INVITED REVIEW

Neutrophils and aquatic pathogens

Kurt Buchmann

Abstract

Introduction: Neutrophilic granulocytes are short-lived cells continuously circulating in the vascular system of vertebrates. They play a basic and decisive role in the innate immune defence of the hosts against all types of pathogenic microorganisms.

Methods: Based on a literature study, the functions of neutrophils and cells with similar functions are described. The study places special emphasis on organisms in the aquatic environment and the pathogens occurring in that particular environment.

Results: The evolutionary origin of this specific cell type is not clear, but its most basic traits (recognition of foreign elements, extracellular trap release, phagocytosis and elimination of ingested material) are found in phagocytes in members of evolutionary ancient invertebrate groups spanning from amoebae, sponges, sea-anemones, molusks (snails and mussels), arthropods (crustaceans and insects) to echinoderms (sea stars and sea urchins). Their functions as innate immune sentinels and effector cells in these groups are well described. Neutrophilic granulocytes with elongated and lobed nuclei (possibly allowing cell movements through narrow extracellular spaces and leaving space for phagosomes) occur in vertebrates including fish, amphibians, reptiles, birds and mammals although the morphology of the nucleus, stainability of cytoplasmic granula, and the antimicrobial armament vary among groups. Following the pathogen invasion of a fish host, the neutrophils migrate from the vascular system into the infection focus. They apply their PRRs (including TLRs) to recognize the invader as non-self, produce netosis by casting extracellular chromatin containing traps in the microenvironment. These nets assist the immobilization of invading microbes and prevents their further spread. The cells attach to and engulf the microbes by phagocytosis, whereafter they eliminate the pathogen in phagolysosomes equipped with a range of killing mechanisms and attract, by release of chemokines, additional immune cells (monocytes, macrophages and lymphocytes) to the site of invasion. Their role in innate immunity of fish hosts towards aquatic pathogens has been elucidated by in vivo and in vitro studies. Neutrophils interact with virus (e.g. IPNV and VHSV), bacteria (e.g. Aeromonas, Vibrio, Edwardsiella, Mycobacterium and Renibacterium) and

Abbreviations: AMP, Antimicrobial peptides; ICAM, Inter-cellular adhesion molecules; IHNV, Infectious haematopoietic necrosis virus; IPNV, Infectious pancreatic necrosis virus; LFA, Leukocyte functional antigens; MHC, Major histocompatibility complex; MMP, Matrix metalloproteinase; MPO, Myeloperoxidase; NET, Neutrophil extracellular trap; NLR, Nod-like receptors; NO, Nitric oxide; PAMPs, Pathogen-associated molecular patterns; PMN, Polymorphonuclear neutrophilic leukocytes; PRRs, Pathogen recognition receptors; RER, Rough endoplasmic reticulum; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; TLRs, Toll-like receptors; VHSV, Viral haemorrhagic septicemia virus.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. Parasite Immunology published by John Wiley & Sons Ltd.
Polymorphonuclear neutrophilic leukocytes (PMNs or simply neutrophils) play together with macrophages a basic and central role in the innate immune defence in vertebrates. Neutrophils are phagocytes known from fish, amphibians, reptiles, birds and mammals. Neutrophil leukocytes in fish show a morphology, function and behaviour of neutrophils, which in many aspects is known in other vertebrates, suggesting that this cell type is an indispensable part of also the piscine immune system. It is commonly believed that PMNs are short-lived and die after exerting their function at the infection focus. However, zebrafish model studies have indicated that some of these cells may leave and migrate back to the vascular system.

The cells contain, apart from the elongated and/or lobed nucleus and cytoplasmic granula, a series of organelles including the mitochondria, Golgi apparatus, ribosomes and rough endoplasmic reticulum (RER). Its origin and evolution may have roots also in invertebrate phyla in which innate immune cells play a central role in host defence. These phagocytes are continuously circulating in the vascular system, from where they are ready to enter certain sites of infection and/or tissue injury. Subpopulations of neutrophils may serve different purposes ranging from immediate response and elimination of pathogens at points of entry to a strict survey and signalling function in the circulation. It can be hypothesized if the

 parasites, including monogeneans (Gyrodactylus), cestodes (Diphyllobothrium), trematodes (Diplostomum) and ciliates (Ichthyophthirius and Philasterides). Despite the decisive function of neutrophils in innate immunity and early protection, the excessive production of ROS, RNS and NETs may lead to pathological disturbances in the host, which are exacerbated if the pathogens evolve immune evasion mechanisms.

**Conclusion:** Neutrophils in aquatic organisms play a central role in innate immunity but may serve as a toll and a support in acquired protection. The strong impact of the cellular reactions not only on pathogen but also on host tissues emphasizes that an optimal immune reaction is balanced, involves targeted and specific effector mechanisms, which leaves a minimum of collateral damage in host organs.

**KEYWORDS**
innate immunity, NET, neutrophil, phagocytosis, ROS

**1 | INTRODUCTION**

**1.1 | Cell morphology and size**

**FIGURE 1** The piscine neutrophilic granulocytes in circulation (NC) next to red blood cells (RBC) in a capillary, neutrophils adhering to the endothelium (NA), during the process of extravasation (NE), in the epidermis (NEP) and during phagocytosis and production of phagolysosomes (NPP) seen in the cytoplasm. An infection focus is indicated showing NET formation with entrapment of microorganisms after a breach of the epidermis with mucous cells (MC) whereafter pathogens (PA) invade the host from the external environment.
primary and secondary vascular systems with different leukocyte composition support this differential function. The size of the cells vary according to their activity. When kept in solution, they are rounded and less than 10 m in diameter, whereas they enlarge when they spread their pseudopodia when attaching to a substrate. Along with macrophages, lymphocytes and thrombocytes, the neutrophilic granulocytes may dominate around both acute and chronic infections in both skin, gills and internal organs. The migration of these cells from the circulation and into specific tissues is termed extravasation and diapedesis (Figure 1). The need for a cell type such as the neutrophilic granulocyte to move from the circulation through narrow intercellular spaces may explain the peculiar nucleus shape. The elongated and lobed shape will facilitate the movements and even make more space for uptake of foreign elements by phagocytosis and formation of phagolysosomes. The communication to and control of the cell behaviour is effected by signal molecules, which may be delivered by other host cells (e.g. macrophages and epithelial cells) or pathogens invading the host organism. The signal molecules (including chemokines and cytokines) interact with receptors located on the PMN membrane, which induce a chemotraction process recruiting neutrophils to the site. This will secure abundance of neutrophils at sites where various pathogens may have entered the host. A characteristic trait of PMNs is their ability to cast extracellular chromatin containing traps (NETs) which immobilize microbes in their surroundings. The release of a range of chemokines explains not only the chemotactic effect but also immunomodulatory influence on other leukocyte types. Based on pathogen recognition receptors (PRRs) on the surface of the neutrophils, the phagocytosis process can be initiated by direct binding to the pathogen, which will be engulfed (internalized in a membrane bound vesicle) and isolated in a vacuole, with a content at low pH, termed a phagosome. This may fuse with lysosomes (originating from the endoplasmic reticulum and Golgi bodies) present in the cytoplasm, whereby the micro-organism is directly exposed to the lysosomal effector molecules such as enzymes, antimicrobial peptides (AMPs), and N- and O-reactive radicals. This will in most cases lead to a fast killing of the engulfed micro-organisms, unless these have evolved mechanisms to resist the attack. Neutrophilic leukocytes are abundant in fish and their interaction with invading pathogens have been documented for a wide array of aquatic pathogens. Following or during the process of performing the microbe-elimination process, the neutrophils may succumb in an apoptotic process (and removed by macrophages and dendritic cells) although it has been reported that neutrophils in zebrafish may return to the circulation.

1.2 | Regulation of neutrophil responses

The neutrophil function involves a wide array of receptors, adhesion molecules and effector molecules but some factors act as central regulators and thereby play a pivotal role. The gene termed Rac2, encoding a Rho small GTPase, plays a central role in neutrophil function in zebrafish. The authors inhibited the gene and found a dose-dependent decrease in respiratory burst, NET release and phagocytosis assays, which suggests that the gene has a similar role as in human neutrophils. Vertebrate neutrophil receptors and surface molecules include TLRs, MHC class I, cell-adhesion molecules, leukocyte functional antigens LFA-1, LFA-2, LFA-3, glycoproteins, intercellular adhesion molecules (ICAMs) which allow the neutrophils to interact with self and non-self molecules in their surroundings. A central function is the ability to leave the circulation and enter infection foci. This endothelium–leukocyte interaction makes use of integrin family molecules. For attraction of other cell types, a production of the cytokine IL-8 is central. The process comprises first the contact between the neutrophil and the endothelial cell layer in the capillaries. The endothelial cells display adhesion receptors (selectins), which will interact with integrin bound to the neutrophil membrane. Following binding the cell will cross the endothelial cell layer, the basal membrane and enter the site of injury or infection. Once present in the focus area, the cell may release chemokines/ cytokines, produce NETs, release N- and O species, complement factors and AMPs such as cathelicidins and release of chemokines/ cytokines attracting other immune cells. Indications for occurrence of these processes in infected fish surfaces may be derived from the immune gene expression following bacterial and parasitic infection of gills and skin of fish. Thus, genes encoding IL-8 (cytokine/ chemokine attracting neutrophils and other leukocytes), cathelicidin 1 and 2 (antimicrobial peptides), lysozyme and complement factors are expressed following Vibrio anguillarum exposure of rainbow trout gills. External environmental factors influence the neutrophil response as temperature is a main regulator of fish physiology due to the poikilothermic nature of this animal group. Some studies have shown a temperature-dependent positive correlation between peripheral blood neutrophil concentrations in Atlantic salmon blood between 10 and 18°C. However, due to the different ecology of various species ranging from a warm water species such as common carp to the cold water species such as Atlantic salmon, the picture may change. Carp showed higher neutrophil concentrations at lower temperatures suggesting a need to elevate innate response at low temperatures at which the adaptive immune reaction is depressed.

1.3 | Effector molecules and mechanisms in piscine neutrophils

The combat of infections in vertebrates involving neutrophils include acidification, NET formation, production of reactive oxygen and nitrogen radicals, antimicrobial peptides and enzymes including lysozyme and elastase. The headkidney in fish is the central immune organ and the source of the myeloid cell lines, including the neutrophils (Figure 2). The presence of a primary and secondary vascular system in fish may play a role in immune cell trafficking to and from infection foci and secondary immune organs. It has been the general opinion that following exertion of their killing processes, the cells went through an apoptosis event. However, indications for a recirculation of the cells in zebrafish have been presented. Neutrophils from a selected range of fish species have been investigated for their production and expression of the mentioned important immune
FIGURE 2 Schematic overview of neutrophil production in the headkidney of a fish. The cell circulation and function in fish primary and secondary vascular systems.

2 | ORIGIN AND EVOLUTION

The neutrophilic granulocyte is in principle a phagocyte with the ability to phagocytose foreign objects, including various types of microorganisms, and eliminate these by use of several killing mechanisms. This ability to ingest and kill external subjects is an evolutionary early trait met in protozoans, such as amoebae and ciliates, and further developed in animal sponges, cnidarians, flatworms, annelids, molluscs, arthropods, echinoderms, chordates, agnathans and gnathostome vertebrates. Even in the most basic and primitive animal, the amoeba, basic functions are met, including recognition of foreign elements, attachment, phagocytosis, lysis of internalized particles, killing of micro-organisms. The ability to recognize PAMPs by PRRs and initiate production of antimicrobial killing mechanisms, including antimicrobial peptides, N- and O species, suggests that the function of neutrophilic leukocytes is evolutionary ancient and indispensable for survival of the host. The neutrophilic granulocyte in vertebrates may not be considered a direct homolog to the phagocytes seen in various invertebrate lineages. Rather, the variety of invertebrate cell functions is very high, and during evolution, some cell types and some functions were lost, whereas some basic biochemical functions have been retained and have found application in vertebrate lineages. Aquatic pathogens have thereby interacted with phagocytes throughout evolution for at least one billion years and numerous immune evading mechanisms of invading pathogens against phagocytosis and elimination have been developed. Many of these functions have been described not only in aquatic animals (such as cartilaginous and teleost fishes) but also in semi- and holo-terrestrial amphibians, reptiles, birds and mammals. Neutrophils dominate among leukocytes in the blood of most mammals, whereas merely a few per cent of the circulating leukocytes in teleosts are neutrophils. Thus, also monocytes play a major role when non-mammalian hosts are responding to bacterial infections. The segmented nucleus is a characteristic feature of these cells. Avian and amphibian neutrophils possess a multilobed nucleus, whereas some reptiles and fish often exhibit a more mononuclear shape. The function of this peculiar nucleus morphology may be a higher nuclear flexibility and cell motility when migrating through tissues and elevates space in the cytoplasm for phagosomes. The cytoplasm contains granules, which in contrast to eosinophils and
macrophages do not stain with eosin and haematoxylin. Birds, reptiles, amphibians and fish do, however, also have neutrophil-like cells which stain with eosin. These cells are termed heterophils.

2.1 | Invertebrate cells with neutrophil characters

2.1.1 | Amoeba

The ancient nature of the ability to recognize foreign elements, trap and phagocytose them is exemplified by protozoans. Amoebae possess membrane bound receptors supplying the protozoan with an ability to recognize Gram-positive and Gram-negative bacteria, respectively, \(^{22,23}\) whereafter effector molecules contribute to elimination of intruders. Thus, the production of reactive oxygen radicals and the formation of extracellular traps by amoebae are central elements in sequestration and killing of bacteria. \(^{4}\)

2.1.2 | Animal sponges (Porifera)

Animal sponges are basic organisms presenting a relatively low level of organization. Some of the cell types carry receptors which can bind to foreign elements, and of these, the so-called Nod-like receptors (NLRs), bind to intracellular antigens. \(^{24}\) Others bind to extracellular elements such as fungi and bacteria. Binding of the antigen to the receptor initiates signal transduction, \(^{25}\) which may lead to the production of a series of protective molecules such as fibrinogen-like proteins \(^{26}\) and perforin. \(^{27}\) The ability of the freshwater sponge *Eunapius carteri* to combat intruding elements was elucidated by Mukherjee et al. \(^{28,29}\) showing a marked phagocytic and cytotoxic response, including the production of reactive oxygen radicals and lysozyme.

2.1.3 | Cnidarians (Anthozoa)

The cnidarians comprise very diverse lineages ranging from parasitic myxozoans to free-living organisms such as jellyfish and sea anemones. The group members possess various cell types and among these some carry immune functions. \(^{30}\) Some of the cells are amoebocytes with a well-developed ability to recognize, respond and phagocytose. One important and basic way to respond is based on the presence of TLRs on the cell surface. \(^{31}\)

2.1.4 | Flatworms (Platyhelminthes)

The flatworm group is composed of several taxa of both parasitic and free-living species. Well-known parasitic groups are trematodes (flukes), cestodes (tapeworms) and monogeneans. They inhabit various aquatic microhabitats (gastrointestinal tract, skin, gills) in their hosts, all of which are rich in micro-organisms. Thus, a trematode in the gut of various types of vertebrates encounter numerous different bacterial taxa associated with the intestinal mucosa and the gut content. A well-developed immune system in these flatworms are therefore indispensable. However, our knowledge on the flatworm response system has mainly been established through studies on planarians, which are free-living. Planarians possess phagocytes, \(^{32}\) termed reticuloocytes, \(^{33}\) exhibit chemotactic responses, and have the ability to phagocytose foreign objects, including bacteria. \(^{34}\) The subsequent process involves the fusion of lysosomes with the phagosomes which allow enzymes to degrade the ingested material. It is further hypothesized that the phagocytes produce and liberate perforins, which may directly kill invading microbes. \(^{35}\)

2.1.5 | Annelids (Polychaeta, Oligochaeta and Hirudinea)

Annelids are segmented worms counting thousands of species and in all the subgroups, Polychaeta, Oligochaeta, Hirudinea, functional immune systems have been documented. Oligochate immune cells, the coelomocytes, are providing these worms with a protective immune response. Thus, earthworms possess hyaline and granular amoebocytes performing phagocytosis and encapsulation of invading microbes, \(^{36}\) whereas the earthworm eleocyte is non-phagocytic but produce bactericidal molecules. \(^{37}\) The coelomocytes display TLRs and may be cytotoxic, \(^{38}\) based on the production coelomic cytolytic factor (CCF) from the coelomic fluid of earthworms. \(^{39}\)

Functional comparative physiological and behavioural studies on phagocytes (isolated from humans and earthworms), when exposed to *Escherichia coli* and *Staphylococcus aureus* bacteria, have added to the notion that the neutrophils carry ancient traits seen early in evolution. \(^{40}\) Another annelid group is the leeches, worms which are mainly known as blood sucking parasites on vertebrates, although we know that a range of species are non-parasitic. Members of this invertebrate group possess a circulatory system with the presence of relatively well-characterized haemocytes. Although it is not possible to identify a neutrophilic granulocyte in the leeches, it is clear that functions associated with this vertebrate granulocyte are to some extent found associated with the leech haemocyte. Different granulocytes, termed granulocyte type I and II, have been identified as important actors in the cellular defence of leeches. The granulocyte type II recognizes, responds and migrates towards intruding foreign elements and shows similarities with neutrophils. \(^{41}\)

2.1.6 | Molluscs (Gastropoda, Bivalvia, Cephalopoda)

Gastropods (snails and slugs) in freshwater, brackish and marine environments have central positions and roles in transmission of mainly digenean trematodes. These parasites, of which many reach another and often sexually mature stage in fish, amphibians, reptiles, birds and mammals, are dependent on the development of larval stages in the snails. Snails become invaded by miracidia, released from hatching
eggs, whereafter other larval stages (sporocysts and/or redia) develop in muscle tissue or organs such as the hepatopancreas. Within these new infective motile and infective larvae, termed cercariae, are formed and released whereafter new hosts (e.g. fish) become infected, when their skin become penetrated. Schistosome cercariae may directly penetrate the skin of humans and mature in the portal veins or bladder wall of the host. To sustain the existence of a trematode species, the immune response of the hosts must not be too effective at the early stage of infection. The first intermediate host, the snail, responds primarily to the early and primary infection with miracidia by raising an immediate cellular reaction, which involves a migration of cells towards the intruder. The parasite resist the attack, and instead of eliminating the parasite, it is rather encapsulated by the snail haemocytes. Myeloperoxidase MPO occurs in the cytoplasmic granules in haemocytes (neutrophil-like cells) in this animal groups. Later, the snail will be able to respond with a relatively effective and often protective response involving humoral factors.

2.1.10 | Crustaceans

This group comprises shrimps, crabs and copepods which mainly occur in the aquatic environment. However, members of this large invertebrate taxon do not only occupy the aquatic environment (freshwater, brackish and marine waters) but may even be terrestrial. Beside the production of a range of humoral molecules, the cellular immunity in crustaceans play a central role for the defense against microbes. Recognition and phagocytosis and killing of invaders are the first response of the cells which may confer some immunity towards reinfection. The antimicrobial armament consists of reactive oxygen and Nitrogen species (hydrogen peroxide, nitric oxide, superoxide anion, hydrochloric acid and hydroxyls) and a prophenoloxidase system securing melanization around the intruder. Even small crustaceans such as the copepod *Macrocyclops albidus* raise immunity against parasites including the cestode *Schistocephalus solidus*. Another crustacean, the isopod woodlouse *Porcello scaber*, possesses specific receptors on their haemocytes, which respond to certain heat-killed bacteria in vitro and elevates the phagocytic activity against previously encountered bacteria.

2.1.11 | Echinoderms

This group, considered highly developed among the invertebrates, often termed proto-chordates, comprises among others seastars, sea cucumbers and sea urchins are central organisms in the history of basic studies of phagocytes. The father of cellular immunity, the Russian zoologist Élie Metchnikoff (1845–1916), observed already in the 19th century that the coelomocytes of sea stars are able to encapsulate and/or phagocytose foreign elements including bacteria. It had previously been observed that leukocytes could perform phagocytosis but the cellular interactions in these invertebrates, resembling inflammation in vertebrates, were found surprising. Further studies have shown that the echinoderm
phagocytes produce reactive oxygen radicals, haemolysin, agglutinins, phenoloxidase and antimicrobial peptides. Echinoderm genomes investigated show a richness of receptors making this group of animals well suited for recognition of foreign material. Thus, the sea urchin Stronglylocentrotus purpuratus carry at least 221 TLR genes, Mesocentrotus franciscanus possess 276 TLR genes and S. fragilis 238 TLR genes. Despite the lack of evidence for occurrence of cytoplasmic granules in the echinoderm haemocytes (and thereby evidence for a close neutrophil-like cell type), the production of these other elements may add to the notion that vertebrate neutrophils find corresponding biochemical analogues in invertebrates.

2.2 | Chordates carrying neutrophilic granulocytes

On the border between invertebrates and vertebrates, the less developed chordates, comprising cephalochordates and urochordates, exhibit a display of cell types. Among the different haemocyte cell types in the tunicate Botryllus schlosseri, the morula cell produces a series of lectins and complement factors. Also, Amphioxus (Branchiostoma lanceolatum) is equipped with haemocytes involved in host defence.

3 | NEUTROPHILS IN FISH INTERACTING WITH AQUATIC PATHOGENS

Numerous studies have been performed on the responses of fish leukocytes towards aquatic pathogen counting virus, bacteria and parasites. Most of the experiments have been performed with a crude isolation of leukocytes from headkidney, spleen or peripheral blood. Many of these cells were not further characterized before use in the assays and may comprise both neutrophils and monocytes/macrophages/monocytes, but it is worth noting that in those studies, clear responses were described towards virus, bacteria and parasites. Isolation of neutrophils from peripheral fish blood or headkidney is performed by use of a discontinuous Percoll gradient. The cells can then be kept in short-term culture in tissue culture wells, used for motility/migration assays or spread on microscope slides for staining (histochemical/immuno-histochemical) and subsequent microscopic evaluation. Studies on the behaviour of the cells following stimulation with various chemo-attractants or immune stimulants may be conducted use of sub-agarose migration and Boyden chamber techniques. The cytoplasmic granules of the classical neutrophils will not stain with standard histological stains eosin and haematoxylin. However, in some species of fish (as may be found in some amphibians, reptiles, birds and mammals), the neutrophil granula stain with eosin, and the cells are therefore termed pseudo-eosinophils or heterophils. Fish neutrophils are probably equipped with membrane surface TLRs. Toll-like receptor agonists extended the lifespan up to 10 d of gilthead seabream (Sparus aurata L.) acidophilic granulocytes, considered, in this fish species, as a functional equivalent of the mammalian neutrophil. The agonists applied sustained a long lasting induction of gene expression (proinflammatory cytokines), phagocytosis and respiratory burst.

3.1 | Virus

The viral disease IPN is caused by the birna-virus infectious pancreatic necrosis virus. It infects salmonids and cause severe disease and mortality among susceptible fish. Neutrophilic granulocytes take part in the host response against this pathogen as illustrated by enumerating neutrophils in salmon after exposure to a virulent strain of IPNV, eliciting 50% mortality in the cohabitated fish. The neutrophils occurred in significantly lower levels in infected fish compared to controls, which indicated that neutrophils in salmon are highly influenced by the infection. Severe problems in fish populations are also caused by rhabdovirus in fish. Cyprinids are susceptible to the spring viraemia of carp (SVC) virus, and salmonids suffer from Egtved disease (VHS) caused by viral haemorrhagic septicaemia virus (VHSV) infections. A closely related rhabdovirus is infectious haematopoietic necrosis virus (IHNV) causing a corresponding disease in salmonids. In a zebrafish model, it was shown that neutrophils in the fish host gather around endothelial cell layers infected by VHSV and clearly interact with the virus. It occurs that merely a subpopulation of the neutrophil supra-population migrates to and takes action in the infection focus. During the interaction with infected cells, it was observed that other neutrophils seem to circulate undisturbed in the vicinity without being attracted. Is corresponds to the existence of neutrophil subsets with different functions in mammals including humans.

3.2 | Bacteria (Gram-positive)

The protection afforded by neutrophils in fish infected with Mycobacterium marinum has been demonstrated by using a zebrafish model. The function of the neutrophils was to remove M. marinum-infected macrophages, as the neutrophils did not interact directly with mycobacteria at the initial infection site. Rather, they were attracted to dying macrophages which they effectively cleared by phagocytosis. Bacterial kidney disease (BKD) is caused by other Gram-positive bacteria Renibacterium salmoninarum. During infections of salmonids, the pathogen dominates in the kidney due to the high vascularization of this organ, but the bacteria occur also in other organs. Following the infection of macrophages, R. salmoninarum survives in the cytoplasm or in phagolysosomes and proliferates in its protected intracellular space, whereas neutrophils appear to be more hostile to the bacteria. Complement mediated opsonization of these bacteria by preincubation in host serum augments phagocytosis.
3.3 | Bacteria (Gram-negative)

*Aeromonas* is a genus of Gram-negative bacteria comprising several species with a significant pathogenic potential. Within this genus, *A. salmonicida* is a major pathogen of Atlantic salmon (*S. salar*). Isolated salmon neutrophils were able to phagocytose bacteria, both opsonized and non-opsonized, but opsonization with immune sera increased the response. Likewise, the production of reactive oxygen species and induction of Boyden chamber migration of salmon neutrophils were enhanced by pretreatment of bacteria with serum. Another bacterial fish pathogen within this genus is *A. veronii* infecting a range of host species, including goldfish (*Carassius auratus*). Neutrophils from this host species infiltrate inflammatory sites and take part in the induction and regulation of acute inflammatory responses. The authors isolated neutrophils from goldfish and detected in vitro migration of the cells towards *A. veronii*. A marked respiratory burst involving production of reactive oxygen radicals in the cells was measured. The neutrophils also internalized dead or dying macrophages previously infected with this bacterial pathogen.

*Edwardsiella tarda* is a Gram-negative bacterial pathogen infecting a range of fish species and resulting in severe disease in aquacultured fish. Isolated neutrophils from tilapia were readily attracted in vitro by chemo-attractants released by the bacterium and shown to play a role central role in the protection of tilapia against bacterial invasion. An immunosuppressive cortisol treatment of the fish resulted in lower neutrophil activity and elevated susceptibility to infection. Neutrophil extracellular traps in Tongue sole were demonstrated to entrap *Edwardsiella* bacteria as a first step towards immobilization of the invading bacteria.

3.4 | Parasites: Protozoans

The cellular response of various freshwater fish species to young trophonts of the freshwater ciliate *I. multifiliis* in the epidermis shows that various types of leukocytes are attracted to the site of invasion. Classical histopathological investigation of common carp infected with *I. multifiliis* trophonts have demonstrated infiltration of neutrophilic granulocytes around the encapsulated parasite in the fish epidermis. By the use of transgenic zebrafish, expressing GFP in the neutrophils (whereby the live cells can be easily detected with an 488 nm excitation wave-length), the densities and migration of these cells in the vascular system can easily be monitored. Neutrophils readily detect an invasion of the epidermis and migrates towards an invading theront (infective stage) of the parasitic ciliate *I. multifiliis*. The dynamics and kinetics of neutrophils were further monitored during an infection/reinfection event. Immediate diapedesis of neutrophils from the vascular system to the invasion site preceded accumulation of neutrophils in the infection focus. Although the fish established an adaptive response (including antibody gene expression) following the initial infection, the neutrophils seemed to play a major role during reinfection. Neutrophils are also central elements in fish responding to another protozoan, the scuticociliate *Philasterides dicentrarchi*. This protozoan is highly pathogenic and able to penetrate skin and gills of the host fish and migrate to internal organs including the central nervous system. Several species including seabass and turbot suffer from infections. One of the response mechanism activated upon infection in turbot is the neutrophilic response. Injection of *P. dicentrarchi* into the body cavity of turbot elicited a massive recruitment of neutrophils to the site, where the cells contributed to extensive coagulation and clot formation leading to immobilization of the parasites.

3.5 | Monogeneans

The mainly ectoparasitic flatworms within the class Monogenea are highly specific parasites on fish. They establish, especially when occurring in high number on the fish surface, a strong inflammation in skin, fins and/or gills of the fish host. In vitro exposure studies using rainbow trout leukocytes have demonstrated that the skin parasitic *Gyrodactylus derjavinioides* becomes colonized and subsequently killed by the cells.

3.6 | Cestodes

Rainbow trout leukocytes react to antigens from the cestode *Diphyllobothrium dendriticum* with polarized migrations and when directly exposed to host cells, the cestode larva becomes encapsulated by various cell types, including neutrophils. *Ligula intestinalis* is a common parasite in the roach body cavity but may also penetrate the musculature which attract various leukocytes, which lead to encapsulation by host cells, among others the neutrophilic granulocytes.

3.7 | Trematodes

Eyeflukes within the genus *Diplostomum* are prevalent in freshwater fish, both in natural waters and in fish farms. Fish become infected following penetration of their external surfaces (skin, fins and gills) by cercariae released from freshwater snails (mainly within the family Lymnaeidae). The cellular immune response capacity of rainbow trout, involving cytotoxic mechanisms, was described by *in vitro* studies. Isolated trout headkidney leukocytes, with inclusion of neutrophils, were incubated with young *Diplostomum metacercariae* resulting in colonization and killing of the parasites.

4 | PATHOGEN EVASION OF NEUTROPHIL IMMUNE MECHANISMS

The neutrophils represent one of the first immune elements activated when pathogens invade the host organism. It is evident that such a cellular barrier prevents extensive pathogen colonization.
of the host until other innate and adaptive immune responses are operating. However, the effectiveness of neutrophils is less than absolute, and often at least a subpopulation of the invading microorganisms survive during their first attack. If this first line of defence would be too effective, we would not have any pathogens present in the host species and this clearly not the situation. The different pathogens apply a series of counteractive systems for survival in the hostile microhabitat created by the incoming neutrophils. A simple survival strategy is displayed by the parasitic ciliate *I. multifiliis* penetrating the epidermis of naïve freshwater fish. Neutrophils are clearly recruited to the infection focus, neutrophils are clearly attempting to attach to the trophont by extending their pseudopodia but the early trophont simply ingests the neutrophilic granulocyte, which subsequently can be seen in food vacuoles in the parasitic organism. More advanced evasion mechanisms will include disguise of their surface PAMPs and inhibition of the central parts of the neutrophil armament: mobility, phagocytic capability, NET formation, production of ROS and RNS, complement activation, production of AMPs and perforins. Atlantic salmon neutrophils are less responsive to virulent strains compared to less virulent *A. salmonica* compared to less virulent forms. Comparative experimental in vitro studies suggested that phagocytosis, motility, complement activation and production of reactive oxygen radicals were functions inhibited by the virulent bacterium. It is also conceivable that the Trojan horse strategy applied by terrestrial animal neutrophils is applicable for aquatic animals. The pathogen, such as *Brucella abortus*, is taken up by the neutrophilic granulocyte which subsequently induce apoptosis in the cell. Thereafter, macrophages engulf the dead cells carrying bacteria and distribute the pathogens systemically in the host. This abuse of the first-line cellular defence system may be present in fish as well.

5 | CONCLUSION

Neutrophilic granulocytes play a major role for protection of fish against aquatic pathogens. The precise evolutionary origin of the neutrophil granulocyte is elusive, but strong evidence suggest that the defence armament of this cell type has an ancient origin. Phagocytes in the early protozoans and metazoa are able to differentiate self from non-self and are able to colonize and engulf the invading foreign elements. Granulocytes with a phagocytic potential and killing mechanisms have been described in the majority of the invertebrate taxons, ranging from amoebae, animal sponges, coelenterates, molluscs, crustaceans and insects. The true neutrophilic granulocyte is a basic element of the innate immune system of all vertebrates and when various viral, bacterial and parasitic pathogens invade fish the neutrophil granulocyte becomes active as the first cellular against the intruder. Evidence suggests that different subpopulations of these granulocytes exist in a host individual, some take action in the local combat of pathogens at the infection focus, whereas others remain as constant surveying cells in the vascular systems. The cell is equipped with a range of PRRs recognizing PAMPS. They produce NETs, ROS, RNS, perforins and AMPs which enables the host to limit the spreading and injuries induced by the pathogens. The cell communicates extensively with other parts of the innate and adaptive immune system by production of chemokines and cytokines. The protective effect of the neutrophil activity is not absolute, whereby subpopulations of the pathogens may survive until adaptive immune reactions have been established in the host.

ACKNOWLEDGEMENTS

The author is employed by the University of Copenhagen.

CONFLICT OF INTEREST

The author declares that he has no conflicts of interests.

PEER REVIEW

The peer review history for this article is available at https://publon.com/publon/10.1111/pim.12915.

ORCID

Kurt Buchmann https://orcid.org/0000-0002-2747-237X

REFERENCES

1. Fingerhut L, Dolz G, de Buhr N. What is the evolutionary fingerprint in neutrophil granulocytes? Int J Mol Sci. 2020;21:4523.
2. Ferguson HW. Systemic Pathology of Fish. Scotian Press, ; 2006:367.
3. Mathias JR, Perrin BJ, Liu T, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebras, *J Leukoc Biol*. 2006;80:1281-1288.
4. Zhang X, Soldat T. Of amoebae and men: extracellular DNA traps as an ancient cell-intrinsic defence mechanism. *Front Immunol*. 2016;7:269.
5. LvG J. The dynamics of neutrophils in zebrafish (*Danio rerio*) during infection with the parasite *Ichthyophthirius multifiliis*. *Fish Shell Immunol*. 2016;55:159-164.
6. LvG J, Korbut R, Jeberg S, Kania PW, Buchmann K. Association between adaptive immunity and neutrophil dynamics in *Danio rerio* infected by a parasitic ciliate. *PLoS One*. 2018;13(9):e0203327.
7. Rasmussen KJ, Steffensen JF, Buchmann K. Differential occurrence of immune cells in the primary and secondary vascular systems in rainbow trout, Oncorhynchus mykiss (Walbaum). *J Fish Dis*. 2013;36:675-679.
8. Silva MT. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol*. 2010;87:93-106.
9. Pijanowski L, Golbach L, Kolaczewska E, Scheer M, Verbong-van Kemenade BML, Chadowska M. Carp neutrophilic granulocytes form extracellular traps via ROS-dependent and independent pathways. *Fish Shell Immunol*. 2013;34:1244e1252.
10. Zhao M-L, Chi H, Sun L. Neutrophil extracellular traps of *Cynoglossus semilaevis*: production characteristics and antibacterial effect. *Front Immunol*. 2017;8(290):1–9.
11. Chen T, Li Y, Sun R, et al. Receptor-mediated NETosis on neutrophils. *Front Immunol*. 2021;12: 775267.
12. Costa-Ramos C, do Vale A, Ludovico P, dos Santos NMS, Silva MT. The bacterial exotoxin AIP56 induces fish macrophage and neutrophil apoptosis using mechanisms of the extrinsic and intrinsic pathways. *Fish Shell Immunol*. 2011;30(1):173-181.
13. Tell RM, Kimura K, San Pali D. Rac2 expression and its role in neutrophil functions of zebrafish (*Danio rerio*). *Fish Shell Immunol*. 2012;33:1086-1094.
14. Kurogi J, Iida T. Impaired neutrophil defense activities and increased susceptibility to Edwardsielliosis by Cortisol Implantation in Tilapia. *Fish Pathology*. 2002;37(1):17-21.

15. Karami AM, Mathiesssen H, Ødegård J, et al. Detecting a major QTL for *Vibrio anguillarum* resistance in rainbow trout. *Front Genet*. 2020;11:607558.

16. Petersen EF, Bjlerew I, Hagland TJ, Wergeland HI. Effect of seawater temperature on leucocyte populations in Atlantic salmon postsmolt. *Vet Immunol Immunopathol*. 2005;106:65-76.

17. Engelsma M. Multiple acute temperature stress affects leukocyte populations and antibody responses in common carp. *Cyprinus carpio* L. *Fish Shellfish Immunol*. 2003;15:397-410.

18. Wen L-L, Zhao M-L, Chi H, Sun L. Histones and chymotrypsin-like elastases play significant roles in the antimicrobial activity of tongue sole neutrophil extracellular traps. *Fish Shell Immunol* 72:470-476.

19. Wu X, Zeng H, Cai L, Chen G. (2021) Role of the extracellular traps in the immune defense of the planaria *Dugesia dorotocephala*. *Hydrobiologia*. 1995;305:189-196.

20. Wiens M, Korzhev M, Krasko A, et al. Innate immune defense of the planarian *Suberites domuncula*. *Mediated activation of fibrinogen related protein and epidermal growth factor gene expression*. *Eur J Biochem*. 2004;271:1924-1937.

21. Wiens M, Korzhev M, Krasko A, et al. Innate immune defense of the sponge *Suberites domuncula* involves induction of a perforin-like molecule. *J Biol Chem*. 2005;280:27949-27959.

22. Mukherjee S, Ray M, Ray S. Phagocytic efficiency and cytotoxic responses of Indian freshwater sponges. *Eunapius carteri* cells isolated by density gradient centrifugation and flow cytometry: a morpho-functional analysis. *Zoology*. 2015;118:8-18.

23. Mukherjee S, Ray M, Ray S. Shift in aggregation, ROS generation, antioxidative defense, lysozyme and acetycholinesterase activities in the cells of an Indian freshwater sponge exposed to washing soda (Sodium carbonate). *Comp Biochem Physiol C Toxicol Pharmacol*. 2016;187:19-31.

24. Detournay O, Schnitzler CE, Poole A, Weis VM. Regulation of cnidarian-dinoflagellate mutualisms: evidence that activation of a host TGF beta innate immune pathway promotes tolerance to the symbiont. *Dev Comp Immunol*. 2012;38(4):525-537.

25. Franzenburg S, Franze S, Kunzel S, Baines JP, Domazet-Loso T, Bosch TCG. My D88-deficient Hydra reveals the ancient function of TLR signaling in sensing in sensing bacterial colonizers. *Proc Natl Acad Sci USA*. 2012;109(47):19374-19379.

26. Ishi S, Sakurai T. Food ingestion by planarian intestinal phagocytic cells? A study by scanning electron microscopy. *Hydrobiologia*. 1991;227:179-185.

27. Murita M. Structure and function of the reticular cell in the planaria *Dugesia dorotocephala*. *Hydrobiologia*. 1995;305:189-196.

28. Morita M. Phagocytic response of planarian reticular cells to heat-killed bacteria. *Hydrobiologia*. 1991;227:193-197.

29. Maciel EL, Oviedo NJ. Phagocytinminthes; molecular dissection of the planarian innate immune system. In Cooper EL (ed.). *Advances in comparative immunology*. Springer Nature, pp. 95-115. 2018.

30. Fuller-Espie SL. Vertebrate cytokines interleukin12 and gamma interferon, but not interleukin10, enhance phagocytosis in the annelid *Eisenia hortensis*. *J Inv Pathol*. 2010;104:119-120.

31. Oppen B, Bogȁr N, Heidt D. Revising lyssin expression of earthworm coelomocytes. *Dev Comp Immunol*. 2013;39:214-218.

32. Engelmann P, Kiss J, Csöngvei V. Earthworm leukocytes kill Hela, HEp-2, PC-12 and PA31 cells in vitro. *J Biochem Biophys Meth*. 2004;61:215-227.

33. Beschin A, Bilej M, Brys L. Convergent evolution of cytokines. *Nature*. 1999;400:627-628.

34. Kokhanuk B, Bodo K, Sétaló G Jr, Nemeth P, Engelmann P. Bacterial engulfment mechanism is strongly conserved in evolution between earthworm and human immune cells. *Front Immunol*. 2021;12:735541.

35. De Eguileor M, Grimaldi M, Tettamanti G, Valvassori R, Cooper EL, Lanzavecchia G. Different types of response to foreign antigens by leech leukocytes. *Tissue Cell*. 2000;32:40-48.

36. Sminia T, van der Knaap WP, Boerriort-Barendsen LH. Peroxidase-positive blood cells in snailes. *J Reticuloendothel Soc*. 1982;31:399-404.

37. Pinaud S, Portela J, Duval D, et al. A shift from cellular to humoral responses contributes to innate memory in the vector snail *Biomphalaria glabrata*. *PLoS Pathog*. 2016;1:18:1005361.

38. Soudant P, Chu FLE, Valey A. Host-parasite interactions: Marine bivalve molluscs and protozoan parasites *Perkinsus* species. *J Inv Pathol*. 2013;114:196-216.

39. Troncone L, Lisa ED, Bertappe P. Morphofunctional characterization and antibacterial activity of haemocytes from *Octopus vulgaris*. *J Nat His*. 2015;49:1457-1475.

40. Baxter RHG, Contet A, Krueger K. Arthropod innate immune system and vector-borne diseases. *Biochemistry*. 2017;56:907-918.

41. Browne N, Heelan M, Kavanagh K. An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. *Virulence*. 2013;4:597-603.

42. Rodrigues J, Brayner FA, Alves LC, Dixit R, Barillasmury C. Host-parasite interaction reveals an ancient function of the complement system. In Cooper EL (ed.). *Advances in comparative immunology*. Springer Nature, pp. 95-115. 2018.

43. Pham LN, Dionne MS, Shirasu-Higa M, Schneider DS. A specific primed immune response in *Drosophila* is dependent on phago-cytes. *PLoS Pathog*. 2007;3:e26.

44. Roth O, Kurtz J. Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse *Porcellio scaber*. *Tissue Cell*. 2009;33(11):1151-1155.

45. Hautoin C. The scope of the crustacean immune system for disease control. *J Invertebr Pathol*. 2012;110:251-260. doi:10.1016/j.jip.2012.03.005

46. Kurtz J, Franz K. Innate defense: evidence for memory in invertebrate immunity. *Nature*. 2003;425:37-38.

47. Gross PS, Al-Shariff WZ, Clow LA, Smith LC. Echinoderm immunity and the evolution of the complement system. *Dev Comp Immunol*. 1999;23:429-442.

48. Arrizza V, Schillachi D. Echinoderm antimicrobial peptides, Chapter 12. In: Ballardin L, Cammarata M, eds. *Lessons in immunity*. Academic Press, Elsevier; 2016:159-176.

49. Buckley KM, Rast JP. Dynamic evolution of Toll-like receptor multi-gene families in echinoderms. *Front Immunol*. 2012;3:136.
56. Franchi N, Ballarin L. Morula cells as key hemocytes of the lectin pathway of complement activation in the colonial tunicate Botryllus schlosseri. *Fish Shellfish Immunol*. 2017;63:157-164.

57. Rhodes CP, Ratcliffe NA, Rowley AF. Presence of coelomocytes in the primitive chordate Amphioxus (Branchiostoma lanceolatum). *Science*. 1982;217:263-265.

58. Sepulcre MP, López-Munoz A, Angosto D, García-Alcazar A, Meseguer J, Mulero V. TLR agonists extend the functional lifespan of professional phagocytic granulocytes in the bony fish gillhead seabream and direct precursor differentiation towards the production of granulocytes. *Mol Immunol*. 2011;48:846-859.

59. Rønneseth A, Pettersen EF, Wergeland H. Neutrophils and B-cells in blood and head kidney of Atlantic salmon (Salmo salar L.) challenged with infectious pancreatic necrosis virus (IPNV). *Fish Shellfish Immunol*. 2013;20:610-620.

60. Marana MH, Schmidt JG, Biacchesi S, Lorenzen N, Jørgensen LVG. Zebrafish (Danio rerio) larvae as a model for real-time studies of propagating VHS virus infection, tissue tropism and neutrophil activity. *J Fish Dis*. 2021;44:563-571.

61. Christoffersson G, Phillipson M. The neutrophil: one cell on many mission or many cells with different agendas? *Cell Tissue Res*. 2018;371:415-423.

62. Yang C-T, Cambier CJ, Davis JM, et al. Neutrophils exert protection in the early tuberculous granuloma by oxidative killing of mycobacteria phagocytosed from infected macrophages. *Cell Host Microbe*. 2012;12:301-312.

63. Rose AS, Levine RP. Complement mediated opsonization and phagocytosis of Renibacterium salmoninarum. *Fish Shellfish Immunol*. 1992;2:223-240.

64. Lamas J, Ellis AE. Atlantic salmon (Salmo salar) neutrophil responses to Aeromonas salmonicida. *Fish Shellfish Immunol*. 1994;2:223-240.

65. Havixbeck JJ, Rieger AM, Churchill LJ, Barreda DR. Neutrophils exert protection in early Aeromonas veronii infections through the clearance of both bacteria and dying macrophages. *Fish Shell Immunol*. 2017;63:18-30.

66. Hines RS, Spira DT. Ichthypophthiriasis in mirror carp. II. Leukocyte response. *J Fish Biol*. 1973;5:527-534.

67. Cross ML. Localized cellular responses to Ichthypophthirius multifilis – Protection or pathogenesis? *Parasitol Today*. 1994;10:364-368.

68. Blanco-Abad V, Noia M, Valle A, et al. The coagulation system helps control infection caused by the ciliate parasite Philasterides dicentrarchi in the turbot Scophthalmus maximus (L.). *Dev Comp Immunol*. 2018;2018(87):147-156.

69. Buchmann K, Bresciani J. Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean Gyrodactylus derjavini. *Dis Aquat Org*. 1999;35:13-22.

70. Sharp GJE, Pike AW, Secombes CJ. Leukocyte migration in rainbow trout (Oncorhynchus mykiss (Walbaum)): optimization of migration conditions and responses to host and pathogen (Diphyllobothrium dendriticum (Nitzsch) derived chemotactants. *Dev Comp Immunol*. 1991;15:295-305.

71. Sharp GJE, Pike AW, Secombes CJ. Sequential development of the immune response in in rainbow trout (Oncorhynchus mykiss (Walbaum, 1792) to experimental plerocercoid infections of (Diphyllobothrium dendriticum (Nitzsch, 1824). *Parasitology*. 1992;104:169-178.

72. Hoole D. Ligula intestinalis (L.) (Cestoda: Pseudophyllidea): an ultrastructural study of the cellular response of roach fry, Rutilus rutilus L., to an unusual intramuscular infection. *J Fish Dis*. 1989;12:523-528.

73. Buchmann K, Bresciani J. Parasitic infections in pond-reared rainbow trout Oncorhynchus mykiss in Denmark. *Dis Aquat Org*. 1997;28:125-138.

74. Duan Y, Jørgensen LVG, Kania PW, Al-Jubury A, Karami AM, Buchmann K. Eye fluke effects on Danish freshwater fish: field and experimental investigations. *J Fish Dis*. 2021;44(11):1785-1798.

75. Whyte SK, Chappell LH, Secombes CJ. Cytotoxic reations of rainbow trout Salmo gairdneri Richardson, macrophages for larvae of the eyefluke Diplostomum spathaceum (Digenea). *J Fish Biol*. 1989;35:333-345.

76. Gutierrez-Jimenez C, Mora-Cartin R, Altamirano-Silva P, et al. Neutrophils as Trojan horse vehicles for Brucella abortus macrophage infection. *Front Immunol*. 2019;10:1-8.

How to cite this article: Buchmann K. Neutrophils and aquatic pathogens. *Parasite Immunol*. 2022;44:e12915. doi:10.1111/pim.12915