



Fish Macrophages

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Abstract

Fish macrophages arise from haematopoietic progenitors in the head kidney and differentiate into tissue macrophage subtypes and/or self-maintaining resident populations. New insights into the ‘forms’ and functions of fish macrophages are provided by in vitro studies of macrophages purified by density gradients/adherence, as well as immortal macrophage-like cell lines and prolonged culture of primary macrophages. Polarisation states observed in mammalian macrophages, with associated changes in molecular and behavioural properties across a spectrum of two extremes, termed M1 and M2, also likely exist in fish macrophages. There is evidence that these different states are underpinned by immunometabolic changes. With the current advances in transcriptome sequencing, markers for macrophages and macrophage subtypes are slowly but definitively emerging in fish species. An ever-increasing toolbox of transgenic zebrafish lines allows for the elucidation of the multiple roles of macrophages in disease models, providing a more detailed insight into their in vivo function in fish.

Keywords

Macrophages · Innate immunity · Fish · Zebrafish · Disease models

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Abbreviations

angptl4	angiopoietin-like 4
arg	arginase
BCG	Bacillus Calmette-Guérin
cAMP	cyclic adenosine monophosphate
cfms	csf-1 receptor
CRISPR	clustered regularly interspaced short palindromic repeats
csf-1	colony-stimulating factor-1 (see also M-CSF)
cxcl11	C-X-C motif chemokine 11
dpf	days post-fertilisation
eGFP	enhanced green fluorescence protein
FACS	fluorescence-activated cell sorter
GAL4	galactokinase 4 transcription factor (see also UAS for combined system)
Hif1a	hypoxia-inducible factor 1 alpha
hpf	hours post-fertilisation
hsp70	heat shock protein 70
ifn γ	interferon gamma, cytokine
il-10	interleukin-10
il-1b	interleukin-1 beta, cytokine
il-4	interleukin-4, cytokine
il-6	interleukin-6, cytokine
inos	inducible nitric oxide synthase
irf8	interferon regulatory factor 8, transcription factor
irg1	immune-responsive gene 1
LPS	lipopolysaccharide
M1	type 1 macrophage
M2	type 2 macrophage
m-csf	macrophage-colony-stimulating factor (see also CSF-1)
mfap4	microfibril-associated protein 4
mm	<i>Mycobacterium marinum</i>
mpeg-1	macrophage-expressed gene 1
mrc1b	mannose receptor 1b
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
nos	nitric oxide synthase
rag	recombination-activating gene
ROS	reactive oxygen species
sp1b	proto-oncogene b
TALEN	transcription activator-like effector nucleases
TB	tuberculosis
TCA	tricarboxylic acid cycle

tg	transgenic
tgfb	transforming growth factor beta
tnfa	tumour necrosis factor alpha
UAS	upstream-activating sequence (see also GAL4 for combined system)

6.1 Introduction

For a long time, immunologists made a clear distinction between cellular immunity and humoral immunity. Cellular immunity is mostly linked to nonspecific innate immunity. Humoral immunity is considered synonymous with antigen-specific acquired immunity, based on Emil Behring and Paul Ehrlich's identification of antibodies which neutralise microbial toxins. To date, these two distinctive arms of the immune system are considered as highly intertwined, partly overlapping, with mutually beneficial activities (Kaufmann 2019). Elias Metchnikoff laid the basis for innate immunity by identifying phagocytic cells which engulf and destroy invading pathogens and conceived the concept of phagocytosis as a process of uptake of particles or microbes rich in food after studying simple organisms such as starfish larvae. He described macrophages and microphages (now called neutrophils) as highly motile phagocytic cells which migrated to sites of foreign body insult. Whereas antibodies are only found in vertebrates, macrophages or macrophage-like cells are present in primitive animals such as starfish alongside vertebrates, and clearly pre-date the development of lymphocytes and associated acquired immune responses.

Macrophage polarisation is a process by which macrophages change their molecular and behavioural properties across a spectrum of two extremes; termed M1 and M2 (Xue et al. 2014). M1 macrophages are commonly associated with the presence of T helper-1 cytokines such as IFN γ , whereas M2 macrophages are commonly associated with the presence of T helper-2 cytokines such as IL-4. Macrophages are evolutionary conserved cell types that evolved more than 500 million years ago (Epelman et al. 2014; Barreda et al. 2016), predating the development of lymphocytes. It is therefore plausible that the initial trigger for 'macrophage polarisation' could rely primarily on sensing microbes and other innate danger signals, not requiring the presence of T-cell derived cytokines. It is possible that the M1-M2 dichotomy could be an evolutionary conserved, intrinsic property of macrophages associated with transitions from healing (M2) to inflammation (M1) (Mills and Ley 2014). The 'macrophage first' view is based on the fact that the ability of macrophage-like cell types to phagocytose foreign objects, and repair cellular damage, already existed in primitive animals.

Not surprisingly, fish macrophages with polarised profiles like mammalian M1 and M2 also exist. Common carp (*Cyprinus carpio*) macrophages can adopt an inflammatory (M1-like) phenotype, characterised by elevated nitric oxide (NO) production in response to lipopolysaccharides (LPS) (Wiegertjes et al. 2016). These macrophages can also develop an anti-inflammatory (M2-like) profile in response to the second messenger, cyclic

adenosine monophosphate (cAMP) (Joerink et al. 2006; Hodgkinson et al. 2017). The need for T-cell-derived cytokines to stimulate the polarisation of macrophages may be less obvious for fish than for mice (Forlenza et al. 2011; Wiegertjes et al. 2016). This takes us from the idea of dichotomous ‘Th1 and Th2 driving’ to the ‘macrophages first’ hypothesis.

Our knowledge of fish macrophages is rapidly advancing, but not at the same pace for all fish species of interest. Progress can be hampered by the diversity of fish, as well as the techniques employed that do not always address all aspects of the immune system. Evolutionary distant families, such as the Salmonidae (e.g. Atlantic salmon, rainbow trout), Cyprinidae (e.g. common carp, zebrafish), Ictaluridae (e.g. channel catfish) and Perciforms (e.g. sea bass, grouper) are commonly studied. It is therefore not surprising that macrophages can behave differently between these diverse groups. Zebrafish (*Danio rerio*) deepen our level of understanding of fish macrophages, especially with the advent of immune transgenic lines, allowing in vivo labelling of specific cell types/organ systems with fluorescent proteins. Investigation of innate immune cells is simplified in larval zebrafish, as before 5dpf there are only two immune cell lineages, the macrophages and the neutrophils. Rag-positive T cells appear at around 5dpf, but functional adaptive immunity, with antibody-producing B lymphocytes, does not develop until 2–3 weeks post-fertilisation (dependent on laboratory conditions) (Willett et al. 1997). The widespread use of zebrafish creates a level playing field, allowing for in-depth studies of macrophage function in a single fish species.

6.2 Macrophage Development

Macrophages arise from haematopoietic progenitors in the bone marrow, which differentiate into tissue macrophage subtypes, such as Kupffer cells in the liver, alveolar macrophages in the lung, microglia cells in the central nervous system and specialised macrophages in the spleen. It was originally proposed that tissue macrophages derive from circulating blood monocytes (van Furth et al. 1972). Now the evidence points to tissue macrophages being seeded during primary haematopoiesis, resulting in self-maintaining resident populations of macrophages (Ginhoux and Jung 2014). All macrophage subtypes are important for homeostasis and play an intricate role in the immune system.

In adult zebrafish, macrophages arise from haematopoietic progenitors in the head kidney and tissue macrophage development appears to be an evolutionary conserved process (Soza-Ried et al. 2010). Survival, proliferation, differentiation and functionality of most cells of the macrophage lineage are governed by colony-stimulating factor-1 (CSF-1), or macrophage-colony-stimulating factor (M-CSF). This cytokine, acting through its cognate receptor, is expressed almost exclusively on committed myeloid precursors and derivative macrophage populations. Goldfish (*Carassius auratus*) CSF-1 appears to be a proficient macrophage growth factor, although possibly with a different role in macrophage

differentiation (Hanington et al. 2009; Rieger et al. 2014; Hodgkinson et al. 2015; Barreda et al. 2016).

Early studies into zebrafish blood lineages were performed using light microscopy, immunohistochemistry and flow cytometry (Herbomel et al. 1999; Lieschke et al. 2001; Hsu et al. 2004). Monocytes and macrophages in adult zebrafish are primarily derived from the head kidney marrow, the site of haematopoiesis (Hsu et al. 2004). In embryos, macrophage precursors first develop at around 20 h post-fertilisation in the anterior lateral plate mesoderm tissue (Herbomel et al. 1999). These so called ‘primitive macrophages’ seed the macrophages resident in the body tissues; for example, by invading the brain tissue where they differentiate into microglia. Primitive macrophages are gradually replaced during embryonic development by those derived from definitive haematopoiesis, which begins at 24hpf in the caudal haematopoietic tissue (CHT) (Lieschke et al. 2001). Tissue-specific macrophages are also found in zebrafish larvae, most notably brain-resident macrophages, known as microglia (Peri and Nüsslein-Volhard 2008). Zebrafish microglia play a similar role in the homeostatic maintenance of the fish brain, as in mammals, and have the same ramified morphology when at rest. In zebrafish, the roles of microglial clearance of apoptotic cells (Hamilton et al. 2020), synaptic maintenance and regeneration (Kyritsis et al. 2012) and vascular maintenance (Fantin et al. 2010) can all be studied. It is reasonable to assume that many macrophage subtypes play an intricate role in fish immune systems.

6.3 How to Study Fish Macrophages

Traditionally, *in vitro* studies of fish macrophages are performed with primary cell cultures, enriched for macrophages by density gradients and/or adherence, in line with human primary blood studies. From the 1980s onwards, macrophages of many different fish species have been isolated using primary culture methods for *in vitro* studies on fish immunology (Villena 2003). Typically, (head) kidneys are gently passed through a sterile nylon mesh, after which cell suspensions are placed on top of a density layer (e.g. Percoll). After centrifugation, the interface layer is collected, washed, counted and seeded, at optimal concentrations, in cell culture medium. From such studies, it is clear that fish macrophages have the capacity to phagocytose and have microbicidal activity, in part mediated by oxygen and nitrogen radicals. Oxygen radical production is determined by colorimetric quantitation of the respiratory burst, using either single point measurements (e.g. by quantification of reduction of a yellow tetrazolium dye to a blue formazan) or real-time measurements (e.g. by quantification of luminol-enhanced chemiluminescent emission). Nitrogen radical production can be determined by a ‘Griess’ reaction, measuring the accumulation of nitrite over a period of a few days following initial stimulation. These *in vitro* studies contribute greatly to our knowledge of the mechanisms of antimicrobial immunity in fish macrophages (Grayfer et al. 2018).

6.3.1 Macrophage Cell Lines

One limitation of studying fish macrophages is the lack of available immortalised cell lines. For mammalian systems, these immortalised cell lines enable simplified in vitro assays to be used, without the need for primary cultures. Occasionally, the development of leukocyte cell lines is reported; however, this is not repeatedly cited in the literature, therefore it cannot be regarded as a regularly used and well-established tool. This is with the notable exception of cell lines developed from rainbow trout (RT) (*Oncorhynchus mykiss*) (Bols et al. 2017). The monocyte/macrophage cell line, RTS11, is one of several cell lines developed from rainbow trout and part of an informally shared 'invitrome'. In the early stages of establishment, RTS11 cells require 30% foetal bovine serum, which can be halved for routine growth, and it is one of only a few cell lines from rainbow trout that can be grown in suspension. RTS11 cells are used to study interactions with *Flavobacterium psychrophilum*, for example, showing that live bacteria alter cellular morphology, stimulate cytokine expression and impair phagocytic activity. This provides an example of how an in vitro macrophage-like cell line can help in understanding the pathogenesis of an important bacterium (Semple et al. 2020). In addition to rainbow trout, TO and SHK-1 are two cell lines derived from the head kidney of Atlantic salmon (*Salmo salar*). In the resting state, they express different immune relevant genes suggesting that they have potentially different functional properties: SHK-1 shares characteristics with dendritic cells, whilst the TO cell line shows characteristics more traditionally associated with macrophages (Collet and Collins 2009). The SHK-1 cell line is used to study interactions with *Piscirickettsia salmonis*, for example, showing that this bacterium, which infects and survives in its host cell, induces a global shutdown of translation during intracellular growth, resulting in decreased cell viability after 10 days (Zúñiga et al. 2019). Most recently, two macrophage-like cell lines were established from the head kidney of a marine fish species, the large yellow croaker (*Larimichthys crocea*) (Cui et al. 2020). Overall, although few in number, fish macrophage-like cell lines allow simplified in vitro assays to be developed to aid our understanding of cell-pathogen interactions.

6.3.2 In Vitro-Derived Macrophage Cell Cultures

Studies on the function of fish macrophages are greatly facilitated by standardised procedures for prolonged in vitro culture of primary cells. In particular, goldfish and common carp macrophages grown in the presence of relatively high serum concentrations can survive for at least 6–8 days. It is well known, also for mammalian macrophages that, macrophages but not lymphocytes selectively survive for longer in vitro (Carr 1973). The presence of progenitor/stem cells in primary kidney macrophage cultures from goldfish allows for studies of myelopoiesis, indicating the development of three distinct subpopulations in response to endogenous macrophage growth factors. Self-renewal is promoted by endogenous macrophage growth factors (Belosevic et al. 2006) in a culture

system, including three subpopulations of macrophage development, namely progenitor cells, monocytes and mature macrophages. These primary kidney macrophage cultures are particularly informative when it comes to the elucidation of the complex mixture of cytokines that regulate progressive and selective macrophage development, from progenitor cells to fully functional mature macrophages *in vitro* (Katzenback et al. 2016). Similar studies in common carp (Joerink et al. 2006; Wentzel et al. 2020a) show that, amongst head kidney leukocytes kept for several days *in vitro*, the lymphocytes die off and the remaining cells differentiate into head kidney-derived macrophages, with the ability to phagocytose and produce oxygen and nitrogen radicals.

6.3.3 Transgenic Zebrafish

Studies on fish macrophages are significantly advanced by the development of immune cell-specific transgenic lines in transparent zebrafish. These lines, in combination with genetic manipulation via clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 and drug treatments, allow investigation of macrophage phenotypes and function *in vivo*. The monocyte/macrophage lineage is one of the first blood cell types for which transgenic lines have been generated. The *Tg(spi1b:eGFP)* and *Tg(spi1b:GAL4, UAS:eGFP)* transgenic lines label the myeloid precursors of macrophages and neutrophils, but lack the specificity to distinguish between the two (Ward et al. 2003; Hsu et al. 2004; Peri and Nüsslein-Volhard 2008). The first transgenic line that specifically labelled cells of the macrophage lineage in larvae is the *Tg(mpeg.1:GFP)* driven by the *mpeg.1* promoter (Ellett et al. 2011). This line is extensively used in the field to label macrophage populations in disease model studies, despite the fact that we do not fully understand what the biological function of *Mpeg.1* is. The *mpeg.1:GFP* line has been followed by a number of transgenic lines, using the promoters of *cfms* and *mfap4* (Gray et al. 2011; Walton et al. 2015) amongst others. The availability of multiple transgenic lines, with different promoters and fluorescent proteins, allows for outcrossing into transgenic lines of other cell types/organ systems. These are combined to produce powerful *in vivo* models for studying the roles of macrophages in diseases. These have been added to in recent years by functional transgenic lines of important macrophage signalling systems. These include transgenic lines of cytokines/immune stimuli, (including *il-1beta* (Nguyen-Chi et al. 2014; Ogryzko et al. 2019), *tnfa* (Marjoram et al. 2015; Nguyen-Chi et al. 2015), *nfb* (Feng et al. 2012) and *irg1* (Sanderson et al. 2015)). This ever-increasing toolbox of transgenic lines is allowing for the unprecedented dissection of the roles of macrophages, in multiple *in vivo* models, throughout the course of disease pathogenesis (Fig. 6.1).

The genetic tractability of zebrafish allows for manipulation of macrophage populations *in vivo*. Morpholinos are antisense oligonucleotides that block translational start and splice sites, knocking down specific *gene* expression. The *spi1b* morpholino allows for knock-down of macrophage (and neutrophil) populations in the developing zebrafish larvae, though these effects are transient (Liongue et al. 2009). Morpholinos have been joined

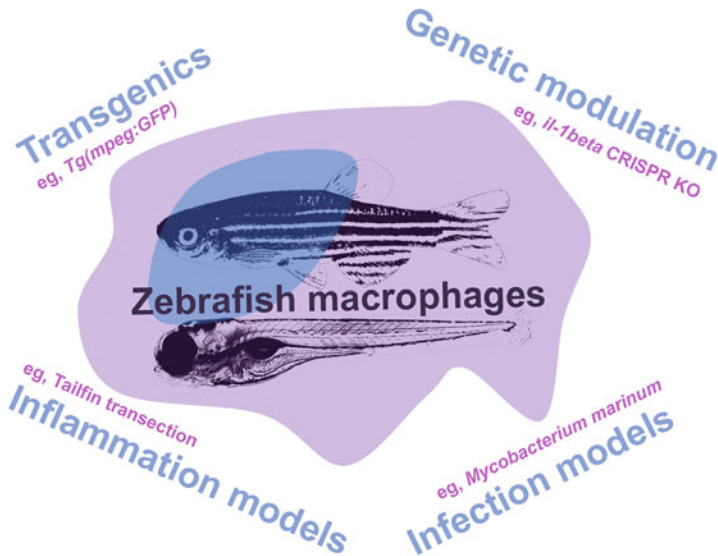


Fig. 6.1 The zebrafish toolbox to dissect the roles of macrophages in disease

recently by full zebrafish knockout lines that are now easier to generate due to TALEN and CRISPR-Cas9 technology. The *irf8* knockout line allows for depletion of macrophage populations, while stimulating neutrophil production (Li et al. 2011; Shiao et al. 2015). Mutants of important macrophage cytokines (eg *il-1beta*) are now available, to modulate macrophage behaviour (Ogryzko et al. 2019). Transgenic lines also allow for manipulation of macrophage and immune cell populations, in order to elucidate their effects on models of disease. Macrophage-driven *Gal4* transcription factor, in combination with a *UAS-nitroreductase-mCherry* expressing transgenic, allows for macrophage ablation and enables assessment of the macrophage contribution to disease (Gray et al. 2011; Prajsnar et al. 2012). Macrophage ablation can also be achieved by utilising their predisposition for high levels of phagocytosis/endocytosis. Liposome- encapsulated clodronate disodium (a bisphosphonate toxic to cells intracellularly), when injected into zebrafish larvae, allows for the temporal depletion of the macrophage population. These macrophage depletion and modulation tools can be used in combination with transgenic lines, to understand the roles of macrophages in development and disease processes.

6.4 Macrophage Polarisation

Macrophages are essential innate immune cells, involved in host defence that can play contrasting roles by initiating and sustaining inflammation. They can also play an important role in the resolution of inflammation and tissue regeneration. Under these conditions, different microenvironments drive macrophages to display a range of effector functions

which tailor immune responses to either combat pathogens, or repair damage. Small variations in microenvironments can lead to an array of macrophage phenotypes (Xue et al. 2014), with the most polarised termed M1 and M2. Traditionally, M1 macrophages are associated with microenvironments influenced by T helper-1 responses (hence M1) and produce pro-inflammatory cytokines, reactive oxygen species (ROS) and nitrogen species, such as nitric oxide (NO). In contrast, M2 macrophages are commonly associated with environments influenced by T helper-2 responses (hence M2) and produce anti-inflammatory products and factors associated with wound healing (Mills et al. 2000). Both M1 and M2 macrophages metabolise the same amino acid, L-arginine. M1 macrophages do so with the help of inducible nitric oxide synthase (iNOS, or NOS2), to produce inflammatory NO. In contrast, M2 macrophages use the enzyme arginase to produce proline and polyamines, important for wound healing processes such as cell proliferation and collagen production, during extracellular matrix regeneration (Vincendeau et al. 2003). The competition for L-arginine in macrophages, between iNOS and arginase is referred to as the ‘arginine fork’.

6.4.1 M1 and M2 Macrophages of Fish

The arginine fork is used as a common readout for fish macrophage polarisation. The arginine forks’ intrinsic capacity to polarise is determined by the differential activity of iNOS versus arginase, as measured by end products, such as nitrite (M1) and urea (M2). Such colorimetric assays typically measure accumulation of stable nitrite (NO₂⁻) in the culture medium by a Griess reaction, or urea in cell lysates as part of an arginase assay. Much knowledge of macrophage polarisation comes from studies in cyprinid fish, such as goldfish and common carp, aided by prolonged *in vitro* cultures.

NO synthases display a large scale of parallel evolution (Moroz and Kohn 2011). Mammalian vertebrates have neuronal, endothelial and inducible isoforms of NOS, while fish only seem to express neuronal and inducible NOS, which they use to catabolise arginine to nitric oxide and citrulline (Andreakis et al. 2011). M1 macrophages abundantly express the enzyme iNOS. Simultaneous generation of NO radicals and reactive oxygen species, including the radical superoxide (O₂⁻), can lead to the formation of peroxynitrite (ONOO⁻), for example. Peroxynitrite is a powerful oxidant and the resulting tyrosine nitration can be considered as a hallmark of tissue injury and a (bio)marker for nitrosative stress, linked with inflammation, in common carp (Forlenza et al. 2008) and zebrafish (Elks et al. 2013, 2014). The combined generation of reactive nitrogen and reactive oxygen species helps define M1 as ‘kill/fight type’ of macrophage, with (*gene*) expression of *inos* (*nos-2*) as a marker for M1 macrophages in fish.

Alongside its role in the urea cycle, arginase regulates cellular arginine and ornithine catabolism to deliver ornithine for processes of extracellular matrix synthesis and polyamine synthesis, important for organogenesis and wound healing (Dzik 2014). Most eukaryotic cells have a polyamine transporter system on their cell membrane that facilitates

the internalisation of exogenous polyamines; a system highly active in rapidly proliferating cells (Wang et al. 2003). Mammalian vertebrates have two arginase isoforms that both catabolise arginine, but differ in cellular expression and cell-type-specific regulation (Jenkinson et al. 1996). In ureotelic animals that excrete excess nitrogen as urea (e.g. mammals), Arg-1 is expressed primarily in the liver and is a cytoplasmic enzyme central to the hepatic urea cycle, whereas Arg-2 is a mitochondrial enzyme that is expressed in almost all organs. Most fish species are ammoniotelic and thus do not excrete urea through the kidneys, instead they excrete ammonia mainly through the gills. Common carp also express two arginase isoforms and both appear to be mitochondrial enzymes (Joerink et al. 2006). The generation of arginase activity and downstream production of collagen and polyamines help define M2 as a 'healing type' of macrophage, with arginase as a robust marker for M2 macrophages of fish.

6.4.2 Studies into M1 and M2 Markers

Measuring increased *gene* expression of inos or arginase can be used as a first marker for macrophage polarisation in fish, but often more than one *gene* variant will need to be examined because of whole genome duplications (WGD). All teleosts are believed to have gone through three rounds (3R) of WGD. As a result of 3R duplications, zebrafish often, but not always, express two copies of the *genes* found in mammalian vertebrate genomes; e.g. zebrafish express two copies of *nos-2*, but only single copy of *arg1* and *arg2*. Some fish families have even undergone an additional round of duplication (4R), including the Salmonidae (Berthelot et al. 2014) and the Cyprinidae (Ohno 1967). An accurate interpretation of immune responses by measuring *gene* expression requires knowledge of the degree of functional divergence between duplicated *genes* (Petit et al. 2017). This is the case even if closely-related fish species, such as zebrafish and common carp, show extraordinary levels of synteny (Henkel et al. 2012). Next generation sequencing (NGS) increasingly assists with interpreting the expression of duplicated *genes*, thus defining their role in immune responses.

Syntenic analysis in zebrafish shows conserved synteny for *nos-2b* (Lepiller et al. 2009), whose function also appears well conserved (Poon et al. 2008; Hegedus et al. 2009). Head kidney-derived macrophages of common carp, when stimulated with LPS and polarised into M1 phenotypes are characterised by a high up-regulation of *nos-2b*, but not *nos-2a* (Wentzel et al. 2020b). This suggests that *nos-2b* *gene* expression might be a robust marker for M1 macrophages of (cyprinid) fish, while the role for *nos-2a* remains less clear. In mammals, Arginase-1 is located in the cell cytosol and is mainly expressed in the liver, taking part in the urea cycle and specifically ammonia detoxification. Arginase-2 is located in the mitochondria and could be considered most relevant for extracellular matrix regeneration. In fish, both *arginase-1* and *arginase-2* contain a mitochondrial targeting sequence making it uncertain as to which *gene* variant would be the best to measure with respect to M2 (Joerink et al. 2006). However, in common carp head kidney-derived macrophages,

arginase-2 seems to be most highly regulated, when challenged with cAMP (Wentzel et al. 2020b). There is further and compelling evidence of macrophage subtypes in zebrafish larvae, present before the development of adaptive immunity. In a tailfin transection inflammation model in *tg(tnfa:GFP)* transgenic zebrafish larvae, *tnfa*-expressing macrophages could be purified by fluorescence-activated cell sorting (FACS). Subsequent RNAseq analysis showed that the *tnfa*-expressing macrophages had a pro-inflammatory profile, suggesting an M1-like profile (Nguyen-Chi et al. 2015) (Fig. 6.2). Furthermore, at later stages of wounding, macrophages that were *tnfa*-negative showed signs of an anti-inflammatory profile, including expression of chemokine receptors that, at least in mammals, are linked with M2 macrophages (Nguyen-Chi et al. 2015). This provides *in vivo* evidence for the presence of M1 macrophages during early stages of inflammation, whereas M2 healing macrophages become more prevalent during wound healing stages.

Using well-described stimuli, LPS (M1) and cAMP (M2), NGS-based analysis of the transcriptional profile of M1- and M2-like polarised macrophages from common carp, shows that transcriptional profile stimuli display a high degree of affinity with those of polarised mammalian macrophages. Amongst well-known M1 marker *genes*, those highly expressed are interleukin-1 β (*il1b*), inducible nitric oxide synthase (*inos*) and serum amyloid A (*saa*). Well-known M2 markers that are highly expressed include the tissue inhibitor of metalloproteinases (*timp2b*), transglutaminase (*tgm2b*) and arginase (*arg2*) (Wentzel et al. 2020b). Measuring increased *gene* expression of *inos* or *arginase* can be used as a first and indicative marker for macrophage polarisation in fish (Tables 6.1 and 6.2), but true evidence for different macrophage phenotypes may require more than one single marker.

It is possible that even more information can be obtained by determining those *genes* that are up-regulated or down-regulated in one macrophage subset and—at the same time—show opposite regulation in the other subset (Wentzel et al. 2020b). Based on such criteria, many more potential marker *genes* of M1 macrophages can be identified, for example, ROS-induced heat-shock protein 70 (*hsp70*), inflammatory response-linked immune response *gene* 1 (*irg1*) and the chemokine *cxcl11* (also identified as a M1 marker in zebrafish) (Lu et al. 2017). Based on the same criteria, additional marker *genes* for M2 macrophages include the mannose receptor (*mrc1b*) and the vascular growth factor angiopoietin-like 4 (*angptl4*). Putative marker *genes*, as listed in Table 6.1, may assist in defining a broader set of comprehensive markers for gene expression studies, as opposed to single *genes* (*inos*, *arginase*), to discriminate between polarisation states. No matter what, the correct interpretation of NGS-based information, such as these transcriptome profiles from *in vitro* polarised macrophage subsets, will require follow-up studies to understand the biological effects. These studies should preferably be *in vivo*, possibly using transgenic zebrafish larvae.

M2 macrophages can achieve their polarisation states by deactivation of a pro-inflammatory profile. Fish macrophages can be deactivated by cytokines, such as transforming growth factor-beta (*tgf- β*) or interleukin-10 (*il-10*). In fact, TGF- β signalling, rather than microbial stimuli, could be the oldest evolutionarily trigger to initiate M1/M2

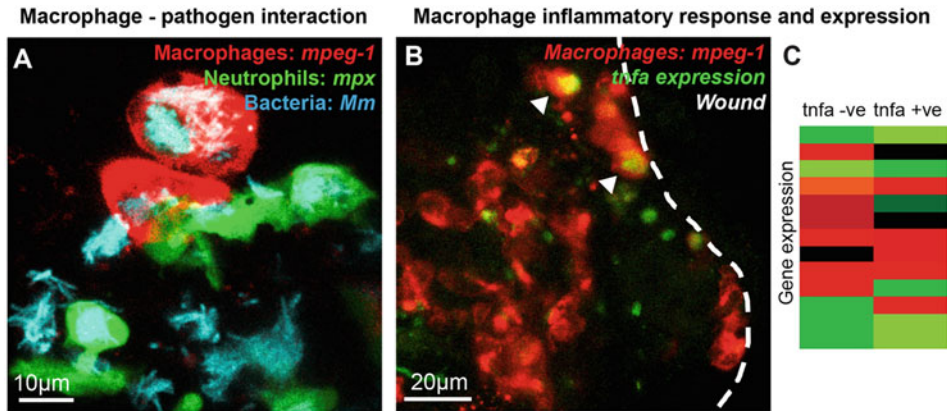


Fig. 6.2 Zebrafish disease models allow macrophage host-pathogen interactions and gene expression changes to be observed during challenge. (a) Fluorescent confocal micrograph of a *Mycobacterium marinum* infected transgenic that labels macrophages in red and neutrophils in green allowing observation of host-pathogen interactions in vivo. (b) Fluorescent confocal micrograph of red macrophages at a tailfin transection site (dotted line), with a population of them expressing *tnfa:GFP* (white arrowheads). (c) Hypothetical gene expression analysis that could be done with FACS-purified macrophages from B with or without *tnfa:GFP* expression, similar to that described in Nguyen-Chi et al. (2015)

macrophage polarisation (Dzik 2014). Both, recombinant Tgf- β and Il-10 attenuate LPS-stimulated inflammatory gene expression in monocytes/macrophages in grass carp (*Ctenopharyngodon idella*) (Wei et al. 2015). In vitro, recombinant Il-10 from goldfish (Grayfer et al. 2011) and common carp (Piazzon et al. 2015) reduces radical production and transcription of pro-inflammatory cytokines, pushing macrophages towards the M2 spectrum. These findings suggest that subtle differences in polarisation states exist in fish macrophages.

An interesting question is whether or not macrophages once polarised, can repolarise. Metabolic reprogramming has been linked to an inability to repolarise from M1 to M2. This is because mouse M1 macrophages do not regain their oxidative capacity upon repolarisation, whereas M2 are able to repolarise into M1 macrophages (Van den Bossche et al. 2016). In apparent contrast, an in vivo tracking experiment in zebrafish larvae, using transgenic *mpeg1+/tnfa+* macrophages recruited to an injury site shows that this M1 phenotype could be reverted to an intermediate phenotype at a later point in time (Nguyen-Chi et al. 2015). Whether the apparently different outcome in mice and fish is due primarily to differences in experimental set-up, or if it indicates more flexibility in the function of polarised macrophages in fish, remains to be determined.

Table 6.1 Gene profile associated with fish M1 macrophages. A list of 10 genes amongst the most highly expressed in LPS-stimulated (M1) macrophages of common carp (Wentzel et al. 2020b). Note the presence of typical M1 markers such as *nos2*, and the high number of pro-inflammatory cytokines

Gene	Gene description	Protein function
<i>il12p35</i>	Interleukin 12 subunit alpha (p35)	Pro-inflammatory cytokine.
<i>il1β</i>	Interleukin 1 beta	Pro-inflammatory cytokine. Also found in M2 macrophages of carp, but lower expressed.
<i>agrn</i>	Agrin	Extracellular-matrix protein involved in monocyte/macrophage survival and phagocytosis.
<i>saa</i>	Serum amyloid A protein	Acute phase protein, chemotactic to phagocytes and inducer of pro-inflammatory cytokines.
<i>cox2</i>	Prostaglandin-endoperoxide synthase 2a	Also known as Cox-2. Increased expression in M1 macrophages.
<i>nos2b</i>	Nitric oxide synthase 2b	Production of antimicrobial nitric oxide. Well-characterised M1 marker.
<i>il6</i>	Interleukin-6	Pro-inflammatory cytokine
<i>irg1</i>	Immune responsive gene 1	High expression in M1 macrophages contributes to metabolic reprogramming.
<i>cxcl13</i>	C-X-C motif chemokine ligand 13	Up-regulated in M1 macrophages
<i>cxcl8ll</i>	C-X-C motif chemokine ligand 8 like 1	Teleost-specific Cxcl8-like cytokine. Recruits neutrophils through Cxcr2.

6.4.3 Immunometabolism

Immunometabolism refers to changes in intracellular metabolic pathways in immune cells, including macrophages that alter their function in a complex interplay between immunity and metabolic reprogramming. Immunologically distinct macrophages may also be metabolically distinct and reprogrammed to enhance opposing pathways of energy (O'Neill et al. 2016). Whereas M2 macrophages rely primarily on oxidative phosphorylation, like many cells in homeostasis, in contrast M1 macrophages are the ones that are 'different' and show metabolic reprogramming towards glycolysis. In M1 macrophages, two 'breaks' in the tricarboxylic acid (TCA) cycle occur and this metabolic reprogramming towards glycolysis supports several inflammatory functions. The first break leads to a shuttling of citrate and succinate out of the mitochondria. Intracellular citrate contributes to the production of NO and ROS and to fatty acid synthesis for membrane and granule formation, whereas succinate contributes to ROS production and can stabilise hypoxia-inducible factor (HIF)1- α . Further release of succinate in the extracellular microenvironment can act as an alarmin. The second break in the TCA cycle leads to an inhibition of the electron transport chain in the mitochondria, mediated by both NO and itaconate, the latter produced from citrate with an enzyme encoded by immune response gene 1 (*IRG1*)

Table 6.2 Gene profile associated with fish M2 macrophages. A list of 10 genes amongst the most highly expressed in cAMP-stimulated (M2) macrophages of common carp (Wentzel et al. 2020b). Note the presence of typical M2 markers such as *arginase2*, and genes associated with angiogenesis and matrix remodelling

Gene	Gene description	Main Function
<i>cyr61ll1</i>	Cysteine-rich angiogenic inducer 61 protein-like protein 1	Extracellular matrix protein involved in angiogenesis and regulation of matrix remodelling.
<i>timp2b</i>	Tissue inhibitor of metalloproteinase 2b	Involved in extracellular matrix remodelling. Decreased in M1 and increased in M2 macrophages.
<i>tgm2b</i>	Transglutaminase 2b protein	Enzyme important in apoptotic cell clearance by phagocytosis. Well-known M2 marker.
<i>arg2</i>	Arginase 2	Arginase 1 is the canonical M2 marker in murine M2 macrophages.
<i>vegfaa</i>	Vascular endothelial growth factor Aa	Signalling protein involved in angiogenesis and tissue generation. Up-regulated in M2 macrophages.
<i>hbegfb</i>	Heparin-binding EGF-like growth factor b	Growth factor in early stages of wound healing. Promotes angiogenesis.
<i>angptl4</i>	Angiopoietin-like 4	Prevents the formation of lipid-laden giant cells. Associated with anti-inflammatory macrophages.
<i>ppap2b</i>	Phosphatidic acid phosphatase type 2B	Lysophosphatidic acid (LPA) inhibitor. Induced by VEGF and involved in angiogenesis.
<i>tdh</i>	L-threonine dehydrogenase	Converts L-threonine into glycine, which modulates macrophage activity.
<i>crema</i>	cAMP-responsive element modulator a	Binds cAMP response element and different splice variants. Modulates transcription.

(Van den Bossche et al. 2017; O'Neill and Artyomov 2019). First indications are that immunologically distinct macrophages of fish could also be metabolically distinct. M1 macrophages of common carp can alter their energy metabolism in a manner similar to those of mice, showing altered oxidative phosphorylation and glycolysis. Common carp M2 macrophages rely on oxidative phosphorylation, without reprogramming towards glycolysis (Wentzel et al. 2020a), with metabolic reprogramming appearing to be conserved.

6.5 In Vivo Disease Models

Zebrafish are a pivotal and widely used preclinical model of human and fish diseases, since the last 20 years (Renshaw and Trede 2012; Jørgensen 2020). Their transparent larvae and genetic tractability enable in vivo microscopy of intact organ systems, which twinned with their small size and high fecundity, have led to their expansive use as a model organism in laboratories across the globe. Originally used as a model for developmental biology in the

1980s and 1990s, they are now used as a model for a plethora of fish and human diseases. Thanks to the availability of transgenic lines and the ability to sort immune cells to assess *gene* expression, they are extensively used as models to understand the roles of macrophages in a wide range of disease settings (Fig. 6.2).

6.5.1 Zebrafish Models of Inflammation

Zebrafish are used as injury and sterile wound models, in part due to their highly regenerative properties, which enable visualisation of the complete healing process over just a few days post-challenge. Macrophages play important roles after tissue injury, caused by either exogenous factors or disease pathogenesis. Post-damage, macrophages protect against invading pathogens, but also play critical roles in tissue repair and regeneration. A widely used zebrafish model of inflammation is sterile transection of the tailfin to induce an inflammatory response (Renshaw et al. 2006; Yoo and Huttenlocher 2009). There are a number of variations in this model, (including a nick model (Yoo and Huttenlocher 2009), a more severe cut that dissects the end of the notochord (Renshaw et al. 2006), and combination with infection (Schild et al. 2020)), but all are common in their induction of an inflammatory response. Neutrophils are some of the first immune cells to reach the wound site after a few minutes, but they are closely followed by macrophages, recruited over the first 2–4 h (Loynes et al. 2010). Neutrophil numbers quickly reduce as inflammation resolves after 24 h post-wound (hpw), both via apoptosis in situ and reverse migration away from the site (Elks et al. 2011). However, macrophage numbers do not decrease, and persist at the injury site beyond 24hpw, playing important roles in efferocytosis (the clearing of apoptotic cells, including dead neutrophils) and subsequent wound healing (Loynes et al. 2010). When macrophage numbers are depleted, then the wound does not heal properly, mirroring the effects on wound healing observed in human diseases where macrophages are perturbed.

Wound models in zebrafish expand to other models beyond the tailfin, including heart, retina and somite injury to investigate the roles of immune cells in healing and regeneration (MacDonald et al. 2015; Gurevich et al. 2018; Marín-Juez et al. 2019; Bevan et al. 2020). The zebrafish is capable of injured heart regeneration, without scar formation, and the roles of macrophages in the regeneration process are becoming a field of intense study. Injury in the muscle block allows for the study of the important process of revascularisation and angiogenesis, a process in which macrophage involvement is critical, presumably in an M2 type response (Gurevich et al. 2018). Wound models are also used in combination with a wide range of disease models, including models of perturbed glucose signalling (diabetes), models of cystic fibrosis and foreign body response, to investigate the roles of immune cells in these processes (Olsen et al. 2010; Gurevich et al. 2019; Bernut et al. 2020).

6.5.2 Zebrafish Models of Infectious Pathogens

A critical function of macrophages is the handling of pathogens by phagocytosis and subsequent degradation, mechanisms that are subverted by many pathogens. As antimicrobial infections are on the increase worldwide, due to the emergence of antimicrobial resistance, there is a pressing need to understand and target these mechanisms. Understanding these processes in macrophages *in vivo* is a critical step towards new treatments. Some of the first disease models in zebrafish were infection models (Rougier et al. 1996; LaPatra et al. 2000; Neely et al. 2002; Davis et al. 2002; van der Sar et al. 2003). Zebrafish infectious disease models make significant contributions to our understanding of the macrophage response to pathogens and, in particular, shed light on how pathogens differentially interact with macrophages and evade their killing mechanisms (Rosowski 2020).

A widely used infection model in zebrafish is the use of *Mycobacterium marinum* (Mm) as a model of mycobacterial disease (most notably of human tuberculosis, but also as a model of Mm infection of commercial fish species) (Hodgkinson et al. 2019). The zebrafish Mm model was driven forward in the early 2000s due to a pressing need for accessible *in vivo* models of tuberculosis (Davis et al. 2002; Meijer et al. 2004). Unlike some other human infections, murine models of TB are hindered because mice are not natural hosts of human *Mycobacterium tuberculosis* (Mtb), and fail to produce all aspects of the hallmark granuloma response. Mm is the closest genetic relative of the human Mtb complex and can infect humans (leading to a condition called fishtank granuloma), where it causes granuloma formation in peripheral tissues, hindered only by its intolerance to temperatures above 28 °C. Mycobacteria are intracellular pathogens that can escape almost every step of the macrophage killing process in the right conditions (Chai et al. 2020). Macrophages are the primary cell type in the granuloma and as such have been a focus of zebrafish Mm research over the last 15 years plus (Davis et al. 2002; Volkman et al. 2004; Clay et al. 2007; Torraca et al. 2015; Rougeot et al. 2019). Antibody staining confirms the heavy involvement of macrophages in granuloma formation, showing that the presence of macrophages is sufficient for granuloma formation to begin, without the need for adaptive immunity (Davis et al. 2002). Genetic depletion experiments show macrophages to be critical players in Mm control, but also essential players in infection dissemination and granuloma formation (Clay et al. 2007). Zebrafish models show that there are several signalling pathways and factors involved in macrophage control of Mm, including Tnf α , Il-1B and autophagy components (Clay et al. 2008; van der Vaart et al. 2014). However, important findings in zebrafish models show that Mm can circumvent key macrophage defence mechanisms, including the production of reactive oxygen and nitrogen species (Elks et al. 2013, 2014; Roca and Ramakrishnan 2013). Elegant genetic work, where macrophages are FACS purified at different stages after infection and RNAseq performed, has identified a plethora of potentially new macrophage genes involved in TB pathogenesis (Rougeot et al. 2019). In combination with signalling transgenic lines, this demonstrates that although the macrophage pro-inflammatory response is initially present after infection,

this is circumvented by bacteria that allow for granuloma formation (Lewis and Elks 2019; Ogryzko et al. 2019). Then, at the granuloma stage the pro-inflammatory factors are once again activated in macrophages. These new lines of evidence are leading to the identification of novel therapeutic targets against TB. These include hypoxia inducible factors, which are able to increase the early pro-inflammatory response, including *il-1beta* and *tnfa*, to aid the clearance of bacteria by innate immune cells (Lewis and Elks 2019; Ogryzko et al. 2019). Aside from the natural fish pathogen *Mm*, zebrafish models of an increasingly growing list of pathogens including bacteria (e.g. *shigella*/*edwardsiella*/*staphylococcus*/*porphyromona*/*burkholderia*), fungi (e.g. *cryptococcus*/*candida*/*aspergillus*), parasites (e.g. trypanosomes) and viruses (e.g. *chikungunya*/*sindbis*) are being utilised to uncover the roles of macrophages in disease pathogenesis (Torraca et al. 2014).

6.6 Future Perspectives

An interesting area of research is the assumed important role of macrophages in trained immunity. Trained immunity is a form of innate immune memory providing increased nonspecific immune responses to subsequent infection, which is based on enhanced inflammatory and antimicrobial properties of innate immune cells (Netea et al. 2016). In food animals, such as fish, the concept of enhancing the innate immune system is not new but few studies have purposefully investigated innate training (Petit and Wiegertjes 2016; Byrne et al. 2020).

Early studies in fish already indicate that injection of brook trout (*Salvelinus fontinalis*) with modified Freund's complete adjuvant containing killed *Mycobacterium butyricum*, increases protection and induces a long-lasting increase in phagocytic and bactericidal activity of peritoneal macrophages (Olivier et al. 1986). Further, vaccination of fish with BCG (the *Bacillus Calmette-Guérin* vaccine against tuberculosis based on an attenuated strain of *M. bovis* and frequently used to study trained immunity in humans (Kleinnijenhuis et al. 2012) provides cross-specific protection in several fish species, including Japanese flounder (*Paralichthys olivaceus*), amberjack (*Seriola dumerili*) (Kato et al. 2010, 2011, 2013) and zebrafish (Oksanen et al. 2013).

Purposely investigated studies in fish have used in vitro cultures of head kidney-derived macrophages from common carp. Following a resting period of 6 days, comparable to the experimental set-up used to study trained immunity in human monocytes (Bekkering et al. 2017), a 2 h in vitro exposure to a soluble nucleotide binding and oligomerisation domain (NOD)-specific ligand, or to soluble β -(1,3/1,6)-glucan, resulted in carp macrophages that displayed typical features of trained immunity for a period of at least 6 days (Petit et al. 2019). Unstimulated, but trained, macrophages displayed increased phagocytosis and elevated constitutive gene expression of the cytokines *il-6* and *tnfa* and a metabolic shift from oxidative phosphorylation towards glycolysis, which could be measured as increased production of lactate. The underlying mechanisms of trained immunity are as yet not fully understood in fish. In mammalian systems, these processes rely on long-lived epigenetic

modifications that persist for longer periods, even after removal of the training stimulus. For example, β -glucan-induced trained immunity of human monocytes has been associated with modifications of histone (H)3 activation and repressor markers at promotor sites of cytokine genes, such as IL-6 and TNF α , and appears key to the altered long-lived inflammatory response associated with trained immunity (Saeed et al. 2014). Areas of research which combine trained immunity with immunometabolic control (Riksen and Netea 2020) are of high interest for fish macrophages, because of the expected conservation of metabolic reprogramming (Wentzel et al. 2020a).

Last but not least, *in vitro* evidence for the effects of trained immunity alone is never easy to show in animals that also have adaptive immunity. Zebrafish could prove especially informative for such *in vivo* studies, owing to the availability of *rag*^{-/-} mutant lines that do not have a functional B- and T-cell response and therefore no functional adaptive immune response (Tokunaga et al. 2017). There are some data on *rag*^{-/-} zebrafish, showing that these fish have a constitutively heightened innate immune activity and increased survival 8 weeks post-exposure to an attenuated non-virulent strain of *Edwardsiella ictaluri* (Tokunaga et al. 2017). This could be interpreted as the first line of experimental *in vivo* evidence of trained immunity in fish.

6.7 Conclusions

In conclusion, fish macrophages are functionally similar to their mammalian counterparts, with the ability to polarise into different activation states, playing important roles in infection response and wound repair. The conservation of function is demonstrated in a wide range of fish species by *in vitro* immunology, performed on various primary cell cultures. Without validated specific markers for fish macrophages, it remains difficult to assess the purity of *in vitro* cell cultures. Although a repertoire of conserved genes for mononuclear phagocyte subsets seems to exist and thus could constitute a list of candidates for relevant markers (Vu Manh et al. 2015), this list is based on a meta-analysis combining cell sorting and comparative transcriptomic analysis and requires confirmation by functional evidence. Studies on the evolution of macrophages suggest that key processes, such as phagocytosis, contribute to the regulation of the inflammatory response since hundreds of millions of years of evolution (Barreda et al. 2016). While purification, differentiation and culture techniques vary across species, the importance of the arginine fork and nitrosative response as markers of activation states is demonstrated in most species studied. More recently, the expansion of zebrafish studies, with all the genetic and microscopy tools available in a single species, unifies groups around the world studying macrophages in fish to use similar techniques and tools. This illuminates further the roles of macrophages in homeostasis and disease, often in the absence of adaptive immunity. There is still much to understand in terms of the variety of macrophage phenotypes and function in fish species, especially in diverse tissue microenvironments.

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