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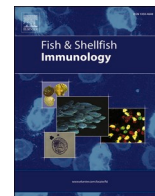
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## What can we learn about fish neutrophil and macrophage response to immune challenge from studies in zebrafish

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### ABSTRACT

Fish rely, to a high degree, on the innate immune system to protect them against the constant exposure to potential pathogenic invasion from the surrounding water during homeostasis and injury. Zebrafish larvae have emerged as an outstanding model organism for immunity. The cellular component of zebrafish innate immunity is similar to the mammalian innate immune system and has a high degree of sophistication due to the needs of living in an aquatic environment from early embryonic stages of life. Innate immune cells (leukocytes), including neutrophils and macrophages, have major roles in protecting zebrafish against pathogens, as well as being essential for proper wound healing and regeneration. Zebrafish larvae are visually transparent, with unprecedented *in vivo* microscopy opportunities that, in combination with transgenic immune reporter lines, have permitted visualisation of the functions of these cells when zebrafish are exposed to bacterial, viral and parasitic infections, as well as during injury and healing. Recent findings indicate that leukocytes are even more complex than previously anticipated and are essential for inflammation, infection control, and subsequent wound healing and regeneration.

### 1. Introduction

Most fish live throughout life in water, in which they are constantly in close contact with potential pathogens. One millilitre of sea water could contain 10 million viruses, one million bacteria and about 1000 small protozoans and algae [1], and some of these are harmful for fish. Similarly, zebrafish are exposed to a range of pathogens in their freshwater habitats, including rice fields of South Asia, before they develop a mature adaptive immune system 4 weeks after hatching [2,3]. Therefore, a system to constantly survey potential dangers is essential. This system must be able to immediately combat dangerous organisms as well as send signals to other compartments in the body, to enhance systemic responses. This system is called the innate immune system and is found with different degrees of sophistication in all animals [4,5].

Teleosts (bony fish), which represent half of all vertebrate species, diverged evolutionarily from mammals 450 million years ago [6] and today we know of more than 33,000 different species, which makes it the most diverse group of vertebrates [7]. Following the divergence, a whole genome duplication took place in fish in the Mesozoic era (252–66 million years ago) [8]. Gene duplications have also occurred, dramatically increasing the number of genes including those in pathogen recognition (eg, toll-like receptors, TLRs). Therefore, fish may have a

more diverse innate immune system compared to mammals, who rely to a higher degree on the adaptive arm of the immune system [6,9]. This frames the innate immune system as an essential mechanism for fish and highlights the importance of understanding the different components, pathways and processes. In this review, we will focus on cells of the innate immune system and more specifically on two types of leukocytes: neutrophils and macrophages. These cells are professional phagocytes and have key roles in combatting pathogens, signalling to the rest of the immune system, repairing wounds, and regenerating tissue [10].

Neutrophils and macrophages have pattern recognition receptors (PRRs) on their cell surface [10,11]. These receptors recognise pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and when activated, a cascade of reactions is initiated leading to (mostly) appropriate responses [12]. The nature of the response depends on whether bacteria, virus, parasites or an injury is the cause of activation [12]. Obtaining detailed knowledge on these mechanisms as well as understanding the difference from mammals improves our understanding of immunological reactions and evolution of the immune system. Furthermore, this knowledge contributes to the development of strategies to manage fish disease in the fisheries/production systems with regard to improvement of prophylaxis and control measurements.

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The zebrafish has been used as a model organism since the 1980s when George Streisinger fought for people's acceptance of the value of this model [13]. As with many other pioneers, he died before the recognition of the usefulness of the model impacted the scientific world and since the 1990s the number of zebrafish publications has increased dramatically (Fig. 1).

The zebrafish larval model, combined with transgenic lines labelling immune cell populations [14,15], has allowed high resolution 4D microscopy, which has shed light on the complexity of the neutrophil and macrophage response to wounding and infection in fish. However, zebrafish are a single species and, much like using murine models for human disease where there are important differences in immune responses, care must be taken when extrapolating findings to other species due to the huge diversity between fish species.

## 2. Fish innate immune systems

Fish have both innate and adaptive immunity but rely more on innate immunity to maintain homeostasis compared to higher vertebrates due to their constant close contact with pathogens through the environment and their exposure to pathogens from the early embryonic stage [9]. Fish possess primary and secondary lymphoid organs as higher vertebrates, except that they lack classical bone marrow and lymph nodes [9,16]. The functions of these organs are therefore covered by other tissues such as the head kidney and spleen [17]. They do, however, have melanomacrophage centers and rodlet cells not encountered in higher vertebrates [16]. They are a diverse group of animals, which is reflected in the different composition and mechanisms of the immune system. A fascinating example of this diversity is found in the Atlantic cod, which lacks the major histocompatibility complex II (Mhc II), a protein believed to be crucial for adaptive immunity and survival from disease [18]. Despite the lack of Mhc II, the fish survive perfectly well. It has been hypothesised that some of the Mhc I gene copies they possess have evolved to include some of the functional roles of Mhc II [19]. Fish have more genes represented in the innate immune system compared to mammals that have a higher complexity of their adaptive immune system [6].

The innate immune system is classically divided into three

compartments: physical/surface barriers, cellular and humoral components [5,20]. It can be furthermore divided into constitutive and inducible, where the constitutive immune limiting response acts quickly with ligand binding to receptors and inducible acts slower but more strongly, sometimes causing immunopathology and tissue damage [4, 21]. PRRs are an essential part of the innate responses and amongst them are toll like receptors (TLRs) which are found both on neutrophils, macrophages and other innate immune cells. PRRs recognise PAMPs and DAMPs and upon activation, an inflammatory response is initiated. Adaptive immunity and antibody production is dependent on direction from the innate immune system [21]. The adaptive immune system may in some fish species play a minor role, which was demonstrated in a study where inhibition of the adaptive arm of the immune system (B and T cells) in *rag*<sup>-/-</sup> zebrafish did not result in decreased protection following re-infection with a bacterium [22]. The Atlantic cod is another example of a fish species that may rely more on innate functions. It lacks Mhc II but has many more genes for TLRs [18,23]. It is known to have a poor antibody response [23,24] but is capable of producing specific immunoglobulins probably with cross presentation from Mhc I [19].

Neutrophils and macrophages are professional phagocytes meaning that they are efficient ingesters of microorganisms and cell debris upon recognition by receptors [25,26]. Macrophages are also professional antigen presenting cells (APCs) and present antigens to T cells on MHC II molecules. Other cell types are furthermore important in innate responses such as granulocytes, red blood cells, thrombocytes, B cells and subtypes of T cells [21].

Fish mucosal surfaces such as skin, fins, gills, intestine, nasal cavity and the newly described Nemausean lymphoid organ (NELO) [27] are immunologically active barriers representing the first line of defence in protection from various insults such as pathogen attack. These surfaces are covered in mucus, which has antimicrobial properties, and are inhabited by beneficial microorganisms (called the microbiome). Therefore, it is key to be able to differentiate "friend" from "foe". Neutrophils and macrophages are essential players in this differentiation. The cells are activated by, amongst other things, complement factors, which are components of the mucosal barriers and are activated by pathogens. A study by Earley et al. (2018) showed that complement genes were down-regulated and that the composition of the microbiome in the intestine changed with the depletion of interferon regulatory factor 8 (Irf8) dependent macrophages, illustrating the impact macrophages may have on homeostasis of the fish [28]. Keratocytes, which are found in the outer epidermal layer, are also phagocytotic and may play important roles in pathogen phagocytosis before dying and being sloughed off from the epidermis [21].

The function of the complement system in an immunological reaction is opsonisation, inflammation and killing of pathogens with the formation of a membrane attack complex [29,30]. The system consists of plasma proteins primarily produced in the liver reacting in a cascade manner with direct or indirect killing of pathogens as a consequence. The system can be activated in three different ways: 1) classical (activated by antibody-antigen complexes), 2) lectin (activated by lectins) and 3) alternative pathway (activated by lipopolysaccharides present on some pathogen surfaces) [29,30]. The complement system is important for the activation of neutrophils and macrophages. Activation of the system can result in attraction of neutrophils and macrophages and stimulate phagocytosis and can also induce inflammation [29,30]. This system is a good example of the expanded use of innate responses in fish compared to mammals, since many of the complement factors have diversified into different isoforms with functional diversification as a result [31]. Many genes of the complement system in mammals have homologs in teleosts [31]. Activation of complement factor C5a (chemoattractant) in the classically activated and alternative pathway activates macrophages and neutrophils [32]. C3b is the most important factor for opsonizing pathogens, which stimulates neutrophils and macrophages to phagocytose the opsonized objects [33,34].

More information on other components of the innate immune system

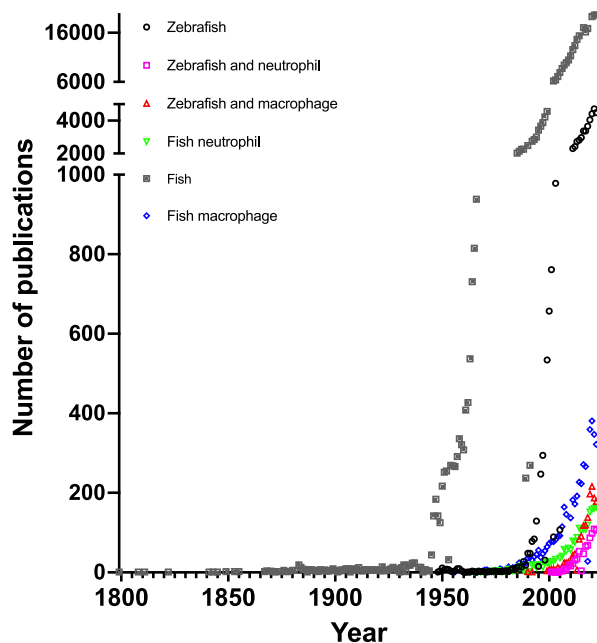


Fig. 1. Using Pubmed the number of publications is depicted showing the result of searches with the words "fish", "zebrafish", "fish macrophages", "zebrafish macrophages", "fish neutrophils", "zebrafish neutrophils" over the years.

can be found in the rest of this special issue and in e.g. Dalmo et al. (2022) [21].

### 3. Zebrafish neutrophils

#### 3.1. Neutrophil development

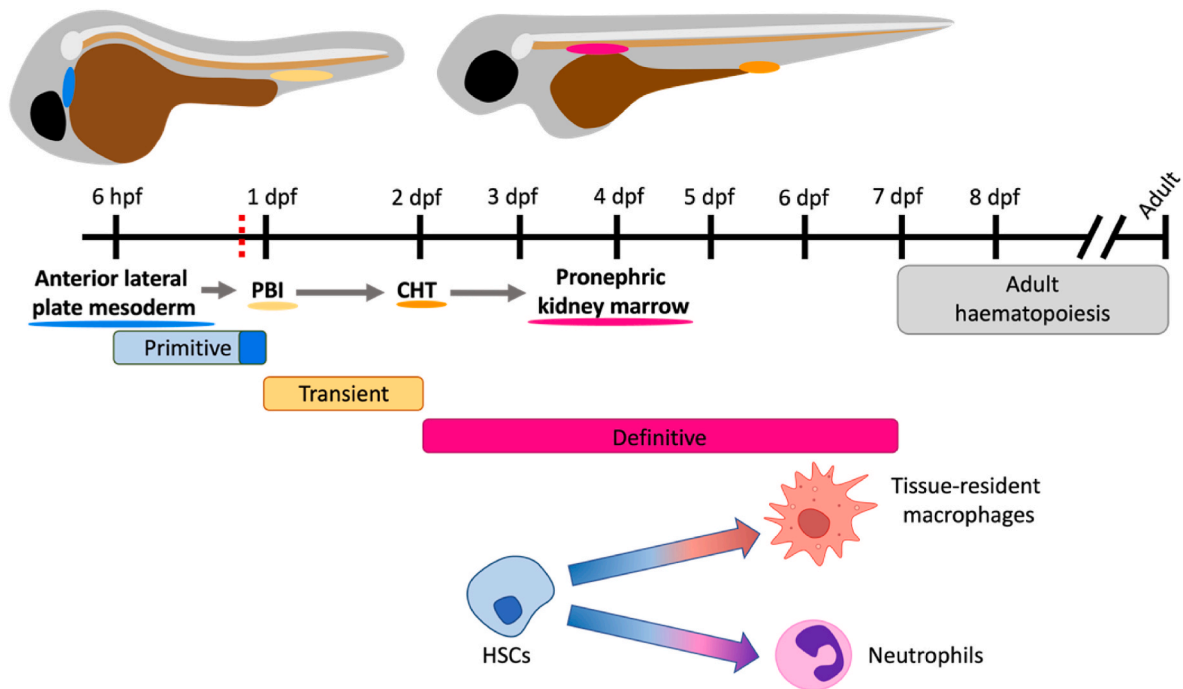
Neutrophils are the primary white blood cell to respond to infection or injury in order to restore homeostasis within an organism [35]. Haematopoiesis is the development of all blood cells, including those of the myeloid and erythroid lineages, and this process is conserved between vertebrates [36–39]. In zebrafish and other teleosts, neutrophils develop through two distinct waves of haematopoiesis (Fig. 2); primitive haematopoiesis, around 24 h post fertilisation (hpf) [40], develops spatially distinct populations of myeloid (neutrophil, mast cell, and macrophage) and erythroid cells, with this then followed by a definitive wave of haematopoiesis, at 48–72hpf, which produces all sub-types of blood cells, including lymphoid cells [41]. During the initial phase in fish, primitive neutrophils are produced in the rostral blood island (RBI) [25,37,42,43] and are believed to be immature neutrophils due to the lack of granule Sudan Black staining, an indication of neutrophil maturation [44]. A transient wave of erythromyeloid progenitor-derived neutrophils are subsequently formed in the posterior blood island (PBI) [45], which further develops into the caudal haematopoietic tissue (CHT). Haematopoietic stem cells migrate to the CHT and mature neutrophils that stain positive for both neutrophil and granule markers, myeloid peroxidase and Sudan Black, develop from this population [44, 46].

##### 3.1.1. Neutrophil maturation

As the first recognisable cells of the neutrophil lineage (myeloblasts) mature, they sequentially develop different granules, in a process known as granulopoiesis [47]. These granules are essential for the successful

function of neutrophils during an inflammatory response [48]. Primitive neutrophils are not classed as mature neutrophils due to the lack of granule markers. However, neutrophils derived from haematopoietic stem cells (HSC) during the definitive wave of haematopoiesis express many granule types [49,50]. There are three categories of granules; primary (azurophilic) granules containing myeloperoxidase, lysozyme and elastases, secondary (specific) granules containing matrix metalloproteinases such as collagenase, and tertiary granules containing gelatinase. Human neutrophil maturation during granulopoiesis is well documented [50,51], however, less is documented about the individual stages of zebrafish neutrophil development and there are some differences. The early zebrafish myeloblast becomes the promyelocyte, similar to human neutrophil progression. It has a rounded nucleus but lacks the coarse, azurophilic primary granules characteristic of human promyelocytes. As neutrophil maturation progresses, the cells become smaller in size, the chromatin condenses and divides into lobes, and the cytoplasm acquires the characteristic granules of mature neutrophils [52]. The adult zebrafish kidney and spleen contain neutrophils at various stages of development, including promyelocytes with a large, round nucleus and diffuse nuclear chromatin, and maturing neutrophils that have segmented nuclei with two or three lobes [52]. In larval zebrafish, most neutrophils have a kidney-shaped nucleus, and about 15–25% have a bilobed nucleus, but only rarely have a multilobed nucleus [44,53–56].

As neutrophils mature during larval stages into adulthood, their nuclear envelope changes in morphology [57]. Zebrafish neutrophils contain a polymorphic nucleus [55,57] as seen in mammals, such as mice and humans. Like their human counterparts, zebrafish neutrophils have segmented nuclei; however, their nuclei are divided into two or three lobes instead of the five lobes typically found in human neutrophils [52].



**Fig. 2. Summary of neutrophil and macrophage ontogeny in zebrafish.** The anterior lateral plate mesoderm (blue) develops at 6 hpf, where myeloid cell precursors originate. At 20–22 hpf, the primitive wave of haematopoiesis occurs. From 24 hpf and onset of circulation, the PBI (yellow) develops, and the transient wave of haematopoiesis begins. The PBI then transitions into the CHT (orange) at 2 dpf, marking the start of the definitive wave of haematopoiesis, with HSCs differentiating into tissue-resident macrophages and mature neutrophils. From 4 dpf, the pronephric kidney marrow (pink) begins to develop, resulting in a gradual replacement of the embryonic haematopoietic system and onset of adult haematopoiesis at 7 dpf. Abbreviations: hpf, hours post-fertilisation; dpf, days post-fertilisation; PBI, posterior blood island; CHT, caudal haematopoietic tissue; HSCs, haematopoietic stem cells.

### 3.1.2. Neutrophil production and lifespan

In mammals, neutrophils are found predominantly in the circulation, waiting for inflammatory cues. In humans, neutrophils make up around 40–70% of circulating leukocytes [51]. Zebrafish and other teleosts differ, as fewer than 5% of neutrophils are in the circulation [25,44,58–61]. During larval stages, zebrafish neutrophils reside throughout the body within tissues and in the CHT until the kidney develops [25,62–64] - this is the site of granulopoiesis in adulthood, ready for deployment of neutrophils to an inflammatory stimulus. The lifespan of neutrophils can vary depending on the environment in which they find themselves and is difficult to study due to their very short lifespan once removed from tissue microenvironments in e.g. *ex vivo* experimentation. Transparent zebrafish larvae are a tractable model to investigate neutrophil lifespan *in vivo*, with one study in the zebrafish [65] measuring the half-life of tissue neutrophils in 3 days post fertilisation larval zebrafish to be 5 days. These neutrophils are likely to be both primitive and definitive but there is no evidence to suggest that they differ in function. In mammals, circulating neutrophils are documented as having a half-life of 6–12 h [66,67], although it has been suggested that it may be more similar to that measured *in vivo* in zebrafish, around 5 days [68]. This lifespan can be significantly altered due to an inflammatory stimulus and remains controversial due to a lack of *in vivo* models where these studies are possible.

### 3.2. Neutrophil responses to immune challenge

It is well established that following an inflammatory stimulus, neutrophils become primed and activated, ready to respond to invading pathogens [69–71]. One of the first responses of the neutrophil following stimulation, is movement out of the circulation or through tissues using adhesion molecules [69,70]. This process is largely dependent on CD11/CD18 activation [72]. Any tissue damage, be it caused by a mechanical wound or invading pathogen, triggers an increase in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [73] at the damaged site, to which the activated neutrophil can respond. A hydrogen peroxide gradient forms, emanating from the wound site, which is thought to be the primary signal for neutrophils to begin migrating to the wound in zebrafish. It has been identified that the Src family kinase Lyn acts as an endogenous H<sub>2</sub>O<sub>2</sub> sensor that initiates neutrophil recruitment to the wound in zebrafish larvae [74]. Other signals are also generated following tissue damage or infection which facilitate the migration of neutrophils through tissues. DAMPs, including DNA, proteins, extracellular membrane components [75] as well as chemokines such as Cxcl1 and Cxcl2 [76,77] are detected by neutrophils, promoting further neutrophil recruitment.

As mentioned previously, H<sub>2</sub>O<sub>2</sub> and other signals such as DAMPs promote neutrophil migration through the tissue to an area damaged by mechanical injury or pathogen invasion. Zebrafish, just like in humans, have an initiation phase of inflammation that is characterised by the increased accumulation of neutrophils to a focal point which peaks at around 4–6 h post injury or infection [14,78]. The migration of neutrophils to sites of challenge in zebrafish includes a behaviour known as neutrophil swarming, first observed in murine models of inflammation [79–82]. Swarming is defined as the gradual accumulation and cluster formation in a highly coordinated manner of chemotaxis following injury [79] or infection [80]. Lämmermann et al. (2013) showed that leukotriene B4 (LTB4) is a key driver of neutrophil swarming acting as a relay signal [81]. Since then, swarming has been identified in zebrafish following tailfin injury or infection [79,80] with a role of LTB4 conserved [79,80]. Swarming has been observed in sterile tail-fin amputation [79] and in otic vesicle infection [79]. In tailfin transection it was shown that neutrophils form a swarm around a “pioneer” neutrophil that is often the first neutrophil to reach the tailfin wound. Pioneer neutrophils undergo a form of lytic cell death that resembles neutrophil extracellular trap (NET) formation. Neutrophils respond to bacteria injected into the otic vesicle, with this recruitment being

amplified by a second wave of recruitment and dense clusters being formed [79], another hallmark of the swarming process. Poplimont (2020) has shown that a cell contact-dependant mechanism through calcium alarm signals enhances swarming and aids bacterial clearance, however the full molecular mechanism and *in vivo* purpose of swarming remains unclear [80].

### 3.2.1. Neutrophil function at inflammatory sites

**3.2.1.1. Phagocytosis.** Once recruited to sites of inflammation or infection, neutrophils can perform a variety of roles to combat pathogens and/or remove cell debris caused by tissue injury. One key mechanism is through phagocytosis of the pathogen or damaged cells to remove it from the host tissue and prevent further tissue damage through necrosis or dissemination of the pathogen. Zebrafish have been used to investigate phagocytosis extensively using a wide variety of bacterial and fungal pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Penicillium marneffeii*, at different infection sites such as Duct of Cuvier, caudal vein or somatic muscle [15,83,84]. Various transgenic fluorescent reporter lines, such as *Tg(mpx:GFP)i114* to study neutrophils and *Tg(mpeg:mCherry-CAAX)sh378* to study macrophages [85,86] have allowed visualisation of bacterial engulfment and acidification of phagosomes in live infected zebrafish larvae. Neutrophils predominantly phagocytose bacteria present in tissues, rather than systemic infections in the bloodstream, where phagocytosis is often performed by macrophages [87]. *Staphylococcus aureus* is a common opportunistic pathogen that is phagocytosed by neutrophils; however, the bacteria utilise the host autophagy machinery to evade intracellular killing mechanisms [88]. The neutrophil can provide an intracellular niche for bacterial dissemination rather than bacterial killing [84,88]. Often, the type of infection and subsequent phagocytosis, can determine whether this is beneficial or detrimental to the host.

**3.2.1.2. NETs.** Neutrophil extracellular trap (NET) formation is a mechanism by which neutrophils are able to capture pathogens and facilitate their destruction [66,89]. In addition to DNA and histones [89], NETs contain proteins from azurophilic (primary) granules such as neutrophil elastase, cathepsin G, and myeloperoxidase, as well as proteins from specific (secondary) granules and tertiary granules, such as lactoferrin and gelatinase. This killing process has been observed and studied using mammalian neutrophils *in vitro* [90–93], however *in vivo* models to visualise NETs are less readily available, with very few murine or fish models able to observe this process.

Palic et al. (2007) were the first to demonstrate NET formation from zebrafish whole kidney assays, as well as in the adult fathead minnow, another teleost family member [94,95]. They identified extracellular DNA and localisation of myeloperoxidase following stimulation in neutrophil cell suspensions. More recently, models using zebrafish larvae have identified *in vivo* NET release following exposure to wounding and bacterial or fungal infection [79,96,97]. In larvae, following tailfin injury, NET release was identified in pioneer neutrophils during the swarming response [79]. In the context of infection, there is evidence that NET release may be linked to neutrophil pyroptosis, a regulated pro-inflammatory mechanism of cell death. Pyroptosis, where the formation of plasma membrane pores results in cell explosion, and NETosis share common pathways and both processes can be activated within neutrophils following infection, enhancing the efficiency of clearing bacterial or fungal pathogens in zebrafish larvae [96,97]. The release of NETs, or NETosis, is traditionally thought to be a form of cell death, however there are now studies that suggest viable NET release occurs, with neutrophils still able to phagocytose once NETs have been released [98,99], however this is yet to be confirmed in zebrafish models. The occurrence of this event is infrequent and difficult to capture in live fish, therefore better tools and transgenics need to be developed to investigate real-time NET release during an inflammatory

response.

**3.2.1.3. Degranulation.** Degranulation is one of the mechanisms by which neutrophils are able to release anti-microbial components [48] in the extracellular matrix, via NET release or into the phagosome following phagocytosis of bacteria. The different types of granules within neutrophils have a variety of antimicrobial substances that can be released [100]. There are very few zebrafish models that look at neutrophil degranulation, and those that do are primarily based on *ex vivo* whole adult kidney assays [94]. Myeloperoxidase release, an indicator of primary granule degranulation, can be detected from whole zebrafish kidneys following stimulation with inflammatory mediators in an *in vitro* setting, therefore provides useful, yet limited information. However, tools are developing to investigate this process using whole larvae imaging, including fluorescent probes and transgenic lines [101, 102]. Using fluorescent probes, granule release can be detected following phagocytosis of zymosan particles, accumulating in the phagosome, which is highly similar to human neutrophil degranulation. But determining which specific subtypes of granules are involved in neutrophil degranulation still warrants further study as these probes do not target myeloperoxidase-positive primary granules. Transgenic reporters, such as *Tg(lyz:Hsa.MPO-mEmerald,cm1c2:EGFP)sh496* [102], may prove useful in studying neutrophil granule dynamics *in vivo*. One study suggests that bacterial contact is not essential for degranulation in neutrophils to occur [103]. Two days post notochord infection with *E. coli*, and long after bacterial clearance, further neutrophils are recruited late to the site of infection. These myeloperoxidase-negative neutrophils accumulate along the notochord, and along with extensive tissue damage, this indicates that degranulation has occurred despite no direct contact or engulfment of bacteria. These differences indicate further research is required to fully investigate degranulation in zebrafish during an infection or inflammatory response.

### 3.2.2. Resolution of inflammation

The process of inflammation is essential to protect organisms from infection and tissue damage, both of which can lead to severe illness, surgery or even death if left uncontrolled [104]. Inflammation itself, however, is a process that can also be uncontrolled and cause tissue damage and disease. In 1982, Metchnikoff identified the beneficial aspects of inflammation and highlighted the importance of neutrophils and macrophages in the maintenance of tissue homeostasis (Discussed by Medzhitov, (2010) [105]). For successful inflammation resolution, neutrophils must be removed from the inflammatory site [106]. In zebrafish, key processes that assist in neutrophil removal, apoptosis [107] and reverse migration away from sites of inflammation [108], have been described.

**3.2.2.1. Apoptosis.** Apoptosis, or programmed cell death, is a key mechanism by which neutrophils die and are removed once they have completed their role at the inflammatory site. This process of neutrophil death and subsequent uptake by macrophages, or efferocytosis [109–111], was historically thought to be the only mechanism in humans by which inflammation resolved. However, zebrafish studies have shown that although neutrophil apoptosis and efferocytosis contribute to inflammation resolution, it is only a small proportion of neutrophils that are removed from inflammatory sites this way [112, 113]. Nonetheless, this process can be manipulated in zebrafish to delay or promote neutrophil survival during the inflammatory response through manipulation of key genes in the apoptosis pathway, for example caspases [112,114], or survival factors, such as hypoxia inducible factors (Hif) [115–117]. Apoptosis can be easily detected within zebrafish using whole-mount antibody staining, such as TUNEL, anti-caspase 3 staining or through the use of apoptotic report lines, e.g. *Tg(mpx:FRET)sh237* [79,112,118–121]. These pathways are conserved between humans and zebrafish and therefore the study and

manipulation of neutrophil apoptosis using zebrafish remains a very important area of research to opens up new opportunities for the development of novel therapeutics promoting inflammation resolution.

**3.2.2.2. Reverse migration.** Reverse migration is the movement of leukocytes away from a site of stimulation or in the opposite direction to the net leukocyte population [122]. The discovery of reverse migration of neutrophils was first identified in a zebrafish model [108,121] and since then, has been identified as a mechanism also applicable to murine and human neutrophils [123–126]. Wounding studies in larval zebrafish have previously shown that neutrophils primarily undergo reverse migration away from sites of inflammation as the key mechanism of inflammation resolution [108,127,128]. Following neutrophil recruitment to tailfin transection, reverse migration of neutrophils can be seen as early as 2–3 h post injury [108,120,121], prompting further studies identifying entry back into the circulation [108,129]. Interestingly, macrophages may also play a role in promoting the reverse migration of neutrophils [130,131]. Initially, it was thought that macrophage contact was necessary, however, several studies have shown neutrophils still exhibit reverse migration in the absence of macrophage contact. One study [130] found that 61.7% of neutrophils reverse migrated after direct contact with macrophages, however the remaining ‘untouched’ neutrophils were also able to reverse migrate. This was also confirmed in *irf8* morphants, where macrophage numbers are drastically reduced, where 35% of neutrophils reverse migrated. It has previously been shown that 71% of reverse migrated neutrophils did not encounter macrophages and 65% of the reverse migrated neutrophils did directly contact a macrophage [131]. These findings and others indicate that reverse migration of neutrophils can be influenced by cell-cell contact [130], the release of soluble factors e.g. PGE<sub>2</sub> and LXA<sub>4</sub> into the surrounding tissue environment [131], or changes in gene expression within neutrophils e.g. Hif expression [120], the Cxcl12/Cxcr4 axis [132] or the Cxcr2/Cxcl8 axis [133,134]. Furthermore, neutrophil phenotype before and after reverse migration (in terms of neutrophil responses and antibacterial effects) were not significantly different from neutrophils that had not been recruited to a wound site [128]. The optical clarity and advanced imaging techniques available in the zebrafish have allowed this process to be observed and studied in detail, however there is still much to learn about the mechanism, as well as the advantages or disadvantages reverse migration of neutrophils may have to the host. There are many studies that suggest there are different beneficial or detrimental effects of reverse migrated neutrophils. Neutrophil reverse migration can promote inflammation resolution locally by removing the neutrophils from the inflamed site but there is also the possibility of reverse migrating neutrophils entering the circulatory system, leading to the potential of systemic spread of inflammation or infection [135], however, this is yet to be determined in zebrafish models.

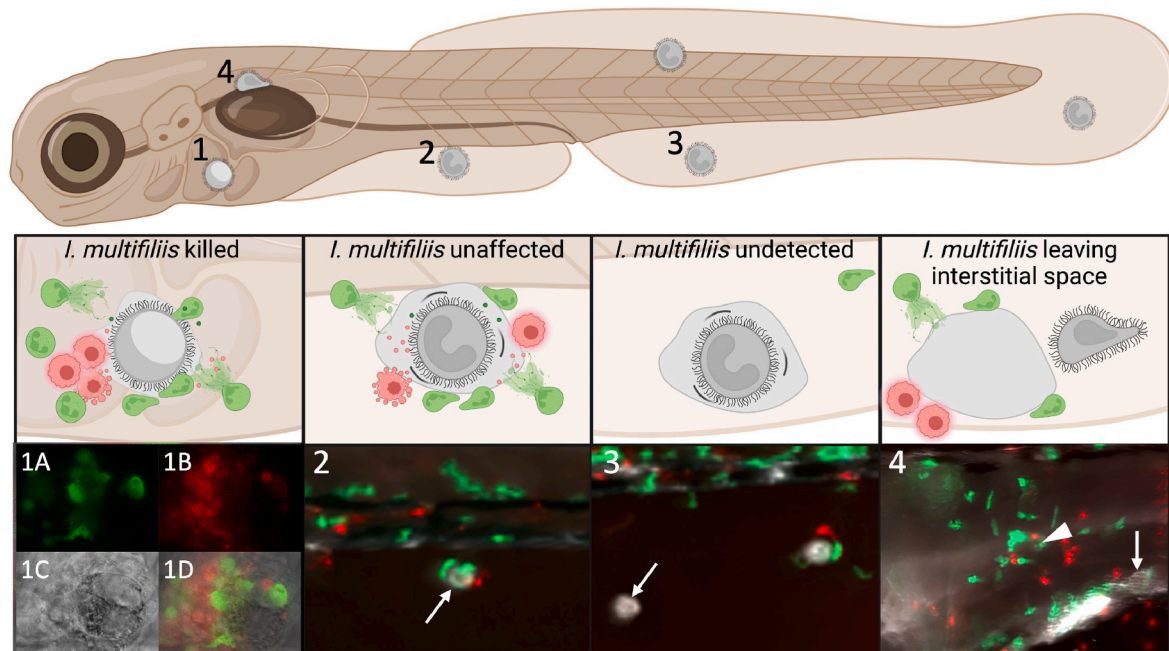
### 3.2.3. Neutrophil responses towards a fish parasite

The zebrafish has been used as a suitable model to investigate host-parasite interactions, focusing on neutrophil and to a lesser degree macrophage responses, when infected with the ciliated protozoan fish parasite *Ichthyophthirius multifiliis*. This parasite is an important pathogen and infects almost all species of freshwater fish on a worldwide scale and causes high morbidity and up to 100% mortality in fish production systems [136]. In the parasitic stage of its direct life cycle, it infects surface tissues such as the skin, gills and fins and settles above the basal lamina and is covered by one to a few fish cell layers. It creates an interstitial space in which it continuously rotates and moves around [137]. Here, it starts to feed from fish material and grows up to 1 mm in size becoming visible as a white spot to the naked eye – hence the name *white spot disease* [136,138,139]. Fish are able to acquire immunity against the parasite and therefore, a lot of research has gone into elucidating the immune responses responsible for protection [137,

139–158]. In immune fish, the parasites penetrate the surfaces but are prematurely forced to exit within a few hours. Responses of naïve and immunised fish have been compared and it is known that antibodies and a Th2-like response play a major role in adaptive immunity [139,146,147,159,160] but also complement [140,150,161] and cellular responses [148,156,162,163] play a part. An inflammatory response is dominant in naïve fish if they receive a high infection pressure [164], while more subtle infections induce more local responses. Neutrophils and macrophages have been described to be nearby the parasites and the interstitial space using histological techniques [162,165]. Due to the “snapshot” nature of histology analysis, the dynamic roles of these cells during parasitic infection were not established. They were never found touching the parasite and the immune cells were often found disintegrated [162,166]. Due to the nature of the infection under the surface of the skin, the infection is accessible to *in vivo* microscopy in most fish. Therefore, to increase the resolution of the roles of these cells, the infection has been applied to a zebrafish neutrophil reporter line, where host-parasite interactions directly can be observed. Adult zebrafish have a level of natural immunity towards the parasite, but persistent infection can be achieved when zebrafish are exposed to stressful conditions (e.g. overcrowding) [136]. In adult zebrafish, neutrophils were found to play a major role in both naïve and immune fish and were found accumulated at infection sites even after the parasites had prematurely exited the immune fish [148]. It was furthermore found that two days after infection, parasites in naïve fish ingested and neutralised neutrophils and thereby reduced immune responses both directly and indirectly by blocking the signals that the neutrophils were supposed to have sent to the rest of the immune system [148]. In zebrafish larvae, the behaviour of neutrophils, macrophages and the parasite have been studied [137]. The authors found that in early-stage infection, both neutrophils and macrophages often accumulated around the parasites (Fig. 3). Neutrophils were especially active in their migration around the interstitial space. The parasite rotated continuously, probably representing an immune evasive behaviour preventing innate immune cells from reaching and damaging the parasite. In the study, a timelapse video illustrated the

death of a parasite, which may have been caused by neutrophil and macrophage attack [137]. Neutrophils exhibited balloon like structures, which may be an indication of NETs, when approaching the parasite. On a global scale, the gene for the macrophage marker *mpeg1.2* was found to be upregulated in larvae 8 h post infection further supporting their involvement in the disease. The authors discussed the different scenarios regarding the interaction of parasites and neutrophils and macrophages (Fig. 3). It was concluded that the neutrophil and macrophage response together with other innate mechanisms was critical to limit the severity of the disease demonstrating the importance of these cells during parasite attack.

The importance of innate immune cells in protection against *I. multifiliis* has also been reported in other fish species such as rainbow trout [163], carp [140,156] and channel catfish [167]. In carp, neutrophils were recorded as first responders to the infection and were followed by other cell types such as granulocytes, eosinophils and basophils [156]. In naïve carp parasite exit points were dominated by neutrophils whereas, in immune carp, more macrophages were found illustrating a switch in the response [162]. Besides directly killing the parasite as suggested in Mathiessen et al. (2023) [137], these cell types are also involved in various other immunological processes such as cytokine and chemokine production, phagocytosis, initiating the complement response, attraction, activation and migration of cells, all processes that aid fish in fighting the disease. In rainbow trout [163] and common carp [141] an increase in serum amyloid A (Saa) following infection has been reported. In mammals, SAA, which has chemoattractant properties towards cells such as T-lymphocytes and neutrophils [163], is synthesised by macrophages [168]. Saa have been found upregulated more than 1600 times in skin of carp following an *I. multifiliis* infection suggesting its involvement in the anti-parasitic response [141]. Furthermore, a downregulation of the neutrophil-expressed complement factor I in blood have been observed while an upregulation in skin was reported [140] suggesting the migration of neutrophils to the site of infection. This was also demonstrated in another study where C-lectin expression increased from blood



**Fig. 3.** Different scenarios observed in zebrafish larvae infected with the fish parasite *Ichthyophthirius multifiliis*. 1) Still image from a timelapse video taken with a confocal microscope from Mathiessen et al. (2023) [137]: Neutrophils (1A) and macrophages (1B) are surrounding a parasite, which just died seconds before (1C). 1D is a merge of 1A-C. Images 2–4 have been captured with a stereo microscope: 2) a parasite is surrounded by neutrophils and macrophages but was unaffected for the 5 h it was imaged (white arrow), 3) a parasite is not surrounded by phagocytes (white arrow) and 4) a parasite (white arrow) has left the interstitial space (white arrowhead) which was surrounded by neutrophils and macrophages. Green cells are neutrophils, red cells macrophages and grey cells parasites.

to skin, suggesting that a migration of neutrophils to site of infection might have occurred [141]. Those studies illustrate the importance of neutrophils in direct response towards a parasite but also in regulating the overall fish immune response.

The TLR signalling pathway has been found activated together with neutrophil migration as a first response to *I. multifiliis* in Tibetan highland fish (*Gymnocypris przewalskii*) [169]. TLRs also activate production of inflammatory cytokines in macrophages. Expression of the pro-inflammatory cytokine *il1 $\beta$*  is increased early after infection [142, 167, 170] and may drive macrophage production of various components such Cox-2 and Mhc II [171]. Cox-2 is suggested to be an enhancer of inflammation in the initial phase of infection [172] whereas Mhc II is linked to adaptive immunity [163]. Additionally, pro-inflammatory cytokines produced by macrophages stimulate the synthesis of macrophage-derived chemokines as well as improving the macrophages phagocytic capacity [173]. An infection with *I. multifiliis* stimulates the production of the chemokine Cxcl8 (Il8) [137, 170, 174, 175], which acts as a chemoattractant for neutrophils to the site of injury. The increase of *cxcl8* followed an increase of *il1 $\beta$*  [174]. The chemokine receptor *cxcr1* was also found elevated following an *I. multifiliis* infection [142, 175] correlating well with the regulation of its ligand, Cxcl8. The interaction between Cxcl8 and Cxcr1 promotes chemotaxis as well as phagocytosis [176]. Other chemokines and receptors such as Cxcl12 and C-C motif chemokine receptor like 1 were found upregulated in the Tibetan highland fish [169], which may further suggest that immune cells are important mediators of protection against this parasite; however, the genes responsible for initiating the response might differ between fish species. *I. multifiliis* infection in channel catfish resulted in an early upregulation of *thr1* and *thr9* [158, 167, 177]. This strongly supports immediate activation of TLRs inducing an immune response of innate origin. TLRs are also involved in the activation of different downstream signalling pathways such as NF- $\kappa$ B and MAPK through tumour necrosis factor receptor-associated factor 6 (Traf6) and transforming growth factor- $\beta$ -activated kinase 1 (Tak1) both of which is expressed by macrophages [178, 179]. Studies have shown that Traf6 and Tak1 deficient macrophages cannot activate NF- $\kappa$ B and MAPK resulting in a lack of inflammatory cytokines [180]. Both *traf6* and *tak1* have been found upregulated in grass carp following *I. multifiliis* infection [181] indicating the importance of macrophages in the production of cytokines through TLRs.

### 3.3. Neutrophils and tissue regeneration

#### 3.3.1. Tailfin regeneration

Zebrafish possess a remarkable ability to regenerate several organs, including heart, fin and spinal cord [134, 182–184] with evidence building that the innate immune system can influence these regenerative capabilities. Many models now exist in the zebrafish to investigate the regeneration process [185] and especially for studying the roles of the immune cells [186]. In zebrafish larvae, it takes around 4–5 days from tail amputation until complete regeneration [182]. With the use of zebrafish reporter lines with fluorescent immune cells, it is possible to visualise the behaviour of the cells during injury and regeneration [186]. Additionally, as the regenerated tail and the uninjured tail appears similar even after several injuries, regeneration studies can be performed repeatedly on the same animals [187]. Following tissue damage, neutrophils and macrophages are responsible for removal of cell debris and foreign organisms and, through this process, assist in regeneration of damaged tissue [188]. Neutrophils have been shown to be the dominant cell type during the first immediate inflammatory response and are responsible for removal of cell debris and microorganisms [186]. The removal and killing of organisms by neutrophils are facilitated through different anti-microbial properties such as phagocytosis, production of reactive oxygen species and extracellular traps [189]. These anti-microbial reactions are suitable for clearing an infection. However, for regeneration purposes, they can be a challenge

both in the short and long term [188]. In the first phase after injury, neutrophils appear to inhibit regeneration. This has been documented by a decreased regeneration time following neutrophil ablation [186] indicating a neutrophil-specific role in tissue regeneration. However, the non-specificity of the anti-microbial properties of neutrophils can be detrimental to the fish tissue itself [190]. When either apoptosis or reverse migration are impaired, neutrophils remain in the regenerating area for an extended period, resulting in a delay in the regeneration [116, 188].

## 4. Zebrafish macrophages

### 4.1. Macrophage development

Macrophages are a key phagocytic leukocyte population that have a wide variety of roles, from infection clearance to tissue repair and regeneration [115, 183, 191]. Herbolme and colleagues initially defined zebrafish macrophage development and function by exploiting the transparent nature of embryos using light microscopy methods [192]. Initially identified based on their amoeboid and highly dynamic morphology, larval macrophages were observed to patrol the blood vessels. Importantly, these cells demonstrated phagocytic ability through clearing of apoptotic debris and by responding to bacterial pathogens, behaviours that are homologous to human macrophages [192]. In mammals, macrophages, along with dendritic cells (DCs), are considered key APCs of the innate immune system [193]. Although adult zebrafish are known to have antigen-presenting DCs, it is unclear as to precisely when mature dendritic cells develop in the larval zebrafish [194, 195]. Recent transcriptomic work identified a clear distinction between macrophages and DCs in adult zebrafish, with DCs being the cell type enriched in MHC-II antigen-presenting molecules [196]. These data suggest that DCs, rather than macrophages, are the primary APCs in zebrafish. Furthermore, as zebrafish do not develop a functional adaptive immune system until beyond 3 weeks post-fertilisation, the need for DCs to bridge the gap between the innate and adaptive immune system is not required [197], challenging the canonical view that the adaptive immune response is needed to drive macrophage polarisation [26]. Consequently, this dogma poses that the innate immune response is able to initiate polarisation to directly respond to a range of stimuli, using T helper cells to amplify, stabilise and co-ordinate this response in a positive feedback loop [26, 198–200].

Since early identification and early descriptions of zebrafish macrophages, the development of transgenic tools to visualise and manipulate macrophages *in vivo* has provided exciting experimental opportunities to uncover new observations about macrophage development and behaviours in health and disease, many of which have since been shown in human systems [78, 115, 201, 202]. Current macrophage zebrafish transgenic reporters include the widely used pan-macrophage *mpeg1.1:mCherry*, *mfap4:tdTomato*, *fms:mCherry* promoter driven lines, which have been extensively used as tools for macrophage behavioural studies *in vivo* [203–207]. However, used alone, transgenic lines do not conclusively differentiate between macrophages and monocytes, nor tissue-resident and monocyte derived macrophage populations [201, 202, 208, 209]. Despite these challenges, there is emerging evidence that supports the presence of monocytes and monocyte-derived macrophages in zebrafish. Using transgenic lines such as *mpeg1:mCherry*, Moyses and Richardson identified a small population of cells hypothesised to be monocytes in an adult zebrafish heart injury model [210]. In larvae, Ccr2+ putative monocytes, but not tissue-resident macrophages, are recruited during mycobacterial infection [211, 212]. However, further work is needed to characterise whether cells that differentially express Ccr2 are true populations of monocytes and macrophages [211–213]. Whilst the current literature likely focusses on the role of tissue-resident macrophages in zebrafish as a consequence of this limitation, there is a growing body of evidence revealing the heterogeneity of these populations both in adults and larvae, some of which will be discussed in

section 4.1.2 [214].

#### 4.1.1. Macrophage ontogeny

In zebrafish, myeloid cell precursors originate from the anterior lateral plate mesoderm, where they migrate to the yolk sac before extravasating and tissue colonisation (Fig. 2) [192,215,216]. This primitive wave occurs as early as 20 hpf, prior to the presence of other leukocytes, such as neutrophils [192]. At 24 hpf, with the onset of blood circulation, the transient definitive wave of haematopoiesis occurs in the PBI, where primitive erythromyeloid progenitors differentiate and primitive macrophages begin to circulate in the blood flow [45,192,215–219]. The PBI transitions into the CHT at 48 hpf, by which point the progenitor population is replaced by HSCs, enabling the second wave of definitive haematopoiesis [45,192,215–219]. During the second wave, HSCs differentiate into macrophage precursors which invade and seed tissues throughout the zebrafish, differentiating into tissue-resident macrophages, including microglia, the specialised macrophages that patrol the central nervous system (CNS) [45,217,220,221]. The pronephric kidney marrow develops from 4 days post fertilization (dpf) and becomes the adult haematopoiesis site from approximately 7 dpf. Migration of HSCs to the developing kidney results in the gradual replacement of the embryonic haematopoietic system [36,215,218]. Comparative descriptions of the origins and timings of the innate immune systems between fish and mammals have been reviewed extensively elsewhere [195,197].

#### 4.1.2. Tissue-resident macrophages

Zebrafish macrophage transgenic reporter lines have been used alongside transcriptomic methods to allow macrophage-specific transcriptomic studies, which have identified distinct tissue-specific macrophage populations in zebrafish, with gene expression signatures found to be conserved with mammals [203–207]. Zebrafish microglia (brain resident macrophages) share significant homology with human microglia. Whilst there are microglia-specific transgenic lines (e.g.: *ApoE:GFP*), most of the literature examining microglia interactions use lines that do not differentiate macrophages and microglia (i.e.: *mpeg:mCherry*) by focusing on the brain [183,222,223]. By isolating *mpeg*+ cells from zebrafish heads, microglia gene expression has been interrogated, identifying many genetic similarities between larval zebrafish microglia and adult human microglia [206]. Furthermore, the heterogeneity seen in human microglia has also been functionally identified in adult zebrafish, emphasising a high degree of conservation between species [207,224]. Other tissue resident macrophage populations are beginning to be identified in zebrafish, with Williams et al., recently combining various spatial transcriptomic techniques to identify the transcriptome of hepatic non-parenchymal cells in an unbiased way and Zhou et al., revealing tissue resident macrophage heterogeneity [196,205]. Furthermore, proteogenomic atlases have identified conserved Kupffer cell (liver resident macrophages) profiles between zebrafish and a range of vertebrates, including human (macaques, pig, hamster, chicken, and human) [205]. Most of the studies of tissue resident macrophages are performed on larvae, due to their transparent nature allowing high-content imaging of macrophage populations. Translating transgenic line research into adult zebrafish is challenging due to the loss of transparency and loss of specificity of these genetic markers in adulthood. For example, *mpeg*+ cells in adult zebrafish have recently been identified to also include a subpopulation of B lymphocytes and metaphocytes, limiting the use of this line to examine macrophages beyond larval stages [225,226]. Nevertheless, these datasets highlight how zebrafish have *bona fide* tissue-resident macrophages which behave and have genetic profiles homologous to their human counterparts.

### 4.2. Macrophage responses to immune challenges

#### 4.2.1. Macrophage polarisation

Macrophage polarisation is a process by which macrophages

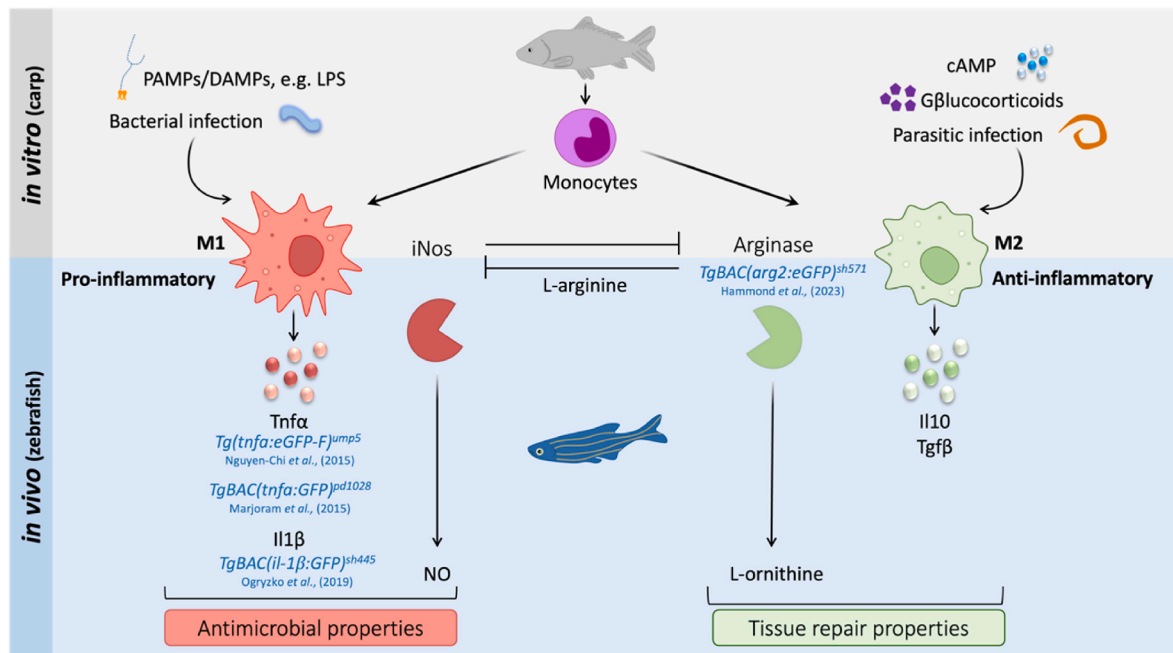
plastically change their phenotype to adapt to their microenvironment in homeostasis and disease. Macrophage plasticity is conserved in fish, with carp monocytes able to polarise towards M1 (pro-inflammatory) or M2 (wound healing) phenotypes from monocytes (M0) *in vitro*, with pro- and anti-inflammatory marker expression akin to mammals (Fig. 4) [203,227,228]. Due to the large blood volume of larger fish such as carp, they have proven useful models for examining macrophage polarisation in fish, as monocytes can be isolated and cultured to obtain macrophages *in vitro* [228–230]. Markers for M1 and M2 activation states have been identified in macrophages from various teleost fish species, including the common carp, grass carp, ayu and spotted green pufferfish [228–230]. Furthermore, qPCR analyses have found that fish macrophages express M2-associated genes following glucocorticoid exposure (in zebrafish) and infection with the parasite *Lepeophtheirus salmonis* (in Atlantic salmon), like human macrophages [231,232].

One of the advantages of using zebrafish as a model organism to study immune activation is their transparency, as it allows us to examine zebrafish macrophage polarisation using transgenic lines and imaging/transcriptomics analysis [115,203,233]. Isolation and RNAseq analysis of macrophages from transgenic zebrafish (*mpeg1:mCherry*) confirmed that the genetic signature of pro-inflammatory and anti-inflammatory macrophages is broadly conserved with humans [204]. Similar to mammalian macrophages, infection or injury in zebrafish results in macrophage expression of pro-inflammatory markers such as *Tnf $\alpha$* , *Il1 $\beta$*  and inducible nitric oxide synthase (*iNos*) [115,234,235].

#### 4.2.2. Using zebrafish reporters to delineate macrophage phenotypes *in vivo*

Pro-inflammatory polarisation of macrophages has become well-characterised in larval zebrafish, and zebrafish models have shed light on their activation *in vivo*. Several zebrafish reporter lines have been generated that have allowed examination of macrophage polarisation over the time course of injury and infectious pathogenesis. To enable real-time visualisation of pro-inflammatory macrophages, promoter driven *Tnf $\alpha$*  transgenic lines have been generated and are now well-characterised reporters that robustly label pro-inflammatory macrophages [203,242]. Using *tnf $\alpha$ :GFP* lines to examine macrophage dynamics *in vivo*, it was found that pro-inflammatory macrophages (*Tnf $\alpha$* +) arrive at wound and infection sites, later switching phenotype to a potential anti-inflammatory phenotype characterised by changes in macrophage shape (amoeboid to elongated) and behaviour (direct to reverse/circular) [191,203,243]. Additional pro-inflammatory transgenic lines which have been used to visualise and characterise pro-inflammatory macrophages in zebrafish include *il-1 $\beta$ :GFP* [115]. Macrophage pro-inflammatory nitric oxide (NO) balance is governed by two enzymes, *iNos* and arginase (that both use L-arginine as a substrate), and they are often used as dichotomous markers for mammalian M1 and M2 phenotypes respectively *in vitro* and were found to mark carp M1 and M2 populations in a similar way [244]. In zebrafish, the activity of *iNos* has been measured using an antibody against tyrosine nitrosylation as an indirect measure of macrophage NO [235,245]. As these experiments rely on fixed larvae, the spatial and temporal dynamics of polarised macrophages is lost. Observing anti-inflammatory macrophages in zebrafish *in vivo* is in its infancy, as characterising the anti-inflammatory response has, until recently, relied on analysis of macrophage morphology and extrapolation from data obtained from these pro-inflammatory reporter lines (e.g.: *tnf $\alpha$* -expressing macrophages vs. *tnf $\alpha$* -negative macrophages) [191,203,243]. Recently, a zebrafish transgenic anti-inflammatory reporter (*arg2:GFP*) has been developed, allowing for *in vivo* characterisation of the anti-inflammatory response in real time post immune challenge [233]. A small population of macrophages upregulated *arg2:GFP* at early and later stages post bacterial infection, indicative of anti-inflammatory macrophages during infectious pathogenesis [233].

Although the current transgenic tools available are enabling pro-inflammatory macrophages to be extensively characterised, there remains a gap in our understanding of the macrophage anti-inflammatory



**Fig. 4. Macrophages polarise on a continuum in response to different stimuli.** Macrophages polarise towards an innate-activated pro-inflammatory phenotype following a range of stimuli, including bacterial pathogens (e.g. *Mycobacterium marinum*) and PAMPs/DAMPs such as LPS [234,236]. Pro-inflammatory macrophages express and release pro-inflammatory cytokines such as tumour necrosis factor- $\alpha$  (Tnf $\alpha$ ) and interleukin 1- $\beta$  (Il1 $\beta$ ) [115,234]. A hallmark of pro-inflammatory macrophages is the enzyme iNos, which uses L-arginine as a substrate to produce antimicrobial NO [237]. The antimicrobial immune response of pro-inflammatory macrophages is essential for increased phagocytic activity and consequent pathogen killing. Alternatively, stimuli such as parasitic infection (e.g. *Lepeophtheirus salmonis*) and glucocorticoids can induce anti-inflammatory macrophages [231,232,236,238]. They express and release anti-inflammatory cytokines such as Il10 and transforming growth factor  $\beta$  (Tgf $\beta$ ) [203,239]. The enzyme arginase is an anti-inflammatory macrophage marker, also using L-arginine as a substrate to produce 'healing' factors such as L-ornithine and polyamines [240]. These anti-inflammatory properties enable this macrophage activation state to promote tissue repair and return of homeostasis. References for zebrafish transgenic lines currently in use included within the figure. Abbreviations: PAMPs/DAMPs, pattern-/damage-associated molecular patterns; LPS, lipopolysaccharides; iNos, inducible nitric oxide synthase; NO, nitric oxide. Adapted from Refs. [26,241].

response. Transgenic lines such as *tnfa:GFP* and *arg2:GFP* will allow us to get a more complete understanding of the plasticity of macrophages to polarise (and re-polarise) in response to microenvironmental cues. The interplay of pro- and anti-inflammatory macrophages in zebrafish is a growing field, and with most of the studies mentioned above looking at larval zebrafish prior to the development of the adaptive immune system, zebrafish research is adding to the emerging dogma which challenges the traditional view that adaptive immune T<sub>H</sub>1 and T<sub>H</sub>2 responses are required to drive macrophage polarisation [26].

#### 4.2.3. Macrophage function in infection and injury

Macrophages rapidly respond to immune challenges such as infection and injury, playing key roles in host defence [246,247]. The activation status of macrophages dictates how well zebrafish can respond to these immune challenges and recover and restore tissue homeostasis once the challenge has been cleared. An initial transient pro-inflammatory response is required for phagocytosis of pathogens and tissue debris, switching to anti-inflammatory, wound healing phenotypes at later stages to promote timely resolution of inflammation and tissue repair [191,203,231,234,243,248,249]. Two immune challenges have given important insights into the role of macrophages in these clinically important processes: *Mycobacterium marinum* (bacterial infection) and tail fin transection (injury) models.

**4.2.3.1. *Mycobacterium* infection.** Zebrafish larvae infected with *Mycobacterium marinum* have been successfully used as an *in vivo* tuberculosis model for the last 20 years, with many important observations made on macrophage and mycobacterial interaction [115,211,234,249–253]. Infection with *M. marinum* results in the formation of hallmark, macrophage-driven, aggregates of immune cells around infection (granulomas). The bacterium can survive, replicate and disseminate in

zebrafish macrophages/granulomas, just as in human tuberculosis. Macrophage phagocytosis and activation is essential for proper control of *M. marinum*. Genetic ablation of macrophages by *pu.1* morpholino knockdown greatly increases the bacterial burden of the larvae, though *M. marinum* failed to disseminate to additional tissues in the absence of macrophages, indicating a role for macrophages in bacterial dissemination [254]. The migratory ability of macrophages, such as that governed by the Cxcr3-Cxcl11 chemokine axis, also plays a key role in *M. marinum* dissemination. *cxcr3.2* mutant zebrafish had increased macrophage motility and *M. marinum* dissemination but were also more microbicidal due to enlarged lysosomes [255,256]. Knockdown of Marco, a macrophage scavenger receptor, not only reduced *M. marinum* phagocytosis, but also reduced the pro-inflammatory cytokine profile (e.g. *il1 $\beta$* ), leading to an increase in bacterial burden, indicating a need for macrophage control of infection [249]. Using the pro-inflammatory transgenic lines mentioned previously, macrophages were shown to transcriptionally upregulate *il1 $\beta$ :GFP* and *tnfa:GFP* early in *M. marinum* infection pathogenesis [115,234]. Furthermore, genetic stabilisation of Hif-1 $\alpha$ , a pro-inflammatory transcription factor, not only upregulated expression of these pro-inflammatory signals in macrophages, but also improved *M. marinum* clearance [115,234]. Signalling pathways which underpin macrophage polarisation, such as induction of *tnfa:GFP* via a cyclooxygenase/prostaglandin E2 axis, have also been uncovered by using *tnfa* promoter driven lines as *in vivo* readouts [234]. However, an appropriate pro-inflammatory response is important for infection control. Excessive Tnf $\alpha$  can also result in bacterial dissemination, as it has been associated with necrosis of *M. marinum*-infected macrophages caused by mitochondrial calcium overload [257]. At later stages of infection, *M. marinum* exploits macrophages by polarising them towards a more anti-inflammatory phenotype and preferentially infecting M2-like/anti-inflammatory macrophages as a survival strategy [252].

*M. marinum* can use cell surface lipids to act as a physical barrier to prevent TLR-mediated pro-inflammatory macrophage detection [211]. Instead, *M. marinum* phenolic glycolipids (PGL) allows for infection of anti-inflammatory macrophages, followed by induction of Ccl2 chemokine production to recruit monocytes to facilitate its escape, creating a protective niche that enables bacterial expansion [211,212,247]. Macrophage-expressed gene 1 (*mpeg1*) is a gene encoding perforin-2, a pore-forming protein that is a key player in macrophage bactericidal activity. *mpeg1* expressing macrophages take part in granuloma formation, but *M. marinum* then downregulate the *mpeg1* promoter, possibly to dampen macrophage antimicrobial function [250]. Macrophages within zebrafish granulomas were found to robustly express E-cadherins, demonstrating *M. marinum*-mediated macrophage-to-epithelioid reprogramming, making them inherently less pro-inflammatory [251, 252]. Together, these studies add to the growing body of evidence that *M. marinum* can transcriptionally shift macrophages away from pro-inflammatory phenotypes towards anti-inflammatory, permissive, phenotypes.

**4.2.3.2. Tail fin transection (injury).** To investigate the macrophage response to sterile injury, in the absence of infection, a zebrafish tail fin transection (amputation) model has been widely adopted. To track macrophage recruitment to the tail fin injury site, the *mpeg1:Kaede* line has been adopted, whereby macrophages express the Kaede protein, which photoconverts from green to red fluorescence with UV light. By photoconverting macrophages at the injury site to red, it can then be determined whether a separate, potentially anti-inflammatory, macrophage population arrives at later stages of regeneration. This technique has allowed us to visualise for the first time *in vivo*, that macrophages are recruited to the injury site in a single wave, changing phenotype throughout the healing and regeneration process [243]. By using *tnfa:GFP* transgenic larvae it has been demonstrated in a number of studies that macrophages are rapidly recruited to the injury site where they accumulate, peaking at 6 h post-amputation (hpa), expressing *tnfa* early after injury and downregulating expression at later stages [191,203,243, 258,259]. The initial macrophage pro-inflammatory response seen early after tail fin transection is vital for wound healing and the regenerative success of the tail fin tissue [191]. Interestingly, though *tnfa*-expressing macrophages were observed at the early stages of recruitment and accumulation at the injury site, RT-qPCR analysis of inflammatory genes, such as *tnfa* and *il6*, found that mRNA expression levels at a tissue level remained largely unchanged throughout the regenerative process, though this does not interrogate macrophage expression directly [260]. Using FACs purification, Nguyen-Chi et al., isolated *tnfa*<sup>+</sup> and *tnfa*-negative macrophages, identifying distinct pro- and anti-inflammatory molecular signatures, respectively [203,243].

Evidence for an anti-inflammatory/M2 switch at later stages of tail fin injury is currently limited to indirect observations, but more direct evidence has recently been emerging. Flow cytometry of *tnfa:GFP* larvae confirmed that at later stages of inflammation, only 5.6% of macrophages remained *tnfa*<sup>+</sup>, suggesting a switch towards an M2-like phenotype [203]. These observations correlated with a shift from a pro-inflammatory (*tnfa*, *il1 $\beta$* , *il6*) to an anti-inflammatory (*tgfb1*, *ccr2*) transcriptional profile identified using qRT-PCR of *tnfa*<sup>+</sup> and *tnfa*-macrophages [203,243]. Unbiased transcriptomic analysis suggest that macrophages independently transition through a linear sequence of anti-inflammatory activation states [248]. Recently, an *arg2:GFP* expressing macrophage subpopulation was observed at the tail fin during regenerative stages, the first direct evidence of anti-inflammatory macrophages using an anti-inflammatory transgenic line in zebrafish [233].

#### 4.3. Macrophages and tissue regeneration

##### 4.3.1. Tail fin regeneration

As mentioned in section 3.3, zebrafish are able to functionally regenerate various tissues after injury, in many cases without the formation of a scar [183]. One of the mechanisms for regenerative success may be the biphasic innate immune response to injury, characterised by a transient pro-inflammatory response, which is then replaced by a pro-longed anti-inflammatory response [261,262]. Macrophages play an essential role in the regenerative process [183,191]. Larvae that are depleted of macrophages (e.g. *irf8*<sup>-/-</sup>) have greatly impaired regenerative capacity [191,262]. Whilst one could be forgiven for thinking that anti-inflammatory macrophages are responsible for regeneration, there is a growing body of evidence demonstrating the importance of pro-inflammatory macrophages for successful regeneration [191,203, 243,262,263].

As the larval zebrafish tail fin can regenerate within 3 days, tail fin transection is a widely studied model of zebrafish regeneration, giving new insights into the *in vivo* role of macrophages in this process. Impaired tail fin regeneration was observed following chemical and genetic depletion of macrophages at different time-points to target pro-inflammatory or anti-inflammatory macrophages, demonstrating the importance of both phenotypes in this process [191]. Following the pro-inflammatory stage, *tnfa* negative macrophages accumulate up until 3 days post-amputation, suggesting that anti-inflammatory macrophages are present during the later stages of the regenerative process [191]. *Tnfa* secreted from pro-inflammatory macrophages has proven to be pivotal for tail fin regenerative success, demonstrating the importance of pro-inflammatory macrophages for the regenerative process [191]. Macrophage phenotype must switch away from a pro-inflammatory phenotype at later stages to ensure regenerative success, with excessive pro-inflammatory macrophage signalling resulting in impaired regeneration and collateral tissue damage [184]. A study using mutant zebrafish larvae that have a reduced pool of peripheral tissue-resident macrophages (*csf1ra*<sup>-/-</sup>) suggests that this macrophage population enables efficient tail fin regeneration by dampening the pro-inflammatory environment [214]. Macrophage polarisation can be therapeutically targeted to modulate tissue regeneration. Treatment with Protectin D1, a pro-resolving mediator, accelerated changes in macrophage behaviour, correlating with increased expression of anti-inflammatory markers (e.g. *tgf1*, *ccr2*) identified by RT-qPCR, leading to accelerated tail fin regeneration [184]. All studies to date use *tnfa*-driven transgenic lines to visualise pro-inflammatory macrophages *in vivo*, identifying potential anti-inflammatory macrophage subpopulations through extrapolation of this data (i.e.: *tnfa* negative macrophages) [183,191,262]. Novel transgenics such as the *arg2:GFP* line could allow investigation of both pro- and anti-inflammatory macrophage roles in the finely-tuned responses that allow zebrafish to have this regenerative capacity [233].

## 5. Conclusion

It is clear that fish have an important role to play in driving our understanding of the immune response to external challenges. Zebrafish have proven their usefulness as models of inflammation and infection through a multitude of studies, uncovering novel mechanisms through assays involving *in vivo* imaging, genetic manipulation and drug screening. Exploiting these key advantages of the zebrafish have uncovered the plasticity of fish macrophages and neutrophils, and how they are able to alter their activation status to respond to different immune challenges. The development of transgenic and mutant zebrafish lines provides us with the tools to examine leukocyte pro- and anti-inflammatory signalling in these contexts, allowing us to further understand the dynamic role of these critical innate immune cell populations *in vivo*. Furthermore, the increasing body of evidence demonstrating functional conservation between zebrafish and

mammalian leukocytes, highlights the value of studying zebrafish macrophage and neutrophil biology to understand human disease. There is, however, a lack of studies on the collaboration and interactions of neutrophils and macrophages, and this should be prioritised in the future, as fine-tuning both cell-specific responses could provide more precise therapeutic intervention for human disease.

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## CRediT authorship contribution statement

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## Data availability

No data was used for the research described in the article.

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