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INVITED REVIEW

Neutrophils and aquatic pathogens

Kurt Buchmann

Laboratory of Aquatic Pathobiology, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Correspondence
Kurt Buchmann, Laboratory of Aquatic Pathobiology, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 7, DK-1870, Frederiksberg C., Denmark.
Email: kub@sund.ku.dk

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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, mollusks (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 migrates 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 septicaemia virus.

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

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.[1] Neutrophil leukocytes in fish show a morphology, function and behaviour of neutrophils, which in many aspects is known in other vertebrates,[2,3] suggesting that this cell type is an indispensable part of also the piscine immune system. It is commonly believed that PMNs are shortlived 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.[3]

1.1 | Cell morphology and size

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.[4] These phagocytes are continuously circulating in the vascular system,[5,6] 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

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[7] 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.[2] 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.[1] 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 chemoattraction process recruiting neutrophils to the site.[8] 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.[9,10] 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.[11] This will in most cases lead to a fast killing of the engulfed micro-organisms, unless these have evolved mechanisms to resist the attack.[12] 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.[3]

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.[13] 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.[14] 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.[15] 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.[16] 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.[17]

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.[7] It has been the general opinion that following exertion of their killing processes, the cells went through an apoptosis event.[1] 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

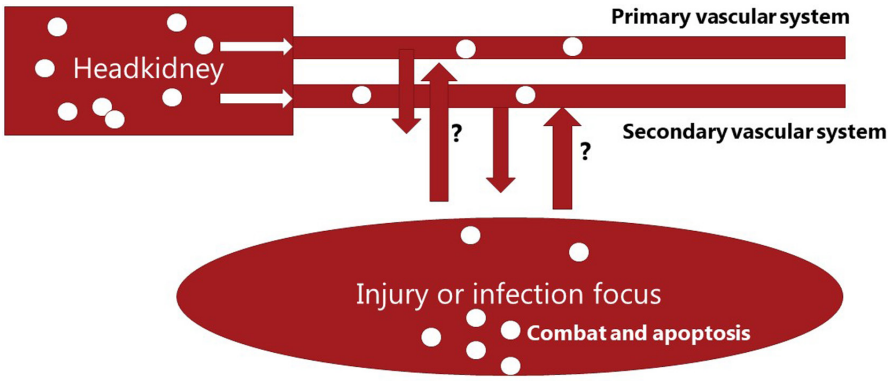

FIGURE 2 Schematic overview of neutrophil production in the headkidney of a fish. The cell circulation and function in fish primary and secondary vascular systems

effector molecules. Additional studies on other species await further efforts, but the impression is that the neutrophil function in fish corresponds, to a high extent, to other vertebrates. NETs are neutrophil extracellular traps which released by neutrophils as an important part of protection against microbial invasion. Neutrophils isolated from the tongue sole *Cynoglossus semilaevis* were chemically induced to produce NETs, and following incubation with different bacterial pathogens (*Pseudomonas fluorescens*, *Vibrio harveyi* and *Edwardsiella tarda*), it was clearly observed that the NETs captured, immobilized and inhibited the bacteria although they were not killed.[10] The neutrophils responsible for NET production produced reactive oxygen species (ROS), nitric oxide (NO) and myeloperoxidase (MPO) as well. Likewise, carp neutrophils show similar responses when *stimulated in vitro* with bacterial, fungal or viral antigens. The antimicrobial effectors associated with the NET formation also comprise a range of other factors. Various enzymes originating from neutrophilic granules, such as myeloperoxidase, neutrophil elastase and matrix metalloproteinase 9 (MMP-9) were shown to be acting with NET in carp,[9] and when the functions of NETs in the tongue sole *Cynoglossus semilaevis* were investigated, it was shown that histones and elastases contribute to the antibacterial effect of the NETs released.[18] Both Gram-negative and Gram-positive bacteria of tongue sole were inhibited. The observation was substantiated by treating NETs with antibodies targeting histones and elastases, which reduced the antimicrobial effect of NETs. It is obvious that the host organisms may benefit from the NET production to eliminate invading microorganisms. However, the immune reactions may show up as a two-edged sword. Evidence indicates that not only neutrophils but also other cell types, including microglial cells in the central nervous system, produce NETs.[19] This raises the possibility that reactions in this sensitive region of the organism may have consequences. By using a rat model, it was demonstrated that NET-formation disrupt cerebrospinal fluid transport. This may elicit oedema in the CNS infected with *Streptococcus pneumonia*, the most common cause of bacterial meningitis, and thereby exacerbate the disease.[20] This unexpected consequence of NET formation may be considered as a serious collateral damage induced by the NETS. DNase treatment disrupting NETs re-established the fluid transport and thereby prevented brain oedema and weight increase. Similar reactions may be expected in fish infected with various pathogens in their CNS.

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.[21] 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.[1] 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.[1] 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 phagcytose 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 socalled 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 (Anthozoans)

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 reticulocytes,[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/[42] Later, the snail will be able to respond with a relatively effective and often protective response involving humoral factors.[43]

2.1.7 | Bivalves (mussels, clams and oysters)

These molluscs employ a series of different cells and tissues in their physiological processes, and haemocytes are by far the most important phagocytic cell type in bivalves. The cells are able to migrate between the circulation and various tissues in which pathogen invasion occurs. One subgroup of cells is comprised by granulocytes with functional similarities to neutrophils in vertebrates. These cells perform phagocytosis and produce reactive O- and N- species in their oxidative burst response. When bivalve haemocytes encounter foreign particles or pathogens, they mount a serial reaction. Cells display chemotaxis, recognition, attachment, internalization, phagosome formation and intracellular degradation. In addition, the haemocytes may also participate in an encapsulation process of the intruder.[44]

2.1.8 | Cephalopods (squids, cuttlefish, octopus and nautilus)

These molluscs are highly developed anatomically and physiologically and display a circulatory system with three hearts, one pumping oxygenated blood and two pumping venous blood towards the gills for oxygenation. The haemocytes found in cephalopods have been classified based on their morphology and motility. Large granulocytes, small granulocytes, hyalinocytes and haemoblasts are recognized in *Octopus vulgaris*.[45] The cells perform recognition, phagocytosis and killing of foreign elements, partly based on their production of N- and O- radicals.

2.1.9 | Arthropods (Crustaceans, Insects)

Haemocytes in the haemocoel and haemolymph of insects are the main cellular component combatting invading micro-organisms by phagocytosis and encapsulation.[46] These cells have several similarities with vertebrate granulocytes[47] are equipped with pattern recognition receptors leaving them ready to attach to, engulf or encapsulate various pathogen, ranging from virus, bacteria, protozoan and metazoan parasites. As insects (e.g. mosquitoes and flies) and chelicerates (e.g. ticks) are vectors for a series of virus, bacteria and parasites, the protection afforded by the cellular response is not always absolute. Otherwise, the life cycle would be broken and the transmitted parasite eradicated. As this is not the case, the invading micro-organisms have developed evading mechanisms allowing the parasites to survive until they become transmitted to the new host. However, despite the innate nature of the cellular response, insects may establish a level of acquired immunity and exhibit a high degree of protection towards a secondary infection as demonstrated for mosquitoes[48] and fruitflies.[49]

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 defence against microbes. Recognition and phagocytosis and killing of invaders are the first response of the cells which may confer some immunity towards reinfection.[50,51] 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*.[52] Another crustacean, the isopod woodlouse *Porcellio scaber*, possesses specific receptors on their haemocytes, which respond to certain heat-killed bacteria in vitro and elevates the phagocytotic activity against previously encountered bacteria.[50]

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.[53,54] Echinoderm genomes investigated show a richness of receptors making this group of animals well suited for recognition of foreign material. Thus, the sea urchin *Strongylocentrotus purpuratus* carry at least 221 TLR genes, *Mesocentrotus franciscanus* possess 276 *TLR* genes and *S. fragilis* 238 TLR genes.[55] 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.[56] Also, *Amphioxus (Branchiostoma lanceolatum)* is equipped with haemocytes involved in host defence.[57]

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/immunohistochemical) and subsequent microscopical 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.[58] 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.[59] 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.[60] 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.[61]

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,[62] 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.[2] Complement mediated opsonization of these bacteria by preincubation in host serum augments phagocytosis.[63]

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.[64] 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.[65] 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 role a central role in the protection of tilapia against bacterial invasion.[14] 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.[10]

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.[66,67] 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*.[5] 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.[6] 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.[68] 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 derjavinoides* becomes colonized and subsequently killed by the cells.[69]

3.6 | Cestodes

Rainbow trout leukocytes react to antigens from the cestode *Diphyllobothrium dendriticum* with polarized migrations[70] and when directly exposed to host cells, the cestode larva becomes encapsulated by various cell types, including neutrophils.[71] *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.[72]

3.7 | Trematodes

Eyeflukes within the genus *Diplostomum* are prevalent in freshwater fish, both in natural waters and in fish farms.[73,74] 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[75] 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.[5] 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 of *A. salmonicida* 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.[64] 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.[76] 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 metazoans 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.

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CONFLICT OF INTEREST

The author declares that he has no conflicts of interests.

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ORCID

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

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