Polarization of immune responses in fish: the ‘macrophages first’ point of view

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Abstract

In this review, we support taking polarized immune responses in teleost fish from a ‘macrophage first’ point of view, a hypothesis that reverts the dichotomous T helper (T_H)1 and T_H2 driving forces by building on the idea of conservation of innate immune responses in lower vertebrates. It is plausible that the initial trigger for macrophage polarization into M1 (inflammation) or M2 (healing) could rely only on sensing microbial/parasite infection or other innate danger signals, without the influence of adaptive immunity. Given the long and ongoing debate on the presence/absence of a typical T_H1 cytokine environment and, in particular, T_H2 cytokine environment in fish immune responses, it stands out that the presence of macrophages with polarized phenotypes, alike M1 and M2, have been relatively easy to demonstrate for fish. We summarize in short present knowledge in teleost fish on those cytokines considered most critical to the dichotomous development of T_H1/M1 and T_H2/M2 polarization, in particular, but not exclusively, interferon- γ and interleukin (IL)-4/IL-13. We review, in more detail, polarization of fish immune responses taken from the macrophage point of view for which we adopted the simple nomenclature of M1 and M2. We discuss inducible nitric oxide synthase, or NOS-2, as a reliable M1 marker and arginase-2 as a reliable M2 marker for teleost fish and discuss the value of these macrophage markers for the generation of zebrafish reporter lines to study M1/M2 polarization in vivo.

Highlights

- Teleost fish macrophages can express M1 and M2 phenotypes
- Inducible nitric oxide synthase, NOS-2, is a marker for fish M1 macrophages
- Arginase-2 is a marker for fish M2 macrophages
- NOS-2 and ARG-2 are interesting candidates for the generation of zebrafish reporter lines

Keywords

Macrophage polarization, fish, zebrafish, iNOS, arginase, LPS

1. Introduction

Traditionally, polarization of immune responses into inflammatory and anti-inflammatory types of immunity have been based on the presence of polarizing cytokine profiles secreted by, among others, T helper 1 (T_H1) and T helper 2 (T_H2) cell types. It is well-accepted that the presence in mice and humans of these polarizing T cell-derived cytokines, including interferon gamma (IFN- γ ; T_H1) and interleukin-4 and -13 (IL-4/-13; T_H2) can drive in vitro cultures of bone marrow-derived macrophages from mice, and differentiated peripheral blood monocytes from humans, into polarized populations referred to as classically activated and alternatively activated, respectively (Martinez et al., 2009). In this concept, classically-activated macrophages are induced and enhanced by an inflammatory T_H1 cytokine environment, whereas alternatively-activated macrophages are induced and enhanced by an anti-inflammatory T_H2 cytokine environment. To mirror the T helper dichotomy (Mills et al., 2000), classically-activated macrophages are also simply referred to as M1 and alternatively-activated macrophages referred to as M2 macrophages.

Over the years the traditional view on the exact role of macrophages in polarized immune responses has been frequently fine-tuned to incorporate innate-activated macrophages next to the IFN- γ driven classically-activated M1 and to incorporate next to IL-4/-13 stimulated M2 macrophages (also called M2a), macrophages co-activated by immune complexes or apoptotic cells (called M2b) or macrophages de-activated by glucocorticoids, transforming growth factor (TGF)- β , or IL-10 (called M2c), the latter also referred to as regulatory macrophages (Mantovani et al., 2004). Recently, the somewhat over-simplified M1/M2 classification has been challenged by transcriptome comparisons of human monocyte-derived macrophages, identifying a large array of macrophage activation states and profiles (Xue et al., 2014). Indeed, M1 and M2 macrophages may simply represent the extremes of the large scale of macrophage activation states (Mills and Ley, 2014). As a reasonable compromise, the use of a common framework for macrophage-activation nomenclature based on i) a defined set of standards encompassing the source of macrophages (e.g. bone marrow-derived versus monocyte-derived), ii) definition of the activators (e.g. IFN- γ versus IL-4/-13), and iii) a consensus collection of markers to describe macrophage activation, would unify experimental standards for diverse experimental scenarios in a more detailed manner (Murray et al., 2014). The latter, however, applies primarily to human and mouse macrophages and thus for this review we have chosen to adopt the definition of M1 and M2 as groups of stimulated macrophages, combining several stimuli able to induce a similar functional profile (Martinez and Gordon, 2014). No matter what, independent of the exact name and number of

macrophage sub-classifications, the consensus is that polarized populations of macrophages are present during T_H -driven immune responses.

One current view is challenging the traditional paradigm of analyzing polarized immune responses by reverting the initial driving forces behind polarized immune responses, IFN- γ and IL-4/-13, and by building on the importance of innate immunity, taking polarized immune responses from a ‘macrophage first’ point of view (Martinez and Gordon, 2014; Mills and Ley, 2014). In this view, M1 or M2 macrophages would differentiate first during an immune response and subsequently direct T lymphocytes to produce T_H1 or T_H2 responses, respectively, to amplify, rather than initiate, M1/M2 macrophage polarization in positive feedback loops (Fig. 1). This would imply that the initial trigger for macrophage polarization could simply rely on sensing microbial/parasite infection or innate danger signals alone, without the necessary influence of adaptive immunity. Of course, the ‘macrophage first’ view does not exclude that T_H1 and T_H2 cytokine profiles amplify macrophage M1/M2 dichotomy, finally resulting in a self-sustaining T_H1 -M1/ T_H2 -M2 polarization during complex immune responses.

It is likely that macrophages are able to perform their main properties including phagocytosis, endocytosis, secretion and microbial killing in the steady state, with M1 and M2 polarizations contributing to a further modulation and tuning of immune responses (Martinez and Gordon, 2014). Also a very attractive point of view is that M2 ‘heal’ states could be the default mode of tissue macrophages with M1 states developing only under critical conditions into a potentially terminal phenotype (Mills and Ley, 2014). In this respect it will be important to examine in future studies exactly which differences exist between steady state and M2 macrophages. It will also be important to determine if all activated macrophages can be grouped within M1 and M2 designations, especially with regard to macrophage phenotypes induced by non-T cell drivers such as those derived from innate immunity. Following the fate of individual macrophages in vivo, for example in live zebrafish (*Danio rerio*) (see also ‘future developments’), may help examine if one-directional transition states from M2 to M1 exist.

It is possible that the M1-M2 dichotomy arose early in evolution, prior to the development of mammalian vertebrates and could be an evolutionary conserved, intrinsic property of macrophages associated with transitions from healing (M2) to inflammation (M1) (Mills and Ley, 2014). Comparative immunologists would tend to favor the ‘macrophage first’ view simply based on the fact that the ability of macrophage-like cell types to phagocytize foreign objects and repair cellular damage already existed in the first primitive animals, whereas the

requirement for cytokine-mediated adaptive immunity developed only later in evolution (Ward and Rosenthal, 2014). Teleost fish are among the evolutionarily oldest vertebrates with both an innate and classical adaptive immune system, and are crucially important for studies on evolutionarily conserved functions of the immune system (Magor and Magor, 2001). Examining macrophage function in teleost fish is highly interesting in particular with respect to determining the effects of T-cell derived cytokines on macrophage polarization. There has been a long-standing debate, which is still ongoing (Yamaguchi et al., submitted 2015), about whether immune responses in fish can be characterized as truly polarized based on the presence/absence of a typical T_H1 cytokine environment and, in particular, a typical T_H2 cytokine environment (Bird et al., 2006; Secombes et al., 2011; Wang and Secombes, 2013). In contrast to the ongoing discussion on the presence of polarized T_H subsets in fish, the presence of macrophages with polarized profiles alike mammalian M1 and M2 (Forlenza et al., 2011; Joerink et al., 2006b; Joerink et al., 2006c) has been relatively easy to demonstrate.

We summarize, in short, the present knowledge in teleost fish on those cytokines considered most critical to the development of M1 and M2, in particular but not exclusively, IFN- γ and IL-4/-13. We discuss, in more detail, polarization of immune responses in fish taken from the macrophage point of view for which, for the purpose of this review, we have chosen to adopt the simple and informative (Martinez and Gordon, 2014), although sometimes confusing (Murray et al., 2014), nomenclature of M1 and M2. We will discuss the use of inducible nitric oxide synthase (iNOS) and arginase which, although sometimes debated (Thomas and Mattila, 2014), have proven useful markers to discriminate between mammalian M1 and M2 (Rath et al., 2014), but also appear useful markers of M1 and M2 of common carp (Forlenza et al., 2011). The enzymes iNOS and arginase, by competing for L-arginine as substrate for both M1 and M2, in a process sometimes referred to as the arginine-fork, determine the balance between ‘inflammatory’ M1 type of macrophages and ‘healing’ M2 type of macrophages. We discuss evidence for the use of iNOS(B), or NOS-2(B), as marker for M1 and the use of arginase-2 as marker for M2 macrophages of teleost fish.

2. M1 macrophages in fish

Notably, macrophages can be activated by microbial infection or innate danger signals without any influence of adaptive immune cells, leading to a form of M1 macrophages also defined as innate-activated (Gordon, 2003). These microbial stimuli can activate macrophages in an innate manner via detection through a large array of pattern recognition receptors (PRRs) (Taylor et al., 2004). Here, it is not the intention to extensively review the detection of

these innate signals by PRRs in fish, for which several reviews exist already discussing the presence and function of, among others, Toll-like and viral nucleic acid receptors (Aoki et al., 2013; Boltaña et al., 2011; Hansen et al., 2011; Kanwal et al., 2014; Palti, 2011; Pietretti and Wiegertjes, 2014; Poynter et al., 2015; Rauta et al., 2014; Zhang et al., 2014). Overall, M1 macrophages can be grouped (Martinez and Gordon, 2014) as to include macrophages activated by bacterial PAMPs, of which probably the most frequently studied is Gram-negative bacterial lipopolysaccharides (LPS), macrophages activated by (microbial ligands in combination with) IFN- γ and macrophages activated by granulocyte macrophage colony stimulating factor (GM-CSF).

LPS from Gram-negative bacteria such as *Escherichia coli* probably are among the best-studied microbial stimuli. Despite the fact that a TLR4 receptor complex, as we know it from mammals, may not be functional in fish (Sepulcre et al., 2009; Sullivan et al., 2009), it is not especially relevant to understanding M1 activation whether LPS is sensed by a true TLR4 receptor complex or by other receptors sensing possible contaminations with peptidoglycan and/or DNA in impure LPS preparations (MacKenzie et al., 2010) as the final activation state will still fit the M1 profile. In mammals, sensing of LPS by the TLR4 complex (Hume, 2015) will lead to activation of the relevant transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) and activator protein 1 (AP-1), and downstream up-regulation of expression of pro-inflammatory cytokines, including IFN- γ and tumor necrosis factor (TNF)- α , resulting in the development of M1-like phenotypes. There are numerous publications that show that in vitro stimulation of fish macrophages with LPS leads to increased respiratory burst activity and associated production of oxygen radicals, production of nitrogen radicals and secretion of pro-inflammatory cytokines (Rieger and Barreda, 2011). Thus, regardless of exactly which receptor senses exactly what compound in LPS preparations and subsequently leads to activation of macrophages, in vitro studies with LPS have clearly proven the existence of innate activated (M1) macrophages in fish.

IFN- γ , as a combination stimulus together with LPS, forms the best characterized inflammatory (M1) side of the balance in the M1/M2 paradigm. LPS+IFN- γ (classically) activated macrophages have higher respiratory burst activity and nitric oxide synthase expression as well as increased ability for antigen presentation (major histocompatibility complex (MHC) class II) and co-stimulation (CD86) than have LPS-only stimulated macrophages (Taylor et al., 2004). Gene expression profiles induced by either LPS or IFN- γ on one hand, or a combination of LPS+IFN- γ on the other, show a large degree of overlap but also enough differences not to consider each of these stimuli homologous, at least not for

human macrophages (Martinez et al., 2006; Nau et al., 2002). The promoter of mouse inducible nitric oxide (iNOS) contains two regions termed RI and RII of which both are mediated by LPS but only RII is mediated by IFN- γ , thus amplifying the effect of LPS (Lowenstein et al., 1993; Xie et al., 1993). The ‘killing’ profiles of innate- and classically-activated macrophages groups them together as M1 macrophages.

Members of the type II IFN family have been well characterized for fish (Wang and Secombes, 2013; Zou and Secombes, 2011). Recombinant grass carp (*Ctenopharyngodon idella*) IFN- γ alone boosts NO production of monocytes/macrophages (Yang et al., 2013) whereas recombinant common carp (*Cyprinus carpio*) IFN- γ , as a combination stimulus with LPS, stimulates a powerful synergistic gene expression of pro-inflammatory cytokines and iNOS, and induces an enhanced respiratory burst activity and nitric oxide production (Arts et al., 2010). Similar to the mammalian immune system, fish T cells and natural killer cells could be likely producers of IFN- γ . Furthermore, recombinant IFN- γ appears to signal via a STAT1-dependent mechanism to increase expression of MHC class I and class II molecules, increase expression of several chemokines and cytokines, among which IL-1 β , IL-6, IL-12 and TNF- α , and increase phagocytosis, oxidative burst and nitric oxide response of several fish species (reviewed by Wang and Secombes, 2013), all characteristics of pro-inflammatory responses linked with M1 macrophages. Similar to the mammalian situation, suppressor of cytokine signaling (SOCS)-1 is important for the control of IFN- γ signaling (Skjesol et al., 2014). It would be interesting, in this context, to study gene expression profiles of LPS-versus LPS + IFN- γ -stimulated fish macrophages in more detail to determine the degree of overlap in activation profile and conservation with mammalian M1 macrophages.

In contrast to mammals, the fish IFN- γ family consist of two members; IFN- γ and IFN- γ -related (IFN- γ rel), although this is probably not of direct relevance to M1/M2 polarization. The distinction made between ‘true’ IFN- γ and IFN- γ rel is primarily based on a lack of nuclear localization signal in IFN- γ rel sequences. Deletion in rainbow trout (*Oncorhynchus mykiss*) IFN- γ of this particular site abolished its ability to induce chemokine expression (Zou et al 2005), in line with the essential role played by mammalian IFN- γ . Recombinant goldfish (*Carassius auratus*) IFN- γ rel elicits a robust but relatively short-lived priming of monocytes for respiratory burst, and down-regulates the priming potential of IFN- γ and of TNF- α (Grayfer et al., 2010; Grayfer et al., 2009). Although knock-down studies in zebrafish indicated partly overlapping functions based on the fact that knock-down of either IFN- γ isoform did not interfere with the ability to clear bacterial infection in vivo (Sieger et al., 2009), recombinant common carp IFN- γ rel fails to prime antimicrobial activity in

phagocytes (Arts et al., 2010). The most detailed studies on IFN- γ rel have been performed on another cyprinid fish species; Ginebra crucian carp (*Carassius auratus langsdorffii*), where two IFN- γ rel genes exist. Recombinant IFN- γ rel1 and IFN- γ rel2 both showed high antiviral activity. The fact that both IFN- γ rel molecules exhibit biological activity as monomers (Shibasaki et al., 2014) and not in the homodimeric conformation usual for IFN- γ , confirms that IFN- γ rel molecules are likely to have biological activity different from IFN- γ . Most probably IFN- γ rel proteins are antiviral proteins without direct effects on M1/M2 polarization in fish.

Once activated, M1 macrophages are characterized by cell-mediated immunity and the capacity to kill ingested and intracellular pathogens; a status driven and/or reinforced, by a series of pro-inflammatory cytokines (Gordon, 2007; Gordon and Taylor, 2005; Mantovani et al., 2004). It is therefore difficult to assign to one of these cytokines in particular a more prominent role as a marker for M1 macrophages, but here we have chosen to shortly discuss the role of TNF- α . Recently, transgenic zebrafish were used as a live model to identify and track, via timelapse microscopy, macrophage subtypes expressing TNF- α (Nguyen Chi et al., 2015). Aseptic wounding and also infection with *E. coli* triggered macrophage recruitment, of which only a subset started to express TNF- α as well as some other pro-inflammatory cytokines, including IL-1 β and IL-6. Of interest, fate tracing of TNF- α (+) macrophages during the time-course of inflammation demonstrated that these pro-inflammatory macrophages converted into other phenotypes. This study elegantly confirms that the diversity and plasticity typical of mammalian macrophages is also common to fish macrophages and may contribute to the ongoing discussion on the existence of one-directional transition states from a M2 to a M1 phenotype, or vice versa.

One complicating factor in studies on the role of TNF- α is that most teleost fish species possess multiple isoforms (Wang and Secombes, 2013; Wiens and Glenney, 2011). Functional studies with recombinant proteins have indicated conserved functions in trout and goldfish (Grayfer et al., 2008; Zou et al., 2003) but also non-conserved functions of TNF- α in seabream and carp (Forlenza et al., 2009a; Roca et al., 2008). So far, most studies have addressed two isoforms (TNF- α 1 and TNF- α 2), but recently a third isoform (TNF- α 3) with only low identities to the other two TNF isoforms was described (Hong et al., 2013). The new ‘type II’ TNF- α has only a short stalk suggesting it might function as a membrane form only, and could be especially interesting because it appears to have a low basal, but relatively early induced gene expression after LPS stimulation of macrophages. It is clear that studying TNF- α in relation to M1 polarization of fish macrophages is an area of research which requires

further investigation of TNF- α isoforms while taking advantage of in vivo studies building on the availability of zebrafish transgenic for TNF- α .

Granulocyte macrophage colony-stimulating factor (GM-CSF, CSF-2) is the latest addition to the M1 category of stimuli (Martinez and Gordon, 2014). GM-CSF is a member of a discrete family of cytokines which also includes IL-3 and IL-5 which regulate the growth, differentiation, migration and effector function activities of many hematopoietic cells and leukocytes with receptors comprised of a cytokine-specific alpha chain (CSF2R α) and a shared beta chain (CSF2-R β) for signaling (Martinez et al., 2009). Although this cytokine family appears very poorly conserved (Huising et al., 2006), the existence of CSF2-R β in fish genomes has been previously described (Liongue and Ward, 2007). Only recently, candidates for IL-3/IL-5/GM-CSF family members have been identified in the elephant shark genome (Dijkstra, 2014) and in genomes of some (cyprinid) fish species (Yamaguchi et al., submitted 2015). This recent identification of candidate GM-CSF-like cytokines in fish genomes opens the way for functional studies on activation of macrophages with recombinant GM-CSF to study the conservation of this form of activation of fish cells into macrophages with M1 profiles.

3. M2 macrophages in fish

M2 macrophages can be generally characterized as having ‘anti-inflammatory’ phenotypes, when developed in the presence of the T_H2 cytokines IL-4 and/or IL-13 (Martinez et al., 2009). Typically, these M2 macrophages show increased arginase activity and produce proteins important for ‘healing’ processes, including the generation of extracellular matrix and polyamines (putrescine, spermidine, and spermine) important for cell growth and division. Whereas the enzyme iNOS is omnipresent in M1 and converts the amino acid L-arginine into L-citrulline and NO, M2 macrophages up-regulate arginase enzymatic activity to compete and convert the same substrate into L-ornithine and urea, counterbalancing the activity of M1. Here it is not the intention to extensively discuss all different stimuli that can lead to the development of mammalian M2 macrophages but rather we will try and put in perspective the relevant data available for fish macrophages that fit M2 phenotypes. Overall, M2 macrophages can be grouped (Martinez and Gordon, 2014) to include i) macrophages activated by IL-4 or IL-13 (also named M2a: Mantovani et al., 2004), ii) macrophages activated during parasite infection with protozoans or helminths either as a combination stimulus with apoptotic cells or immune complexes (also named M2b), iii) macrophages activated by macrophage colony stimulating factor CSF-1 but also iv)

macrophages de-activated by glucocorticoids or by cytokines such as TGF- β or IL-10 (also named M2c or regulatory macrophages).

Stimulation of macrophages with IL-4/-13 forms the best characterized anti-inflammatory (M2) side of the balance in the M1/M2 paradigm. In mammals, IL-4 and IL-13 have overlapping but distinct roles in inflammatory responses to extracellular parasites via production of IgE, differentiation of T_H2 lymphocytes and activation of M2 (Van Dyken and Locksley, 2013). The heterodimeric receptor complex for IL-4 and IL-13 consists of a common receptor subunit (IL-4R α), which is the molecular basis for their overlap in biological functions, and a receptor subunit specific for each cytokine (Mueller et al., 2002). The evolution of IL-4 and IL-13 and their receptor subunits has recently been reviewed (Wang and Secombes, 2015). IL-4 and IL-13 are found side by side in the mammalian genome. Given this genomic organization it seems likely that a single IL-4/13 ancestor gene existed in jawless vertebrates, which has been duplicated in different lineages by whole genome duplication and/or tandem duplication events (Wang and Secombes, 2015). In fish, at least two IL-4/IL-13 genes exist (IL-4/13A and IL-4/13B), both with low homology to IL-4 and IL-13 (Li et al., 2007; Ohtani et al., 2008), of which maybe IL-4/13A shows the most complete synteny with other genes in the T_H2 cytokine complex (Yamaguchi et al., submitted 2015). This could fit the observation that zebrafish injected with recombinant IL-4/13A show increased numbers of DC-SIGN⁺ (CD209) cells and IgZ-2⁺ B cells in peripheral blood (Hu et al., 2010; Lin et al., 2009). Recombinant zebrafish IL-4/13A was shown to bind to the zebrafish IL-4R α chain (Zhu et al., 2012). Of interest, a T cell line (TCR⁺, CD4-1⁺) of common carp was established that expresses IL-4/13B, thus showing a T_H2-like phenotype (Yamaguchi et al., 2013). In vitro testing of supernatants of this cell line on common carp macrophages could verify the ability, or inability, of the second IL-4/13 isoform to drive macrophages to express an M2 phenotype. Without doubt, it would be interesting within the T_H2/M2 context, to study gene expression profiles of IL-4/13-stimulated fish macrophages for the presence of typical M2 markers, including arginase, to determine the degree of conservation in activation profile within the M2 group of macrophages found in a lower vertebrate.

Studies in mice have shown that arginase gene expression and activity can be induced by IL-13 via the increase of intracellular cAMP and tyrosine kinase phosphorylation. Exogenous cAMP can be used for a cytokine-independent activation of M2 macrophages based on the fact that cAMP-mediated activation of protein kinase A can mimic the effect of IL-13 as the primary signaling pathway responsible for the production of arginase (Chang et al., 2000).

Mouse macrophages isolated from the resolving (healing) phase of an acute peritonitis express an M2 phenotype controlled by cAMP and in particular, characterized by an increase in arginase-I and IL-10 and expression of the mannose receptor, the latter considered a typical M2 marker. Apparently, cAMP can control the balance of pro-inflammatory versus anti-inflammatory cytokines once signals that drive inflammation are dampened (Bystrom et al., 2008). Human macrophages can also directly sense extracellular cAMP, inducing a phenotype resembling M2 macrophages. It is likely that a direct influx of cAMP into the cell or activation of adenylyl cyclases via adenosine receptors leads to an increase of intracellular second messenger cAMP, controlling the anti-inflammatory effects on monocytes (Sciaraffia et al., 2014). Macrophages of common carp stimulated with exogenous cAMP increase arginase activity but not NO production, and the induced arginase activity could be specifically inhibited by the NOS inhibitor N(omega)-hydroxy-nor-L-arginine (NOHA) (Joerink et al., 2006c). Therefore, common carp macrophages show the intrinsic capability to polarize into M2-type/healing state as shown by high levels of arginase.

Susceptibility to both protozoan and helminth infections have been linked to the presence of M2 macrophages (Kreider et al., 2007; Raes et al., 2007; Sica and Mantovani, 2012). Exactly which (sub) types develop in vivo depends on parasite virulence, host genotype and infection stage, but also possible co-infections with other parasites such as helminths that can be co-endemic with protozoan parasites (Raes et al., 2007). For fish, there are a number of studies that have measured arginase gene expression during bacterial or parasitic infection (Forlenza et al., 2011), but gene expression studies based on whole organ transcription analysis cannot be more than indicative for the presence of either M1 or M2, although maybe the use of an iNOS/arginase gene expression ratio (Fast et al., 2009) might be considered an interesting approach to quantify macrophage polarization in vivo. Atlantic salmon (*Salmo salar*) infected with salmon louse show an inflammatory pattern of gene expression in damaged skin indicative of T_H2-like responses. Of interest, up-regulation of arginase-1, among others, was seen in the intact skin of infected fish and of the major components of extracellular matrix, TGF- β and IL-10, but only at the adult stage of sea louse infection (Skugor et al., 2008). Taken together with up-regulation of matrix metalloproteinases (MMP), this classifies the wounds afflicted by sea louse as chronic and possibly presents an interesting infection model to study iNOS/arginase gene expression ratio and thus M1/M2 polarization in salmon.

Few studies have addressed macrophage polarization in vivo; some indications exist from our studies on experimental infections of common carp with either of two related

Kinetoplastid protozoan parasites. Infections with *Trypanoplasma borreli* appear characterized by the presence of large numbers of M1 macrophages as deduced from an increase in IFN- γ , TNF- α and iNOS gene expression and associated increase in serum nitrite levels and tissue nitration (Forlenza et al., 2009b; Forlenza et al., 2008; Joerink et al., 2006a; Ribeiro et al., 2010; Saeij et al., 2002; Saeij et al., 2000; Stolte et al., 2008). Experimental infections with *Trypanosoma carassii*, in contrast, do not result in a prominent NO response (Joerink et al., 2007; Saeij et al., 2002) but instead result in elevated levels of arginase enzyme activity, particularly during the later phase of infection (Joerink et al., 2006a). Of interest, ex vivo studies with head kidney leukocytes from *T. borreli*-infected carp, re-stimulated with LPS showed significant amounts of NO production, whereas re-stimulation of head kidney leukocytes from *T. carassii*-infected carp with cAMP show higher levels of arginase, but not vice versa (Joerink et al., 2006a). This suggests that macrophages from *T. borreli*-infected carp could be polarized towards an M1 phenotype, whereas macrophages from *T. carassii*-infected could be polarized towards an M2 phenotype. Overall, it is clear that more experimental studies with parasite-infected fish are required to characterize in vivo activation of M2 in fish.

Macrophage colony-stimulating factor (M-CSF, CSF-1) is the latest addition to the M2 category of stimuli (Martinez and Gordon, 2014). M-CSF is the primary regulator of proliferation, differentiation, and survival of macrophages via its cognate colony-stimulating factor-1 receptor (CSF1-R) (reviewed in Barreda et al., 2004), which leads to activation of STAT6 and other regulators, many of which are also part of the IL-4/-13 signaling pathways. M-CSF appears relatively well-conserved, although rainbow trout, zebrafish and goldfish CSF-1 each have a unique exon/intron structure (Wang et al., 2008). Signaling through CSF1-R mediates the proliferation, differentiation, and activation of macrophages and their progenitors. In goldfish, CSF1-R is expressed on all macrophage sub-populations (progenitors, monocytes, macrophages), and increases with macrophage development (Katzenback and Belosevic, 2012). Recombinant trout CSF-1 promotes the growth of head kidney leukocytes, and up-regulates the expression of CXCR-3 in macrophages, suggesting a role in the trafficking of macrophages to sites of inflammation or injury (Wang et al., 2008). Recombinant goldfish CSF-1 induces the differentiation of monocytes into macrophages and proliferation of monocyte-like cells with pro-inflammatory effects on these cell types (Grayfer et al., 2009). The latter could be abrogated by anti-CSF1-R antibody and also by a soluble CSF1-R, which presence appears to be a mechanism for the regulation of CSF-1 function seemingly unique to fish (Barreda et al., 2005; Hanington et al., 2009; Hanington et al., 2007).

Soluble CSF1-R modulates reactive oxygen production, nitric oxide synthesis, chemotaxis, and phagocytosis in cultured macrophages and plays a role in the control of inflammation in vivo, which includes modulation of macrophage function and recruitment (Rieger et al., 2014; Rieger et al., 2015). Although there is as yet no clear evidence that soluble CSF1-R plays a role in determining fish M2 macrophage phenotypes, it might be interesting to further investigate soluble CSF1-R in the context of macrophage polarization.

Macrophages de-activated by glucocorticoids or by the cytokines TGF- β or IL-10 can also be included in the group of M2(c) macrophages. In lower animals including snails, the expression of arginase can be induced by the presence of parasite antigens and TGF- β leading to collagen synthesis, without the presence of T_H2 cytokines (Dzik, 2014). In fact, it has been suggested that TGF- β -signaling, rather than microbial stimuli, would be the evolutionarily oldest trigger to initiate M1/M2 macrophage polarization (Dzik, 2014). Of the large TGF- β superfamily, TGF- β 1 seems to be the most potent regulator of iNOS (Vodovotz, 1997). The extremely high degree of conservation of TGF- β 1 (Gitelman and Derynck, 1994), suggests that its signaling function in inflammatory responses and tissue necrosis may have been preserved over time (Dzik, 2014). Recombinant grass carp IL-10 and recombinant grass carp TGF- β 1 both attenuate LPS-stimulated inflammatory gene expression in monocytes/macrophages (Wei et al., 2015). Recombinant goldfish IL-10 reduces radical production in monocytes and transcription of specific pro-inflammatory cytokines (Grayfer et al., 2011). Recombinant common carp IL-10 deactivates both neutrophils and macrophages, as shown by inhibition of oxygen and nitrogen radical production as well as reduced expression of pro-inflammatory genes, but also MHC genes involved in antigen presentation. Similar to mammalian IL10, carp IL-10 acts through a signaling pathway involving phosphorylation of STAT-3, ultimately leading to early up-regulation of SOCS-3 expression (Piazzon et al., 2015). It would be of interest to study more extensively the gene expression profiles of TGF- β 1- or IL-10-stimulated fish macrophages in order to examine the degree of conservation in the (de)activation profiles and effects on expression of M2 markers of fish macrophages.

4. The relevance of catabolizing L-arginine

Arginine is a non-essential or conditionally-essential amino acid in mammals but a truly essential amino acid in birds and fish, because the latter animal species cannot synthesize arginine de novo. In mammals, uptake in the small intestine appears to be the major route of citrulline for de novo synthesis of arginine by the kidneys in mammals, resulting in normal

plasma levels of arginine in humans of 100–200 μ M (Windmueller and Spaeth, 1981). Uptake of citrulline in the small intestine usually is sufficient for a healthy adult, but the biosynthetic pathway may not always produce enough arginine during growth or disease (Morris, 2006), in which cases arginine supplementation is also required in humans. Animal feed for birds and fish always need to contain sufficient arginine to maintain a healthy diet. In catfish (*Ictalurus punctatus*), healthy plasma levels are around 100–200 μ M (Buentello and Gatlin III, 2000). Recent experiments indicated that higher levels of dietary arginine could attenuate the inflammatory response induced by LPS in both, chicken (Tan et al., 2014) and teleosts, e.g. Chinese common carp (Jiang et al., 2015). Given the fact that teleosts cannot synthesize arginine de novo, nutritional supplementation can possibly open a route to influence arginine concentrations at the whole animal level, and thereby influence macrophage polarization and fish health.

In general, arginine is preferentially taken up via members of the solute carrier family 7 (SLC7) (Closs et al., 2006). Cationic amino acid transporter-1 (CAT-1), or SLC7-A1, is one of the main transporters for arginine uptake into cells, where arginine is subsequently used for protein synthesis. Murine macrophages constitutively express high affinity SLC7-A1 (CAT-1) but transport arginine via the low affinity SLC7-A2 (CAT-2) transporter when activated (Morris, 2006). Grass carp SLC7-1 expression is down-regulated in fasting animals (Yang et al., 2014), but receptor expression in macrophages is not checked. Clearly, identification and characterization of members of the SLC7 family members relevant to fish macrophages requires further investigation.

Upon arginine transport into cells, mammalian cells can catabolize arginine by four classes of enzymes: nitric oxide synthase (NOS), arginase, arginine decarboxylase (ADC), and arginine:glycine aminotransferase (AGAT) (Wu and Morris, 1998). In macrophages, the enzymes NOS and arginase play a major role in arginine catabolism and thus a central role in the polarization of immune responses and physiology of macrophages associated with NOS (M1) and arginase (M2) activity.

4.1 Inducible nitric oxide synthase (iNOS) as a marker for M1

Most probably, multiple events of parallel evolution of NOS exist which has led to different lineages, with inducible NOS (iNOS) as the most basal ancestral prototype. In invertebrates, nitric oxide acts as a signaling molecule and does not appear to have the cytotoxic function found in vertebrates (Rath et al., 2014). Mammalian vertebrates have three isoforms of NOS: NOS1 or neuronal NOS (nNOS), NOS2 or iNOS, and NOS3 or endothelial NOS (eNOS).

Both nNOS and eNOS are constitutively expressed enzymes that are calcium-dependent. In contrast, iNOS is inducible and independent of calcium because calmodulin binds to iNOS even at low intracellular calcium concentrations (Rath et al., 2014), an effect that probably occurs also in fish (Saeij et al., 2000). Most likely, eNOS originated as the last isoform within the mammalian clade and indeed, eNOS sequences appear absent from teleost fish genomes. NOS catabolizes arginine to nitric oxide and citrulline. M1 macrophages abundantly express the enzyme iNOS, but also express a pathway to catabolize citrulline via the citrulline–NO cycle for resynthesis of arginine, which allows these macrophages to ensure sufficient supply of arginine for prolonged NO synthesis (Rath et al., 2014). Simultaneous generation of NO radicals and reactive oxygen species, including the radical superoxide (O_2^-) can, for example, lead to the formation of peroxynitrite ($ONOO^-$), a powerful oxidant leading to the formation of nitrotyrosine (Radi et al., 2001). Tyrosine nitration can be considered a hallmark of tissue injury and has been used as a specific (bio)marker for nitrosative stress linked with inflammation, a phenomenon also observed in fish (Elks et al., 2014; Forlenza et al., 2008). The combined generation of reactive nitrogen and reactive oxygen species helps define M1 as ‘kill/fight type’ of macrophages with iNOS (NOS-2) being an excellent marker for M1 macrophages of fish.

Teleosts are believed to have gone through three rounds (3R) of whole genome duplications, approximately 360 million years ago (MYA) (Venkatesh, 2003). Some, like the Salmonidae are assumed to have gone through an additional fourth round of duplication (4R) (Berthelot et al., 2014). Likewise, within the Cyprinidae, common carp, but not zebrafish, have gone through an additional duplication round (4R). Indeed, the majority of exons of zebrafish have two hits in the carp genome (Henkel et al., 2012). Common carp and zebrafish are estimated to have diverged approximately 120 MYA (Wang et al., 2012), but have retained extraordinary levels of synteny (Henkel et al., 2012). Whole genome duplications can result in both gene loss and gene gain but there exist significant differences in gene retention for different functional categories of genes between fishes and land vertebrates (Blomme et al., 2006). As a result of 3R duplications zebrafish can express two copies where mammalian vertebrates express a single copy; where human and mouse express a single copy of inducible NOS, zebrafish express two copies of this gene (NOS-2A and NOS-2B). Syntenic analysis shows conserved synteny for NOS-2B, but not NOS-2A, suggestive of gene divergence of NOS-2 genes in zebrafish (Lepiller et al., 2009). Although most research in zebrafish has focused on NOS-2A (Elks et al., 2013; Hall et al., 2012), NOS-2B appears functionally conserved (Hegedus et al., 2009; Poon et al., 2008). Building on our well-characterized in

vitro culture system to polarize common carp macrophages into cells with M1 phenotypes, while using the zebrafish and common carp genomes for gene annotation, we collected these M1 macrophages for RNA analysis and subsequent transcriptome analysis. Head kidney-derived macrophages, when stimulated with LPS, polarized into M1 phenotypes characterized by a high up-regulation of NOS-2B, but not NOS-2A gene expression (Fig. 2). This observation is supported by the fact that phylogenetic and syntenic analyses indicate that NOS-2B, but not NOS-2A, is associated with syntenic genes identified for NOS-2 genes in higher vertebrates (Lepiller et al., 2009), suggesting that NOS-2B might be a robust marker for M1 macrophages of (cyprinid) fish.

4.2 Arginase as a marker for M2

The enzyme arginase is present in bacteria, yeasts, plants, invertebrates, and vertebrates, although phylogenetically, plant arginases are closer to the bacterial agmatinases, which pre-date arginase evolutionary. Arginases from plants and ammoniotelic animals, organisms which excrete soluble ammonia, are localized in mitochondria (Jenkinson et al., 1996). The primordial function of arginase appears to be regulation of cellular arginine and ornithine catabolism, unrelated to the urea cycle, 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 internalization of exogenous polyamines; a system highly active in rapidly proliferating cells (Wang et al., 2003). The generation of arginase activity and downstream production of collagen and polyamines help define M2 as a ‘healing type’ of macrophage with arginase being a robust marker for M2 macrophages of fish.

Vertebrates have a relatively recent arginase gene duplication that, at least in mammals, has led to a cytosolic and a mitochondrial form, named ARG-1 and ARG-2, respectively. Although both isoforms catabolize arginine, they are encoded by two different genes which differ in cellular expression and cell-type-specific regulation (Jenkinson et al., 1996). In ureotelic animals; organisms which 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. Like other vertebrates, teleost fish express ARG-1 and ARG-2, but the presence of mitochondrial targeting sequences in both isoforms (Joerink et al., 2006c) suggests that these enzymes are both of the mitochondrial form. Apparently, arginases function as mitochondrial enzymes in fish, which generally are ammoniotelic (exceptions are marine elasmobranchs,

coelacanths and African lungfishes) and thus do not excrete urea through the kidneys but ammonia mainly through the gills.

Mammalian M2 macrophages express both ARG-1 and ARG-2, but numerous studies have shown that it is the enhanced expression of the cytosolic ARG-1 that depletes these macrophages of intracellular arginine pools leaving less substrate for iNOS (Rath et al., 2014) and thus counteracts the development of M1 macrophages and leads to the production of ornithine and downstream synthesis of several polyamines, characteristic of M2 macrophages. Head kidney-derived macrophages of common carp, when stimulated with cAMP, polarized into M2 phenotypes characterized by a high up-regulation of ARG-2, but not ARG-1 gene expression (Fig. 3), suggesting that ARG-2 might be an excellent marker for M2 macrophages of fish.

5. Future developments

A major limitation of understanding M1/M2 polarization in murine and human models is the lack of in vivo imaging potential. Fish models, such as the zebrafish and medaka (*Oryzias latipes*) have led the way in this respect, with intra-vital, in vivo imaging of immune processes in their embryos being a major advantage of the model due to their small size and transparent tissues (Crespo et al., 2014; Renshaw and Trede, 2012). The zebrafish, especially, has emerged as a useful model for the study of inflammation and infection, with a number of well-characterised models to investigate both processes (Henry et al., 2013; Torraca et al., 2014). Zebrafish embryos have functional macrophages at 1 day post-fertilization (dpf) and neutrophils by 2 dpf. The development of fluorescent reporter lines marking these cell types enables detailed analysis during inflammatory and infection processes (Ellett et al., 2011; Gray et al., 2011; Renshaw et al., 2006). The relative ease of genetic manipulation is a major advantage of the zebrafish model, and the genes for fluorescent proteins (e.g. green fluorescent protein, GFP) can be inserted into the genome using bacterial artificial chromosome (BAC) recombineering, or can be placed under the control of a partial promoter, to give specific expression in the cell type of interest (Mathias et al., 2006; Renshaw et al., 2006). The rapidly-increasing number of well-characterized experimental infection and inflammation models in zebrafish are highly valuable for in vivo investigations of (anti) inflammatory processes.

The complex behaviors of zebrafish macrophage/neutrophil sub-populations observed during inflammation and infection have shown that M1- and M2-like macrophage subsets may indeed also be present in the zebrafish embryo/larvae (Cambier et al., 2014; Ellett et al.,

2011; Herbomel et al., 1999). Given the in vitro findings in (for example) the closely-related common carp this may not be surprising, but these potential subsets have yet to be fully identified and characterized in vivo. The zebrafish has already emerged as being a particularly useful animal model for the study of inflammation and infection in vivo and thus zebrafish may help to examine macrophage polarization, particularly the plasticity of macrophages to switch subtypes during an inflammation/infection response, which so far has proven difficult to observe in vivo. Since the current classification of M1 / classically-activated and M2 / alternatively-activated macrophages have, in the main, arisen from in vitro studies using specific stimuli, zebrafish may help with truly reflecting the complex nature of in vivo environments. Zebrafish models of leukocyte biology are, in general, used from 2 to 5 dpf, while the optical properties of the embryo are optimal and before the onset of functional adaptive immunity. These properties make them an exciting model for investigation of the ‘macrophage first’ hypothesis, while not negating looking later in development when the adaptive immune response is active.

Much of the recent focus in zebrafish has been around TNF- α , as an important cytokine involved in the activation of leukocytes after inflammation/infection challenge and a putative although not unique marker for M1. In a well-characterized zebrafish *Mycobacterium marinum* infection model of tuberculosis, TNF- α is up-regulated in infected macrophages and leads to production of reactive oxygen species to control the infection (Roca et al., 2008). In the *M. marinum* model it has been shown that reactive nitrogen species mediated by NOS-2 aids infection control when stimulated by upregulated hypoxia signaling (via the hypoxia inducible factor-1 alpha, HIF-1 α , transcription factor), evidence that suggests that NOS-2 may have a role in leukocyte subset behavior during zebrafish infection (Elks et al., 2013; Elks et al., 2014). The possibility of developing M1 (e.g. NOS-2) and M2 (e.g. ARG-2) zebrafish transgenic lines using different fluorescent markers is an exciting prospect, as these could be combined in double or triple transgenics with existing marker lines to generate a model in which the plasticity of macrophages over the course of an infection/inflammatory process could be studied in vivo. Their use in well-defined infections, such as the *M. marinum* model, would allow in detail characterisation of macrophage phenotype over the timecourse of an infection, and importantly, realtime observation of one-directional switching events in vivo at the level of individual cells. Crucially, the genetic tractability in the zebrafish embryo model would allow molecular dissection of the pathways involved in any M2 to M1 (or vice versa) switching events. In order for transgenic M1 and M2 reporter lines to be generated a set of reliable, and preferentially evolutionary-conserved, markers for these polarized cell types are

required. In a recent experiment, building on a well-characterized in vitro culture system for head kidney-derived macrophages, we made use of the large size of common carp to collect polarized macrophage sub-types for transcriptome analysis using the largely syntenic zebrafish and common carp genomes for annotation. As previously discussed, microbial ligand LPS polarized these macrophages into M1, whereas extracellular cAMP polarized these macrophages into M2, with NOS-2(B) and ARG-2 as respective markers, each with low *P* value and high fold-change of gene expression. Of course, these preliminary analyses require confirmation and, preferably, expansion with other stimuli such as IFN- γ and IL-4/IL-13. However, the value of the current dataset may be apparent from the confirmation of NOS-2 as marker for M1 and ARG-2 as marker for M2 macrophages of fish.

In addition (data not shown), un-stimulated head kidney-derived carp macrophages express at medium-high levels (1,000-4,000 reads per million) the beta chain shared by the GM-CSF cytokine family and also the common receptor subunit IL-4R α for recognition of IL-4/-13. Expression levels of IL-4R α show a 5-fold up-regulation but only after cAMP stimulation, suggesting cAMP indeed induces a M2-like phenotype in these macrophages. Furthermore, MRC-1B, provisionally annotated as the mannose receptor, has a high basal expression (5,000-25,000 reads per million) in un-stimulated macrophages, shows a 5-fold down-regulation in LPS-stimulated cells but a 3-fold up-regulation in cAMP-stimulated cells. The latter data on MRC-1B suggest that indeed this could be the mannose receptor and a suitable M2 marker for fish macrophages. It is our expectation that although NOS-2 and ARG-2 may prove good markers for M1 and M2 macrophages, respectively, the expression of these molecules may not always be exclusive for these macrophage phenotypes and additional markers (e.g. IL-4R α or MRC1-B for M2) may be required to confirm profiles of polarized macrophages. In zebrafish, the availability of well-characterised macrophage fluorescent transgenic lines, described earlier (MPEG-1 and FMS driven expression), in combination with novel M1/M2 reporter lines, will allow identification of NOS-2 and ARG-2 positive macrophages even if other cell types also express these genes. Our preliminary findings indicate the value of combining in vitro culture systems of polarized head kidney-derived macrophages with the genetic identification of M1 and M2 markers for fish that can subsequently be used in zebrafish to study polarized immune responses in vivo.

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623

Figure legends

Figure 1

‘Macrophage first’ point of view: polarized immune responses are initiated by activation of macrophages and sustained by T_H1 (IFN- γ) and T_H2 (IL-4/13) cytokines.

Microbial stimuli are sensed by macrophages through Toll-like receptors (TLRs) along with CD14, or other pattern recognition receptors (PRR). Sensing of microbial stimuli (e.g. LPS from Gram-positive bacteria) leads to the development of innate-activated M1 macrophages with increased phagocytic activity, production of reactive oxygen species (ROS), nitric oxide (NO) and pro-inflammatory cytokines, among which IFN- γ . This cytokine drives T cells into a T_H1 response, and in a feedback loop IFN- γ induces classically-activated M1 macrophages characterized by a higher expression of MHC class II and co-stimulatory molecules in addition to an increase of the inflammatory functions already described for innate-activated M1 macrophages.

Parasite-related stimuli (e.g. chitin from helminths) are sensed by macrophages, leading to the development of alternatively-activated M2 macrophages. These have increased arginase activity, associated decreased microbicidal activity, and increased production of collagen and polyamines for cell growth and healing processes. T cells are skewed towards a T_H2 profile and in a feedback loop, the T_H2 cytokines IL-4 and/or IL-13 further induce alternatively-activated M2 macrophages which express DC-SIGN as well as higher levels of MHC class II molecules, in addition to an increase of the anti-inflammatory functions already described.

Figure 2

Nitric oxide synthase-2(B) is a marker for M1 macrophages of fish

Common carp head kidney-derived macrophages were stimulated with LPS, or cAMP, and gene expression analyzed by RNAseq (Illumina). Nitric oxide synthase (NOS) has two hits in the zebrafish genome (NOS-2A and NOS-2B) because of a genome duplication event (3R), whereas common carp have two hits for NOS-2A and -2B each in the genome (and transcriptome) because of an additional duplication event (4R). Only NOS-2B, but not NOS-2A is expressed in common carp macrophages. Accordingly, gene expression of NOS-2B was studied by real-time qPCR using gene-specific primers and found highly up-regulated only after stimulation with LPS.

Figure 3

Arginase-2 is a marker for fish M2 macrophages of fish

Common carp head kidney-derived macrophages were stimulated with LPS, or cAMP, and gene expression analyzed by RNAseq (Illumina). Zebrafish arginase (here, ARG-2) has two hits in the common carp genome (and transcriptome) because of an additional genome duplication event (4R). Only ARG-2, but not ARG-1 is expressed in common carp macrophages. Accordingly, gene expression of ARG-2 was studied by real-time qPCR using gene-specific primers and found highly up-regulated only after stimulation with cAMP.

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Figure 1 Macrophage

M1 macrophage

Macrophage

M2 macrophage

Figure 2

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RNA-Seq analysis of NOS-2A

Stimulus	Normalized counts		Fold change	padj
	Control	Stimulated		
cAMP	0.4	0	0	1
LPS	2.3	1.0	0.5	1

RNA-Seq analysis of NOS-2B

Stimulus	Normalized counts		Fold change	padj
	Control	Stimulated		
cAMP	4412 (1835-6988)	3978 (1264-6692)	0.8 (0.7-1)	0.2-1
LPS	2076 (776-3377)	98688 (34485-162891)	46 (44-48)	4.21E-60 - 1.14E-57
Average (Range)				

qPCR analysis of NOS-2B

Phylogenetic analysis of NOS-1 and NOS-2

Figure 3

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RNA-Seq analysis of ARG-2

Stimulus	Normalized counts		Fold change	padj
	Control	Stimulated		
cAMP	1355 (769-1940)	15568 (11003-20133)	16 (6-26)	5.47E-148 - 5.35E-09
LPS	3681 (2156-5205)	5863 (4559-7167)	1.8 (1.4-2.1)	1
Average (Range)				

qPCR analysis of ARG-1 and ARG-2

Phylogenetic analysis of ARG-1 and ARG-2