share this dichotomy, we compared the antimicrobial responses among teleost neutrophils isolated from the kidney hematopoietic compartment and the peritoneal challenge site of goldfish injected with 1 × PBS−/− or zymosan. Neutrophils isolated from the inflammatory site exhibited significantly higher rates of phagocytosis and marked increases in ROS production compared with their hematopoietic counterparts (Fig. 2A and B). As such, we chose to focus on neutrophils isolated from the inflammatory site to examine their contributions throughout the acute inflammatory response.

Figure 1. Intraperitoneal administration of zymosan in goldfish induces marked infiltration of leukocytes that is linked to high levels of proinflammatory mediators. Goldfish were injected intraperitoneally with 2.5 mg of zymosan. Cells were harvested by peritoneal lavage at 0 h (saline alone) and at 8, 12, 18, 24, 36, 48, and 72 h, and counted or used for RNA extraction. A) Cells per lavage at the indicated time points (n = 5). B) Respiratory burst capacity in isolated cells at these time points was determined via DHR staining (n = 5). C) Cytokine levels were measured by quantitative PCR (RQ). Zymosan induced a general increase in proinflammatory cytokine expression levels in the early stages of the time course. Each line represents an individual fish (n = 4). D) Sudan Black staining was used to determine the proportion of neutrophils in the peritoneal exudate at 18 hpi (n = 5). Representative images show neutrophils positive for Sudan Black staining as well as a Sudan Black-negative monocyte/macrophage (M/Mac) and lymphocyte (Lym). All statistical results correspond to a significance level of P < 0.05 using 1-way analysis of variance. *Significantly different from 0 hpi; ‡Significantly different from 18 hpi.
After an increase in CXCL8 expression, neutrophils rapidly infiltrate the site of inflammation and activate potent respiratory burst responses

In mammals, chemotactic mediators such as CXCL8 are produced within inflammatory sites and recruit neutrophils from the circulation [42, 43]. Teleost models, including zebrafish and carp, have also shown CXCL8 to mediate neutrophil recruitment [36, 44, 45]. Using this as our foundation, we were interested in determining whether CXCL8 played a role in our model. Examination of CXCL8 expression during the acute inflammatory response to zymosan-induced peritonitis showed a significant increase at the site of inflammation, peaking at 12 hpi and returning to basal levels by 18 hpi (Fig. 4A). This increase in CXCL8 expression directly preceded the rapid influx of neutrophils into the peritoneum, which peaked at 18 hpi (Fig. 4B).

**Table 1. Primer sequences for quantitative PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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<tbody>
<tr>
<td>EF-1a forward</td>
<td>CCG TCG AGA TCG GCG ATG AGT</td>
</tr>
<tr>
<td>EF-1a reverse</td>
<td>TTG AGA GAS AGC TCG TCG AGG TT</td>
</tr>
<tr>
<td>CXCL8 forward</td>
<td>CTT AGA GTG GAC GCA TGT GAA</td>
</tr>
<tr>
<td>CXCL8 reverse</td>
<td>TGG TGT CTT TAC ACG GTG ATG TTG</td>
</tr>
<tr>
<td>IFN-γ forward</td>
<td>GAA ACC CTA TGG GCG ATC AA</td>
</tr>
<tr>
<td>IFN-γ reverse</td>
<td>GTA GAC AGG CTT CAG CTC AAA CA</td>
</tr>
<tr>
<td>IL-1β forward</td>
<td>GCC CTC CTC AAC TAC ATG TTG</td>
</tr>
<tr>
<td>IL-1β-1 reverse</td>
<td>GTG AGA CAT TAA GCG GCT TCA C</td>
</tr>
<tr>
<td>TNF-α2 forward</td>
<td>TCA TCC CTT ACG AGC GCA TTT</td>
</tr>
<tr>
<td>TNF-α2 reverse</td>
<td>CAG TCA CTT AGC GCA TTG</td>
</tr>
</tbody>
</table>

EF, elongation factor.

**Induction of teleost acute inflammation results in marked mobilization of neutrophils from the hematopoietic compartment to the peripheral blood**

It is widely recognized that many inflammatory reactions are associated with a prompt and selective mobilization of neutrophils from the bone marrow to the inflammatory site in mammals [12–14, 34]. Furthermore, teleost neutrophils have also been shown to mobilize from the hematopoietic compartment in models such as the zebrafish and gilthead seabream [36–40]. Among others, C/EBPα and Nos2α are critical to the initiation of granulopoiesis after infection, and CXCL8-CXCR2 signaling is required for the motility and mobilization of neutrophils from hematopoietic tissues [36, 41]. As the next step in examining the acute inflammatory response in teleost fish, we investigated the contribution of the hematopoietic compartment to the mobilization of neutrophils in goldfish. We found that a dramatic increase in the peritoneal leukocytes (Fig. 1A) correlated with a decrease in total leukocytes within the hematopoietic compartment (Supplemental Fig. 1A). When we examined the number of leukocytes remaining in the hematopoietic compartment, we found that the number of neutrophils decreased dramatically by 70% at 12 hpi (Fig. 3A). This correlated further with a marked early decrease in leukocyte proliferation (8 hpi; Fig. 3B), which had returned to basal levels by 72 hpi. The steady increase in proliferation between 8 and 72 hpi likely contributed to the replenishment of the depleted pool of hematopoietic neutrophils, eventually returning to basal levels at 48–72 hpi.

After an in vivo challenge with zymosan, the proportion of circulating neutrophils increased nearly 10-fold, from <5% at 0 hpi to nearly 50% of circulating leukocytes at 8–18 hpi (Fig. 3C). In contrast, peripheral blood monocytes peaked at 36 hpi (Supplemental Fig. 1C), and peripheral blood lymphocytes had decreased significantly at 8–36 hpi (Supplemental Fig. 1D). The total blood leukocyte counts remained relatively constant throughout the 72-h time course (Supplemental Fig. 1B). However, the significant increase in the proportion of neutrophils within the blood at 8–36 hpi (Fig. 3C) also resulted in a significant increase in total neutrophil numbers, which peaked at 8 hpi (8.4-fold over basal levels; Fig. 3D), identifying a period of neutrophilia within the circulating blood of goldfish after this challenge.

Figure 2. Peritoneal neutrophils display increased phagocytic and respiratory burst capacity compared with their hematopoietic counterparts. Goldfish were injected intraperitoneally with 1 × PBS−/− or 2.5 mg of zymosan (zym). Neutrophils were isolated from the hematopoietic kidney tissue and peritoneal exudate. A) Phagocytic capacity of neutrophils. Solid black bars represent internalization; hatched white bars, surface binding of particles (n = 4). Representative images from ImageStream MKII flow cytometer denote nonphagocytic (n-Ph), surface bound (SB), and internalized (Int) events. B) Respiratory burst capacity of neutrophils (n = 4). All statistical results correspond to a significance level of P < 0.05 using 1-way analysis of variance. *Significantly different from PBS injection (kidney neutrophils); †significantly different from zymosan injection (kidney neutrophils); ‡significantly different from PBS injection (peritoneal neutrophils). PerC, peritoneal cavity.
Together with the presented data, this finding allowed us to define 0–18 hpi as the induction phase of the acute inflammatory response in this self-resolving peritonitis model.

Figure 3. Intraperitoneal administration of zymosan induces an efflux of neutrophils from the hematopoietic tissue into circulation. Goldfish were injected i.p. with 2.5 mg of zymosan for 0, 8, 12, 18, 24, 36, 48, or 72 h. Before kidney isolation, goldfish were injected i.p. with BrdU and incubated for 1 h to allow incorporation of BrdU into tissues. A) Neutrophil numbers in the hematopoietic kidney tissue. Before cell harvesting, kidneys were weighed. Total kidney leukocytes at each time point were counted using a hemocytometer. To account for varying sizes of fish, counts were divided by kidney weight (in mg) to determine leukocytes/mg tissue (n = 4). B) Percentage of proliferating cells within the kidney at each of the indicated times (n = 4). C) Percentage of peripheral blood neutrophils. Fish were killed and bled at 0, 8, 12, 18, 24, 36, 48, and 72 h. Next, peripheral blood smears were stained with Sudan Black. Neutrophils were identified as Sudan Black positive; monocytes and lymphocytes both stained negative. A minimum of 100 cells was counted to determine the proportion of neutrophils in the peripheral blood (n = 4). D) Number of peripheral blood neutrophils (n = 4). All statistical results correspond to a significance level of P, 0.05 using 1-way analysis of variance. *Significantly different from 0 hpi; †significantly different from 8 hpi; ‡significantly different from 12 hpi; and §significantly different from 18 hpi.

Figure 4. Kinetics of CXCL8 expression, neutrophil infiltration, downstream respiratory burst responses, and entry into programmed cell death. Goldfish were injected i.p. with 2.5 mg of zymosan. At 0, 8, 12, 18, 24, 36, 48, and 72 h, peritoneal cells were harvested by lavage. A) CXCL8 levels in total leukocyte population measured by quantitative PCR (RQ) (n = 4). B) Kinetics of neutrophil infiltration. Neutrophils were isolated using Percoll and counted on a hemocytometer (n = 4). C) Respiratory burst responses of peritoneal neutrophils (n = 4). D) Percentage of apoptotic neutrophils isolated from the peritoneal cavity (n = 4). All statistical results correspond to a significance level of P < 0.05 using 1-way analysis of variance. *Significantly different from 0 hpi; †significantly different from 8 hpi; ‡significantly different from 12 hpi; §significantly different from 18 hpi; and ¶significantly different from 24 hpi.
As described, neutrophils isolated from the inflammatory site displayed more prominent antimicrobial responses than did their naive counterparts. To gain additional insights into the antimicrobial potential of neutrophils during the teleost acute inflammatory response, we examined kinetics of ROS production among infiltrated neutrophils. As expected, we found that neutrophils displayed prominent respiratory burst responses on entry into the peritoneal inflammatory site. Similar to the total cell population, respiratory burst responses peaked at 18 hpi (Fig. 4C). However, unlike ROS production by total leukocytes, which declined steadily until 72 hpi (Fig. 1B), the respiratory burst responses of neutrophils had returned to basal levels by 36 hpi (Fig. 4C).

Increase in neutrophil apoptosis marks transition to the proresolution phase

Neutrophil apoptosis and their subsequent uptake by phagocytes is central to the successful resolution of inflammation in mammals [46]. Its contributions to resolution of inflammation in teleosts has previously been documented in a zebrafish embryo tail transection model [21]. Thus, we addressed whether the induction of neutrophil apoptosis might also mark the transition between proinflammatory and proresolution responses in our self-resolving peritonitis teleost model, in which acute inflammation was driven through Dectin-1/TLR-2 activation. We found that the presence of apoptotic neutrophils peaked at 24 hpi, subsequently decreasing to basal levels by 72 hpi (Fig. 4D). This transition followed the induction phase of acute inflammation defined above. Between 24 and 48 hpi, the neutrophil counts remained above basal levels, even as the neutrophil ROS production levels returned to baseline. We hypothesized that this remaining subset of viable neutrophils had shifted toward a proresolving phenotype, actively promoting the resolution of inflammation.

Inflammatory neutrophils can modulate the functional responses of macrophages through soluble factors

Recent studies have highlighted the direct contribution of mammalian neutrophils to the resolution of inflammation by the internalization ACs [9, 10] and the release of lipid mediators [47–49]. To characterize the effect of teleost neutrophils on macrophage functional responses, goldfish neutrophils were stimulated in vivo and isolated from the peritoneum at 18 and 48 hpi, as described in previous sections. Neutrophils were cultured for 2 h in 0.4-μm Transwell inserts (Corning Life Sciences, Corning, NY, USA) in the presence of zymosan or AC to determine whether soluble factors could elicit changes in macrophage responses outside of the Transwell insert (Fig. 5A). We found that when incubated with zymosan, neutrophils isolated 18 hpi induced significant increases in macrophage ROS production compared with neutrophils isolated at 48 hpi (60% vs. 46%, respectively). This suggests that neutrophils from the induction phase of this acute inflammatory response (isolated at 18 hpi) were more suited to activating inflammatory macrophage functions than those from the proresolution phase (isolated at 48 hpi). In contrast, when these induction phase neutrophils were incubated with ACs, they displayed no capacity to reduce macrophage ROS production to below basal levels (Fig. 5A). This differed dramatically from the proresolution neutrophils, which decreased macrophage ROS levels when incubated with ACs (28% vs. 38%, respectively). This suggests that the neutrophils released soluble factors that modulated macrophage function and that the types of soluble factors produced are largely dependent on the phenotype exhibited by isolated neutrophils (proinflammatory or proresolving). In addition, we examined the ability of neutrophils to induce the uptake of apoptotic neutrophils by macrophages. We observed no differences in the ability of macrophages to internalize apoptotic neutrophils after stimulation under these conditions (Fig. 5B).

Lipid mediators represent key immunoregulatory soluble factors released by neutrophils. Thus, we investigated the potential contributions from LTB₄, known to be involved in neutrophil recruitment and activation [34], and LXA₄, an important mediator with proresolving functions [30, 47, 49]. We found that the secretion of the proinflammatory lipid LTB₄ displayed very similar kinetics to those of CXCL8 (Fig. 6A). A significant increase in LTB₄ (~75 pg/5 × 10⁵ neutrophils) was found at 8–12 hpi, followed by

Figure 5. Neutrophil soluble mediators affect macrophage functional responses. Goldfish were injected i.p. with 2.5 mg of zymosan (zym). Cells were harvested by peritoneal lavage at 18 or 48 hpi and run over a 51% Percoll gradient to isolate the neutrophils. Neutrophils were added to the upper chamber of a 6-well Transwell insert. Macrophages were seeded in the lower compartment. Neutrophils were incubated with zymosan, apoptotic neutrophils, or nothing at a 3:1 (particle/neutrophil) ratio. A) Macrophage respiratory burst responses after 2 h of incubation (n = 4). B) Percentage of internalization of apoptotic neutrophils by macrophages after 2 h of incubation (n = 4). Apoptotic neutrophils were generated from neutrophils isolated from the peritoneal cavity at 18 hpi. Representative images from ImageStream MKII analyses showed internalized or surface-bound apoptotic neutrophils. All statistical results corresponded to a significance level of P < 0.05 using 2-way analysis of variance. *Significantly different from 18-h PMN alone; †significantly different from 18-h PMN+zym; ‡significantly different from 18-h PMN+AC; §significantly different from 18-h PMN+zym+AC.
an immediate return to basal levels. In contrast, LXA4 peaked (~300 pg/5 x 10^5 neutrophils) during the putative transition point to the proresolution phase (24 hpi; Fig. 6B). A comparison among the neutrophil, mononuclear cell, and soluble exudate fractions confirmed that neutrophils were the primary producers of LTB4 and LXA4 (Supplemental Fig. 2). The peak LXA4 release at 24 hpi paralleled that of peak neutrophil apoptosis, supporting this as a transition point toward the resolution of acute inflammation. We next determined whether these lipid mediators also induced autocrine effects on neutrophils. We found that stimulation with LTB4 promoted increased respiratory burst responses in both naive neutrophils isolated from the hematopoietic kidney and in inflammatory neutrophils isolated from the peritoneal cavity at 18 hpi. Although a dose-dependent effect was evident in hematopoietic kidney PMN (Fig. 6C), the peritoneal cavity PMN displayed consistently high levels of ROS production (Fig. 6D). This is likely associated with the activated status of inflammatory PMNs infiltrating the peritoneal compartment. Primary macrophages were then stimulated in vitro with LTB4 or LXA4 at varying concentrations for 2 h. Although LTB4 has been shown to activate the intracellular killing mechanisms of murine peritoneal macrophages [50], LTB4 had little effect on teleost primary kidney macrophage responses (Fig. 6E). In contrast, LXA4 tended to decrease macrophage ROS production at all concentrations examined (Fig. 6E). In addition, stimulation with LXA4 resulted in a significant increase in macrophage uptake of apoptotic neutrophils in a dose-dependent manner (Fig. 6F). Macrophages stimulated with the highest concentration of LXA4 (290 pg) displayed a fourfold increase in apoptotic neutrophil uptake compared with unstimulated levels (Fig. 6F).

**DISCUSSION**

The acute inflammatory response requires proper coordination of both pro- and anti-inflammatory responses for effective defenses against infection, the removal of damaged cells, and the initiation of tissue repair processes. Similar to previous studies in mammals that have examined zymosan-induced inflammation [51, 52], i.p. injection of zymosan into goldfish resulted in substantial cellular infiltration and marked increases in the generation of antimicrobial products. Based on this model, 72 h provided an adequate observation period for examination of the induction, regulation, and resolution of acute inflammatory responses [53].

Neutrophils are essential first-line effector cells of innate immunity; their recruitment into peripheral tissues is central to host defense against invading pathogens. In mammals, neutrophils spend most of their lives in the bone marrow, where 5 x 10^10 to 10 x 10^10 new neutrophils are generated each day [11]. A large storage pool of mature neutrophils exists, termed the bone marrow reserve [12–14]. On receiving the appropriate signals,
this pool can be rapidly mobilized to respond to an infection or inflammatory challenge [12–14], resulting in a dramatic increase in the number of circulating neutrophils. Our results are consistent with those from mammals, in which the number of neutrophils within the hematopoietic tissue of goldfish decreased by nearly 70% within 12 hpi. Neutrophil depletion in the hematopoietic compartment led to a rapid increase in peripheral blood neutrophils, with the number of circulating neutrophils increasing 8.4-fold over basal levels. During this period, a dramatic decrease in proliferation also occurred within the hematopoietic compartment. The lack of proliferation, rapid release of neutrophils from the kidney, and increase in peripheral blood neutrophils indicated that a storage pool of neutrophils exists within the hematopoietic tissue of goldfish, similar to that of mammals. However, mammalian neutrophils represent the largest population of circulating leukocytes during homeostasis. This is not the case in teleost fish, in which lymphocytes are the dominant circulating leukocyte [54]. This suggests that teleost fish are far more dependent on the hematopoietic storage pool of neutrophils, which likely represents most of the mature neutrophils that can traffic to and infiltrate the site of inflammation. In mammals, many of the neutrophils infiltrating the site of inflammation are presumably already in circulation. Their hematopoietic storage pool might then exist as a method to replenish peripheral blood neutrophils after depletion. Thus, mammalian neutrophils might be able to respond more rapidly to acute injury or pathogen challenge. This would also come at the expense of 1 regulatory safeguard for the release of these potent effector cells from the hematopoietic compartment.

Diverse chemoattractants, such as CXCL8 and LTB₄ [42–44, 55], can act to recruit neutrophils to a site of inflammation. However, given their destructive potential to host tissues, neutrophil entry must be tightly regulated [35, 56]. In zebrafish, increases in CXCL8 expression can be seen within hours of wounding [44]. Furthermore, CXCL8 is upregulated in response to acute inflammatory stimuli, critical for normal neutrophil recruitment to the wound and resolution of inflammation [44]. Similarly, during induced atopic dermatitis in mice and humans, the influx of neutrophils was largely dependent on the generation of LTB₄ by neutrophils in an autocrine manner [57]. We have shown that both CXCL8 expression and LTB₄ production increase rapidly at the site of inflammation at 8–12 hpi. This provides 2 potential contributors to the rapid recruitment of neutrophils to the site of inflammation in teleosts. Furthermore, the narrow window in which CXCL8 expression and LTB₄ production increased are consistent with the tight regulation of this process from the inflammatory site. Most LTB₄ was produced by neutrophils at 12 hpi (51 pg/5 × 10⁵ cells; Supplemental Fig. 2). In contrast, mononuclear cells (lymphocytes, monocytes, and macrophages) were found to produce low levels of LTB₄ at this time point (14 pg/5 × 10⁵ cells; Supplemental Fig. 2). LTB₄ was shown to increase the production of ROS in both naive and inflammatory neutrophils. Our results highlight the complementary autocrine effect by which lipids can aid neutrophil recruitment throughout the site of inflammation.

We found that neutrophil infiltration into the peritoneal cavity peaked directly after the spike in CXCL8 and LTB₄ at 18 hpi, correlating well with previously published data from Chadzinska et al. [58], in which cellular infiltration peaked between 16 and 24 hpi. Induction of proinflammatory antimicrobial responses correlated well with leukocyte recruitment, because maximal ROS production was observed during peak infiltration (18 hpi). In contrast, neutrophil influx was found to peak at 4–6 hpi in a murine zymosan-induced peritonitis model [53, 59, 60], although neutrophil-specific responses were not examined. A second murine study showed total leukocyte ROS production peaking at 8 hpi [10]. The quicker response time of mammalian neutrophils might be associated with a couple of factors. First, neutrophils are the dominant circulating leukocyte in mammals and thus should be able to respond more quickly to an infection compared with teleost fish, in which they only represent approximately 5% of the circulating leukocytes. Second, resident peritoneal cells in mice might display an enhanced capacity for recruitment to the inflammatory site compared with their teleost counterparts. The induction of inflammation was followed by a controlled resolution phase of inflammation, in which cell counts, ROS production, and the expression of proinflammatory cytokines returned to basal levels at 24–72 hpi.

The resolution of inflammation is critical to maintaining host health. One important step toward resolution is the induction of programmed cell death in inflammatory cells. Given the potentially destructive nature of neutrophils at an inflammatory site, their half-life is very short, and the removal of apoptotic neutrophils by macrophages is believed to be a crucial component driving the resolution of inflammation [61, 62]. It was previously thought that the internalization of ACs was an immunologically neutral event; however, evidence has now indicated that the internalization or binding of apoptotic neutrophils by macrophages is believed to be a crucial component driving the resolution of inflammation [61, 62]. The induction of inflammation was followed by a controlled resolution phase of inflammation, in which cell counts, ROS production, and the expression of proinflammatory cytokines returned to basal levels at 24–72 hpi.

The balance between pathogenic and homeostatic signals is crucial for effective pathogen clearance with minimal damage to surrounding host tissues [66]. Macrophages are well known to internalize ACs and subsequently downregulate their proinflammatory responses [10, 67, 68]. In addition, human and murine neutrophils activated ex vivo possess the capacity to internalize ACs, resulting in decreased ROS production [9, 10]. This establishes mammalian macrophages and neutrophils as active contributors to the resolution of inflammation. We have previously shown this capacity to extend to macrophages, but not neutrophils, in teleost fish [10]. Despite our present findings, it is possible that specific instances could exist when teleost neutrophils do internalize ACs. In 1 case, zebrafish neutrophils were
shown to internalize apoptotic macrophages infected with *Mycobacterium marinum* [41]. However, one would predict significant activation of pattern recognition receptors under these conditions, likely rendering these infected ACs poor contributors to the resolution of inflammation that is normally exhibited during a classic acute inflammatory process.

Our results have also indicated that teleost neutrophils are capable of altering their phenotype throughout the acute inflammatory response, contributing to both the induction and resolution of inflammation. Two phases of the acute inflammatory response in teleost fish became apparent: (1) an induction phase of acute inflammation from 0 to 18 hpi, and (2) a proresolving phase from 24 to 72 hpi (Fig. 7). The period in between (18–24 hpi) represents a transition point between these 2 phases, in which divergent pro- and anti-inflammatory responses overlap. Neutrophils isolated during the induction phase (18 hpi) were only able to induce ROS production in macrophages. However, neutrophils isolated during the proresolving phase (48 hpi) also possessed the capacity to downregulate macrophage ROS production. Although we observed no change in the ability to stimulate an increased uptake of ACs by macrophages, we recognize this might be a possibility under different experimental conditions. We are currently pursuing 2 potential modifications. First, the neutrophils might need to be isolated at a different time point, and second, different types of macrophages (e.g., inflammatory vs. hematopoietic) and/or their activation status might display a differential capacity for induction of apoptotic cell uptake.

We have previously shown that, in the presence of ACs, teleost neutrophils produce soluble factors that can modulate monocyte and macrophage responses [10]. However, these previous experiments were undertaken with neutrophils from the transition point (24 hpi). It has been shown in mammals that lipid mediators are one of the primary soluble factors produced by neutrophils. As such, we examined the production of lipid mediators throughout an acute inflammatory response. We found a shift in the production of LTB4 to LXA4 in neutrophils during the transition phase (18–24 hpi) of acute inflammation (Figs. 6C and 7), further supporting our argument of multiphenotypic neutrophils. As previously mentioned, LTB4 secretion parallels that of increased CXCL8 expression within the inflammatory milieu. Current models propose that LTB4 acts as a secondary chemoattractant important in initiating the inflammatory process, indicating that teleost neutrophils might be actively involved in recruiting more neutrophils to the inflammatory site. Furthermore, LTB4 has been implicated in the activation of neutrophils through BLT1 receptor endocytosis, inducing degranulation and increased respiratory burst responses [69, 70]. However, LXA4 secretion paralleled that of neutrophil apoptosis, peaking at 24 hpi. LXA4 aids in the uptake of ACs by macrophages, playing an important role in the resolution of inflammation [71, 72]. Similarly, we found that macrophage internalization of apoptotic neutrophils increased in a dose-dependent manner, suggesting that neutrophils participate in the resolution of inflammation by stimulating...
macrophages with LXA₄ before entering apoptosis. LXA₄ also contributed to downregulating ROS production in macrophages and inflammatory neutrophils, suggesting this proresolving lipid might play a broader role than previously described.

From our findings, the induction phase of acute zymosan peritonitis lasts from 0 to 18 hpi in teleosts (Fig. 7). It is marked by proresolution period from 24 to 72 hpi, resulting in a return to homeostasis (Fig. 7). Our results provide additional insights into the mechanisms by which neutrophils contribute to the induction and regulation of acute inflammatory processes. Furthermore, it provides a foundation from which we can examine the discrete transition, we observe proinflammatory cytokine expression return to basal levels, a switch in the secretion of lipid mediators from LTβ to LXA₄, and an increase in apoptotic neutrophils. Thus, we hypothesized that the switch in soluble lipid mediators is facilitated, at least in part, by the secretion of PGE₂ from monocytes, macrophages, and epithelial cells, a possibility we are currently pursuing. The final period of acute peritonitis is the proresolution period from 24 to 72 hpi, resulting in a return to homeostasis (Fig. 7). Our results provide additional insights into the mechanisms by which neutrophils contribute to the induction and regulation of acute inflammatory processes. Furthermore, it provides a foundation from which we can examine the discrete differences between mammalian and teleost neutrophils and their various contributions to resolving inflammation.

AUTHORSHIP

J.J.H. conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. A.M.R., M.E.W., J.W.H. conceived and designed the experiments, performed the experiments, and analyzed the data. D.R.B. conceived and designed the experiments, analyzed the data, and wrote the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

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